CHAPTER 5

Isolation, Purification and partial characterization of respiratory Nitrate Reductase (NarGHJI) from *M. tuberculosis* H37Ra.
5.1. Introduction

Nitrate reductase [NaR; EC 1.7.99.4; Nitrate: (acceptor) oxidoreductase] is a membrane-bound enzyme and is present in many anaerobic and facultative anaerobic prokaryotes (1). It plays an important role in energy production when oxygen is not readily available or completely absent in the environment. The purification and properties of Nitrate reductase (NarGHJI) has been subjected to extensive investigation and characterization in *Escherichia coli* and *Bacillus licheniformis* (2,3). The enzyme is composed of three subunits: a catalytic α subunit (NarG) of 112 to 140 kDa with MGD cofactor, a soluble β subunit (NarH) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-4S] centers, and a 19- to 25-kDa membrane biheme b quinol-oxidizing γ subunit (NarI) (4). Soluble α and β subunits are anchored to the cytoplasmic side of the membrane by the γ subunit and can be solubilized by detergents or heat. NarI is heat sensitive and can be lost during the purification procedure, leading to the isolation of a soluble αβ complex that can reduce nitrate with reduced viologens as electron donors. A δ polypeptide (NarJ), which is not part of the final enzyme, seems to participate in the assembly or stability of the αβ complex prior to its membrane attachment (5,6). The enzyme uses the quinol pool as the physiological electron donor and generates a PMF by a redox loop mechanism (7,8). NarI oxidizes quinols at the periplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to NarG, via the Fe-S centers of NarH, to reduce nitrate with consumption of two cytoplasmic protons. The low- and high-potential heme b groups of NarI located at opposite sides of the membrane allow an effective transmembrane electron transfer (9). The enzyme has also been characterized in some other denitrifying and nitrate respiring organisms like, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans* and *Klebsiella aerogenes* but very little is known about its counterpart in *M. tuberculosis* (10-13). Though the enzyme present in the *M. tuberculosis* was found more than 70% homologous to NarGHJI of *E. coli*, yet this genetic difference could bring a substantial divergence to the structure as well as function of the enzyme (14). It was therefore of interest to isolate, purify and characterize this membrane bound NarGHJI from *M. tuberculosis* in order to understand the basic role of this enzyme during anaerobic dormant stage. This could help in understanding the chemical and physical nature of nitrate reductase for establishing the functional structure of nitrate reductase and its relationship with the survival of the organism during latent stage. In this chapter I have described a simple method for the preparation of pure nitrate reductase from *M. tuberculosis* H37Ra, which involves release from membranes by selective detergent and purification by a single gel filtration step. I have also described the partial
characterization of NarGHJI from \textit{M. tuberculosis} H37Ra including its molecular weight, optimal activity conditions, substrate specificity and inhibition kinetics.

5.2. Results

5.2.1. Purification

NarGHJI from \textit{M. tuberculosis} H37Ra was purified by modifying a method published earlier (15). Protein purification was divided into three stages. The stages were preparation of membrane fractions, extraction of enzyme from membranes and final purification. It was important that a rapid, sensitive and specific assay should be available for the detection of protein of interest during purification. A bionebulization-based method of cell lysis was found suitable to break the tough cell wall of mycobacteria and prepare membrane fractions (16). Initially, different agents such as 100mM potassium phosphat buffer, Triton X-100 and Tween 80 were used to extract the membrane protein without losing enzyme activity. Triton X-100 based extraction of nitrate reductase from membrane fractions of \textit{M. tuberculosis} could provide the maximum yield of the enzyme without losing any discernible activity. Ion exchange chromatography with Unosphere Q beads (anion exchanger) was found to finally purify the enzyme to almost homogeneity level with elution at 1.25M NaCl, using fast performance liquid chromatography. The final scheme of the purification after optimization of each of these steps is elaborated below.

