THE UNIVERSITY OF CALGARY

CHEMICAL STUDIES ON THE ACTIVE SITE OF LIPOAMIDE DEHYDROGENASE FROM *ESCHERICHIA COLI* K12

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Chemical studies on the active site of lipoamide dehydrogenase from Escherichia coli K12" submitted by Charles F.B. Holmes in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The pyruvate dehydrogenase multienzyme complex (PD complex) from E. coli K12 comprises: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and lipoamide dehydrogenase (E3). The amino acid sequence of E3 is largely unknown. This thesis embodies a protein chemical study of the active site and catalytic mechanism of E3. Aspects of the active-site coupling mechanism between E2 and E3 were also investigated.

PD complex was purified from E. coli by protamine sulphate precipitation and ultracentrifugation. The [^{14}C]-radiolabelled bifunctional reagent, p-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2$[^{14}C]CONHPhAsO), was used to specifically alkylate an active-site, catalytically essential, histidine residue on E3. This residue, specifically modified in the N^3-imidazole ring position, was identified by high voltage electrophoresis (HVE), amino acid and radiochemical analyses of isolated, [^{14}C]-radiolabelled E3.

E3, inhibited (whilst part of the PD complex) with $BrCH_2$[^{14}C]CONHPhAsO, was isolated and digested with cyanogen bromide (CNBr), which cleaves specifically on the C-terminal side of methionine residues. The digest was mapped by reverse phase high performance liquid chromatography (HPLC). HVE, amino acid and radiochemical analyses of peptide peaks in the digest revealed that the [^{14}C]-radiochemically labelled histidine residue resided (>90%) in the most radioactive peptide, designated CNBr(X). This peptide contained at least 34 amino acids and was apparently the C-terminal peptide of E3, since it

was devoid of homoserine or homoserine lactone (all other peptides contained homoserine and/or homoserine lactone as the C-terminal product of CNBr cleavage). The amino acid sequence of CNBr(X) was largely elucidated (30 residues) by automated Edman degradation. [^{14}C]-Radiolabel was detected in sequencer cycles 14 and 15 (trace) only, inferring that the active-site histidine residue was present in position 14 of the CNBr(X) sequence.

CNBr(X) was directly homologous to the C-terminal region of E3 from pig heart (63%) and human erythrocyte glutathione reductase (42%). The E3 active-site histidine corresponded exactly to a similar essential histidine residue (His-467) in glutathione reductase. The essential histidine in glutathione reductase is held firmly by a short hydrogen bond between the N^1-imidazole ring position and glutamic acid (Glu-472) [Pai and Schulz (1983) J. Biol. Chem. 258, 1752]. These two flavoproteins are known to be mechanistically similar. CNBr(X) also contained glutamic acid, 5 residues downstream of the catalytic histidine, which may serve to position histidine firmly in the active site of E3. This assumption was supported by earlier active-site directed irreversible inhibition studies, whereby only the N^3-imidazole ring position of histidine was alkylated by $BrCH_2$[^{14}C]CONHPhAsO.

The results indicate that a catalytic histidine residue present in the C-terminal region of E3 may be the base proposed by others to be important in the E3 catalytic mechanism. This thesis provides further evidence for an evolutionary relationship between E3 and glutathione reductase from widely differing organisms.

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ABBREVIATIONS

αKG complex	α-Ketoglutarate complex
Ace E	Pyruvate dehydrogenase (E1) gene
Ace F	Dihydrolipoamide acetyltransferase (E2) gene
Aro P	General aromatic amino acid permease gene
ATZ	2-anilino-5-thiazolinone
BAL	British Anti Lewisite (2,3-dithiopropanol)
$BrCH_2[^{14}C]CONHPHAsO$	p-[(Bromoacetyl)amino]phenyl arsenoxide ([^{14}C]-radiolabelled)
CNBr	Cyanogen bromide
CoA	Coenzyme A
DCC	Dicyclohexyl carbodiimide
DTT	Dithiothreitol
E1	Pyruvate dehydrogenase
E2	Dihydrolipoamide acetyltransferase
E3	Dihydrolipoamide or lipoamide dehydrogenase
E.C.	Enzyme catagorization
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavin adenine dinucleotide
GMP	Guanosine monophosphate
GSSG	Oxidised glutathione
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
HVE	High voltage electrophoresis
Lpd	Lipoamide dehydrogenase (E3) gene
M_r	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
Nad C	Quinolinate phosphoribosyl-transferase gene
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$NaDodSO_4$	Sodium dodecyl sulphate

$NaDodSO_4$-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
NEM	N-ethyl maleimide
NMR	Nuclear magnetic resonance
OMe	O-methylated
PD complex	Pyruvate dehydrogenase multienzyme complex
PITC	Phenylisothiocyanate
PMSF	Phenylmethylsulphonyl fluoride
Polybrene	1,5-dimethyl-1,5-diazaundecamethylene polymethobromide
PTC	Phenylthiocarbamyl
PTH	Phenylthiohydantoin
Quadrol	N,N,N^1,N^1-tetrakis (2 hydroxypropyl)ethylenediamine
RP/HPLC	Reverse phase high performance liquid chromatography
Suc A	α-Ketoglutarate dehydrogenase gene
Suc B	Dihydrolipoamide succinyltransferase gene
TCA	Trichloroacetic acid
t_d	Doubling time
TEMED	Tetramethylene diamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TPP	Thiamine pyrophosphate
Tris	Tris (hydroxy amino) methane

Amino Acid Code

Amino acid or residue thereof	Three-letter symbol	One letter symbol
Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H

CHAPTER 1 - INTRODUCTION

Proteins, the most versatile of the macromolecules, play a central role in living systems and are responsible for both the metabolic capabilities and the morphology of organisms. This thesis represents part of an ongoing investigation that seeks to use multienzyme proteins as model systems for studying protein structure and function.

1.2 Multienzyme Proteins

Enzyme nomenclature as applied to organised multienzyme systems has recently been the subject of much debate (Welch and Gaertner, 1979, 1980; Karlson and Dixon, 1979, 1980; Van Döhren, 1980). The generic term "multienzyme protein" describing an enzyme that catalyses different reactions at separate sites has found the largest acceptance. This can be further subdivided into "multienzyme complex", when the association of enzymes is non-covalent, and "multienzyme polypeptide", when it is covalent.

Multienzyme complexes are structural, functional and regulatory units made up of several enzymes. Multienzyme polypeptides may be defined by two characteristic properties. Structurally, they consist of a single type of polypeptide chain, but they have multiple catalytic or binding functions (Kirschner and Bisswanger, 1976).

Multienzyme polypeptides have possibly evolved from independent, small proteins via multienzyme complexes, in which gene fusion has led to the production of one polypeptide chain containing different enzyme activities. Representative examples are found in the pathways of amino acid and fatty acid synthesis. The most well known example of one polypeptide chain containing two enzyme functions is aspartokinase I -homoserine dehydrogenase of *Escherichia coli* (Truffa-Bachi *et al.*, 1974). However, one of the more remarkable cases is fatty acid synthetase from baker's yeast that has two gene loci, fas 1 and fas 2, encoding two polypeptide chains with eight catalytic functions (Kirschner and Bisswanger, 1976; Schweizer *et al.*, 1975).

1.3 Multienzyme Complexes

In general, multienzyme complexes can be envisaged as aggregates of enzymes that catalyse two or more steps in a metabolic sequence. The principal physiological advantages of multienzyme complexes are threefold: (i) enhancement of overall catalytic efficiency, (ii) channelling of reactive intermediates, and (iii) regulation of metabolic fluxes (Hammes, 1982).

The catalytic activity of isolated individual subunits of a multienzyme complex can be enhanced when in association with other complex components. Thus, complex formation provides a means of concentrating catalysts rather than having them randomly distributed in the cell.

The sequestering of reaction intermediates is probably the most common function of multienzyme complexes. Confining intermediates to the complex prevents their destruction by competing chemical reactions and is also clearly advantageous if the intermediate in question is unstable ("Hot potato hypothesis"; Perham, 1982).

Although enhancement of catalytic efficiency and sequestering of reaction intermediates may be envisaged as mechanisms for regulating metabolic fluxes, many of the multienzyme complexes are also regulated by classical allosteric mechanisms such as feedback inhibition and antagonism of specific metabolites.

The many known multienzyme complexes from various sources have been extensively reviewed elsewhere (Adamson, 1981; Reed and Pettit, 1981; Reed, 1974). This study has involved a further investigation of the pyruvate dehydrogenase multienzyme complex (PD complex) from *E. coli*.

1.4 PD Complex from E. coli

In all organisms so far studied, the oxidative decarboxylation of pyruvate to form acetyl-CoA and CO_2, at the expense of NAD^+, is catalysed by the PD complex. The overall reaction catalysed is:

$$\text{Pyruvate} + NAD^+ + CoA \longrightarrow \text{Acetyl CoA} + NADH + H^+ + CO_2$$

In organisms metabolising acetyl-CoA, including E. coli (Fig. 1.4.1), the complex therefore plays a central role in glucose breakdown and fat synthesis. The complex is comprised of multiple copies of three different enzymes, which in order of participation are: pyruvate dehydrogenase (E1) (E.C. 1.2.4.1), dihydrolipoamide acetyltransferase (E2) (E.C. 2.3.1.12) and (dihydro-) lipoamide dehydrogenase (E3) (E.C. 1.6.4.3). In addition to this are a series of cofactors and prosthetic groups, namely: TPP, Mg^{2+}, coenzyme A, lipoic acid, FAD and NAD^{+}.

The regulation of the PD complex in E. coli has been reviewed extensively (Hucho, 1975; Reed, 1974; Reed and Oliver, 1982) and in keeping with its central position in energy metabolism (Fig. 1.4.1), is fairly complex.

A series of metabolites from glycolysis have an activating influence on the complex, whereas citric acid cycle intermediates such as citrate are inhibitory. The complex can also be activated by AMP and inhibited by GMP. Furthermore, as alluded to earlier, the PD complex is subject to allosteric control by pyruvate, which exerts a positive cooperative effect that is made more intense in the presence of the feedback inhibitor acetyl-CoA (Schwartz et al., 1968; Bisswanger and Henning, 1971; Shepherd et al., 1976). The main target for the majority of effectors seems to be the E1 component of the complex. However, it has recently been shown that NADH can exert a negative effect on the complex by altering the redox state of E3 (Wilkinson and Williams, 1981; Schminke-Ott

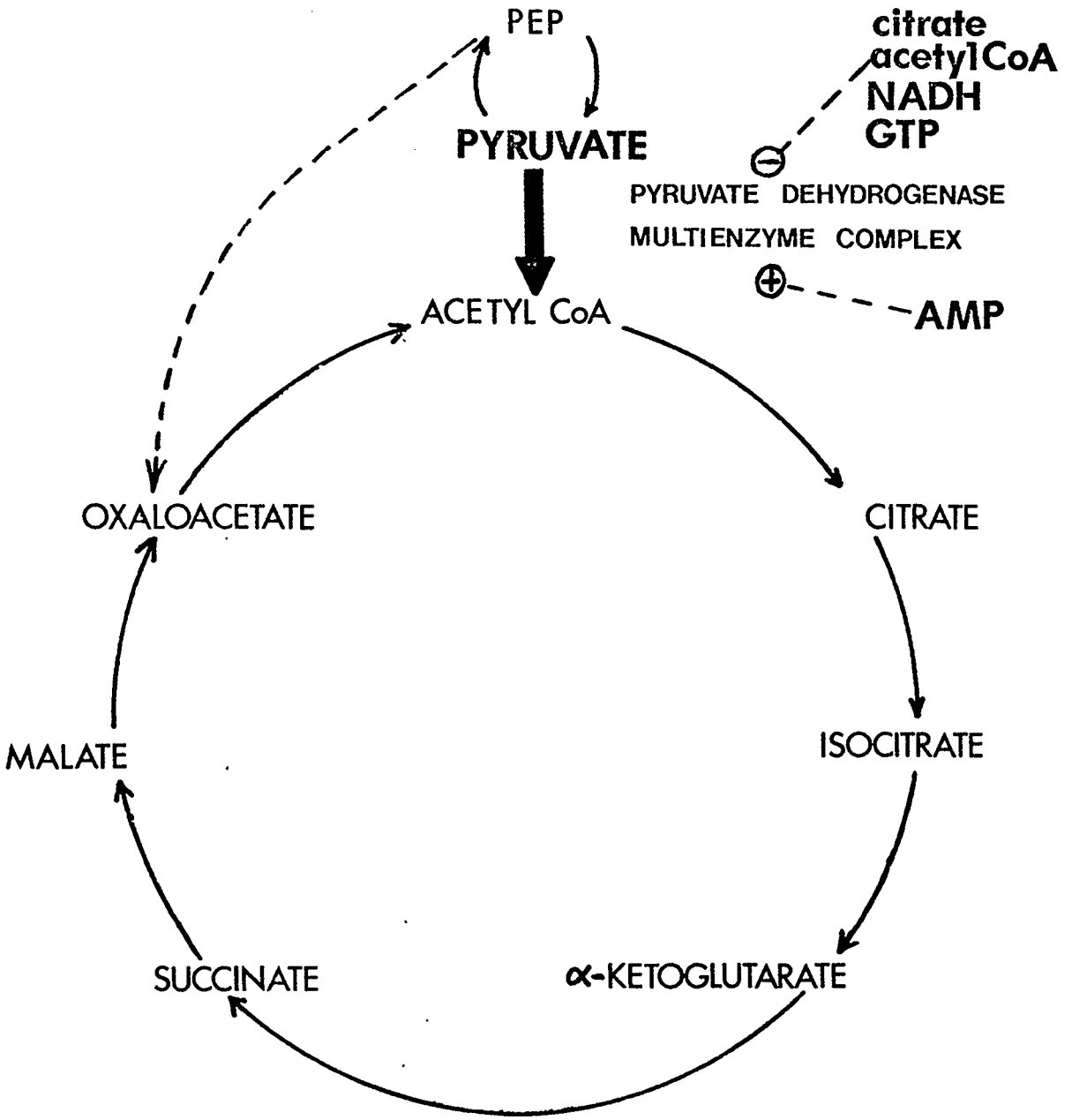


Figure 1.4.1 A diagram illustrating the central metabolic role of the PD complex in *E. coli*.

and Bisswanger, 1981). This latter finding is one example of how the $\frac{NADH}{NAD^+}$ ratio might regulate the tricarboxylic acid (TCA) cycle in *E. coli*.

1.5 Structural Organisation of the PD Complex from E. Coli

The subunit molecular weights (M_r), as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE) (Fig. 1.5.1) of each of the components of the PD complex (E1, E2 and E3), have been estimated with fairly good agreement between various research groups at 96,000-100,000, 78,000-89,000 and 55,000-56,000, respectively (Gebhardt *et al.*, 1978; Perham and Thomas, 1971; Eley *et al.*, 1972; Vogel, 1977; Angelides *et al.*, 1979). The only major point of contention concerns the M_r of the E2 subunit, which may have a molecular weight as low as 64,500, as determined by sedimentation equilibrium analysis (Bliele *et al.*, 1979; Reed and Oliver, 1982).

The overall molecular weight of the PD complex from *E. coli* remains a point of much contention, the most recently published values are: 3.75×10^6 (Vogel *et al.*, 1972); 4.6×10^6 (Eley *et al.*, 1972); 5.25×10^6 5.25×10^6 (Durschlag, 1975); 6.1×10^6 (Danson *et al.*, 1979) and 4.8×10^6 (Angelides *et al.*, 1979). These discrepancies may arise from differences in the stoichiometry of various enzyme preparations. Variability in

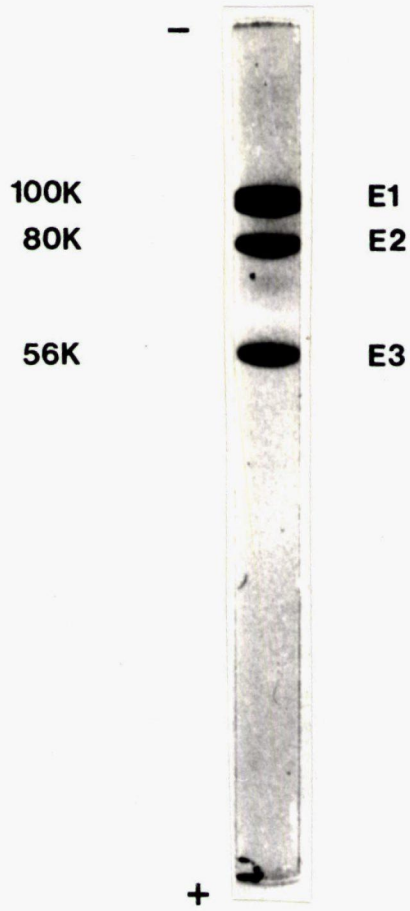


Figure 1.5.1 Subunit composition of PD complex from *E. coli*. Molecular weights of pyruvate dehydrogenase (pyruvate decarboxylase) E1, dihydrolipoamide acetyltransferase (lipoyl transacetylase) E2, and dihydrolipoamide dehydrogenase (dihydrolipoyl dehydrogenase) E3, are given. (Stevenson *et al.*, 1978)

the polypeptide chain stoichiometry might reflect the loss of E1 and E3 subunits from the E2 core during purification of the complex. However, discrepancies may also be the result of variations in the growth conditions of bacteria and state of assembly of the complex (Danson and Perham, 1976), or be the result of methodological errors caused by difficulties in measuring such large molecular weights (Adamson, 1981).

Not surprisingly, estimations of the subunit stoichiometry of the PD complex do not find general agreement. A model involving an overall stoichiometry of E1:E2:E3 ≡ 1:1:0.5, with 24 polypeptide chains of E1, 24 chains of E2 and 12 chains of E3, has found acceptance (Eley *et al.*, 1972; Reed *et al.*, 1975; Angelides *et al.*, 1979; Speckhard and Frey, 1975). An alternative model proposing a polypeptide chain stoichiometry of E1:E2:E3 ≡ 1-2:1:1 has also been proposed (Bates *et al.*, 1975) and substantiated (Bates *et al.*, 1977; Hale and Perham, 1979[a]) in the same laboratory. These authors report a stoichiometry involving 24-48 polypeptide chains of E1, 24 chains of E2 and 24 chains of E3.

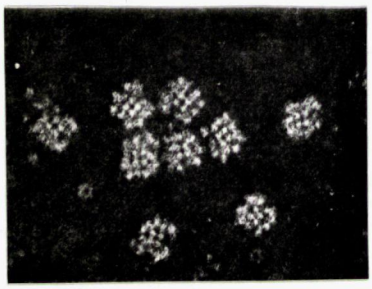
It is, however, generally agreed that the *E. coli* PD complex is organised about a central core comprising an oligomer of E2 to which multiple copies of E1 and E3 are joined by non-covalent bonds. Results of electron microscopic studies (Reed and Oliver, 1968; Oliver and Reed, in press), X-ray crystallographic studies (DeRosier *et al.*, 1971; Fuller *et al.*, 1979) and sedimentation

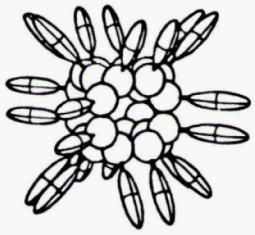
equilibrium molecular weight determinations (Eley *et al.*, 1972; Pettit *et al.*, 1973), have demonstrated that the E2 core consists of 24 apparently identical polypeptide chains arranged in a 432 molecular symmetry to form a cube-like particle (Fig. 1.5.2).

1.6 Reaction Mechanism of the PD Complex from E. Coli

The commonly accepted mechanism of action of the complex is outlined in Fig. 1.6.1. Pyruvate undergoes decarboxylation and oxidation as a result of the reactions catalysed by E1, the pyruvate dehydrogenase component. E1 requires the participation of two cofactors, TPP and lipoic acid, and is the rate determining enzyme in the overall process. The C_2 carbon atom in the thiazole ring of TPP is highly acidic (Breslow, 1958) and readily ionises to form a carbanion that can add to the carbonyl group of pyruvate. Following decarboxylation, a carbanionic 1-hydroxyethyl TPP moiety is formed on E1 and can now function as a nucleophile.

E2 contains lipoic acid, which is covalently bonded to the enzyme by the formation of an amide bond involving the ε-amino group of a lysine residue (Nawa *et al.*, 1960). This lipoyl-lysine unit provides a flexible arm (approximately 14 Å in length), which is thought to be capable of rotating between the catalytic centres of the complex. This allows the 1,2-dithiolane ring of





A B

Figure 1.5.2 Structure of the PD complex from E. coli. Electron micrograph image and interpretive model. (A) Native PD complex (x200,000, Bleile et al., 1979). Two E1 subunits are thought to be distributed on each of the twelve edges of the cube-like E2 inner core. Two E3 subunits are apparently found on each face of the E2 core (24:24:12 stoichiometry, after Eley, 1972; Reed and Oliver, 1982). (B) The quaternary structure of the E2 core (Bleile et al., 1979). Each E2 subunit contains a subunit binding domain (sphere) and an extended lipoyl domain (ellipsoid). The subunit binding domain contains the binding sites for E1 and E3 and the catalytic site for transacetylation (Fig. 1.6.1). The lipoyl domain (M_r=31,600) carries at least two covalently bound lipoyl moieties, and is separated from the subunit binding domain by a trypsin-sensitive "hinge" region (Reed and Oliver, 1982).

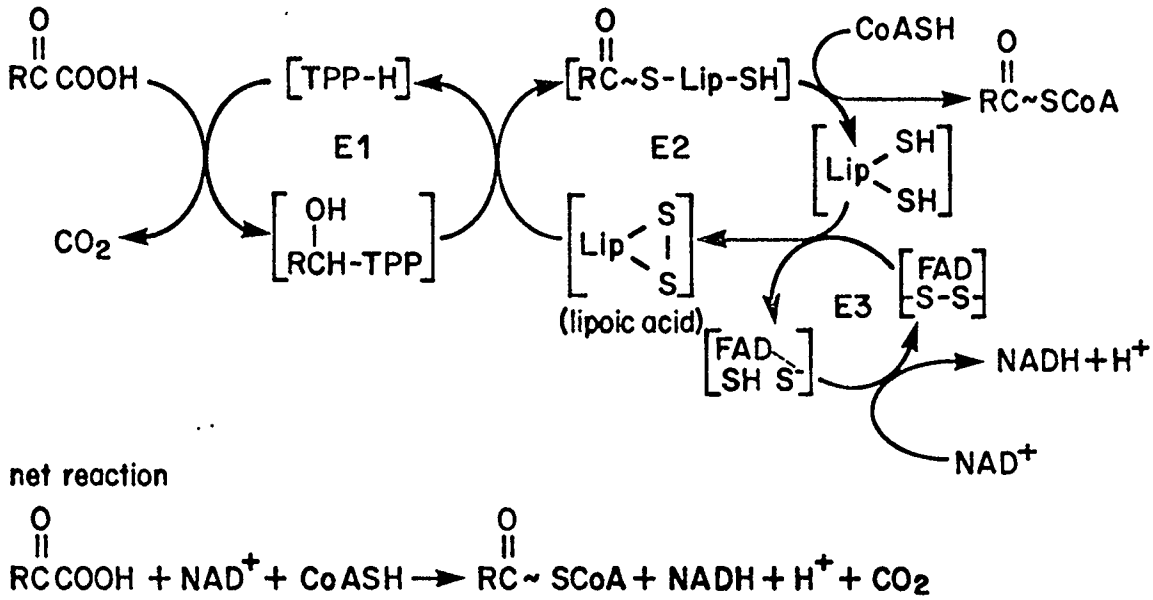


Figure 1.6.1 Reaction mechanism of the PD complex from *E. coli*. E1 refers to pyruvate dehydrogenase (a decarboxylase), E2 is dihydrolipoamide acetyltransferase and E3 is lipoamide dehydrogenase. TPP is thiamin pyrophosphate.

lipoamide to be appropriately positioned at the active site of E1 (Green and Oda, 1961; Koike et al., 1963; Reed and Oliver, 1982).

Nucleophilic addition of the 1-hydroxyethyl TPP moiety to the disulphide group of oxidised lipoamide generates a hemithioacetal. Expulsion of TPP constitutes the actual oxidation step converting the sp^3 carbon of the hemithioacetal to the sp^2 carbon of the acetyl-thioester linkage of S^6-acetyldihydro lipoamide (Fig. 1.6.1). E2 subsequently catalyses the transfer of acetyl groups from the thiol of lipoamide to the thiol of coenzyme A. This transthiolation reaction results in the formation of fully reduced enzyme bound lipoamide and acetyl-CoA. In this form E2 is not competent to carry out another enzyme turnover.

E3, functions to reoxidise dihydrolipoamide back to the active disulphide form (Williams, 1976). In doing this, E3 is in turn left in a reduced state and needs NAD^+ to act as a reoxidant.

The intermolecular coupling of the lipoyl-lysine "swinging arm" and the active-site of E3 provides one of the key focal points of this investigation. It is, therefore, pertinent to examine the function and stoichiometry of the lipoyl-lysine residues on E2 in greater detail.

1.7 Lipoyl-lysine Residues on E2; Stoichiometry and Role in Active-site Coupling

The role of E2 as the structural core of the PD complex from *E. coli* is universally accepted (Reed and Oliver, 1968). However, a clear understanding of the stoichiometry and "raison d'etre" of lipoic acid residues in the active-site coupling mechanism has remained elusive. Reed and co-workers first suggested that the lipoyl-lysine moiety provides a flexible arm of about 14 Å in length (Nawa *et al.*, 1960). They further proposed that this would allow the reactive 1,2-dithiolane ring to rotate among the catalytic sites of E1, E2 and E3, in a "swinging arm" active-site coupling mechanism (Reed, 1974). This concept was strongly supported by experiments using electron spin resonance spectroscopy, which revealed that the lipoyl-lysine moieties possessed a high degree of mobility within the PD complex (Ambrose and Perham, 1976; Grande *et al.*, 1976). However, fluorescence energy transfer measurements (Moe *et al.*, 1974; Angelides and Hammes, 1979) have indicated that the distances between catalytic sites in the *E. coli* PD complex are significantly larger (at least 40 Å) than the distance (14 Å) that could be spanned by a single rotating lipoyl-lysine residue (Shepherd and Hammes, 1977; Scouten *et al.*, 1980), thus necessitating interaction between two or more such moieties.

A substantial body of evidence now suggests that two (Danson and Perham, 1976; Brown and Perham, 1976;

Speckhard *et al*. 1977; Collins and Reed, 1977; Bates *et al*., 1977; White *et al*., 1980) or possibly three (Hale and Perham, 1979a) lipoyl-lysine residues are present per polypeptide chain of E2. It is generally well accepted that each E2 polypeptide chain contains two lipoyl residues that can become reductively acetylated in the presence of pyruvate (Danson and Perham, 1976; Speckhard *et al*., 1977; Collins and Reed, 1977; Shepherd and Hammes, 1977) and can undergo reoxidation by E3 and NAD^+ (Collins and Reed, 1977; Danson *et al*., 1981). Angelides and Hammes (1978) have consequently proposed that a series (handover) interaction between at least two lipoyl moieties is required to link the active sites in the PD complex. This idea appeared to be compatible with the presence of two lipoyl moieties on each E2 subunit, and with the finding that intramolecular transfer of acetyl groups between lipoyl moieties can occur under conditions in which only a few E1 subunits are functional (Bates *et al*., 1977; Collins and Reed, 1977; Cate *et al*., 1980).

Recent experiments (Frey *et al*., 1978; Ambrose-Griffin *et al*., 1980; Akiyama and Hammes, 1980; Stepp et al, 1981) have cast doubt on the probability that the normal catalytic mechanism involves transfer of an acetyl group (and an electron pair) from one lipoyl moiety to a second lipoyl moiety enroute to coenzyme A. However, redox reactions between reduced and oxidised lipoyl moieties, ie. thiol-disulphide exchange, are apparently part of the normal reaction mechanism (Reed and Oliver, 1982).

Further insight into the active-site coupling mechanism has come from limited proteolysis studies on the E2 core of the PD complex (Bleile et al., 1979; Hale and Perham, 1979[b]), demonstrating that the lipoyl-lysine moiety is part of a "super arm", ie. the lipoyl domain. It was proposed that movement of lipoyl domains, and not simply rotation of lipoyl moieties, provides the means to span the distance between catalytic sites on the α-keto acid multienzyme complexes (Stepp et al., 1981; Reed and Oliver, 1982; Perham et al., 1981).

The elegant proton nuclear magnetic resonance (NMR) studies of Perham et al. (1981) have continued to provide support for this proposal. Unexpectedly sharp lines in the proton NMR spectra of the E. coli α-keto acid multienzyme complexes have been attributed to high internal mobility of the lipoyl domains (Perham et al., 1981; Wawrzynczak et al., 1981; Perham and Roberts, 1981). In summary, these investigators have demonstrated that the flexible lipoyl domain has enough mobility to increase the potential distance (2.8 nm) that can be spanned by a single lipoyl-lysine residue to account for the suggested 40 Å distance between PD complex active sites. This clearly eliminates the need for the "handover" mechanism outlined by Angelides and Hammes (1978).

The findings of Reed and Perham on the mobility of lipoyl domains have clearly had to be reconciled with earlier observations on the ability of El to reductively

acetylate two lipoyl moieties on E2. Many workers had already suggested that only one of these two lipoyl-lysine residues is essential for PD complex activity (Frey *et al.*, 1978; Bliele *et al.*, 1979; Ambrose-Griffin *et al.*, 1980). Evidence is now accumulating in favour of a "take over" mechanism in which one lipoyl-lysine residue is able to take over the catalytic role of another lipoyl moiety, if it is inactivated or excised (Berman *et al.*, 1981; Stepp *et al.*, 1981; Ambrose-Griffin *et al.*, 1980).

In summary, it is now clear that one lipoyl-lysine "swinging arm", attached to a highly flexible lipoyl domain on the E2 polypeptide chain, is capable of interacting with the E1, E2 and E3 active sites of the PD complex; therefore, the problem still remains to elucidate the need for two or even three lipoyl moieties on E2. It is becoming clear that the significant mobility of parts of the E2 core may well allow a given E1 active site to be served by more than one lipoic acid residue, possibly by lipoic acid residues bound to different E2 chains in the core of the PD complex (Bates *et al.*, 1977; Cate *et al.*, 1980; Ambrose-Griffin *et al.*, 1980; Berman *et al.*, 1981; Stepp *et al.*, 1981; Perham *et al.*, 1981). By this mechanism the oligomeric E2 core, with its extended lipoyl domains, apparently obviates the necessity for a particular juxtapositioning of the three catalytic components while still ensuring an efficient coupling of their activities.

1.8 Organisation and Expression of the PD Complex Genes of E. Coli K12

The three polypeptide chains E1, E2 and E3 in the α-keto acid dehydrogenase complexes from *E. coli* are encoded by the following structural genes (Table 1.8.1).

Table 1.8.1 Gene-protein relationships of the enzymic components of the α-keto acid dehydrogenase complexes of *E. coli* K12 (Langley and Guest, 1978)

Gene	Component		
ace E	Pyruvate dehydrogenase	E1	PD COMPLEX
ace F	Dihydrolipoamide acetyltransferase	E2	PD COMPLEX
lpd	Dihydrolipoamide dehydrogenase	E3	PD COMPLEX
suc A	α-ketoglutarate dehydrogenase	E1	α-KG COMPLEX
suc B	Dihydrolipoamide transuccinylase	E2	α-KG COMPLEX

All gene components illustrated are transcribed clockwise with respect to the *E. coli* linkage map. The polarities of the ace and suc operons are indicated by the arrows.

In Table 1.8.1, the E1 and E2 structural genes (ace E and ace F) constitute an operon with ace $\overrightarrow{EF}$ polarity, which is very close to the nad C (quinolate phosphoribosyltransferase) and aro P (general aromatic amino acid

permease) genes at 2.6 min on the E. coli linkage map (Guest, 1978). The E3 structural gene (lpd) is linked to the ace F gene thus: nad C - aro P - ace E - ace F - lpd. In contrast, the specific components of the analogous aa-ketoglutarate dehydrogenase complex (aaKG complex) are encoded by the suc A and suc B genes of the suc operon, which is situated at 16.4 min and has the same functional polarity (Guest, 1978). The E3 component of the aaKG complex is encoded by the identical ace F linked lpd gene. This leads us to the question, how is the expression of the single lpd gene geared to the expression of the separately regulated ace and suc operons? Work with mutants has suggested that the lpd gene can be expressed independently of the ace and suc genes (Creaghan and Guest, 1972; Langley and Guest, 1978).

Recent evidence (Guest et al., 1981) has confirmed the existence of a single promoter for the ace E and ace F genes and a separate promoter for the lpd gene. This gene was shown to be transcribed with the same polarity, that is, clockwise with respect to the linkage map. The relative rates of expression of the three PD complex genes from bacterial promoters were estimated as 0.94:1.0:1.4-2.3 (E1:E2:E3) on a molar basis. These results are consistent with an E1:E2 stoichiometry of 1:1 and support the polypeptide chain stoichiometry for the PD complex suggested by Eley et al. (1972). Furthermore,

the excess production of E3 indicates that the lpd promoter functions independently; at least in supplying components for the αKG complex (Guest et al., 1981).

The mode of regulation of the lpd (E3) gene remains a matter for debate. Langley and Guest proposed (1978) that it might be subject to simple autonomous regulation in which its gene product serves as a negatively acting repressor. Other workers have suggested that the lpd (E3) gene is subject to an overall co-ordinate control of the ace region (Schminke-Ott and Bisswanger, 1981).

To date the ace E, ace F, lpd, suc A and suc B genes specifying both the pyruvate dehydrogenase and αKG complexes have been cloned in phage (Guest and Stephens, 1980) and plasmid (Guest et al., 1983) vectors and have almost been completely sequenced (Guest, unpublished results). In particular, E2 of the PD complex has been totally sequenced at the DNA level and contains three 100 amino acid sequences that are homologous (>80%). Clearly, this could be consistent with the presence of three lipoic acid residues on similar sequences; though this is of course not direct evidence as the DNA sequence will not reveal which lysine(s) carries lipoic acid. As mentioned earlier, Hale and Perham (1979[a]) have also suggested that the PD complex may contain three and not two lipoic acid residues per E2 chain. Their suggestion followed measurements of the intrinsic lipoic acid content of the PD complex grown in the presence of ^{35}S.

Clearly these results had to be reconciled with the findings of several workers who have demonstrated that pyruvate can acetylate only two lipoic acid residues per E2 polypeptide chain. The authors consequently proposed a novel mechanism (Hale and Perham, 1979[a]) in which the third lipoyl residue plays an essential role in catalysis. Several experiments reported in this thesis were designed to test this hypothesis and the results clearly refute this proposal.

1.9 Lipoamide Dehydrogenase (E3)

This study involves a detailed investigation of the active-site coupling of E2 and E3 in the PD complex from *E. coli* with a [^{14}C]-radiolabelled bifunctional reagent, p-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2[^{14}C]CO$-NHPhAsO). The use of arsenoxides in protein chemistry has been extensively documented (Robinson, 1980; Stevenson *et al.*, 1978; Adamson, 1981; Adamson and Stevenson, 1981) and these reagents have, as the present study will also show, been of great value in studying the structure-function relationships of several enzymes, because of the affinity of the arsenoxide moiety (R-As=O) for vicinal thiol groups. The mode of action of these reagents will be further discussed in concert with a description of their synthesis in Chapter 2.

The properties of the flavoenzyme lipoamide dehydrogenase have also been well reviewed (Williams, 1976; Adamson, 1981), however, recent evidence has

closely linked the structure and mechanism of this flavoenzyme to that of other dithiol-flavin containing enzymes such as glutathione reductase (Untucht-Grau *et al.*, 1982; Pai and Schulz, 1983; Schulz *et al.*, 1978) and thioredoxin reductase (Williams *et al.*, 1976; Holmgren, 1981; Williams *et al.*, 1982). Chemical modification and amino acid sequence analyses in this study have confirmed and extended these findings.

Lipoamide dehydrogenase from all sources is composed of two identical subunits, each containing one mole of FAD and an active-site redox disulphide bond (Massey and Palmer, 1962; Burleigh and Williams, 1972; Brown and Perham, 1972; Matthews *et al.*, 1974; Williams, 1976). The generally accepted mechanism of action for E3 is illustrated (Fig. 1.9.1). Catalysis in the physiological direction is clockwise and the enzyme shuttles between the oxidized form (E) and two-electron reduced form (EH_2) in a manner consistent with a "ping-pong BiBi mechanism" (Massey *et al.*, 1960; Massey and Veeger, 1961). Further reduction of EH_2 forms EH_4, the four electron reduced enzyme, in which FAD is fully reduced and accordingly the absorption spectrum shown for lipoamide dehydrogenase in *E. coli* (Fig. 1.9.2) is bleached. The presence in the EH_2 spectrum of an additional long wavelength band at 530 nm was interpreted by Massey and Ghisla (1974) to represent a charge transfer interaction between the thiolate anion and FAD. This inference was incorporated by Williams (1976) in his postulated mechanism (Fig. 1.9.1).

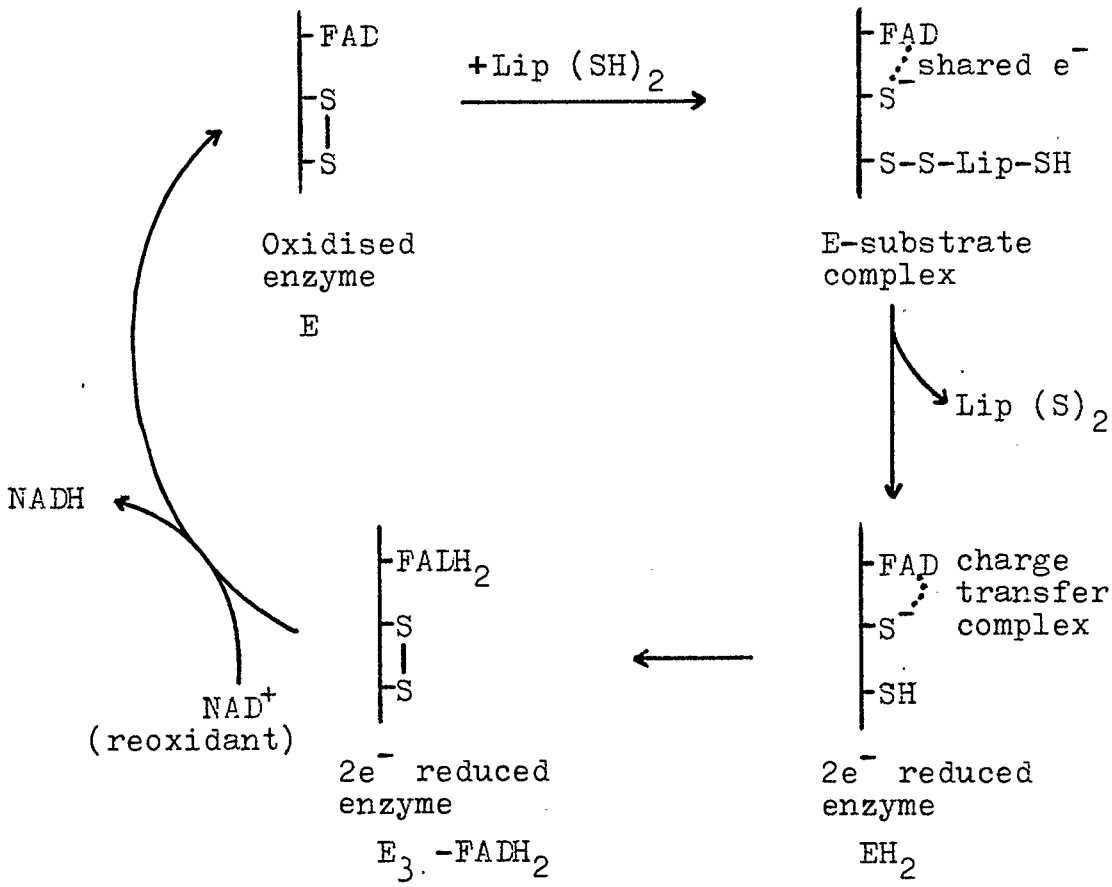


Figure 1.9.1 Catalytic mechanism of lipoamide dehydrogenase from *E. coli*. The presumed pathway of electrons is from dihydrolipoamide to the redox disulphide, via dithiol-disulphide interchange, thence to FAD and finally to NAD^+ (Searls *et al.*, 1961; Thorpe and Williams, 1976; Williams *et al.*, 1976).

