Chapter- 7

Discussion and Conclusion

7. DISCUSSION AND CONCLUSION

ALA, NQSA and $K_3Fe(CN)_6$ being impermeable to cell, were assumed to act at the level of the plasma membrane. These compounds act as natural electron acceptor of transplasma membrane electron transport (transPMET). In transPMET the redox enzymes can transfer electrons from cytosolic electron donors to artificial non-permeable electrons acceptors. The transmembrane enzyme(s) can reduce compounds with a negative redox potential down to -290 mV. The release reduced compounds don't affect the transPMET ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction. The high capacity of LDC to transfer electrons from intracellular donors to extracellular electron acceptors like ALA, NQSA, $K_3Fe(CN)_6$ and oxygen is poorly understood [426, 427]. We have synthesized DTNS as impermeable electron acceptor of transPMET system. In this report, we have presented evidence for the presence of a numerous ectoredox enzymes to donate electron to impermeable electron acceptors with different redox potential. It is apparent that impermeable electron acceptor DTNS could be as useful and as specific as ALA, NQSA and $K_3Fe(CN)_6$. We feel that reagents of this type will be important in determining the position and orientation of redox carriers in transPMET system. In both ALA and DTNS, disulfide bridge is reduced after accepting electrons from transPMET system. They differ from each other in respect to the nature of disulfide bridge. In ALA, disulfide bridge is intramolecularly linked with an aliphatic chain whereas in DTNS disulfide bridge is intermolecularly linked with the aromatic ring. Due to these differences DTNS showed higher affinity and reaction rate compared to ALA (Tables 6.1 and 6.2). It is noteworthy that ALA and DTNS showed same pH optimum, which differ little from the pH optimum of NQSA and $K_3Fe(CN)_6$ (Table 6.1).

It is evident from the observation of Table 6.3 that no significant effect on NQSA reduction was found with cells exposed to Na^+, to K^+ or to other cations substituting for Na^+ and K^+. Our data provide evidence that the reduction of ALA and DTNS requires the presence of K^+ for maximal activity, whereas for the reduction of ferricyanide, presence of Na^+ is essential. Stiles et al. [428] have shown that in *Leishmania donovani* surface membrane, P-type ATPases resembling fungal K^+ and Na^+-ATPases are present. The possibility that these K^+ and Na^+-ATPases are coupled with the transPMET system cannot be ruled out, however. Absence of Cl^- anion has stimulating effect for the

reduction of four electron acceptors. It has already been observed that Cl^- transport plays an important role in LDC in putative pH regulatory mechanism [429]. It appears from these observations and also from the tables 6.5- 6.7 that though ALA and DTNS are both disulfide redox reagents, they are not receiving electrons from the same redox site. Similarly, NQSA and $K_3Fe(CN)_6$ differ from each other with respect to the site of redox interaction. Hence, ALA, DTNS, NQSA and $K_3Fe(CN)_6$ may have different sites of redox interaction.

Table 6.4 shows that the four electron acceptors of transPMET system are considerably stimulated by anaerobiosis. Anaerobic stimulation have placed ALA, DTNS in one group and NQSA, $K_3Fe(CN)_6$ to another group. This makes the main transPMET chain into two divisions and signifies the relative importance of the sites of reduction. The major effect under normal conditions appears to be due to oxygen inhibition of the redox sites leading to ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction (Tables 6.4, 6.8). Anaerobic conditions increased ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction so that dihydrolipoic acid, 5-mercapto- 2-nitroaniline-N-sulphonic acid, hydroquinone of NQSA and $K_3Fe(CN)_6$ production increased sharply, respectively, in the incubation mixtures where the O_2 level is being depleted. The stimulation produced by anaerobiosis is probably due to inability in functioning of oxygen redox site which resulted in an increased channeling of electrons into the ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction pathway; however, it is surprising that $K_3Fe(CN)_6$ did not produce O_2 uptake inhibition in LDC whereas ALA, DTNS and NQSA inhibited the O_2 uptake (Table 6.8), an observation concordant with the results of table 6.4. We have observed that incubation of LDC in presence of $K_3Fe(CN)_6$ resulted in increased uptake of 14C-deoxy-d-glucose (data not presented). It is tempting to speculate that increased influx of d-glucose has generated higher level of NADH/NADPH and consequently higher rate of O_2 uptake, which compensated O_2 uptake inhibition by $K_3Fe(CN)_6$.