**Culture conditions for purification:** \textit{M. tuberculosis} H37Ra culture was grown in minimal medium, supplemented with 5mM of sodium nitrate at 130 rpm and 37\(^{\circ}\)C within a shaker incubator. Cultures were inoculated with 1\% of 1.0 \textit{A}\textsubscript{620} as initial inoculums size and were incubated upto 10 days when it reached to late exponential phase of growth in 1-litre flask. These cultures were kept static for another 2 days for anaerobic induction of NR.

**Cell lysis and preparation of membrane fractions:** All the operations, except where otherwise stated, were performed at 4\(^{\circ}\)C hereafter. Culture was centrifuged at 8000 rpm for 30 minutes to harvest the cells. Cell pellet was washed twice with 50mM Tris HCL buffer of pH 7.5. Between 6 to 8gm packed wet weight of cells was obtained from 3 liters of culture. Cells were resuspended in 10 ml of 100 mM Tris/HCL buffer (pH 8.0) for e gram of pellet. This cell suspension was subjected to bionebulization based cell disruption system (BioNeb Glas-Col, USA) for 10 cycles of 3 minutes each at 200psi (16). Clumps were broken completely after 10 cycles of bionebulization and a homogenized cell suspension was obtained. Lost volume of
suspension during bionebulization was adjusted with same Tris/HCL buffer. 5mM EDTA, 0.5mg/ml lysozyme and 50μg/ml DNAse + RNAse was added to this lysate and slowly stirred for 30 minutes to lyse the remaining intact cells. Further efficiency of lysis was increased with fridge thawing of the suspension for 5 times. Lysate was then centrifuged at 3000 rpm for 5 minutes at room temperature to pellet down the cell debris. Supernatant was centrifuged at 25000 g for 2 hours to collect the membrane fractions at bottom. Enzyme activity was examined in these membrane fractions to ensure that these membrane fractions contain the enzyme in sufficient quantity (Table 5.1).

**Extraction of enzyme from membrane fractions:** Resulting pellet of membrane fractions was washed twice with same Tris/HCL buffer and resuspended in 50mM sod phosphate buffer (pH 7.2) containing 0.1mM of 2-mercaptoethanol and 0.1 mM phenyl methanesulfonylfluoride (PMSF). This suspension was added with Triton X-100 to a final concentration of 2% triton. The mixture was stirred at 100 rpm on a magnetic stirrer for 30 minutes and centrifuged at 25000 g for 2 hours. This supernatant found to contain significant amount of nitrate reductase activity while pellet fraction obtained after this centrifugation did not show any discernible nitrate reductase activity, hence confirmed the effective extraction of the enzyme (Table 5.1).

**Heat treatment:** A heat treatment step commonly used to remove unwanted proteins during the purification of this nitrate reductase was also attempted here (17). After treating the extracted protein sample at 65°C for 7 minutes, sample was kept overnight at 4°C and centrifuged at 15,000 rpm for 60 minutes to remove heat precipitated proteins. This could remove many unwanted proteins and increased the purity of the enzyme. However, inclusion of this also resulted in the loss of one of the subunit from the enzyme confirmed by SDS PAGE done after FPLC (Figure 5.1). Though loss of one of the subunit did not cause any loss of enzyme activity, this heat treatment step was excluded in this protocol to retain all the three subunits of nitrate reductase (NarGHI). Samples were therefore subjected to ion exchange chromatography in the next step itself.
Table 5.1 Purification scheme of nitrate reductase from *Mycobacterium tuberculosis* at different steps.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>%</th>
<th>Activity unitsa (%)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>467</td>
<td>100</td>
<td>1184</td>
<td>2.53</td>
<td>1</td>
</tr>
<tr>
<td>Cell free lysate</td>
<td>351</td>
<td>75.16</td>
<td>872</td>
<td>2.48</td>
<td>0.98</td>
</tr>
<tr>
<td>Crude membrane fraction</td>
<td>98</td>
<td>20.98</td>
<td>790</td>
<td>66.72</td>
<td>3.18</td>
</tr>
<tr>
<td>Triton X 100 extract</td>
<td>32</td>
<td>6.85</td>
<td>722</td>
<td>60.97</td>
<td>8.91</td>
</tr>
<tr>
<td>Pooled Unosphere Q eluted</td>
<td>3.4</td>
<td>0.72</td>
<td>602</td>
<td>50.76</td>
<td>69.96</td>
</tr>
<tr>
<td>Dialyzed and concentrated by filtration</td>
<td>3.1</td>
<td>0.66</td>
<td>589</td>
<td>49.74</td>
<td>75.09</td>
</tr>
<tr>
<td>After Gel filtration and concentration</td>
<td>2.7</td>
<td>0.57</td>
<td>440</td>
<td>37.16</td>
<td>64.41</td>
</tr>
</tbody>
</table>