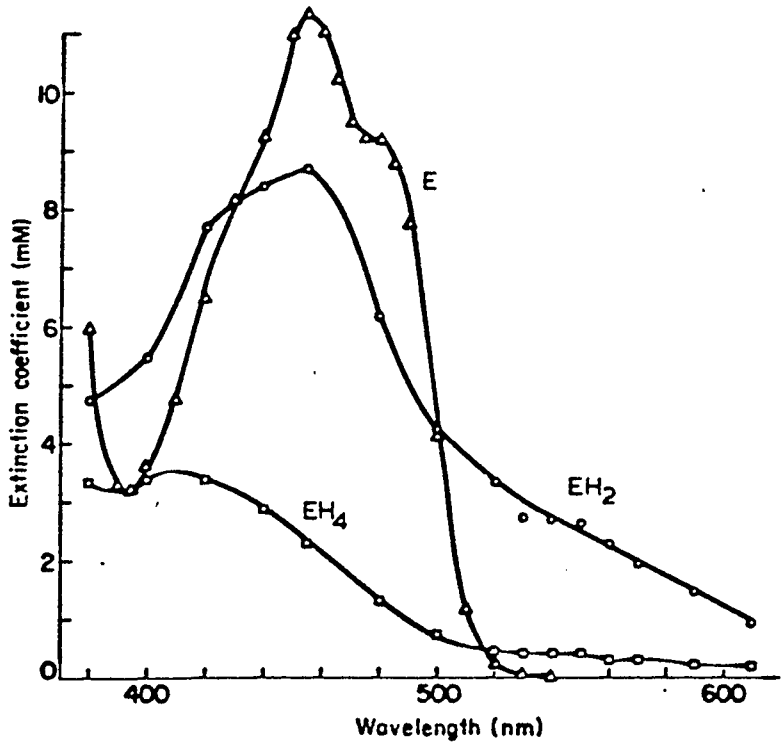


Figure 1.9.2 The visible spectra of lipoamide dehydrogenase from E. coli (Williams, 1976).

E - Absorption spectrum typical of oxidised flavoproteins

EH_2 - Diminished flavin absorbance and additional long wavelength band (≃ 530 nm)

EH_4 - Bleached flavoprotein

The oxidation - reduction potential of E to EH_2 was measured as a function of pH (Matthews and Williams, 1976) and in pig heart E3 it was established that the reduction of E3 by dihydrolipoamide involved the transfer of two protons as well as two electrons from the substrate to the enzyme between pH 5.5 and 7.5. Thus, for the charge-transfer interaction to be due to a thiolate anion interacting with FAD, these workers had to invoke a base which would accept a second proton upon reduction of the enzyme to EH_2 (Matthews et al., 1977). This protonated base could stabilise the thiolate anion by forming a thiolate anion-base pair. A mechanism was described in which deprotonation of dihydrolipoamide (pK_1=9.35) occurs prior to nucleophilic attack by activated dihydrolipoamide on the active-centre disulphide (Matthews et al., 1977; Adamson, 1981).

1.10 Nature of the Proposed Base in the Catalytic Mechanism of E3

The first clue to the identity of the putative base in the E3 catalytic mechanism came from chemical modification studies (Matthews et al., 1977) on the two electron reduced enzyme (EH_2). The reactivity of EH_2 with iodoacetamide was investigated between pH 5.5 and 9.0. Inactivation of E3 did not display a pH profile characteristic of a reaction involving a single thiol, that is, it did not proceed more readily at higher pH values where the thiol is likely to be ionised. Instead, the inactivation revealed a "plateau" of nucleophilic reactivity

in the neutral pH range which appeared to be associated with a group that had a pKa=8.0 (Matthews et al., 1977).

Somewhat earlier than the findings of Matthews, Polgar (1973) described a similar type of inactivation profile for reactions involving the active-site cysteine residue of papain with chloroacetamide. These results were attributed to the formation of an imidazolium/ thiolate ion pair in the catalytic mechanism of the enzyme. Consequently, Matthews (1977) suggested, by analogy, that the putative base (pKa=8.0) in E3 might also be a histidine residue. More detailed information on the presence of a catalytically essential histidine residue in the active site of E3, has come from the chemical modification studies of Adamson and Stevenson (1982), and in the studies described in this thesis.

One of the primary objectives of this study was to conclusively illustrate the involvement of a histidine residue as an essential residue in the catalytic mechanism of E3 by isolating an active-site peptide containing this residue.

1.11 Glutathione Reductase: Structural and Mechanistic Similarity to Lipoamide Dehydrogenase

Glutathione reductase (E.C. 1.6.4.2) is a ubiquitous flavoenzyme consisting of two identical polypeptide chains and two molecules of FAD. The enzyme from human erythrocytes has been crystallised and analysed by X-ray

diffraction at 2 Å resolution (Thieme *et al.*, 1981). The complete amino acid sequence of 478 amino acid residues is also known (Krauth-Siegel *et al.*, 1982; Untucht-Grau *et al.*, 1982) and the dimeric enzyme has a molecular weight of 104,800. The structure of the enzyme is illustrated in Fig. 1.11.1. On the basis of this data, glutathione reductase can be compared structurally and mechanistically with other flavoproteins such as flavodoxin (Mayhew *et al.*, 1975; Smith *et al.*, 1977), D amino acid oxidase (Ronchi *et al.*, 1982; Swenson *et al.*, 1982), p-hydroxybenzoate hydroxylase (Weirenga *et al.*, 1982) and lipoamide dehydrogenase (Williams *et al.*, 1981; 1982).

The similarity between glutathione reductase and lipoamide dehydrogenase is especially pertinent to the studies outlined in this thesis. Both flavoproteins catalyse electron transfer between a pyridine nucleotide and a disulphide, and both contain a cystine residue which undergoes reduction-oxidation during catalysis. The overall reaction catalysed by glutathione reductase is as follows:

$$\begin{array}{l}\text{(GSSG)}\\ \gamma\text{-Glu-S-Gly}\\ \gamma\text{-Glu-S-Gly}\end{array} + \text{NADPH} + \text{H}^+ \rightleftharpoons 2\text{GSH} + \text{NADP}^+$$

In a mechanism analogous to lipoamide dehydrogenase, the enzyme cycles between the oxidised and the two electron-reduced form (EH_2). EH_2 again appears to be a charge transfer complex in which a thiolate anion (residue 63) is the donor and FAD is the acceptor.

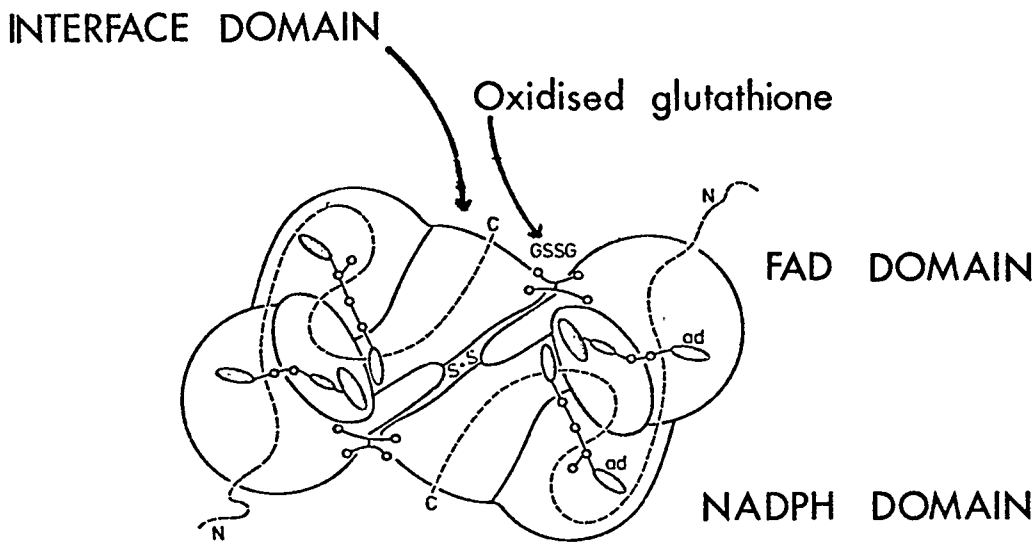
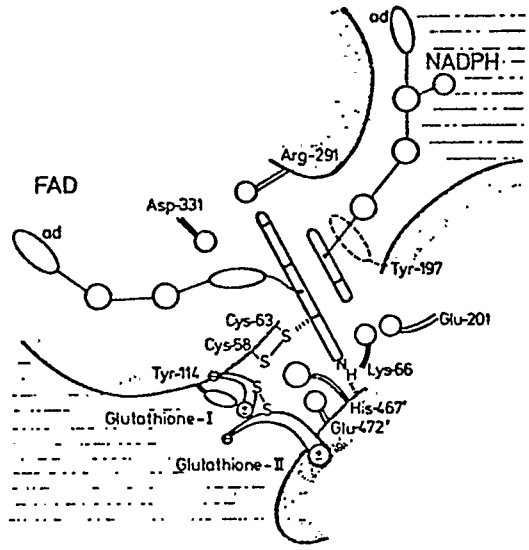


Figure 1.11.1 Structure of dimeric glutathione reductase viewed along the molecular twofold axis. The NH_2 terminal 18 residues are flexible. The remaining polypeptide chain is geometrically organised into four domains. The ligands FAD, NADPH and GSSG are indicated. There exists a single intersubunit disulphide bridge across the molecular axis (Pai and Schulz, 1983).

Each monomer of glutathione reductase consists of four structural domains (Fig. 1.11.1): an FAD binding domain, an NADPH binding domain, a central domain and a subunit interface domain. NADPH is bound in an extended conformation with the nicotinamide ring stacking onto the reverse face of the flavin part of FAD and with adenine located at the protein surface. Histidine-467 is postulated to act as a base in the mechanism, possibly by stabilising the thiolate anion - FAD charge transfer complex (cf. lipoamide dehydrogenase). Evidence for this observation has come firstly from X-ray analysis (Schulz *et al.*, 1978; Pai and Schulz, 1983) of the orientation of the histidine residue in the active centre with respect to FAD, the redox disulphide (cys 58-63), and the sulphur atoms of glutathione (G-S-S-G); also from chemical modification studies (Boggaram and Mannervik, 1978) identifying an essential histidine in the catalytic mechanism. The spatial orientation of histidine-467 in the active site of glutathione reductase is illustrated schematically in Fig. 1.11.2. This histidine residue is part of the interface domain of the monomeric enzyme and is located on the opposite subunit from the other active-site components (Figs. 1.11.1 and 1.11.2). This presumably explains why the enzyme is inactive as a monomer.

In contrast to glutathione reductase, details of the structure and mechanism of E3 are not nearly as well defined. The enzyme from pig heart has yet to be fully sequenced, however, a substantial number of tryptic peptides (equivalent to 36% of the overall protein) have



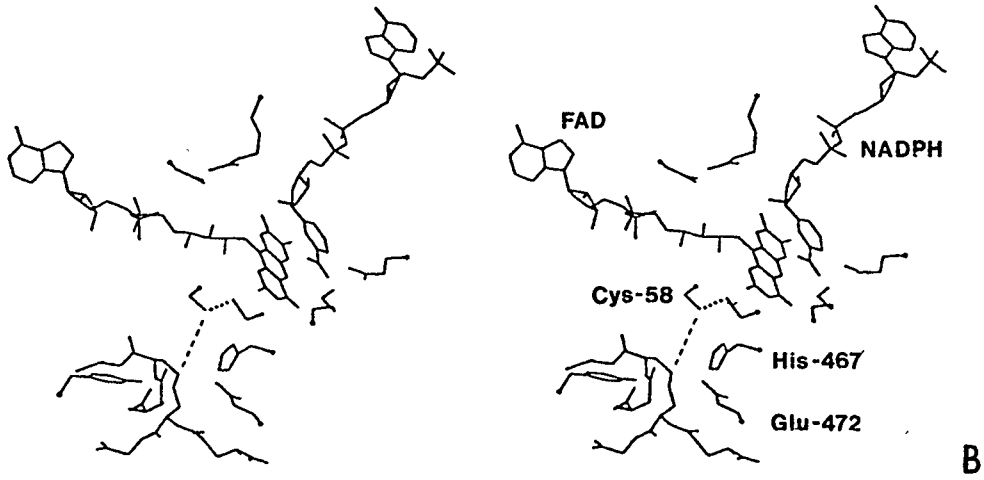


Figure 1.11.2 The catalytic centre of glutathione reductase. (A) Schematic outline of the catalytic centre. The orientation of the imidazole ring of His-467 is 0.36 nm from the redox active disulphide (Cys-58: Cys-63) and 0.36 nm from glutathione. His-467 is held firmly in the active site by a short hydrogen bond between the N^1-imidazole ring position and Glu-472, and by a short hydrogen bond (0.24 nm) between the carbonyl oxygen of His-467 and N^3 in the flavin ring. (B) Stereo drawing of ligands bound at the active site together with important amino acid residues. All C_α -atoms of these amino acids are marked by dots. The bridge Cys-58: Cys-63 is given by a dotted line (Pai and Schulz, 1983).

been isolated, sequenced and placed on the map of glutathione reductase (Williams *et al.*, 1982). The average homology was found to be 40% extending throughout the molecule. Very little sequence data has so far been published on E3 from *E. coli*, with only a fifteen residue sequence surrounding the redox disulphide, the N-terminal residue (Ser) and the COOH-terminal (Lys-Lys) known (Williams, 1976). When comparing the sequence surrounding the redox disulphide with other similar sequences from glutathione reductase and eukaryotic lipoamide dehydrogenase, it is clear that there is a significant degree of homology between these enzymes in this region of the active site (Fig. 1.11.4).

Table 1.11.4 Sequences around the oxidation-reduction active cystine residue in lipoamide dehydrogenase (L.D.) and glutathione reductase (G.R.) isolated from several species (Williams *et al.*, 1982)

Pig heart L.D.	T L G G T C L N V G C I P S K
E. coli L.D.	T L G G V C L N V G C I P S K
Human erythrocyte G.R.	K L G G T C V N V G C V P K K
Yeast G.R.	A L G G T C V N V G C V P K
E. coli G.R.	Q L G G T C V N V G C V P K

The mechanistic and structural similarities between lipoamide dehydrogenase and glutathione reductase that have been outlined, suggest that the overall active-site topography of the two enzymes is similar. With this in mind, many conclusions concerning the mechanism of lipoamide dehydrogenase may be extrapolated back to glutathione reductase.

1.12 Experimental Aims of the Project

The main objective of this work was to investigate the possibility that a histidine residue is involved in the mechanism of lipoamide dehydrogenase from *E. coli*. It was also hoped that by obtaining information on the primary structure surrounding this residue, further conclusions could be drawn about the relationship between glutathione reductase and lipoamide dehydrogenase from both eukaryotes and prokaryotes. The main experimental plan was as follows:

(i) To refurbish the Chemap 450 litre PEC fermentor (Department of Biology, University of Calgary) and to establish a protocol for the growth, in kilogram amounts, of an *E. coli* K12 mutant constitutive for the production of PD complex.

(ii) To purify the PD complex from *E. coli*.

(iii) To synthesize the [^{14}C]-radiolabelled bifunctional arsenoxide, p-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2[^{14}C]CONHPhAsO$).

(iv) To carry out active-site directed inhibition of the PD complex with this reagent, using the [^{14}C]-radiolabelled bromoacetamido moiety to alkylate a residue (most likely histidine), in or near the active site of E3 (Adamson and Stevenson, 1982). This was to be achieved by "a priori" anchoring of the arsenoxide moiety of the reagent to reduced lipoic acid residues on E2, followed by delivery of the bromoacetamido (alkylating) moiety into or near the E3 active site.

(v) To isolate E3 from the PD complex using hydroxylapatite chromatography, and identify the site(s) of alkylation by high voltage electrophoresis (HVE) with radiochemical analysis, and by amino acid analysis with scintillation counting for [^{14}C]-radiolabel.

(vi) To chemically modify the lipoic acid residues on E2 with N-ethyl maleimide (NEM) in a pyruvate dependent reaction, and investigate the effect of this modification on the mode of action of $BrCH_2[^{14}C]$-CONHPhAsO on the PD complex. These experiments were designed for two reasons, firstly, to answer the question; Does NEM-blocked PD complex deliver the [^{14}C]-radiolabelled bromoacetamido moiety of

the bifunctional arsenoxide into the active site of E3? Also, to investigate certain aspects of the stoichiometry of lipoyl-lysine residues on E2.

(vii) The final objective of this study was to isolate a peptide from E3 containing the alkylated (histidine) residue and to deduce as much of the amino acid sequence surrounding this residue as possible.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

Specialised chemicals and materials utilized during this project were obtained from the following sources:

Acetonitrile - HPLC grade (Caledon), acrylamide (Biorad), ammonium persulphate (Canalco), aquasol-2 (New England Nuclear), arginine (Sigma), biofluor (New England Nuclear), bis (N,N^1-methylene bis) acrylamide (Biorad), bovine serum albumin (Sigma), [1-^{14}C] bromoacetic acid (Amersham), S-carboxymethyl cysteine (Sigma), N^1-carboxymethyl histidine, N^3-carboxymethyl histidine, N^1,N^3-dicarboxymethyl histidine (Calbiochem), coenzyme A (PL Biochemicals), coomassie brilliant blue G-250 (Eastman), cyanogen bromide (Aldrich), cysteine HCl (Fisher), 2,3-dithiopropanol, dithiothreitol (DTT), ethylene diamine-tetraacetic acid (EDTA) (Sigma), folin-ciocalteau reagent, glycerol, (Fisher), histidine (Sigma), hydroxylapatite (Biorad), iodoacetic acid (BDH), lima bean trypsin inhibitor (Sigma), 2-mercaptoethanol (Eastman), NAD/H (BDH), N-ethylmaleimide (Pierce Chemicals), 2-propanol-HPLC grade (Caledon), pyruvic acid (Sigma), RNA - yeast (ICN), sequencer reagents (Beckman), silica gel 60 F-254 TLC sheets (Merck), sodium dodecyl sulphate (Pierce Chemicals), tetramethylenediamine (TEMED) (Canalco), thiamine HCl, thiamine pyrophosphate (Sigma),

trifluoroacetic acid (Pierce/Fisher), trypsin-TPCK treated (Seravac), tryptophan, urea (analytical grade) (Sigma).

All nutrients required for the bacterial growth of E. coli K12 were obtained from Difco.

2.2 Experimental Methods

2.2.1 Bacterial Growth of E. Coli K12

The overall protocol developed for the growth of E. coli in kilogram quantities was as follows. Routinely, a culture tube containing 5 mL nutrient broth (bacto-tryptone 10 g/L, bactoyeast extract 10 g/L and NaCl 10 g/L made to volume with tap water) was autoclaved at 15 psi for 20 min, prior to the addition of 1 ampoule of freeze dried bacteria. This suspension was grown for 12 h at 37°C and subcultured onto agar plates to test for purity. (Agar plates were prepared 24 h prior to use by aseptically pouring an autoclaved solution of 15 g agar per L of nutrient broth, whilst still hot, into sterile petri dishes and allowing them to set.) After 24 h at 37°C, the plates were reexamined and viably pure colonies were restreaked onto agar slants, (the preparation of agar slants has been fully described by Miller, 1972 and Adamson, 1981). Cultured slants were incubated for 48 h at 37°C, whereupon the "bacterial mats" from several slants were taken up in saline and used to innoculate a fernbach flask containing 50-100 mL of primary culture

medium. The composition of the growth medium used in the primary large scale culture of E. coli is illustrated in Table 2.2.1.1.

The primary culture was grown for 17 h at 37°C and used to innoculate a further 5-10 L of culture medium contained in shake flasks. These flasks were incubated at 37°C on a New Brunswick Scientific incubator/shaker until early to middle log phase of the bacterial growth and formed the innoculum for the large scale growth of E. coli.

Table 2.2.1.1 Culture medium used for the growth of E. coli K12

Nutrient Source	Reagent	Concentration (g/L)
Sulphur	Na_2SO_4	0.7*
Phosphate	NaH_2PO_4	9.5*
	K_2HPO_4	7.5*
Nitrogen	NH_4Cl	1.5
Trace metals	$MgCl_2$	0.066
	$FeCl_3$	0.0036
	$MnCl_2$	0.0052
	$CaCl_2$	0.064
Carbon	Glycerol	10.0
Auxotrophic requirement	Histidine	0.1
	Tryptophan	0.1
	Arginine	0.2
	Thiamine HCl	0.0001

* Adjust to pH 7.0

2.2.2 Large Scale Growth of *E. Coli* in Chemap PEC 450 L Fermenter

Prior to innoculation, 150-200 L of growth medium (made up to the mark with tap water) was sterilised *in situ* for 1 h at 121°C in the Chemap PEC 450 L fermenter. The medium was cooled to 37°C and 5-10 L of small scale innoculum added (2.5-5.0%). The growth was followed by taking hourly samples and monitoring the increased turbidity at $O.D_{500}$. The pH of the medium was kept near 7.0 by the manual addition of KOH and foaming was controlled by adding conservative amounts of corn oil. Optimal growth conditions involved sparging at 350 rpm and aeration at 2,000 cm^3/min. The cells were harvested at late log phase (18-22 h) after cooling the culture to 4-6°C. Harvesting was performed as rapidly as possible (1 h) in a Sharples super centrifuge. The resultant cell paste was weighed and stored immediately at -20°C.

2.2.3 Purification of PD Complex from E. Coli K12 (Bisswanger 1981)

The first of two procedures utilised to purify the PD complex essentially involved a series of ultracentrifugation steps. The method, reported by Bisswanger in 1981, is slightly faster and has led to the production of purer enzyme than earlier methods (Schminke-Ott and Bisswanger, 1981). The procedure was tried for the first time in our laboratory with a number of slight modifications.

E. coli K12 cells (80-150g) were thawed in 200 mL of 20 mM potassium phosphate buffer (pH 7.0, with 0.9 mg/mL PMSF, 1 mg/mL EDTA and 0.02% sodium azide) overnight at 4°C. The crude suspension was passed through a Manton Gaulin homogeniser for 5 min at 5,500 psi and the cell debris was removed by centrifugation at 26,800 g for 30 min at 4°C. The crude extract was assayed for enzyme activity and protein concentration and the volume recorded. The extract was then diluted to a protein concentration well below 20 mg/mL and ultracentrifuged for 4 h and 150,000 g at 4°C. The yellow gelatinous pellet was resuspended in 50 mM phosphate buffer (pH 7.0 with 0.9 mg/mL PMSF, 1 mg/mL EDTA and 0.02% azide) and clarified at 26,800 g and 4°C for 10 min. This extract was termed "spin 1" and assayed for enzyme activity and protein concentration. These ultracentrifugation clarification and assay procedures were repeated twice until "spin 3" extract was obtained. This solution was allowed to stand overnight on ice.

The next purification step involved fractionation on an hydroxylapatite column (5 x 4 cm). The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0 (containing no additives) at 4°C. The clarified extract, "spin 3", was layered on top of the column and the column was washed with equilibration buffer. Elution was carried out under atmospheric pressure with increasing concentrations of potassium phosphate buffer (pH 7.0) in a step-wise fashion. Phosphate buffer (50 mM) eluted a large breakthrough peak of non-homogeneous

protein, 150 mM phosphate buffer stabilised the baseline and the PD complex was eluted in 275 mM phosphate. Aliquots were taken at various stages during the fractionation procedure for analysis by $NaDodSO_4$-PAGE.

The peak containing PD complex activity was pooled and subjected to a final ultracentrifugation step for 3 h and 150,000 g at 4°C to concentrate the PD complex. The final, bright yellow, gelatinous pellet was carefully redissolved in a minimal amount of 50 mM phosphate buffer and clarified by centrifugation in a clinical centrifuge for 5 min. The resultant PD complex solution was assayed for enzyme activity and protein concentration, then immediately frozen on dry ice and stored at -85°C for up to 3 months.

2.2.4 Purification of PD Complex from *E. Coli* K12 (Reed and Mukherjee, 1969)

The procedure routinely adopted to purify the PD complex was based largely on the method first outlined by Reed and Mukherjee (1969) and since modified by Danson *et al*. (1979) and Adamson (1981). An additional modification of the methods reported by these investigators involved the use of a Manton Gaulin cell homogeniser for rapid and efficient cell breakage of the initial bacterial suspension.

The general procedure was as follows: 500 g of *E. coli* K12 were thawed in 750 mL 20 mM phosphate buffer

(pH 7.0, containing 1 mg/mL EDTA, 0.9 mg/mL phenylmethylsulphonyl fluoride (PMSF) and 0.02% sodium azide) overnight at 4°C. The next day the cells were blended with buffer in a Waring blender at 4°C. Cell breakage was achieved by homogenisation for 4 min in a Manton Gaulin homogeniser at 5,500 psi, collecting the ruptured cell suspension on ice. Cell debris was removed by centrifugation in a Beckman centrifuge (J21; JA20 rotor) at 26,800 g for 30 min. The supernatant was assayed for protein concentration and PD complex activity, the volume measured and the protein concentration diluted to 20 mg/mL with 20 mM phosphate buffer. The pH was adjusted to 6.15 with 1% acetic acid prior to fractionation with protamine sulphate.

Protamine sulphate (2%), pH 6.2, was then added dropwise to the crude supernatant over a period of 1 h (the amount added was equivalent to 0.15 volumes of the supernatant). After further stirring for 10 min, the mixture was centrifuged at 4°C for 20 min at 4,000 g. The white pellet containing nucleic acids was discarded and the supernatant similarly treated with a further 0.03 volumes of protamine sulphate. This procedure was continued until negligible enzyme activity remained in the supernatant (usually after 0.18-0.24 volumes of protamine sulphate had been added).

The yellow-brown precipitate containing PD complex activity was resuspended in 100 mL of 100 mM phosphate buffer (pH 7.0, with 1 mg/mL EDTA, 0.02% azide and 0.9

mg/mL PMSF) by means of a Wheaton glass tube and hand driven pestle. The suspension was stirred for 1 h and then centrifuged at 4°C for 10 min at 4,000 g. The resultant extract was termed "protamine extract", assayed for PD complex activity and the protein concentration was determined. For every gram of protein, 5 mL of a 1% yeast RNA solution (pH 6.2) was added; the purpose being to precipitate any remaining protamine sulphate. This solution was allowed to stand overnight on ice.

The cloudy yellow solution from the previous step was clarified by centrifugation for 15 min at 28,500 g at 4°C. The protein concentration was estimated from the absorbance at 260 and 280 nm (Adam, 1952) and readjusted to approximately 7 mg/mL by the addition of 50 mM phosphate buffer (containing 1 mg/mL EDTA, 0.9 mg/mL PMSF and 0.02% azide, pH 7.0), prior to ultracentrifugation of the clear, yellow solution at 4°C for 4 h and 150,000 g in a Ti 45 Beckman rotor. This resulted in sedimentation of the large PD complex into small bright yellow pellets from which the supernatant was carefully removed. These pellets were resuspended in 50 mM phosphate buffer, pH 7.0, (containing 1 mg/mL EDTA and 0.02% azide) and stirred very gradually at 0°C with a magnetic stirrer.

Routinely this yellow coloured solution was clarified by centrifugation at 28,500 g for 10 min at 4°C and assayed for enzyme activity and protein concentration. The strain of *E. coli* K12 used in these studies

contains only negligible levels of αKG complex activity, consequently, further purification steps were not necessary, as the enzyme was judged to be already virtually pure by $NaDodSO_4$-PAGE. (This observation has also been made by Hale, 1977 and Adamson and Stevenson, 1981.)

Isolated PD complex was aliquoted into 1 mL portions (40-50 mg/mL) and deep frozen immediately on dry ice. PD complex preparations were stored for up to 3 months at -85°C.

2.2.5 Protein and Enzyme Assays

All protein concentration determinations carried out in this study followed the method of Lowry *et al*. (1951) as modified by Cooper (1977) unless otherwise stated. Enzyme assays for PD complex and E3 activities involved a standard NAD^+ reduction assay first described by Reed and Mukherjee (1969), with the slight modifications introduced by Danson and Perham (1976) and Adamson (1981). The general procedure was as follows: stock solutions A-D were prepared prior to use and stored for no longer than a week at 4°C.

Solution A: NAD^+ (2.62 mM), TPP (0.21 mM) and $MgCl_2$ (1.05 mM) in 52.5 mM potassium phosphate buffer, pH 8.0. Solution B: coenzyme A (6.5 mM) and cysteine HCl (130 mM) in deionised water. Solution C: sodium pyruvate

(100 mM) in deionised water. Solution D: dihydrolipoamide (12 mM) in 95% ethanol (Reed *et al.*, 1958; Adamson, 1981).

All assays were conducted in a 1 mL silica cuvette. The assay for PD complex comprised 0.95 mL of solution A, 25 µl solution B and 25 µL solution C, whereas the E3 assay comprised 0.95 mL solution A, 25 µL solution D and 25 µL deionised H_2O. All assays were carried out at room temperature and were started by the addition of enzyme (1 to 20 µL). The increase in absorbance was measured at 340 nm corresponding to the production of NADH. In all assays used, range finding was employed by plotting V_o against enzyme concentration; only appropriate dilutions of enzyme on the linear portion of the plot were used. By measuring Δ O.D$_{340}$ and using an ε_{340} of 6.22×10^3 L $mol^{-1}cm^{-1}$ for NADH, the number of international units (IU) of PD complex or E3 could be calculated. One IU of activity of PD complex and E3 causes the formation of 1 µmole of NADH/min at 25°C.

2.2.6 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

$NaDodSO_4$-PAGE was carried out largely by the methods of Davis (1964), Ornstein (1964) and Laemmli (1970). Stock solutions consisted of the following: Solution A: 30 g acrylamide and 0.8 g bis-acrylamide made up to 100 mL with deionised water. Solution B: 18.15 g Tris dissolved in 50 mL deionised water, this was adjusted to

pH 8.8 with 25 mL of HCl and made up to 100 mL with deionised water. Solution C: 10% $NaDodSO_4$ solution. Solution D: 5 g Tris dissolved in 50 mL deionised water, adjusted to pH 6.7 with 1M HCl and made up to 100 mL with deionised water.

Polyacrylamide gels (7.5%) were prepared as follows: 7.5 mL of solution A was mixed with 22.4 mL of solution B and 20 uL TEMED. This solution was deaerated and 100 uL 10% ammonium persulphate solution was added. The gels were cast immediately in acid washed glass tubes and overlayed with 1-butanol. $NaDodSO_4$ was absent in the actual gel preparations (Wyckoff et al., 1977).

Samples were prepared for electrophoresis by adding an equal volume of the following solution: Glycerol (2.0 mL), solution C (2.0 mL), solution D (100 uL), 2-mercaptoethanol (200 uL) and deionised water (5.7 mL). The samples were then heated to 95°C for 3 min in acid washed ignition tubes. After cooling, 1 drop of 0.1% bromophenol blue tracking dye was added and the denatured samples were applied to gels which had been washed thoroughly to remove the 1-butanol. The samples were stacked at 1 mA per tube for 1 h and then electrophoresed at 2 mA per tube until the tracking dye had reached the end of the gel (usually after 5-8 h).

The upper and lower gel buffers consisted of 100 mL Tris running buffer pH 8.4 (6 g Tris and 28.8 g glycine

made up to 1 L with deionised water), 5 mL solution C and 985 mL deionised water. Following electrophoresis the gels were stained for 3-12 h with Coomassie Brilliant Blue G-250 in 50% methanol/7% acetic acid and destained by diffusion in 7% acetic acid/5% methanol, containing white wool to absorb the dye.

2.2.7 Automated Amino Acid Analyses

Dry protein or peptide samples were hydrolysed in 6 M HCl (Aristar grade) in evacuated, sealed, glass tubes for 24, 42 and 72 h (where applicable) at 110°C. After hydrolysis, the tubes were opened and dried down *in vacuo* over sodium hydroxide pellets. Acid hydrolysates (dried) of peptides obtained from cyanogen bromide cleavage, were treated with mild base (3-5% ammonium hydroxide for 30 min at 25°C) to convert homoserine lactone residues to homoserine.

Amino acid analyses were carried out on a Beckman 121 automatic amino acid analyser by the method of Spackman *et al*. (1958), employing a single column system and detection with ninhydrin (Lindsay and Stevenson, 1976), or with o-pthalaldehyde and liquid scintillation counting, as described by Adamson (1981).

2.2.8 High Voltage Electrophoresis Experiments

HVE of acid hydrolysates was carried out in the vertical strip apparatus first described by Michl (1955).

The buffer systems and coolants were as described by Smillie and Hartley (1966). Acid hydrolysates of purified protein or peptide, suspended in deionised water, were applied to Whatman 3 MM paper at the same spot on the origin as the following standards: N^1 - carboxymethyl histidine, N^3 - carboxymethyl histidine, $N^{1,3}$ - dicarboxymethyl histidine, S - carboxymethyl homocysteine, S - carboxymethyl cysteine and aspartic acid. After soaking in electrophoresis buffer, the ionogram was electrophoresed for 1 h at 3 kV. Amino acids were detected by staining with cadmium-ninhydrin dip reagent (Heilman *et al.*, 1957) and were developed for 20 min at approximately 60°C. The location of the standards were noted and the ionogram was cut into half-inch strips for liquid scintillation counting.

2.2.9 Liquid Scintillation Counting Procedures

Aqueous samples (up to 2 mL) and paper strips from high voltage ionograms were counted in Aquasol 2 (10 mL) on an LKB 1215 Rackbeta liquid scintillation counter. Scrapings from thin layer chromatography (TLC) plates used to check the purity of $BrCH_2CONHPhAsO$ were placed in Biofluor (8 mL) and counted in a similar fashion.

$NaDodSO_4$-PAGE gels were prepared for scintillation counting according to the method of Tishler and Epstein (1968). Slices (0.5 cm) were placed in scintillation vials, dried *in vacuo* and covered with 1.5 mL of 30% hydrogen peroxide. The vials were capped securely and

incubated at 37°C until the gel slices had dissolved (24-48 h). Aquasol 2 (10 mL) was added to each of the solubilised gel solutions and the vials counted as described previously.

Samples from the amino acid analyses of radio-labelled protein or peptide were collected directly into vials and 10 mL of scintillation fluid (containing 0.4% omnifluor, 80% toluene, 15% Beckman biosolve and 5% H_2O) was added to each fraction. Scintillation counting was carried out on a Beckman LS-250 liquid scintillation counter.

2.2.10 Hydroxylapatite Adsorption Chromatography (Isolation of E3 from the PD Complex)

This procedure was employed to purify E3 from both native and arsenoxide inhibited PD complex.

Hydroxylapatite columns were poured from a well mixed slurry containing 10 mM phosphate buffer, pH 7.0, and were equilibrated thoroughly with 10 mM phosphate buffer, pH 7.0, containing 8 M urea until the $O.D_{280}$ was near zero. A solution of PD complex in 10 mM phosphate buffer, pH 7.0, containing 8 M urea, was applied to the column and the column was further washed with 10 mM phosphate buffer containing 8 M urea. Elution of the PD complex components was achieved in a step-wise fashion with increasing concentrations of phosphate buffer (50, 100 and 500 mM, pH 7.0 containing 8 M urea). Phosphate

buffer at 500 mM was usually sufficient to elute E3 as a yellow band. Fractions containing E3 were dialysed exhaustively against several changes of deionised water at 4°C and lyophilised for further use.

Several workers have observed that E3 is remarkably resistant to proteolysis (Searls and Sanadi, 1960; Brown, 1974; Hale and Perham, 1979[a]; Bleile *et al.*, 1979; Adamson, 1981) whereas E2, and to a lesser extent E1, are more readily degraded (Perham and Thomas, 1971; Vogel *et al.*, 1971; Gebhardt *et al.*, 1978; Harrison, 1974; Hale and Perham, 1979[a]; Adamson, 1981). Consequently, prior to application onto hydroxylapatite columns, PD complex was treated with trypsin (10 μg/mL) for 75 minutes, then lima bean trypsin inhibitor was added (30 μg/mL) for 5 min. This solution of the PD complex was then made 8 M in urea and applied to a hydroxylapatite column. E3 was isolated as described earlier.

Prior to use, dialysis tubing was routinely boiled for 3-5 min in 500 mL deionised water containing approximately 0.01% EDTA, washed thoroughly in deionised water and stored at 4°C in 35% ethanol.

2.2.11 Synthesis and Purification of [^{14}C]-Radiolabelled p-[(Bromoacetyl)amino]phenyl Arsenoxide

The [^{14}C]-radiolabelled arsenoxide p-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2[^{14}C]CONHPhAsO$), was synthesized largely by the method of Robinson (1980),

starting with [1-^{14}C]-bromoacetic acid and dicyclohexyl carbodiimide (DCC). In a novel procedure, the reagent was further purified by silicic acid chromatography, which effectively removed any contamination by [1-^{14}C]-bromoacetic acid.

[1-^{14}C]-Bromoacetic acid (0.68 mg, 4.3 μmol, specific activity 52.1 mCi/mmol) in dichloromethane was added to cold, recrystallised bromoacetic acid (10 mg, 0.07 mmol) dissolved in dichloromethane. This solution was added to a solution of 16.5 mg DCC in dichloromethane and stirred for 5 min to promote the formation and precipitation of cyclohexylurea. The cyclohexylurea was removed by filtering through a glass wool plug in a pasteur pipette.

The filtrate, now containing [^{14}C]-radiolabelled bromoacetic anhydride was added to aminophenyl arsenoxide (7.5 mg, 0.04 mmol) dissolved in 20 mL of anhydrous acetone and the mixture stirred for 30 min. Bromoacetyl bromide (11.5 mg, recrystallised before use) was then added, with stirring for 15 min, to scavenge any unreacted aminophenyl arsenoxide. Dilute ammonia (10%) was added and the precipitate of ammonium bromide formed was removed by filtration. The solvent was removed from the filtrate by rotary evaporation. The white solid obtained was washed with cold water to remove unreacted bromoacetic acid and then dried under vacuum.

This initial preparation contained [^{14}C]-radiolabelled p-[(bromoacetyl)amino]phenyl arsenoxide but was

often significantly contaminated (approximately 10-15%) with [1-^{14}C] bromoacetic acid as well as small amounts of unreacted aminophenyl arsenoxide (NH_2PhAsO); therefore, a final purification step involving fractionation on a silicic acid column was frequently employed. A silicic acid column (20 x 0.5 cm) was equilibrated with chloroform: methanol (30:1, V/V) and the arsenoxide preparation was applied in a small volume of the same solvent.

