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Purification and Characterization of NAD(P)H Oxidase from *Giardia lamblia*

by

Yimei Sun

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Purification and Characterization of NAD(P)H Oxidase from *Giardia lamblia*", submitted by Yimei Sun in partial fulfillment of the requirements for the degree of Master of Science.

Dr. K. J. Stevenson, Supervisor
Department of Biological Sciences

Dr. H. Ceri
Department of Biological Sciences

Dr. G. M. Gaucher
Department of Biological Sciences

Dr. F. F. Snyder
Department of Medical Biochemistry

Date: 1 September 1995

ABSTRACT

A new flavoprotein, NAD(P)H oxidase, has been isolated from the protozoan *Giardia lamblia* by salt fractionation, hydrophobic chromatography, hydroxyapatite adsorption and ion-exchange chromatography. Under aerobic conditions, this enzyme catalyzes electron transfer from NAD(P)H to molecular oxygen producing $NAD(P)^+$ and H_2O. The maximum enzyme activity is between pH 6.8 to 7.2. FAD is a required cofactor for this NAD(P)H oxidase but only weakly associates with the apoenzyme. The inhibition studies with trivalent arsenical compounds indicated that cysteinyl residues are present in the active site of this enzyme. The molecular weight of this enzyme was determined to be 41,300 (+/- 3,000) Daltons and no subunit structure has been identified. A sequence of MKVIILGANHG was revealed as the N-terminal sequence for this enzyme.

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To

my parents

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ABBREVIATIONS

A	absorbance
ABTS	2, 2'-azino-di-(3-ethylbenzthiazolin sulfonate)
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
Da	Dalton
DTT	dithiothreitol
EDTA	ethylenediamine tetracetic acid
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
FPLC	Fast Protein Liquid Chromatography
Kav	average distribution coefficient
kDa	kilo Dalton
Log	logarithm
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl flouride
SDS	sodium dodecyl sulfate

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Introduction

1.1.1 Scope of the work and organization of the thesis

The main objective of this work was to isolate NAD(P)H oxidase from *Giardia lamblia* trophozoite. As a result, the purified enzyme can be further characterized to determine the molecular weight, subunit composition, amino acid composition, and the most important, N-terminal amino acid sequence. The revealment of the N-terminal information is the foundation to identify and further characterize the gene encoding this enzyme. Moreover, the elucidation of the gene and following homologous comparison to the flavo oxidoreductases from other species will not only allow us to achieve detail understanding of the catalytic mechanism for this enzyme, but also provide valuable information on ancestral molecular characteristics present in this primitive eukaryotic organism.

This thesis is organized as follows. It starts with an introduction to *Giardia lamblia* and NADH oxidase in Chapter 1; chemicals and culture medium used in this work are listed in Chapter 2; the experimental methods involving cell culturing, purification attempts and characterization studies are presented as three parts in Chapter 3; results and discussion of the work compose Chapter 4; the final Chapter 5 includes conclusion and future perspective.

The following review of the literature includes some background information on *Giardia lamblia* and the bacterial source NADH oxidase.

1.2 Biology and Epidemiology of *Giardia lamblia*

1.2.1 Nomenclature

Giardia is a genus of intestinal protozoan parasite. It was first discovered by van Leeuwenhoek in 1681 [Dobell, 1920] and was further described in great detail by Lambl [Lambl, 1859]. At present, *Giardia* along with the other flagellated human parasites such as: *Trypanosoma, Leishmania, Trichomonas* and *Diemtamoeba*, is placed in the phylum Sarcomastigophora, class Zoomastigophorea and order Diplomonadida [Levine et al., 1980].

On the basis of morphological criteria, a system has been suggested to divide *Giardia* into three species [Filice, 1952]. *G. agilis* infects amphibians; *G. muris* infects rodents, birds and reptiles and *G. lamblia* (also named *G.duodenalis* or *G. intestinalis*) infects mammals including humans, as well as birds and reptiles. More recently, electron-microscopic studies [Erlandsen and Bermick, 1987] and nonmorphological analysis [Nash et al., 1985] such as DNA restriction patterns and surface antigens have further divided *G. lamblia* into several species or subspecies, but the conventional three part division is still a commonly accepted classification system.

1.2.2 Life cycle and cell structure

G. lamblia, like all species within the genus, has a simple life cycle. It has two developmental phases: trophozoite and cyst. The trophozoite is the generative form which inhabits the small intestine of host and multiplies by binary fission, this ac-

counts for the symptoms of giardiasis. The infective cyst mainly exists in the external environment and is relatively resistant to environmental changes, such as dessiccation and cold [Jarroll et al., 1980].

Each cyst contains two trophozoites. When a cyst is ingested, the acidic environment of the host stomach initiates the excystation process and enzymatic digestion, possibly by trypsin and chymotrypsin in the intestine, completes this process to release trophozoites [Farthing, 1984]. On the other hand, some of the trophozoites are induced to encyst by primary bile salt, fatty acids as well as alkaline pH [Gillin et al., 1987, Gillin et al., 1988]. The cysts are passed in the feces which can infect new hosts primarily through water contamination, thus the cycle is repeated.

G. lamblia trophozoite has a shape like a flattened tear drop with approximate size of 12-15 μm by 6-8 μm and 1-2 μm thick [Feely et al., 1984]. The internal structure of trophozoite consists of a funis, two median bodies shaped like a claw hammer, a ventral disk at the anterior portion of the ventral surface and four pairs of cadually directed flagella which are arranged in bilateral symmetry. The flagellar motion is proposed to provide the necessary hydrodynamic force for attaching to the intestinal mucosa of the host [Holberton, 1974].

The ventral disk of *G. lamblia* is a concave structure and is composed of microtubules, cross bridges attached to the microtubules and microribbons which contain *Giardia* specific proteins-giardiasis [Peattie et al., 1989, Crossley and Holberton, 1983, Crossley and Holberton, 1985]. The function of the ventral disk associates with the attachment of the organism to the host's intestine [Erlandsen and Feely, 1984].

The median body of *Giardia* lies transversely in the mid-portion of the cell body and its morphological difference has been used to distinguish Giardia species

[Filice, 1952]. The function of the median body remains elusive, but its large composition of microtubules suggests that it may be functionally related to the ventral disk [Crossley et al., 1986, Feely et al., 1982].

Internally, *G. lamblia* contains two nuclei symmetrically placed on either side of the midline. Studies by using radioactive isotope [Wiesehahn et al., 1984] as well as fluorescent staining [Kabnick and Peattie, 1990] revealed that both nuclei are identical considering replicational ability and transcriptional activity. However, whether there is any exchange of DNA between the two nuclei and what is the selective advantage of containing two nuclei are still unknown.

Other organelles present include lyososomal vacuoles containing a variety of hydrolytic enzymes [Lindmark, 1988, Feely and Dyer, 1987], short rough endoplasmic reticulum and free ribosomes [Feely et al., 1984]. However, many of the common eukaryotic organelles including mitochondria, smooth endoplasmic reticulum, peroxisomes and nucleoli have not been identified in *G. lamblia.* A Golgi apparatus has only been found during the encystation process [Reiner et al., 1990].

The *Giardia* cyst is about 5 μm by 8 μm and is surrounded by a 0.3 μm thick wall [Sheffield and Bjorvatn, 1977]. The major sugar component of the cyst wall is galactosamine in the form of N-acetylgalactosamine [Jarroll et al., 1989]. The vacuoles, ribosomes, flagellar axonemes and fragments of ventral disk are found inside the cyst wall [Sheffield and Bjorvatn, 1977, Erlandsen and Feely, 1984]. Most cysts contain four nuclei.

1.2.3 Metabolism

Metabolism in *G. lamblia* has only been intensively investigated after the development of axenic medium for the growth of trophozoite in 1976 [Meyer, 1976]. Consistent with the absence of mitochondria and hydrogenosomes, studies of the carbohydrate metabolism demonstrated that the *G. lamblia* trophozoite lacks Krebs cycle, cytochrome-mediated electron transport and oxidative phosphorylation [Lindmark and Jarroll, 1980, Weinbach et al., 1980]. Energy is produced by fermentation. Simple sugars, primarily glucose, are converted to pyruvate by the Embden-Meyerhoff pathway and pyruvate is further metabolised incompletely to organic products and CO_2.

Recent studies [Paget et al., 1993] found that the end products of carbohydrate metabolism vary considerably with the change of O_2 concentration. Under strict anaerobic conditions, alanine is the major product, whereas addition of low concentration of O_2 (<0.25 μM) stimulates ethanol production and decreases alanine production. Further increasing O_2 concentration to above 46 μM gives acetate as the predominant product. It has been suggested that this shift of carbon flux may allow *Giardia* trophozoite to cope with the host's intestinal O_2 concentration changes which fluctuate between 0 to 60 μM [Atkinson, 1980]. But to answer how this metabolic flux is modulated requires more investigations.

Besides the production of alanine, the *de novo* synthesis of amino acids has only been observed for valine [Paget et al., 1993]. It seems that *G. lamblia* trophozoite obtains most of the amino acids by scavenging from the environment. It is not known whether amino acids can be used as a potential energy source as observed in the mammalian system.

As mentioned above, this organism generates energy by substrate-level phosphorylation. Further evidence has shown that pyrophosphate acts as the direct energy source [Mertens, 1993]. Although an electron transport protein, ferredoxin, has been purified, little is known about the electron transport system in *G. lamblia.* It has been proposed that this organism has a particular respiratory chain different from that in mitochondria and consists of iron-sulfur proteins and flavins [Lindmark and Jarroll, 1980, Weinbach et al., 1980].

Studies of nucleic acid metabolism in *G. lamblia* by several research groups [Wang and Aldritt, 1983, Aldritt et al., 1985, Berens and Marr, 1986] [Jarroll et al., 1987] revealed that this organism lacks *de novo* synthesis of both purine and pyrimidine and the acquirement of nucleotides depends on salvage of exogenuous nucleic acid. Furthermore, it was pointed out that *G. lamblia* takes up pyrimidines from medium by active transport mechanism and uses different sites for importing cytosine and thymidine [Jarroll et al., 1987].

Jarroll and colleagues [Jarroll et al., 1981] first employed radiolabeled lipid precursors to examine the lipid metabolism pattern in *G. lamblia.* They discovered that this organism is incapable of synthesizing cellular phospholipids or sterols *de novo* and utilizes preformed fatty acids as well as cholesterol in the growth medium. Although it is not clear how much individual contribution from free fatty acids or esterified fatty acid derivatives to the lipid synthesis, trophozoites have been shown to incorporate exogenuous phosphotidylcholine [Farthing et al., 1985] as well as free fatty acids such as palmitic acid and arachidonic acid [Blair and Weller, 1987]. Moreover, these free fatty acids can be esterified into phospho- and neutral-lipids.

In summary, *G. lamblia* trophozoite lacks *de novo* synthesis of both lipids and nucleosides. It relies on salvage pathways to obtain purines, pyrimidines, fatty acids

and most of the amino acids. Simple sugars are the major source of energy and pyrophosphate, obtained from substrate-level phosphorylation, serves as direct energy form. The electron transport system probably consists of iron-sulfur proteins and flavins. This primitive metabolism system in *G. lamblia* corresponds to its parasitic life and is consistent with being considered as an ancestral eukaryote.

1.2.4 Genetics

The understanding of *G. lamblia* genetics at the molecular level is just at its initial stage. The results and conclusions presented in the following parts are achieved from limited experimental data available to date in the literature.

The genome complexity of *G. lamblia* is estimated by *Cot* analysis ranging from 3×10^7 to 8×10^7 bp [Nash et al., 1985, Boothroyd et al., 1987]. The G+C content of the genome is proposed to be 42% by Nash's group [Nash et al., 1985] or 48% by Boothroyd's group [Boothroyd et al., 1987], whereas the nucleotide sequence of the gene encoding rRNA consists of 75% G+C [Healey et al., 1990]. The analysis of protein-coding gene sequences so far reveals a G+C content ranging from 49% to 65% [Adam et al., 1988, Aggarwal et al., 1989, Aggarwal et al., 1990, Baker et al., 1988, Gillin et al., 1990, Kirk-Mason et al., 1988, Kirk-Mason et al., 1989] [Mowatt et al., 1991, Peattie et al., 1989, Yee and Dennis, 1992].

At present, researchers have not reached agreement on the number of chromosomes in each nucleus of *G. lamblia*. Light microscopic observation of fluorescent stained nuclei suggested that each nucleus of *G. lamblia* is haploid containing four condensed chromosomes [Kabnick and Peattie, 1990]. In contrast, pulse-field separation of intact chromosomes and densitometric scanning indicated that each trophozoite con-

tains approximately 30 to 50 chromosomal DNA molecules and is probably polyploid. Further studies involving different approaches are required to resolve these conflicting data.

The rRNA of *G. lamblia* consists of 28S, 16S and 5.8S subunits which are smaller than those of typical eukaryotes [Edlind and Chakraborty, 1987]. Healey and colleagues [Healey et al., 1990] revealed the complete nucleotide sequence of rRNA coding gene and found this 5,566 bp coding unit is tandemly repeated in the genome. The small subunit rRNA of *G. lamblia* possesses special characteristic and is an important element in determining the evolutionary position for this organism. Sequence analysis of 16S rRNA coding gene in *G. lamblia* demonstrates greater similarity to those of archaebacterium than do the sequences from other eukaryotes [Sogin et al., 1989]. As a result, *G. lamblia* is considered as the earliest eukaryote branch diverged from prokaryote.

Since only a small amount of protein-coding genes have been illustrated from *G. lamblia*, our knowledge of its transcription and translation mechanisms is limited. A possible TATA box has been identified in the genomic DNA sequence [Kirk-Mason et al., 1989, Gillin et al., 1990, Aggarwal et al., 1990] and is located 9 to 134 bp upstream from the initiation codon. However, it is still unknown if this putative TATA box is the RNA polymerase recognition site.

The present data from the analysis of translation show an extremely short 5' leader sequence in *G. lamblia* mRNA [Aggarwal et al., 1990, Aggarwal et al., 1989] [Gillin et al., 1990, Peattie et al., 1989, Yee and Dennis, 1992]. This suggests that the ribosome binding site on mRNA is closely linked with the initiation of translation. Nash and Mowatt [Nash and Mowatt, 1992] also found that the 3' untranslated region of mRNA in this organism is relatively short compared to those seen in the

other eukaryotes. A consensus motif of AGTA/GAAC/T consistently appears 6 to 9 nucleotides beyond the termination codon, which is further separated by 7 to 10 bases from the beginning of the polyA tail. This consensus sequence was thus proposed as a polyadenylation signal in *G. lamblia* [Peattie et al., 1989]. To date, no introns have been reported in *G. lamblia.*

In summary, genetic studies of *G. lamblia* are only at beginning. More studies are required to achieve a better understanding about how this organism manipulates its genetic information.

1.2.5 Epidemiology

G. lamblia is the most commonly isolated intestinal parasite in humans and is especially prevalent among children in developing countries [Farthing et al., 1986, Flores et al., 1983]. The infection of *G. lamblia* is usually acquired through ingestion of cysts, although ingestion of large amount of trophozoites can also lead to infection [Meyer and Jarroll, 1980]. Humans infected with *G. lamblia* may develop symptoms such as diarrhea and weight loss, whereas some infections are asymptomatic [Turner, 1985].

The cyst infects its host through a variety of different vehicles. The most common source is through contaminated water which is a notable cause of giardiasis in travelers [Istre et al., 1984, Navin et al., 1985]. Studies have shown that simple chlorination treatment will not kill the cyst in water. At present, the most effective method to remove *Giardia* cyst from drinking water is through sand filtration [Logsdon et al., 1979].

Direct faecal-oral transmission is a major source of giardiasis in children attending

day-care centers or schools. The prevalence rate for North American school-age children ranges from 4-20% [Woo and Paterson, 1986], whereas in developing countries such as in Bangladesh, the rate can be as high as 40% [Gilman et al., 1985]. For the day-care center children, the prevalence of giardiasis is at particularly high risk where the rate can reach 90% [Turner, 1985].

A less common food-borne giardiasis has also been reported by several research groups [Osterholm et al., 1981, Petersen et al., 1988, White et al., 1989]. These outbreaks are probably caused through contamination of freshly prepared food by an infected food handler.

1.2.6 Evolutionary studies

To reconstruct the origin of life and to elucidate the relationships among various life forms in the earth has been an aged subject for scientists. But it was not until the recent advent of nucleic acid sequencing technology, which brought a revolution in Biology, that the evolutionary studies became approachable and reliable. At present, rRNA sequence has been accepted as a common and crucial criteria to measure phylogenetic relationships [Woese, 1987]. As the amount of rRNA sequence data increases, scientists are gaining a clearer insight into the linkage among organisms.

The living world was divided by Chatton [Chatton, 1937] into two kingdoms called Prokaryote and Eukaryote on the basis of the presence of a true nucleus. Later, Woese and Fox [Woese and Fox, 1977] further divided Prokaryote into two groups: Eubacteria and Archaebacteria composed of methanogens, sulfur metabolising thermoacidophiles and halobacterium. No matter which system is accepted, it is universally believed that eukaryotes evolved from primitive organisms. But how this evolution

proceeded is still not very clear. Two different hypothesises have been advanced so far to offer explanations. The "serial endosymbiosis" theory suggests that the first eukaryote appeared is an aerobe [Margulis, 1981]. In contrast, Cavalier-Smith [Cavalier-Smith, 1975, Cavalier-Smith, 1987] attemped to link eukaryotes to prokaryotes through an anaerobic eukaryote. Moreover, *G. lamblia* has been proposed as this "missing link" [Kabnick and Peattie, 1991].

As an eukaryote, *G. lamblia* has well defined nucleus as well as cytoskeleton, but lacks many characteristic eukaryotic structures such as mitochondria, smooth endoplasmic reticulum and nucleoli [Feely et al., 1984]. In addition to its extremely primitive structure, the sequence analysis of its 16S rRNA demonstrated that *G. lamblia* shares more sequence similarity with prokaryotes than does the available rRNA sequence from any other eukaryote [Sogin et al., 1989]. Thus, *G. lamblia* is evolutionarily closer to prokaryotes than are any other eukaryotes and is probably the first emerged eukaryote during the evolution. The phylogenetic position of *G. lamblia* is shown in Figure 1.1 [Kandler, 1992]. More studies are being focused on constructing the universal phylogenetic tree. One of the goals of studying NAD(P)H oxidase in *G. lamblia* is to obtain the gene sequence corresponding to this enzyme and to perform homologous comparison for the purpose of elucidating the phylogenetic position of *G. lamblia* from the point of protein evolution.