*1 Unit = 1 μM NO₂ produced per minute at 37°C.

*Protein concentration were determined by using the BioRad protein assay kit with bovine serum albumin as standards (BioRad Laboratories, Hercules, CA, USA).

**Ion exchange chromatography:** Ion exchange chromatography was used for purification of this enzyme from *M. tuberculosis*. Unosphere Q anion exchanger column (size 26mm X 10 cm) was attached with the basic system (BioRad, Biological Duoflow) for fast performance liquid chromatography (FPLC). Extracted protein solution was injected into the column already, equilibrated with 50mM of sodium phosphate buffer (pH 7.2). The column was washed sequentially with 50 ml of the above buffer, with a linear gradient of 0-2.0M of NaCl in the above buffer at a flow rate of 1ml/min. The A₂₈₀, conductivity, pH and nitrate reductase activity were measured on all fractions and SDS-PAGE analysis was performed for fractions of interest. Active fractions from the column were pooled, dialyzed for 12 hours against the above phosphate buffer and concentrated by filtration with 3Kd filter (AMIKON, Millipore). The profile of protein in active fractions confirmed the preparation of an almost homogenous nitrate reductase in SDS-PAGE (Fig. 5.1). The purification achieved by this fast flow column was more than 60 fold and with a yield of about 50% (Table 5.1). The peak nitrate reductase activity eluted at salt
concentration of 1.25M NaCl in the gradient (Fig. 5.2). The fractions with the activity were eluted in the salt concentration range between 1.35 to 1.15 M NaCl concentrations. The pooled fractions which contained more than 20% of the peak of activity yielded a total volume of 3.6ml. The specific activity of enzyme was increased from 22.55 to 177.1 units/mg of protein after this chromatography. There is a clear protein band seen at the expected molecular weight range in lane 7 of the SDS PAGE which corresponds with the elution of nitrate reductase activity at peak (Fig. 5.1).

Fig. 5.1 SDS-PAGE analysis during different steps of purification of nitrate reductase. A and B shows the purification of enzyme including and excluding the heat treatment step respectively. Molecular weight markers used in the lane 1 of A and B were: β- galactosidase (Mr 117 Kd), phosphorylase b (Mr 97 Kd), bovine serum albumin (Mr 66 Kd), ovalbumin (Mr 45 Kd) and soybean trypsin inhibitor (Mr 21). Lane 2,3,4,5 and 6 of A were loaded respectively with crude cell lysate, membrane extract, heat treated extract at 65°C, heat treated extract at 70°C, FPLC peak activity fraction of heat treated sample. Lane 2,3,4 and 5 of B were loaded respectively with concentrated FPLC peak activity fraction, duplicate of lane 2, membrane extract, crude cell lysate. 20μl of protein sample having concentration of at least 200μg/ml was loaded and stained with comassie blue.
Fig. 5.2 Profile of nitrate reductase elution from the Unosphere Q fast flow anion exchange chromatography column. The peak of nitrate reductase activity was eluted at salt concentration of 1.25mM NaCl in 50mM sodium phosphate buffer of pH 7.2. Salt concentration, conductivity, $A_{280\text{nm}}$ and $A_{260\text{nm}}$ monitored along with time with corresponding number of fraction as denoted in the figure.