Elution was carried out with chloroform: methanol (30-40:1, V/V) under atmospheric pressure and 0.2 mL fractions collected (flow rate 0.3-0.6 mL per h). The progress of the purification could be followed by spotting 10 µL of each fraction on silica TCL sheets and chromatographing the sheets in a solvent system consisting of butanol: acetic acid: water (1:1:1, V/V/V).

The final $BrCH_2[^{14}C]CONHPhAsO$ preparation was characterised by TLC and melting point analyses. The overall purification scheme is illustrated in Fig. 2.2.11.1. The R_f value was determined to be 0.86 by TLC analysis and compares very favourably with previous results in this laboratory (Robinson, 1980). The overall yield for the synthesis was 40-50% and the compound had a melting point of 196°C which was identical to the melting point value described for the arsenoxide in earlier syntheses (Robinson, 1980).

The radiochemical purity of the final arsenoxide preparation was determined by TLC in butanol: acetic

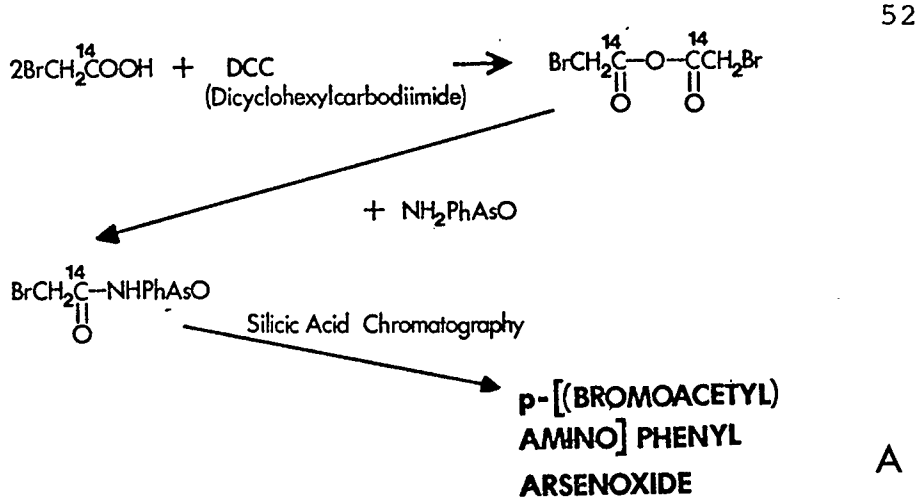
acid: water (1:1:1,V/V/V). After the runs were finished, the TLC plates were dried and the reagent visualised under UV light by its absorption at low wavelengths (Fig. 2.2.11.1). The location of radioactivity was determined by scraping 0.5 cm bands from the TLC plate followed by liquid scintillation counting as described previously.

The specific activity of $BrCH_2[^{14}C]CONHPhAsO$ was determined by liquid scintillation counting of known amounts of radioactive material as described by Robinson (1980) and Adamson (1981).

2.2.12 Inhibition Experiments

Stock solutions of $BrCH_2[^{14}C]CONHPhAsO$ were stored in 95% ethanol at a concentration of 10 mM in the deep freeze at -20°C. 2,3-Dithiopropanol (BAL) was stored as a 100 mM solution in 95% ethanol at -20°C for up to 1 month.

The amounts of PD complex and arsenoxide used for particular investigations during the course of this work varied greatly. The procedure for a typical inhibition experiment, was as follows: 3 vials containing 10 mg PD complex in 1 mL of 10 mM phosphate buffer, pH 7.0, were incubated at 0°C. Enzyme assays for PD complex and E3 activity were carried out with appropriate range finding, as discussed previously. Bifunctional arsenoxide (0.1 mM) was added to vials 1 and 2, which were labelled



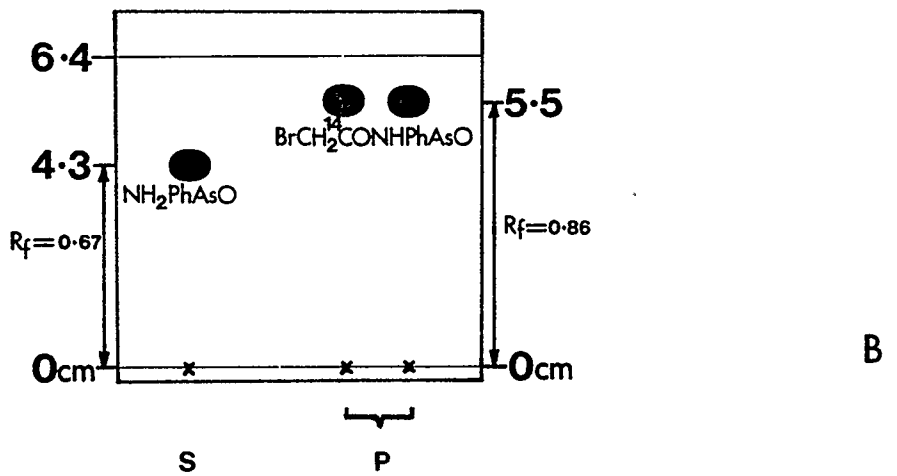


Figure 2.2.11.1 (A) Schematic outline of the synthesis of [^{14}C]-radiolabelled p-[(bromoacetyl) amino] phenyl arsenoxide. (B) TLC analysis of the final product was performed on Merck silica gel sheets using a solvent system comprised of butanol: acetic acid: water (1:1:1, V/V/V).

P - Duplicate samples of the final preparation of $BrCH_2[^{14}C]CONHPhAsO$

S - p-aminophenyl arsenoxide (NH_2PhAsO) standard

R_f - Ratio of distance travelled by a spot and the solvent

"arsenoxide test" and "arsenoxide control", respectively. An equivalent amount of 95% ethanol was also added to vial 3 and this was labelled "cofactor control".

All three vials were incubated at 0°C for 15 min and then reassayed for enzyme activity. In all the experiments described in this thesis, both PD complex and E3 activities remained at 95-100% after this stage. At time zero, cofactors for the PD complex were added to vials 1 and 3, and an equivalent amount of water was added to vial 2.

The course of the arsenoxide inhibition was followed by assaying each reaction vessel for PD complex and E3 activities every 15 min until the reactions were complete. BAL was then added (in 8-12 fold excess) to vials 1 and 2 to remove unreacted arsenoxide. "Inhibited" PD complex (vial 1) and "control" PD complex (vial 2) were reassayed for enzyme activity after 10 min and then dialysed exhaustively against several changes of 20 mM phosphate buffer, pH 7.0, at 4°C.

The mode of action of $BrCH_2[^{14}C]CONHPhAsO$ and BAL on the PD complex from _E. coli_ will be discussed more fully in the forthcoming chapters of this thesis.

2.2.13 Reduction and S-Carboxymethylation of E3

The procedure adopted for the reduction and S-carboxymethylation of E3 was based largely on that of Crestfield *et al*. (1963), with the modifications suggested by Perham (1978).

E3 (10 mg) was dissolved in a solution containing 3 mL 0.1 M Tris HCl (pH 8.6) and 3.61 g urea. The solution was made up to 7.5 mL with deionised water (making it 8 M in urea), flushed with N_2 and stirred at room temperature for 30 min.

An aliquot (150 µL) of a fresh 0.1 M stock solution of DTT was then added to the protein solution (final concentration 2 mM), which was again stirred, flushed with N_2 and incubated at 37°C for 90 min. A further aliquot (70 µL) of a freshly prepared 0.5 M solution of iodoacetic acid (final concentration 4.5 mM) was then added and the solution flushed thoroughly with N_2 once more, prior to incubation in the dark at 37°C for 40 min.

The course of the alkylation reaction was monitored by taking small samples at periodic intervals and adding them to tubes containing 5% nitroprusside. As the reaction progressed, the initial purple colour (indicative of the presence of free -SH groups) was gradually lost with little change being observed after 35-40 min. To terminate the reaction, the pH of the protein solution

was lowered to 3.0 with 12 M HCl and a drop of 2-mercaptoethanol added to react with any excess iodoacetic acid. The solution was dialysed exhaustively against deionised water at 4°C and then lyophilised for future use.

2.2.14 Cyanogen Bromide (CNBr) Cleavage of E3

The experimental protocol for CNBr cleavage of E3 followed closely the method of Perham (1978). Freeze dried, reduced and carboxymethylated E3 (10-50 mg) was dissolved in 70% formic acid (0.6-3.0 mL) and 2 volumes of water added to give a final protein concentration of approximately 5 mg/mL. With great care, a weight of CNBr equivalent to that of the protein (usually 40-50 mg) was added to the protein solution in the fume hood. This solution was then bubbled with N_2 and left securely sealed for 24 h at room temperature.

After 24 h, 10-20 volumes of water were added to the flask and the solution was freeze dried. Lyophilisation was repeated once more from water and the freeze dried digest stored for future use at -20°C.

The success of the CNBr cleavage was assessed by $NaDodSO_4$-PAGE on 10% polyacrylamide gels and also by amino acid analysis of an acid hydrolysate of the digest.

2.2.15 Enzymatic Cleavage of E3 CNBr Peptides

Tryptic digests of E3 peptides were carried out according to Perham (1978). Peptide samples (0.01-0.2 μmol) were dissolved in 200 μL of 0.5% ammonium bicarbonate (fresh, pH 8.0). Enzyme solution (10 μL, 1 mg/mL) was added and the reaction vial incubated at 37°C for 4 h. The products were recovered by lyophilisation.

2.2.16 High Performance Liquid Chromatography (HPLC) of E3 Peptides

All HPLC experiments concerning the isolation of peptide fragments in this thesis were carried out on a Varian 5000 liquid chromatograph, equipped with a Valco AH60 injector and a variable wavelength UV-50 detector system. Elution profiles were monitored with a Beckman chart recorder and peaks were collected manually.

In this study, two different column systems were used, a Varian cyanopropyl (CNAQ) reverse phase column (30 cm x 4 mm) with Brownlee CNAQ guard column, and a Varian micropak C_{18} reverse phase column with Brownlee RP-18 guard column. Aqueous trifluoroacetic acid (TFA, 0.1%) was used as the starting solvent in binary solvent systems that employed up to 60% acetonitrile or 2-propanol as limiting solvents. To help maximise peptide solubility in the limiting solvent, acetonitrile and 2-propanol also contained 0.1% TFA. The most common

procedure utilised was the one developed for the isolation of an active-site histidine peptide on the CNAQ column system. The general method was as follows:

E3 (50-55 nmol) was reacted with CNBr as previously described, the digest dissolved in a minimal amount of 0.1% TFA and "spin filtered" using Bioanalytical Systems Inc. MF1 centrifugation tubes in a clinical centrifuge. An aliquot (80 µL, 10 nmol), of peptide digest was injected onto the column and the column developed with increasing concentrations of acetonitrile (containing 0.1% TFA). The relevant peptides were isolated with the following programme: time 0 min (100% TFA, 0% acetonitrile), time 3 min (100% TFA, 0% acetonitrile), time 24 min (93% TFA, 7% acetonitrile), time 28 min (73% TFA, 27% acetonitrile), time 43 min (70% TFA, 30% acetonitrile), time 47 min (70% TFA, 30% acetonitrile), time 78 min (40% TFA, 60% acetonitrile), time 83 min (40% TFA, 60% acetonitrile), time 90 min (100% TFA, 0% acetonitrile).

Peaks were monitored at 206-210 nm and collected manually, prior to recovery by lyophilisation.

2.2.17 Automated Amino Acid Sequence Analysis

All automated sequence analyses in this thesis were carried out on a Beckman 890C liquid phase sequencer, equipped with a "cold trap" and a P-6 "sequemat" autoconverter. The design concepts of the sequencer are

based on the automated phenylisothiocyanate (PITC) N-terminal sequential degradation procedure for proteins, developed by Edman and Begg (1967) and the manual Edman degradation method (Edman, 1949). The overall procedure consists of three operations (Fig. 2.2.17.1): coupling of phenyl isothiocyanate (PITC) to the N-terminus of the protein or peptide, cleavage of the N-terminal residue as a 2-anilino-5-thiazolinone (ATZ) derivative with anhydrous heptafluorobutyric acid (HFBA) and conversion of the ATZ amino acid derivative to the corresponding phenylthiohydantion (PTH) amino acid with methanolic HCl. These procedures have been extensively reviewed by Niall (1973, 1977), Waterfield and Bridgen (1975), and Edman and Henschen (1975). The sequencing programme used in this study was developed by T. Carne and D. Burke in the Division of Biochemistry with the assistance of D. McKay from the Department of Medical Biochemistry and included the following features.

5% Phenylisothiocyanate (PITC) in heptane (as the coupling reagent), 0.1 M Quadrol pH 9 (non-volatile coupling buffer), benzene (for precipitation of PTC-peptide and extraction of excess PITC), ethyl acetate (for extraction of quadrol and PITC breakdown products), anhydrous HFBA (for cleavage of N-terminal amino acids as ATZ derivatives), butyl chloride and DTT (for the extraction of cleaved products). ATZ derviatives extracted with butyl chloride, were converted to their respective PTH amino acids in the "sequemat" autoconverter, with methanolic HCl at 65°C.

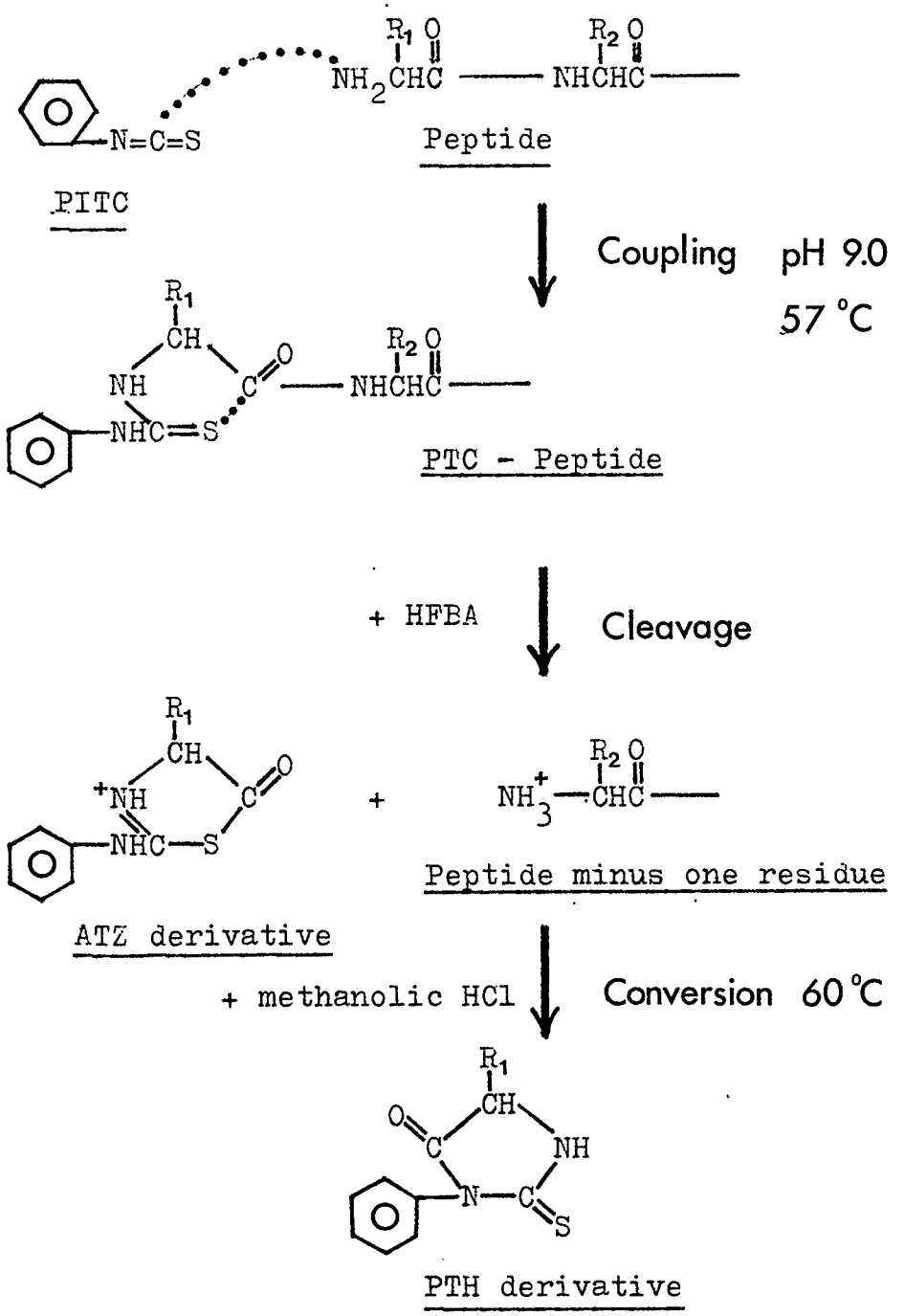


Figure 2.2.17.1 Three stage Edman degradation (Edman, 1949; Allen, 1981).

PITC - phenylisothiocyanate

HFBA - heptafluorobutyric acid

ATZ - 2-anilino-5-thiazolinone

PTH - phenylthiohydantoin

The coupling and cleavage reactions are carried out in a cylindrical glass spinning cup. Solutions and solvents enter through feed lines at the bottom of the cup and are spread as thin films on the cup wall thus allowing a high surface area for reactions to take place. This arrangement allows the spinning film to be extracted by an immiscible solvent sliding over its surface to the top of the cup. The extracting solvent is then scooped off to the fraction collector or to waste. To reduce the washout of the peptide sequenced in this study, the film stabilising substance, Polybrene, was applied to the walls of the spinning cup in a "precycling stage" as follows: 2 mg Polybrene and 50 nmol Gly-Gly were taken up in 400 µL H_2O and added to the sequencer. Four cycles of degradation were performed, with the aim of blocking any reactive groups in the polymer capable of interfering with the coupling and cleavage reactions.

Routinely 10-50 nmol "CNBr peptide" were dissolved in 400 µL 0.1% TFA and applied to the sequencer. The identification of PTH amino acids was carried out by HPLC on a 5 µ Ultrasphere-ODS (250 x 4.6 mm) reverse phase, C_{18} column with a pre-column (45 x 4.6 mm), both at 45°C, and a binary solvent system, consisting of A: 5% tetrahydrofuran in 5.25 mM acetate, pH 5.15 and B: 90% CH_3CN, 10% tetrahydrofuran. This column system was installed on a Varian 5000 liquid chromatograph, with Vista 401 control module and a UV-50 variable wavelength detector system. The PTH amino acids from each sequencer cycle were monitored at 269 nm and the majority were identified

by careful comparison of their retention times with those of standard PTH derivatives. PTH S-carboxymethylcysteine, PTH aspartic acid and PTH glutamic acid were all detected by HPLC analysis of their respective methyl ester derivatives (following esterification by methanolic HCl during the conversion reaction).

Alternatively, PTH amino acids were back hydrolysed to their respective "free" amino acids. The conditions for this were typically: 5.7 M HCl and 0.1% $SnCl_2$ at 150°C for 4 h (Mendez and Lai, 1975). The free amino acids were detected by amino acid analysis as described previously.

Certain PTH amino acids were not readily detectable by either of these methods because of difficulties inherent in the automated Edman procedure. During the conversion reaction PTH serine and PTH cysteine undergo possible β-elimination followed by further reactions and thus could not be identified. PTH threonine undergoes β-elimination to a lesser extent. Small quantities of PTH threonine are detectable, firstly by HPLC analysis and also by back hydrolysis to α-aminobutyric acid which is detected by amino acid analysis. PTH arginine and PTH histidine are recovered from the sequencer in extremely low yields, however, the latter derivative could be identified (albeit in small quantities) by back hydrolysis followed by amino acid analysis. The low yields of PTH arginine and PTH histidine may be due to their poor

extractability (both side chains are positively charged) into butyl chloride as ATZ derivatives following the cleavage reaction (Niall, 1973) and/or into methylene chloride as PTH derivatives following the conversion reaction.

CHAPTER 3 - RESULTS AND EXPERIMENTAL RATIONALE

3.1 Growth of E. Coli K12 Mutant

The first objective of this project was to develop a large scale facility for the growth of an *E. coli* K12 mutant constitutive for the production of PD complex. The procedure now routinely adopted has been fully outlined in Chapter 2.

The growth characteristics of *E. coli* K12 cells grown in both large and small scale cultures are compared in Fig. 3.1.1. Growth of *E. coli* K12 in cultures of 150-200 L was achieved with a shorter lag phase and a decreased doubling time. These characteristics reflect careful optimization of the growth conditions in the PEC fermenter. For instance, the high degree of agitation and aeration employed ensured that the distribution of growing *E. coli* cells remained as uniform as possible throughout the medium, thus facilitating greater contact with available nutrients.

The protocol developed has enabled *E. coli* K12 cells to be grown in kilogram quantities with a doubling time of approximately 60 min. Typical yields of bacteria were 9 g (wet cell paste) per L of growth medium (ie. 1.8 kg per 200 L culture).

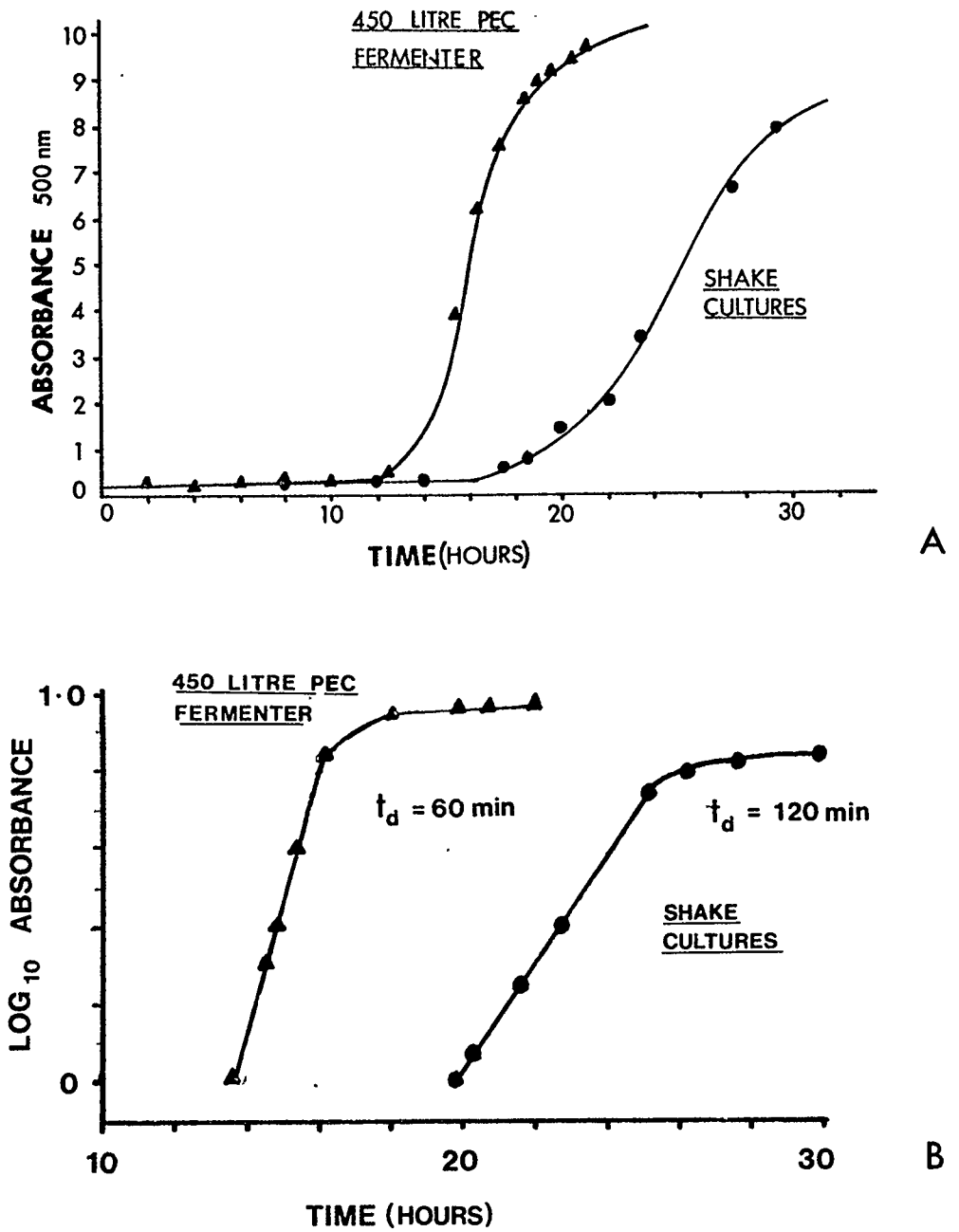


Figure 3.1.1 Growth of *E. coli* K12. (A) Growth characteristics of the bacterium in large and small scale culture, monitored by the increase in absorbance at 500 nm. (B) A plot of $\log_{10}$ absorbance versus time, that readily illustrates the doubling time t_d (time taken for the cell population to double) for both cultures during the exponential growth phase.

3.2 Purification of PD Complex from E. Coli K12

3.2.1 Cell Breakage

E. coli cells were routinely ruptured at high pressures by Manton Gaulin homogenisation. The efficiency of cell breakage is illustrated in Fig. 3.2.1.1. One pass through the homogeniser was sufficient to achieve breakage of a majority of the cell population (greater than 80% efficiency). A further pass, after cooling to 4°C, rendered the breakage virtually complete.

3.2.2 Purification of PD Complex from *E. Coli* K12 (Bisswanger, 1981)

Two independent methods for the purification of PD complex from *E. coli* K12 were evaluated. The first of these was described by Bisswanger (1981) and has been purported to result in a rapid isolation of highly pure PD complex. This was especially noteworthy as several workers have shown that E2 is extremely susceptible to proteolysis during other established procedures for isolating the PD complex (Perham and Thomas, 1971; Vogel *et al.*, 1971; Gebhardt *et al.*, 1978; Adamson, 1981).

The procedure was found to be straightforward and convenient. The key purification step involved fractionation on hydroxyapatite and is illustrated in Fig. 3.2.2.1. The overall results achieved are shown in Table 3.2.2.1.1. The PD complex was purified fiftyfold

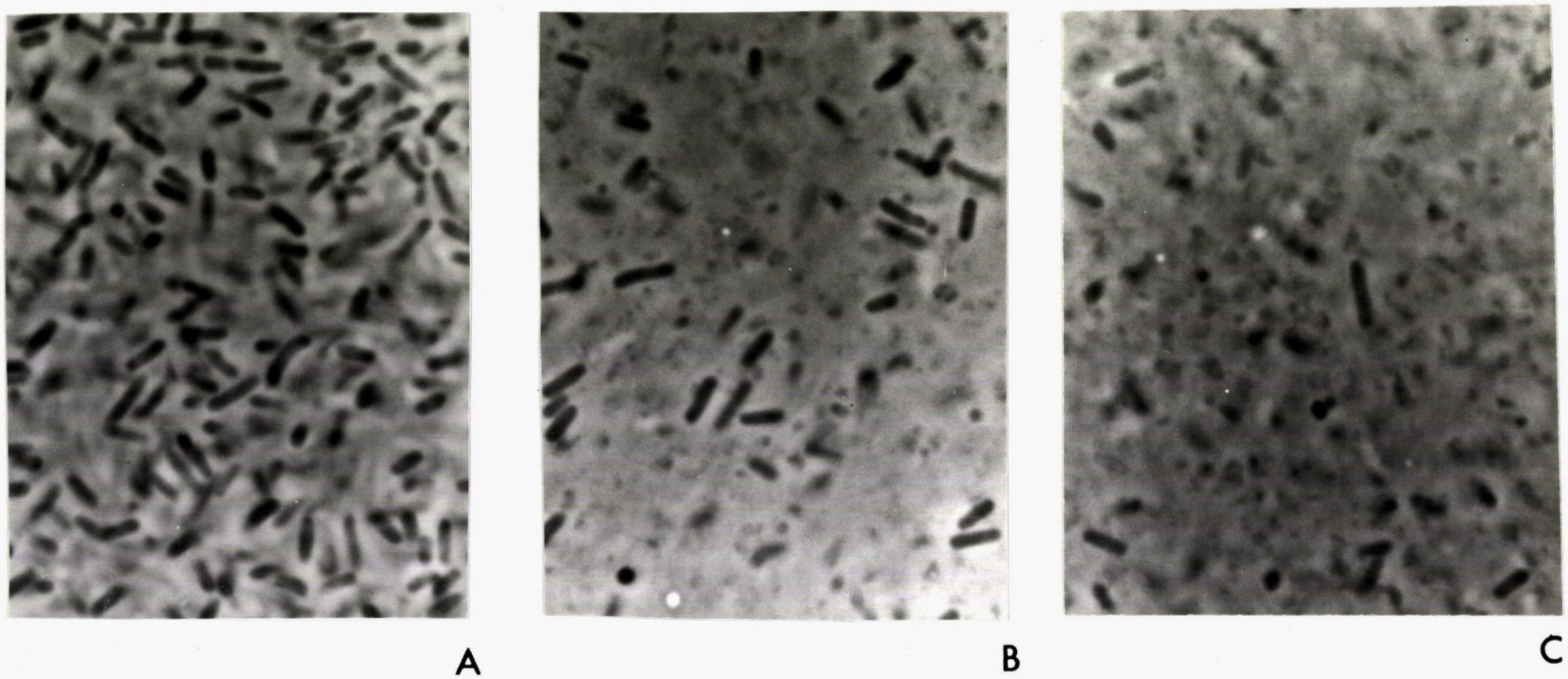


Figure 3.2.1.1 Estimation of the efficiency of cell breakage by Manton Gaulin homogenisation. Equivalent aliquots of whole and broken cell suspension were added to one drop of glycerol on a glass slide and covered with a cover slip. Photographs were taken at a magnification of 2000x on a Zeiss phase contrast microscope. (A) Whole cell suspension. (B) and (C) Broken cell suspensions after one and two passes through the homogeniser, respectively. **(Prepared by K.J.Stevenson)**

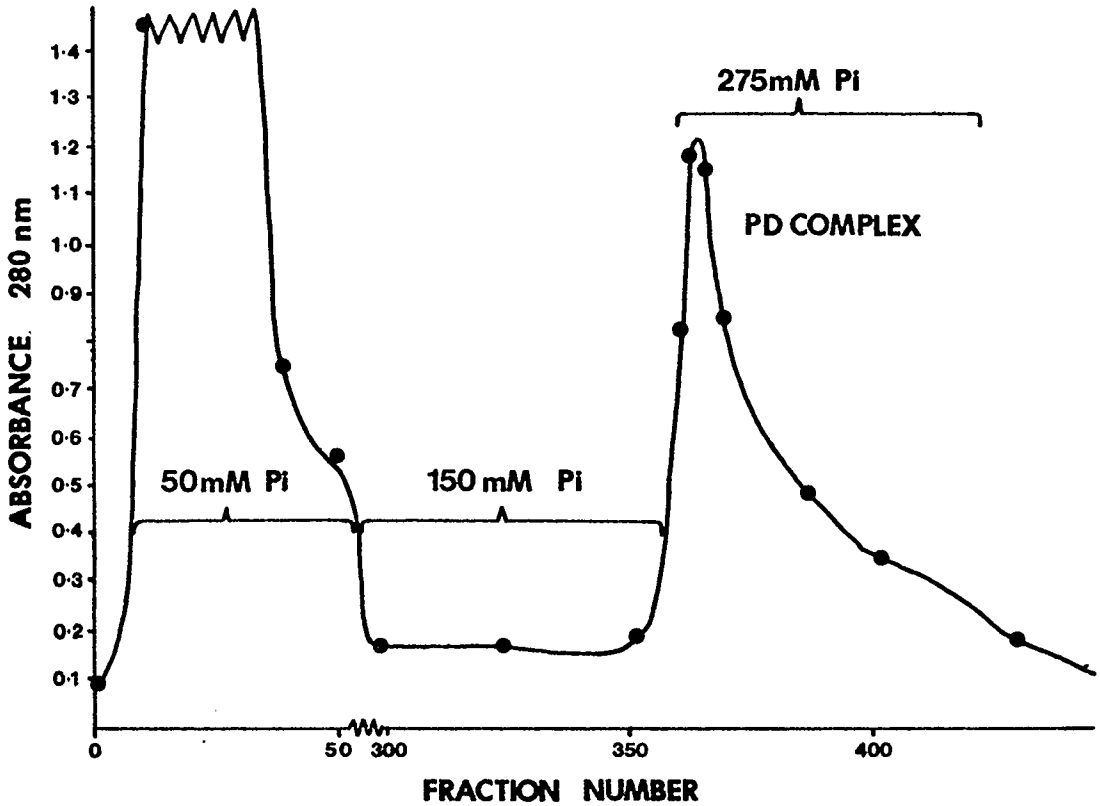


Figure 3.2.2.1 Fractionation of the PD complex on hydroxylapatite. Crude PD complex was applied to a hydroxylapatite column (5 x 4 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0, at 4 °C. Elution was carried out in a step-wise fashion as shown. Flow rate was 15 mL/h and 1 mL fractions were collected. For further experimental details see Section 2.2.3.

Table 3.2.2.1.1 Purification of PD complex from *E. coli* by the method of Bisswanger(1981)

Procedure	Fraction Number	Volume (mL)	Unit/ mL	Total Units	Protein (mg/mL)	Specific Activity	Yield (%)	Purification
Crude extract	1	210	6.4	1350	17.5	0.4	100	1
Ultra-centrifugation 1	2	50	22.5	1125	5.6	4.0	83.3	10
Ultra-centrifugation 2	3	50	16.3	820	2.6	6.3	61.8	15.8
Ultra-centrifugation 3	4	50	11.7	585	1.9	6.2	43.2	15.5
Hydroxylapatite fraction	5	17	11.3	190	0.57	19.7	14.1	49.3
Final PD complex	6	2	40.2	80	2.0	20.1	6.0	50.2

The specific activity is presented as units/mg of protein.

and was essentially pure as judged by $NaDodSO_4$-PAGE on 7.5% polyacrylamide gels (Fig. 3.2.2.2.).

Despite the highly pure preparations obtained, this method was found to have severe limitations. Successive ultracentrifugation in steps 1, 2 and 3 drastically limited the amount of E. coli cells that could be processed in one batch. This problem was exacerbated by consistently low overall yields of the PD complex. Typically, no more than 60 μg PD complex could be obtained per g of bacteria.

In view of the need for large amounts of PD complex to complete the experiments described in this study, the Bisswanger procedure for isolating PD complex was not routinely adopted in this laboratory.

3.2.3 Purification of PD Complex from E. Coli K12 (Reed and Mukherjee, 1969)

Representative results achieved when the PD complex was purified from E. coli using the method of Reed and Mukherjee are shown in Table 3.2.3.1.1. Typically, PD complex with a specific activity in excess of 20 units per mg of protein was prepared in good yield (1 mg PD complex per g of bacteria). In relation to the amount of PD complex prepared, this method was clearly superior to the isolation described by Bisswanger in that sevenfold more E. coli cells could be processed in the same length of time with a fifteenfold greater yield. Homogeneity

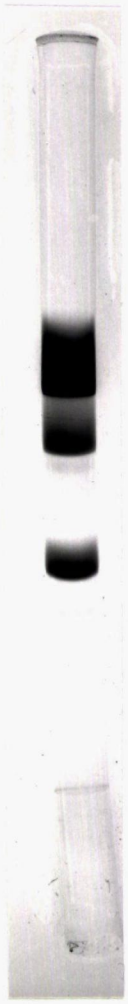


Figure 3.2.2.2 $NaDodSO_4$-PAGE of PD complex purified by the method of Bisswanger (1981). Electrophoresis was performed on 7.5% polyacrylamide gels. For further experimental details, see Section 2.2.6.

Table 3.2.3.1.1 Purification of PD complex from *E. coli* by the method of Reed and Mukherjee (1969)

PROCEDURE	STEP No.	VOL (ml.)	UNITS / ml	TOTAL UNITS	PROTEIN mg/ml	UNITS / mg prot.	YIELD %	PURIFI-CATION
CRUDE EXTRACT	1	890	23·7	21,100	26·5	0·9	100	1
0·15 VOLUME PROTAMINE SULPHATE SUPERNATANT	2	1,000	20·9	20,900	10·0	2·1	98	2·4
PROTAMINE EXTRACT	3	200	67·5	13,510	11·0	6·1	64	6·9
ULTRACENTRI-FUGATION	4	13	803·9	10,450	36·5	22·0	49·5	25

was assessed by $NaDodSo_4$-PAGE on 7.5% acrylamide gels (Fig. 3.2.3.1) and compared very favourably with similar analyses of the PD complex prepared by the Bisswanger technique. Provided the isolation was carried out as swiftly as possible and freshly prepared PD complex was immediately frozen in a dry ice bath, proteolysis of E2 was found to be minimal. In view of the negligible levels of αKG complex present, further purification by isoelectric precipitation was not found to be necessary. This was significant as it has been shown that substantial proteolysis can occur during the time taken to manipulate this extra purification stage (Adamson, 1981).

In summary, the Reed and Mukherjee procedure was routinely adopted throughout this thesis as a method for the isolation of PD complex from E. coli K12.

3.3 Synthesis of [^{14}C]-Radiolabelled Arsenoxide, $BrCH_2[^{14}C]CONHPhAsO$

Syntheses were carried out as previously described in Section 2.2.11. Silicic acid chromatography significantly improved the purity of the final arsenoxide preparation and results from a typical experiment are illustrated in Fig. 3.3.1. This additional purification stage often resulted in a substantial decrease in yield of approximately 10-30%. However, this was more than compensated for by the increase in purity achieved. TLC and radiochemical analysis of arsenoxide preparations

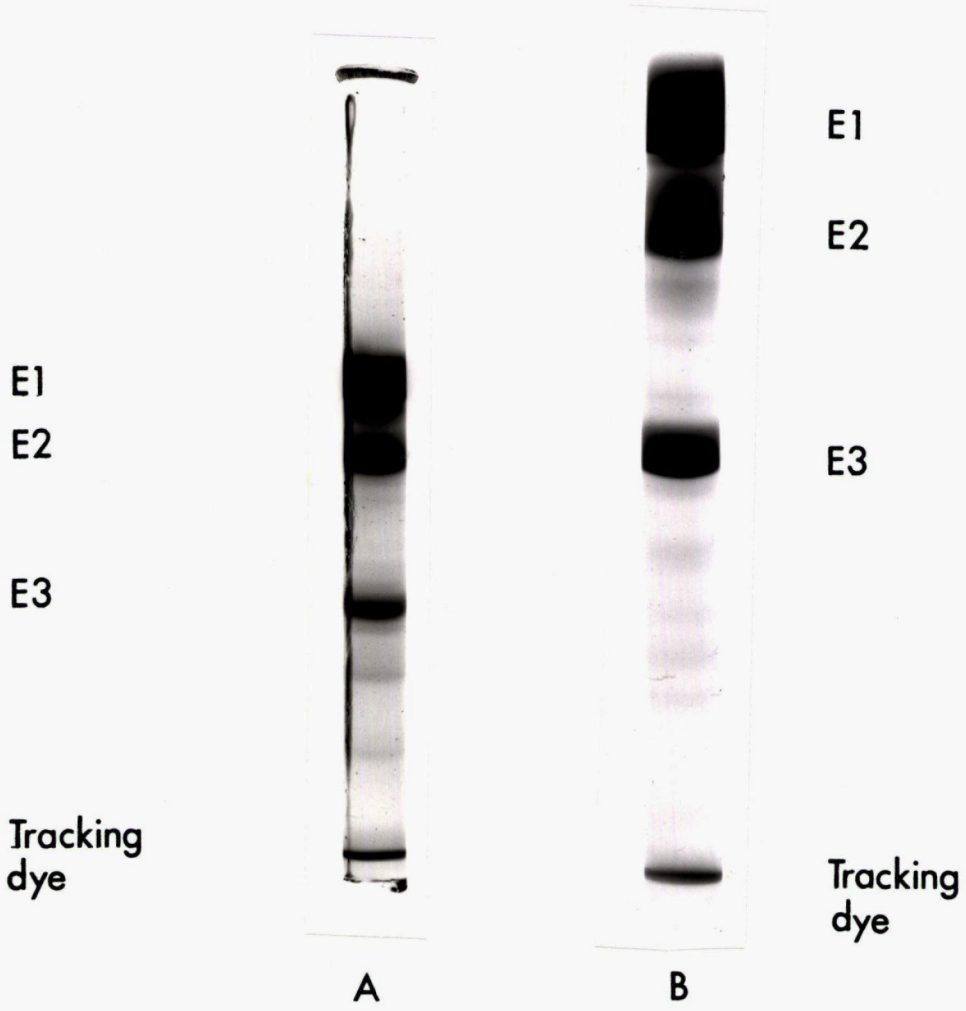


Figure 3.2.3.1 $NaDodSO_4$-PAGE of two different preparations of PI complex, prepared the method of Reed and Mukherjee (1969). For methods of preparation, refer to Section 2.2.6.