1.3 NADH Oxidase

1.3.1 General description

NADH oxidase (NADH: acceptor oxidoreductase, EC 1.6.99.3) is a flavoenzyme which catalyzes electron transfer from NADH to an acceptor to regenerate NAD^+. Whereas this enzyme from mammalian mitochondria has been well defined as an es-

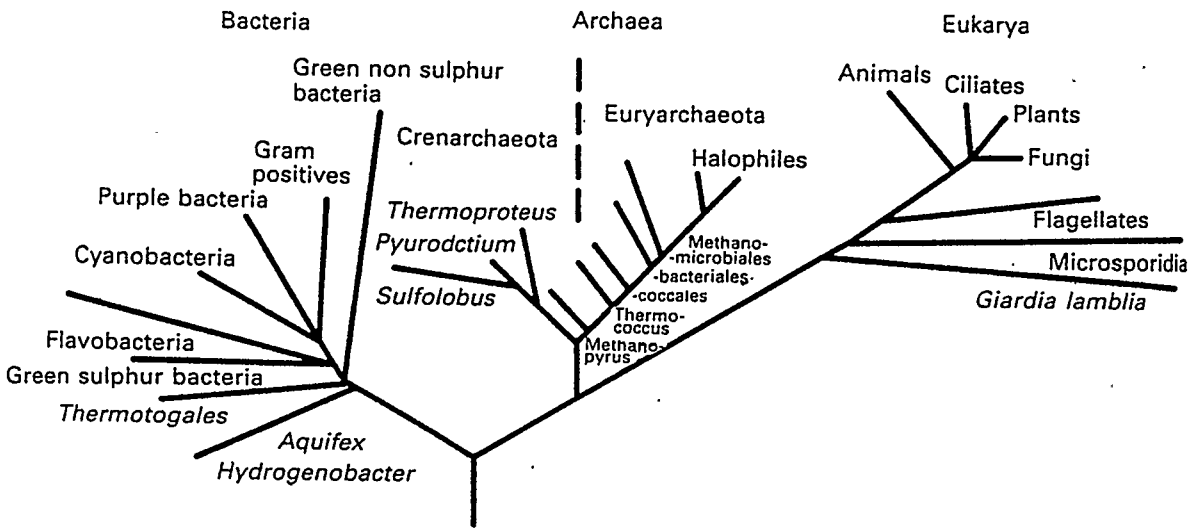

Figure 1.1. Universal phylogenetic tree showing the division of organisms into three domains and the evolutionary position of *G. lamblia* [Kandler, 1992].

sential part of the O_2-reducing electron transport chain which usually couples with oxidative phosphorylation [Hatefi and Stiggal, 1976], much less attention has been paid to the studies of this enzyme from microorganisms. Nevertheless, NADH oxidase has been identified and isolated from several bacterial species to date. These include facultative anaerobic bacterium *Amphibacillus xylanus* EP01 [Niimura et al., 1993], *Streptococcus faecalis* [Hoskins et al., 1962, Schmidt et al., 1986, Dolin, 1955], *Leuconostoc mesenteriodes* [Koike et al., 1985] and *Acholeplasma laidlawii* [Reinards et al., 1981]; the anaerobic extreme thermophile *Thermoanaerobium brockii* [Maeda et al., 1992]; the aerobic thermophiles *Thermus aquaticus* YT-1 [Cocco et al., 1988] and *Thermus thermophilus* HB8 [Park et al., 1992b]; and the aerobic bacteria *Bacillus megaterium* [Saeki et al., 1985].

The NADH oxidases isolated from the above bacterium utilize molecular oxygen as electron acceptor to oxidize NADH. The physiological role of the enzyme in these microorganisms, especially in the anaerobes, is uncertain. However, some explanations have been attempted. Since these bacterium lack cytochrome-mediated electron transport system, the oxidation of NADH by the NADH oxidase regenerates reducing power NAD^+ which may further facilitate the substrate-level phosphorylation as well as the maintenance of intracellular redox balance [Ahmed and Claiborne, 1989a, Niimura et al., 1993]. Moreover, the presence of this enzyme in the anaerobic bacterium possibly acts as a scavenger to remove harmful oxygen from the cellular environment to protect cell growth [Maeda et al., 1992].

1.3.2 Properties of bacterial NADH oxidase

NADH oxidases purified from the above bacterial species can be divided into two groups on the basis of product discrepancy. The enzymes from aerobically cultured

S. faecalis and *L. mesenteriodes* catalyze the direct four-electron transfer producing H_2O as a final product, on the other hand, the enzymes from the other bacterium involve two-electron reduction of O_2 to H_2O_2. The H_2O-forming NADH oxidases may consist high redox-potential flavin cofactors compared to the H_2O_2-forming NADH oxidases which accounts for this discrepancy.

In addition to utilizing molecular oxygen, these enzymes have been shown to possess the capability of transferring electrons from NADH to various electron acceptors such as dichloroindophenol, ferricyanide, cytochrome C, p-nitroblue tetrazolium and methylene blue. These compounds either mimic or are electron acceptors for enzymes involving respiration pathway such as NADH dehydrogenase. However, the common inhibitors for respiratory pathway including EDTA, NaN_3 and KCN had no effect on these bacterial NADH oxidase activity, except for the metal-containing enzyme derived from *A. laidlawii.* Therefore, these bacterial NADH oxidases are different from those enzymes in respiratory NADH dehydrogenase system and form a new group of enzyme. Why the bacterial NADH oxidases can reduce such a broad range of compounds is probably due to their structural similarity to those functional related flavoenzymes.

As for the electron donor, however, these microbial NADH oxidase are more specific for utilizing β-NADH as substrate. Although some enzymes from extreme thermophiles exhibit oxidation towards β-NADPH and α-NADH, the activity is much lower than that with β-NADH. Obviously, β-NADH is more rationally to be the natural substrate for these enzymes. In bacteria, β-NADH is mainly produced during glycolysis. Because of the absence of a respiratory electron transport chain, the existence of NADH oxidase in these microorganisms regenerates NAD^+ from NADH, thus allowing more efficient energy production through pathways involving substrate-level

phosphorylation [Niimura et al., 1993, Ahmed and Claiborne, 1989a].

A non-covalently bound flavin cofactor is involved in the electron transfer process. All the purified enzymes show the characteristic spectrum of flavoproteins with double absorption peaks at 380 nm and 450 nm. FAD has been proven to be the common prosthetic group for bacterial NADH oxidases, except for the enzyme from *A. laidlawii* which appeared to be a membrane-bound metallo-flavo protein containing FMN, nonheme iron, copper and labile sulfur as its redox center. It is also the only exception in the subunit composition. Unlike the other members, which are either monomer or identical polymer, it consists of three different size subunits of 65 kDa, 40 kDa and 19 kDa. It is not clear how these three subunits interact with each other to form a functional NADH oxidase. The size of NADH oxidase varies among the bacterium. The smallest enzyme derived from *T. thermophilus* is a monomer of 26.8 kDa, whereas the largest one from *T. brockii* consists of six identical subunits of 71.3 kDa. However, other than these two extreme cases and the oligomer composing enzyme from *A. laidlawii*, the five other bacterial NADH oxidases are comparatively consistent considering their subunit composition. They are either dimers or tetramers with subunit ranging from 48.9 kDa to 55 kDa.

Usually each enzyme subunit contains one FAD prosthetic group as electron transfer intermediate. But the NADH oxidase from *T. brockii* has two FAD molecules in each of its six subunits. Moreover, an iron-sulfur cluster, probably in the form of Fe_2S_2, has been identified in each subunit. No explanation has been proposed for this unusual structure. As for the other metal-containing NADH oxidase from *A. laidlawii*, the distribution of FMN cofactor among three different size subunits is not confirmed.

The flavin cofactor interacts with apoenzyme through non-covalent binding. In

contrast to other flavoproteins which contain a tightly bound cofactor, the flavin prosthetic group in several bacterial NADH oxidases is only weakly held by the enzyme. During the isolation of NADH oxidase from *S. faecalis, T. aquaticus, B. megaterium* and *T. thermophilus*, the addition of free flavin has been shown to be essential for retrival of maximum enzyme activity. This observation can be explained by the loss of the flavin cofactor during the purification procedures due to its weak association with the corresponding enzyme.

The genes encoding NADH oxidase have been cloned and sequenced from *S. faecalis* [Ross and Claiborne, 1992], *T. thermophilus* [Park et al., 1992a], *T. brockii* [Liu and Scopes, 1993] and *A. xylanus* [Niimura et al., 1993]. The typical FAD and NAD(P)-binding domains have been identified in all deduced amino acid sequences of these four proteins. Among them, Ross and Claiborne [Ross and Claiborne, 1992] have performed a detailed analysis on the derived 446 amino acid sequence of NADH oxidase from *S. faecalis*. Three motifs, corresponding to two $\beta\alpha\beta$ folds and one β sheet, are found in each subunit of this dimeric enzyme and are involved in adenine nucleotide-binding as well as flavin-binding. The region from Lys2 to Arg33 forms a $\beta\alpha\beta$-fold which is designated to bind the ADP moiety of FAD. However, this segment deviates from the consensus ADP-binding fingerprint sequence at the second glycine position. Instead of the "invariant" glycine triad (Gly-X-Gly-X-X-Gly) [Wierengar et al., 1986], the second glycine is replaced by threonine. Another domain corresponding to the second FAD-binding site is located from Thr271 to Asp281. This region is highly consistent with the sequence of TXXXXhyhhGD (h represents small hydrophobic amino acid and y is an aromatic residue) reported by Eggink and colleagues [Eggink et al., 1990]. It forms a β-sheet structure binding the flavin moiety of FAD. The second $\beta\alpha\beta$-fold spans from Arg149 to Asp180 and involves adenine

nucleotide-binding of NADH. This region possesses many common characteristics which are highly conserved in NADH-specific disulfide reductases such as lipoamide dehydrogenase [Westphal and Dekok, 1988]. A number of amino acids presented in NADPH-specific enzymes are replaced and the arrangement of amino acids benefits to NADH-binding. This well explains the preference for NADH over NADPH as electron donor by this enzyme as mentioned before. However, no suggestion with regard to the accommodation of electron acceptors such as O_2 has been proposed. A future X-ray structural analysis of NADH oxidase may be helpful for solving this unknown.

In addition, Ross and Claiborne [Ross and Claiborne, 1992] have also attempted to provide rational explanation for the observed lability of this enzyme towards the essential requirement of additional free flavin cofactor for its maximum activity. According to their proposal, Cys8 within the FAD-binding $\beta\alpha\beta$-fold domain is responsible for this unusual weak association between FAD prosthetic group and NADH oxidase apoenzyme. The easy oxidation of this specific cysteinyl residue may contribute to the loss of FAD cofactor during the purification procedures. In addition to Cys8, the replacement of the second glycine in the glycine triad may also play a role in the weak FAD-binding. To affirm the above assumptions, site-directed mutagenesis studies at these two amino acid positions are required to compare the impact on cofactor association by replacing them with other amino acids.

Two amino acid residues, His10 and Cys42, are also identified as the important catalytic groups, whose functions will be discussed in the following section. The C-terminal of the polypeptide was proposed to maintain the dimeric structure of this NADH oxidase.

1.3.3 Catalytic mechanism

Generally, the understanding of the catalytic mechanism for this group of enzymes is limited. Data have been mainly achieved from inhibition studies and spectrophotometric analysis. Some analysis results achieved from different bacterial NADH oxidases are even contradictory. It is possible that this class of enzymes may not possess an unique catalytic scheme. Nevertheless, a reasonable explanation for the catalytic reaction involving four-electron transfer has been proposed by Claiborne's group through their five years of studies on *S. faecalis*. This model will be presented in detail.

Despite the difference in products, all these bacterial NADH oxidases are capable of transferring electron to O_2 potentially via superoxide radical O_2^-. In order to determine the property of reaction intermediate, superoxide dismutase was used to dismute the superoxide radical [McCord and Fridovich, 1969]. The activity of NADH oxidases from *A. laidlawii* and *T. brockii* was inhibited by the superoxide dismutase, indicating the involvement of superoxide radical as an electron transfer intermediate for these two metal-sulfur containing proteins. On the contrary, superoxide dismutase had no effect on the enzyme reaction of the rest of members. Therefore, superoxide radical is not involved in the electron transfer process for these enzymes. The above two opposite results towards superoxide dismutase effect suggest that different catalytic pathways may exist between metal-sulfur containing enzymes and metal-deficient enzymes. This is probably due to the differences of redox centers. Whereas the metal-sulfur containing NADH oxidase employ iron-sulfur cluster in the form of Fe_2S_2 as part of the catalytic center, no metal ion participates at all in electron transfer catalyzed by the non-metal containing enzymes.

Nevertheless, a cysteinyl residue is regarded as the most potential candidate to act as the non-flavin redox group, either in the form of complex or derivatives. Thiol reagents such as $HgCl_2$ and iodoacetic acid derivatives have been shown to inhibit some of the NADH oxidases through inactivation of the redox active cysteine group. Although the enzymes from *A. laidlawii* and *T. thermus* seem inert to iodoacetamide or iodoacetate treatments, it can not exclude the possible existence of a complex form of cysteinyl residue which is unreactive towards a thiol reagent.

Identification of the redox active cysteinyl residue(s) has only been achieved for the NADH oxidase from *S. faecalis*. Actually, this is the only protein so far which has been extensively studied in this family. As previously described, this particular NADH oxidase consists of two identical subunits, each containing one FAD prosthetic group. It catalyzes direct four-electron reduction of O_2 to H_2O without the involvement of a superoxide radical. Thiol reagents and hydrogen peroxide inhibit the enzyme activity which has led to the first proposal that a redox-active disulfide may be involved in the electron transfer [Schmidt et al., 1986] as observed with most of the flavo oxidoreductases. However, anaerobic reduction of the enzyme with 1eq of NADH/FAD only regenerated one additional DTNB-reactive thiol group, in contrast to two expected thiol groups from the putative redox active disulfide. Furthermore, sequence analysis of this enzyme can not prove the presence of a disulfide.

Ahmed and Claiborne concluded from their anaerobic titration studies and suggested that a single cysteinyl residue may function as redox-active group in each subunit [Ahmed and Claiborne, 1989a, Ahmed and Claiborne, 1989b]. In addition, they identified this residue as Cys42. It is interesting to point out that this redox-active residue, according to their studies, exists as an unusual stabilized cysteine-sulfenic acid (Cys-SOH) in the native, oxidized form of enzyme. Therefore, each subunit of

this NADH oxidase consists of one FAD cofactor and one cysteine derivative as its catalytic centre.

Despite the apparent identity between the two subunits, the active centers are not functionally equivalent. Anaerobic reduction of the enzyme with sodium dithionite, a thiol reducing reagent, yielded a three-electron/FAD or six-electron/dimer stoichiometry in contrast to the expected four-electron/FAD [Ahmed and Claiborne, 1989a, Ahmed and Claiborne, 1989b]. In other words, dithionite titration can only cause the complete reduction of one of the subunits referred as the EH_4 form in which both FAD and cysteine-sulfenic acid are in their reduced form, whereas the other subunit is only partially reduced to its EH_2 form consisting of reduced $FADH_2$ and the original state of cysteine-sulfenic acid. This EH_2/EH_4 form enzyme can not be further reduced to EH_4/EH_4 state. Although Ahmed and Claiborne did not mention the significance of this non-functional equivalence between the two cysteine derivatives, it is possible that the reduction of cysteine-sulfenic acid in one subunit by NADH somehow retards the formation of reduced cysteine derivative in the other subunit, this, in turn, only allows one subunit to carry out the catalytic function instead both of them function simultaneously. However, there should not be any preference about which particular subunit performs the catalytic function. At present, it is not clear why only one of the identical subunits is involved in the electron transfer during a single reaction. If there is any cooperation between the two subunits needs further investigation.

Ahmed and Claiborne have also shown that the off-rate for bound NAD^+ is extremely slow and NAD^+ dissociation occurs after the oxygen incorporation [Ahmed and Claiborne, 1989a, Ahmed and Claiborne, 1989b]. It forms a very stable complex with the reduced state of the enzyme. The binding site for NAD^+ is local-

ized proximate to the FAD-binding domain for the isoalloxazine moiety. The tightly bound NAD^+ is proposed to facilitate intramolecular electron transfer for reduction of the reaction intermediate (hydroperoxide) as well as regeneration of cysteine-sulfenic acid. In this case, NAD^+ is not only a product but also serves as an effector.

In addition to Cys42, another amino acid residue identified as His10 is proposed to serve as an acid-base catalyst in the active site. This is a very common characteristic among disulfide oxidoreductases to employ an active site residue, usually histidine or serine, as proton donor or acceptor [Karplus and Schulz, 1987, Schierbeek et al., 1989, Mulrooney and Williams, 1994, Rietveld et al., 1994]. Although this residue is not directly involved in electron transfer, it plays a vital role in carrying on the reaction. It has been shown that the replacement of an acid-base catalyst (His439) with an alanine residue in *E. coli* glutathione reductase resulted in more than a 600-fold decrease in the enzymatic reaction rate [Rietveld et al., 1994]. The function of His10 in *Streptococcus* NADH oxidase is probably to protonate the hydroxy group of hydroperoxide to release H_2O product.

The foregoing led Ahmed and Claiborne [Ahmed and Claiborne, 1989b] to suggest a plausible catalytic scheme for *Streptococcus* NADH oxidase as shown in Figure 1.2. However, more studies are needed to determine if this scheme applies to the other bacterial NADH oxidases as well.

1.3.4 Homologous comparison

The gene sequences for microbial NADH oxidases have been revealed by different research groups from *S. faecalis, T. brockii, T. thermophilus* and *A. xylanus* [Ross and Claiborne, 1992, Liu and Scopes, 1993, Park et al., 1992b]

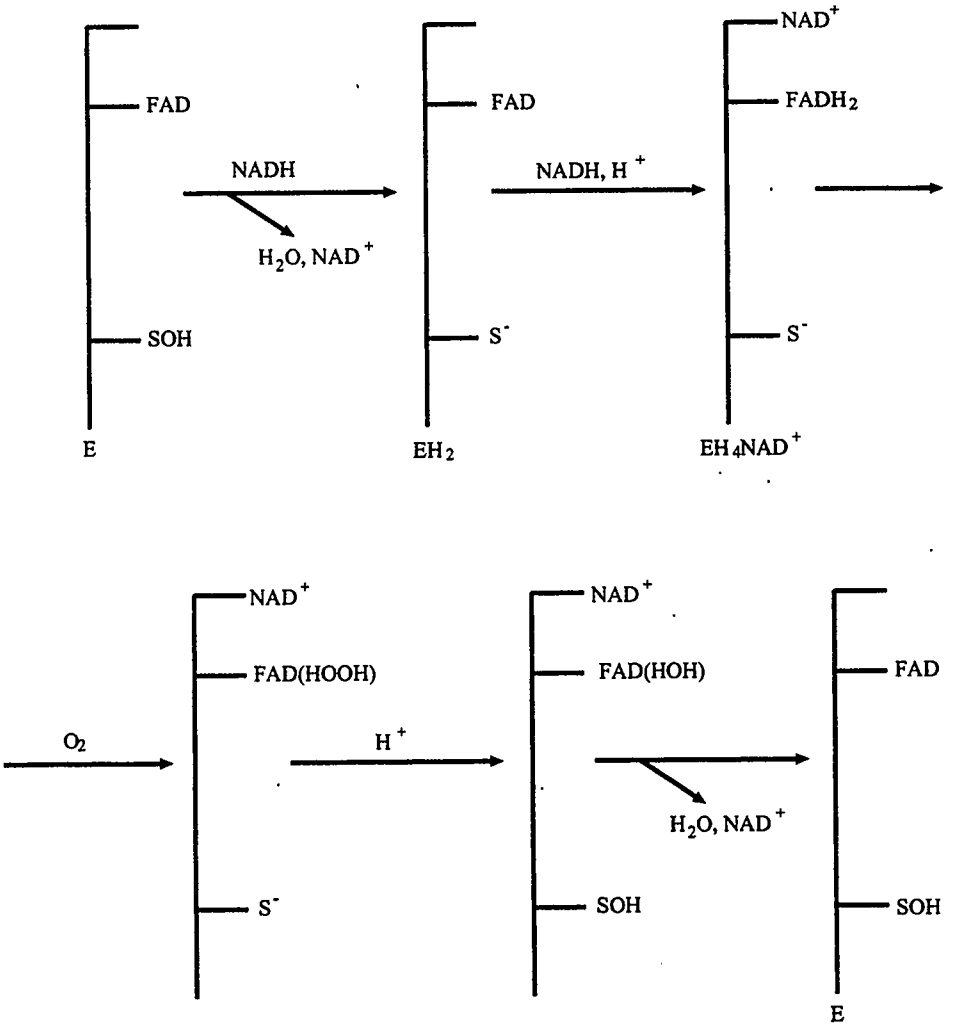

Figure 1.2. The catalytic scheme for NADH oxidase from *Streptococcus faecalis* proposed by Ahmed and Clairborne [Ahmed and Clairborne, 1989b].