5.2.2. Determination of Molecular Weight

MW of nitrate reductase was determined by gel filtration chromatography using a Sephacryl S-300 beads column, pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.2), containing 0.1mM 2-mercaptoethanol and 0.1mM PMSF. Purified protein at 1mg/ml concentration was applied to the column and buffer was pumped through the column at 0.25ml per minute and fractions each of 0.5 ml were collected. In order to find out the molecular weight of the purified protein, first molecular weight markers were passed through the column and a standard curve was generated with the elution volume. Based on this standard curve, the molecular weight of nitrate reductase was found to be 214Kd (Fig. 5.3). This data was also in confirmation with the
denatured SDS-PAGE data, which indicated to have three subunits of about 125, 60 and 25 in the enzyme preparation respectively for NarG, H and I (Fig. 5.1) (18,19).

\[
y = -0.8795x + 3.4952 \\
R^2 = 0.9945
\]

![Graph showing the relationship between Log of Mr and Ve/Vo.](image)

Fig. 5.3 Standard curve of the elution of molecular weight markers from non denaturing sephacryl 300 size exclusion chromatography column. The markers include; thyroglobulin (670Kd), bovine gamma globulin (158Kd), ovalbumin (44Kd), and myoglobulin (17Kd). Nitrate reductase activity peak was eluted at Ve/Vo of 1.3243 corresponding to a molecular weight of 214 Kd.

### 5.2.3. Stability, Effect of pH, temperature and metal ions on activity of enzyme

Stability of the enzyme was first examined in various buffers, and other stabilizing agents to determine its native environment and optimum preservation conditions. Among different buffers, it was found to be most stable in 50mM sodium phosphate buffer (pH 7.2). Addition of glycerol in the buffer increased the longevity of the enzyme’s activity compared to other stabilizers such as PMSF, β-MSH, DTT, threonine, cysteine, leupeptin and dithionite (Fig. 5.4) (20). Without addition of glycerol there was a consistent loss of activity of pure enzyme with time and the loss was minimized with addition of 2% glycerol (Fig. 5.5A and B). PMSF (10 μM) and β-MSH (10 μM) were also added in the enzyme preparation to avoid the proteolytic degradation.

Nitrate reductase activity was optimal at pH 7.0 (Fig. 5.6). It maintained more than 50% of the optimal activity from pH 5 to 9 and still detectable activity in the range of 4-12. This
indicated the enzyme has ability to adapt to a wide range of pH and functioning in environment at different physiological pHs. The optimal temperature of the enzyme was found to be 45°C and more than 50% of it was retained in the range of 20-60°C (Fig. 5.7). Activity was almost completely lost at below 10°C and above 70°C. In case of *E. coli* nitrate reductase, optimal temperature was reported to be 23°C (21). This indicated that the extreme temperature adaptability and thermo tolerant nature of *M. tuberculosis* nitrate reductase.

Different metal ions were examined thereafter to determine their effect on nitrate reductase activity. There was no significant effect seen of any of the metal ions on nitrate reductase activity. Some favorable effect by Mo⁵⁺ and W⁵⁺ could be seen by only 11-20% at concentration of (10nM) (Fig. 5.8).

![Fig. 5.4](image)

**Fig. 5.4** Effect of different agents on stability of nitrate reductase. The effect on nitrate reductase activity was measured after 24 hours of addition of these agents to enzyme solution. The concentration used for gycerol was 2% and 0.1mM for other agents. Where NP Buf = sodium phosphate buffer, PMSF= phenyl methane sulfonyl fluoride, β-MSH = 2-mercaptoethanol, DTT = dithiothreitol, Gly = glycerol, Cys = cysteine, Thr = threonine, Lp = Leupeptin. Results are mean +/- SD of three identical experiments.
Fig. 5.5 Stability of nitrate reductase activity of purified enzyme without addition of glycerol (A) as compared to with addition of 2% glycerol (B). The reaction was carried out at pH 6.8, 37°C and 60 minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100μM Methyl Viologen, 10μM Clealand reagent, 20μg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