Antimycin A has been shown to be a potent inhibitor of respiratory chain; the site of action has been placed at the level of Qi site binding and blocking electron transfer from the heme center to ubiquinone [430]. Table 6.5 shows that the ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase system is inhibited significantly in different order by antimycin A. Antimycin A showed complete inhibition of O_2 uptake in LDC (Table 6.8). Thus, on the basis of such observations, it would appear that ALA, DTNS, NQSA, $K_3Fe(CN)_6$ and

oxygen are reduced by the involvement of a b-cytochrome in unrecognized component of the transPMET system. It is obvious from these results that the site of antimycin An inhibition will be before the ALA, DTNS, NQSA, $K_3Fe(CN)_6$ and O_2 redox sites. The effects of HQNO, rotenone and capsaicin reported in tables 6.5 and 6.8 are of interest in view of the above conclusion. HQNO was found to be a potent inhibitor of electron transport. The locus of the action appears to be the same as, or proximate to, that of antimycin A [430]. The inhibition by HQNO of ALA reduction was much less potent than by antimycin A. It is not likely that b-cytochrome involved in ALA reduction is identical with the b-cytochrome of DTNS, NQSA and $K_3Fe(CN)_6$ reductase complex. HQNO inhibition of trans-PMET system has placed ALA and DTNS in one group and NQSA, $K_3Fe(CN)_6$ to another group. Rotenone and capsaicin are among the most widely used inhibitors of mitochondrial and bacterial electron transport because they selectively inhibit the oxidation of NADH-linked substrates [431, 432]. The binding site of rotenone responsible for inhibition of NADH oxidation in complex I of mitochondrial electron transport chain is localized in the O_2 site of the dehydrogenase and not between NADH and flavoprotein [433]. The effect of these inhibitors on the reductase activity is summarized in Tables 6.5 and 6.8. Rotenone was found to be a potent inhibitor of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase activity, but O_2 uptake was only partially inhibited. This observation supports the idea that transmembrane electron transport depends on the production of a cytosolic reducing agent, such as NADH, the oxidation of which liberates electrons which travel to the outside of the cell via a transPMET chain. Partial inhibition of O_2 uptake by rotenone is suggestive to the presence of an O_2 redox site not sensitive to rotenone. Capsaicin, a new naturally occurring inhibitor of proton-pumping NADH quinone oxidoreductases (NDH-1) present in the respiratory chain of various species of bacteria and mitochondria, can be divided into two groups [434, 435]. One group of enzymes that bear the energy-coupling site is designated as NDH-1 and the other group that does not as NDH-2. In response of capsaicin to four electron acceptors they can be divided into highly sensitive NQSA, $K_3Fe(CN)_6$ reductase group and less sensitive ALA, DTNS group (Fig. 6.1.A, Table 6.5). Effect of capsaicin on O_2 uptake inhibition was similar to rotenone (Table 6.8). Here, it is noteworthy that capsaicin do not interrupt rotenone insensitive electron transfer from NADH [436]. The sensitivity to ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction inhibition in LDC by capsaicin supports

the notion of the presence of an energy-coupling site (NDH-1) in transPMET system. This finding correlates the inhibition of plasma membrane NADH oxidase activity Capsaicin caused a dose-dependent decline in LDC growth that closely paralleled its inhibition of reductase activities. These results suggest that transPMET helps to maintain a stable redox environment required for LDC viability.

