Chapter 2

Review of Literature
2.1 Apicomplexan parasites

Despite significant advances in biomedical researches that have been produced hundred of drugs that are safe and effective against bacteria, viruses, fungi, prions and parasites, infectious diseases are still a major cause of death, disability, social and economic upheaval for millions around the world. Parasitic diseases caused by protozoan parasites of the phylum apicomplexa are amongst the most prevalent worldwide both in human and livestock (Levine 1988). Apicomplexan parasites are the large phylum of unicellular, obligate intracellular organisms of great medical importance. They involve the human pathogens Plasmodium spp. with Plasmodium falciparum responsible for tropical malaria (Maitland et al. 2003) and Toxoplasma gondii, an opportunistic pathogen of immunocompromised individuals (transplant recipients and AIDS patients) and common cause of many congenital diseases (toxoplasmosis), together affecting several hundred million people and animal worldwide with severe clinical symptoms (Dubey 1993; Tenter et al. 2000). Recently Toxoplasma gondii infection has also been implicated as a possible cause of psychological disorder like schizophrenia (Cohen 1999). In some areas of the world, more than 50% of human beings are infected with Toxoplasma gondii (Tenter et al. 2000). In addition, several apicomplexan parasites cause great commercial losses by infecting economically important animals.

2.2 Apicoplast

The life cycle, habitat and route of transmission of apicomplexa parasites vary greatly however, they have a unique organelle (micronemes, dense granules, rhoptides) which fulfill vital functions in cell motility, cell adhesion, parasite entry and exit and protein secretion (Blackman and Bannister 2001). These organisms have another unique organelle, namely cryptic plastid or appropriately called apicoplast (Wilson 2002; Foth and McFadden 2003). Apicoplast has received great interest among investigators because of two reasons. First, it provides evidence for a secondary endosymbiotic event in apicomplexa whereby their common ancestor engulfed an algal organism, giving rise to the organelle’s four membrane visible by electron microscopy (Roos et al. 1999; Fast et al. 2001). Second, the plastid like organelle offers a new and promising target for rational drug design, because it is assumed to contain a number of unique metabolic pathways not found in mammalian hosts (McFadden and Waller 1997).
With one exception, all apicomplexan seem to have retained the plastid which is still reflected by the occurrence of four membranes surrounding the apicoplast of extant apicomplexa (McFadden and Waller 1997; Zhu et al. 2000). Although the apicoplast has its own 35kb circular genome which encodes for either ribosomal proteins or proteins involved in the replication of the episomal genome (Roos et al. 1999) but, are not sufficient to constitute the whole organelle and to provide insights into its experimentally proven essential function (Fichera and Roos 1997). It is therefore obvious that nuclear encoded genes must exist that code for apicoplast localized proteins, similar to the situation in chloroplast and plants (Martin et al. 1998). The reduced size of the apicoplast genome with respect to plant and algal plastids suggest that the loss of genes encoding apicoplast proteins or their progressive transfer to the nucleus has taken place. These nuclear encoded proteins are transported to the apicoplast via N-terminal bipartite targeting sequence, which is both necessary and sufficient to transport proteins in to apicoplast through all four membranes (Roos et al. 1999; DeRocher et al. 2000; Waller et al. 2000; He et al. 2001). As this organelle is indispensable for the survival of parasite and host cell is deficient of such organelle, it makes apicoplast and protein present therein parasite specific target for chemotherapy (Fichera and Roos 1997). It is also presumed that the apicoplast would have different physiology than mammalian hosts as this organelle is decent from algal plastids. Apicoplast is assumed to contain a number of unique metabolic pathways not found in the vertebrate host making it an ideal “playground” for those interested in drug targets. Amongst them are enzymes of the plant-like fatty acid biosynthesis machinery, enzymes of the non mevalonate isoprenoid biosynthesis pathway and part of heme biosynthesis pathway. From their presence in the apicoplast it can be concluded that fatty acid and lipid biosynthesis seems to be a major function of apicoplast. Another recently describe enzyme, Ferredoxin NADP⁺ reductase and its redox partner ferredoxin, point to another interesting organelle specific biosynthetic pathway, namely [Fe-S] cluster biosynthesis (Seeber 2003; Seeber et al. 2005).