Fig. 5.6 Effect of pH on nitrate reductase activity of purified enzyme. The activity was assayed in different pH phosphate buffers. The reaction was carried out 37°C and 60 minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100μM Methyl Viologen, 10μM Clealand reagent, 20μg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.
Fig. 5.7 Effect of different temperature on nitrate reductase activity of purified enzyme. The reaction was carried out at pH 6.8, 60 minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100μM Methyl Viologen, 10μM Clealand reagent, 20μg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

Fig. 5.8 Effect of different metal ions on nitrate reductase activity of purified enzyme. 10μl of 10μM solution of each metal ion solution was added in the assay reaction just before addition of enzyme. The reaction was carried out at pH 6.8, 37°C and 60 minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100μM Methyl Viologen, 10μM Clealand reagent, 20μg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.
5.2.4. Substrate specificity of nitrate reductase

Nitrate reductase activity was assayed against a concentration range of 0 to 100mM of nitrate in the reaction to determine the affinity of enzyme for substrate. Maximum activity of enzyme could be achieved at a concentration of 1.28mM (Fig. 5.9). $K_m$ for nitrate was found to be 380µM as determined from Hans-Wolf plot. These low $K_m$ values indicate high affinity of the enzyme towards nitrate (Fig. 5.10). Along with methyl viologen, which is commonly used as electron donor in the reaction, some other substrates were also examined for their ability to be used as artificial electron donor in the reaction catalyzed by nitrate reductase. It was found that benzyl viologen could also effectively support the purified enzyme activity (data not shown). Other electron donors such as NADH, NADPH, reduced glutathione, succinate, fumarate and formate could not support the reaction. The $K_m$ values of methyl viologen and benzyl viologen were calculated to be 60 and 80µM respectively (Fig 5.11 and 5.12). As compared to the $K_m$ values obtained in nitrate reductases of other prokaryotes, the $K_m$ values for nitrate, methyl viologen and benzyl viologen were very similar and indicated the high affinity and efficiency of *M. tuberculosis* nitrate reductase (Table 5.2) (22).

![Nitrate reductase activity of purified enzyme at different nitrate concentrations.](image)

Fig. 5.9 Nitrate reductase activity of purified enzyme at different nitrate concentrations. The reaction was carried out at pH 6.8, 37°C and 60 minutes incubation time in a final assay volume of 2ml containing 20mM Sodium Phosphate buffer, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.
Fig. 5.10 Hans-Wolf plot for determination of the $K_m$ of nitrate for nitrate reductase. Where $V =$ Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20$\mu$g/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37$^\circ$C and with incubation time of 60 minutes. Results are mean $\pm$ SD of three identical experiments. Other details of the experiment are described in materials and method section.

Fig. 5.10 Hans-Wolf plot for determination of the $K_m$ of Methyl Viologen for nitrate reductase. Where $V =$ Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20$\mu$g/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37$^\circ$C and with
incubation time of 60 minutes. Results are mean +/- SD of three identical experiments. Other details of the experiment are described in materials and method section.

Fig. 5.10 Hans-Wolf plot for determination of the \( K_m \) of Benzyl Viologen for nitrate reductase. Where \( S = \) Concentration of Benzyl Viologen in mM, \( V = \) Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20\( \mu \)g/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8, 37\(^\circ\)C and with incubation time of 60 minutes. Results are mean +/- SD of three identical experiments. Other details of the experiment are described in materials and method section.