[Niimura et al., 1993]. It has been suggested that all the flavoproteins may have originally derived from a common ancestral adenine nucleotide-binding protein [Ross and Claiborne, 1992]. In order to search for the linkage among flavoproteins, the sequencing data were subjected to analysis.

The result are surprising for the thermophiles. Except for the common dinucleotide-binding fingerprints, no significant sequence homology has been found between the two thermal NADH oxidases and other proteins [Liu and Scopes, 1993] [Park et al., 1992b]. The thermophilic bacterium have possibly developed very special protein structures during evolution to cope with their extreme environment. Therefore, their protein composition may deviate from the others for the sake of thermal stability.

In contrast, the sequence analysis for the enzyme from *S. faecalis* [Ross and Claiborne, 1992] and *A. xylanus* [Niimura et al., 1993] shows limited but significant sequence homology with flavo disulfide oxidoreductases. The amino acid sequence of *A. xylanus* NADH oxidase possesses 51.2% and 72.5% sequence identities to those of alkyl hydroperoxide reductase F52a from *Salmonella typhimurium* [Tartaglia et al., 1990] and the NADH dehydrogenase from an alkalophilic *Bacillus sp.* [Xuemin et al., 1991], respectively, regardless of the distance among three species. The two $\beta\alpha\beta$ folds involving ADP-binding sites share 22.5% sequence homology with the NADH oxidase from *S. faecalis.*

The analysis on *Streptococcus* NADH oxidase was performed by Ross and Claiborne [Ross and Claiborne, 1992]. They compared this protein with a large range of flavo disulfide oxidoreductases. It was found that this enzyme shares 44% identical sequence with the NADH peroxidase obtained from the same strain [Ross and Claiborne, 1991]. Moreover, the regions involving dinucleotide-binding and catalytic function are strik-

ingly resemble. Not only do they possess the same active site residues identified as Cys42 and His10, but also employ similar catalytic schemes. These two enzymes can be considered extremely closely related. On the other hand, the *Streptococcus* NADH oxidase exhibits a considerable level of homology in protein sequence with a group of dinucleotide-requiring disulfide oxidoreductases, including trypanothione reductase, lipoamide dehydrogenase, mercuric reductase, alkyl hydroperoxide reductase and glutathione reductase. Although the overall sequence identity is not remarkable, ranging from 29% with *Straphylococcal* mercuric reductase [Laddaga et al., 1987] to 17% for alkyl hydroperoxide reductase.F52a from *Salmonella* [Tartaglia et al., 1990], the sequences associated with dinucleotide-binding are highly conserved.

The flavo proteins mentioned above are from different species which are taxonomically distant from *S. faecalis* and *A. xylanus*. However, the two bacterial NADH oxidases show considerable sequence homology with these flavoproteins, especially in the regions involving dinucleotide-binding. Furthermore, despite the difference in substrate specificity, all these enzymes contain FAD as a prosthetic group and catalyze electron transfer with the participation of adenine dinucleotide. Based on the above findings, the flavo oxidoreductases are thought to have evolved from a common ancestral dinucleotide-binding protein and diverged in function during the evolutionary process. As more and more sequence data are revealed, we will see clearer linkage among the flavoproteins.

In conclusion, studies on microbial NADH oxidase are only at a preliminary stage. Some results to date are even contradictory and more research is required to clarify these observations. Hopefully, studies on this enzyme from *G. lamblia* may help to achieve a better understanding about this protein family.

CHAPTER 2

MATERIALS

2.1 Reagents

All the common laboratory chemicals were obtained from Fisher, Sigma or BDH. Casein hydrolysate and NCTC vitamin mix were obtained from Gibco-BRL. Yeast extract and dextrose were obtained from BDH. CLEX was supplied by Dextran Product Limited. Penicillin G and streptomycin sulfate were from Sigma. NADH, NADPH, FAD, FMN, catalase, peroxidase and ABTS were obtained from Boehringer Mannheim. Protein molecular weight markers and precast Phastgel 8-25 for PAGE were obtained from Pharmacia. All Procion dyes were obtained from Imperial Chemical Corporation. The reagent of p-arsanilic acid was purchased from Eastman Kodak. The chromatography matrices of Octyl-Sepharose 4B, Sepharose 6B, Thiopropyl-Sepharose 6B, Mono Q and Superose 12 were obtained from Pharmacia. Micro-prep ceramic hydroxyapatite was obtained from Bio-Rad.

2.2 Cell Strain

Giardia lamblia strain WB was initially obtained courtesy from the laboratory of Dr. Ceri (Department of Biological Sciences, The University of Calgary).

2.3 Growth Medium

G. lamblia trophozoites were cultured *in vitro* in modified TYI-S-33 medium [Keister, 1983]. The ingredients and their relative quantities in one liter of the medium

Table 2.1. Composition of the modified TYI-S-33 medium for *Giardia lamblia*

Ingredient	Quantity/per litre of media
Casein hydrolysate	20.0 g
Yeast extract	10.0 g
Dextrose	10.0 g
NaCl	2.0 g
K_2HPO_4	1.0 g
KH_2PO_4	0.6 g
L-cysteine	2.0 g
L-ascorbic acid	0.2 g
Ferric ammonium citrate	22.8 mg
NCTC vitamin mix	30.0 ml
CLEX	100.0 ml
Milli Q H_2O	870.0 ml

are listed in the Table 2.1.

The medium without NCTC vitamin mix and CLEX was first adjusted to pH 6.8 with 5 M NaOH and sterilized by passing through 0.22 micron hydrophilic membrane filter (Millipore) with a Millipore prefilter. The NCTC vitamin mix and CLEX, which are sterile commercially, were then added directly to the filtered solution. To inhibit the growth of microorganisms other than *G. lamblia*, the antibiotic solution containing penicillin G (5.88×10^5 units) and streptomycin sulfate (0.588 g) was added through Millipore syringe filter to each litre of the medium [Meyer, 1976].

CHAPTER 3

METHODS

3.1 Cell Culture, Harvest and Breakage

G. lamblia trophozoites were propagated in the modified TYI-S-33 medium referred to the "growth medium" in the previous Chapter. The cultures were incubated in autoclaved 50 ml screw cap culture tubes at 37°C.

The *G. lamblia* strain was maintained by regularly subculturing the trophozoites. When the cell population reached late log phase (72 to 96 hours), 3 ml of the old culture was transferred to another sterile 50 ml tube containing 47 ml of fresh medium. This procedure was repeated every three to four days.

To collect cells, large scale cell culturing was involved which required inoculation of cells in one 500 ml rolling bottle (Bellco) and forty 50 ml culture tubes. Cell harvesting was pursued during the late log phase of growth to obtain maximum yield. This process involved cold-shocking the cultures on ice for 10 to 15 minutes followed by centrifugation at $500 \times g$ in a Sorvall GS3 rotor at 4°C. The cell pellet was then washed twice with phosphate buffered saline (containing 77 mM NaCl, 32 mM KH_2PO_4 and 47 mM K_2HPO_4) at pH 6.8 and subjected to centrifugation at $500 \times g$ using a Sorvall SS34 rotor at 4°C. The collected cells were suspended in 15-20 ml of phosphate buffered saline containing 7% glycerol and stored at -70°C until needed.

Cell breakage of *G. lamblia* was achieved by sonication or French press. The frozen

cells were thawed at 4° and pelleted by centrifugation as described above. The cells were resuspended in 50 mM potassium phosphate buffer at pH 7.0. For small volume (1.0-1.5 ml) of cell suspension, a Sonifier Cell Disrupter (Model-W350) was used in a pulsed mode and 40% duty cycle. The cell suspension was subjected to two 15 second cycles of sonication with a 15 second interval of cooling on ice to prevent overheating. Cell breakage using French press was performed on a large volume of sample. The cells were disrupted by passing through the press three times at a pressure of 1000 to 1500 psi. The protease inhibitor PMSF (phenylmethylsulfonyl fluoride), previously dissolved in ethanol as 100 mM stock solution, was added into the homogenate immediately after the first cycle of cell breakage to a final concentration of 0.1 mM. The cell debris was removed by centrifugation at 20,000 rpm for 30 minutes using Sorvall SS34 rotor at 4°C. The supernatant, referred as crude extract, was retained for analysis and purification.

3.2 Enzyme Assay

The routine enzyme assay for NAD(P)H oxidase was performed at room temperature in 50 mM potassium phosphate buffer at pH 7.0. A total volume of 1.0 ml assay mixture contains 0.2 mM NADH or NADPH, 0.02 mM FAD and 50-200 μl of fraction sample from different purification stages. The enzyme activity was measured with a Hitachi Spectrophotometer (Model U-2000) by following the decrease of absorbance of NAD(P)H at 340 nm over two minutes. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 μmol of NADH per minute at room temperature. The specific activity referred to units per mg protein. The extinction coefficient for NADH at pH 7.0 is 6.22×10^3 $M^{-1}cm^{-1}$. The assays for enzyme characterization were carried out under special conditions which will be specified separately.

3.3 Protein Assay

The protein concentration was determined using Bio-Rad protein assay kit based on the method of Bradford [Bradford, 1976]. Bovine serum albumin was used as protein standard at concentration of 0.2, 0.4, 0.6, 0.8 mg/ml for the standard assay, or 2.0, 4.0, 6.0, 8.0, 10.0 μg/ml in the microassay. The fraction samples from crude extract or different purification steps were diluted to appropriate concentrations if necessary and subjected to the standard assay or the microassay depending on their concentrations. For the standard assay, 100 μl of each standard or sample was used to mix with 5$\times$ diluted dye reagent. In the microassay, which was applied for measuring low concentration samples, 800 μl of each standard or sample was taken to mix with 200 μl of dye reagent. The above reaction mixtures were incubated at room temperature for 15 minutes to develop color change which were subsequently measured at 595 nm with the spectrophotometer. The standard curve was prepared by using absorbance values versus concentrations for bovine serum albumin standards. Comparison to the standard curve provided the protein concentrations for fraction samples.

3.4 Purification of NAD(P)H Oxidase

3.4.1 Purification attempt with synthesized matrices

3.4.1.1 Trivalent arsenical-agarose matrix

The purification of NAD(P)H oxidase from *G. lamblia* was initially attempted with synthesized matrices including trivalent arsenical-agarose matrix and several dye-ligand matrices. All the matrices were synthesized successfully in our laboratory.

The arsenical matrix (Sepharose-S-$CH_2CONHPhAsBr_2$) was obtained by coupling

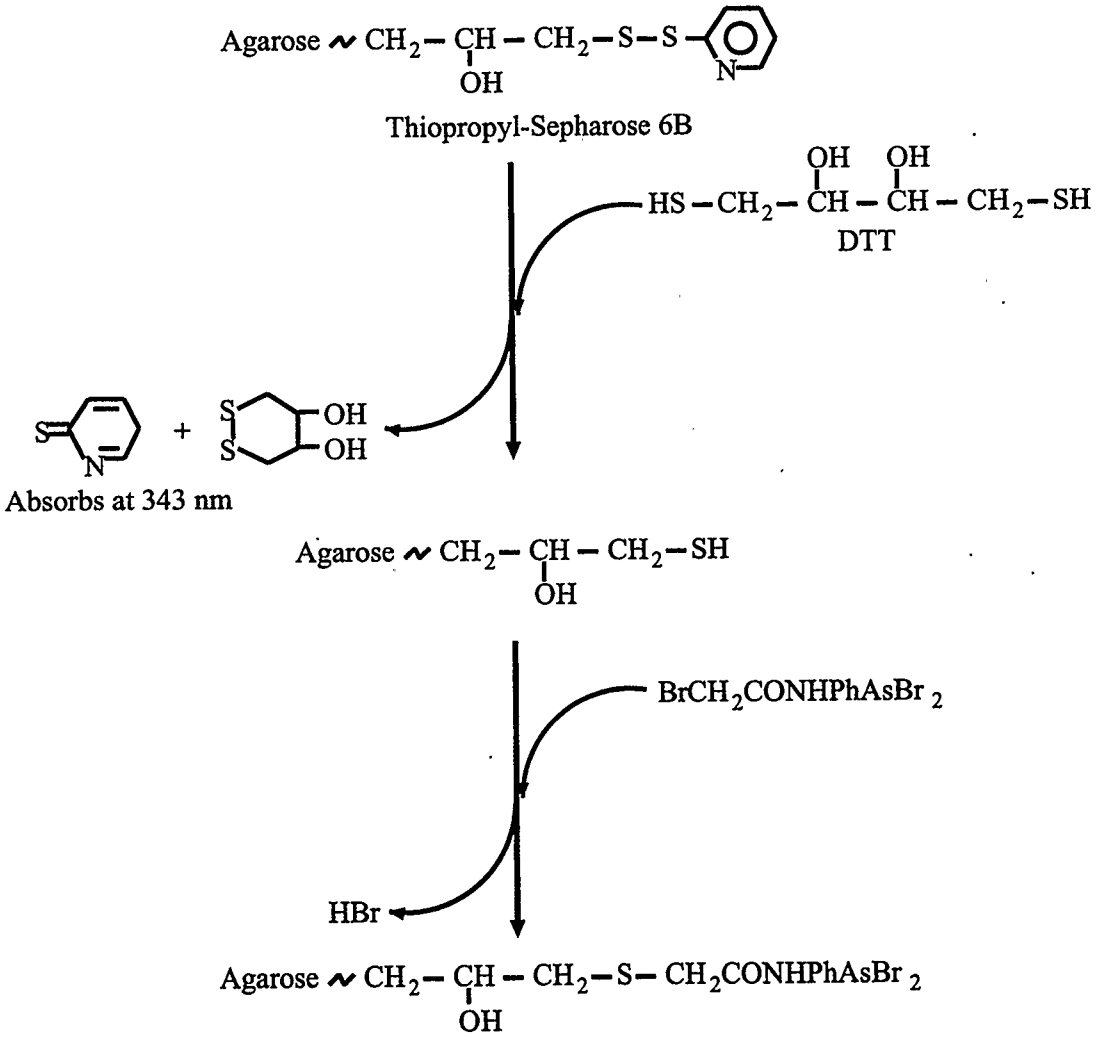

Figure 3.1. Synthesis of trivalent arsenical-agarose matrix using Thiopropyl-Sepharose and trivalent arsenical.

the bifunctional arsenical compound $BrCH_2CONHPhAsBr_2$, which was synthesized from p-arsanilic acid and bromo acetyl bromide following the method described by Robinson [Robinson, 1980], to Thiopropyl-Sepharose 6B (Pharmacia). The Thiopropyl-Sepharose has been commercially made in the activated disulfide form possessing thiopyridine. This activated Sepharose matrix was first reacted with dithiothreitol (DTT) in a ratio of 120 μmol DTT per g of Sepharose in 50 mM potassium phosphate buffer at pH 7.5 to produce a terminal reactive thiol group on the agarose and release 2-thiopyridone stoichiometrically. The thiol content of the matrix was determined by measuring the production of 2-thiopyridone which absorbs at 343 nm with an extinction coefficient of 8.08×10^3 $M^{-1}cm^{-1}$. The free thiol form of the matrix was washed repeatedly with water followed by 30% ethanol. The bifunctional reagent $BrCH_2CONHPhAsBr_2$, dissolved in 30% ethanol, was added to the matrix in 1.5-fold excess over the amount of thiol groups on the matrix. After mixing the two reactants for 24 hours to allow maximum coupling, the matrix was washed throughly with 30% ethanol to remove any unreacted arsenical. Finally, iodoacetamide was added in excess (equals to the amount of thiol groups on the matrix) and mixed for 2 hours to block any remaining free thiol groups on the matrix. Washing with water removed the excess reagent and the matrix was ready for use. The diagram in Figure 3.1 shows the entire process for this synthesis of the trivalent arsenical matrix. All the above reactions were carried out at room temperature.

To analyze the capacity of the arsenical-agarose matrix, 1.0 ml of newly synthesized matrix was first washed thoroughly with 50 mM potassium phosphate buffer at pH 8.0 and mixed with 1.0 ml of this buffer containing excess amount (50 μmol) of dihydrolipoamide, a dithiol compound which is capable of binding to the matrix by forming a cyclic dithioarsine derivative with the arsenical moiety of agarose. The re-

maining (uncomplexed) dihydrolipoamide in solution was removed by filtrationn and quantitated by its thiol content using 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in 50 mM potassium phosphate buffer at pH 8.0. DTNB reacts quantitatively with thiol groups in dihydrolipoamide to produce thionitrobenzoate anions which absorb strongly at 412 nm with an extinction coefficient of 1.36×10^4 $M^{-1}cm^{-1}$. For each mol of dihydrolipoamide, two mol of thionitrobenzoate anions are produced from the reaction with DTNB. Thus, the amount of free dihydrolipoamide could be determined using a spectrophotometer. This, in turn, provided the amount of dihydrolipoamide bound to the arsenical matrix. Since dihydrolipoamide reacted with the arsenical groups stiochiometrically, the content of arsenical groups on the matrix could be inferred.

The synthesized arsenical-agarose was utilized in the initial attempts to purify the NAD(P)H oxidase. After washing repeatedly with 50 mM potassium phosphate buffer at pH 7.0, 1.5 ml of the matrix was placed in a 15 ml screw cap tube and the crude extract (1.5 ml) was added. This suspension was then mixed using a wrist shaker at 4°C for 6 hours. Following centrifugation to sediment the matrix, the supernatant was assayed for enzyme activity. The matrix was then washed with 50 mM potassium phosphate buffer at pH 7.0 to remove any unbound proteins. The effluent was also subjected to enzyme assay. No enzyme activity for the NAD(P)H oxidase was detected in the proteins which did not attach to the matrix. To desorb the proteins complexed to the arsenical matrix, the buffer containing 50 μmol dihydrolipoamide was added. Following a two hour incubation at 4°C, the matrix was centrifuged so that an aliquot of the supernatant could be removed for the enzyme activity assay. A control sample was prepared in parallel by mixing crude extract with Sepharose 4B and the same steps mentioned above were also performed on this sample.