2.3 Ferredoxin NADP⁺ Reductase

Ferredoxin (flavodoxin)-NADP(H) reductases (FEC 1.18.1.2) are ubiquitous flavoenzymes that deliver NADPH or low potential one electron donor (Ferredoxin, flavodoxin) to redox based metabolisms in plastids, mitochondria and bacteria (Carrillo and Ceccarelli 2003). Recently existence of Fd/FNR redox system has also been reported in apicoplast of the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum*.
(Vollmer et al. 2001; Pandini et al. 2002b). In chloroplast and cyanobacteria, FNR is responsible for the electron transfer from photosystem I to NADP+, for the provision of reducing equivalents needed in the Calvin cycle for CO₂ fixation (Arakaki et al. 1997). The reaction catalyzed by FNR, a FAD-containing protein is the following:

\[
\text{FNR} \\
2 \text{Fd} (\text{Fe}^{12}) + \text{NADP}^+ + \text{H}^+ \quad \text{\rightarrow} \quad 2 \text{Fd} (\text{Fe}^{3+}) + \text{NADPH}
\]

Since NADP⁺ reduction requires a hydride ion and Fd a [2Fe-2S] protein, is a one electron carrier, FNR functions as a transducer between monoelectronic and bielectronic flow. It thereby exploits the properties of its FAD prosthetic group, which can exist in both the semiquinone (1e⁻) and the dihydroquinone (2e⁻) redox state. In nonphotosynthetic plastids, electron flows in reverse direction from NADPH to Fdox, yielding Fdred (Bowsher et al. 1993), which then serves as a reductant for various enzymes. A provisional list of enzymes of plant, dependent on reduced ferredoxin is given in Table 1.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferredoxin-thioredoxin reductase</td>
<td>Redox regulation</td>
<td>(Schurmann 2002)</td>
</tr>
<tr>
<td>Choline monoxygenase</td>
<td>Osmotic protection</td>
<td>(Burnet et al. 1995)</td>
</tr>
<tr>
<td>Glutamate synthase</td>
<td>Amino acid synthesis</td>
<td>(Vanoni and Curti 1999)</td>
</tr>
<tr>
<td>Heme oxygenase</td>
<td>Chromophore metabolism</td>
<td>(Cornejo et al. 1998)</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>Nitrogen metabolism</td>
<td>(Muramoto et al. 2002)</td>
</tr>
<tr>
<td>Nitrite reductase</td>
<td>Nitrogen metabolism</td>
<td>(Rubio et al. 1996)</td>
</tr>
<tr>
<td>Pheophorbide a oxygenase</td>
<td>Chromophore metabolism</td>
<td>(Schmitz and Boehme 1995)</td>
</tr>
<tr>
<td>Phytochromobilin synthase</td>
<td>Chromophore metabolism</td>
<td>(Pruzinska et al. 2003)</td>
</tr>
<tr>
<td>Δ9 Stearoyl desaturase</td>
<td>Fatty acid modification</td>
<td>(Kohchi et al. 2001)</td>
</tr>
<tr>
<td>Sulfite reductase</td>
<td>Sulfer metabolism</td>
<td>(Thompson et al. 1991)</td>
</tr>
<tr>
<td>Adrenodoxin reductase</td>
<td>Steroid hydroxylation in mammalian mitochondria</td>
<td>(Nakayama et al. 2000)</td>
</tr>
</tbody>
</table>

The two redox systems (photosynthetic and nonphotosynthetic) are highly similar, but are finely tuned in the redox potential of their prosthetic groups (FAD and [2Fe-2S]) to accomplish their different metabolic tasks. FNR from Toxoplasma gondii and others apicomplexa are phylogenetically more closely related to non-photosynthetic FNR isoforms than the chloroplast and cyanobacterial FNRs implicating that reduced Fd might also be the product of this peculiar FNR (Vollmer et al. 2001).
2.4 Evidences for backward reaction