TTFA is a well-known metal coordinate compound of lipophilic nature [437] and has been shown to be a potent inhibitor for succinate-coenzyme Q reductase of complex II in aerobic tissues such as bovine heart [438, 439]. It is of interest to ascertain the inhibition of transPMET reductase by this compound. As shown in Tables 6.5 and 6.8, about 50% to 85% of reductase activities were inhibited by 1.16 mM to 2 mM TTFA. In contrast, mammalian succinate-coenzyme Q reductase was inhibited about 90% by less than 0.35 mM TTFA [440]. Since TTFA inhibits the reoxidation of the iron–sulphur center (S-3) of bovine complex II [441, 442], the sensitivity of LDC transPMET system towards TTFA may indicate a pathway for electron transfer involving iron– sulphur center. This finding correlates the inhibition of cell surface iron-sulphur center with inhibition of growth by TTFA (Fig. 6.2.B). Effects of mitochondrial complex IV inhibitor cyanide on transPMET reductase system are also included in Tables 6.5 and 6.8. Cyanide showed very strong inhibitory effect of NQSA and $K_3Fe(CN)_6$ reductase system, whereas it showed moderate inhibitory effect on ALA, DTNS and O_2 uptake. The inhibition produced by cyanide is probably due to the inhibition of a heme containing cytochrome. Strikingly, however, cyanide failed to inhibit O_2 uptake completely (Table 6.8) and the extent of inhibition is correlated with the ALA and DTNS reductase inhibition (Table 6.5). Thus, on the basis of such observations, it would appear that NQSA and $K_3Fe(CN)_6$ are reduced by an interaction with cyanide-sensitive cytochrome system and ALA, DTNS reduction inhibition by a cyanide-sensitive factor which forms a separate branch pathway. Cyanide insensitive respiration occurs in various plants and protozoa [443]. An alternative terminal oxidase, cytochrome O, has been found in several kinetoplastid flagellates [444]. The possible explanation of the incomplete inhibition of whole cell respiration is the relatively low sensitivities of the leishmanial enzymes and participation of oxygen in other cellular reactions.

The magnitude of O_2 uptake inhibition by ALA, DTNS and NQSA can be correlated with the anaerobic stimulation of ALA, DTNS and NQSA (Tables 6.4 and 6.8). Anaerobiosis

stimulated ALA, DTNS and NQSA reduction 48%, 44% and 97%, respectively. At the same concentration of ALA, DTNS and NQSA oxygen uptake was inhibited 54%, 52% and 71% respectively. Lack of O_2 uptake inhibition by ferricyanide may be explained by the enhanced rate of glucose uptake in presence of ferricyanide. We, therefore, conclude that transPMET system is intimately linked with the O_2 reduction site as terminal electron acceptor. Tables 6.6 and 6.8 gives the results obtained by adding p-chloromercuribenzene sulfonic acid on ALA, DTNS, NQSA, $K_3Fe(CN)_6$ reductase system and O_2 uptake. The importance of sulfhydryl groups for ALA, DTNS, $K_3Fe(CN)_6$ reductase system and O_2 uptake is shown by the almost complete inhibition produced by nonpermeable PCMBS. In contrast, NQSA reductase system was only partially inhibited by PCMBS. Other sulfhydryl group(s) inhibitors, which can be conveniently discussed in this section, are cadmium chloride and sodium arsenite that blocks the vicinal sulfhydryl groups. Tables 6.6 and 6.8 give the results of the effects of cadmium chloride and sodium arsenite on the transPMET reductase system. It can be seen that cadmium chloride and sodium arsenite can distinguish between the reductase systems and suggest the importance of vicinal sulfhydryl groups for different reductase systems (Table 6.6). These vicinal sulfhydryl groups appear to be involved in disulfide–dithiol interchange activity that intimately involve in redox signaling in biological processes [445, 446].