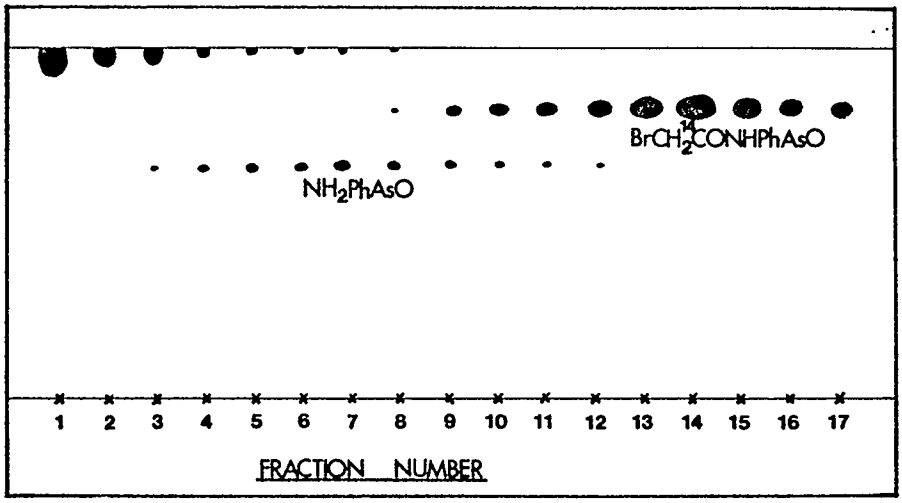


Figure 3.3.1 TLC analysis of $BrCH_2[^{14}C]CONHPhAsO$ purified on a silicic acid column. The column was eluted with chloroform: methanol (30:1, V/V). Eluted fractions were analysed on TLC with a butanol: acetic acid: water (1:1:1, V/V/V) solvent system. For further details see Section 2.2.11.

before and after silicic acid chromatography typically revealed an increase in radiochemical purity in excess of 10%. This reflected the loss of the major contaminant, $BrCH[^{14}C]COOH$. These results are illustrated in Fig. 3.3.2.

The specific activity of $BrCH_2[^{14}C]CONHPhAsO$ determined by the methods described in Section 2.2.11 varied with each different synthesis, but was typically between 2,500 and 7,000 dpm/nmole. A typical synthesis resulted in the preparation of 2-3 mg (7-10 μmol) of $[^{14}C]$-radiolabelled bifunctional arsenoxide.

3.4 Inactivation of the PD Complex with $BrCH_2[^{14}C]$ CONPhAsO: Selective Modification of a Histidine Residue in the Active Site of E3

3.4.1 Experimental Rationale

Bifunctional reagents can be used to introduce both inter-and intramolecular cross-links into proteins. This study has made extensive use of the $[^{14}C]$-radiolabelled heterobifunctional arsenoxide, $BrCH_2[^{14}C]CONHPhAsO$. This reagent contains two reactive groups ($BrCH_2CONH$-R and R-AsO) sufficiently different to permit well controlled sequential reactions of each group. The arsenoxide moiety reacts specifically with vicinal thiols to form a stable, cyclic dithioarsinite complex; a reaction which can be reversed by adding BAL. In contrast, the bromoacetyl moiety can readily alkylate nucleophilic side

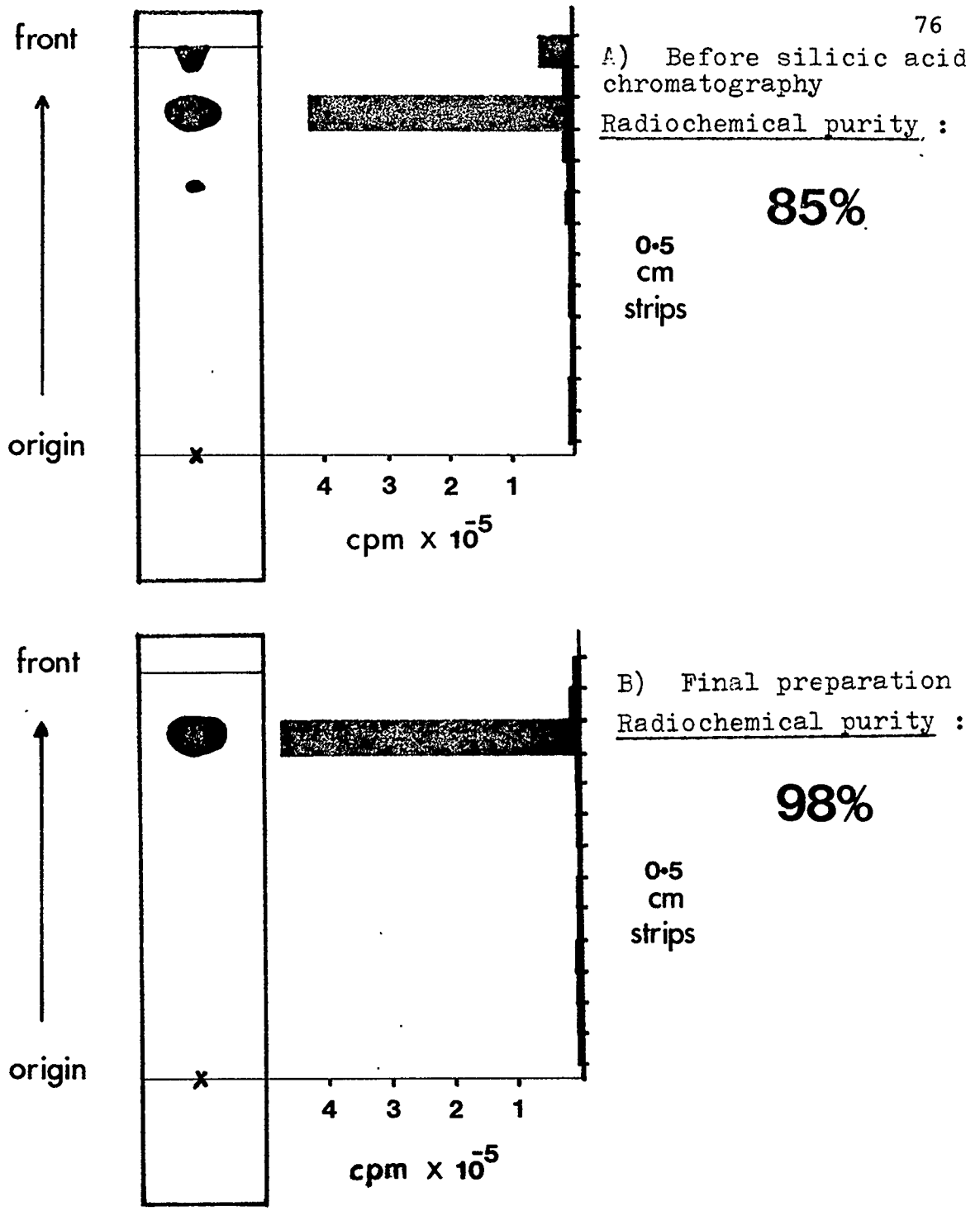


Figure 3.3.2 TLC/radiochemical analyses of $BrCH_2[^{14}C]CO$-NHPhAsO before and after silicic acid chromatography. For experimental details, see Section 2.2.11 and Figure 2.2.11.1.

chains occurring in proteins in an irreversible manner similar to that of bromoacetamide ($BrCH_2CONH_2$).

It has been shown that E3 can be inhibited, whilst part of the PD complex, by $BrCH_2CONHPhAsO$. The bromoacetamido moiety of the reagent is delivered into the active site of E3 by arsenoxide modified dihydrolipoyl residues on E2 (Stevenson et al., 1978; Adamson and Stevenson, 1981).

In the presence of pyruvate, TPP, Mg^{2+} and coenzyme A (El-mediated lipoyl system), the lipoyl residues on E2 become fully reduced. Under these conditions, the arsenoxide moiety on $BrCH_2CONHPhAsO$ initially forms a stable dithioarsinite complex with the reduced lipoyl residues. In keeping with the commonly accepted "swinging arm" mechanism of the PD complex (Fig. 1.6.1), the "anchored" reagent is delivered into or near the active site of E3 where an irreversible alkylation takes place. These modifications result in the intermolecular cross-linking of E2 and E3.

Reduced lipoic acid can be reformed, and E2 regenerated, upon addition of excess BAL, which forms a more stable five membered ring compound with the arsenoxide (Peters et al., 1945). However, E3 remains covalently modified by virtue of the interaction of the alkylating moiety ($BrCH_2CONH-R$) with a residue at or near the active site of E3. This process of inhibition is outlined in Fig. 3.4.1.1.

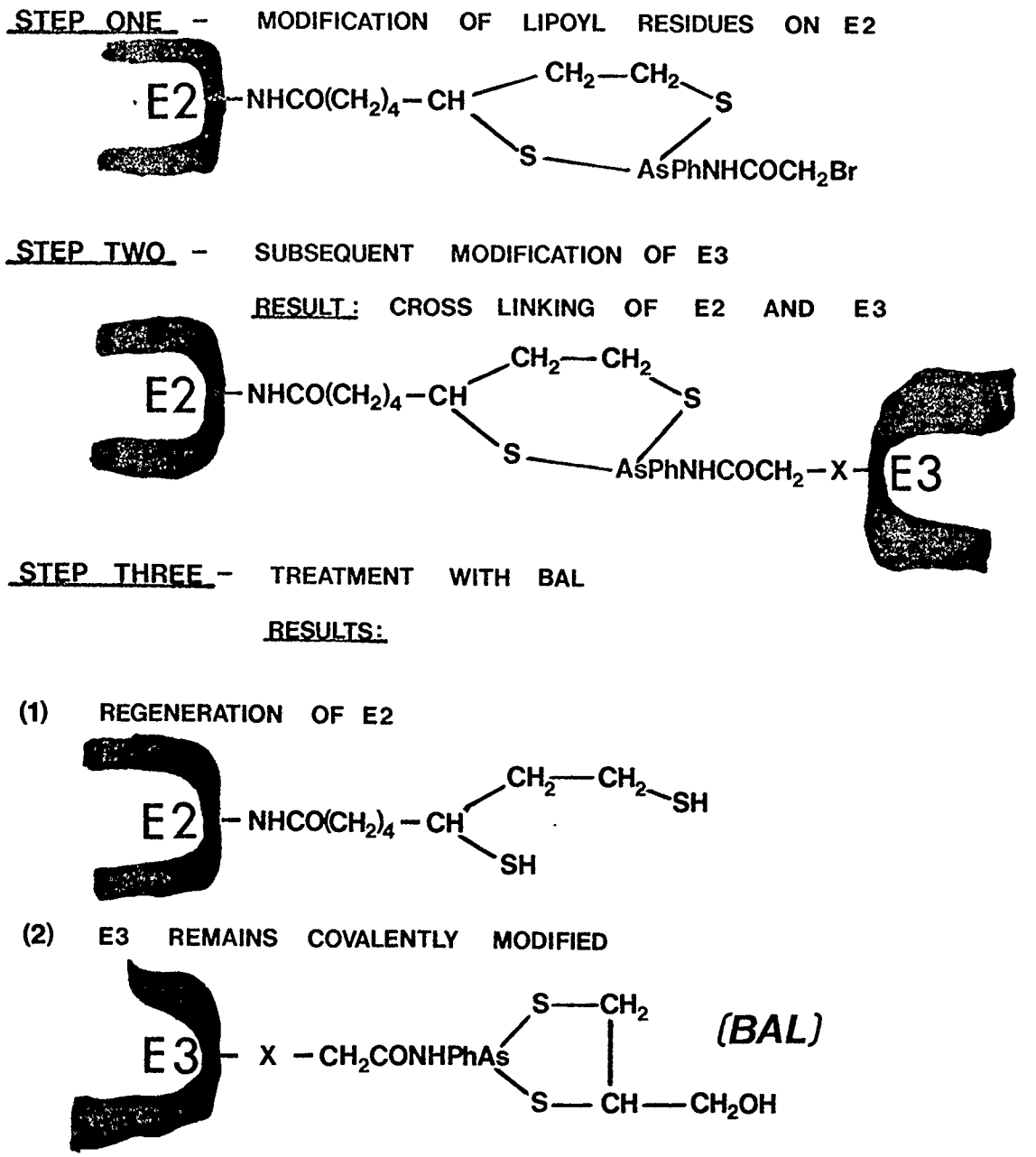


Figure 3.4.1.1 A scheme illustrating the inactivation of PD complex with $BrCH_2[^{14}C]CONHPhAsO$ in the E1-mediated lipoyl system (pyruvate, TPP and coenzyme A) or the E3-mediated lipoyl system (NADH only). PD complex is inactive since alkylation of E3 is irreversible.

In summary, both the PD complex and E3 are inactivated because of the irreversible modification of E3 (Stevenson et al., 1978; Adamson and Stevenson, 1981). In the reaction scheme described, reduction of E2 lipoyl residues is achieved by using the E1-mediated lipoyl system. Provided NAD^+ is absent from the experiment, the lipoyl residues will remain reduced (Stevenson et al., 1978). Alternatively, the reversibility of the NAD^+ reduction step (E3 catalysis) in the PD complex mechanism allows reduction of the lipoyl residues in the presence of an excess of NADH and this is referred to as the E3-mediated lipoyl system (Fig. 1.6.1).

Experiments performed in this laboratory on the PD complex with [^{14}C]-radiolabelled bifunctional arsenoxide, in the E1-mediated lipoyl system only, have provided evidence for the alkylation of a histidine residue in or near the active site of E3 (Adamson, 1981).

These findings were confirmed and also extended by investigating the inhibition of PD complex with $BrCH_2$[^{14}C]CONHPhAsO in both the E1 and E3-mediated lipoyl systems. Results from both sets of experiments provided further evidence for the involvement of an essential histidine residue in the catalytic mechanism of E3.

3.4.2 Inhibition of PD Complex with $BrCH_2$[^{14}C] CONHPhAsO: E1-Mediated Lipoyl System

This experiment was carried out largely as described in Section 2.2.12, and the results are illustrated in Fig. 3.4.2.1. In order to minimise any nonspecific

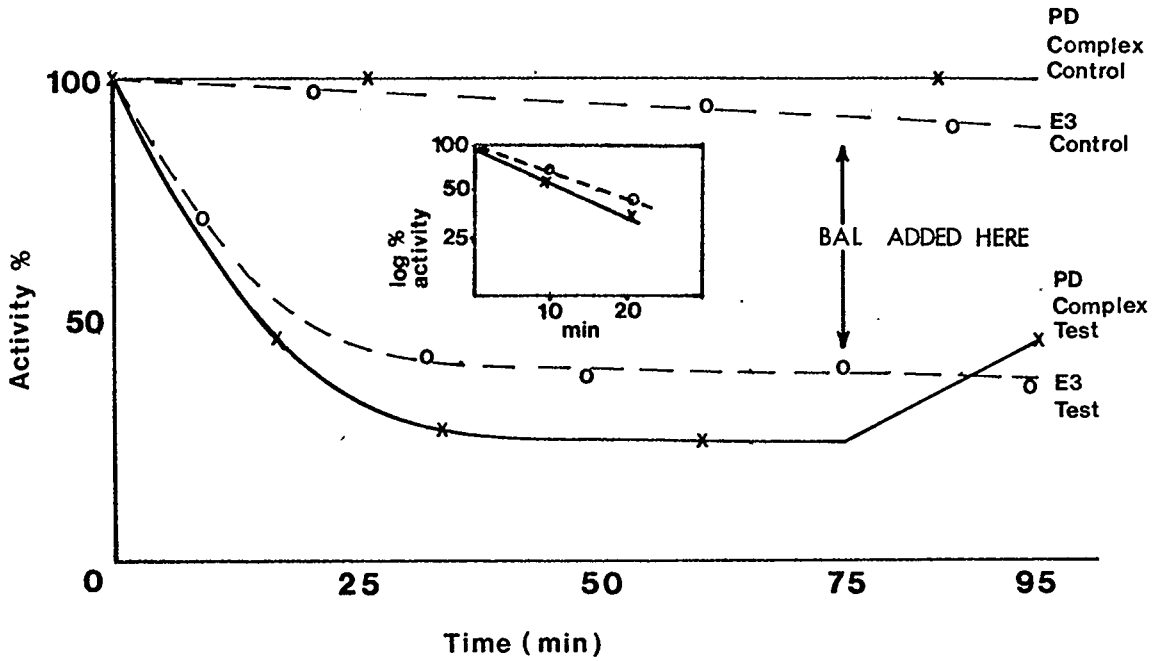


Figure 3.4.2.1 Inhibition of PD complex (10.2 mg/mL) with $BrCH_2[^{14}C]CONHPhAsO$ (0.15 mM) in the E1-mediated lipoyl reduction system. To begin the reaction, cofactors (TPP, 0.55 mM; Mg^{2+}, 6.2 mM; pyruvate, 4.5 mM; and coenzyme A, 0.15 mM) were added to 1 mL PD complex and 0.15 mM $BrCH_2[^{14}C]CONHPhAsO$, this reaction mixture was termed PD complex test. The PD complex control contained 1 mL PD complex and 0.15 mM $BrCH_2[^{14}C]CONHPhAsO$ only. BAL (1.5 mM) was added after 75 min to both samples, as described in Section 2.2.12. (x——x = PD complex activity, o---o = E3 activity).

alkylations by the bromoacetamido moiety of $BrCH_2[^{14}C]$-CONHPhAsO, the molar ratio of reagent to protein was kept as low as possible (Adamson, 1981; Adamson and Stevenson, 1982). Preliminary experiments were carried out to determine the minimum amount of arsenoxide that could be used to significantly inhibit both PD complex and E3 activities. PD complex (10.2 mg in 1 mL) was inhibited with $BrCH_2[^{14}C]$CONHPhAsO (0.15 mM) at 0°C for 75 min. The PD complex and E3 activities remaining were 25% and 40%, respectively. The addition of BAL (1.5 mM) to the sample caused reactivation of PD complex activity to about the level of E3 inhibition. Throughout the experiment, a control vial containing PD complex, $BrCH_2[^{14}C]$CONHPhAsO and no cofactors largely retained both E3 and PD complex activities. Both the control and test samples were exhaustively dialysed and frozen, as described previously in Section 2.2.12.

3.4.3 $NaDodSO_4$-PAGE and Radiolabel Analysis of Inhibited PD Complex (E1-Mediated System)

$NaDodSO_4$-PAGE and radiolabel analyses were carried out on both "$[^{14}C]$-arsenoxide inhibited" and "control" PD complex from the previous experiment. Polyacrylamide gels were sliced and analysed for radioactivity as described in Section 2.2.9, and the results are illustrated in Fig. 3.4.3.1. The $[^{14}C]$-radiolabel was primarily associated with the E3 component in the inhibited PD complex. These findings are clearly supportive

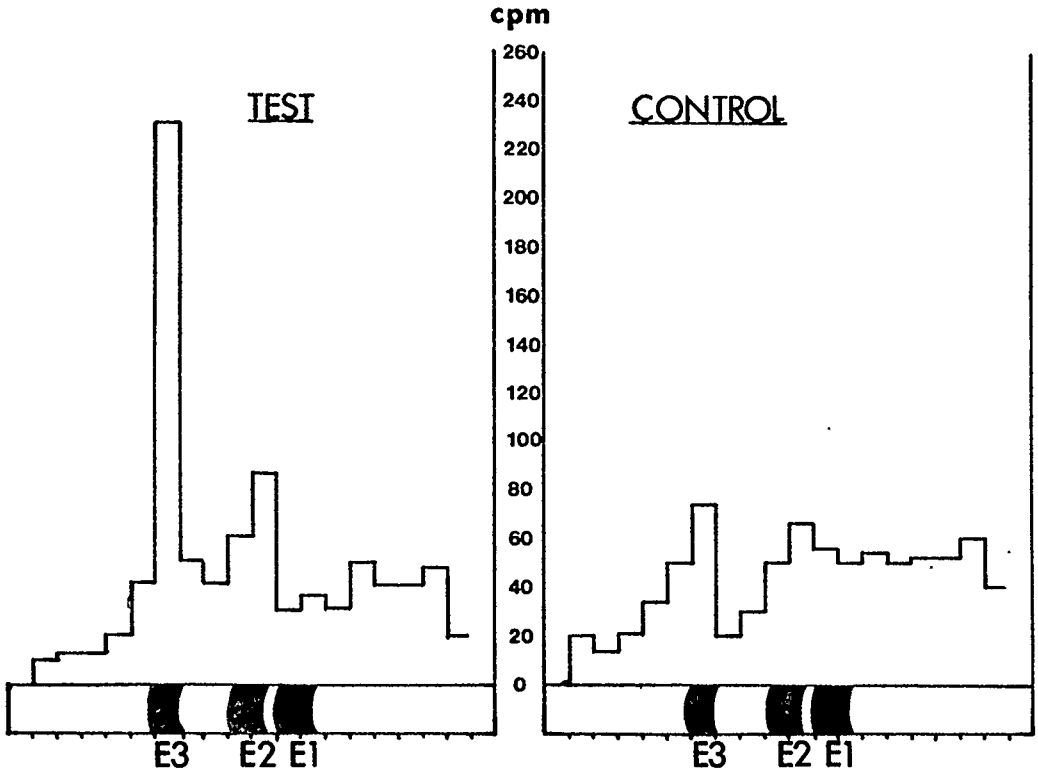


Figure 3.4.3.1 $NaDodSO_4$-PAGE and [^{14}C]-radiolabel analysis of PD complex, inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the E1-mediated lipoyl reduction system. Test :- PD complex inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the presence of cofactors (TPP, Mg^{2+}, pyruvate, coenzyme A). Control :- PD complex incubated with $BrCH_2[^{14}C]CONHPhAsO$ only. In both cases, 50 µg of PD complex was applied to 7.5% polyacrylamide gels. The gels were sliced in 0.3 cm sections and counted for radioactivity.

of the proposed mode of action of the bifunctional arsenoxide on the PD complex. The [^{14}C]-radiolabel present in the E3 component of "control" PD complex is due to non-specific alkylations on E3 by the bromoacetamido moiety of the bifunctional arsenoxide, whereas the [^{14}C]-radiolabel in the E3 component from "test" PD complex is attributable to a specific irreversible alkylation in or near the active site of E3. With a view to identifying the site(s) of alkylation on E3, this component was isolated from the PD complex by hydroxylapatite chromatography.

3.4.4 Isolation of E3 from the PD Complex

E3 was isolated from PD complex, previously treated with trypsin, by employing hydroxylapatite chromatography. Stepwise elution with increasing concentrations of phosphate buffer (pH 7.0, 8 M in urea) was used to fractionate E3 from other PD complex components. E3, inhibited with $BrCH_2$[^{14}C]CONHPhAsO, and "control" E3 were isolated as shown in Figs. 3.4.4.1 and 3.4.4.2, respectively. Full experimental details are described in Section 2.2.10. In both cases, E3 was assessed to be virtually pure (ie. free of other PD complex components) by the criteria of $NaDodSO_4$-PAGE on 7.5% polyacrylamide gels.

The yield of E3 inhibited with $BrCH_2$[^{14}C]CONHPhAsO, as isolated from the PD complex by hydroxylapatite chromatography, was markedly lower than that of "control"

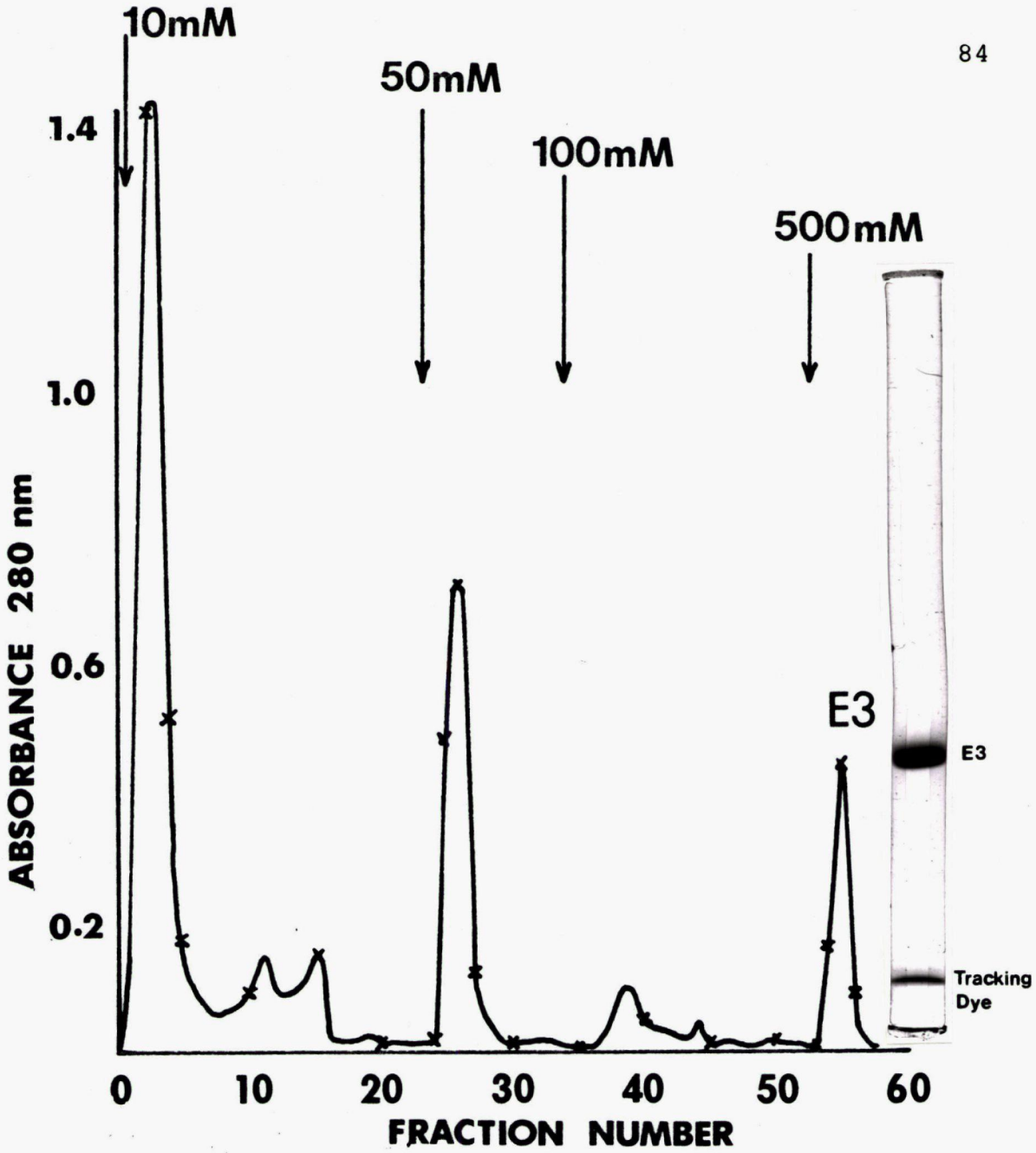


Figure 3.4.4.1 Isolation of E3 from PD complex inhibited with $BrCH_2[^{14}C]CONHPhAsO$ (E1-mediated lipoyl system). E3 was isolated from 10 mg PD complex on a hydroxylapatite column. The concentrations illustrated refer to sodium phosphate buffer adjusted to pH 7.0 and made 8 M in urea. The arrows denote the stepwise change in the concentration of buffer used to elute the column. The flow rate was 1.5 mL/h. Fraction volumes were 1.2 mL. $NaDodSO_4$-PAGE of isolated E3 was on 7.5% polyacrylamide gels.

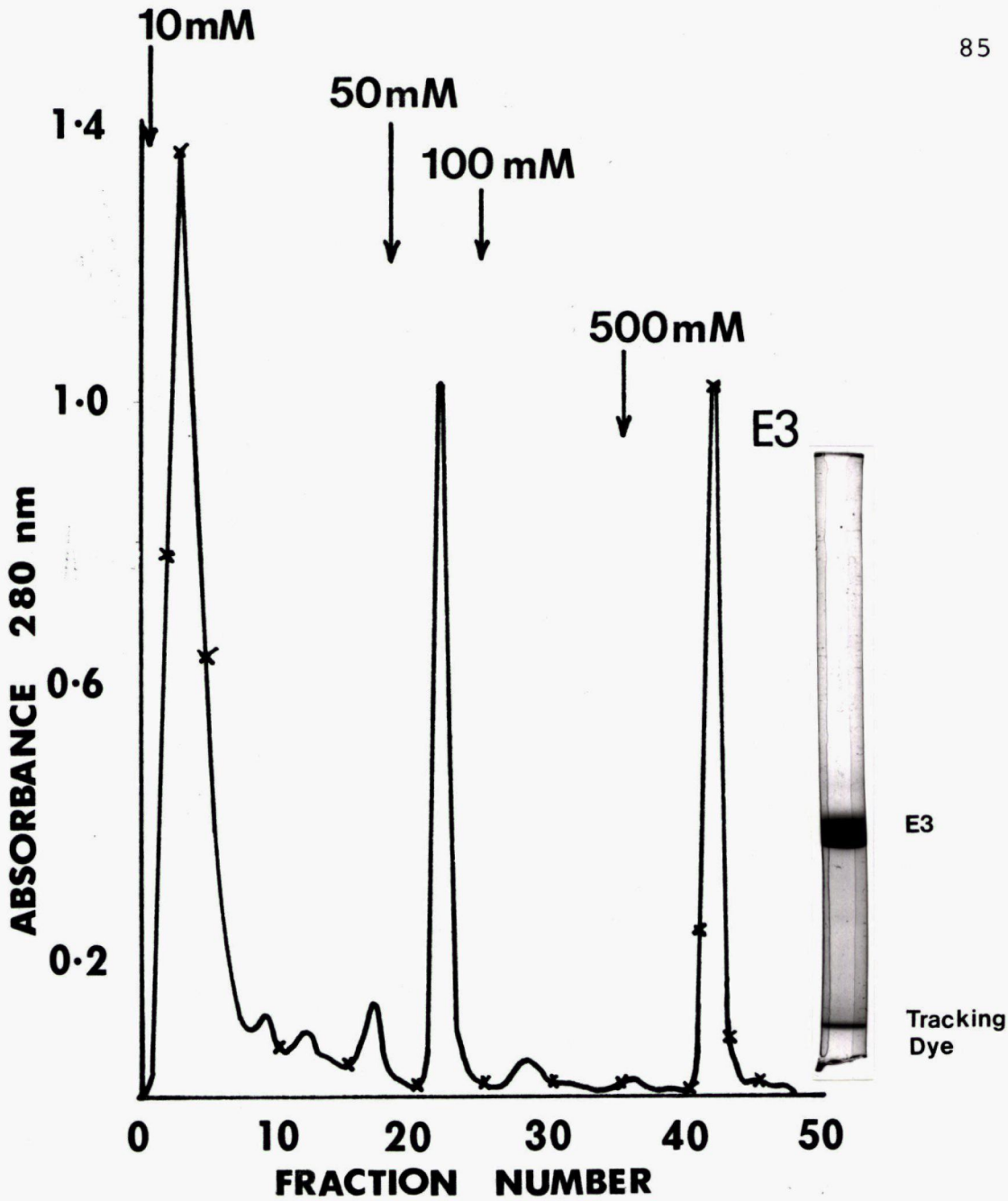


Figure 3.4.4.2 Isolation of E3 from control PD complex incubated with $BrCH_2[^{14}C]CONHPhAsO$ only. E3 was isolated from 10 mg PD complex on a hydroxylapatite column. The concentrations illustrated refer to sodium phosphate buffer, adjusted to pH 7.0 and made 8 M in urea. The arrows denote the stepwise change in the concentration of buffer used to elute the column. The flow rate was 1.6 mL/h. Fraction volumes were 1.2 mL. $NaDodSO_4$-PAGE of isolated E3 was on 7.5% polyacrylamide gels.

E3 isolated from native PD complex in a similar procedure. Typically, the yield of E3 in the inhibited, substrate-reduced, sample was between 55-65% of E3 in the control. The decreased yield of E3 inhibited with $BrCH_2[^{14}C]CONHPhAsO$ has been attributed to three possible factors: (i) denaturation of substrate-reduced E3 in the presence of 8 M urea leading to a decreased affinity for the hydroxylapatite matrix, (ii) the increased susceptibility of substrate-reduced E3 to proteolytic digestion with trypsin and (iii) loss of affinity for hydroxylapatite caused by the arsenoxide modification. E3 is known to be stable in 8 M urea (Adamson, 1981). Indeed under these conditions, PD complex components are readily dissociable whilst E3 remains active. This explains why the isolation of E3 on hydroxylapatite in the presence of 8 M urea is effective. However, several workers have noted that if the active-site redox disulphide of E3 is reduced, denaturation in 8 M urea occurs far more readily (Massey, 1960; Adamson, 1981). A consequence of this may be a decreased affinity of substrate-reduced E3 for hydroxylapatite. Also, de Kok *et al*. (1981) have shown that oxidised E3 from *Azotobacter vinelandii* is resistant to proteolytic degradation by trypsin whereas substrate-reduced E3 is not. The compact structure of the oxidised enzyme presumably becomes more accessible to trypsin upon reduction of the redox desulphide. The persistence of substrate-reduced E3 up to the stage of proteolytic digestion of the PD complex with trypsin (in 8 M urea) may therefore lead to a loss of intact E3.

Experiments to be reported later in this thesis (Section 3.5.6) have shown that factors (i) and (ii) are probably the principal reasons for the low yield of E3 inhibited with [^{14}C]-radiolabelled bifunctional arsenoxide.

3.4.5 Identification of Chemically Modified Residues on E3 (El-Mediated System)

HVE (pH 6.5) was performed on an acid hydrolysate of E3 inhibited with [^{14}C]-bifunctional arsenoxide and the results are illustrated in Fig. 3.4.5.1. By far the majority of the [^{14}C]-radiolabel was found to be associated with N^3-carboxymethyl histidine. A smaller amount of radioactivity was present on the origin; this was largely attributable to insoluble material. HVE and radiochemical analysis of an acid hydrolysate of an equal amount of "control" E3, revealed negligible radioactivity throughout the ionogram (ie. no section greater than 30 cpm). This finding corresponded to the minimal pattern of radiolabelling associated with "control" E3 as analysed by $NaDodSO_4$-PAGE (Fig. 3.4.3.1). These results illustrate an almost exclusive alkylation of histidine in the N^3 position on the imidazole ring. No radioactivity was associated with N^1-carboxymethyl or $N^{1,3}$-dicarboxymethyl histidine. These findings are consistent with a modification by the bromoacetamido moiety of the bifunctional arsenoxide. Under the conditions of strong acid hydrolysis prior to HVE, the bifunctional arsenoxide attached to E3 (as E3-X-CH_2[^{14}C]CONHPhAs=BAL) would be

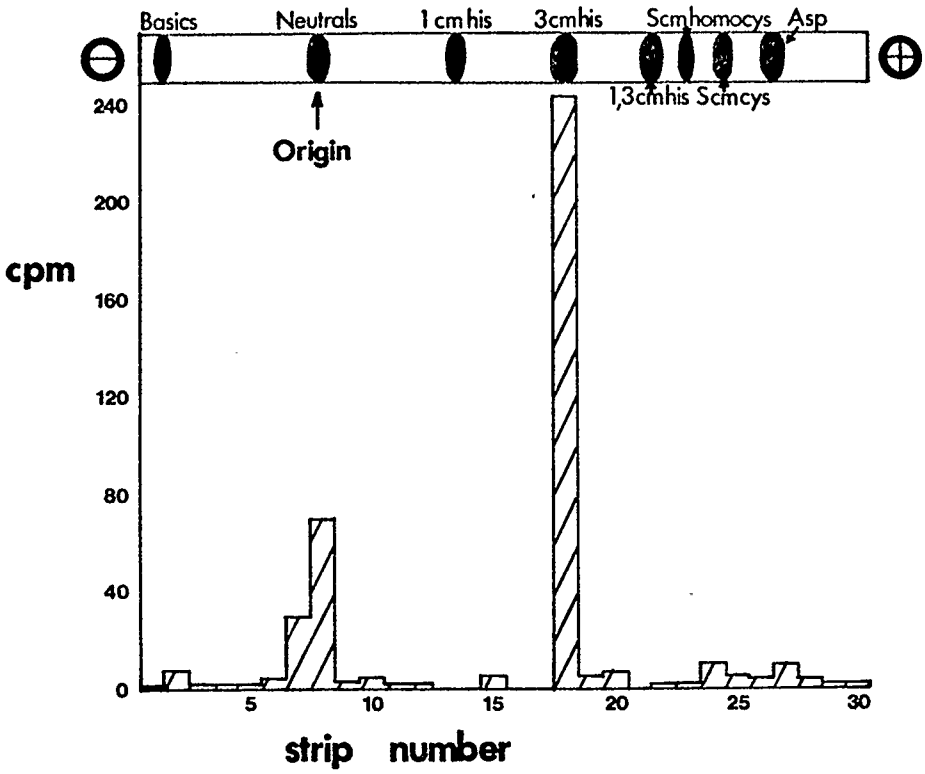


Figure 3.4.5.1 Identification of radiolabelled, carboxymethylated amino acid derivatives in the acid hydrolysate of E3, inhibited with $BrCH_2[^{14}C]CONHPhAsO$ (whilst part of the PD complex) in the E1-mediated lipoyl reduction system. High voltage electrophoresis and scintillation counting of 0.5 inch paper strips were carried out as described in Sections 2.2.8 and 2.2.9. 1 cm his is N^1-carboxymethyl histidine, 3 cm his is N^3-carboxymethyl histidine, 1,3 cm his is $N^{1,3}$-dicarboxymethyl histidine, S cm cys is S-carboxymethyl cysteine, S cm homocys is S-carboxymethyl homocysteine.

cleaved at the acid labile amide bond (-CONH), cf. Fig. 3.4.1.1, thus leaving the residue(s) modified by the reagent in the form of a [^{14}C]-radiolabelled carboxymethyl derivative.