Anaerobic photoreduction studies of *Toxoplasma gondii* FNR shows opposite behavior to that of photosynthetic FNRs. In case of *Spinach oleracea* FNR (SoFNR) NADP\(^+\) was reduced first, where as in case of TgFNR FAD was nearly fully reduced before NADPH accumulated (Pandini et al. 2002b). Low redox potential (-310 to -450mV) is characteristics to classical plant type [2Fe-2S] Fds. As shown in figure 1 The redox potential of the root type Fd is more positive than leaf type Fd, thus favoring the direction of electron flow from NADPH to Fd in nonphotosynthetic tissues while the lower redox potential of leaf type ferredoxin support a higher photosynthetic activity with electron flow in opposite direction (Hanke et al. 2004a). The redox potential of *Plasmodium falciparum* Fd (-266mV) and *Toxoplasma gondii* Fd (-290mV) is even more positive than those of root Fds. (Pandini et al. 2002b; Kimata-Ariga et al. 2007a). These values are similar to that of mitochondrial adrenodoxin (-250mV) (Couture et al. 2001), another protein containing a [2Fe-2S] cluster, which functions as an electron carrier for cytochrome P\(_{450}\)S. The higher redox potential appears to be characteristic feature of the Fd/FNR couple in the apicoplast. This yields reduced Fd, which could then serves as electron donor to a number of reductive pathways in the apicoplast yet to be identified.

![Diagram](image)

**Figure 1**: Comparison of calculated potentials with known measurements of NADPH and maize and *T. gondii* proteins: maize leaf Fd (Matsumura et al. 1999); maize root Fd (Akashi et al. 1999); leaf FNR and root FNR (Aliverti et al. 2001); *T. gondii* FNR (Pandini et al. 2002b). This figure is adopted from research article published by Kimata et al (2007).
2.5 Sequence analysis

A significant homology of TgFNR sequence with plant and algal counterparts over the entire sequence was observed with the most conserved residues being the binding sites for cofactor FAD and NADP (H). The most prominent difference is the large insertions of the 31 amino acids located immediately N-terminal of the FAD binding site (Bednarek et al. 2003) and replacement of positively charged Lys75, which is conserved in all species except *Toxoplasma gondii*, the green algae and root isoforms. Lys 75 has been shown to be essential for Fd binding (Martinez-Julvez et al. 1998).

**Figure 2:** Sequence alignment diagram of amino acid sequences of FNRs from various sources. The sequence position identical residues are shown in white color with red background and the position that is homologous is shown in red color with white background.
Apicomplexan FNRs are characterized by small insertion sequences which is absent in all plant type FNRs (Vollmer et al. 2001). Homology based modeling of TgFNR with three dimensional structure of maize non-photosynthtic FNR from root isoforms as template (Aliverti et al. 2001) and shows that the overall structure of TgFNR is quite similar to that of the root FNR, and the insertion would loop out without disturbing the FAD binding site (Bednarek et al. 2003). Studies with the deletion mutants of T. gondii FNR showed that such a species specific sub-domain does not have major function in protein folding and stability. Rather, it participates in Fd binding, significantly increasing the stability of the protein-protein complex and improving the catalytic efficiency of the enzyme in the electron transfer to the Fd. On the other hand, the 20-25 N-terminal residues of the polypeptide chain, which in plant leaf FNRs has been shown to stabilize the Fd-FNR complex, do not have this role in TgFNR (Pandini et al. 2006). Quite unexpectedly, TgFNR now undoubtedly has been shown to belong to the nonphotosynthetic isoforms class of FNRs, however it does not possess a disulfide bond which is peculiar feature of this class of enzyme (Aliverti et al. 2001).

2.6 Structural and functional portraits of Ferredoxin NADP+ Reductase

Three dimensional structures have been determined for eight distinct FNRs: four leaf-type enzymes (spinach, corn, pea, and paprika), one root- type enzyme (corn), one cyanobacterial (Anabaena variabilis), one bacterial type (E.coli), and more recently one apicomplexan enzyme (Plasmodium falciparum). In addition, a number of structures have been determined for site directed mutants of the FNRs from spinach, pea, and A. variabilis (Karplus and Faber 2004; Milani et al. 2007). Despite ample variations in amino acid sequences the chain topology of all plant type FNR proteins (plastids, bacterial and apicomplexan) are highly conserved with most difference occur at the loops between the invariant secondary structure elements (Karplus et al. 1991; Correll et al. 1993; Karplus and Bruns 1994; Aliverti et al. 1995; Bruns and Karplus 1995; Serre et al. 1996; Ingelman et al. 1997; Sridhar Prasad et al. 1998; Deng et al. 1999; Mayoral et al. 2000; Dorowski et al. 2001; Kurisu et al. 2001; Hermoso et al. 2002).