3.4.1.2 Dye-ligand Sepharose matrices

Dye-ligand chromatography can be a highly effective form of affinity chromatography for isolation of nucleotide-requiring proteins. The proteins bind to the dye-ligand matrix due to the structural similarity between their nucleotide cofactor and the synthetic dye. Since NAD(P)H oxidase consists of flavin cofactor and utilizes NAD(P)H as substrate, it was likely that this enzyme would bind to the dye-ligand matrix. The preparation of the dye-ligand matrix followed the method proposed by Lowe and colleagues [Lowe et al., 1980]. Simply, preswollen Sepharose 6B (Pharmacia) matrix was suspended in 0.2 M Na_2CO_3 solution at pH 11. The Procion dye dissolved in the same Na_2CO_3 solution was added to the Sepharose suspension. After incubation at 59°C for 40 minutes, the reaction mixture was left at room temperature for 24 hours to allow for maximum coupling. Finally, the matrix was washed throughly with water to remove any unreacted dye molecules. Various Procion dyes (Cibacron Blue 3GA, Procion Red HE-3B, Procion Red HE-7B, Procion Yellow MX-R, and Procion Orange MX-G) were successfully coupled to the Sepharose matrix. To evaluate the effectiveness of the Procion-Sepharose matrix on the isolation of NAD(P)H oxidase, a column (0.9 × 10 cm, Pharmacia) was prepared using about 5 ml of each matrix. The column was equilibrated with 50 mM potassium phosphate buffer at pH 7.0, and a small amount (about 2 ml) of crude extract was applied to the matrix and the unbound proteins were eluted with the same buffer. The desorption of bound proteins was tried with different solutions. The buffer containing 2 mM NADH or 1 mM FAD as mobile ligand was used to desorb any nucleotide-requiring proteins bound to the matrix. In addition to this, high salt buffer (buffer containing 1 M KCl) was employed to release bound proteins by disrupting the ionic bonds between the proteins and the matrix. The above chromatography procedures were carried out at

4°C. Samples of non-binding proteins and proteins desorbed from Procion-Sepharose were analyzed for NAD(P)H oxidase activity.

3.4.2 Overview of purification procedures

After many successful or disappointing attempts, an appropriate purification approach has been achieved. NAD(P)H oxidase was isolated from *G. lamblia* by ammonium sulfate fractionation, Octyl-Sepharose hydrophobic chromatography, Hydroxyapatite adsorption and finally via ion-exchange chromatography on a Mono Q matrix. The following sections will provide detail descriptions on each of these procedures. It should be pointed out that this NAD(P)H oxidase is very labile. The stabilization studies (Section 3.5.3) performed prior to the purification demonstrated that the enzyme activity was easily lost during storage. Because of the lability of this enzyme, all the following purification operations were performed at 4°C in the presence of 0.5 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). All chromatography matrices except for Mono Q were set up in Amicon adjustable columns.

3.4.3 Salt fractionation

Salt fractionation using ammonium sulfate is a very simple yet effective method to fractionate proteins. It is commonly adopted as the initial step for purification. Pilot experiments were first implemented to determine the optimum range of salt fractionation using ammonium sulfate at pH 7.0. Sufficient ammonium sulfate (solid) was added individually into seven 1.0 ml volume of crude extract samples to bring the ammonium sulfate saturation to 10%, 20%, 30%, 40%, 50%, 55% and 60%, respectively. Solublization of the ammonium sulfate led to protein precipitation and

the samples were centrifuged at 12,000 rpm for 30 minutes using a microcentrifuge (Eppendorff 5415C) to remove the precipitate. The supernatant was analyzed for both protein absorbance at 280 nm and NAD(P)H oxidase activity. The obtained data were plotted versus percent salt saturation. This provided information on the salting-out range for NAD(P)H oxidase under these conditions. These procedures also gave an indication of the extent of protein precipitation. For large scale preparations, solid ammonium sulfate was added into crude extract (15-20 ml) to achieve a final 25% saturation. Because of the acidic property of ammonium sulfate, 1 M KOH was used to maintain the solution pH at 7.0. After centrifugation at 15,000 rpm for 30 minutes in a Sorvall SS34 rotor, the supernatant was assayed for NAD(P)H oxidase activity and the protein concentration was determined. This sample was subjected to further purification on Octyl-Sepharose. A small portion (0.5 ml) was removed and retained for later analysis by SDS-PAGE.

3.4.4 Hydrophobic chromatography on Octyl-Sepharose 4B matrix

Hydrophobic chromatography separates proteins on the basis of their surface hydrophobicity. Three Pharmacia hydrophobic matrices, including Octyl-Sepharose 4B, Phenyl-Sepharose CL-4B and Butyl-Sepharose 4B, were examined for their effectiveness. Octyl-Sepharose 4B matrix provided the most effective performance for isolating NAD(P)H oxidase and was selected. A hydrophobic chromatography column was prepared using about 30 ml of Octyl-Sepharose 4B matrix. Following column equilibration with 50 mM potassium phosphate buffer at pH 7.0 containing 25% ammonium sulfate, the supernatant from ammonium sulfate treatment was applied to the column. The flow rate was maintained at 1 ml per minute (Gilson minipuls 2 pump), and a Bio Rad fraction collector (Model 2110) was used to collect samples with 2.0 ml

volume. The starting buffer, containing 25% ammonium sulfate, was passed through the column first to remove any proteins which did not bind to the matrix. Subsequent to this high salt wash, 25 mM potassium phosphate buffer at pH 7.5 was applied to desorb the hydrophobic proteins bound to the matrix. All collected fractions were analyzed for enzyme activity and absorbance at 280 nm. The activity for NAD(P)H oxidase was mainly detected in the low salt desorption fractions, although some of the passing-through fractions showed slight activity. Fractions with high enzyme activity were pooled and 0.5 ml of this sample was retained for SDS-PAGE. Before applied to hydroxyapatite matrix, the pooled sample was desalted with Amicon centriprep-30 to remove ammonium sulfate.

3.4.5 Hydroxyapatite adsorption chromatography

Hydroxyapatite has been widely used for separation of proteins and nucleic acids since the work of Tiselius in 1956 [Tiselius et al., 1956]. It has incredibly high capacity and is less time-consuming compared to other chromatography methods. Micro-prep ceramic hydroxyapatite (Bio-Rad) was used for its physical and chemical stability, which allows a good flow rate under pressure. To secure the best performance, buffers required for this chromatography step were degassed before use. A 30% slurry was prepared by suspending 3 g of hydroxyapatite dry powder in 25 mM potassium phosphate buffer at pH 7.5. The slurry supplied about 5 ml of hydroxyapatite matrix in the column. Ten column volumes (50 ml) of 25 mM potassium phosphate buffer at pH 7.5 was allowed to pass through the matrix for equilibration. The desalted sample from the hydrophobic chromatography was then applied to this hydroxyapatite column. After removal of non-interacting proteins with the loading buffer, a linear gradient of 25 mM to 500 mM potassium phosphate at pH 7.5 was created by mixing

15 ml of each buffer to desorb proteins bound to the column, including NAD(P)H oxidase. The flow rate during the whole process was controlled at 1 ml per minute, whereas the eluted fractions were collected in 2 ml volume. After analysis for enzyme activity and absorbance at 280 nm, the fractions with high enzyme activity and low protein content (low absorbance reading at 280 nm) were combined for further purification on Mono Q matrix. Before being applied to the Mono Q column, the pooled sample was desalted using Amicon centriprep-30 to bring the ionic strength to 25 mM potassium phosphate at pH 7.5. A small portion (0.5 ml) of this sample was removed to be analyzed by SDS-PAGE.

3.4.6 FPLC on Mono Q matrix

As a final purification step, ion-exchange chromatography was performed on a Mono Q matrix using Pharmacia Fast Protein Liquid Chromatography (FPLC) system. Mono Q matrix is an anion exchanger which adsorbs negative charged proteins through ionic interaction. The desorption of proteins is achieved by applying a gradient of high salt to the column. The Mono Q column was first equilibrated with 25 mM potassium phosphate at pH 7.5. The flow rate was adjusted to 1 ml per minute and the fraction collector was set at 0.5 ml per minute which gave a 0.5 ml fraction size. Prior to sample injection, the desalted sample obtained from the hydroxyapatite was centrifuged at 20,000 rpm for 10 minutes in a Sorvall SS34 rotor to remove particles. The sample was applied to the matrix through a super loop supplied by Pharmacia. The non-negative charged proteins were eluted from the column with 25 mM potassium phosphate starting buffer at pH 7.5. A nonlinear salt gradient from 0 to 1 M KCl in 25 mM potassium phosphate buffer at pH 7.5 was sebsequently applied to desorb proteins attached to the matrix. The formation of salt gradient was designed

as follows. The increase of KCl concentration from 0 to 500 mM took 40 minutes, whereas only 5 minutes was allowed to further reach to 1 M KCl. NAD(P)H oxidase was eluted from this column by salt gradient. Fractions with enzyme activity were examined individually by SDS-PAGE.

3.5 Characterization of NAD(P)H Oxidase

3.5.1 SDS-PAGE

To evaluate the above purification results, SDS-PAGE was performed on a Pharmacia PhastSystem in the presence of β-mercaptoethanol and SDS [Laemmli, 1979]. Samples from different purification stages were desalted and concentrated or, alternatively, diluted to bring their absorbance at 280 nm near 3. This treatment was performed using Amicon microcon-10. To each 3 μl of these samples, equal volume of 2$\times$ sample buffer was added. The sample buffer contains 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 2.5% SDS, 5.0% β-mercaptoethanol and 0.01% bromophenol blue. A sample of low molecular weight standard (Pharmacia) was also prepared. After boiling for 5 minutes, the samples were loaded on precast Phastgel 8-25 (stacking gel contains 8% T and 3% C; separating gel consists of 8 – 25% T and 2% C) through a sample applicator consisting of eight 1 μl wells. The running buffer for PhastSystem was cast into agarose strips consisting of 0.2 M tricine, 0.2 M Tris at pH 8.1, 0.55% SDS and 2% agarose. The electrophoresis was run for 75 Vh and the gel was developed using Coomassie Blue staining technique according to the manufacturer's direction. The fractions corresponding to one single band on SDS-PAGE gel were combined for further characterization. The molecular weight for denatured NAD(P)H oxidase was also determined from the SDS-PAGE by comparing its migration distance to those of the molecular weight standards. These standards include phosphorylase B (94,000

Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), soy bean trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da). The migration distance for each standard was plotted against the logarithm value of its molecular weight to generate a standard curve. Referred to this curve, the molecular weight for denatured NAD(P)H oxidase could be estimated from its migration distance.

3.5.2 Determination of native molecular weight

To determine the native molecular weight of NAD(P)H oxidase, gel filtration chromatography was carried out on a Superose 12 column via the Pharmacia FPLC system. A single running buffer, 50 mM potassium phosphate at pH 7.0, was used in the chromatography process. The flow rate was maintained at 0.5 ml per minute and 0.5 ml size fractions were collected. Samples applied to the column were in 200 μl volume to avoid overloading the column. The column was calibrated with Pharmacia molecular weight standards including Blue Dextran (2,000,000 Da), ferritin (440,000 Da), catalase (232,000 Da), aldolase (158,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), chymotrypsinogen A (25,000 Da), and ribonuclease A (13,700 Da). An additional standard of ATP was also required for column calibration. The standards were run through the column individually to prevent any interaction among them. Both freshly prepared crude extract and purified enzyme were subjected to gel filtration chromatography for molecular weight determination. The reason to use two fraction samples is to examine if there is any affect by purification process on the subunit composition for NAD(P)H oxidase. The elution volumes for both samples were the same. Since the elution volume for a protein is related to its size [Ackers, 1970], the native molecular weight for NAD(P)H oxidase could be determined by compar-

ing to a standard curve derived from the calibration proteins. For each standard protein and NAD(P)H oxidase sample, an average distribution coefficient (Kav) was calculated according to the formula of $Kav = (Ve - Vo)/(Vt - Vo)$, where Ve is the elution volume for a protein; Vo is the void volume of the column determined by the elution volume of Blue Dextran; Vt is the total volume of the column obtained from the elution volume of ATP. A standard curve was established by plotting the Kav values for the protein standards versus the logarithms of the corresponding molecular weights. Referring to this standard curve, the native molecular weight for NAD(P)H oxidase could be determined from its calculated Kav value.

3.5.3 Stabilization studies

Before the purification efforts were carried on, crude extract was subjected to various conditions to search for the optimum combination in stablizing NAD(P)H oxidase activity. Three samples were prepared by diluting crude extract 20 fold with 50 mM potassium phosphate buffer at pH 7.0; the buffer plus 0.1 mM PMSF; and the buffer in the presence of 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol. All the samples were stored at 4°C. Nitrogen gas was allowed to bubble through the sample prepared with buffer containing 0.1 mM PMSF. An aliquot of each sample was removed for enzyme activity assay after 0, 1, 2, 5, and 24 hours of storage. The relative enzyme activity, which is the percentage of enzyme activity compared to the starting sample, was plotted against the corresponding time of storage to determine the effect of different conditions on NAD(P)H oxidase stability. The stabilization studies were also performed on the crude extract containing either 1 mM DTT or 1 mM EDTA by following the enzyme activity change over time.

3.5.4 pH profile

To determine the pH profile of NAD(P)H oxidase activity, four different buffer systems were used to cover a pH range of 5.5 to 10. Acetic acid was adjusted with sodium hydroxide to pH 5.5 and 6.0. Potassium phosphate was used within pH range of 6.0 to 8.0. From pH 7.5 to 8.5, Tris/HCl was used, whereas glycine/HCl was applied to the alkaline pH range of 8.0 to 10. All the buffers were made in 50 mM concentration. The effect of pH change on NAD(P)H oxidase activity was shown by performing the enzyme assay under different pH conditions such as 5.5, 6.0, 6.3, 6.5, 6.8, 7.0, 7.2, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The measurement of enzyme activity for this study deviated from the standard assay on the buffer system. Unlike the standard enzyme assay, appropriate buffer was used to maintain the assay mixture at a certain pH value as mentioned above. *G. lamblia* crude extract was the source of NAD(P)H oxidase.

3.5.5 Salt effect on NAD(P)H oxidase

Two types of potassium salts-potassium phosphate and potassium chloride were employed to determine the optimum salt concentration for NAD(P)H oxidase. Solutions at pH 7.0 were prepared from both salts to provide the following salt concentrations: 25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 400 mM, 500 mM, 600 mM, 800 mM, 1.0 M, 1.2 M, 1.4 M, 1.5 M, 1.6 M, 2.0 M and 2.5 M. NAD(P)H oxidase was brought to the above salt concentrations by incubating 100 μl of crude extract with 860 μl of the corresponding buffer for 30 minutes. With addition of 20 μl of 10 mM NADH and 20 μl of 1 mM FAD to the incubation mixtures, NAD(P)H oxidase activity could be measured under different salt concentrations.

3.5.6 Flavin effect on NAD(P)H oxidase

The addition of FAD in the enzyme assay mixture was found to increase the reaction rate. To monitor the effect of flavin on NAD(P)H oxidase activity, both enzyme assays in the presence of and without FAD were performed on fraction samples collected from all purification stages. The mononucleotide form of flavin-FMN was also examined for its impact on NAD(P)H oxidase by replacing FAD with FMN in the enzyme assay mixture.

3.5.7 Oxygen effect on NAD(P)H oxidase

G. lamblia is classified as an anaerobe which requires O_2-deficient environment for its maximum growth. This fact, obviously, makes people argue about the utilization of O_2 by NAD(P)H oxidase. Although it is difficult to determine exactly which compound is the physiological electron acceptor for this NAD(P)H oxidase, it is easy to clarify whether this enzyme can utilize O_2 by performing the enzyme assay under anaerobic conditions. However, the spectrophotometer for anaerobic analysis is not available. Instead of pursuing anaerobic conditions, oxygen was partially removed from the enzyme assay mixture by bubbling nitrogen gas through the assay solution before the addition of NADH. The activity obtained from this assay was compared to the enzyme activity under normal conditions.

3.5.8 Analysis of product

The possible formation of H_2O_2 was examined by both catalase and peroxidase catalyzed reactions. With the addition of about 60 U catalase (Boehringer Mannheim) to the enzyme assay mixture, the potential presence of H_2O_2 could be visiually de-

tected by the formation of O_2 bubble. Besides this method, a peroxidase-ABTS coupled reaction was also performed to examine whether H_2O_2 is produced from the reaction catalyzed by NAD(P)H oxidase. By adding 100 μl of 20 mM 2, 2'-Azino-di-(3-ethylbenzthiazolin sulfonate) (ABTS) (Boehringer Mannheim) and 30 U peroxidase (Boehringer Mannheim) to the enzyme assay mixture, the presence of H_2O_2, if produced, could be identified by the formation of blue color.

3.5.9 Kinetics studies

NAD(P)H oxidase from *G. lamblia* is capable of oxidizing both NADH and NADPH. The Km values for the enzyme at pH 7.0 were determined for both compounds by performing the enzyme assays in duplicate at different substrate concentrations. The initial velocities for enzyme reaction were obtained from each substrate at the concentrations of 0.0075 mM, 0.010 mM, 0.0125 mM, 0.025 mM, 0.05 mM and 0.1 mM. A graph was generated by plotting initial velocity versus initial velocity divided by substrate concentration. The Km values were obtained from this graph by Eadie-Hofstee analysis.

3.5.10 Inhibition studies with trivalent arsenical compounds

The inactivation of NAD(P)H oxidase by trivalent arsenical was performed with p-aminophenyl dichloroarsine ($H_2NPhAsCl_2$) and p-bromoacetylaminophenyl dichloroarsine ($BrCH_2CONHPhAsCl_2$). Each compound, previously dissolved in ethanol, was added into crude extract to a final concentration of 0.1 mM. After incubating for 1.5 hours, an aliquot was removed from the sample to monitor the enzyme activity. The rest of the sample was further incubated for another 1.5 hours after the addition of DTT to 1 mM concentration. The enzyme activity was measured

again after this process. A crude extract sample containing 5% ethanol, which equals to the ethanol concentration in the sample with arsenical, was prepared as a parallel control.

3.5.11 N-terminal amino acid sequence analysis

The sequence analysis for N-terminal amino acids was performed by the Protein Sequencing Facility at the University of Calgary. Simply, the sequencing process includes the generation of phenylthiocarbamoyl-amino acid (PTC-amino acid) from the N-terminus of the protein by Applied Biosystem's Gas Phase Protein Sequencer, the automatic transformation of PTC-amino acid to PTH-amino acid (phenylthiohydantoin-amino acid), and the identification of PTH-amino acid by HPLC system on Applied Biosystem's 120 Phenylthiohydantoin Analyzer. Three enzyme samples obtained from separate purification runs were subjected to this analysis.