Table 5.2 \( K_m \) of nitrate, MV, BV determined for NR purified from \emph{M. tuberculosis} H37Ra compared with previously reported values for \emph{E. coli}.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) for \emph{M. tuberculosis} NR</th>
<th>( K_m ) for \emph{E. coli} NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>380( \mu )M</td>
<td>330( \mu )M</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>60( \mu )M</td>
<td>29( \mu )M</td>
</tr>
<tr>
<td>Benzyle viologen</td>
<td>80( \mu )M</td>
<td>34( \mu )M</td>
</tr>
</tbody>
</table>
5.2.5. Inhibition kinetics of Nitrate reductase

Inhibition characteristics of the purified enzyme were determined by applying the known inhibitors of nitrate reductase to the reaction in dose dependent manner. Azide which was used for the inhibition of nitrate reductase in the whole cell culture showed a similar pattern of inhibition on isolated enzyme (23). Lineweaver-Burk plot drawn from the results obtained from different concentration of azide at varied concentrated of nitrate indicated the typical competitive inhibition of the enzyme (Fig 5.13). Thiocyanate on the other hand showed an un-competitive type of inhibition of nitrate reduction without being affected by nitrate concentration in the reaction (Fig. 5.14).

![Lineweaver-Burk plot showing competitive inhibition of purified nitrate reductase by azide.](image)

Fig. 5.13 Lineweaver-Burk plot showing competitive inhibition of purified nitrate reductase by azide. The concentration of 5μM (■), 20μM (▲) and 50μM (●) of azide resulted in subsequent decrease in the inhibition constant of 3.1, 2.2 and 1.7mM respectively. Experiments was carried out at 20μg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8, 37°C and with incubation time of 60 minutes. Different doses of inhibitor were added just after addition of enzyme in the reaction mixture. Results are mean +/- SD of three identical experiments.
Fig. 5.14 Lineweaver-Burk plot showing un-competitive inhibition of purified nitrate reductase by thiocyanate. The concentration of 5mM (■), 20mM (▲) and 50mM (●) of thiocyanate resulted in subsequent increase in the inhibition constant of 0.8, 3.0 and 4.7mM respectively. Experiments was carried out at 20μg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8, 37°C and with incubation time of 60 minutes. Different doses of inhibitor were added just after addition of enzyme in the reaction mixture. Results are mean +/- SD of three identical experiments.

5.2.6. UV-VIS absorption spectroscopy of Nitrate reductase

Absorbance spectra of the purified nitrate reductase in UV-Visible region are shown in figure 5.15. The position of maximum absorbance is seen in the range of 240-290nm having three different peaks in this region (Fig. 5.15A). After addition of dithionite, there was a new peak seen with λ_{max} at 340nm which is generally seen with the reduced form of an enzyme (5.15B). Dithionite which provides a reducing environment may cause a different rearrangement of iron and sulfur clusters within the enzyme subunits (24,25). There could also be seen a change of absorbance pattern showing decrease in the peak at 250nm and increased peak at 275nm after addition of dithionate which is being under characterization process in the future laboratory work.
Fig. 5.15 UV-Visible spectra of purified nitrate reductase before (A) and after addition of dithionite (B). A baseline correction was done for the buffer solution in which the enzyme was kept before taking the spectra. Enzyme concentration used for spectra was 200μg/ml and 10μM of dithionite was added in the enzyme solution.
5.3. Materials and Methods

5.3.1. Bacterial strains, media, culture conditions, buffers and solutions

*M. tuberculosis* H37Ra (ATCC 25177), an avirulent strain, was used as source of nitrate reductase (NarGHJI). Recipes for media, buffers, solutions used in the production, purification and characterization of nitrate reductase were as per described previously (21,22).

5.3.2. *In vitro* Nitrate reductase assay

Nitrate reductase activity was assayed by measuring the reduction of nitrate to nitrite with methyl viologen as the electron donor (15). The assay mixture contained 0.5ml of 100mM sodium phosphate buffer (pH 7.1); 0.1ml of 100mM sodium nitrate; 0.2ml of 0.05% methyl viologen; 10μl of 10mM Cleland’s reagent and 1.1ml of water plus enzyme. To start the reaction, 0.1ml of a solution containing 0.8% sodium bicarbonate and 0.8% sodium dithionite was added to the assay mixture and it was gently swirled until uniformly blue. After incubation for 60 minutes at 37°C, the reaction was stopped by mixing rapidly in a Vortex mixer until the blue color had disappeared. Nitrite was then determined by the following the diazo coupling procedure (26). Two milliliters of a 1% solution of sulfanilic acid in 20% HCl was added to the assay mixture and it was mixed thoroughly. Then, 2 ml of a 0.129% solution of N- naphthylethylenediamine diHC1 was added and, after 10 minutes, the absorbance at 540 nm was measured. Of the above reagents, the dithionite- bicarbonate solution and the methyl viologen were prepared fresh daily and the Cleland’s reagent was kept frozen. All other reagents were stable indefinitely. A unit of activity is defined as the production of 1μmole of NO₂ per min at 37°C. Specific activity is expressed in units per mg of protein.