2.7 The overall structure

In spite of their different origin all the FNRs display similar structural topology including two structural domains (Figure 3). The N-terminal FAD- binding domain and C-terminal NADP" -binding domain. The FAD domain hosts a β-barrel built by two
perpendiculars three-stranded anti-parallel beta sheets (β1β2β3 and β3β4β6) and a single α-helix, nestled between β5 and β6. The C-terminal domain hosts a five stranded parallel β-sheet (β9β8β7β10β11), surrounded by seven α-helix (αB-αH). The first crystal structure of an apicomplexan FNR from *P. falciparum* has been recently been reported for *P. falciparum*. The overall topology of the enzyme is similar to that of plant type FNRs but PfFNR displays structural properties likely to be unique for plasmodial reductases. The PfFNR crystal accommodates four molecules in the asymmetric unit, the crystal lattice packing gives rise to an open tetramer. Within such tetramer, covalent dimer based on the oxidation of residue Cys99 in two opposite subunits. Studies in the solution show that NADP+ as well as 2'-phospho AMP, promotes the disulfide stabilizes dimer. The isolated dimer is essentially inactive but full activity is recovered up on disulfide reduction (Milani et al. 2007).

*Figure 3: Three-dimensional structure of* **Plasmodium falciparum** **Ferredoxin NADP**+ **reductase monomeric form.** (A) The diagram was produced using the program UCSF Chimera. The structure of the enzyme is color coded by N-terminal FAD binding domain (purple) and C-terminal NADP+ binding domain (olive green) and FAD molecule sandwiched between two domains (yellow). (B) Detailed view of the isoalloxazine ring system in PfFNR displaying relevant interactions with active site amino acid residues.

As most apicomplexan enzymes PfFNR contains two major insertion of 28 and 5 amino acid residues respectively. The longer insertion falls just before FAD binding motif and shorter is few amino acids after NADP+ binding GTG sequence motif. The first insertion sequence is reflected to disordered loop and second insertion occurs in a wide surface loop
also displaying conformational flexibility. The structural data results are in full agreement with the data obtained by limited proteolysis of TgFNR as most of the protease cleavage site identified in TgFNR fall in these sequence insertion elements (Pandini et al. 2006).

2.8 FAD binding

The FAD molecule is present in all crystal structures of FNR as it is present as a very tight binding prosthetic group (kd~ 3nM; (Zanetti et al. 1982). The FAD group is extended along the large cleft between the two domains (Karplus et al. 1991; Correll et al. 1993; Karplus and Bruns 1994; Bruns and Karplus 1995; Deng et al. 1999; Aliverti et al. 2001; Dorowski et al. 2001; Kurisu et al. 2001) with the isoalloxazine ring system stacked between the aromatic side chains of two tyrosine residues (On the Si-face, which rests against the protein backbone) of the C-terminal Tyr 314 (on the Re-face which is outward and readily available for interaction with NADP(H) in spinach FNR). The FAD binding site in plastids FNRs is quite highly conserved for the FMN half of the prosthetic group. In contrast, the adenosine portion of FAD shows significant variations in its position of binding. There is also a mobility gradient along the FAD molecule itself, with the isoalloxazine moiety being the best ordered portion (at the centre of the active site), the pyrophosphate being somewhat more mobile, and the adenosine portion being highly mobile. This gradient is seen in all of the crystal structures FNRs (Ceccarelli et al. 2004). Even though there is a high degree of structural conservation in the plant type FNR family, FAD does not adopt a unique conformation in all members.

The overall FAD binding mode described for PfFNR is essentially conserved in Zea mays FNR (ZmFNR) and Spinach oleracea FNR (SoFNR). Presence of modified flavin, as reported for the recombinant form of TgFNR that contains variable amount of 6-OH-FAD could thus be excluded in PfFNR (Pandini et al. 2002a). The main conformational differences affecting only the protein surface location of the adenosine moiety (displaced by 1.7Å in ZmFNR and by 5Å in SoFNR). Such surface location accounts for the mobility of the FAD adenosine end observed in crystal structure of PfFNR and in Zm FNR. Given the location of the FAD adenine ring far from the active site region, such structural variations should bear little relevance for PfFNR catalysis. Overall the interaction of FAD cofactor and FNRs at active site is predominantly occurs through hydrogen bonding, van der Waal contacts and pi-pi interactions which are essential for proper orientation of FAD cofactor and intern necessary for optimum functioning of the enzyme (Milani et al. 2007).
2.9 Reaction rates and catalytic mechanism