The results provide strong evidence for the involvement of a histidine residue in the catalytic mechanism of E3 and are consistent with the previous findings of Adamson and Stevenson (1982).

3.4.6 Inhibition of PD Complex with $BrCH_2[^{14}C]$ CONHAPhAsO: E3-Mediated Lipoyl System

PD complex (12 mg) was inhibited with 0.5 mM $BrCH_2[^{14}C]$CONHPhAsO, in the manner described previously (Section 2.2.12), with the exception that 2 mM NADH was used to reduce the lipoyl residues on E2 (E3-mediated lipoyl system). The results from this experiment are illustrated in Fig. 3.4.6.1.

Preliminary inhibition experiments were carried out in the E3-mediated lipoyl system in which the arsenoxide to protein ratio was identical to that described earlier for E1-mediated experiments. However, under these conditions, the rate of E3 inactivation was consistently found to be much slower (typically only 30% E3 inactivation was achieved). These results (data not shown) confirmed similar findings by Adamson and Stevenson (1981) working with non-radioactive bifunctional arsenoxide in the E3-mediated lipoyl system. Partial interference with the active-site directed alkylation of E3 by NADH bound at the active site may account for this phenomenon.

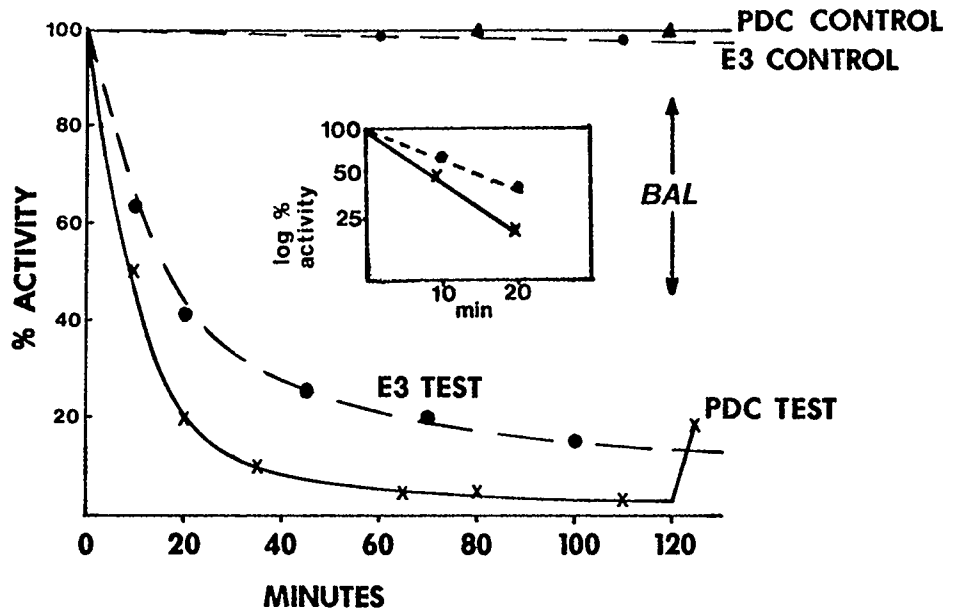


Figure 3.4.6.1 Inhibition of PD complex with $BrCH_2[^{14}C]CONHPhAsO$ in the presence of NADH (E3-mediated lipoyl reduction system). PD complex (1 mL, 12 mg/mL) was incubated with 0.50 mM $BrCH_2[^{14}C]CONHPhAsO$ and the reaction started with the addition of 2 mM NADH. This sample was labelled PD complex test. The reaction was terminated with 5 mM BAL after 120 min, as shown. The control sample contained PD complex and $BrCH_2[^{14}C]CONHPhAsO$, as above, but did not have NADH added. (▲—▲ = PD complex control, x—x = PD complex test, ●---● = E3 activity, test and control samples).

In the experiment illustrated in Fig. 3.4.6.1., the arsenoxide concentration was increased approximately threefold from 0.15 mM to 0.50 mM, with the result that both PD complex (95%) and E3 (85%) were strongly inhibited. A tenfold excess of BAL over arsenoxide was used to terminate the reaction after 120 min, with the result that the PD complex was reactivated to about the level of E3 inhibition (as observed earlier). A control vial containing only 12 mg PD complex and 0.50 mM arsenoxide retained both PD complex and E3 activities after 120 min.

The inhibited and control samples of PD complex were labelled "arsenoxide test" and "arsenoxide control" respectively, and were exhaustively dialysed, as described previously.

3.4.7 $NaDodSO_4$-PAGE and Radiolabel Analysis of Inhibited PD Complex (E3-Mediated System)

$NaDodSO_4$-PAGE and radiolabel analyses of "inhibited" and "control" PD complex from the previous experiment were performed in an analogous manner to those described for the PD complex inhibited in the E1-mediated lipoyl system (Sections 2.2.6, 2.2.7 and 3.4.3) and the results are illustrated in Fig. 3.4.7.1. [^{14}C]-Radiolabel above the level of the control was primarily associated with E3 in the PD complex inhibited with $BrCH_2[^{14}C]CONHPhAsO$. This finding is in excellent agreement with earlier results for the inhibition of PD complex in the E1-mediated system.

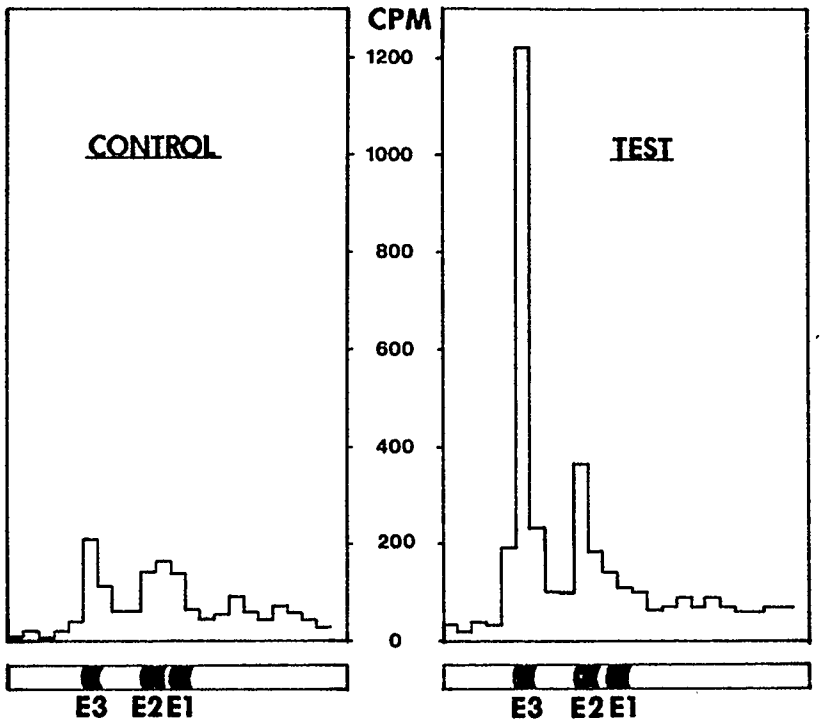


Figure 3.4.7.1 $NaDodSO_4$-PAGE and [^{14}C]-radiolabel analysis of PD complex inhibited with $BrCH_2$[^{14}C]CONHPhAsO in the E3-mediated lipoyl reduction system. Test :- PD complex inhibited with $BrCH_2$[^{14}C]CONHPhAsO in the presence of NADH. Control :- PD complex incubated with $BrCH_2$[^{14}C]CONHPhAsO only. In both cases, 50 µg of PD complex was applied to 7.5% polyacrylamide gels. The gels were sliced in 0.3 cm sections and counted for radioactivity.

In contrast to inhibition of the PD complex by $BrCH_2[^{14}C]CONHPhAsO$ in the E1-mediated system, a significant amount of $[^{14}C]$-radiolabel was also consistently found in E2. Since $NaDodSO_4$-PAGE revealed that no cross-linked E2 and E3 was present after BAL treatment (ie. arsenoxide modification of the reduced lipoyl residues on E2 had been fully reversed), it appears most likely that the $[^{14}C]$-radiolabel associated with E2 is attributable to an irreversible alkylation(s) by the bromoacetamido moiety of the bifunctional arsenoxide. Lipoyl residues are known to interact with the active site of E2 in keeping with the accepted PD complex mechanism (Fig. 1.6.1), thus arsenoxide modified lipoyl residues on E2 must also be expected to interact with the active site of E2 (this is in addition to their interaction with the active site of E3 and alkylation of a histidine residue).

The absence of $[^{14}C]$-radiolabel in E2 in the control PD complex sample (which contains oxidised lipoyl residues) suggests that any alkylation occurring on E2, in the PD complex sample inhibited with $BrCH_2[^{14}C]$-CONHPhAsO, may well be mediated via the reduced lipoyl residues. These observations imply that the radiolabelling of E2 may include the direct alkylation (by the $BrCH_2[^{14}C]CO$- moiety of the bifunctional arsenoxide) of a residue(s) in or near the active site of E2.

$NaDodSO_4$-PAGE analysis of PD complex inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the E1-mediated lipoyl system,

revealed the presence of only a very small amount of [^{14}C]-radiolabel in E2. A possible explanation for this could be that in this system (which contains pyruvate, TPP, Mg^{2+} and coenzyme A), coenzyme A is bound at the active site of E2 and thus might protect susceptible residues from alkylation. In the E3-mediated system however, only NADH is required to reduce the lipoyl moieties on E2 and thus, the active site of E2 would be openly available to alkylation by the bromoacetamido moiety of $BrCH_2$[^{14}C]CONHPhAsO.

Further evidence for the irreversible modification of the active site of E2 during the E3-mediated inhibition of the PD complex will be presented later in this thesis and the implications of these findings fully discussed in the next chapter.

3.4.8 Isolation of [^{14}C]-Radiolabelled E3 from the PD Complex

E3 was isolated from (i) the PD complex inhibited in the E3-mediated system by $BrCH_2$[^{14}C]CONHPhAsO and from (ii) PD complex control, by hydroxylapatite chromatography in the presence of 8 M urea (Section 2.2.10). The procedure adopted and results achieved were virtually identical to those described for the isolation of E3 in Section 3.4.4, and will therefore, not be considered in detail here. The E3 samples isolated in this experiment were judged to be pure by $NaDodSO_4$-PAGE on 7.5% polyacrylamide gels.

3.4.9 Identification of Chemically Modified Residues on E3 (E3-Mediated System)

Chemically modified residues on E3 inhibited by $BrCH_2[^{14}C]CONHPhAsO$, were identified by HVE (pH 6.5) and $[^{14}C]$-radiochemical analysis of an acid hydrolysate of the enzyme, as described in Sections 2.2.8, 2.2.9 and 3.4.5. The results, illustrating the location of the $[^{14}C]$-radiolabel in both inhibited and control E3 samples, are presented in Figs. 3.4.9.1 and 3.4.9.2.

The majority of the $[^{14}C]$-radiolabel was again associated with N^3-carboxymethyl histidine and the results from this series of experiments compare favourably with previous results found for the E1-mediated inhibition of E3 (Section 3.4.5). In the presence of excess NADH, the inactivation of PD complex and E3 appears once more to be attributable to anchoring of the -AsO moiety of the bifunctional arsenoxide to reduced lipoyl residues on E2, followed by delivery of the bromoacetamido moiety into E3 where irreversible alkylation of a histidine residue occurs in or near the active site.

Some $[^{14}C]$-radiolabel, albeit in smaller amounts, was also associated with S-carboxymethyl homocysteine (Fig. 3.4.9.1) in the acid hydrolysate of modified E3 and may reflect modification of a methionine residue(s) near the active site of E3. In this case, the alkylated methionine residue(s) in E3 (prior to acid hydrolysis) would be expected to exist as an S-alkyl methionine

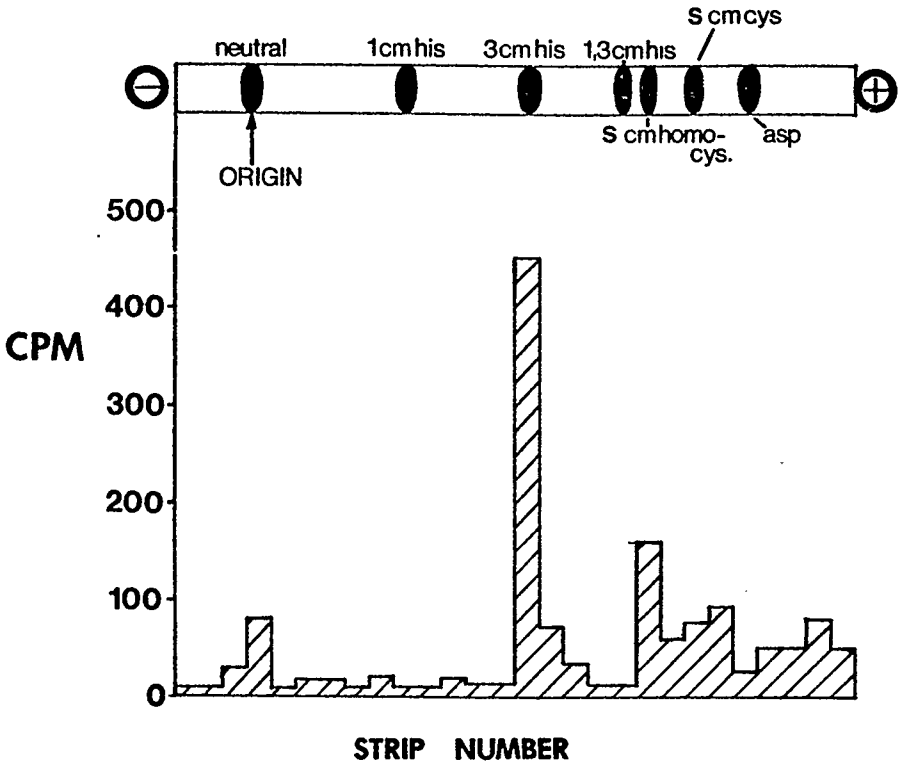


Figure 3.4.9.1 Identification of radiolabelled, carboxymethylated amino acid derivatives in the acid hydrolysate of E3 inhibited with $BrCH_2[^{14}C]CONHPhAsO$ (whilst part of the PD complex) in the E3-mediated lipoyl reduction system. High voltage electrophoresis and scintillation counting of 0.5 inch paper strips were carried out as described in Sections 2.2.8 and 2.2.9. The abbreviations used for carboxymethylated amino acid standards are presented in Fig. 3.4.5.1.

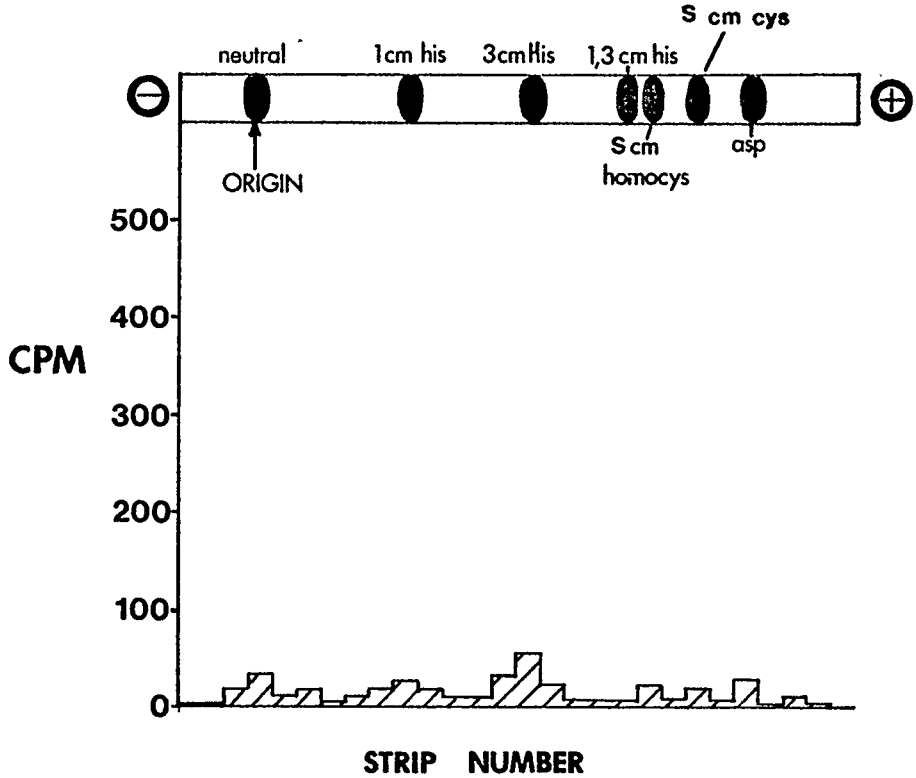


Figure 3.4.9.2 Identification of radiolabelled, carboxymethylated amino acid derivatives in the acid hydrolysate of E3. E3 was isolated from the PL complex control exposed to $BrCH_2[^{14}C]CONHPhAsO$ in the absence of NADH. High voltage electrophoresis and scintillation counting of 0.5 inch paper strips were carried out as described in Sections 2.2.8 and 2.2.9. The abbreviations used for carboxymethylated amino acid standards are presented in Fig. 3.4.5.1.

sulphonium ion. This compound is very unstable and decomposes in strong acid to a variety of compounds, one of which is S-carboxymethyl homocysteine (Gundlach *et al.*, 1959).

3.5 Clarification of the Mode of Action of $BrCH_2[^{14}C]CONHPhAsO$ on the PD Complex From *E. Coli*: Does E2 Contain a Third Non-acetylatable Lipoic Acid Residue?

3.5.1 Introduction

At the outset of the studies outlined in this section, it was hoped to design a series of experiments that would critically test the previously outlined rationale for the mode of action of the bifunctional arsenoxide on the PD complex. The stoichiometry of lipoyl-lysine residues on E2 and their role in the active-site coupling of E2 and E3 was reviewed earlier (Sections 1.6, 1.7 and 1.8). It is well accepted that each E2 polypeptide chain contains two lipoyl residues that can become reductively acetylated in the presence of pyruvate (for references, see Section 1.7). Furthermore, two lipoic acid residues per E2 chain can be specifically radiolabelled with $[^{14}C]$-NEM in a pyruvate dependent reaction (Brown and Perham, 1976; Danson and Perham, 1976). The acetylated lipoyl moieties on E2 contain an S^6-acetyl dihydrolipoyl moiety which possesses a free thiol group at position S^8 (Gunsalus *et al.*, 1956). This S^8-thiol group reacts specifically with NEM to form an irreversible thioether bond. These reactions are outlined schematically in Fig. 3.5.1.1.

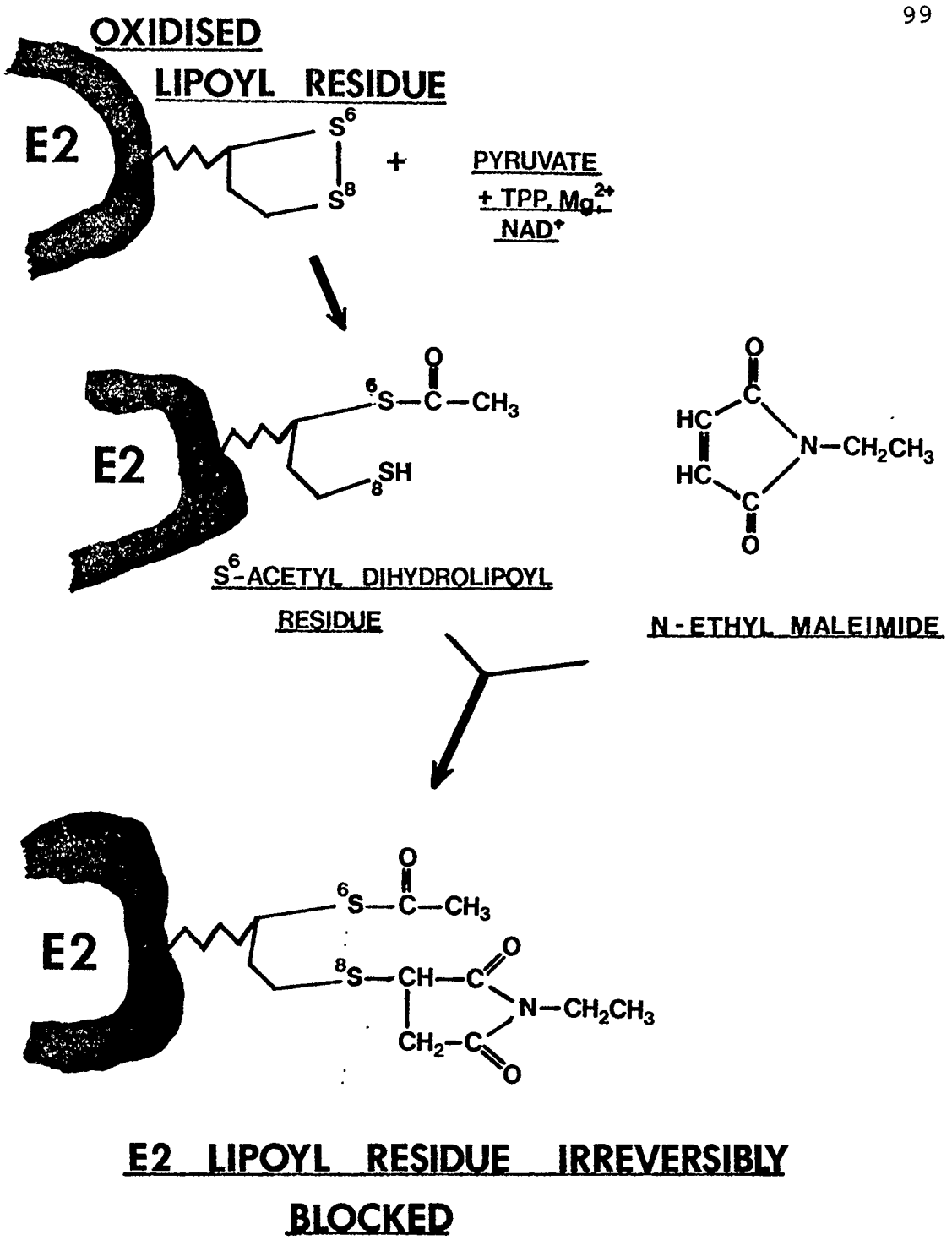


Figure 3.5.1.1 A scheme illustrating the modification of PD complex with NEM in the presence of pyruvate.

NEM inhibition of the PD complex (in the presence of pyruvate) therefore provides a very convenient way of irreversibly blocking the lipoyl residues on E2. These "NEM modified" lipoyl residues do not contain a vicinal dithiol group and are unreactive towards the arsenoxide moiety of $BrCH_2[^{14}C]CONHPhAsO$.

The previously outlined reaction scheme (Fig. 3.4.1.1) for the mode of action of $BrCH_2[^{14}C]CONHPhAsO$ on the PD complex envisages "a priori" anchoring of the arsenoxide to reduced lipoyl residues on E2. This is followed by delivery of the bromoacetamido moiety of the reagent into or near the active site of E3 where irreversible alkylation of a histidine residue ensues.

According to this scheme, PD complex possessing lipoyl residues blocked with NEM should not be able to "anchor" the bifunctional reagent via the AsO- moiety, nor deliver the $BrCH_2[^{14}C]CO$- moiety into the active site of E3. Under these circumstances E3 ought to remain fully active and no incorporation of $[^{14}C]$-radiolabel should occur.

If the inactivation of E3 by $BrCH_2[^{14}C]CONHPhAsO$ does not require initial "anchoring" of the arsenoxide moiety to lipoyl residues on E2, then inactivation and/or $[^{14}C]$-radiolabelling of E3 in the NEM-blocked PD complex would be expected to occur.

3.5.2 Experimental Rationale

In order to test this hypothesis, NEM-blocked PD complex was incubated with $BrCH_2[^{14}C]CONHPhAsO$ in the

presence of excess NADH. Using this approach it was also possible to investigate other aspects of the E2-E3 active-site coupling mechanism, namely to gain further insight into the stoichiometry of lipoyl residues on E2. The experimental rationale for this is as follows. As discussed in Sections 1.7 and 1.8, the exact stoichiometry of E2 lipoyl residues remains controversial. Recently, Hale and Perham (1979[a]) obtained evidence for the presence of three lipoyl residues per E2 polypeptide chain (Section 1.8). To reconcile this finding with the generally well accepted theory that only two lipoyl residues could be reductively acetylated by pyruvate, they proposed a novel mechanism in which the third residue is non-acetylatable (i.e. does not interact with E1 directly to yield an S^6-acetyl lipoyl residue) and serves only to pass reducing equivalents from acetylatable lipoyl residues directly into the active site of E3. This scheme is outlined diagrammatically in Fig. 4.4.1.

Clearly, if there is a third non-acetylatable lipoyl residue present on E2 that functions in the normal catalytic mechanism of the PD complex, it would be expected to remain oxidised in the presence of pyruvate (Figs. 1.6.1 and 3.5.1.1) and as such would be unreactive towards NEM. In the presence of excess NADH (E3-mediated lipoyl system) however, the third lipoyl residue would become reduced (via E3-mediated NADH dependent reduction) and would then be in a position to bind the -AsO moiety of the bifunctional reagent. The bromoacetamido moiety in turn could now be delivered into or near the active site of E3.

In summary, if E2 contains a third non-acetylatable lipoyl residue, then under the conditions of the above experiment, significant inactivation and [^{14}C]-radio-labelling of E3 would be expected.

3.5.3 Inhibition of PD Complex with NEM

PD complex was pre-incubated with pyruvate, TPP, Mg^{2+} and NAD^{+} for 0.5 min at 0°C to convert oxidised lipoyl residues on E2 to their S^{6}-acetyl dihydrolipoyl derivatives (Figs. 1.6.1 and 3.5.1.1). The PD complex was then rapidly inactivated in the presence of NEM (Fig. 3.5.3.1) whilst the activity of E3 remained largely unaffected. These results are in accordance with the well accepted reaction scheme outlined in Fig. 3.5.1.1 and are in good agreement with the results reported by others (Grande *et al.*, 1975; Brown and Perham, 1976; Danson and Perham, 1976; Ambrose and Perham, 1976; Collins and Reed, 1977; Henderson *et al.*, 1979; Ambrose-Griffin *et al.*, 1980; Adamson, 1981). NEM-inhibited PD complex was denoted "test" and dialysed exhaustively overnight to remove unreacted NEM.

3.5.4 Inhibition of NEM-inhibited and Native PD Complex by $BrCH_2$[^{14}C]CONHPhAsO

NEM-blocked PD complex was incubated with $BrCH_2$-[^{14}C]CONHPhAsO in the presence of excess NADH at 0°C, as illustrated in Fig. 3.5.4.1. This sample was denoted "test". Native PD complex (dialysed overnight at 4°C

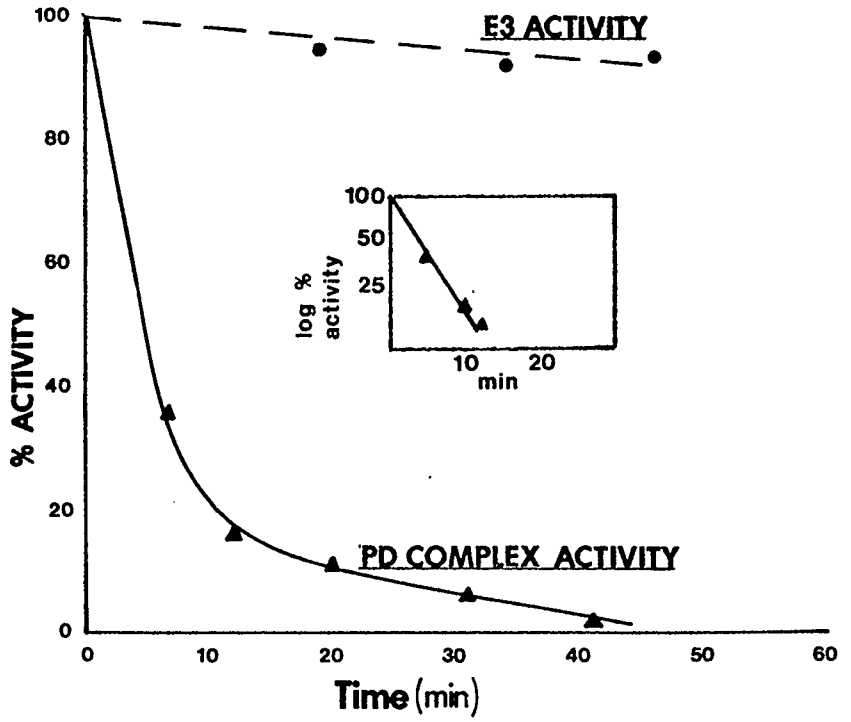


Figure 3.5.3.1 Pyruvate dependent inhibition of PD complex by N-ethyl maleimide (NEM). PD complex in sodium phosphate buffer (pH 7.0, 10 mg/mL) was incubated with pyruvate (2 mM), TPP (0.5 mM), Mg^{2+} (5 mM) and NAD^+ (1 mM) at 0 °C for 30 sec then NEM (1 mM) was added. The solution was flushed with N_2 prior to and at intervals during the incubation. PD complex and E3 enzyme assays were carried out as described in Section 2.2.5.

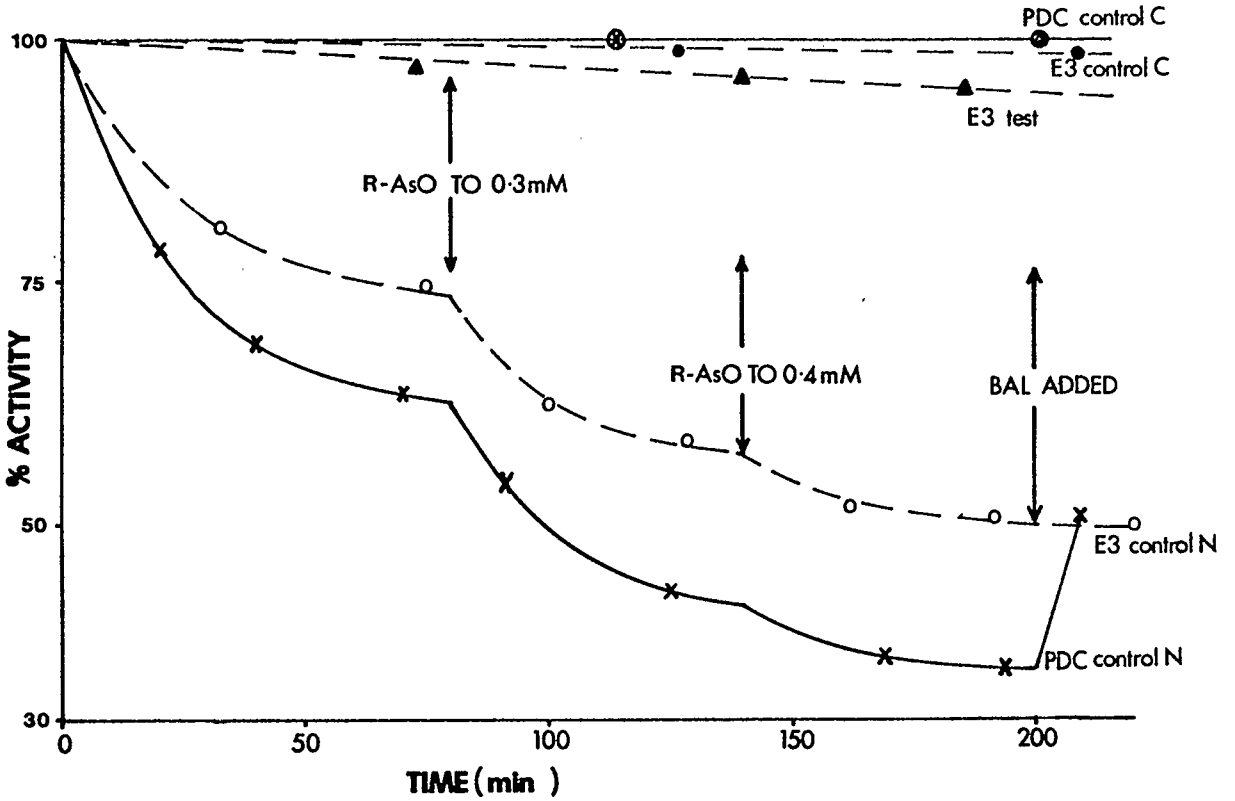


Figure 3.5.4.1 Incubation of N-ethyl maleimide inhibited PD complex with $BrCH_2[^{14}C]CONHPhAsO$ in the presence of NADH. Experimental procedure was as described in Section 2.2.13. Test refers to NEM-blocked PD complex (10 mg/mL) containing $BrCH_2[^{14}C]CONHPhAsO$ (initially 0.2 mM) and NADH (2 mM). Control N refers to native PD complex containing $BrCH_2[^{14}C]CONHPhAsO$ (initially 0.2 mM) and 2 mM NADH. Control C refers to native PD complex incubated with $BrCH_2[^{14}C]CONHPhAsO$ (initially 0.2 mM) in the absence of NADH. The concentration of $BrCH_2[^{14}C]CONHPhAsO$ in test, control N and control C samples was increased, as shown, to 0.40 mM after 140 min. BAL (4 mM) was added to each of these samples after 200 min (x---x = PD complex (PDC) activity in control N sample, o---o = E3 activity in control N sample, ▲---▲ = E3 activity in test sample, ●---● = E3 activity in control C sample, ⊗---⊗ = PD complex (PDC) activity in control C sample).

without additives) was also incubated with [^{14}C]-bifunctional arsenoxide in the presence of excess NADH. This sample was denoted "control N" and was used to obtain a "normal" profile for the inhibition of PD complex in the E3-mediated lipoyl system. A further control consisted of native PD complex, incubated with [^{14}C]-bifunctional arsenoxide only, and was denoted "control C".

As illustrated in Fig. 3.5.4.1, native PD complex (control N) lost 50% of its E3 activity and 65% of PD complex activity after 180 min. The amount of $BrCH_2$[^{14}C]CONHPhAsO present was increased carefully from a low starting concentration in an attempt to maximise the specificity of the reaction. Native PD complex incubated only with $BrCH_2$[^{14}C]CONHPhAsO (control C), retained both PD complex and E3 activities as shown (Fig. 3.5.4.1). NEM-blocked PD complex largely retained its E3 activity despite the presence of $BrCH_2$[^{14}C]CONHPhAsO and excess NADH.

These experiments demonstrate that NEM modification of the dihydrolipoyl residues on E2 prevents the inhibition of E3 by $BrCH_2$[^{14}C]CONHPhAsO. The findings are consistent with the proposed mode of action of $BrCH_2$[^{14}C]CONHPhAsO on the PD complex, as outlined previously in Fig. 3.4.1.1.

The failure to inactivate E3 in the "test" sample presumably because of the inability of acetylated, NEM-blocked PD complex to "anchor" and deliver $BrCH_2$[^{14}C]-

CONHPhAsO into the active site of E3, still had to be correlated with the pattern of [^{14}C]-radiolabelling in the "test", "control N" and "control C" samples of PD complex. Accordingly, each of these samples were exhaustively dialysed, subjected to NaDodSO$_4$-PAGE and analysed for the presence of [^{14}C]-radiolabel.

3.5.5 NaDodSO$_4$-PAGE and [^{14}C]-Radiolabel Analysis of NEM-blocked PD Complex, Treated with BrCH$_2$[^{14}C]-CONHPhAsO (E3-mediated System)

The results from NaDodSO$_4$-PAGE analyses of "test" (acetylated, NEM-inhibited PD complex treated with BrCH$_2$[^{14}C]CONHPhAsO in the presence of NADH), "control N" (native PD complex inhibited with BrCH$_2$[^{14}C]CONHPhAsO in the presence of NADH) and "control C" (native PD complex incubated with BrCH$_2$[^{14}C]CONHPhAsO only) samples are illustrated in Fig. 3.5.5.1.

In "control N", the majority of the [^{14}C]-radiolabel was associated with E3, although a significant proportion of radioactivity was also present in E2. These results correlate well with earlier studies (Section 3.4.7). The "test" PD complex sample was virtually devoid of [^{14}C]-incorporation and was very similar to "control C".

These results provide strong evidence in favour of the proposed mode of action on the PD complex. The absence of [^{14}C]-radiolabel in the E3 component of NEM-blocked PD complex is consistent with the fact that this

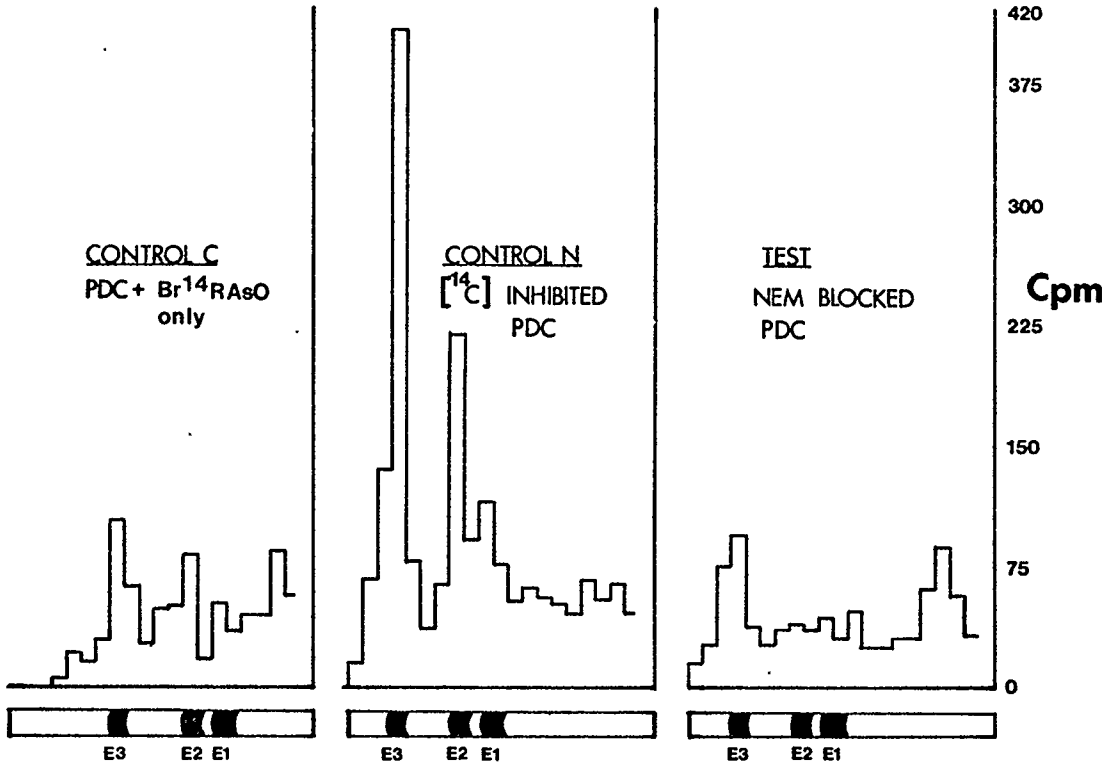


Figure 3.5.5.1 $NaDodSO_4$-PAGE and [^{14}C]-radiolabel analysis of acetylated, N-ethyl maleimide inhibited PD complex (PDC) incubated with $BrCH_2$[^{14}C]CONHPhAsO (Br^{14}RAsO) in the E3-mediated lipoyl reduction system. The definitions of control C, control N and test samples are presented in Fig. 3.5.4.1. 100 µg of each sample were applied to 7.5% polyacrylamide gels. The final gels were sliced in 0.3 cm sections and counted for radioactivity.

component remained fully active in the presence of $BrCH_2[^{14}C]CONHPhAsO$. This illustrates a probable correlation between the inactivation of E3 (whilst still part of the PD complex) and the modification of an active-site histidine residue.