Another important finding is that valinomycin, which creates a specific K^+ permeability and monensin, which creates a specific Na^+ permeability had marked effects on redox enzymes of transPMET system (Table 6.7). It should also be emphasized that the O_2 uptake by LDC was more sensitive to valinomycin than monensin. Promastigotes in the insect gut and in the blood stream are exposed to an environment whose pH is neutral and K^+ is low. Promastigotes then invade the macrophages via the recruitment of lysosomes, whose environment is acidic (pH 4.5–5.5). The parasite then escapes from the parasitophorus vacuole to the cytosol to multiply as amastigote [85]. In the cytosol of the host cell, the pH is neutral (pH 7.0–7.2), the concentration of K^+ is high (135 mM) and that of Na^+ is low (15 mM). L. donovani likely developed effective mechanisms to efficiently cope with the extremes in environments it experiences. These mechanisms would likely involve ion pumps and carriers located on the parasites plasma membrane. Since both K^+ and Na^+ ion permeability affects the transPMET system, we may presume that transPMET system regulate K^+ and Na^+ ion permeability. Ponte-Sucre et al. have

reported that the K^+ channel blockers 4-aminopyridine and glibenclamide impaired growth and survival of *Leishmania* [447]. When we evaluated the effect of 4-aminopyridine on transPMET, it was observed that 4-aminopyridine strongly inhibited ALA and ferricyanide reduction, whereas DTNS and NQSA reduction was stimulated substantially. Valinomycin-induced respiration inhibition of LDC appears to be due to loss of cytoplasmic K^+. Obliteration of growth was also observed in the presence of K^+ sparing Na^+ channel blocker amiloride (Fig. 6.2.D). The protonophore and uncoupler FCCP inhibits ALA, DTNS and $K_3Fe(CN)_6$ reductase activity. In contrast, NQSA reductase activity was not affected at all (Table 6.7). Since FCCP prevents the formation of pH gradient it appears that ALA, DTNS and $K_3Fe(CN)_6$ reduction by transPMET system is linked with proton translocation.

Tricyclic antipsychotic drug trifluoperazine showed strong inhibition on ALA, DTNS reductase activity and O_2 uptake, whereas it showed moderate inhibition on NQSA reductase activity. In contrast, trifluoperazine showed strong stimulation on $K_3Fe(CN)_6$ reductase activity (Tables 6.5 and 6.8). In light of the current observations, trifluoperazine modulate multidrug resistance by inhibiting P-glycoprotein ATPase [448-450]. In plant mitochondria trifluoperazine, inhibition of electron transport and adenosine triphosphatase activity has been identified [451]. There are a number of reports in the literature of plant-like traits associated with *Leishmania* spp. [452]. Based on these observations, it is tempting to speculate that transPMET system in LDC may be linked with multidrug resistance conferred by glycoprotein. Evidence suggests the presence of a 105-kDa P-type ATPase on *Leishmania donovani* plasma membrane that is mechanistically similar to other P-type enzymes of higher eukaryotes.

Taken in total, these inhibitor/stimulator studies illustrate the presence of different redox sites for four electron acceptors. The possibility that there is more than one site of electron egress to the electron acceptor cannot be ruled out, however. The redox chain appears to be branched at several points, which incorporates b-cytochromes, cyanide sensitive hemoprotein, cyanide insensitive oxygen redox site, NADH linked energy coupling site as judged by capsaicin inhibition, iron–sulphur center and K^+/Na^+ channel. Electron transfer in the transPMET system requires an energized state of the membrane. Capsaicin of the vanilloid type showed its similarity in inhibition with transformed

mammalian cells [453]. Resistance to normal mammalian cell inhibition by capsaicin forms the basis for transPMET as an antileishmanial drug target.

Promastigote form of *L. donovani*, is agglutinated specifically with concanavalin A. Cumulative results demonstrate that ligands similar to α-D-mannosides are constituents of *L. donovani* pellicular membranes [423]. Based on that observation plasma membrane of *L. donovani* promastigote were isolated by concanavalin-A. Peroxidase and oxidase enzymes were selectively labeled with radio labeled iodine (^{125}I) at low temperature. This iodination resulted that more than 90% labeled were recovered in the membrane pellet (pellet I). To remove the bound concanavalin-A, the pellet I was homogenized with α-methyl mannoside. The plasma membrane retained in the supernant was centrifuged to produce the pure plasma membrane (pellet IV). Three enzymes (acid phosphatase, cytochromes c oxidase and NADPH-cytochrome c reductase) were used to assay the purity of plasma membrane fraction. It was concluded that among the three marker enzymes, the acid phosphatase activity was found mostly in plasma membrane fraction.