Batie and Kamin (Batie and Kamin 1984a; b) formulated the first detailed pathway for the FNR-mediated electron transfer between NADP (H) and ferredoxin, using data from binding equilibria, steady-state kinetics and rapid mixing experiments. Go’mez-Moreno and coworkers have also studied the electron transfer to and from flavodoxin (Hurley et al. 1999; Martínez-Julve et al. 1999). The reverse reaction, that is the electron transport from NADPH to Fd (or Fld), is routinely measured in vitro through a coupled assay, using cytochrome c (cyt c) as final electron acceptor-

\[
\text{NADPH} + 2\text{Fd (Fe}^{3+}\text{)} \rightleftharpoons \text{NADP}^+ + \text{H}^+ + 2\text{Fd (Fe}^{3+}\text{)}
\]

\[
\text{Fd (Fe}^{3+}\text{)} + \text{cyt c (Fe}^{3+}\text{)} \rightleftharpoons \text{Fd (Fe}^{2+}\text{)} + \text{cyt c (Fe}^{2+}\text{)}
\]

Although the reversibility of reaction is well documented (Batie and Kamin 1984a; b; Hurley et al. 2002; Carrillo and Ceccarelli 2003) the forward and backward components of NADPH synthesis and Fd reduction follow different kinetic mechanisms when measured in vitro. Electron transfer from reduced Fd to NADP\(^+\) fits a compulsory ordered pathway involving formation of a ternary complex (with the pyridine nucleotide acting as leading substrate), where as the reverse reaction conforms a two step transfer “ping-pong” mechanism (Forti and Sturani 1968; Batie and Kamin 1984a; b). Fd and NADP(H) display reciprocal positive cooperativity during FNR catalysis, resulting from a decrease in the binding affinities to corresponding binding complexes (Batie and Kamin 1984a; b). Apparently, interactions of the two substrates with FNR are too strong to allow rapid turnover and demand to the contribution of an induced fit step to accomplish the rates of the steady-state catalysis. In particular, The NADP\(^+\) binding facilitates the dissociation of Fdox from its complex with FNR\(_{\text{ox}}\) and FNR\(_{\text{red}}\). These are the rate limiting steps of the overall reactions.

A schematic representation of in vivo catalytic cycle Chloroplast Fd-FNR system is shown IN figure 4.

2.10 Ferredoxin binding

Association of FNR and Fd is transient and steered by electrostatic interactions between complementary patches of basic and acidic residues that decorate the active site regions in the flavoenzyme and the Fe-S protein, respectively (De Pascalis et al. 1993; Jelesarov et al. 1993). The binary complexes of the oxidized FNR and Fd could be resolved by X-Ray crystallography for both the Anabaena and maize couple (Morales et al. 2000;
Kurisu et al. 2001). The resulting structure revealed that Fd binds to the concave region of the FAD domain of maize FNR, burying an accessible area of \( \sim 800\text{Å}^2 \) in each partner, which represents about 5% and 15% of the total surface areas of the FNR and Fd respectively (Kurisu et al. 2001). These polar groups play a major role in determining the relative orientation of the two electron carriers in the initial non productive complex (De Pascalis et al. 1993; Hurley et al. 1996). Attainment of the functional conformations competent for electron exchange requires further fine adjustment, stabilized by combination of well-defined hydrogen bonds, salt bridges, van der Waals interactions and hydrophobic packing forces originating from the dehydration of the protein-protein interface (De Pascalis et al. 1993; Hurley et al. 1999; Ullmann et al. 2000; Martinez-Julvez et al. 2001; Hurley et al. 2002).