5.3.3. SDS-PAGE

SDS-PAGE was performed as described by Laemmli, under reducing condition using a miniprep protein gel apparatus (Amersham Biosciences) (27). Gels were 0.5mm thick and contained 12.5% polyacrylamide in the separating gel. Protein samples were mixed 1:1 with 2X sample buffer and heated to 95°C for 5 minutes prior to loading. Standards used were BioRad’s high molecular weight markers diluted 1:50 in the buffer. Standards include: b-galactosidase (119000) phosphorylase b (Mr 97500) bovine serum albumin (Mr 66200), ovalbumin (Mr 45000) and soybean trypsin inhibitor (Mr 21500). Gels were electrophoresed at constant current of 40 milliampere at room temperature and stained with BioRad’s Commassie blue stain kit. Native
PAGE was performed with similar running conditions except the addition of SDS and heat treatment of samples.

5.3.4. Unosphere Q anion exchange chromatography

Protein sample was loaded at 1ml per minute flow rate on the Unosphere Q fast flow column which had been pre-equilibrated with 50mM sodium phosphate buffer (pH 7.2) and fractions of 0.3 ml each were collected. After washing 3 column volumes of sodium phosphate buffer (pH 7.2) at 10ml per minute flow rate, baseline absorbance 280 nm was returned to zero and protein was eluted with a 100ml linear gradient of 0 to 2.0 M NaCl in 50mM sodium phosphate buffer (pH 7.2) at a flow rate of 1 ml per minute. Column was washed with 3 column volumes of 50mM sodium phosphate (pH 7.2) buffer with 2M NaCl at a flow rate of 10ml per minute.

5.3.5. Determination of molecular weight of native nitrate reductase

Estimation of the molecular weight of the nitrate reductase was made by gel filtration chromatography on the sephacryl 300 column. A standard curve of the log of the molecular weight versus elution volume was produced using 5 molecular weight standards. Molecular weight standards used were: blue dextran (Mr 2000000), beta amylase (Mr 200000), alcohol dehydrogenase (Mr 150000), bovine serum albumin (Mr 66000) and carbonic anhydrase (29000). Standards (500µg of each protein in a total volume of 0.5ml) were loaded on the column which had been pre-equilibrated with 50mM sodium phosphate buffer.

5.3.6. Enzyme Kinetics

For all the experiments related to kinetics studies of enzyme such as effect of temperature, pH, metal ions, inhibitors, substrate specificity, etc, 10µl of purified enzyme having a concentration of 200 µg/ml enzyme was added in the reaction. Reaction was carried out at pH 6.8, temperature 37°C and incubation time of 60 minutes, once this was standardized. Substrates, inhibitors and other modulators were added after the addition of enzyme in the assay. Frozen enzyme kept in aliquots, added with 2% glycerol, 10µM PMSF and 10µM β-MSH, was thawed and dilute to 200µg/ml in sodium phosphate buffer (50mM pH 7.2) before use.
5.4. Discussion