Hagerhall reported that menaquinone was an obligatory redox mediator of bacterial electron transport [49]. In mammalian system, the localization of ubiquinone-10 in the plasma membrane, the extraction and reactivation experiments of Sun *et. al.* are strong evidences for the implication of ubiquinone-10 as a functional member of the plasma membrane electron-transfer system [11]. Thus this study was undertaken to find out the quinone(s) function in the transplasma membrane electron transport (transPMET) system of *L. donovani* promastigote (Table 4). UV inactivation of napthoquinone co-enzyme resulted in either inhibition or stimulation of reduction rate. Previously oxygen was identified as physiological electron acceptor in the transPMET system of *L. donovani* promastigote [21]. UV exposed *L. donovani* promastigote cells also showed parallel stimulation (Fig. 6) but UV exposed cells inhibited ALA reduction. In conjugation with MOON and octadecylamine more reduction rate for ALA and ferricyanide was observed in UV exposed *L. donovani* cells. HQNO a quinone analog inhibitor showed potent inhibition to the above reduction rates. These observations support the role of redox potential of mobile electron carriers for redox couple in transPMET system. It therefore also suggested that at least some of the electron donor and/or acceptor enzyme complexes are present in the transPMET system.

Most primary ion pumps use the energy provided by the hydrolysis of ATP to energize ion-transport process across cell membranes. Accordingly, 'ATPase' has been found in all organisms studied so far. Several families of ATPase, which can be distinguished by their transport mechanism, their quaternary structure and their sensitivity towards specific inhibitors, have been found in various membranes and cell compartments. Pederson and Carafoli introduced the term P-type, F-type and V-type ATPases [248]. P-type ATPases (P-ATPase) have a phosphorylated transitional stage; F-type ATPases (F-ATPases) are primarily used in ATP synthesis and, therefore, are usually referred to as 'F-ATP synthesis'; V-type ATPase (V-ATPases) are generally and functionally related to F-ATPases but function only in ATP breakdown. To this classification scheme ATP-Binding Cassette (ABC) transporters (or traffic ATPases) have also been added. ABC transporters, which have been found in all living organisms, mediate uptake or efflux of a verity of solutes at the expense of ATP [454].

The use of specific inhibitors has made it possible not only to make quick assignments of unknown ATPases but also to study their functions in the corresponding membrane. Thus, ortho-vanadate is a well-known inhibitor for P-ATPases, ABC-transporters, whereas F-ATPases are inhibited by ventrericidin and DCCD. Bafilomycins are high affinity inhibitors of V-ATPases [455]. This class of antibiotics allows a clear distinction between the three types of ATPases. F-ATPases are not affected, whereas P-ATPases are inhibited in the micro molar and V-ATPases in the nano molar concentration range.

Membrane bound proton translocating inorganic pyrophosphatases (H^+-PPase; EC 3.6.1.1) belong to a category of proton pumps, distinct from F-, P-, and V-ATPases, which utilize pyrophosphate hydrolysis as the driving force for H^+ movement across biological membranes [456]. H^+-PPase have been characterized both at biochemical and genetic levels in various higher plants [457, 458], a few eubacteria [459-461], and in an archaea [462] and some human pathogenic protozoa [463].

Inorganic pyrophosphate was for a long time believed to be merely a byproduct of biosynthetic reactions (synthesis of nucleic acids, coenzymes, proteins, activation of fatty acids and isoprenoid synthesis) subject to immediate hydrolysis by inorganic pyrophosphatases. From the early 1960s, however, data have accumulated suggesting an important bioenergetics and regulatory rate for this compound [464]. In plants [465] and

in some parasitic protozoa, pyrophosphate is used in place of ATP as an energy donor in several reactions, such as the pyrophosphate- phosphofructokinase of *Trichomonas vaginalis* [466], *Entamoeba histolytica* [467], *Giardia lamblia* [468], *Naegleria fowleri* [469], *T. gondii* [470], *Eimeria tenella* [471]; the pyruvate phosphate dikinase of *Giardia lamblia*[472], *Entaamoeba histolytica* [473]; or the phosphoenol pyruvate carboxy-transphosphorylase [474] and the pyrophosphate-acetate kinase [475] of *Entaamoeba histolytica*. It has been postulated that pyrophosphate analogs could be selective inhibitors of some of these parasite enzymes [476].