Recently, the molecular interactions between *Plasmodium falciparum* Fd/FNR system has been studied by NMR chemical shift perturbation analysis and site directed mutagenesis. Five ionic bridges stabilized the leaf complex and among them two salt bridge Fd-
Glu65/FNR-Lys91 and Fd-Glu66/FNR-Lys88 were shown to dominantly contribute to the electrostatic interaction of Fd and FNR (Akashi et al. 1999; Kurisu et al. 2001)(Kurisu et al., 2001; Akashi et al., 1999). In root complex, on the other hand three ionic bridge of Fd-Glu30/FNR 156, Fd-Glu56/FNR310 and Fd-Asp57/FNR Lys84 together with several hydrogen bonding pairs stabilizes the relative orientation of proteins (Hanke et al. 2004b). With regards to Plasmodium falciparum proteins, the studies show that the combination of acidic residues of Asp26/Glu29/Glu34 conferred the large contribution to the interaction with PfFNR in both kinetic and physical binding analyses, in decreasing order. The acidic residues Asp65/Glu66 are also important but to the lesser extent than Asp26/Glu29/Glu34. On the other hand the contribution of Glu92/Asp93/Asp97 appears to be small. In this context, the interaction mode of PfFd/FNR may be more similar to root complex than leaf complex, which appears to be reasonable if the interaction mode is related to the direction of physiological electron transfer (Kimata-Ariga et al. 2007b).

2.11 NADP (H) binding

Charge interactions between FNR residues and the 2'-phosphate group specific of NADP(H) have been usually regarded as responsible for preference of this nucleotide over NAD(H) (Bruns and Karplus 1995; Serre et al. 1996; Medina et al. 2001; Tejero et al. 2003) but unexpected contributions to coenzyme recognition were found to be also provided by residues that interact with the pyrophosphate bridge (Hermoso et al. 2002; Tejero et al. 2003) and by the carboxyl terminal phenolic side chain (Piubelli et al. 2000). The increase in affinity caused by replacements of the corresponding tyrosine by nonaromatic residues was so dramatic that the resulting FNR mutants were able to avidly incorporate NADP⁺ during biosynthesis in E. coli and kept it bound through the purification and crystallization steps (Deng et al. 1999). Once the carboxyl terminal residue is replaced, the electrostatic interactions at the 2'-phosphate group of NADP⁺ are no longer sufficient to discriminate between the coenzymes, and FNR becomes an efficient NAD(H) oxidoreductase (Piubelli et al. 2000).

Based on these data, Hermoso et al. have proposed a sequential recognition pathway in which the dinucleotide binds first in a nonproductive manner through its 2'-P-AMP portion to a large cavity on the FNR surface (Hermoso et al. 2002). Then the bound coenzyme and the adjacent regions of the protein undergo structural rearrangements leading to a more tight NADP⁺ conformation and reorganizing the FNR cavity to match the charge and shape of the
AMP portion of the substrate. Finally, tyrosine displacement would allow the nicotinamid to approach the flavin for hydride transfer. The movement of this carboxyl terminal residue appears to be essential for catalysis, but the driving force for such displacement remains unknown. Dorowski et al. have proposed that Fd binding might contribute to this process by nestling the tyrosyl phenol group into a hydrophobic pocket of the iron-sulfur protein, facilitating nicotinamide docking and establishing a loosely bound complex compatible with turnover. Interactions with both Fd and NADP(H) are indeed accompanied by small structural changes in the complexed FNR relative to the free enzyme (Bruns and Karplus 1995; Deng et al. 1999; Morales et al. 2000; Dorowski et al. 2001; Kurisu et al. 2001; Hermoso et al. 2002). The correlation between these conformational movements and the induced-fit component of the kinetics remains yet to be elucidated.

Figure 5: Schematic representation of the bipartite NADP(H) binding mode to ferredoxin NADP⁺ reductase. The model is based on the properties of a tyrosine to serine site directed mutant of pea FNR bound to NADP (H) (Deng et al. 1999; Mayoral et al. 2000). Site A and N represent the adenosine and the nicotinamide binding regions respectively, in the active site of FNR. This figure is adopted from review review article published by Carrillo et al (2003).

Turnover numbers in the range of 200-600 s⁻¹ have been reported for Anabaena and chloroplast FNR (Medina et al. 1998; Carrillo and Ceccarelli 2003). Recently the catalytic properties of Chlamydomonas reinhardtii flavoenzyme have been determined, and shown to be in same range (kcat= 90-174s⁻¹) (Decottignies et al. 2003). Bacterial reductases on the other hand, appear to be much less active (Table 2). The decline in catalytic competence, relative to its plastidic and cyanobacterial homologues, is entirely accounted for by a drastic decrease in the kcat value, while the Km for NADP(H), Fd and Fld remain in the low micromolar ranges for all reductases (Carrillo and Ceccarelli 2003). Wan and Jarrett have measured the anaerobic oxidation of NADPH by E.coli system made up of FNR and either
ferredoxin or any of the two bacterial flavodoxins (Wan and Jarrett 2002). The obtained absolute rates were 0.15 s⁻¹ and about 0.004 s⁻¹, respectively. The low efficiency is therefore intrinsic to the reductases itself and not caused by faulty reduction of cyt c acceptor. Similar low activities have been obtained with *A. vinelandii* (Isas and Burgess 1994) and *Rhodobacter capsulatus* reductases (Bittel et al. 2003).