3.5.12 Amino acid composition

The elucidation of amino acid composition for NAD(P)H oxidase was also performed by Protein Sequencing Facility at the University of Calgary. The purified enzyme was subjected to acid hydrolysis at 150°C by 6 M HCl in the presence of 0.1% β-mercaptoethanol for one hour *in vacuo*. The hydrolysate was vacuum dried and redissolved in pH 2.2 citrate buffer. The amino acid analysis was carried on an Alpha-2 Plus LKB Amino Acid Analyzer using ninhydrin as colorimetric reagent.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Purification of NAD(P)H Oxidase

4.1.1 Purification attempts with synthesized matrices

4.1.1.1 Purification attempt with arsenical-agarose matrix

Since most of the flavo oxidoreductases discovered employ two cysteinyl residues as the catalytic groups, it is highly possible that the NAD(P)H oxidase from *G. lamblia* possesses a similar structure. With this possibility, a synthesized arsenical-agarose matrix was constructed to purify the NAD(P)H oxidase from crude extract. The principle of this separation is reversible covalent chromatography. Proteins containing two spacially close cysteinyl residues will specifically attach to the immobilized trivalent arsenical on the matrix by forming reversible cyclic dithioarsine covalent bond. With the addition of dithiol reagents such as dihydrolipoamide, the covalently bound proteins can be released from the matrix. Figure 4.1 shows the principle of this chromatography.

The arsenical-agarose matrix was first analyzed with dihydrolipoamide to determine the trivalent arsenical content. The analysis indicated that approximately 27 μmol of arsenical groups were present per ml of hydrated matrix. Therefore, the synthesis of the arsenical-agarose was successful and this matrix was used to isolate NAD(P)H oxidase from *G. lamblia*.

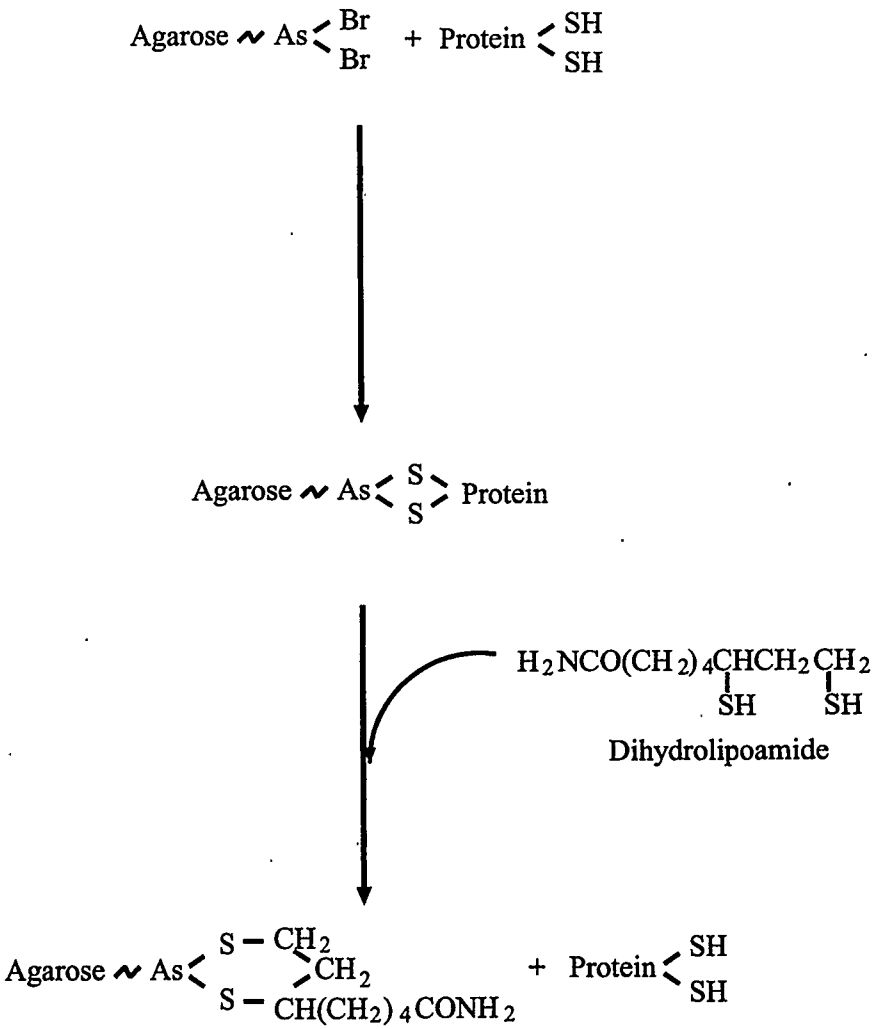

Figure 4.1. Isolation of protein containing vicinal cysteinyl residues with covalent chromatography using synthesized trivalent arsenical-agarose matrix.

The covalent attachment of NAD(P)H oxidase to the arsenical matrix was highly successful. After incubating the crude extract with the arsenical-agarose matrix for 24 hours, all enzyme activity was removed from the supernatant, whereas the parallel control incubated with agarose alone only showed only a limited loss of activity over the same time period. This suggested that the loss of activity in the supernatant after incubation with the arsenical-agarose was due to the covalent attachment of the enzyme to the matrix.

However, the desorption of the enzyme from the matrix turned out to be very problematic. Although the measurement of A_{280} revealed that dihydrolipoamide desorbed some proteins bound to the matrix, the proteins did not express NAD(P)H oxidase activity. NAD(P)H oxidase could still remain bound to the matrix. One possible reason for this unsuccessful desorption is that, since the vicinal thiols in the enzyme are believed to be located in its catalytic site, dihydrolipoamide may not have reached the arsenical-enzyme complex to interact with the arsenical and release the enzyme. The steric hindrance imposed by the arsenical arm attached to the agarose may be the problem. Another desorption condition was attempted with the usage of 3 M urea to partially denature the NAD(P)H oxidase. The idea was to expose the interaction sites between the enzyme and the arsenical, thus allowing dihydrolipoamide to compete with the enzyme for interacting with the arsenical and result in the removal of the enzyme. The measurement of A_{280} revealed that this procedure removed some additional proteins from the matrix, however, no NAD(P)H oxidase activity was detected. Whether or not the enzyme of interest was removed by this method is unknown since no enzyme activity was detected after urea was removed by dialysis. Because of the problem in desorption, this matrix was not further used. The behavior of the enzyme towards this matrix, however, indicated the potential existence of two

spacially close cysteinyl residues in the active site of NAD(P)H oxidase.

4.1.1.2 Purification attempt with dye-ligand matrices

Affinity chromatography using dye-ligand matrices has been widely applied to the isolation of dinucleotide-requiring proteins. The proteins are adsorbed on to the matrix due to the structural similarity between their cofactors and the dye. Since both substrate (NADH and NADPH) and cofactor (FAD) for NAD(P)H oxidase are dinucleotides, this chromatography method was investigated.

The coupling of the Procion dye to the agarose matrix was easily demonstrated by the colour change on the matrix. Overall, the synthesis of dye-ligand matrices using a variety of Procion dyes were successful. However, none of the matrices was proven to be very effective in the combined adsorption and desorption of NAD(P)H oxidase. The majority of proteins in the crude extract, including NAD(P)H oxidase, did not adsorb to most of the dye-ligand matrices and were eluted directly by the loading buffer. Although the adsorption of the enzyme on some dye-ligand matrices was observed, the desorption of an active enzyme was never obtained. Desorptions with 1 mM NADH, 1 mM FAD or high salt (1 M KCl) resulted in the elution of small amount of proteins but no NAD(P)H oxidase activity was detected.

Since no significant purification could be obtained with several Procion dye-agarose matrices, the dye-ligand chromatography method was discontinued. It is not clear why these dye-ligand matrices performed so poorly with NAD(P)H oxidase.

4.1.2 Purification of NAD(P)H oxidase

The NAD(P)H oxidase was eventually isolated from *G. lamblia* by a sequential application of salt fractionation, Octyl-Sepharose hydrophobic chromatography,

hydroxyapatite adsorption chromatography, and FPLC using a Mono Q matrix. The result from each step is presented sequentially in the following parts.

The cell breakage was obtained by three passes through the French Press or via sonication. No intact cells in the cell lysate were observed under microscopic examination. After centrifugation to separate cell debris, the NAD(P)H oxidase activity was identified in the supernatant. The addition of Triton X-100 to the cell debris did not release any additional enzyme activity. This indicates that the NAD(P)H oxidase is unlikely to be a membrane-bound enzyme. The cell-free supernatant, referred as the crude extract, was subjected to the following methods.

Salt fractionation using ammonium sulfate was attempted as the initial step to obtain a fraction enriched in NAD(P)H oxidase activity. The pilot experiment demonstrated that the NAD(P)H oxidase was precipitated from the crude extract within $40-60\%$ range of ammonium sulfate saturation. However, during the actual purification, the crude extract was only brought to 25% ammonium sulfate saturation so as to precipitate some proteins but retain the enzyme in the supernatant in the presence of a reasonably high salt concentration. This step resulted in a modest 1.6-fold of purification with an 80% yield.

The supernatant of 25% ammonium sulfate was directly subjected to hydrophobic chromatography on Octyl-Sepharose 4B matrix. The proteins were eluted from the column by washing the matrix with 50 mM potassium phosphate buffer at pH 7.0 containing 25% ammonium sulfate followed by 25 mM potassium phosphate buffer at pH 7.5. The elution profile for this hydrophobic chromatography is illustrated in Figure 4.2. It should be noted that the activity for the NAD(P)H oxidase was not distributed at an unique elution position from the Octyl-Sepharose 4B matrix. The NAD(P)H oxidase activity was mainly detected in the fractions desorbed by

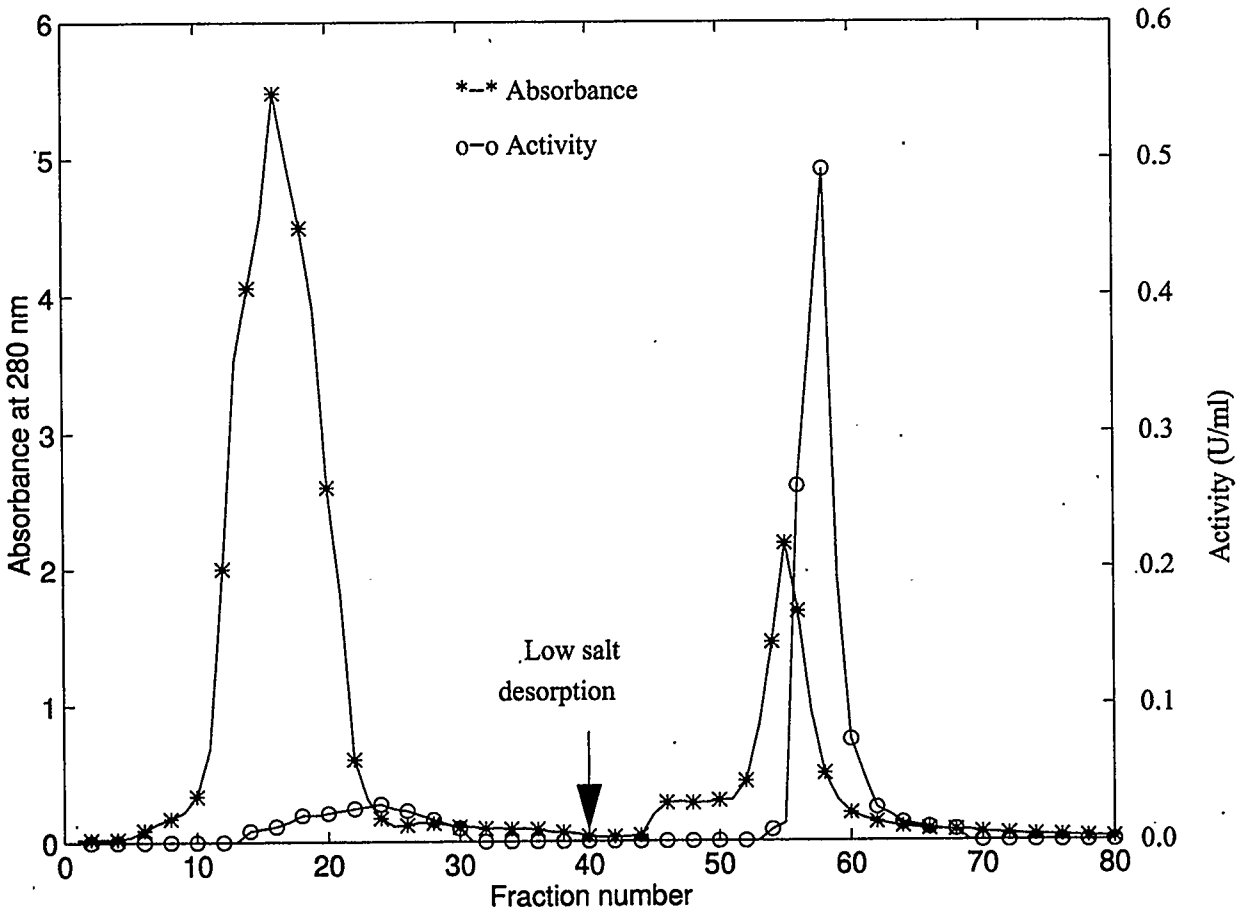

Figure 4.2. The elution profile of hydrophobic chromatography on Octyl-Sepharose 4B matrix. The NAD(P)H oxidase was eluted from the matrix with 25 mM potassium phosphate buffer at pH 7.5.

the buffer containing no ammonium sulfate. However, some of the break-through fractions also showed slight activity toward the oxidation of NAD(P)H. The possibility of overloading the matrix was excluded by reapplying the break-through fractions to a recycled Octyl-Sepharose matrix and again a non-adsorbed NAD(P)H oxidase activity was observed. The detection of enzyme activity at different elution stages suggests that more than one protein in *Giardia* crude extract may be capable of oxidizing NAD(P)H. However, only one of the protein fractions, which accounted for the majority of NAD(P)H oxidase activity, was subjected to further purification and analysis. Thus, only those fractions desorbed with low salt buffer and containing high enzyme activity were collected for next purification step. The yield from the Octyl-Sepharose hydrophobic chromatography was 24% with a 3-fold of purification.

The sample collected from the hydrophobic chromatography was desalted with Amicon centriprep-30 to remove ammonium sulfate before being applied to the hydroxyapatite matrix. At pH 7.5 and ionic strength of 25 mM potassium phosphate, the NAD(P)H oxidase was adsorbed to hydroxyapatite matrix, whereas many proteins were not adsorbed and eluted directly with the equilibrating buffer. A linear gradient of 25 mM to 500 mM potassium phosphate was used to desorb proteins bound to the hydroxyapatite matrix. The desorption of NAD(P)H oxidase was observed after the potassium phosphate concentration reached 300 mM. The elution profile for this hydroxyapatite adsorption chromatography is demonstrated in Figure 4.3. The fractions possessing high enzyme activity and low protein content were combined for the final purification step. The hydroxyapatite adsorption chromatography resulted in 9-fold of purification and 50% yield.

The final purification step was performed on a Mono Q anion-exchange matrix using a Pharmacia FPLC system. Before being applied to the Mono Q matrix, the sam-

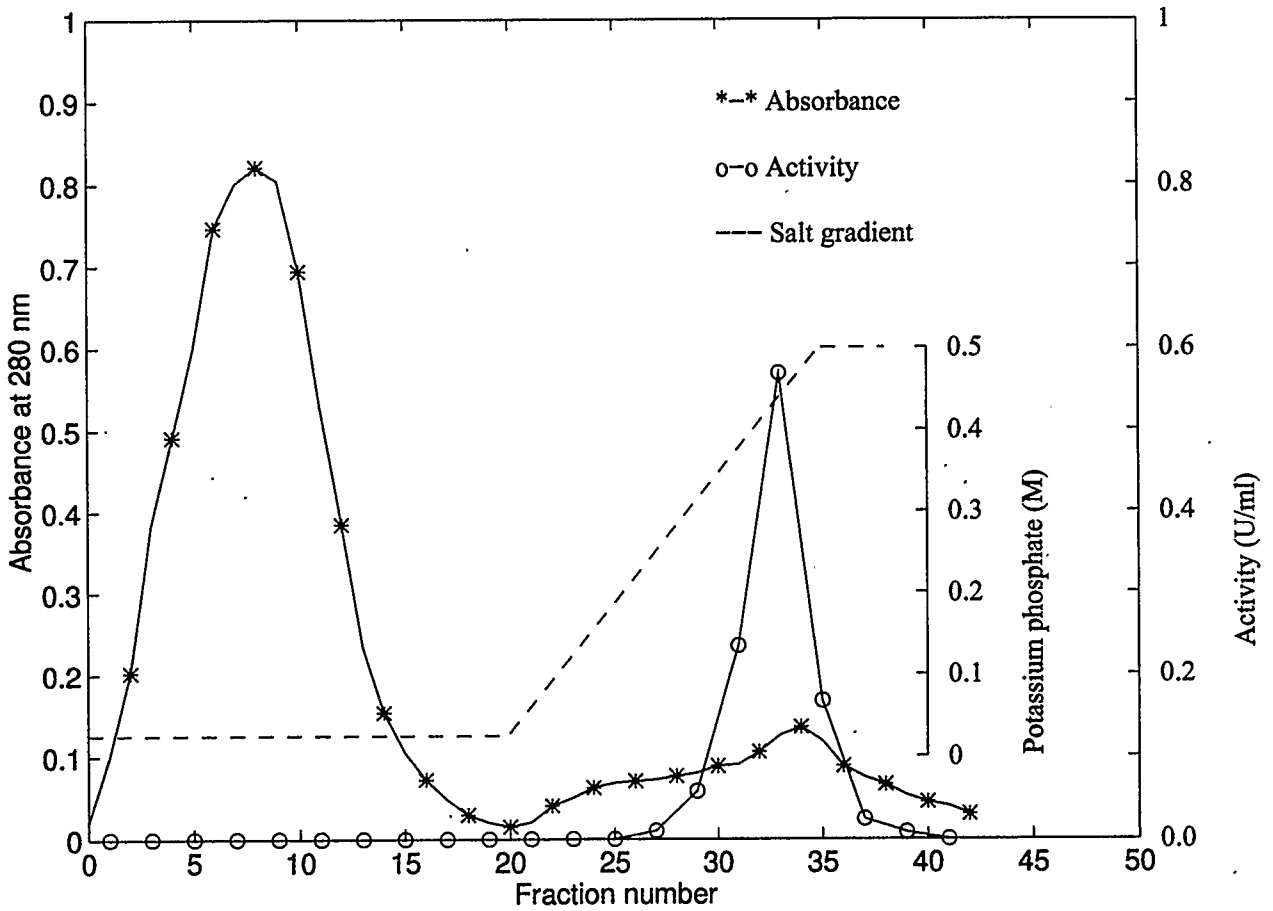

Figure 4.3. The elution profile of hydroxyapatite adsorption chromatography. The NAD(P)H oxidase was eluted from the matrix with potassium phosphate gradient.

ple obtained from the hydroxyapatite column was desalted with Amicon centriprep-30 to reduce potassium phosphate concentration to 25 mM. The elution profile using a potassium chloride gradient is illustrated in Figure 4.4 for this ion-exchange chromatography. Several protein peaks, which were nicely separated, can be identified in this profile. Among them, NAD(P)H oxidase activity was associated with the major protein peak desorbed by potassium chloride gradient from 350-420 mM. Two fractions showed high enzyme activity and were analyzed individually by SDS-PAGE. Both of them appeared to be homogeneous and were combined for N-terminal amino acid sequence analysis. A 50% yield and 2.5-fold of purification was achieved from this ion-exchange chromatography.