In this thesis an ATPase and PPase activity has been characterized in permeabilized promastigotes of *L. donovani* .Our results on the general properties of the membrane ATPase with reference to cation requirements, pH optimum, and sensitivity to inhibitors are in agreement with those of Zilberstein and Dwyer [477]. The Mg^{2+}- dependent activities of ATPase and PPase differed little in optimal conditions. ATPase and PPase functioned best at pH 7 (Fig. 6.17). Optimal substrate concentration for ATPase and PPase was 0.5mM ATP and 1mM Mg^{2+}. Strikingly when ATP and Mg^{2+}concentration and PPi and Mg^{2+} concentration was increased at a ratio of 1:2, activities were decreased progressively. Lack of saturation kinetics appears to be due to increasing Mg^{2+} concentration. Increasing Mg^{2+} concentration inhibits ATPase and PPase activity. Stimulation of ATPase activity either by Na^+ or K^+ in comparison to ATPase activity in presence of both Na^+ and K^+ suggest the presence of a Na^+- stimulated and K^+-stimulated ATPase activity and the absence of Na^+, K^+-ATPase activity (Table 6.11).

Our observation was supported by the work of Stiles *et. al.*[428]. Similarly, PPase showed it's to highest activity in presence of K^+. Na^+ appears to be inhibitory for PPase activity (Table 6.11). ATPase and PPase activity was strongly inhibited by –SH group inhibitors Hg^{2+}. Similarly nonpermeable -SH group inhibitor PCMB and APAOS inhibited ATPase and PPase activity moderately (Table 6.13). From this observation it can be concluded that exoplasmic -SH groups of plasma membrane are involved in ATPase and PPase activity. PPase activity was strongly inhibited by fluoride ion, a property found in all types of PPase. Strikingly enough, ATPase activity was also partially inhibited in digitonin permeabilized LDC (Table 6.14). Fluoride is not an inhibitor of ATPase or ATP synthase [478]. Inhibition of ATPase activity by fluoride suggests the involvement of PPase activity in the rate of ATPase. This observation was

further strengthened when imidodiphosphate, a specific inhibitor of PPase, stimulated ATPase activity (Fig. 6.25). Similarly specific F-ATPase inhibitor sodium azide stimulated ATPase activity (Fig. 6.20). At 20μM concentration of DCCD, 80% inhibition of PPase and 40% inhibition of ATPase were achieved (Fig. 6.23), which again supports the involvement of ATPase in PPase activity. DCCD is known to inhibit plasma membrane H^+-PPase activity [479]. Triethyltin bromide an inhibitor of F-ATPase showed profound inhibition on ATPase activity, but little on PPase (Fig. 6.27). Quercetin, an inhibitor of F-ATPase, showed strong inhibition on ATPase activity, but little on PPase activity (Fig. 6.24). Sodium orthovanadate (Fig. 6.21) and verapamil (Fig. 6.26) are inhibitors of P-type ATPases and they showed strong inhibition on ATPase and PPase activity. Similarly trifluoroperazine, an inhibitor of P-type ATPase showed profound inhibition on PPase activity.

From these findings on transPMET system in *Leishmania donovani* promastigotes, it may be concluded that ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reductase activities are linked with either ATP hydrolysis or ATP synthesis along with either PPi hydrolysis or PPi synthesis. Here it is worth to note that transPMET system in mammalian cells failed to show any ALA, DTNS, and NQSA reductase activity and $K_3Fe(CN)_6$ reductase activity is not coupled with ATPase / ATP synthase activity. *Leishmania* cells differ from the mammalian cells on the above aspects. These differences can be exploited for rational chemotherapy of leishmaniasis.

In conclusion, analysis of their roles in parasite survival and pathogenesis is likely to lead to the discovery of novel targets for antileishmanial chemotherapy.