### Table-2

<table>
<thead>
<tr>
<th>Property</th>
<th>Plastidic FNR class</th>
<th>Bacterial FNR class</th>
<th>Apicomplexan FNR class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototype</td>
<td><em>S. oleracea</em></td>
<td><em>A. vinelandii</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>3-D structure</td>
<td>Two domain</td>
<td>Two domain</td>
<td>Two domain</td>
</tr>
<tr>
<td></td>
<td>FNR</td>
<td>FNR</td>
<td>FNR</td>
</tr>
<tr>
<td>Sheet-loop-sheet</td>
<td>present</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>FAD conformation</td>
<td>extended</td>
<td>folded</td>
<td>extended</td>
</tr>
<tr>
<td>C-terminal residue (interacting with flavin)</td>
<td>aromatic</td>
<td>aliphatic</td>
<td>aromatic</td>
</tr>
<tr>
<td></td>
<td>200-600</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (s⁻¹)</td>
<td>photosynthesis</td>
<td>nitrogenase</td>
<td>anaerobic metabolism</td>
</tr>
<tr>
<td>Metabolic commitment (typical)</td>
<td>nitrite</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Metabolic rates in s⁻¹ (typical)</td>
<td>200-500</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

In the long term of evolution from their common origin, plant type FNR proteins have undergone multiple modifications to face the metabolic challenges posed by host organisms displaying very different lifestyle. The selective pressure optimized catalytic efficiency only to those enzymes that had operates with in very rapid pathways such as oxygenic photosynthesis (k<sub>cat</sub> = 200-600 sec⁻¹). Catalytic improvement was accomplished by retaining subtle changes in secondary structure and FAD conformation that led to progressive increase in turn over numbers (extended FAD conformation, aromatic stacking of the isoalloxazine ring system and high specific activity). Other FNR forms involved in less demanding pathways (nitrogen fixation, nucleotide reduction and amino acid synthesis etc.) display only fraction of these rates. It is possible that structural constraints (folded FAD structure and low catalytic competence) that prevented the development of highly active bacterial reductase
could have some type of compensatory effect on these enzymes (i.e. enhance stability, facilitated folding and assembly). Apicomplexan FNRs having catalytic efficiency is intermediate between plant type and bacterial type of FNRs and very much similar to that of mitochondrial type of FNRs although the fate of reducing equivalents delivered by apicomplexan FNRs is still unclear (Ceccarelli et al. 2004).

2.12 Perspectives of the present study

One of the classic important issues in protein folding and stability is the relative role of non-covalent interactions whose cumulative effect not only responsible for determination of specific three dimensional structure of proteins but also their mutual shuffling regulate the enzyme function and interaction with other protein molecules. Understanding these interactions in the proteins is not only an academic challenge but also has enormous applications for the pharmaceutical and biotechnological industries. Ionizable groups play an important role in determining the protein structure (Perutz 1978) and their functional activity (Sharp and Honig 1990). These amino acid residues are also source of the pH dependence of protein stability (Matthew and Gurd 1986; Allewell and Oberoi 1991; Yang and Honig 1993). It is therefore essential that protein maintain the appropriate side-chain ionization. What are the forces that govern the protein stability of the proteins and how they disrupted or strengthened by solvent conditions is a problem that is under intense scrutiny (Dill 1990; Makhatadze and Privalov 1995). In present investigation, we have made an attempt to evaluate the relative contributions of various types of non covalent interactions in stabilization of native conformation and conformational stability of TgFNR by modulation of environmental pH, salts and chaotropic agents like GdnHCl or urea. Furthermore, significant development in this area of research is only restricted to small proteins or domains of larger proteins but many more questions are still unanswered for multidomain or multimeric proteins. Folding/ unfolding studies on TgFNR not only extend our studies on multidomain proteins but also provide valuable information and direct characterization of folding/unfolding behavior of entire molecule which is necessary for complete understanding of the folding mechanism of larger proteins.