The results also argue against the presence of a third, catalytically active, non-acetylatable lipoyl residue on E2. Clearly, had this residue been present, excess NADH would have rendered it reduced and reactive towards $BrCH_2[^{14}C]CONHPhAsO$ (cf. Section 3.5.2). Under these conditions, significant inactivation and $[^{14}C]$-radiolabelling of E3 would have occurred in NEM-blocked PD complex treated with $BrCH_2[^{14}C]CONHPhAsO$.

The absence of $[^{14}C]$-radiolabel in the E2 component from NEM-blocked PD complex (treated with $BrCH_2[^{14}C]$-CONHPhAsO), indicates that the observed $[^{14}C]$-radiolabelling of E2 in native PD complex, inhibited by $BrCH_2[^{14}C]CONHPhAsO$, is indeed mediated via the lipoyl residues on E2.

3.5.6 Isolation of E3 from "Control C", "Control N" and "Test" PD Complex Samples

E3 was isolated from "control C", "control N" and "test" PD complex samples. The isolation procedures involved fractionation on hydroxylapatite in the presence of 8 M urea and were virtually identical to the E3 isolation experiments already described (Section 2.2.10 and

Figs. 3.4.4.1 and 3.4.4.2). The isolation profiles achieved are illustrated in Fig. 3.5.6.1. Purity of the final E3 preparations was assessed by $NaDodSO_4$-PAGE on 7.5% polyacrylamide gels.

The isolation of E3 by hydroxylapatite chromatography (Sections 3.4.4 and 3.4.8) has previously revealed that the yield of substrate-reduced E3 (inhibited with $BrCH_2[^{14}C]CONHPhAsO$) is always substantially lower than that found for control E3 when both enzymes are isolated from the PD complex. This phenomenon has previously been attributed to a decrease in affinity of substrate-reduced E3 for the hydroxylapatite matrix (Section 3.4.4). A careful examination of the yields of E3 from "control C", "test" and "control N", isolated in a similar fashion by hydroxylapatite chromatography (Fig. 3.5.6.1), has allowed this assumption to be critically tested.

The average yield of E3, inhibited with $BrCH_2[^{14}C]$-CONHPhAsO (control N), as determined in two separate experiments, was 62% compared to uninhibited E3 (control C). The average yield of E3, isolated from NEM-blocked PD complex (test) was 75% compared to uninhibited E3 (control C).

E3 isolated from NEM-blocked PD complex was reduced with excess NADH but was not modified with $BrCH_2[^{14}C]$-CONHPhAsO. This sample therefore provided an effective control parameter for estimating the effect of the specific alkylation of E3 on the affinity of the enzyme

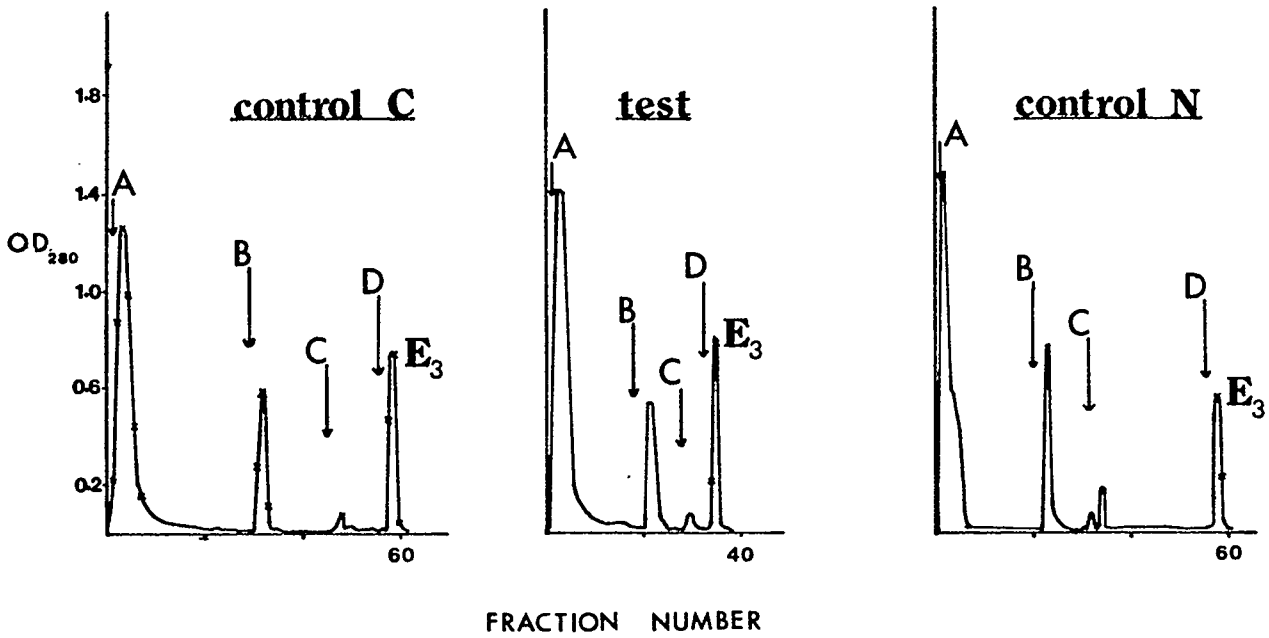


Figure 3.5.6.1 Isolation of E3 from controls and modified PD complex using hydroxylapatite chromatography. E3 was isolated from 10 mg control C, control N and test PD complex samples, pre-treated with trypsin, as described in Section 2.2.10. A,B,C and D refer to 10,50,100 and 500 mM sodium phosphate buffer (pH 7.0, 8 M in urea), respectively. Column flow rates varied from 1.5-3.0 mL/L. Fraction volumes were 1.2 mL. The elution position of E3 is analogous to previously described isolation experiments, Figs. 3.4.4.1 and 3.4.4.2. The definitions of control C, control N and test PD complex samples are presented in Fig. 3.5.4.1.

for hydroxylapatite. The results suggest that the major cause of the low yields of purified E3, inhibited with $BrCH_2[^{14}C]CONHPhAsO$, arises from substrate-reduction of the redox disulphide of E3, as discussed in Section 3.4.4.

The overall decrease in yield directly attributable to the chemical modification of E3 was approximately 13% and this was judged to be sufficiently low enough not to invalidate experiments undertaken to quantitate the extent of the modification of E3 (Section 3.6).

3.5.7 HVE and [^{14}C]-Radiochemical Analyses of E3 Isolated from "Test", "Control N" and "Control C" Samples of PD Complex

HVE at pH 6.5 and [^{14}C]-radiochemical analyses of acid hydrolysates of E3 isolated from "control C" and "test" PD complex samples revealed the presence of negligible amounts of [^{14}C]-radiolabel, whereas a similar amount of an acid hydrolysate of E3 from the "control N" PD complex sample contained a significant amount of [^{14}C]-radiolabel that was predominantly associated with N^3-carboxymethyl histidine (data not shown). The overall pattern of [^{14}C]-radiochemical labelling in this sample was virtually identical to that described for the earlier analysis of an acid hydrolysate of E3, inhibited with $BrCH_2[^{14}C]CONHPhAsO$ (whilst part of the PD complex) in the E3-mediated lipoyl system (Fig. 3.4.9.1).

3.6 Large Scale Inactivation of the PD Complex with $BrCH_2[^{14}C]CONHPhAsO$, Isolation and Amino Acid Analysis of $[^{14}C]$-Radiolabelled E3

3.6.1 Experimental Rationale

The experimental data reported in this section describe the large scale isolation of $[^{14}C]$-radiolabelled E3 and quantitation of the degree of alkylation of histidine by amino acid analysis. Quantities of $[^{14}C]$-radiolabelled E3 were then utilized for commencement of HPLC experiments to isolate a peptide containing the putative, active-site histidine residue (Section 3.7).

3.6.2 Inactivation of PD Complex with $BrCH_2[^{14}C]$-CONHPhAsO (El-Mediated System): Isolation and Amino Acid Analysis of $[^{14}C]$-Radiolabelled E3

PD complex (160 mg) in 20 mM phosphate buffer, pH 7.0 (4mL), was incubated with 0.3 mM $BrCH_2[^{14}C]CONHPhAsO$ (specific activity 3 x 10^3 dpm/nmol) in the presence of pyruvate (18 mM), Mg^{2+} (24.8 mM), TPP (2.2 mM) and coenzyme A (0.6 mM) at 0°C. Under these conditions, PD complex and E3 activities were rapidly lost and the reaction was terminated after 45 min by the addition of BAL (3 mM) which returned PD complex activity to about the level of E3 inactivation (48%). These results were in close agreement with those found for the small-scale inhibition of PD complex described in Fig. 3.4.2.1. A control vial of PD complex, incubated with $[^{14}C]$-radiolabelled bifunctional arsenoxide only, lost negligible PD complex and E3 activities.

E3 was isolated from PD complex, inhibited with $BrCH_2$ [^{14}C]CONHPhAsO, and from "control" PD complex by hydroxylapatite chromatography. Both E3 samples were subsequently acid hydrolysed as described previously (Section 2.2.10, Figs. 3.4.4.1 and 3.4.4.2). HVE and [^{14}C]-radiochemical analyses once again showed histidine to be uniquely modified in the sample of E3 that was inhibited with $BrCH_2$[^{14}C]CONHPhAsO.

Aliquots from both "inhibited" and "control" E3 samples were then subjected to automated amino acid analysis and liquid scintillation counting (as described in Sections 2.2.8 and 2.2.9). In addition to normal amino acids, the following modified amino acid standards were also run on the amino acid analyser: S-carboxymethyl cysteine, N^3-carboxymethyl histidine, N^1-carboxymethyl histidine and S-carboxymethyl homocysteine. Results from these analyses confirmed that the majority of [^{14}C]-radiolabel (65%) was associated with N^3-carboxymethyl histidine. In keeping with findings previously described in this thesis, no formation of N^1-carboxymethyl histidine occurred. The remainder of [^{14}C]-radiolabel could not be attributed to the presence of any other modified residues with the exception of S-carboxymethyl cysteine (7%). A similar amount of "control" E3 contained negligible amounts of [^{14}C]-radiolabel.

These results confirmed previous HVE and [^{14}C]-radiolabel analyses (Section 3.4.5). Because the

specific activity of $BrCH_2[^{14}C]CONHPhAsO$ was known, the extent of the modification of N^3-carboxymethyl histidine was quantitated as 0.37 ± 0.15 residues per mol of E3 (Table 3.6.2.1). The amount of E3 present in each analysis was determined from the quantitation of glutamic acid and glycine assuming 49 and 52 residues per mol of E3, respectively, as indicated in Table 3.6.2.1.

Assuming that alkylation of a histidine residue in an E3 molecule leads to 100% inhibition of that molecule, we would expect a total of 0.48 residues of acid stable N^3-carboxymethyl histidine to be present in the acid hydrolysate of E3 that was inhibited to a level of 48% residual activity.

During the purification of $[^{14}C]$-radiolabelled E3 on hydroxylapatite, the specific alkylation of E3 appeared to be responsible for a loss in yield of 10-15% (Section 3.5.6) compared with substrate-reduced E3 (non-inhibited). The quantitation of the extent of the modification of N^3-carboxymethyl histidine may therefore be an underestimation because of the possible loss of fully modified E3 on hydroxylapatite chromatography. With this in mind and allowing for the experimental error involved in this determination (Adamson and Stevenson, 1982), there is good agreement between the degree of modification of histidine as N^3-carboxymethyl histidine and the overall loss of E3 activity.

Table 3.6.2.1 Amino acid composition of lipoamide dehydrogenase

Sample	[^{14}C] -E3 [a] composition (E1-mediated system)	[^{14}C] -E3 [a] composition (E3-mediated system)	E3 [b] composition (Adamson, 1981)
Aspartic acid	42.3 (42)	42.3 (42)	41
Threonine	28.6 (29)	26.6 (27)	25
Serine	15.9 (16)	16.4 (16)	16
Glutamic acid	48.9 (49)	49.2 (49)	49
Glycine	52.4 (52)	51.7 (52)	52
Alanine	51.8 (52)	52.3 (52)	52
Valine	41.8 (42)	42.7 (43)	45
Methionine	8.2 (8)	8.1 (8)	8
Isoleucine	31.6 (32)	32.2 (32)	34
Leucine	35.3 (35)	33.2 (33)	33
Tyrosine	7.3 (7)	7.0 (7)	8
Phenylalanine	14.0 (14)	14.0 (14)	14
Histidine	11.9 (12)	13.6 (14)	13
Lysine	33.2 (33)	36.5 (37)	37
Arginine	17.2 (17)	16.1 (16)	16
N^3-carboxy-methyl histidine	0.37	0.3	-

Analyses were based, where possible, on the amounts of stable amino acids such as glycine, alanine and glutamic acid (Adamson, 1981; Williams, 1976).

a - [^{14}C]-radiolabelled E3 isolated on hydroxylapatite in phosphate buffer, pH 7.0, 8 M urea, after tryptic digestion of intact PD complex

b - Isolated on hydroxylapatite in phosphate buffer, pH 7.0, 8 M urea, with no prior tryptic digestion of intact PD complex

3.6.3 Inactivation of PD Complex with $BrCH_2[^{14}C]$-CoNHPhAsO (E3-Mediated System): Isolation and Amino Acid Analysis of $[^{14}C]$-Radiolabelled E3

PD complex (160 mg) in 20 mM phosphate buffer, pH 7.0, (4 mL), was incubated with 0.8 mM $BrCH_2[^{14}C]$CO-NHPhAsO (specific activity 3 x 10^3 dpm/nmol) in the presence of excess NADH (2.5 mM) at 0°C. Under these conditions, PD complex and E3 activities were lost quite rapidly and the reaction was terminated after 55 min by the addition of BAL (8 mM), which returned PD complex activity to about the level of E3 inactivation (50%). A control vial of PD complex incubated with $BrCH_2[^{14}C]$CO-NHPhAsO only, lost negligible PD complex and E3 activities.

E3 was again isolated from PD complex inhibited with $BrCH_2[^{14}C]$CONHPhAsO and from "control" PD complex, by hydroxylapatite chromatography. Aliquots of these preparations were hydrolysed to free amino acids with strong acid (6 M HCl) (Section 2.2.10). HVE and $[^{14}C]$-radiochemical analyses revealed that the $[^{14}C]$-radiolabelling of E3 was qualitatively identical to that found for the small scale inhibition described previously in Section 3.4.9, Figs. 3.4.9.1 and 3.4.9.2. Aliquots of acid hydrolysed E3 (from inhibited and control samples) were then subjected to automated amino acid analysis and liquid scintillation counting. The majority of $[^{14}C]$-radiolabel was again found to be associated with N^3-carboxymethyl histidine (60%), however, a significant proportion of $[^{14}C]$-radiolabel (25%) was found in advance of S-carboxymethyl cysteine on the amino acid chromatogram.

The radioactivity observed in advance of S-carboxymethyl cysteine may be attributed to the presence of acidic methionine sulphonium salt breakdown products: glycollic acid ($HOCH_2[^{14}C]COOH$) and methyl mercapto acetic acid ($CH_3S-CH_2[^{14}C]COOH$). Alkylation of methionine residues on E3 by $BrCH_2[^{14}C]CONHPhAsO$ would result in the formation of S-BAL-arsenophenylamidomethyl methionine sulphonium salt (Fig. 3.6.3.1).

During strong acid hydrolysis, this salt is unstable and should give rise to the acid breakdown products mentioned, as well as S-carboxymethyl homocysteine ($HOOC[^{14}C]CH_2SCH_2CH_2CH(NH_3^+)COOH$). $[^{14}C]$-Radiolabel appeared to be associated with S-carboxymethyl homocysteine when inhibited E3 (acid hydrolysed) was subjected to HVE and radiochemical analysis (cf. Fig. 3.4.9.1). However, the presence of $[^{14}C]$-radiolabelled S-carboxymethyl homocysteine was never detected on automated amino acid and radiochemical analyses. Thus the modification of methionine by $BrCH_2[^{14}C]CONHPhAsO$ remains open to question. A central objective of this study was to purify a peptide from E3 that contained the putative active-site histidine residue. Consequently, the possibility of a methionine modification occurring on E3, when the PD complex was inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the E3-mediated lipoyl system, was not actively pursued.

Quantitation of the degree of modification of histidine revealed the presence of 0.3 ± 0.15 residues of

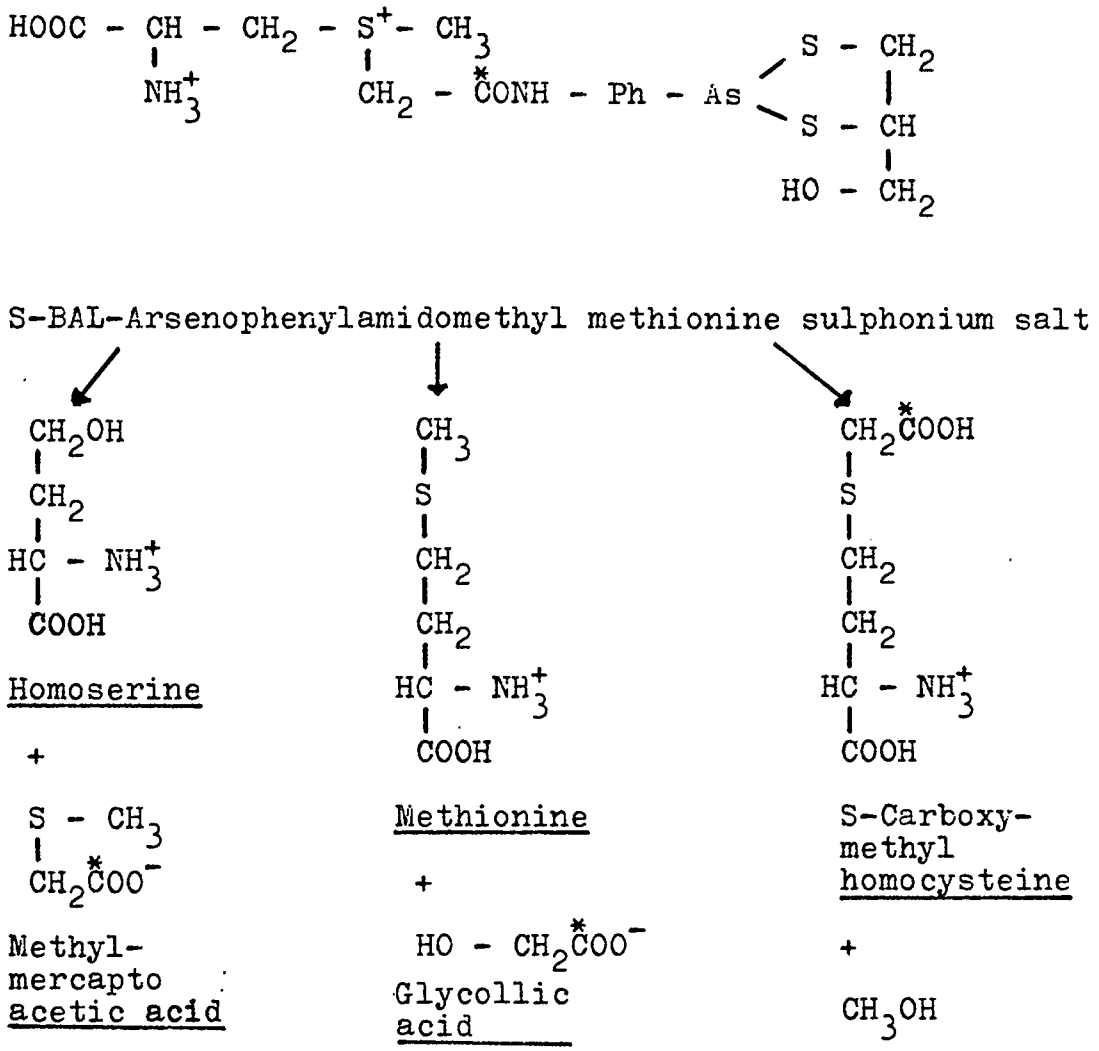


Figure 3.6.3.1 Decomposition of S-BAL-Arsenophenylamidomethyl methionine sulphonium salt in 6 M HCl (110 °C, 24 h) (Gurd, 1967; Stevenson and Smillie, 1970). [^{14}C]-Radiolabelled carbon is marked(*). The amide bond (-CONH-) present in the methionine sulphonium salt is labile under strong acid conditions.

N^3-carboxymethyl histidine per mol of E3 that was inactivated to 50% residual activity. Most of the loss in biological activity that occurs when E3 is inactivated with $BrCH_2[^{14}C]CONHPhAsO$ in the E3-mediated lipoyl system is attributable to the irreversible alkylation of a histidine residue. Although the value of 0.3 residues may be an underestimate as discussed previously, the loss in biological activity may also be due in part to the modification of another residue(s). Although a small amount (less than 0.05 residues per mol of E3) of S-carboxymethyl cysteine was detected in the acid hydrolysate of E3 (inhibited with $BrCH_2[^{14}C]CONHPhAsO$), this does not satisfactorily account for the additional loss of E3 activity observed. The remaining loss of activity may therefore have been due to modification of a methionine residue on E3. As mentioned previously, a detailed investigation of such a possibility was beyond the scope of this study.

The overall amino acid compositions of $[^{14}C]$-radiolabelled E3, from both E1- and E3-mediated lipoyl systems, are illustrated in Table 3.6.2.1. The analyses compare very favourably with previous amino acid composition data on E3 (Adamson, 1981; Williams, 1976). The findings described in this section provided further evidence for the role of a histidine residue in the catalytic mechanism of lipoamide dehydrogenase. A histidine residue in or near the active site of E3 was modified by the action of the bromoacetamido moiety of $BrCH_2$-$[^{14}C]CONHPhAsO$ when the PD complex was incubated with

this reagent in the presence of pyruvate, TPP, Mg^{2+} and coenzyme A (E1-mediated lipoyl system) or excess NADH (E3-mediated lipoyl system). The radiolabelling of histidine was more specific in the E1-mediated lipoyl system. Therefore, experiments designed to isolate an E3 peptide containing this residue, were conducted on E3 that had been inhibited (whilst part of the PD complex) with $BrCH_2[^{14}C]CONHPhAsO$ in this lipoyl system.

3.7 Isolation of a Peptide from E3 Containing the Putative Catalytic Histidine Residue

3.7.1 Experimental Rationale

Glutathione reductase from human erythrocytes has been shown to possess a catalytically essential histidine residue at position 467 of the 478 residue polypeptide chain (Krauth-Siegel *et al.*, 1982; Pai and Schulz 1983; Boggaram and Mannervik, 1978). In view of the catalytic and structural similarities between glutathione reductase and lipoamide dehydrogenase (pig heart) it was considered likely that a similar essential histidine residue, if present in lipoamide dehydrogenase, would also reside in the C-terminal regional of the enzyme. With this in mind, CNBr was used to cleave the E3 polypeptide chain. This reagent has been shown to specifically cleave polypeptides at methionine residues (Gross and Witkop, 1967). The overall reaction is illustrated schematically in Fig. 3.7.1.1. CNBr cleaves on the C-terminal side of methionine to form peptides ending in homoserine lactone

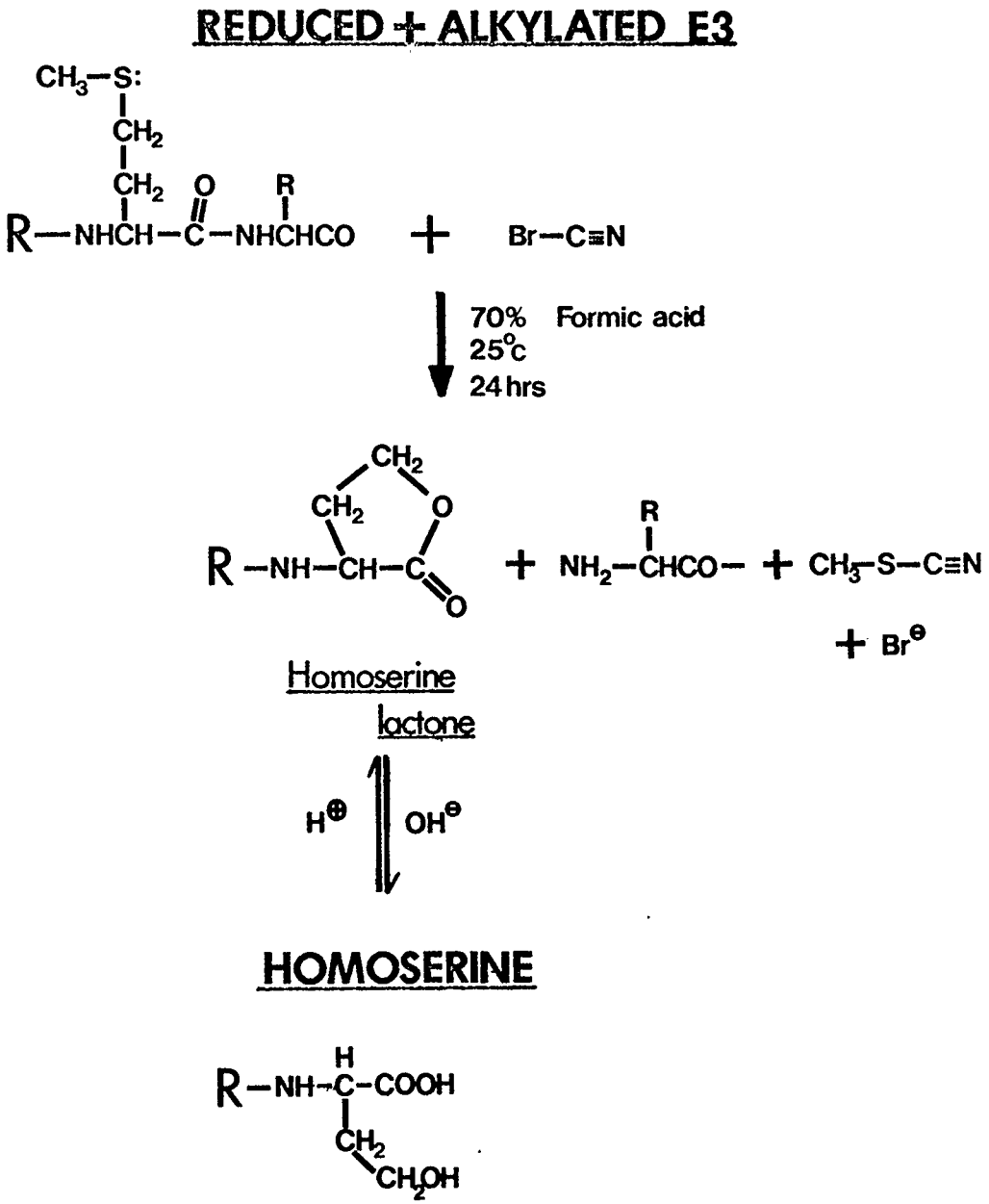


Figure 3.7.1.1 Cleavage of reduced and alkylated E3 with cyanogen bromide.

and homoserine, with the exception of the C-terminal peptide of the protein which can be readily identified by amino acid analysis since it contains no homoserine or homoserine lactone.

The isolation of E3 peptides generated by CNBr cleavage was accomplished by employing reverse phase high performance liquid chromatography techniques (RP/HPLC). These methods utilise a nonpolar stationary phase and a polar mobile phase. For the separation of peptides, the stationary phase usually consists of long chain alkyl groups bonded to small ($\leq$ 10 μm diameter) regular, rigid silica microspheres. These bonded silicas are fully porous (pore diameters 60-300 Å) and are packed in stainless steel columns (typically 10 mm x 25 cm). The mobile phase is usually a mixed organic/aqueous solvent. The large backpressure created by these columns have meant that high pressures (30-300 atm) are required to attain reasonable flow rates. RP/HPLC offers great advantages in resolution, speed and ease of sample recovery. In this study CNBr peptides from E3 were isolated using a cyanopropyl stationary phase and a mobile phase consisting of either acetonitrile/0.1% TFA or 2-propanol/ 0.1% TFA.

3.7.2 Cleavage of E3, Inhibited with $BrCH_2[^{14}C]CONHPhAsO$, with Cyanogen Bromide

$[^{14}C]$-Radiolabelled E3 was reduced with DTT and alkylated with iodoacetic acid (twofold excess over DTT)

as described in Section 2.2.14. Reduced and alkylated E3 was then digested with CNBr (Section 2.2.14). The success of CNBr cleavage was estimated by subjecting a sample of the CNBr digest of E3 to acid hydrolysis and automated amino acid analysis (Section 2.2.7). According to the reaction scheme outlined in Fig. 3.7.1.1, successful cleavage of E3 should involve cleavage of all methionine residues to homoserine lactone and homoserine. Consequently, by quantitating the amount of methionine present in the CNBr digest, the cleavage efficiency could be estimated. By this method, 0.1-1.0 methionine residues remained per mol of E3 after CNBr treatment. Assuming a normal E3 methionine content of 8 residues per mol (results from 3 separate analyses), this was equivalent to a cleavage efficiency of 88-99%. In addition, $NaDodSO_4$-PAGE was occasionally used to confirm the absence of uncleaved E3 in the CNBr digest (ie. no 56,000 M_r band attributable to intact E3 remained after CNBr treatment, data not shown).

3.7.3 HPLC Analysis of Peptides Generated by CNBr Cleavage of [^{14}C]-Radiolabelled E3

E3 contains 8-9 residues of methionine per mol of enzyme (Williams, 1976), and assuming cleavage with CNBr to have been largely complete, approximately 9-10 peptides were expected in the CNBr digest. Early attempts to resolve these peptides involved the use of RP/HPLC with an octadecyl (C_{18}) bonded stationary matrix (Section 2.2.16) and various mobile phases, including

acetonitrile or 2-propanol (both containing 0.1% TFA). These experiments were largely unsuccessful, presumably because of the relatively large size and hydrophobicity of the CNBr peptides. These problems were ultimately overcome by using a cyanopropyl (CNAQ) RP/HPLC column system. This column provided a bonded matrix that was much less hydrophobic than the octadecyl (C_{18}) matrix. Consequently, the hydrophobic CNBr peptides generated from E3 were less strongly adsorbed to the CNAQ column and more readily desorbed by 2-propanol and/or acetonitrile.

The optimal separation of peptides generated by CNBr cleavage of [^{14}C]-radiolabelled E3 is illustrated in Figs. 3.7.3.1 and 3.7.5.1. The system described, employed a mobile phase of acetonitrile/0.1% TFA and a gradient of acetonitrile/0.1% TFA to 0.1% TFA applied as shown in Figs. 3.7.3.1 and 3.7.5.1. The gradient was adjusted at various points throughout the elution profile to improve the resolution of individual peptides. In an initial analysis, all effluent from the column was collected in 2.2 mL fractions and analysed for radioactivity, as shown in Fig. 3.7.3.1. The majority of [^{14}C]-radiolabel was associated with a peak termed CNBr(X), smaller amounts of radioactivity were also associated the breakthrough peak (A) and the larger peptides in the overall digest (B, C and D) which eluted at higher concentrations of acetonitrile. The overall recovery of [^{14}C]-radiolabel from the CNAQ column varied from 40-50%. Acid hydrolysates of peaks A, CNBr(X), B, C

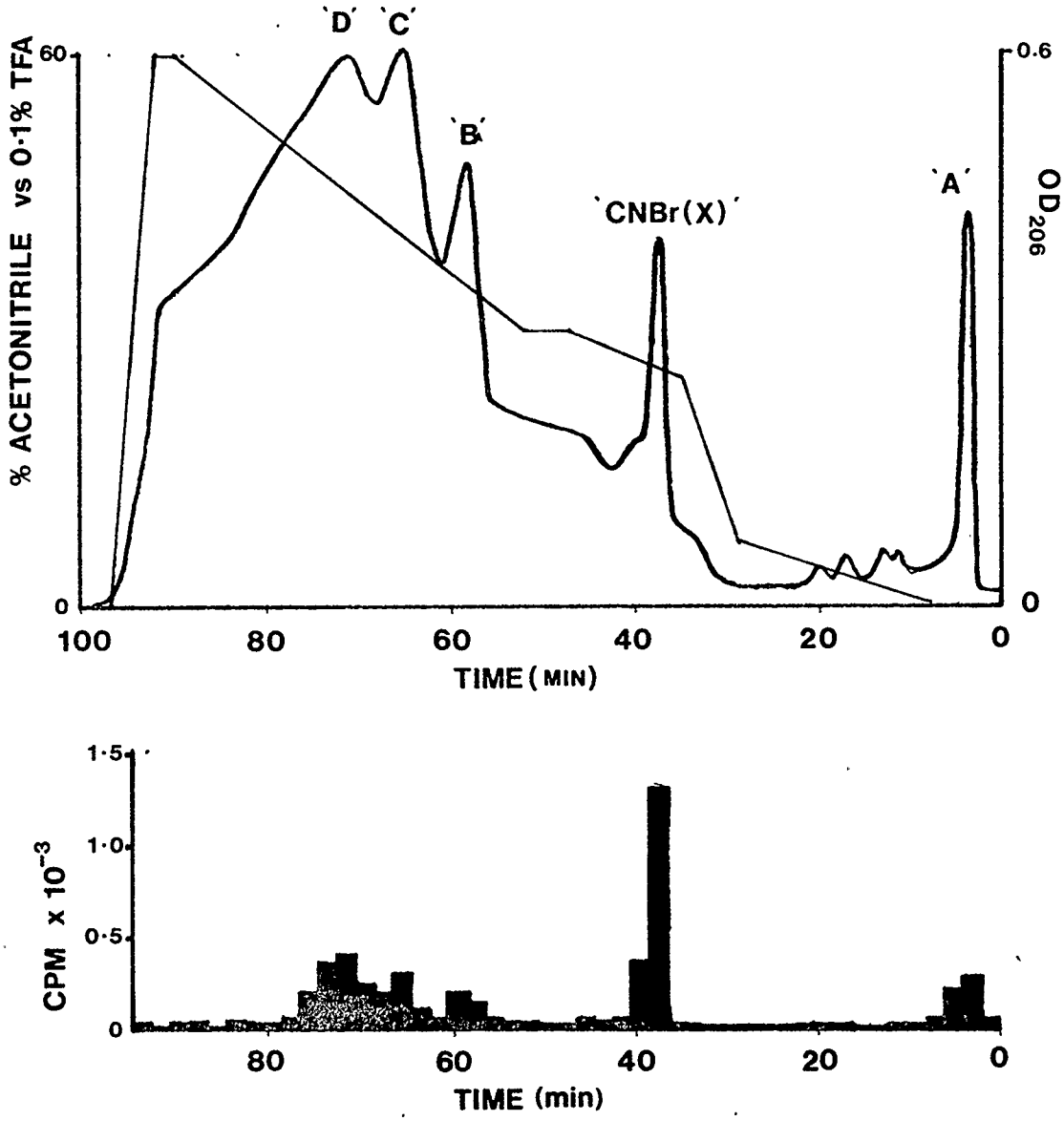


Figure 3.7.3.1 Reverse phase HPLC analysis of CNBr peptides from [^{14}C]-radiolabelled E3. The E3 digest (7 nmol) dissolved in 0.1% trifluoroacetic acid (TFA) was injected onto a Varian CNAQ column and peptides eluted with increasing concentrations of acetonitrile/0.1% TFA. The experimental procedure is fully described in Section 2.2.16. Peptides were monitored at 206 nm and peak collection was made by hand. Samples (2.2 mL) were collected every 2 min and counted for radioactivity. Peaks A,B,C,D and CNBr(X) are described in the text.

and D were analysed for the presence of modified amino acid residues by using HVE and [^{14}C]-radiochemical analysis (Section 2.2.8 and 2.2.9).

3.7.4 Identification of [^{14}C]-Radiolabelled Residues in Peptides Isolated from CNBr Digested E3 (Inhibited with [^{14}C]-Bifunctional Arsenoxide)

HVE and [^{14}C]-radiochemical analyses of peak A from the overall CNBr digest of E3 revealed the absence of any radioactivity associated with N^3-carboxymethyl histidine. The majority of [^{14}C]-radiolabel, albeit in small amounts (Fig. 3.7.3.1), appeared to be associated with S-carboxymethyl cysteine.

CNBr(X) was the most strongly [^{14}C]-radiolabelled peak in the digest (Fig. 3.7.3.1), due to the exclusive modification of a histidine residue(s) in the N^3 position of the imidazole ring by the bromoacetamido moiety of $BrCH_2$[^{14}C]CONHPhAsO. The specific labelling of the CNBr(X) peptide is illustrated in Fig. 3.7.4.1.

HVE and [^{14}C]-radiochemical analyses of peaks B, C and D revealed a nonspecific pattern of radiolabelling, although peaks C and D did contain small amounts of [^{14}C]-radiolabelled N^3-carboxymethyl histidine ($\leq$ 10% of that found in the CNBr(X) peptide); these samples were most likely contaminated with E3 protein and peptide that had remained undigested by CNBr.

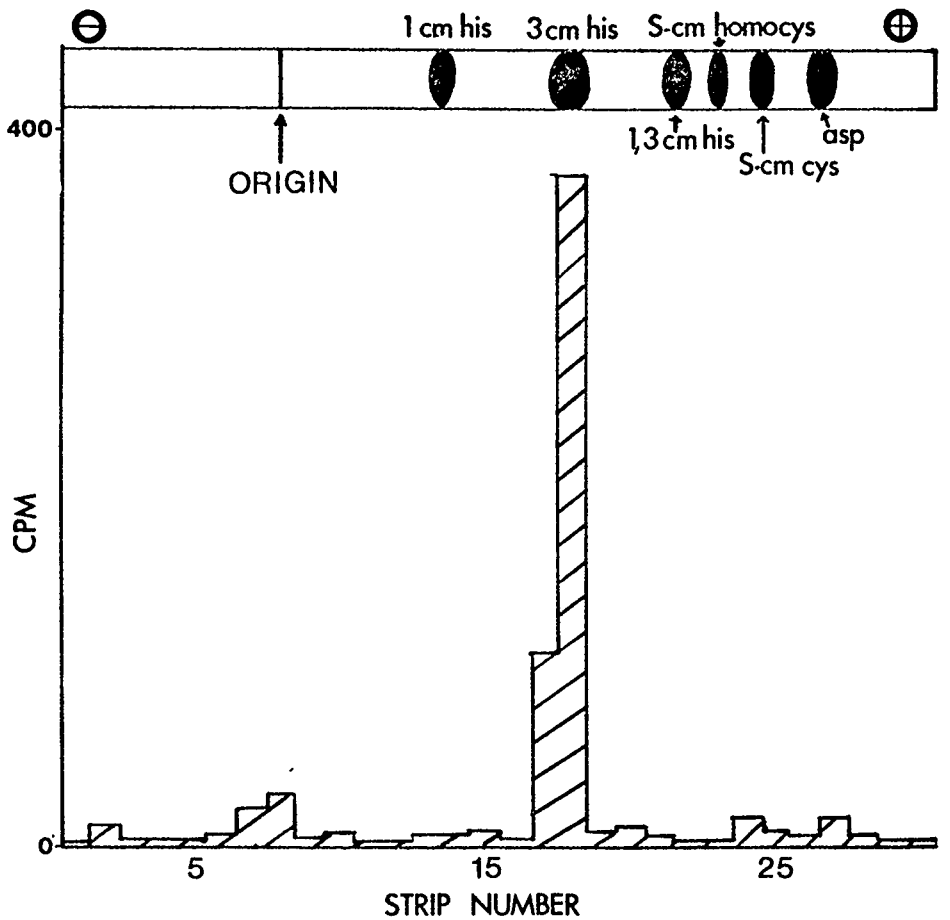


Figure 3.7.4.1 High voltage electrophoresis and radiochemical analysis of an acid hydrolysate of the CNBr(X) peptide. The experimental procedure was as described in Sections 2.2.8 and 2.2.9. Abbreviations for carboxymethylated amino acids are presented in Fig. 3.4.5.1.