Nitrate reductase (NarGHJI), belongs to an important family of reductases and plays a key role of energy generation in a wide variety of bacteria (28). These organisms which contain this enzyme are either facultative anaerobes or obligatory anaerobes and generally reside in environments where nitrate is readily available. There were certain differences seen among these organisms for the structural as well as chemical properties in nitrate reductase. It was surprising initially that *M. tuberculosis*, which was known to be an obligate aerobe, contained this enzyme (29). However, once the concept of hypoxic dormant stage in mycobacteria was proved, it was accepted as a facultative anaerobe which can use nitrate in either limited or complete absence of oxygen (30). Although it is almost a decade since the availability of genome draft revealing that *M. tuberculosis* contains this respiratory nitrate reductase, yet the attention towards the study of this enzyme in isolation could not be attracted so far (14). As our results and earlier findings strengthened this enzyme becoming a possible drug target against dormant tubercle bacilli, an elaborated study on the enzyme in isolation became imminent to better understand the role of this enzyme in pathogenicity before taking it up as drug target studies (23,29). It was reasonably more complex to purify nitrate reductase from *M. tuberculosis* due to its slow growing nature, tough wall to break and the membrane bound nature of the enzyme. A successful purification of nitrate reductase from *M. tuberculosis* H37Ra in this study provided a major step forward isolating and purifying enzymes from wild type strains of mycobacteria. In this purification we could also retain all three subunits of enzyme which was not shown in many of the organisms (3,10,11). There were some noticeable differences seen as well in the characteristics of enzyme compared to nitrate reductase of other organisms. Although *M. tuberculosis* is a slow growing organism in nature, nitrate reductase was found to have much higher specific activity of 190 units/mg protein compared to the fast growing *E. coli*, where it is only 76 units/mg protein in the purified enzyme preparation (Table 5.1) (11).

The molecular size of the enzyme was nearly similar to what was reported in other organisms (Fig. 5.1 and 5.3) (12). However, the native form of the enzyme was found here to remain in monomer form showing 220 Kd band in the gel exclusion chromatogram, which was seen to be tetramer form with MW ~880Kd in the native gel exclusion chromatogram of protein other organisms (17). The subunits of enzyme were also found to have a more or less similar molecular weight to what was reported for other bacterial nitrate reductase subunits. It is not very clear about the association of NarI in the purified preparation in preventing them to form the tetramer.
Comparative molecular weight of the enzyme as determined by gel exclusion chromatography subunit indicated the existence of monomer form in both preparation of enzyme with and without containing NarI (Fig 5.3). This data point out towards a different subunit association of *M. tuberculosis* nitrate reductase as compared to the nitrate reductases of other organisms (13,17).

Addition of glycerol in the enzyme solution resulted in increased stability of enzyme, hence enzyme’s activity could be preserved for longer time, which also suggested the possible hydrophobic nature of the enzyme (Fig 5.4 and 5.5). The pH and temperature studies of nitrate reductase also indicated the wide range of adaptability to pH and temperature which indicated its ability to adapt according to different physiological conditions as the pathogen is supposed to face changing environment during the course of disease and particularly during the latent stage (Fig. 5.6 and 5.7). The $K_m$ values of Nitrate, MV and BV determined for *M. tuberculosis* nitrate reductase were quite similar to that reported for *E. coli* and other organisms (5.9, 5.11 and 5.12) (10). The competitive and non-competitive nature of inhibition of this enzyme by azide and thiocyanate respectively, was expected based on their well known mode of action and indicated similar binding sites available in this enzyme as well (Fig 5.13 and 5.14). Presence of an oxidized and reduced form similar to other nitrate reductases was seen by UV-Visible spectra (Fig. 5.15) (24,25).

Electron paramagnetic resonance, differential light scattering, small angle X-ray scattering, chorionic dichroism and X-ray crystallography in the subsequent studies are being carried out in our laboratory to understand the physical and chemical nature of the enzyme in detail. An enzyme based microplate nitrate reductase assay is also being developed in the laboratory which could be effectively used to search novel anti- tuberculosis molecules targeting nitrate reductase of *M. tuberculosis*.

Altogether, this work and the subsequent characterization studies should contribute to the understanding of the bioenergetics mechanism mediated by NarGHJI and allow us to explore the development of new anti-mycobacterial agents that target key stage of latency in *M. tuberculosis*. 


5.5. References

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