Samples from each purification step were analyzed on SDS-PAGE gel to monitor the progress of purification and examine the homogeneity of the final product. The SDS-PAGE results are illustrated in Figure 4.5. Although similar amounts of proteins were applied to the gel, the staining intensity indicates that some excessive dilution appears to have occurred in the sample of 25% ammonium sulfate supernatant. It can be seen that the final product obtained from the above purification procedures only consists of NAD(P)H oxidase represented as the single protein band on the SDS-PAGE gel. The purification progress is listed in Table 4.1. It seems that the hydroxyapatite adsorption chromatography is the most effective purification step which accounted for a 9-fold of purification. Interestingly, this is in keeping with the purification of other flavoenzymes which consistently adsorb to hydroxyapatite. The sample collected from the hydrophobic chromatography was concentrated by the hydroxyapatite matrix and a large amount of proteins other than NAD(P)H oxidase were removed by this adsorption chromatography. This is also reflected in Figure 4.5. Although the purification fold obtained from the Mono Q ion-exchange

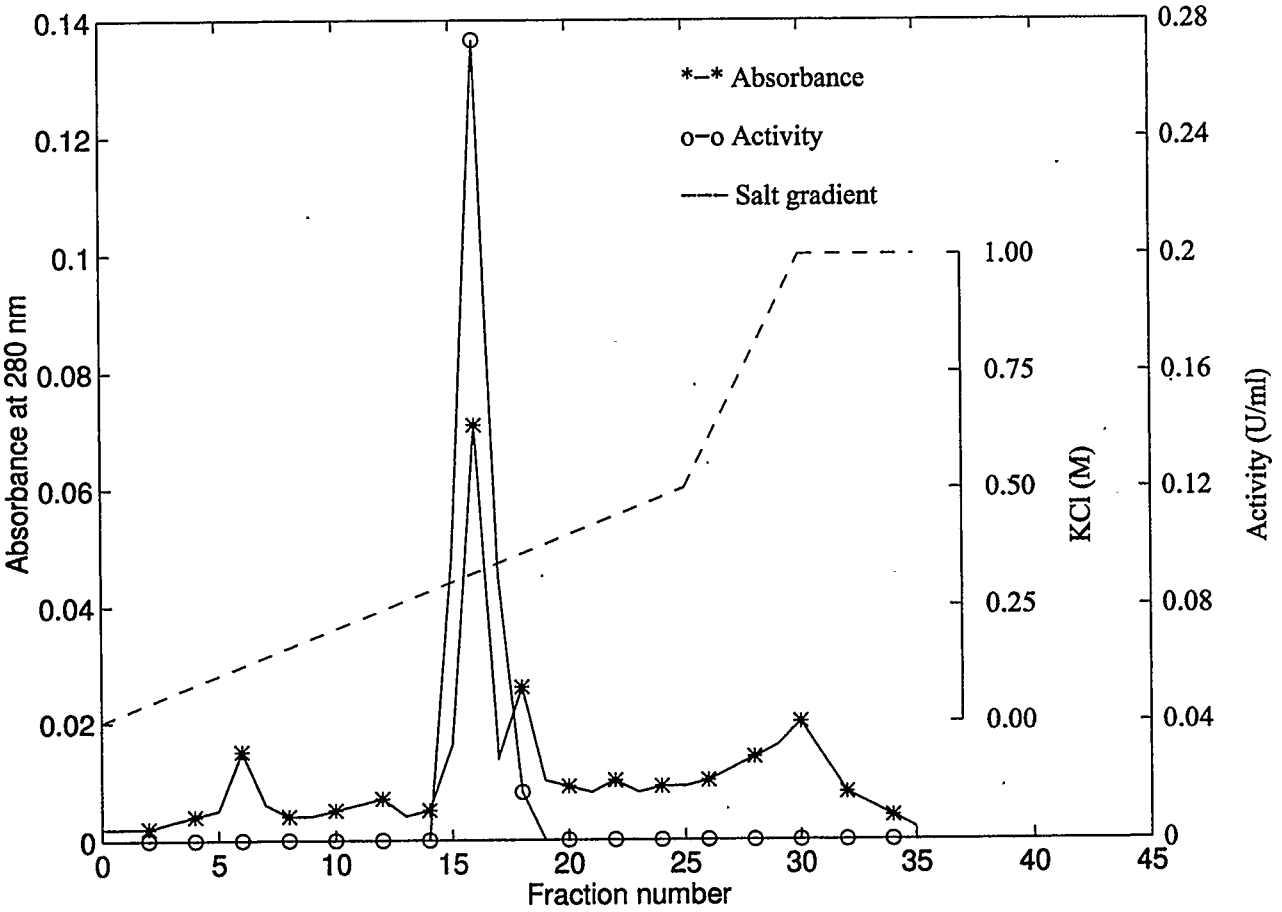

Figure 4.4. The elution profile of ion-exchange chromatography on Mono Q matrix. The NAD(P)H oxidase was eluted from the matrix with KCl gradient.

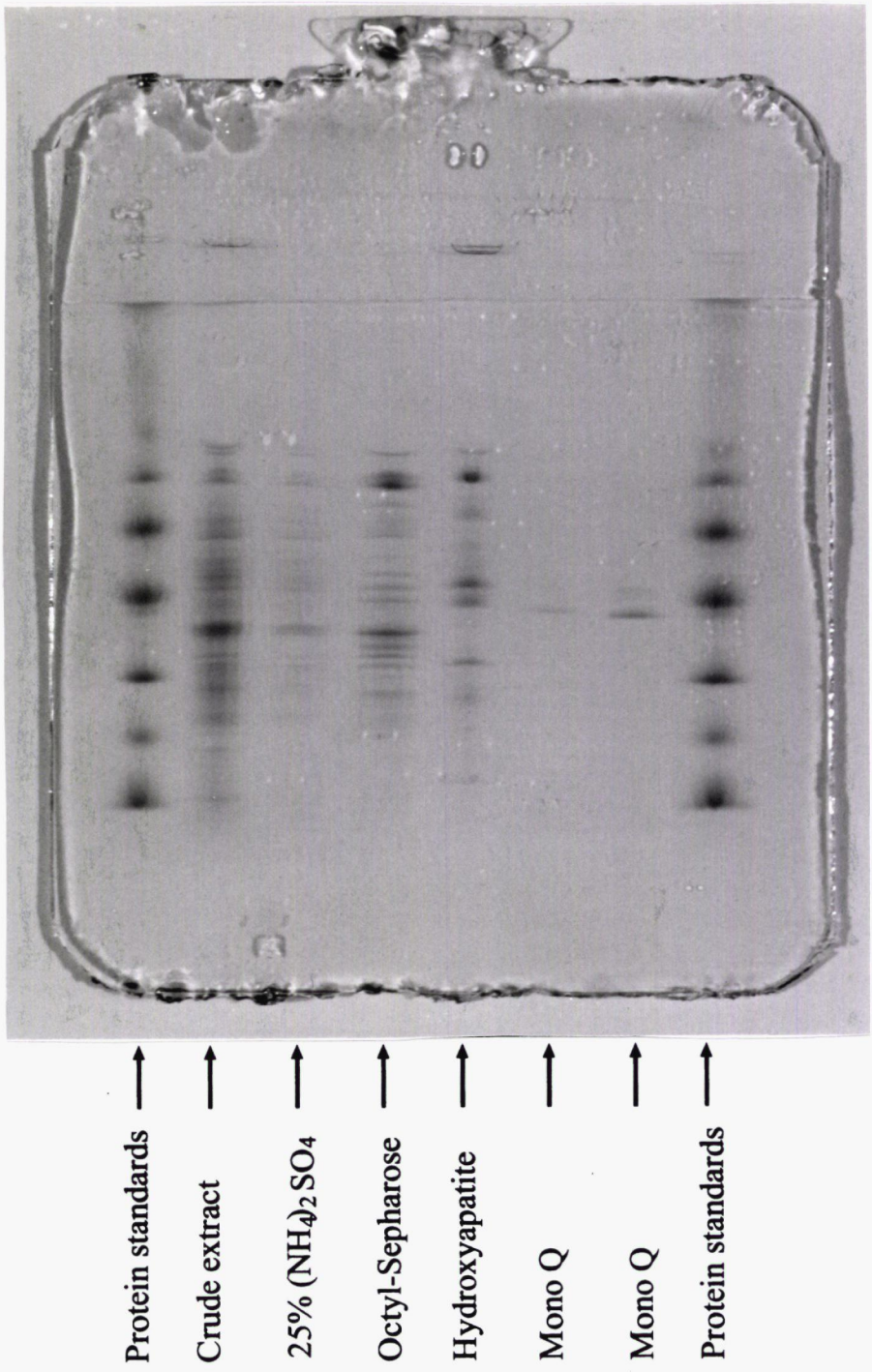

Figure 4.5. SDS-PAGE of fractions with NAD(P)H oxidase activity collected from each purification procedure.

chromatography is not impressive, this was a vital step to achieve pure NAD(P)H oxidase. It is through this step that the NAD(P)H oxidase was finally separated from all other proteins. The overall recovery of NAD(P)H oxidase was 5% with a 108-fold of purification. Generally, the yield and the purification fold are comparatively low. Part of the reason for this is that the enzyme activity is not stable. Reduction of the isolation time may be helpful to improve the yield. The separation of unknown NAD(P)H oxidizing enzyme(s) also accounted for the low yield from the hydrophobic chromatography. The property of the unknown protein(s) needs further investigation.

Table 4.1. Purification of NAD(P)H oxidase from *Giardia lamblia.*

Fraction	Volume (ml)	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Yield	Purification fold
1. Crude extract	15.0	64.5	8.75	0.14	100%	1.0
2. 25% $(NH_4)_2SO_4$	14.0	34.9	7.50	0.22	80%	1.6
3. Octyl-Sepharose	7.0	2.70	1.80	0.67	19.2%	5.0
4. Hydroxyapatite	5.0	0.15	0.90	6.00	10.3%	44.0
5. Mono Q	2.0	0.03	0.44	14.60	5.0%	108.0

4.2 Characterization of NAD(P)H Oxidase

4.2.1 Determination of molecular weight and subunit composition

The molecular weight for NAD(P)H oxidase and its subunit composition were determined from both SDS-PAGE and gel filtration chromatography. The standard curve generated from SDS-PAGE using protein standards is illustrated in Figure 4.6. Based on the migration distance on SDS-PAGE gel, the molecular weight for NAD(P)H oxidase was estimated to be 40,900 (+/- 2,000) Da from the standard graph. This value is for the enzyme subunit due to denaturation by SDS,

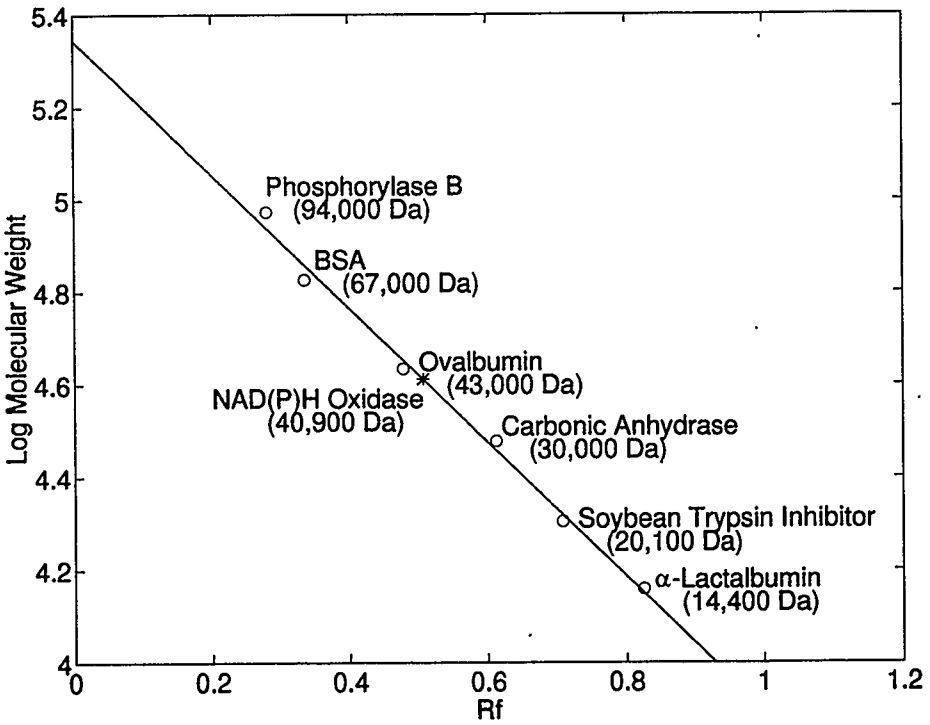

Figure 4.6. Standard curve obtained from SDS-PAGE using low molecular weight protein standards for determination of the subunit molecular weight for NAD(P)H oxidase.

β-mercaptoethanol and heat.

To determine the native molecular weight for NAD(P)H oxidase, gel filtration chromatography using FPLC was performed on Superose 12 matrix under non-denaturing conditions. Both freshly prepared crude extract and purified enzyme samples were subjected to this analysis. It required exactly the same amount of buffer for both samples to elute NAD(P)H oxidase from the Superose 12 column. This means there is no alteration for NAD(P)H oxidase in terms of size by the purification process. The enzyme does not have any associations with other proteins. Various protein standards with molecular weights ranging from 13,700 Da to 440,000 Da were used to generate a standard curve as shown in Figure 4.7. The native molecular weight for NAD(P)H oxidase determined from this standard curve was 41,300 (+/- 3,000) Da.

The two values obtained from SDS-PAGE and gel filtration chromatography are very close. It can be concluded that the NAD(P)H oxidase isolated from *G. lamblia* is a monomer with molecular weight of 41,000 (+/- 3,000) Da. Compared to the NADH oxidases obtained from bacterium [Niimura et al., 1993, Schmidt et al., 1986, Dolin, 1955, Koike et al., 1985, Reinards et al., 1981, Maeda et al., 1992] [Cocco et al., 1988, Park et al., 1992b, Saeki et al., 1985], this monomeric structure was not common and has only been found in the enzyme from *T. thermophilus* [Park et al., 1992b]. The size of this *Giardia* enzyme is smaller than most of the bacterial NADH oxidases and is reasonably similar to the subunit size (48,900 Da) of *Streptococcus* NADH oxidase [Schmidt et al., 1986]. Since the molecular weights determined from the crude extract and for the purified enzyme were the same, no significant effects on the enzyme's structure were introduced by the purification procedures.

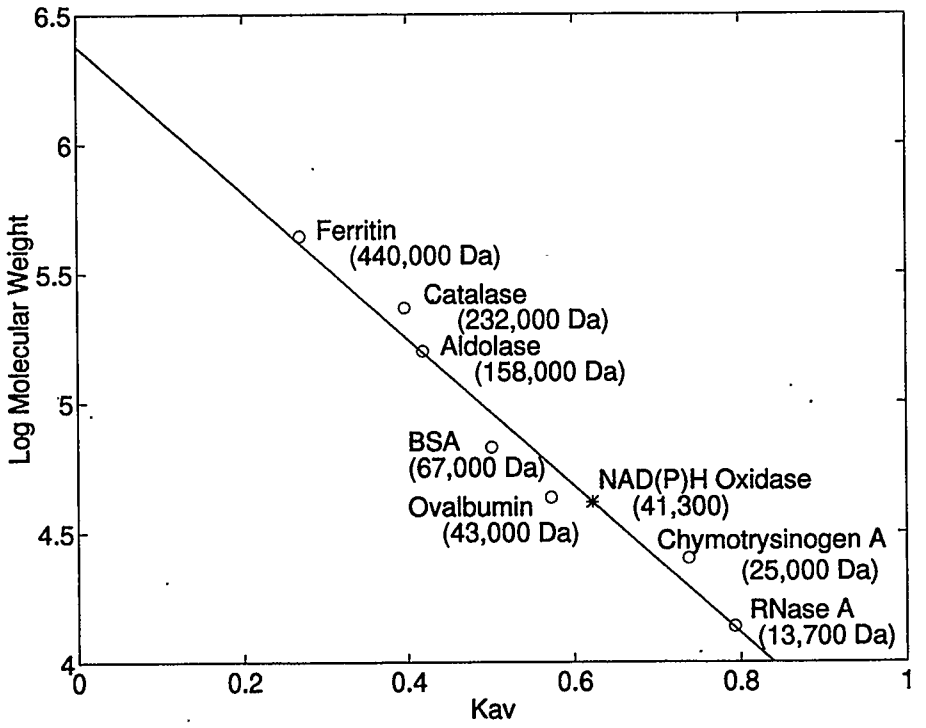

Figure 4.7. Standard curve obtained from Superose 12 gel filtration chromatography using wide range of molecular weight protein standards for determination of the native NAD(P)H oxidase molecular weight.

4.2.2 Stabilization studies

The NAD(P)H oxidase activity is not very stable especially in crude extract. Storage of crude extract at -20°C for 24 hours usually resulted in a 30 – 40% loss of the enzyme activity. In order to try to stabilize the enzyme activity, antioxidants and common protease inhibitors were added to the crude extract. Figure 4.8 demonstrates the enzyme activity change in diluted crude extract over time under three conditions. Surprisingly, no improvement on the enzyme stability was observed under any of the conditions applied. The loss of enzyme activity in three samples followed exactly the same pattern. Half of the enzyme activity was lost within 2 hours of storage at 4°C, whereas further storage up to 24 hours only caused an additional 10% enzyme activity decrease. The enzyme in dilute crude extract was highly unstable at 4°C and tended to lose 60% activity no matter what stabilization efforts were applied. The enzyme activity change in the sample containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol was also plotted against the incubation time in the logarithm form (Figure 4.9). In this graph, the decrease of the enzyme activity follows two different rates.