3.7.5 Improved HPLC Resolution of [^{14}C]-Radiolabelled CNBr(X) Peptide and of Smaller Peptides in the CNBr Digest of E3

HPLC analyses of different CNBr digests were highly reproducible; the smaller, less hydrophobic peptides in the digest were largely resolved into 5 peaks at low % acetonitrile versus 0.1% TFA ratios by employing a shallow gradient.

When 2-propanol/0.1% TFA was used as the organic mobile phase, the CNBr(X) peptide eluted as a single, broadly resolved peak in about 25% of the organic solvent in the gradient. Resolution of this peak was improved by employing acetonitrile/0.1% TFA as an organic mobile phase (Fig. 3.7.3.1) and by flattening the gradient before and through the elution of the peptide (Fig. 3.7.5.1). Elution of the CNBr(X) peptide with acetonitrile required slightly higher concentrations of the organic solvent (26-28%). This was presumably due to the lower hydrophobicity of acetonitrile compared to 2-propanol. Acetonitrile was also superior to 2-propanol as a limiting solvent owing to its greater transparency which allowed smaller quantities of digest to be monitored at lower detector wavelengths.

HPLC analysis of CNBr peptides from nonradiolabelled E3 showed that the unmodified CNBr(X) peptide was largely unresolved from the [^{14}C]-radiolabelled peptide. Apparently the peptide was too large (34-36 residues, M_r

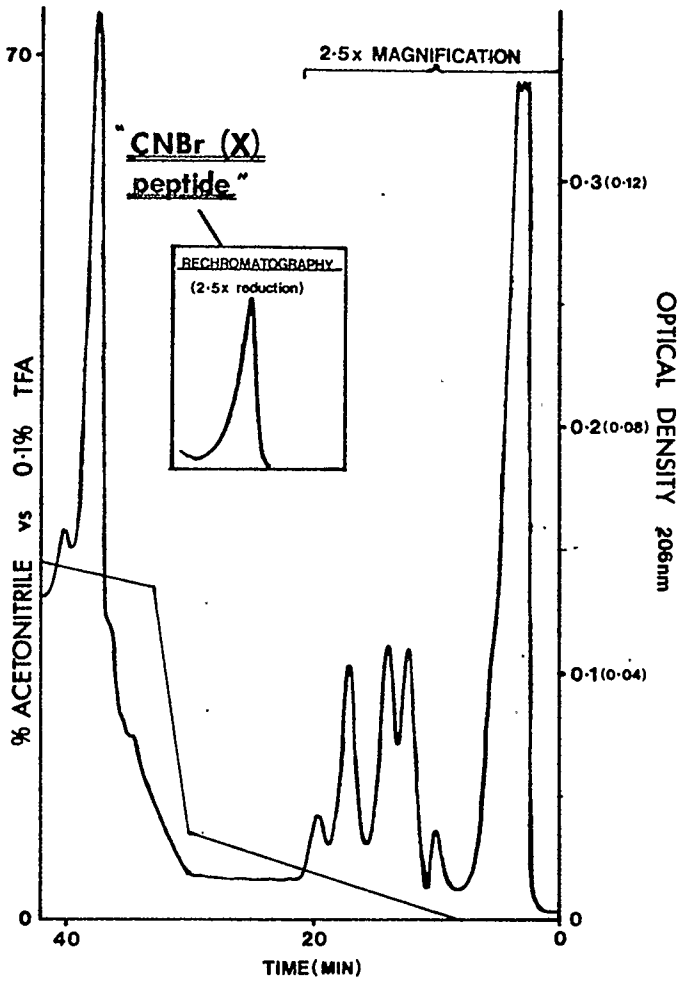


Figure 3.7.5.1 Reverse phase HPLC analysis of the smaller CNBr peptides of [^{14}C]-radiolabelled E3 at higher resolution. The E3 digest (6 nmol) was dissolved in 0.1% trifluoroacetic acid (TFA) and injected onto a Varian CNAQ column (flow rate 1.1 mL/h). The smaller CNBr peptides were eluted with low concentrations of limiting solvent (acetonitrile containing 0.1% TFA). The CNBr(X) peptide eluted in about 27-28% acetonitrile (0.1% TFA) versus 0.1% TFA. A smaller, largely nonradioactive peak consistently eluted after the CNBr(X) peptide. The CNBr(X) peptide rechromatographed as a single peak using the same HPLC elution programme. Further experimental details are described in Section 2.2.16.

3600-3900, Section 3.7.6) for its resolution on the CNAQ column system to be adversely affected by the unique modification of a histidine residue with $BrCH_2[^{14}C]CO$-NHPhAsO.

The isolation of the CNBr(X) peptide was hampered by the presence of an unidentified peak eluting at a slightly higher concentration of acetonitrile (29%). This peak may have been attributable to a smaller, hydrophobic peptide (Fig. 3.7.5.1). In a preliminary experiment HPLC column effluent was collected every 15 seconds as the CNBr(X) peptide and trailing peak were eluted. Liquid scintillation counting of these fractions showed that the vast majority of radioactivity was located at the tip of the CNBr(X) peak. The trailing peak appeared to contain little radioactivity.

The larger, more hydrophobic peptides in the digest (peaks B, C and D) were only partially resolved with the HPLC system described and possible ways to improve the isolation of these peptides will be discussed in Chapter 4.

In summary, the CNBr(X) peak was the only peptide in the digest to contain significant amounts of radioactivity. The $[^{14}C]$-radiolabel in the CNBr(X) peptide was solely attributable to the presence of histidine alkylated with $BrCH_2[^{14}C]CONHPhAsO$.

3.7.6 Amino Acid Composition of [^{14}C]-Radiolabelled CNBr(X) Peptide

The amino acid composition of the CNBr(X) peptide was determined in two separate experiments on different CNBr digests of E3 in which the enzyme was inhibited with [^{14}C]-radiolabelled bifunctional arsenoxide to 50% and 55% residual activity, respectively. The amino acid analyses and liquid scintillation counting were carried out as described in Sections 2.2.8 and 2.2.9. Virtually all the [^{14}C]-radiolabel (>95%) was associated with N^3-carboxymethyl histidine, thus confirming earlier HVE and [^{14}C]-radiochemical analyses of the peptide (Fig. 3.7.4.1).

The amount of N^3-carboxymethyl histidine present per mol of peptide was estimated to be 0.25 and 0.20 ($\pm$ 0.15) residues for analyses 1 and 2, respectively. When the overall data from these two analyses was averaged, the minimum total amino acid composition of the peptide was about 34 residues corresponding to a molecular weight of approximately 3,650 daltons (Table 3.7.6.1).

Careful inspection of the amino acid composition data revealed that the peptide contained approximately integral amounts of the more stable amino acids such as ala, asp, glu, gly, phe and lys. This finding, together with the fact that the CNBr(X) peptide rechromatographed on HPLC as a single peak (Fig. 3.7.5.1), gave a fairly good, though by no means conclusive, indication of the

Table 3.7.6.1 Amino acid composition of CNBr(X) peptide

Amino acid	Analysis 1	Analysis 2	Average Composition	
G	2.6	3.3	(2.95)	3
A	4.1	4.0	(4.05)	4
S-CM C	0.9	0.6	(0.75)	1
D	3.0	3.2	(3.1)	3
E	2.9	3.4	(3.15)	3
I	1.7	1.9	(1.8)	2
L	2.5	2.7	(2.6)	3
T	1.9	2.0	(1.95)	2
H	1.5	1.5		2
N^3-CM H	0.15 [a]	0.2 [b]		
P	1.8	1.6	(1.7)	2
S	1.4	1.7	(1.55)	2
V	1.7	2.0	(1.85)	2
F	1.1	1.0	(1.05)	1
K	3.0	3.2	(3.1)	3
R	0.4	0.6	(0.5)	1
HSe/ HSe lactone	-	-	none	
			Total	34

Analyses 1 and 2 were carried out on acid hydrolysates of CNBr(X) peptide, isolated from different CNBr digests of [^{14}C]-radiolabelled E3. HSe refers to homoserine, N^3-CM H refers to N^3-carboxymethyl histidine and S-CM C refers to S-carboxymethyl cysteine. The data are based, where possible, on the amounts of stable amino acids present i.e. ala and asp (4 and 3 residues/mol CNBr(X) peptide, respectively). Tryptophan content was not determined.

b - E3 inhibited to 50% residual activity with $BrCH_2[^{14}C]CONHPhAsO$

a - E3 inhibited to 55% residual activity with $BrCH_2[^{14}C]CONHPhAsO$

purity of the isolated peptide. An additional striking feature of the amino acid analysis results was that the CNBr(X) peptide appeared to be totally devoid of any homoserine or homoserine lactone. Preliminary amino acid analyses of all other peaks in the HPLC peptide map revealed the presence of homoserine and/or homoserine lactone. This indicated that the CNBr(X) peptide was the C-terminal peptide of lipoamide dehydrogenase.

The absorbance of the [^{14}C]-radiolabelled CNBr(X) peptide at 206 nm was higher than that expected when compared with other peptides in the digest. This is apparently attributable to the strong absorbance of the S-BAL arsenophenylamidomethyl moiety attached to histidine. Preliminary experiments showed that $BrCH_2CONHPhAsO$ (dissolved in acetonitrile) absorbs strongly in the region 200-210 nm. This absorption was further enhanced by the addition of BAL.

3.8 Amino Acid Sequence Analysis of CNBr(X) Peptide

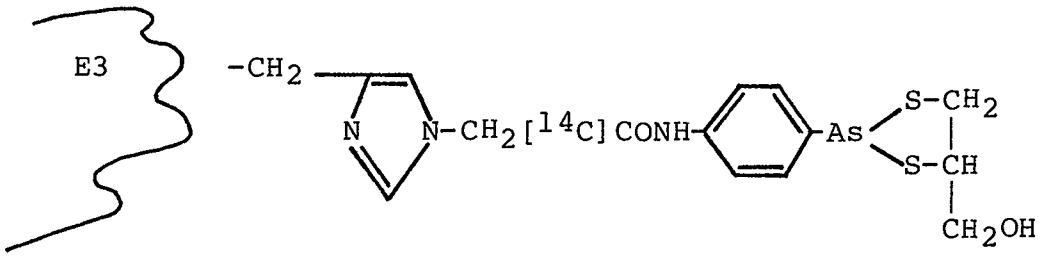
3.8.1 Introduction

Sequential Edman degradation of the CNBr(X) peptide was performed using the "spinning-cup" sequencer methodology first described by Edman and Begg (1967) and reviewed in Section 2.2.17.

Two independent analyses were carried out on samples of CNBr(X) peptide isolated in separate experiments. In

the first sequence run, the [^{14}C]-radiolabelled peptide (8-10 nmol) was degraded for 37 cycles and 50% of the PTH derivative(s) from each cycle analysed by HPLC (see Section 2.2.16). The remaining 50% of each PTH derivative was then subjected to scintillation counting. In this manner 25 out of a possible 34 amino acids were assigned a position in the sequence of the peptide (data not shown). Owing to technical difficulties, it was not possible to identify arginine, histidine or serine at this stage (as discussed in Section 2.2.17). Sequencer run number two was carried out on a larger amount of peptide (20 nmol). The results obtained were in excellent agreement with the previous analysis and are presented in Fig. 3.8.1.1 and Table 3.8.1.1.1. In this experiment, 20% of each PTH derivative was analysed by HPLC and a further 20% subjected to scintillation counting to detect [^{14}C]-radiolabel. The remainder was stored for possible back hydrolysis (with 6 M HCl) to the respective free amino acid.

Previous experiments in this study have described the specific radiolabelling of a histidine residue in or near the active site of E3 and the purification of a peptide containing this residue. The nature of this modification is illustrated below:



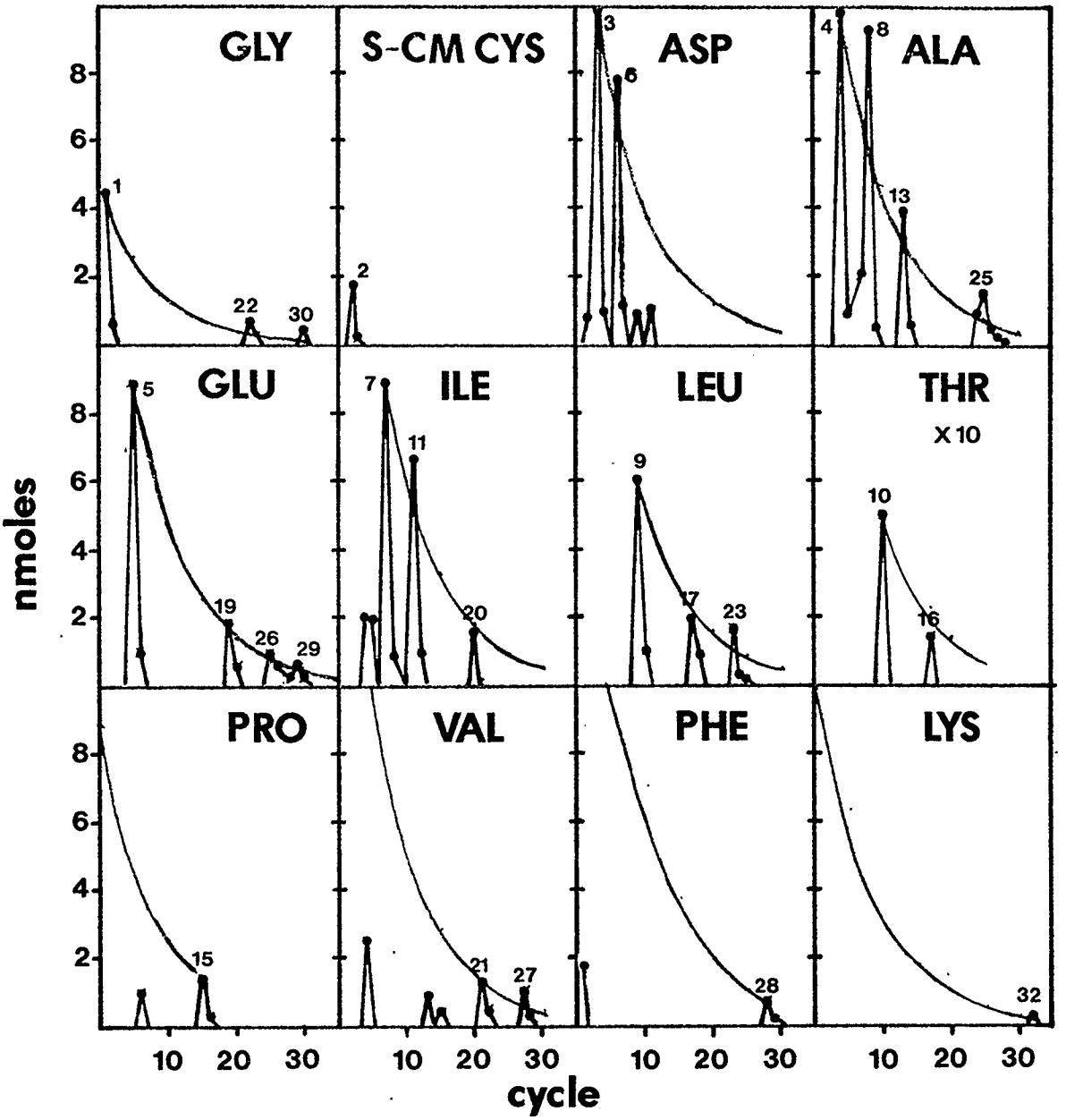


Figure 3.8.1.1 Automated amino acid sequence analysis of the CNBr(X) peptide. The peptide (approximately 20 nmol) was degraded for 38 cycles and each cycle analysed for PTH derivatives by HPLC (Section 2.2.17). The yields recovered from the sequencer are illustrated. Each curved line represents the theoretical yield of a particular PTH amino acid at every degradation cycle. Each line is based on a calculated repetitive yield of 88% (as described in the text) normalised to the first significant PTH amino acid peak. A PTH amino acid was assigned to a position in the CNBr(X) sequence if its yield at that cycle was close to or above the normalised curve. The probable assignment of PTH amino acids to positions in the sequence are illustrated.

Table 3.8.1.1.1 Assignment of amino acid residues to the sequence of the CNBr(X) peptide

Cycle Number	Assignment		PTH Amino Acids (pmol)
1	G	(Gly)	G(4,300), F(1,700)
2	C	(Cys)	S-CMC(1,700), G(600)
3	D	(Asp)	D(10,070), S-CMC(140)
4	A	(Ala)	A(9,900), D(1,000), V(2,500)
5	E	(Glu)	E(8,840), A(900), I(1,300)
6	D	(Asp)	D(7,750), P(1,000), E(800)
7	I	(Ile)	I(8,950), D(1,200), A(2,000)
8	A	(Ala)	A(9,200), I(800)
9	L	(Leu)	L(6,000), A(500), D(900)
10	T	(Thr)	T(500), L(1,000)
11	I	(Ile)	I(6,650), D(1,000)
12	H	(His)	H(300), I(1,000)
13	A	(Ala)	A(3,800), V(900)
14	H/H*	(His)	H*(100), H(100), A(500)
15	P	(Pro)	P(1,300), V(430)
16	T	(Thr)	T(140), P(300)
17	L	(Leu)	L(1,900)
18	-		L(1,000)
19	E	(Glu)	E(1,840)
20	I	(Ile)	I(1,500), E(600)
21	V	(Val)	V(1,300)
22	G	(Gly)	G(630), V(500)
23	L	(Leu)	L(1,600), G(300)
24	-		L(300), A(900)
25	A	(Ala)	A(1,430), L(200)
26	E	(Glu)	E(900), A(400)
27	V	(Val)	V(1,000), E(600), A(200)
28	F	(Phe)	F(700), V(300), E(250), A(100)
29	E	(Glu)	E(470), F(150), V(50)
30	G	(Gly)	G(300), E(300), F(90)
31	-		G(100)
32	K	(Lys)	K(200)
33	-		-
34	-		D(130)

H* represents the [^{14}C]-radiolabelled histidine previously identified by HVE, amino acid and radiochemical analysis of the CNBr(X) peptide.

The [^{14}C]-radiolabel was shown to be attached to histidine in the CNBr(X) peptide by HVE/radiochemical analysis and amino acid analysis/scintillation counting for [^{14}C]-radiolabel. Radiochemical analyses of all PTH derivatives obtained from the sequencer enabled the [^{14}C]-radiolabelled histidine derivative to be placed in the sequence of the CNBr(X) peptide. In both sequence runs, the only radioactive cycles corresponded to positions 14 and 15 (trace) in the CNBr(X) sequence (Fig. 3.8.2.1 and Table 3.8.1.1.1).

3.8.2 Elucidation of the Amino Acid Sequence Surrounding [^{14}C]-Radiolabelled Histidine in the CNBr(X) Peptide

The assignment of a particular amino acid residue to a position in the sequence of the CNBr(X) peptide was rationalised as follows.

The N-terminal residue of the CNBr(X) peptide was determined to be glycine. Although the first cycle from the Beckman sequencer also contained PTH phenylalanine, the amount present (1,700 pmol) was only 28% compared with PTH glycine (4,300 pmol). Furthermore glycine is known to be only partially cleaved (60-70%) during the Edman degradation (Niall, 1973; Tarr 1977). Cysteine was assigned to position 2 since cycle 2 contained only PTH (OMe) S-carboxymethyl cysteine and a small amount of PTH glycine (overlap from the previous sequencer cycle). Position 3 was determined to be aspartic acid, since

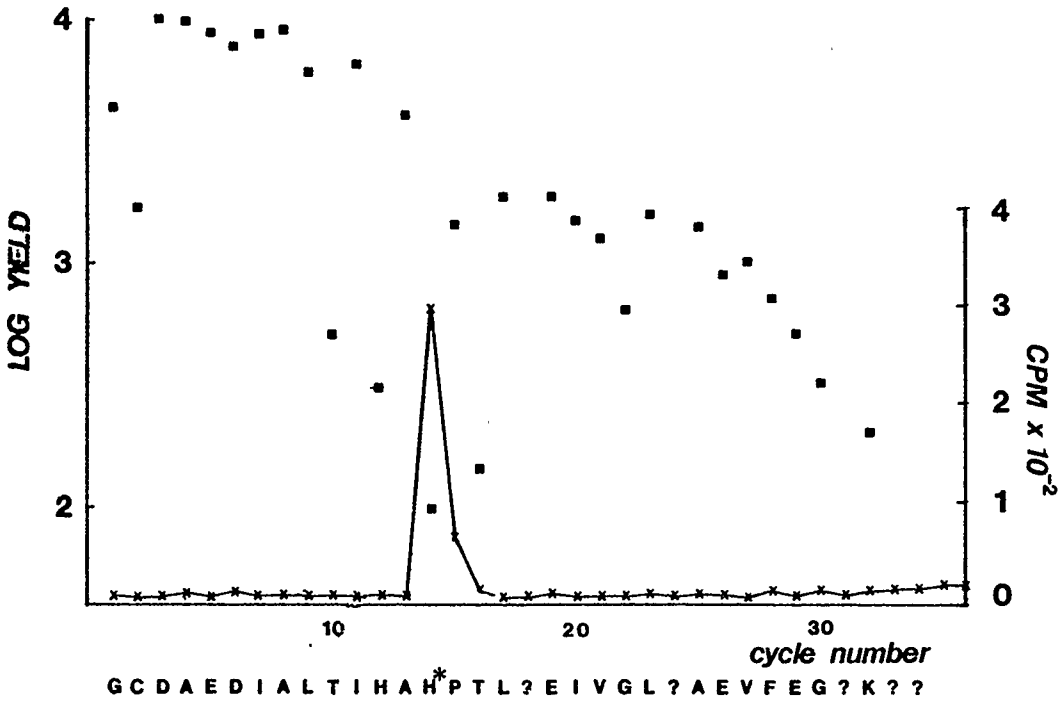


Figure 3.8.2.1 Placement of [^{14}C]-radiolabelled, alkylated histidine in the amino acid sequence of the CNBr(X) peptide. ■ - Represents the log (yield in pmol) of PTH derivative. x—x - Represents the radioactivity found at each cycle of the sequencer analysis. H* - Represents the histidine residue modified with $BrCH_2$[^{14}C]CONHPhAsO, as determined by HVE, amino acid and radiochemical analysis of the CNBr(X) peptide.

cycle 3 contained PTH (OMe) aspartic acid (10,070 pmol), a trace of PTH (OMe) S-carboxymethyl cysteine as overlap from cycle 2 and no other contaminating PTH amino acid derivatives. Cycle 4 contained mostly PTH alanine (9,900 pmol), PTH (OMe) aspartic acid (overlap from cycle 3) and PTH valine (2,500 pmol). Since the ratio of PTH valine to PTH alanine was 20:80, alanine was assigned to position 4. Cycles 5, 6 and 7 each contained primarily one PTH derivative (Table 3.8.1.1.1), the ratios of contaminating PTH derivative to assigned PTH derivative were 13:87, 11:89 and 18:82, respectively. Therefore, glutamic acid, aspartic acid and isoleucine were assigned to positions 5, 6 and 7 in the amino acid sequence. Alanine was assigned to position 8 since cycle 8 contained PTH alanine and a trace of PTH isoleucine (overlap from cycle 7) only. Cycles 9 and 11 predominantly consisted of PTH leucine and PTH isoleucine, respectively, therefore leucine and isoleucine were assigned to these positions in the peptide sequence. Cycles 10 and 16 each contained small amounts of PTH threonine as well as PTH leucine and PTH proline, which were "overlaps" from cycles 9 and 15, respectively. The assignment of threonine to positions 10 and 16 in the sequence was corroborated by back hydrolysis (with 6 M HCl) of 80% of the PTH derivative(s), obtained in cycles 10 and 16 from the sequencer, to α-aminobutyric acid (detected by amino acid analysis Section 2.2.17).

HPLC analysis of 10% of the PTH derivative(s) from cycle 12 revealed the presence of PTH isoleucine (1,000

pmol overall) which was attributed to "overlap" from cycle 11, no other PTH derivatives were detectable. Back hydrolysis and amino acid analysis of a further 70% of this cycle revealed the presence of histidine, albeit in low yield (300 pmol overall). Recovery of PTH histidine from the Beckman Sequencer has always been very poor (Section 2.2.17), therefore, histidine was tentatively assigned to position 12 in the peptide sequence. Cycles 13 and 15 contained predominantly PTH alanine (3,800 pmol) and PTH proline (1,300 pmol), respectively, though the yield of each PTH derivative was low. The low yield of PTH proline is most easily rationalised since this residue (an amino acid) is known to be cleaved with difficulty during the Edman degradation, especially if the subsequent residue is histidine, threonine, glutamic acid or aspartic acid (Tarr, 1977). The low yield of PTH alanine in cycle 13 may be partly explained by the nature of the amino acid residue in position 14 of the sequence. Analysis of each sequencer cycle for radioactivity revealed that [^{14}C]-radiolabelled histidine (100 pmol) was uniquely present in cycle 14. Back hydrolysis and amino acid analysis of this cycle also revealed the presence of histidine (100 pmol). Amino acid analysis of the CNBr(X) peptide had previously showed that the CNBr(X) peptide contained 0.2 residues per mol. Therefore, the yield of PTH histidine was low when compared with that of [^{14}C]-radiolabelled PTH histidine. Apparently histidine, alkylated with $BrCH_2$[^{14}C]CONHPhAs-BAL, is more easily extracted into organic solvents and is therefore recovered in greater yield following cleavage and conversion reactions in the sequencer and sequemat, respectively (Section 2.2.17).

Tarr (1977) has described the adverse effect of histidine (present in position 14 in 80% of the CNBr(X) peptide) on the cleavage of a preceding residue during the Edman degradation. Furthermore, the presence of a large, hydrophobic arsenoxide-BAL moiety attached to histidine in position 14 (in 20% of the CNBr(X) peptide) may interfere with coupling and cleavage reactions in the vicinity of this residue. These factors may account for the low yield of PTH alanine in cycle 13.

By knowing the yield of a given PTH residue at two different cycles during the sequencer run, the repetitive yield (R) between these cycles can be calculated:

$$R = \left(\frac{B}{A}\right)^{\frac{1}{N_B - N_A}}$$

Where B = percentage of residue at step B, A = percentage of residue at step A, N_A is the numerical position of residue A and N_B is the numerical position of residue B.

Using this procedure, the overall repetitive yield between cycles 5 and 29 (both glutamic acid) was 88%. A plot of log (yield in pmol) versus cycle number (Fig. 3.8.2.1) revealed a distinct drop in yield between cycles 12 and 18 in the sequencer analysis (an almost identical pattern was also apparent in the sequencer run on 10 nmol CNBr(X) peptide).

The repetitive yield up to cycle 11, as judged by the yields of isoleucine at cycles 7 and 11, was approximately 93%. The repetitive yield from cycles 21 (val) to cycle 27 (val) was 98%. The CNBr(X) peptide was sequenced between cycles 17 and 27 with good repetitive yield enabling the latter half of the peptide sequence to be largely elucidated. Cycles 17 and 19 contained only PTH leucine (1,900 pmol) and PTH glutamic acid (1,840 pmol), respectively. Cycle 18 contained PTH leucine, however the amount present was approximately half of the expected value (assuming a repetitive yield of approximately 98% and knowing the yield of the previous cycle) and was probably "overlap" from the preceding residue. Further analysis of this cycle by back hydrolysis and amino acid analysis failed to identify any amino acid present at this position in the sequence.

The identities of cycles 20-32 are illustrated in Table 3.2.1.1.1. Position 24, 31, 33 and beyond, remained unassignable.

Overlap of PTH derivatives from one cycle to the next was approximately 10% during the first few cycles of the sequence run, this steadily increased throughout the run and was often in excess of 30% by cycle 30. Furthermore, the yields of PTH derivatives began to drop appreciably after cycle 27. Consideration of these factors meant that amino acids were assigned positions in the overall sequence with increasing uncertainty toward the C-terminus of the CNBr(X) peptide.

The yield of PTH glycine in cycle 1 of the sequence analysis of the CNBr(X) peptide was 4,300 pmol. Therefore, since 20 nmol CNBr(X) peptide containing 7,500 cpm of [^{14}C]-radiolabel was applied to the sequencer, the initial yield of peptide was 22%.

The overall sequence obtained is in good agreement with the amino acid composition described previously (cf. Table 3.7.5.1), the amounts of val(2), gly(3), ala(4), his(2), S-CM cys(1), thr(2), phe(1) and leu(3) are identical in both cases. In the sequence, 5 positions were unidentified, this corresponds to 6 residues from the amino acid composition which have yet to be accounted for, namely: pro(1), ser(2), arg(1) and lys(2). Further discrepancies involve glutamic acid (3 residues in the composition versus 4 already placed in the sequence) and isoleucine (2 residues in the composition versus 3 already placed in the sequence). The low value of isoleucine in the amino acid composition may be explained by the presence of an ile-val bond in the peptide that would be resistant to acid hydrolysis, however the discrepancy in the number of glutamic acid residues is at present unclear. In view of these discrepancies, interpretation of the amino acid composition data may have led to an underestimation of the true length of the C-terminal CNBr(X) peptide which could actually be 36-37 amino acid residues in length.

Cycles 1, 4, 5, 6, 7, 9, 11, 13 and 15 were found to contain varying amounts of contaminating PTH amino acids.

The average contamination was approximately 15-18% and although undesirable, this was judged not to have interfered with the assignment of amino acid residues to these positions in the sequence of the CNBr(X) peptide.

However, the presence of these contaminating residues probably indicated that small quantities of a smaller peptide had copurified with the CNBr(X) peptide (see Section 3.7.5, Fig. 3.7.5.1) and therefore, may have been responsible for discrepancies arising in the amino acid composition data.

3.8.3 Treatment of CNBr(X) Peptide with Trypsin

The C-terminal residues of lipoamide dehydrogenase from *E. coli* are known to be lys-lys (Williams, 1976). This satisfactorily accounts for the two extra lysine residues not placed in the amino acid sequence but found in the amino acid composition of the peptide.

Preliminary experiments (data not shown), in which the CNBr(X) peptide was incubated with trypsin (see Section 2.2.15) suggested that the peptide was apparently unaffected by the proteolytic enzyme (i.e. it had the same retention time on HPLC analysis as the untreated peptide). The fact that the CNBr(X) peptide was largely not cleaved by trypsin argues for the placement of the previously unaccounted for arginine residue near the C-terminus of the peptide, since the presence of a trypsin

resistant arg-pro bond has been ruled out between residues 1 to 29. The CNBr(X) peptide presumably contains 3 lysine residues clustered near the C-terminus, therefore, even if tryptic cleavage at lysine and arginine did occur the peptide generated would be largely unaltered in size and would be expected to have a retention time similar to untreated CNBr(X) on HPLC analysis.

In summary, the C-terminal peptide containing a catalytically essential histidine residue was largely sequenced. The position of the histidine residue in the peptide was located by virtue of the [^{14}C]-radiochemical label attached to this residue during the inactivation of E3. The sequence surrounding this active-site residue was homologous to an active-site sequence in the C-terminal region of glutathione reductase (Fig. 4.5.1). The position of the active-site histidine residue in the CNBr(X) peptide from E3 corresponds exactly to histidine-467 which has been implicated as an essential catalytic residue in the mechanism of glutathione reductase (Boggaram and Mannervik, 1978; Krauth-Seigel *et al.*, 1982; Pai and Schulz, 1983).

The homology of the CNBr(X) peptide to glutathione reductase and pig heart lipoamide dehydrogenase will be fully discussed in the next chapter.

3.9 Isolation of Additional CNBr Peptides from E3

In earlier experiments in which the [^{14}C]-radiolabelled CNBr(X) peptide was purified from E3 (Section 3.7), it was not possible to fully resolve the smaller

CNBr peptides that eluted in low concentrations of % acetonitrite versus 0.1% TFA (cf. Fig. 3.7.5.1). These peptides were more efficiently resolved by using a similar HPLC programme with the exception that 2-propanol containing 0.1% TFA was used as the limiting solvent in place of acetonitrile. Using this approach, six peptides were resolved and were rechromatographed as single, symmetrical peaks (Fig. 3.9.1). Each peptide is currently being characterised in this laboratory, prior to amino acid sequence analysis.

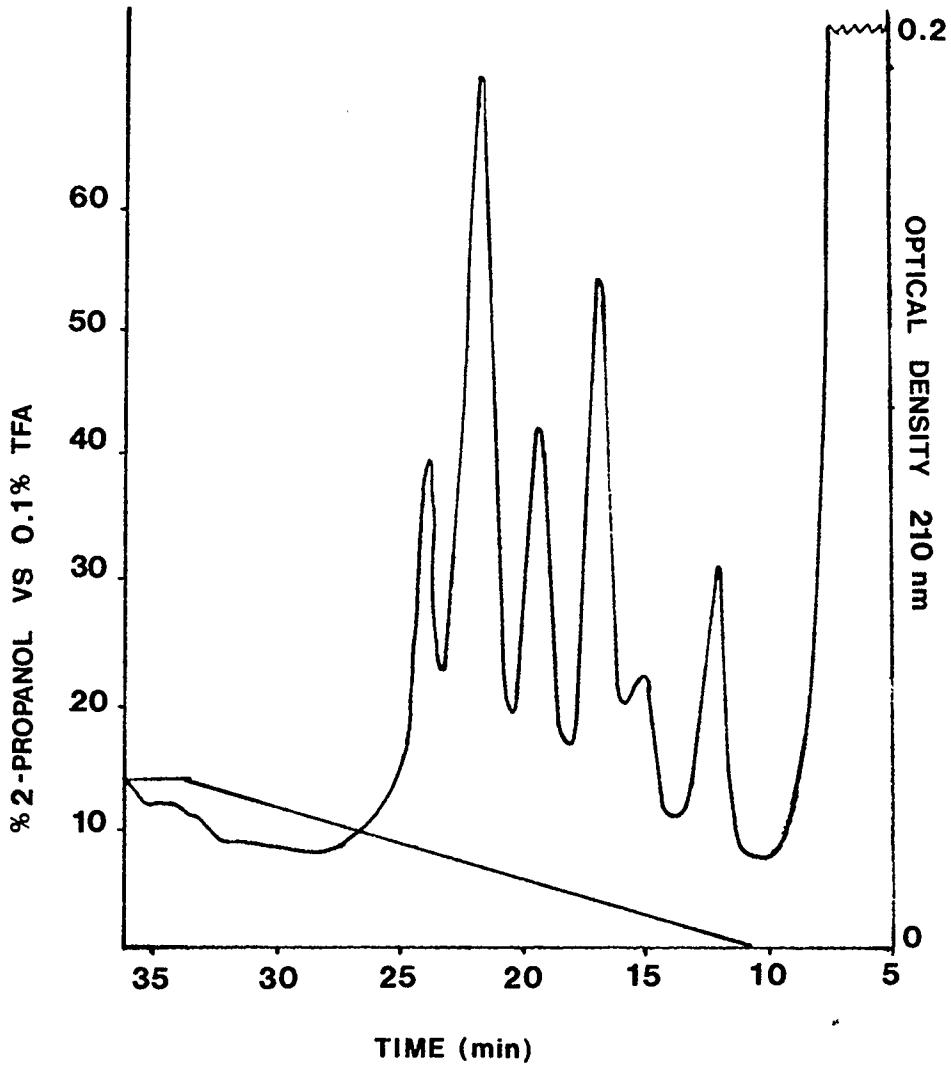


Figure 3.9.1 Improved reverse phase HPLC analysis of small CNBr peptides from E3. The digest (10-20 nmol) was dissolved in 0.1% TFA and analysed on a Varian CNAQ column. Several peptides in the digest were resolved in low concentrations of 2-propanol/0.1% TFA as a limiting solvent system. They are assumed to be quite small since they each have a relatively low absorbance at 210 nm. The HPLC column flow rate was 0.7 mL/h.

CHAPTER 4 - DISCUSSION

4.1 Growth of E. Coli K12

A protocol for the large scale growth of a mutant strain of *E. coli* K12 cells, constitutive for PD complex production, was developed in the initial stages of this study. Efficient oxygenation and agitation of the growing bacterial culture were found to be limiting factors in the scale up of *E. coli* growth to a culture volume of 200 L. Since *E. coli* is a facultative anaerobe, insufficient oxygenation can cause the bacterial culture to grow anaerobically.

The anaerobic growth of *E. coli* cells in the 450 L fermenter (from a small innoculum grown aerobically) is characterised by a greatly lengthened lag phase, a slower exponential phase and a rapid decrease in the pH of the medium. The slower growth rate presumably reflects the decreased efficiency with which the bacterium can generate ATP anaerobically, whilst the fall in pH is due to the increased production of organic acids, such as lactate. In future experiments, incorporation of an extra oxygen supply into the fermenter vat may enable the scale of growth to be improved to 400 L.

4.2 Purification of PD Complex from E. Coli

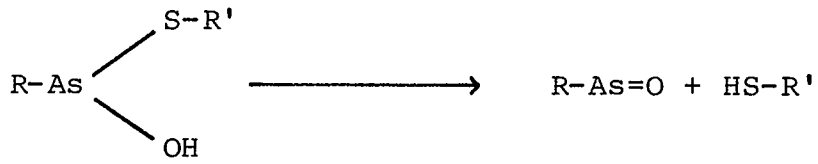
Of the two methods evaluated for isolation of the PD complex from *E. coli*, the procedure devised by Bisswanger

(1981) was useful only when small quantities of PD complex were required. The method described by Reed and Mukherjee (1969) gave rise to pure PD complex in superior yields. The results obtained compared very favourably with the findings of other workers (Perham, 1982; Adamson, 1981; Stepp *et al.*, 1981). The ability of the *E. coli* K12 mutant to overproduce PD complex was exemplified by the low purification factor (25-50) needed to satisfactorily isolate the enzyme system by either method.

4.3 Arsenoxide Inhibition Studies

In the PD complex, nucleophilic side chains capable of reacting with the bromoacetamido moiety of this reagent include cysteine, histidine, lysine, methionine and tyrosine. The possible sites for arsenoxide action are the reduced lipoyl-lysine residues of E2 or the reduced active-site disulphide of E3.

The mechanism by which mono-substituted arsenoxides (R-As=0) react with vicinal thiol groups is poorly understood at present. Webb (1966) has suggested that the hydrated species of the arsenoxide ($R\text{-}As{=}O \xrightarrow{H_2O} R\text{-}As(OH)_2$) may be the actual reactant in aqueous solutions. Parker and Karash (1959) have shown that the structure R-AsOH(SR') rapidly decomposes to reform the arsenoxide



Consequently, the reaction of R-AsO with monothiols is unlikely. In contrast R-AsO has been shown to form stable complexes with thiol groups when they are arranged close enough for ring formation to occur.