The stability of the enzyme in undiluted crude extract stored at -20°C was also investigated in the presence of 1 mM DTT or 1 mM EDTA. A sample of crude extract in 50 mM potassium phosphate buffer at pH 7.0 was set as control. The change of enzyme activity in each sample was monitored up to 96 hours. After the initial 24 hours of storage at -20°C, a 30% decrease of enzyme activity was observed for all three samples with or without the presence of DTT or EDTA. However, further storage of the sample containing 1 mM DTT to 96 hours at -20°C only caused additional 10% enzyme activity loss, whereas the control sample and the sample containing 1 mM EDTA hardly show any activity (10% of initial activity) over the same period. The

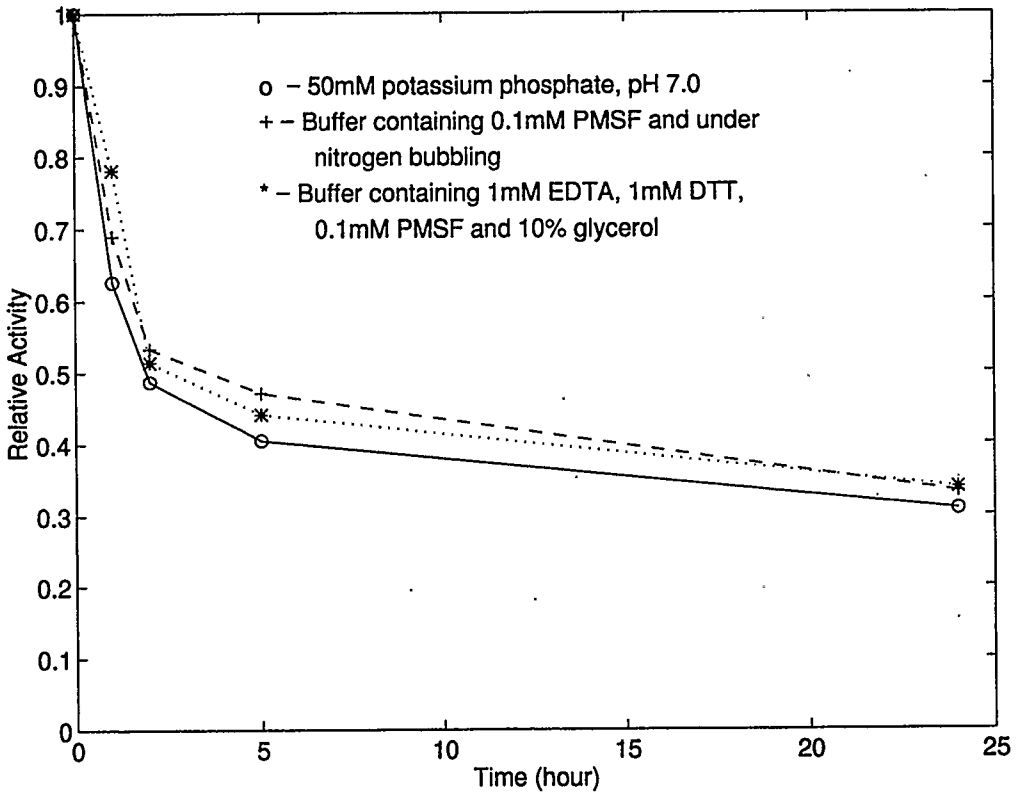

Figure 4.8. Comparison of the stability of NAD(P)H oxidase activity in three crude extract samples which were diluted individually with 50 mM potassium phosphate at pH 7.0, the buffer containing 0.1 mM PMSF with the presence of nitrogen bubbling and the buffer containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol. The enzyme activities after different incubation time at 4°C were compared to the enzyme activity at time zero.

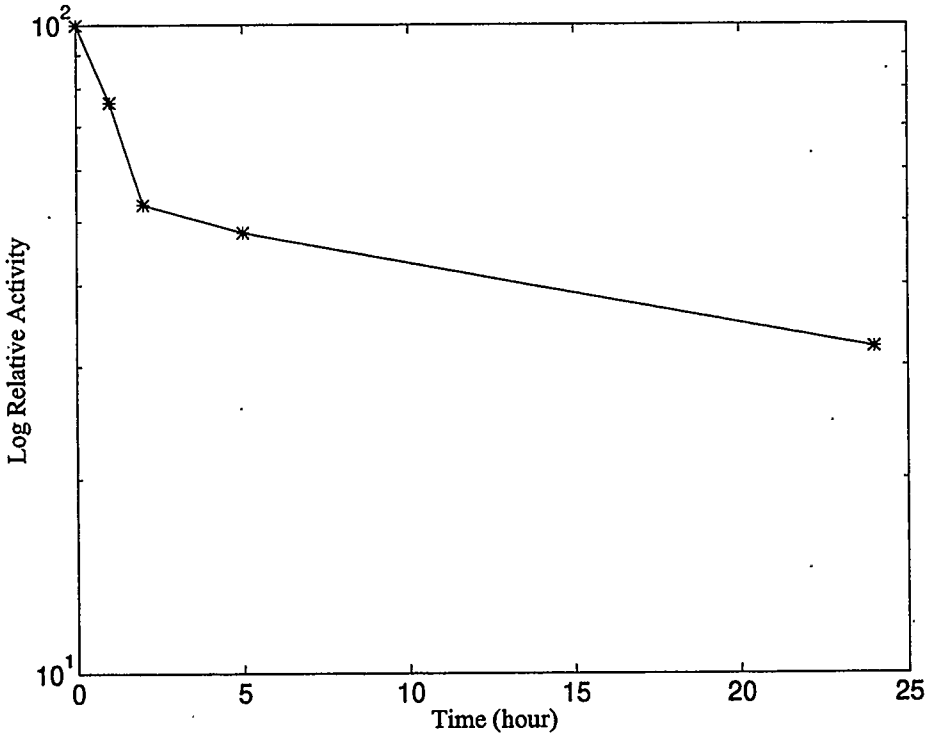

Figure 4.9. The logarithm plot of NAD(P)H oxidase activity in the crude extract sample diluted with 50 mM potassium phosphate at pH 7.0 containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol. The enzyme activities after different incubation time at 4°C were compared to the enzyme activity at time zero.

presence of DTT stabilized the enzyme activity in terms of long term storage. On the basis of this observation, DTT was added into all buffers used for purification.

The rapid loss of NAD(P)H oxidase activity in the crude extract could arise from the proteolysis. Although PMSF and EDTA were used, only certain types of proteases were inhibited by these two reagents. PMSF is most effective on serine protease and EDTA is specific for metallo proteases. Other types of proteases, such as thiol proteases, are normally not affected by PMSF or EDTA thereby allowing them free access to susceptible enzymes in the crude extract. The function of these noninhibited proteases could result in the loss of NAD(P)H oxidase activity in the crude extract.

Compared to the crude extract, the purified form of NAD(P)H oxidase was found to be much more stable. In the presence of 1 mM DTT and 0.1 mM PMSF, the purified enzyme could be stored up to one week at -20°C with less than 20% enzyme activity loss.

This dramatic improvement of enzyme stability in the purified sample compared to the crude extract implies that there could be more than one enzyme in *Giardia* crude extract that can oxidize NAD(P)H but possess different properties. If this is true, the observed total enzyme activity towards NAD(P)H in the crude extract is actually contributed by different proteins. One of the proteins associated with the NAD(P)H oxidase activity is comparatively stable. Besides this enzyme, there is at least another protein in *Giardia* crude extract which possesses unstable activity in utilizing NAD(P)H and is primarily responsible for the enzyme activity loss in the crude extract as shown above. Although nothing is known about such a protein(s), the existance of another NAD(P)H-utilizing enzyme(s) (other than the purified NAD(P)H oxidase) in *Giardia* has been observed in the elution profile from the hydrophobic chromatography. In the Octyl-Sepharose hydrophobic chromatography, the NAD(P)H-oxidizing

enzymes in crude extract were separated into two different groups corresponding to their hydrophobicity (refer to Section 4.1.2). The enzyme ultimately purified to homogeneity as NAD(P)H oxidase was comparatively hydrophobic and was only eluted from the Octyl-Sepharose matrix by the buffer containing no ammonium sulfate. In addition, a low level of NAD(P)H-utilizing activity was also detected in the fractions which were eluted unretarded from the matrix. No further studies have been pursued on these less hydrophobic proteins. However, at least one more protein besides the purified NAD(P)H oxidase in *Giardia* crude extract is capable of consuming NAD(P)H and this activity was probably vulnerable to some unknown factors which resulted in the decrease of enzyme activity as described above. More studies are required to clarify the properties of the new protein(s), however, it is not the issue for this thesis.

4.2.3 pH profile

Since the functions of the catalytic groups (such as acid-base catalyst and redox active residue) in flavo oxidoreductases are pH dependent, the enzyme activity is affected by pH change. A pH profile (Figure 4.10) was established for the NAD(P)H oxidase by monitoring the enzyme activity under different pH conditions. The optimum pH for this enzyme ranges from 6.8 to 7.2 which is observed in most of the bacterial NADH oxidases. This indicates that the catalytic residues in this group of enzymes require neutral conditions for their maximum functions. The decrease of NAD(P)H oxidase activity under acidic conditions was slightly more dramatic than those under alkaline conditions. Future studies of the catalytic mechanism for this enzyme may be helpful to explain this difference.

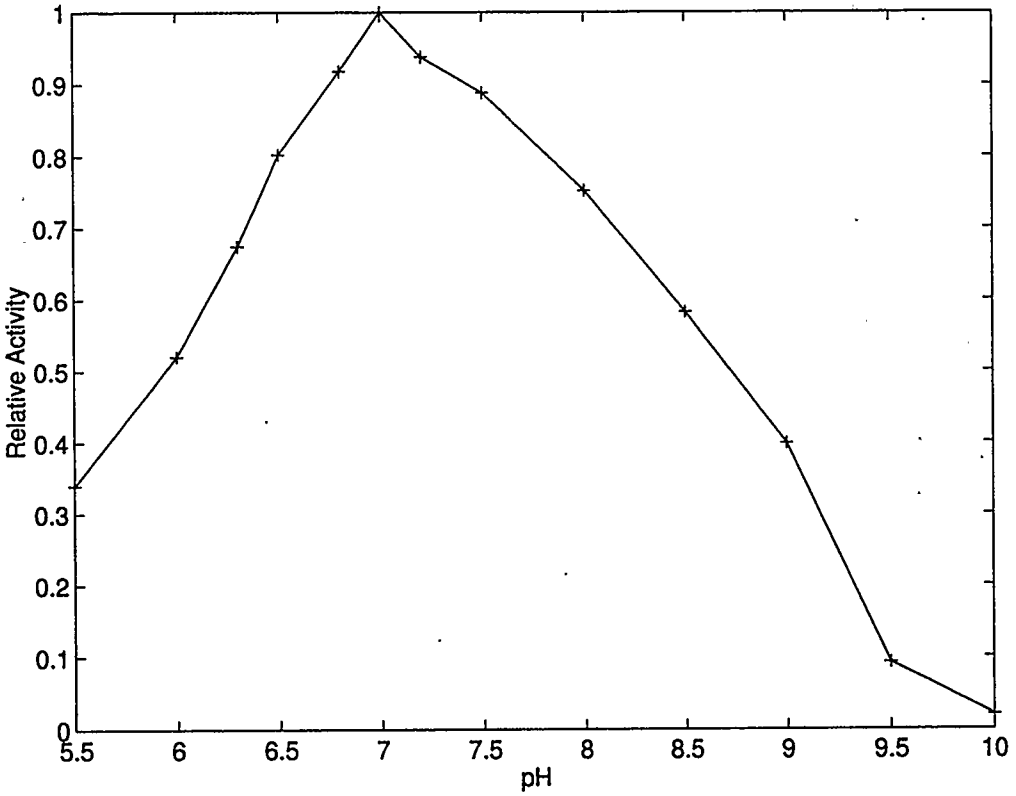

Figure 4.10. The effect of pH change on NAD(P)H oxidase activity. The enzyme activities under different pH conditions were converted to relative activity compared to the enzyme activity at pH 7.0.

4.2.4 Salt effect on NAD(P)H oxidase

The effect of salt concentration on NAD(P)H oxidase in crude extract was demonstrated in Figure 4.11 using both potassium phosphate and potassium chloride. At pH 7.0, the maximum enzyme activity for the NAD(P)H oxidase was observed at salt concentration between 50 mM to 150 mM towards either salt. Neither lower or higher salt concentration benefits to the enzyme reaction. When the salt concentration is above 300 mM, the change of activity for NAD(P)H oxidase follows different patterns for the two salts. With the increase of potassium chloride concentration up to 2.5 M, the enzyme activity kept decreasing. In contrast, increase in potassium phosphate concentration to 2.5 M did not cause additional enzyme activity loss, the enzyme activity was sustained at 60% relative to its value at 50 mM salt concentration. This means the NAD(P)H oxidase is more susceptible to the effect of chloride anion than to phosphate. High concentration of phosphate may stabilize the enzyme conformation through increasing the hydrophobic interaction within the protein.

4.2.5 Flavin effect on NAD(P)H oxidase

The NAD(P)H oxidase requires the presence of free FAD for its maximum activity at all stages of purification. By adding 0.02 mM FAD to the enzyme assay mixture, the increase in the reaction rate ranged from 1.5-fold to above 10-fold as the purification proceeded. Replacing FAD with FMN only slightly increased the reaction rate. This dramatic impact of FAD on the NAD(P)H oxidase indicates that FAD is an essential cofactor for this enzyme. However, it only weakly associates with the NAD(P)H oxidase and is easily removed from the enzyme during the purification procedures. This necessary requirement for additional flavin has also been

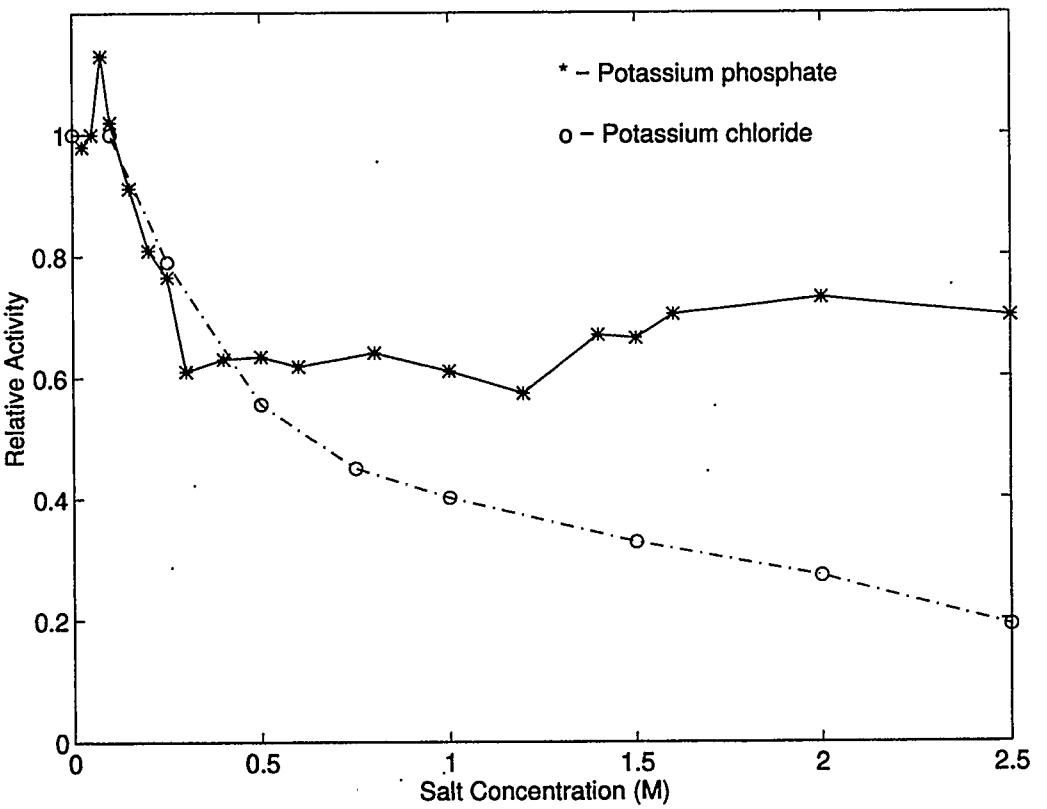

Figure 4.11. The effect of salt concentration change on NAD(P)H oxidase activity using potassium phosphate and potassium chloride. The enzyme activities at different salt concentrations were converted to relative activity compared to the enzyme activity at 50 mM salt concentration.

observed for the NADH oxidases obtained from *S. faecalis* [Schmidt et al., 1986], *T. aquaticus* [Cocco et al., 1988], *B. megaterium* [Saeki et al., 1985] and *T. thermophilus* [Park et al., 1992b]. It is an unusual property for flavoproteins. In most of the flavoproteins, the flavin prosthetic group is tightly attached to the protein through noncovalent interactions. There are well defined domains in flavoproteins to accommodate the flavin cofactor. Some variations in these specific sites will affect the interaction between the apoenzyme and its cofactor. The analysis of the FAD-binding domains in *Streptococcus* NADH oxidase has proposed that a labile amino acid residue-Cys8 may account for the easy loss of FAD cofactor [Ross and Claiborne, 1992]. However, whether this explanation applies to the *Giardia* NAD(P)H oxidase and other bacterial NADH oxidases mentioned above needs more investigation.

It has been proposed that all the flavo oxidoreductases may have originated from a common ancestral adenine nucleotide-binding protein and acquired their specific functions later [Ahmed and Claiborne, 1989b]. In the first flavoprotein, the flavin cofactor was likely noncovalently linked to a protein. It is reasonable to assume that the ancestral flavin-protein complexes are only weakly held together in the form of flavoproteins. The *Giardia* NAD(P)H oxidase, as well as the above bacterial NADH oxidases, contains loosely associated flavin cofactor which suggests that they are potential ancient flavoproteins. In future, homologous comparison of the flavoprotein sequences will provide more information on the evolutionary studies with regard to the flavoproteins.

4.2.6 O_2 requirement

Partial removal of O_2 from the enzyme assay mixture resulted in a 3-fold decrease of reaction rate. This indicates that O_2 is necessary for the reaction catalyzed by

the NAD(P)H oxidase under current conditions. Nevertheless, it is not clear whether O_2 or another unknown compound is the physiological oxidant for this enzyme. The utilization of O_2 seems contradictory with the anaerobic property of *Giardia lamblia.* It is hard to imagine any real function for an O_2-consuming enzyme in *G. lamblia* if classified as obligate anaerobe. However, the requirement of low O_2 tension for its optimum axenic culturing as well as the presence of trace O_2 under its natural growing conditions for *G. lamblia* in mammalian intestine negates the obligate anaerobic definition for this organism and enables O_2 to be a potential candidate as the natural electron acceptor for NAD(P)H oxidase. At this point, it is unrealistic to determine exactly which compound is utilized by this enzyme to regenerate $NAD(P)^+$ during the physiological process, nevertheless, this experiment proves that O_2 is a possible choice.