The reactions of mono-substituted arsenoxides with the PD complex from E. coli have been extensively studied in this laboratory (Stevenson et al., 1978; Adamson and Stevenson, 1981). The organic arsenoxide NH_2PhAsO has a considerably higher affinity for dihydolipoyl-lysine residues on E2 than the reduced active-site disulphide on E3. The complex formed between dihydolipoyl-lysine residues and the reagent is a six-membered ring structure. This structure is presumably more thermodynamically stable than the larger complex formed between the reagent and the reduced active-site disulphide on E3, in which the thiol groups are separated by four amino acid residues (Brown and Perham, 1972; Burleigh and Williams, 1972). The specific interaction of the arsenoxide moiety of $BrCH_2[^{14}C]CONHPhAsO$ with reduced lipoyl groups on E2 is therefore well defined.

It has been proposed (Stevenson, 1978) that the PD complex is inhibited by $BrCH_2[^{14}C]CONHPhAsO$ in a manner consistent with the delivery of the bromoacetamido moiety of the reagent into the active-site of E3 by arsenoxide modified dihydrolipoyl residues on E2. The irreversible inactivation of E3 was attributed to the alkylation of an active-site residue (Section 3.4.1, Fig. 3.4.1.1). When E3, inhibited with $BrCH_2[^{14}C]CONHPhAsO$ (E1-mediated

lipoyl system), was isolated from the PD complex, the predominant site of alkylation on E3 was identified as a histidine residue. The degree of histidine modification correlated well with the original loss of E3 activity (Adamson and Stevenson, 1982). These findings were confirmed and extended in the initial part of this study.

In $NaDodSO_4$-PAGE and [^{14}C]-radiolabel analyses of PD complex, inhibited with $BrCH_2$[^{14}C]CONHPhAsO (E1-mediated lipoyl system), the radioactivity was predominantly associated with E3. E2 contained a small amount of [^{14}C]-radiolabel, whilst E1 remained virtually unmodified. According to the accepted reaction mechanism, lipoyl-lysine residues on E2 interact with the active sites of E1, E2 and E3 in the PD complex. The selective radiolabelling of E3 is attributable to the specific modification of a histidine residue in or near the active site, however, the virtual absence of radiolabel in E1 and E2 is more difficult to rationalise. In the E1-mediated lipoyl system, coenzyme A bound at the active site of E2 may protect nucleophilic residues from modification with $BrCH_2$[^{14}C]CONHPHAsO. Since an extensive lipoyl-lipoyl interaction network for the exchange of electrons and possibly acetyl groups is present in the PD complex (Reed and Oliver, 1982; Hackert _et al._, 1983), the small level of radioactivity present in E2 may reflect alkylation of lipoyl groups present on one or more other subunits.

In this study no direct interaction between modified lipoyl-lysine residues and E1 could be demonstrated.

This suggests that lipoyl residues may not interact directly with E1 during catalysis. This finding is consistent with an earlier suggestion (Bleile *et al.*, 1981), that an amino acid side chain on an E2 lipoyl domain may participate in the acetyl group transfer from E1 to E2 bound lipoyl-lysine residues. Alternatively, structural features of the lipoyl domain itself may facilitate binding of lipoyl residues to E1 (Adamson, 1981). A clear distinction cannot be made between these two models, however, subsequent work (Perham *et al.*, 1981; Stepp *et al.*, 1981; Reed and Oliver, 1982; Hackert *et al.*, 1983) has revealed that active-site coupling within the PD complex relies heavily upon the flexibility of entire lipoyl domains. This mobility allows E1 to be serviced by at least two entire lipoyl domains and not just by lipoyl-lysine residues.

The inhibition of the PD complex with $BrCH_2[^{14}C]$-CONHPhAsO in the presence of excess NADH (E3-mediated lipoyl system) was investigated. The inactivation of E3 in this system was found to be slower than in the E1-mediated system, accordingly increased amounts of $BrCH_2[^{14}C]$CONHPhAsO were needed to obtain an equivalent level of E3 inhibition. These findings were consistent with preliminary results obtained previously in this laboratory with nonradioactive $BrCH_2$CONHPhAsO (Adamson, 1981). The presence of NADH, bound close to the active site of E3 may well interfere with the alkylation of histidine by $BrCH_2[^{14}C]$CONHPhAsO.

Amino acid and radiochemical analyses of E3, inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the E3-mediated lipoyl system, revealed that the loss of E3 activity was primarily attributable to the alkylation of a histidine residue. However, the $[^{14}C]$-radiolabelling of E3 was not as selective as E3 inhibited with $BrCH_2[^{14}C]CONHPhAsO$ in the E1-mediated lipoyl system. Partial modification of a methionine residue in or near the active site of E3 may also account for some loss of E3 activity. Amino acid and radiochemical analyses of E3 inhibited with $BrCH_2[^{14}C]CONHPhAsO$ provided evidence for the presence of acidic radioactive products (methylmercapto acetic acid and/or glycollic acid) attributable to the possible breakdown of a $[^{14}C]$-radiolabelled methionine sulphonium salt (Fig. 3.3.1).

HVE and radiochemical analyses of an acid hydrolysate of inhibited E3 contained a radioactive peak that was apparently attributable to S-carboxymethyl homocysteine, however a more thorough investigation by amino acid analysis, coupled with scintillation counting of column effluent, failed to identify any S-carboxymethyl homocysteine in the E3 hydrolysate.

Modification of a methionine residue on E3 with $BrCH_2[^{14}C]CONHPhAsO$ gives rise to the S-BAL-arsenophenyl amidomethyl methionine sulphonium salt (Fig. 3.3.1) which, after treatment with strong acid can decompose as shown. Adamson and Stevenson (1981) have pointed out the difficulty in trying to predict the relative quantities

of these breakdown products. For instance, steric or inductive effects brought about by the BAL-arseno adduct may minimise fragmentation of the $-S-CH_3$ bond. This may account for the lack of S-carboxymethyl homocysteine in the hydrolysis of inhibited E3 in which other methionine sulphonium salt breakdown products are visualised. Despite this, the possible modification of a methionine residue in the vicinity of the E3 active site must remain a matter for further investigation.

During inactivation of the PD complex with $BrCH_2$-[^{14}C]CONHPhAsO in the E3-mediated lipoyl system, the amount of [^{14}C]-radiolabel incorporated into E2 was considerably higher than in the El-mediated system. Adamson (1981) has shown that the alkylation of neighbouring lipoyl groups by (nonradioactive) bifunctional arsenoxide modified dihydrolipoyl-lysine residues, is roughly equivalent in both systems. The best explanation for the observed increase in [^{14}C]-radiolabel is that an alkylation took place involving a residue(s) at or near the active site of E2. It should be noted that in the E3-mediated lipoyl system, NADH is used to generate reduced lipoyl residues on E2; consequently, no coenzyme A will be bound at the E2 active site. In view of the intricate way in which the active sites of each component subunit are coupled in the PD complex, it has previously been impossible to introduce a radioactive "tag" into the E2 active site. Alkylation of the E2 active site with $BrCH_2$[^{14}C]CONHPhAsO may eventually facilitate the isolation of an active-site peptide.

In summary, the irreversible alkylation of a histidine residue at the active site of E3 was achieved by the inhibition of PD complex with $BrCH_2[^{14}C]CONHPhAsO$ in both E1- and E3-mediated lipoyl systems. These experiments provided further proof of the role of an imidazole side chain in the catalytic mechanism of E3.

4.4 Does E2 Contain a Third, Non-acetylatable Lipoyl Residue that Participates in Catalysis?

Further illustration of the proposed mechanism of E3 inhibition (whilst part of the PD complex) by $BrCH_2[^{14}C]CONHPhAsO$, has come from experiments (Section 3.5) in which N-ethyl maleimide was used to irreversibly modify lipoyl groups on E2. The inability of NEM inhibited lipoyl groups to deliver $BrCH_2[^{14}C]CONHPhAsO$ into the active site of E3 was illustrated by the total absence of $[^{14}C]$-radiolabelled histidine in E3 and by the observation that E3 remained fully active. These findings indicate a possible correlation between the alkylation of a histidine residue in or near the active site of E3 and the loss of E3 activity. Apparently, the inactivation of E3 by $BrCH_2[^{14}C]CONHPhAsO$ is dependent upon "a priori" anchoring of the arsenoxide moiety to reduced lipoyl groups on E2.

Recently, Hale and Perham (1979[a]) proposed that each E2 subunit in the PD complex contains three lipoyl-lysine residues, one of which is non-acetylatable. This residue was envisaged to interact with E2 and E3 only (i.e. no

contact with E1) and could serve to pass reducing equivalents from lipoyl groups on E2 into the active site of E3 (Fig. 4.4.1).

The ability of E2 dihydrolipoyl-lysine residues (reduced with excess NADH) to deliver the bromoacetamido moiety of $BrCH_2[^{14}C]CONHPhAsO$ into the active site of E3 has previously been demonstrated in this study (Sections 3.4 and 4.3). In this inhibition scheme, lipoyl-lysine residues on E2 are initially reduced irrespective of whether they are acetylatable or not (cf. E1-mediated lipoyl system where only acetylatable lipoyl residues may later become reduced in the presence of pyruvate and coenzyme A). It was therefore possible to critically test the hypothesis put forward by Hale and Perham (1979[a]), by investigating the effect of irreversibly blocked lipoyl groups on the inhibition of E3 in the E3-mediated lipoyl system.

A third non-acetylatable residue, if present on E2, would remain oxidised in the presence of pyruvate (Figs. 1.6.1 and 3.5.1.1) and would therefore be unreactive towards NEM. In the presence of NADH this putative third lipoyl residue would be reduced and should then be able to bind $BrCH_2[^{14}C]CONHPhAsO$ and deliver the reagent into the active site of E3 (Fig. 4.4.1). However, since no inactivation nor radiochemical labelling of E3 was observed under these conditions, it must be concluded that the presence of a third, catalytically active, non-acetylatable lipoyl residue on E2 is unlikely. This

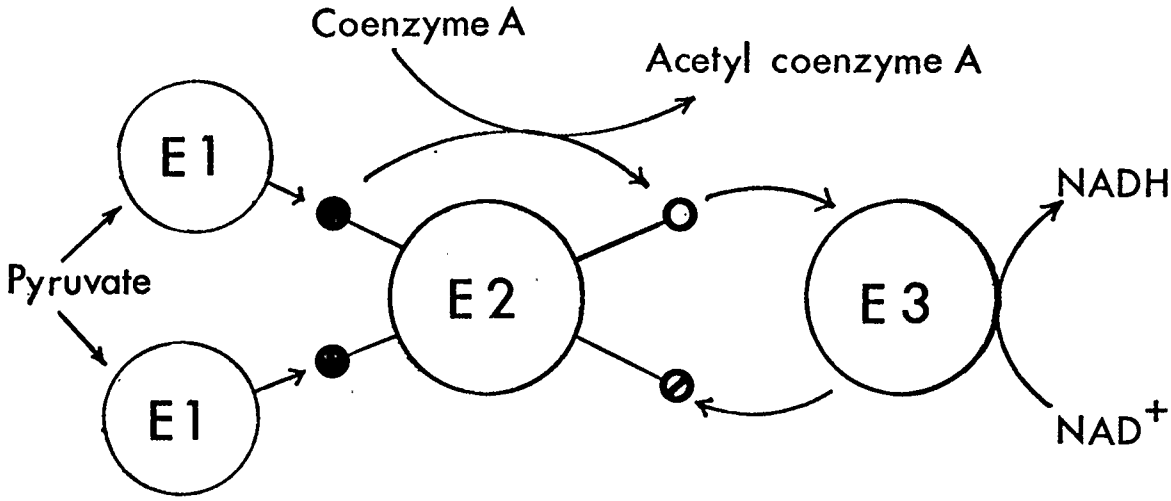


Figure 4.4.1 A possible scheme for the interaction of active sites in the PD complex, assuming that there are three lipoyl - lysine residues per E2 chain (Hale, 1977; Adamson, 1981).

● - S^6-Acetyl dihydrolipoyl-lysine residue
○ - Dihydrolipoyl-lysine residue (fully reduced)
⊘ - Lipoyl-lysine residue (fully oxidised)

assertion is supported since acetylated PD complex (reduced with NADH) is not inhibited by sodium arsenite (normally a potent inhibitor of reduced lipoyl groups) (Adamson, 1981).

The results of chemical modification studies on the PD complex reported in this thesis, are consistent with the most recent model for active-site coupling within the PD complex proposed by Reed and co-workers (Stepp et al., 1981; Reed and Oliver, 1982; Hackert et al., 1983). This model (Fig. 4.4.2) emphasises movement of lipoyl domains, and not simply rotation of lipoyl-lysine residues, to span the physical gaps between catalytic sites on the complex. Lipoyl groups accept acetyl moieties from E1 and, after reduction by coenzyme A, then interact directly with E3 to become reoxidised.

Interestingly, each E2 subunit has been found to contain only two acetylatable lipoyl-lysine residues (Section 1.7). Experiments reported in this thesis refute the possibility that a third non-acetylatable lipoyl residue, if present, participates directly in the overall PD complex mechanism. In view of the ability of the highly flexible E2 lipoyl domains to service E1, E2 and E3 subunits within the PD complex, the need for such a third lipoyl-lysine residue remains difficult to rationalise.

4.5 Experiments to Isolate a Peptide on E3 Containing the Putative Catalytic Histidine Residue

Reverse phase HPLC has been of great use in purifying small peptides obtained by extensive enzymatic

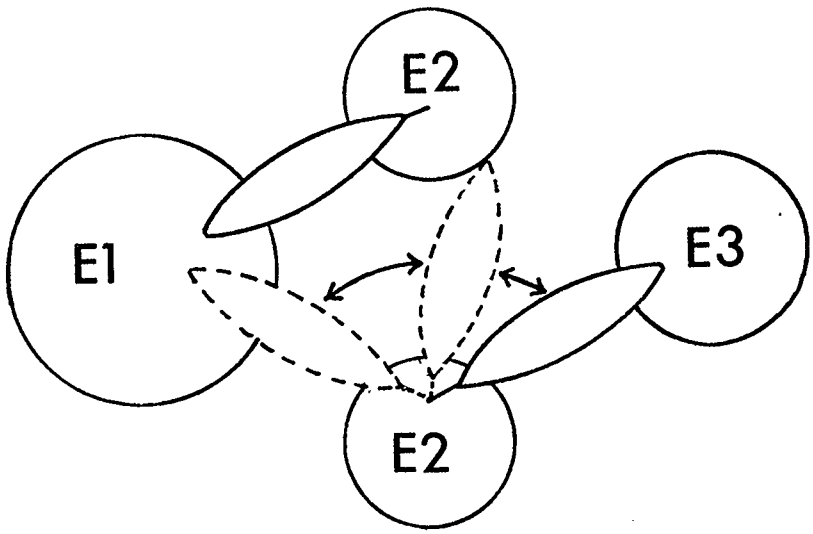


Figure 4.4.2 A model illustrating a postulated active-site coupling mechanism in the PD complex (Stepp et al., 1981). Each E2 subunit is represented by a sphere (subunit binding domain) and its attached ellipsoid (lipoyl domain). The active site of E2 is on the subunit binding domain. Each E1, E2 and E3 subunit is serviced by at least two lipoyl groups that reside on two separate lipoyl domains. The E1, E2 and E3 subunits need not be adjacent in the PD complex, since the length of the entire lipoyl domain (greater than 60 Å) is sufficient to allow its covalently attached lipoyl groups to reach distant catalytic sites on the complex.

proteolysis (Naider et al., 1979; Schroeder et al., 1979; O'Hare and Nice, 1979; McMillan et al., 1979; Wilson et al., 1979; Williams et al., 1981; Mahoney and Hermodson, 1980). The purification of large polypeptides (> 30 residues) has been considerably more difficult because of their insolubility in most solvents and their tendency to form aggregates.

In this study, peptides obtained from CNBr cleavage of [^{14}C]-radiolabelled E3 were readily dissolved in 0.1% (V/V) TFA in water. Aqueous TFA is particularly suitable as a starting solvent system for reverse phase HPLC analysis as it is volatile and essentially transparent in the UV absorption range of the peptide bond (205-230 nm). Most peptide HPLC systems reported to date, employ either 1- or 2- propanol, ethanol, methanol or acetonitrile as the organic solvents. The order of effectiveness in eluting peptides from a reverse phase HPLC column is: 1-propanol $\geq$ 2- propanol $\geq$ ethanol aa acetonitrite >> methanol (Wilson et al., 1981; Mahoney and Hermodson, 1980).

The peptides produced from a CNBr digest of [^{14}C]-radiolabelled E3 were first analysed on a Varian C_{18} micropak reverse phase column, however, using this system it was not possible to resolve the peptide mixture. Furthermore, the yield of [^{14}C]-radiolabel from the column was less than 10%. The most successful method for the fractionation of the peptide digest employed a less hydrophobic cyanopropyl stationary phase together with a

binary solvent system. This consisted of 0.1% TFA as the starting solvent and a linear gradient of acetonitrile/ 0.1% TFA as the limiting solvent (Figs. 3.7.3.1 and 3.7.5.1).

In view of its greater hydrophobicity, 2- propanol was found to be more effective in eluting the very large polypeptides in the CNBr digest of E3 (data not shown). Since the CNBr(X) peptide was the only peptide in the digest significantly modified with $BrCH_2[^{14}C]CONHPhAs$-BAL, a further investigation of the larger E3 polypeptides was not undertaken in this study.

When considering the size of the CNBr(X) peptide, its absorbance at 206 nm during HPLC analysis was unexpectedly high in comparison to other peptides in the digest (Fig. 3.7.3.1). Preliminary experiments indicated that the compound $BrCH_2CONHPhAs$-BAL (synthesized in acetonitrile) absorbs strongly in the 200-210 nm wavelength region. The additional absorbance of the CNBr(X) peptide was consistent with the results of HVE, amino acid and radiochemical analyses (Fig. 3.7.4.1; Table 3.7.6.1) which showed that the CNBr(X) peptide contained a histidine residue specifically modified with $BrCH_2$-$[^{14}C]CONHPhAs$-BAL. Amino acid analysis of the CNBr(X) peptide revealed that it contained at least 34 amino acids but was devoid of homoserine or homoserine lactone. This indicated that the CNBr(X) peptide was the C-terminal peptide of lipoamide dehydrogenase. The amount of $[^{14}C]$-radiolabelled histidine present per mol of

CNBr(X) peptide was 0.20 residues per mol of CNBr(X) peptide. This value was lower than expected since E3 had previously been shown to contain 0.37 residues of radiochemically modified histidine per mol.

Due to the unusual chemical modification of the CNBr(X) peptide, namely the introduction of a 10-15 Å apolar moiety, the low yield of [^{14}C]-radiolabelled CNBr(X) peptide with respect to unmodified peptide is difficult to interpret. The [^{14}C]-radiolabelled peptide was largely not resolved from unlabelled peptide. Presumably, the size of the CNBr(X) peptide tended to override any effect the arsenical modification might have had on the affinity of a single peptide molecule for the cyanopropyl stationary phase. An important column parameter affecting the behaviour of peptides on reverse phase HPLC column systems is the pore size of the silica support (van der Rest _et al_., 1980; Lewis _et al_., 1980; Pearson _et al_., 1980; Regnier, 1983; Hermodson and Mahoney, 1983). Hydrophobic interactions (in 0.1% aqueous TFA) between apolar arsenical moieties in two or more CNBr(X) peptide molecules may have led to aggregation of the [^{14}C]-radiolabelled peptide. The CNAQ (cyanopropyl) column utilised in this study had a 60 Å pore size. Since denatured CNBr(X) peptide (34 residues) would possess an extended length in excess of 100 Å, aggregation may have impeded penetration of the [^{14}C]-radiolabelled peptide into support pores on the HPLC column. A CNAQ column with a 300 Å pore size has recently become available and may improve the recovery of large peptides such as the CNBr generated C-terminal peptide from lipoamide dehydrogenase.

The inorganic support material present in reverse phase HPLC columns can be more than a passive carrier for the bonded phase (Pearson and Regnier, 1981; Regnier, 1983). Since the inorganic support-bonded phase composite apparently functions together in the separation of polypeptides, possible interaction of the chemically modified histidine moiety (His - $CH_2[^{14}C]$CONHPhAs-BAL) with the inorganic support material of the CNAQ column could not be ruled out during the isolation of the CNBr(X) peptide.

4.6 Amino Acid Sequence Analysis of the CNBr(X) Peptide

Reverse phase HPLC and/or back hydrolysis with amino acid analysis were used to identify the PTH amino acids produced by automated Edman degradation of the $[^{14}C]$-radiolabelled CNBr(X) peptide (Figs. 3.8.1.1, 3.8.2.1 and Table 3.8.1.1.1). By these methods the amino acid sequence of 30 residues in the C-terminal region of E3 was determined with varying certainty.

The repetitive yields of each sequencer cycle began to fall substantially beyond cycle 28 (Fig. 3.8.2.1), consequently the assignment of amino acids to positions 29, 30 and 32 must be considered highly tentative. No sequence data were obtainable beyond position 32 in the peptide. It was likely that as the sample size of the CNBr(X) peptide became reduced to less than six residues, the mechanical and extractive losses of the peptide from the spinning cup reaction vessel became limiting. This

would give rise to falling repetitive yields and eventually washout of the remaining 2-5 residues of the peptide (Niall, 1975, 1977; McKay, 1983).

The CNBr(X) peptide appeared to be contaminated with small quantities of a hydrophobic peptide, as suggested by HPLC analysis (Fig. 3.7.5.1) and evidenced by the presence of additional PTH amino acids in sequencer cycles 1, 4, 5, 6, 7, 9, 11, 13, 15 and 16 (tentative sequence F--VIPA-D-D-V-VP). The average ratio of contaminating PTH amino acid to assigned PTH amino acid was approximately 15:85. As an approximate guideline, several workers have described 70-80% as the minimum peptide purity from which useful sequence information can be derived (Konigsberg and Steinman, 1977; Allen, 1981). As indicated in Fig. 3.8.1.1, the amount of contaminating peptide present did not appear to interfere with the elucidation of the sequence of the CNBr(X) peptide.

In view of the fact that no homoserine or homoserine lactone was detected in amino acid analyses of the CNBr(X) peptide, an alternative explanation for the observed contamination in the early sequencer cycles may be micro-heterogeneity in the sequence of the CNBr(X) peptide. This would be surprising since no such heterogeneity has been detected in E3 from pig heart (Williams, 1983).

The [^{14}C]-radiochemically labelled histidine residue was assigned to position 14 in the sequence of the

CNBr(X) peptide. It might be argued that the [^{14}C]-radiochemically labelled histidine residue may have been attributable to the smaller contaminating peptide and not to the C-terminal CNBr(X) peptide. This possibility would be inconsistent with the HPLC data (Fig. 3.7.5.1, Section 3.7) since the smaller peptide eluting just after the CNBr(X) peptide was virtually nonradioactive. Furthermore, the tentatively deduced sequence of the contaminating peptide did not show any distinct homology to any region of glutathione reductase. In view of the known similarity in mechanism and primary structure between this enzyme and E3 from pig heart, an active-site histidine peptide might reasonably be expected to be homologous to a corresponding region in glutathione reductase. Elucidation of the complete nucleotide sequence of E3 from _E. coli_ K12 has almost been completed (Guest, unpublished results) and will critically test this assertion.

Low recovery of the PTH derivative from the first cycle is a commonly reported phenomenon in the automated sequence analysis of proteins and polypeptides (Allen, 1981) and is poorly understood at present. Sequence analyses of pure myoglobin standards prior to analysis of the CNBr(X) peptide also illustrated low initial yields (25%). Glycine residues present in the CNBr(X) peptide and in myoglobin have been consistently sequenced in low yield (55-65%), presumably because of the poor conversion of the ATZ glycine derivative to the corresponding PTH derivative during the Edman degradation (Section 3.8.2). The poor recovery of PTH glycine may have led to an underestimate of the actual initial yield of peptide.

The yield of PTH aspartic acid in cycle 3 was 10,070 pmol, therefore, the amount of peptide actually being sequenced during a sequencer run may have been nearer 50%. Allowing for a repetitive yield of 93% up to sequencer cycle 14, we might expect in excess of 700 cpm [^{14}C]-radiolabel (corresponding to modified histidine) in cycle 14. The amount detected was 300 cpm, however this discrepancy has been clarified. Space filling models suggest that the chemically modified histidine residue may be capable of sterically hindering the Edman degradation of modified CNBr(X) peptide in the vicinity of position 14. Steric hindrance may therefore be a significant factor in the low recovery of [^{14}C]-radiolabelled histidine as a PTH amino acid derivative.

4.7 Conclusion

This study provided further evidence for the involvement of an active-site histidine residue in the catalytic mechanism of lipoamide dehydrogenase from *E. coli*. Experiments in which acetylated PD complex was irreversibly inhibited with N-ethyl maleimide, then treated with $BrCH_2$[^{14}C]CONHPhAsO, illustrated the novel intermolecular active-site coupling mechanism between E2 and E3 subunits within the multienzyme complex. These studies showed that when the lipoyl groups present on E2 are acetylated and irreversibly modified with NEM, the PD complex (in the presence of NADH) is unable to anchor $BrCH_2$[^{14}C]CONHPhAsO via reduced lipoyl groups nor deliver this reagent into the active site of E3. These findings

suggest that E2 is unlikely to contain a catalytically active non-acetylatable lipoyl residue that interacts with E3. If present, this residue would have anchored $BrCH_2[^{14}C]CONHPhAsO$ via the arsenoxide moiety, and then delivered the $[^{14}C]$-radiolabelled bromoacetamido moiety into the active site of E3.

The primary sequence of at least twenty-seven amino acid residues surrounding an E3 active-site histidine residue was determined by the automated Edman degradation of a peptide produced from CNBr cleavage of the $[^{14}C]$-radiochemically labelled enzyme. This peptide (CNBr(X)) was assumed to be the C-terminal CNBr peptide since it contained no homoserine or homoserine lactone and was homologous to the C-termini of glutathione reductase (Krauth-Siegel _et al._, 1982) and pig heart lipoamide dehydrogenase (Williams _et al._, 1982; Williams, 1983). This homology is illustrated in Fig. 4.7.1.

Williams and co-workers (1982) have found an extensive sequence homology between pig heart lipoamide dehydrogenase and human glutathione reductase. Nine tryptic peptides (172 residues) from pig heart lipoamide dehydrogenase were directly homologous (40%) with glutathione reductase. The homology extended throughout the molecule indicating a probable evolutionary relationship between these two mechanistically similar enzymes (Williams _et al._, 1982).

Studies presented in this thesis have confirmed that eukaryotic glutathione reductase is also mechanistically

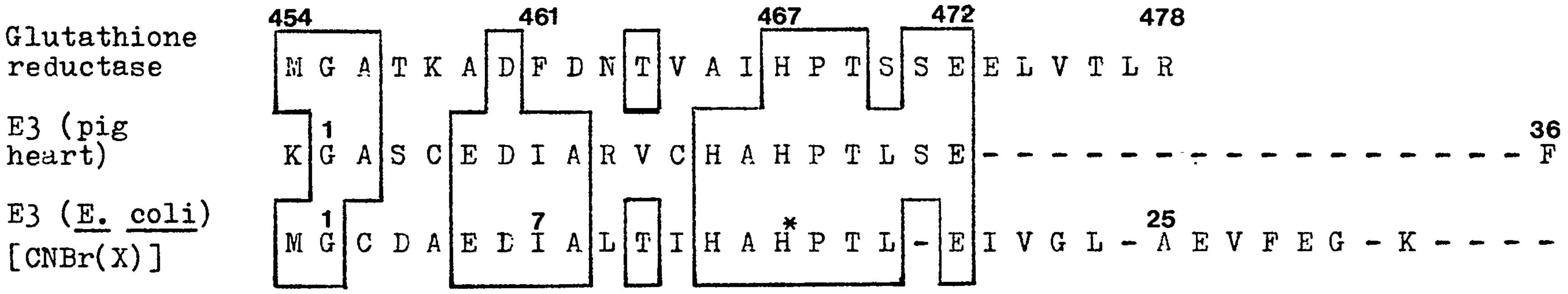


Figure 4.7.1 Homology of CNBr(X) peptide to the C-terminal region of glutathione reductase from human erythrocytes (Krauth-Siegel *et al.*, 1982), and lipoamide dehydrogenase from pig heart (Williams *et al.*, 1982; Williams, 1983). The E3 sequences have been numbered 1-36 as shown. The pig heart E3 sequence is known to extend 11 residues beyond the C-terminus of glutathione reductase. H* represents the catalytic histidine in *E. coli* E3 identified by [^{14}C]-radiochemical labelling.

similar to lipoamide dehydrogenase from a prokaryote. The first 19 amino acid residues in the amino acid sequence of the CNBr(X) peptide (preceded by methionine) were directly homologous (42%) with residues 454 to 472 in the C-terminal region of glutathione reductase. This part of the CNBr(X) peptide sequence was also directly homologous with the C-terminal region of lipoamide dehydrogenase from pig heart (63%).

Allowing for the interchange of amino acid residues with aliphatic side chains and for exchange of glutamic acid with aspartic acid, the homology between the C-terminal region of glutathione reductase (26 residues) and the corresponding sequence in lipoamide dehydrogenase (*E. coli*) is 50%. This finding provides further evidence for an evolutionary relationship between glutathione reductase and lipoamide dehydrogenase from widely differing organisms.

The active-site histidine residue which participates in the catalytic mechanism of lipoamide dehydrogenase corresponds exactly to histidine-467 in glutathione reductase. The role of histidine-467 as an essential catalytic residue in glutathione reductase has been extensively described, on the basis of chemical modification studies and X-ray diffraction analyses (Boggaram and Mannervik, 1978; Thieme *et al.*, 1981; Schulz *et al.*, 1978; Pai and Schulz, 1983).

The most impressive homology between lipoamide dehydrogenase and glutathione reductase exists in a particularly hydrophobic sequence of amino acids surrounding the catalytic histidine residue. The amino acid sequence from position 7 to 23 in the CNBr(X) peptide has a hydrophobicity index, calculated according to Segrest and Feldmann, of 2.2 (Segrest and Feldmann, 1974). This finding is consistent with previous experiments performed in this laboratory, in which the differential useage of organic and inorganic monofunctional arsenoxide reagents showed that the active site of E3 was relatively hydrophobic (Adamson, 1981). Allowing for conservative replacements, this hydrophobic sequence shows a 67% and 75% homology with similar sequences in glutathione reductase and pig heart lipoamide dehydrogenase, respectively. This once again suggests that structural knowledge of the active site of glutathione reductase may be applied to lipoamide dehydrogenase.

In a situation where thiol and imidazole side chains are present in the same environment, such as the active site of substrate-reduced lipoamide dehydrogenase, the former side chain, regardless of its ionisation state, would be expected to be the more reactive entity toward $BrCH_2[^{14}C]CONHPhAsO$. The finding that a histidine residue is uniquely modified suggests that the $[^{14}C]$-radiolabelled arsenoxide must selectively encounter the histidine residue. The N^3 position in the imidazole ring is specifically modified (N^1-alkylated histidine has

never been detected), which suggests that this residue is firmly held in the active site of E3 with the N^3 position orientated towards the incoming substrate.

Comparison of the amino acid sequences surrounding the active-site histidine residue in both glutathione reductase and lipoamide dehydrogenase reveals a satisfying rationale for the singular modification of histidine in the N^3 position with $BrCH_2[^{14}C]CONHPhAsO$. Recent high resolution X-ray diffraction analyses of glutathione reductase have shown that the essential histidine residue-467 in glutathione reductase is indeed firmly held at the active site by means of a short, strong hydrogen bond from glutamic acid residue-472 stretching to the N^1 position of the histidine imidazole ring. Furthermore, the hydrogen attached to N^3 in the isoalloxazine ring of FAD forms a short hydrogen bond to the peptide carbonyl group of histidine-467. The presence of proline in position 468 presumably helps add rigidity to the polypeptide chain in the vicinity of the catalytic histidine residue. Clearly, histidine is firmly held in the active site of glutathione reductase with the N^3 position of the imidazole ring presumably orientated towards incoming glutathione.

In a situation virtually analogous to glutathione reductase, the residues surrounding the active-site histidine residue in lipoamide dehydrogenase are highly conserved. Proline, threonine and glutamic acid are present in identical positions relative to the essential

histidine residue. This is not surprising in view of the similarity in catalytic mechanism between these two enzymes. We may therefore assume that, as in glutathione reductase, the catalytic histidine residue in lipoamide dehydrogenase from E. coli, is firmly held at the active site of the enzyme probably by similar forces to those found in the active site of glutathione reductase. This presumably explains why only the N^3 position of the imidazole ring is ever modified by $BrCH_2[^{14}C]CONHPhAsO$. The possible orientation of the essential catalytic histidine residue in the active site of lipoamide dehydrogenase is illustrated in Fig. 4.7.2.

The amino acid sequence studies reported in this thesis provide good evidence at the molecular level to support the original hypothesis by Matthews (1977) on the catalytic mechanism of E3. It was proposed that the primary encounter of an E2 dihydrolipoyl-lysine residue is with a base which serves to deprotonate the lipoyl group, thereby generating a thiolate anion, which in turn can attack the redox disulphide of E3. In glutathione reductase the imidazole ring of histidine-467 contacts the sulphur of cysteine-58 and the sulphur of glutathione-I (Pai and Schulz, 1983). The substitution of alanine for isoleucine in the position prior to the active-site histidine residue in lipoamide dehydrogenase (pig heart and E. coli) may facilitate a less hindered contact between N^3 in the imidazole ring of histidine and bulky dihydrolipoyl-lysine residues on E2 (Fig. 4.7.1).

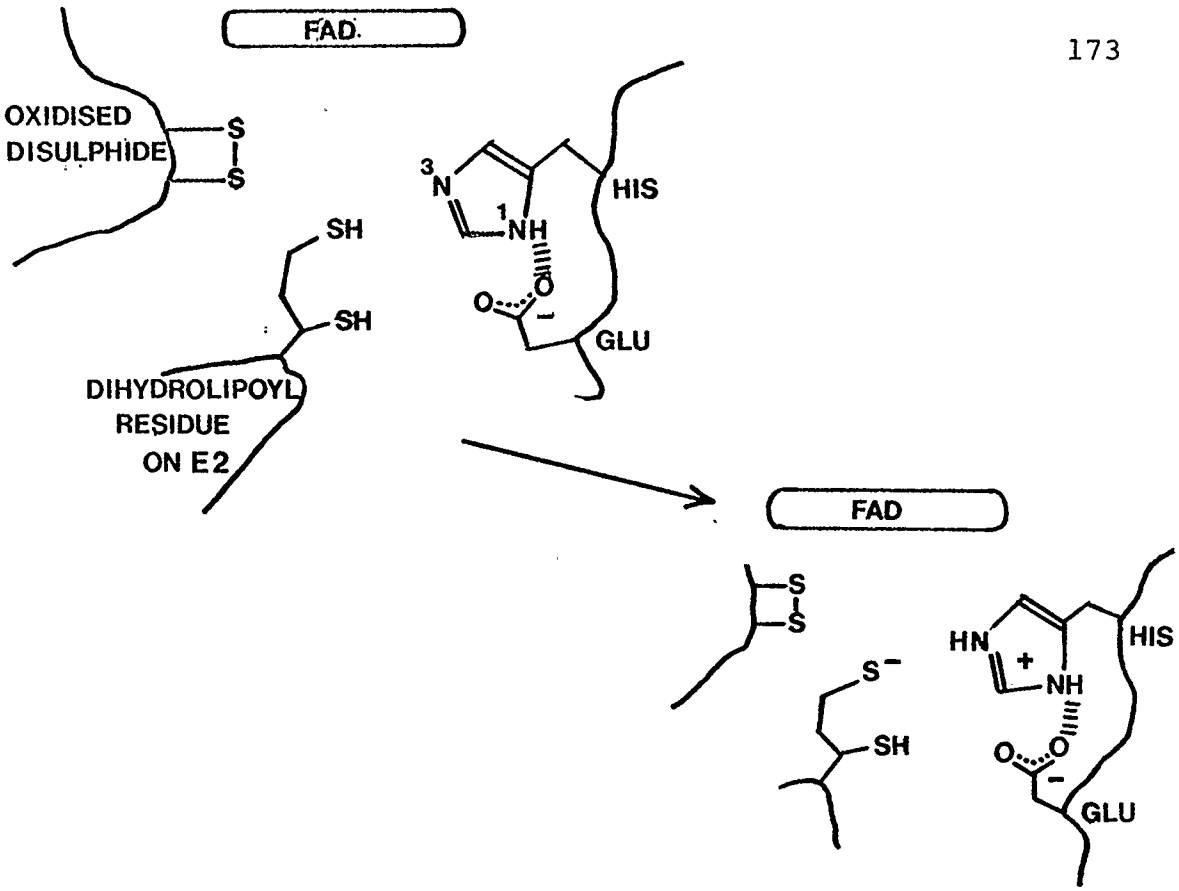


Figure 4.7.2 Possible orientation of the essential catalytic histidine residue in the active site of E3 from *E. coli*. Histidine acting as a base is positioned firmly in the active site by a hydrogen bond between a glutamic acid residue and the N^1-imidazole ring position. The N^3-imidazole ring position is orientated so as to facilitate a primary encounter with an incoming dihydrolipoyl-lysine residue. Deprotonation of the lipoyl group generates a thiolate anion, electrons then pass to the redox active-site disulphide via dithiol-disulphide interchange, thence to FAD and finally to NAD^+, as described in Fig. 1.9.1 (Williams *et al.*, 1976; Matthews *et al.*, 1977).

Although the mechanism of lipoamide dehydrogenase is essentially preserved in glutathione reductase, both substrate specificities have changed. An important difference between the two mechanisms is that, whereas in two-electron reduced lipoamide dehydrogenase the presence of NAD^+ changes the redox potential of FAD relative to the dithiol so that electrons pass to the FAD, no such change is observed in glutathione reductase. This evolution allows glutathione reductase to function physiologically in the opposite chemical direction (Williams *et al.*, 1982). The structural basis of this difference is, as yet, unknown.

The studies presented in this thesis have provided further evidence for an evolutionary relationship between glutathione reductase and lipoamide dehydrogenase. Both enzymes are present in *E. coli* suggesting that they diverged rather early. At the present it is not known if lipoamide dehydrogenase predates glutathione reductase, however the former enzyme is found in the anaerobe *Peptococcus glycinophilus* which is known from 16S RNA sequences to be an ancient species (Fox *et al.*, 1980). Glutathione reductase is thought to have arisen in response to the advent of oxygen. Recently, lipoamide dehydrogenase has been found in methanogenic archaebacteria (Danson, 1983). It will be of interest to determine whether or not glutathione reductase is present in such an organism, whose physiology is more suited to an atmosphere containing carbon dioxide and some hydrogen but little or nor oxygen.

Isolation and sequencing of peptides derived from archaebacterial lipoamide dehydrogenase and comparison of the primary structure of the enzyme with known sequences in E. coli lipoamide dehydrogenase and eukaryotic glutathione reductase, could provide further insight into the evolutionary relationship of these two enzymes. Since a catalytically essential histidine residue has been shown to reside in the C-terminal region of lipoamide dehydrogenase from E. coli, the isolation and sequencing of the C-terminal CNBr peptide of this enzyme from archaebacteria may therefore provide a convenient starting point for an interesting evolutionary study.

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