4.2.7 Product analysis

The bacterial NADH oxidases can be divided into two groups in terms of the reduced product, which are H_2O-producing NADH oxidase and H_2O_2-producing NADH oxidase. The possible generation of H_2O_2 by *Giardia* NAD(P)H oxidase was negated by both catalase assay and peroxidase-ABTS coupled analysis. Therefore, the *Giardia* NAD(P)H oxidase catalyzes four-electron transfer producing H_2O and NAD^+ as final products similar to the NADH oxidases obtained from *S. faecalis* [Schmidt et al., 1986] and *L. mesenteriodes* [Koike et al., 1985]. The overall reaction is: $2NAD(P)H + O_2 + 2H^+ - -- > 2NAD(P)^+ + 2H_2O$

4.2.8 Kinetics studies

Unlike other dinucleotide-utilizing enzymes, the NAD(P)H oxidase from *G. lamblia* is capable of oxidizing both NADH and NADPH almost equally well. Kinetics studies were performed on this enzyme at pH 7.0. From Eadie-Hofstee analysis, the Km values for this enzyme were estimated to be about 10 μM for NADH (Figure 4.12) and 25 μM for NADPH (Figure 4.13). Because the enzyme activity is not stable, no Vm values were obtained. However, it can be seen that the reaction rates for this enzyme utilizing either substrate are the same. There is almost no preference for NADH or NADPH. This is a very interesting finding for a dinucleotide-utilizing enzyme. Usually, an enzyme is either more specific for NADH or for NADPH because the enzyme's active site is arranged in such way that only one of them can be accommodated properly. Although some of the bacterial NADH oxidases such as the enzymes from *L. mesenteroides* [Koike et al., 1985], *B. megaterium* [Saeki et al., 1985], and *T. thermophilus* [Park et al., 1992b] exhibit utilization toward NADPH, the activity is over an order of magnitude lower than that with NADH as substrate judged from the reaction rates and Km values. It is certainly worthwhile to determine what makes the *Giardia* NAD(P)H oxidase so special regarding its substrate utilization. The sequence which reveales the dinucleotide-binding domains in this NAD(P)H oxidase will be a useful contribution to this question.

4.2.9 Inhibition studies with trivalent arsenical compounds

Since the *Giardia* NAD(P)H oxidase may employ two active site cysteinyl residues to transfer electrons as do most of the flavo oxidoreductases, the inhibition studies with trivalent arsenical compounds were performed on this enzyme to explore its

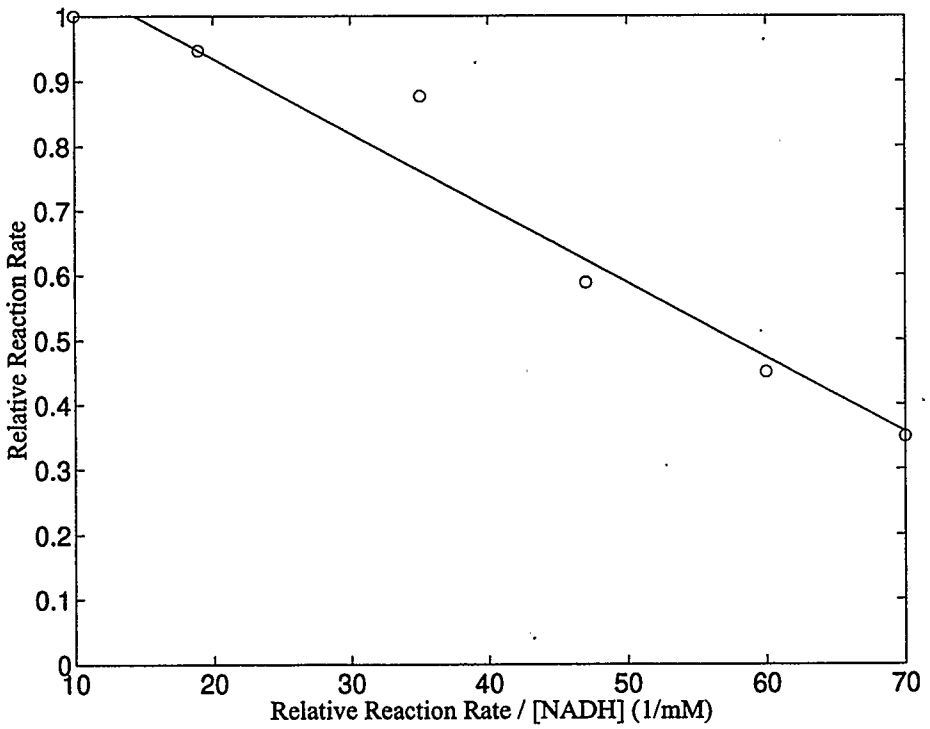

Figure 4.12. Determination of Km value for NAD(P)H oxidase using NADH as substrate. The reaction rates at different NADH concentrations were converted to relative reaction rates compared to the reaction rate at 0.2 mM NADH concentration.

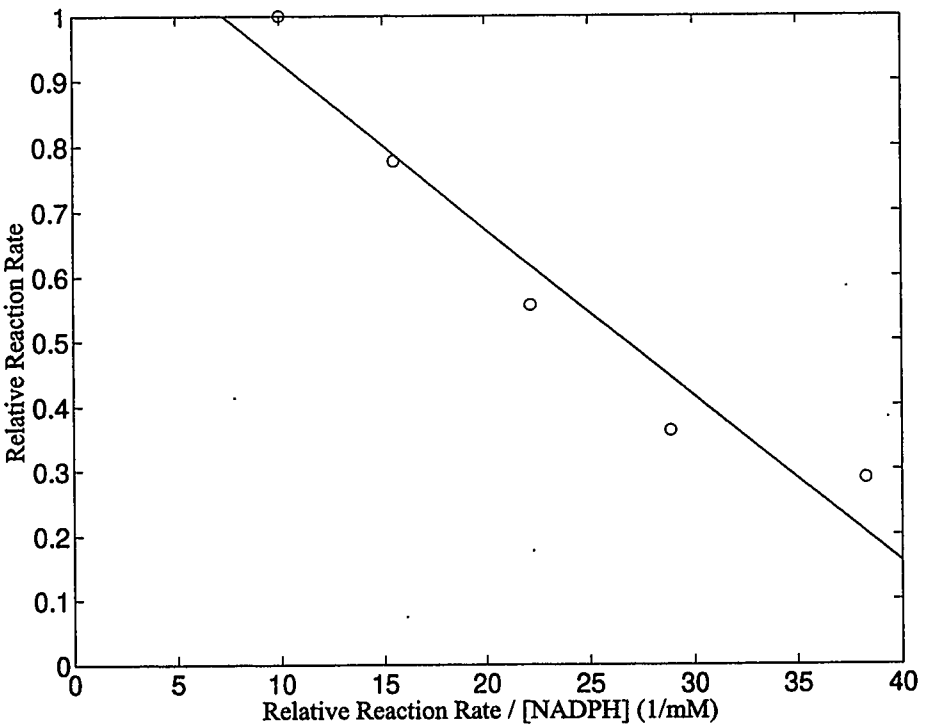

Figure 4.13. Determination of Km value for NAD(P)H oxidase using NADPH as substrate. The reaction rates at different NADPH concentrations were converted to relative reaction rates compared to the reaction rate at 0.2 mM NADPH concentration.

active site. The trivalent arsenical compound can interact with any protein possessing two spacially close cysteinly residues to form reversible cyclic dithioarsenite covalent bonds. If these cysteinyl residues are involved in the enzyme's catalytic process, which is the case for most of the flavo oxidoreductases, the protein-arsenical complex should not show any activity. However, with the addition of dithiol reagents such as DTT, the arsenical compound can be detached from the protein and form cyclic dithioarsenite complex with the dithiol reagent instead. Therefore, the cysteinyl residues on the enzyme should recover their function upon the addition of dithiol reagent. The principle of this inhibition and reactivation process was applied in the purification attempt using arsenical-agarose matrix (Section 4.1.1). The overall reactions for this inhibition studies are similar to that described in Figure 4.1 except that the arsenical compound is not immobilized on the agarose matrix.

Two arsenical compounds, a monofunctional $H_2NPhAsCl_2$ and a hetero-bifunctional $BrCH_2CONHPhAsCl_2$, have been used for the inhibition studies. Both of them caused 80% loss of the NAD(P)H oxidase activity in the crude extract, whereas the control sample containing 5% ethanol only showed slight decrease of the enzyme activity. Upon the addition of DTT, no reactivation was achieved for the sample inhibited by $BrCH_2CONHPhAsCl_2$. However, this can be explained if an alkylation rather than (or in addition to) a dithioarsenite formation occurred. The bromo acetamide moiety in the bifunctional compound can modify a cysteinyl residue or other active site residue through alkylation and results in irreversible loss of enzyme activity. The addition of DTT to the enzyme inhibited by $H_2NPhAsCl_2$ retrieved 20% of activity. This recovery rate is unexpectantly low for the monofuctional reagent and is probably due to the accessibility limitation of DTT to the enzyme's active site. Nevertheless, it can still be concluded that cysteinyl residues are involved

in the catalytic process for this NAD(P)H oxidase.

4.2.10 N-terminal amino acid sequence

The sequence analysis of three preparations of NAD(P)H oxidase revealed the following N-terminal amino acid sequence: Met-Lys-Val-Ile-Ile-Leu-Gly-Ala-Gln-His-Gly-. Because of the low repetitive yield for each cycle during three runs, no further sequence could be achieved. The search of Gene Bank revealed that this *Giardia* NAD(P)H oxidase shares a 63% homology over the above eleven amino acids with the NADH peroxidase from *Streptococcus faecalis*, a flavo enzyme which catalyzes the reduction of H_2O_2 to H_2O by oxidizing NADH [Ross and Claiborne, 1991]. It is not surprising that these two enzymes may possess a high level of homology because they are closely related in terms of the cofactor and catalytic function. Although no homology has been found between the *Giardia* NAD(P)H oxidase and the bacterial NADH oxidases on this short N-terminal sequence, it is believed that these enzymes will show high level of identity if more amino acid sequence is disclosed.

4.2.11 Amino acid composition

The amino acid composition of NAD(P)H oxidase is shown in Table 4.2. Although no cysteinyl residues were identified from this analysis, it can not exclude the existence of cysteinyl residues in the enzyme. The cysteinyl residue is susceptible to the acid hydrolysis and can not be detected without a modification. The alkylation of the cysteinyl residue using thiol reagent, such as iodoacetic acid, will generate stable S-carboxymethyl cysteine which is detectable on the amino acid analyzer. Sequence revealment of the NAD(P)H oxidase coding gene will also provide reliable information on the amino acid composition of this enzyme.

Table 4.2. The amino acid composition of NAD(P)H oxidase from *Giardia lamblia*

Amino acid	Composition (Mole %)
Asp	11.87
Thr	5.91
Ser	6.36
Glu	11.02
Pro	3.27
Gly	10.45
Ala	7.66
Val	6.73
Met	2.47
Ile	7.09
Leu	7.35
Tyr	3.52
Phe	3.34
His	3.79
Lys	5.53
Arg	3.66

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Giardia lamblia has been well known for a long time as a protozoan parasite that can cause intestinal infection in mammals. However, it was not until the recent discovery of its small subunit rRNA sequence that *G. lamblia* became important in evolutionary studies. The special characteristic of its 16S rRNA as well as its extreme primitive cell structure suggest that *G. lamblia* is an early branching derivative from the eukaryotic lineage. Because of this phylogenetic position, *G. lamblia* is potentially important for studying ancestral molecular characteristics present in the earliest eukaryotic organisms. The enzymes from *G. lamblia* are also a valuable resource for the investigation of protein evolution via homologous studies.

To date, *G. lamblia* has been studied mainly from a clinical point of view. Only a small number of proteins have been isolated and characterized from this organism and most of them are surface antigens or structural proteins. A glutamate dehydrogenase is the only enzyme from *G. lamblia* known so far that has been completely sequenced [Yee and Dennis, 1992]. This thesis work has led to the discovery of a new enzyme-NAD(P)H oxidase from *G. lamblia.* The isolation of the enzyme was accomplished successfully by salt fractionation, hydrophobic chromatography on Octyl-Sepharose matrix, hydroxyapatite adsorption chromatography and ion-exchange chromatography via Mono Q matrix. The purified NAD(P)H oxidase appeared to be a monomer

with molecular weight of 41,000 (+/-3,000) Daltons. Under aerobic conditions, this enzyme is capable of oxidizing both NADH and NADPH without requiring any other substrate. The optimum pH for the enzymatic reaction is between 6.8 to 7.2.

The reaction involving NADH is more common and it is catalyzed by NADH oxidase. In mammalian mitochondria, the oxidation of NADH by oxygen is part of the reactions carried by the electron transport chain and is usually coupled with the oxidative phosphorylation. However, *G. lamblia* is believed to lack both cytochrome-mediated electron transport and oxidative phosphorylation [Lindmark and Jarroll, 1980, Weinbach et al., 1980]. Therefore, the NAD(P)H oxidase in *Giardia* is unlikely to play the same role as the mitochondria enzymes. The oxidation of NADH has also been detected in several bacterium as discussed in Chapter 1. The *Giardia* NAD(P)H oxidase is indeed similar to the bacterial NADH oxidases considering the following facts. They all catalyze the direct reduction of oxygen to produce NAD^+. The electron transfer process requires the involvement of a non-covalently bound flavin cofactor. Moreover, all of these enzymes are not linked with oxidative phosphorylation. Therefore, the *Giardia* NAD(P)H oxidase and the NADH oxidases obtained from various microorganisms form a new group of enzymes whose functions are under investigation.

As mentioned previously in Chapter 1, there are two types of NADH oxidases: H_2O-forming NADH oxidase and H_2O_2-forming NADH oxidase. The NAD(P)H oxidase isolated from *G. lamblia* catalyzes four-electron transfer with H_2O as a product. It together with the NADH oxidases obtained from *S. faecalis* [Schmidt et al., 1986] and *L. mesenteriodes* [Koike et al., 1985] functions as a H_2O forming enzyme. Oxygen is completely reduced to H_2O by these three enzymes.

Despite the difference of the reduction products, all the bacterial NADH oxidases are specific in utilizing NADH as electron donor. NADPH is either not oxidized or not preferred. In contrast, the *Giardia* NAD(P)H oxidase is capable of oxidizing both NADH and NADPH with almost no preference. This non-selectivity towards the dinucleotide substrate is uncommon for a dinucleotide-utilizing enzyme. It suggests that the active site of the NAD(P)H oxidase may possess some special characteristics to allow the accommodation of both NADH and NADPH individually. To prove this hypothesis, the amino acid sequence of the NAD(P)H oxidase is required. Moreover, this dual functionality for NADH and NADPH is beneficial to an organism that requires an environment of low oxygen content or no oxygen. Both NADH and NADPH can be utilized to lower the oxygen content. Therefore, this NAD(P)H oxidase may play an important protection role in the survival of *G. lamblia.*

As observed in the bacterial NADH oxidases, a flavin cofactor is involved in the electron transfer process catalyzed by the *Giardia* NAD(P)H oxidase. The addition of free FAD has been found to be essential for the NAD(P)H oxidase to retrieve its maximum activity at any stage of the purification process. This means that FAD is the required cofactor for the *Giardia* NAD(P)H oxidase. However, the FAD prosthetic group in *Giardia* NAD(P)H oxidase is only weakly held by the enzyme and was easily removed from the enzyme during the purification procedures. This loosely adsorbed flavin cofactor has also been reported in the NADH oxidases obtained from *S. faecalis* [Schmidt et al., 1986], *T. aquaticus* [Cocco et al., 1988], *B. megaterium* [Saeki et al., 1985] and *T. thermophilus* [Park et al., 1992b]. It is an unusual observation for a flavoprotein to require additional flavin for its maximum activity. In most of the flavoproteins, the flavin cofactor is strongly attached to the apoprotein through non-covalent interactions with the dinucleotide-binding domains on the protein. As

for the *Giardia* NAD(P)H oxidase and the above bacterial NADH oxidases, the flavin-binding sites on these enzymes may not possess all of the characteristics which are present in the majority of flavoproteins and are required for the strong flavin-protein interaction. The analysis of the FAD-binding domains in the *S. faecalis* NADH oxidase has proposed that the oxidation of a labile amino acid residue-Cys8 may account for the easy loss of the flavin cofactor in this enzyme [Ross and Claiborne, 1992]. At present, it is not clear what cause the weak association between the *Giardia* NAD(P)H oxidase apoenzyme and its FAD cofactor. However, the revealment of the protein sequence for this enzyme in future and further analysis of the flavin-binding domains will be helpful for answering this question.

It has been proposed that all the flavo oxidoreductases may have derived from a common ancestral adenine nucleotide-binding protein and developed their specific functions later [Ross and Claiborne, 1992]. To search for the potential ancestral flavoprotein, attentions should be paid to the proteins which contain loosely associated flavin cofactor for the following reason. When a flavin group was initially linked to a protein to form the first flavin-protein complex (flavoprotein), it is highly possible that this complex was not very stable. Therefore, the *Giardia* NAD(P)H oxidase and the above bacterial NADH oxidases are potential primitive flavoproteins because of their unusual property towards the flavin cofactor binding.

In addition to the FAD prosthetic group, the inhibition studies performed with the monofunctional trivalent arsenical compound and the following reactivation attempt with dithiothreitol have proven that at least two cysteinyl residues are present in *Giardia* NAD(P)H oxidase and are important for the catalytic function for this enzyme. Although a cysteine-sulfenic acid has been proposed as the catalytic group in the *S. faecalis* NADH oxidase [Ahmed and Claiborne, 1989a,

Ahmed and Claiborne, 1989b], the *Giardia* NAD(P)H oxidase is unlikely to have the same catalytic mechanism. Instead, it is highly possible that this NAD(P)H oxidase may employ a redox active disulfide to transfer electrons as found in most of the flavo oxidoreductases.

Under the assay conditions, oxygen was used by *Giardia* NAD(P)H oxidase to accept electrons from NADH or NADPH. However, whether oxygen is the natural substrate for this enzyme is uncertain. The presence of this particular NAD(P)H oxidase in *G. lamblia* regenerates reducing power $NAD(P)^+$ which may further facilitate energy production through the pathways involving substrate-level phosphorylation.

A short sequence of 11 amino acids has been revealed from the purified *Giardia* NAD(P)H oxidase by N-terminal amino acid sequence analysis. This revealment provides fundamental information for further studying the NAD(P)H oxidase from a molecular biology approach. Based on the obtained amino acid sequence, an oligonucleotide probe can be designed to identify the gene encoding NAD(P)H oxidase from both *G. lamblia* genomic DNA and cDNA libraries. Future work may start with the characterization of genomic DNA and cDNA clones of the NAD(P)H oxidase gene. This will result in identification of the gene sequence corresponding to the NAD(P)H oxidase which can be directly converted to the amino acid sequence. With the knowledge of the protein sequence, more analysis can be performed on the NAD(P)H oxidase to further understand this enzyme with respect to its active site structure, catalytic mechanism, physiological function and evolutionary relation to other flavoproteins. The regulation mechanism of this *Giardia* NAD(P)H oxidase gene is another interesting topic to study.

In summary, this thesis presents a successful method for isolating a new enzyme-NAD(P)H oxidase from *Giardia lamblia.* The basic information about the properties

of this NAD(P)H oxidase has been obtained from the characterization studies performed in this work.

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