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Structure-function relationship in leishmanial globin coupled soluble adenylate cyclase

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Abstract
Leishmania promastigotes inhabit the midgut of sandfly, where they are densely packed together and the environment of these promastigotes is then likely to become hypoxic or even anoxic. Interestingly, *Leishmania* can survive in low oxygen concentrations, which is an exception from *Trypanosoma brucei*. Although some transcriptional regulators in mammals help to respond in adaptive responses during hypoxia, *Leishmania* lacks hypoxic inducible factor (HIF) as well as transcriptional regulation. Thus, the underlying mechanism still remains unclear. Recently, our laboratory has discovered a globin coupled heme containing adenylate cyclase from *L. major* called HemAC-Lm, which is likely to function in cellular adaptability under various O_2 limiting conditions. HemAC-Lm proteins usually consist of an N-terminal heme-containing O_2 sensor domain and a C-terminal adenylate cyclase domain. The N-terminal heme-containing O_2 sensor domain has two globin domains (globin-A and globin-B). Biochemical studies of wild type and mutant proteins further suggest that the oxygenated form of heme is only present in globin-A domain, leading to maximum cAMP production. Furthermore, the data from HemAC-Lm knock down, as well as overexpressed cells, suggest that cAMP generation shows stimulatory, as well as inhibitory role in cell proliferation during normoxia. In addition, O_2-dependent cAMP signaling via protein kinase A plays a fundamental role in cell survival through suppression of oxidative stress under hypoxia.

Keywords: *Leishmania*, Heme protein, Hypoxia, Adenylate cyclase, Oxygen sensor proteins

Introduction

Adaptation to a changing environment is the basic characteristic of all living organisms. Several sensor proteins thus have evolved with time to detect and respond to environmental changes. All organisms express heme sensor proteins to regulate small gaseous molecules like O_2, CO and NO that regulate numerous important cellular pathways (Girvan and Munro, 2013). In the past few years, the heme-based sensor field bloomed with the discovery of several new heme-binding sensory domains including heme nitric oxide/oxygen (H-NOX) binding domains, heme PAS domains, CooA proteins, heme-binding SCHIC domains, heme GAF domains, and sensor globin domains. Generally, heme based sensors consist of a sensor domains that link to the transmitter domains with variety of biological functions like histidine kinases, phosphodiesterases, DNA-binding domains, guanylate cyclases, diguanylate cyclase, and methyl accepting chemotaxis protein (Farhana *et al.*, 2012). Globin-coupled sensor (GCS) proteins were first discovered in bacterial aerotaxis before the discovery of globin-coupled heme containing adenylate cyclase (HemAC-Lm) from the unicellular eukaryotic organism *Leishmania*, GCSs are known to exist only in the prokaryotic world.

Adenosine 3′,5′-cyclic monophosphate (cAMP) is a vital signaling molecule that acts as a key second messenger in diverse biological functions, including proliferation, survival, differentiation, migration and programmed cell death. cAMP is universally generated by adenylate cyclase (AC), which catalyzes the cyclization of ATP to cAMP. AC is regulated by various molecules including bicarbonate, calcium, and hormones (Hanoune and Defer, 2001; Steegborn, 2014). Several heme based oxygen sensor proteins are known to be associated with cyclic nucleotide synthesis, including heme containing diguanylate cyclase (Hem-DGC), heme based YddV and BPeGreg (globin coupled diguanylate cyclase from *Bordetella pertusis*). HemAC-Lm is the only characterized heme containing oxygen regulated AC. However, the cAMP signaling pathways in protozoan parasites, belonging to the order Kinetoplastida differ a lot from their mammalian hosts. Genomic analyses suggest that these parasites lack heterotrimeric G protein, conventional transcription factors, as well as classical cAMP effectors, such as cAMP gated channels or cAMP secretory channels, although they do have the receptor-type ACs that are topologically similar to GC-coupled receptors of higher eukaryotes. Both ACs belong to a family of class III AC which are widely distributed from unicellular to multicellular organisms, forming the biggest family of ACs (Salmon *et al.*, 2012). Throughout this mini-review, the globin-coupled heme containing adenylate cyclase from *Leishmania major* will be presented from a structure-function point of view, emphasizing its contribution to the parasite survival under limited oxygen conditions.

Primary Structure of HemAC-Lm

HemAC-Lm is 616 amino acids long and is composed of N terminal globin-A (78-209 amino acid) domain, followed by globin-B (210-360 amino acid) domain which is linked with C terminal adenylate cyclase (361-616) domain (Figure 1A). Both globinA and globinB domains share sequence and structural homology with other globin domains, including mammalian myoglobin, cytoglobin and neuroglobin. However, when compared to hemoglobin and myoglobin they lack the D helix and have a shortened E helix (Sen Santara *et al.*, 2013). The absolutely conserved residues in all globins are the proximal His in the F helix, Pro in C helix, and Phe in the CD1 region (J Mol Biol 196(1):199–216). All three residues, Pro115, Phe121, and His161, are conserved in this enzyme. A key feature of Globin-A is its six-coordinate heme structure in the ferrous states, with the proximal histidine (His161) and an endogenous unknown distal residue (Roy *et al.*, 2014). Furthermore, SWISS-MODEL protein modeling also predicts His311 that acts as the proximal iron-coordinating ligand in globin B but lacks the distal His residue which is crucial for stabilizing ferrous-O_2 state. Globin B domain is linked to the C terminus AC domain, which contains characteristic lysine (Lys-427) and aspartic acid (Asp-508) as conserved residues for catalysis (Roy *et al.*, 2015).

Ligand Binding Characteristic and Mechanism of Catalysis

Biochemical and spectroscopic characterization of full length wild type, truncated and site-specific mutant proteins have provided the role of globin-A connecting globin-B domain in catalysis. This study also characterizes the specific amino acid(s) or region(s) of HemAC-lm responsible for heme binding, and the mechanism by which the heme and connecting globin-B domain regulates AC activity. Characteristic absorption spectra of full length HemAC-Lm is similar to oxygen-bound heme proteins with absorption maxima of 414 (Soret), 575 (α-band), and 538 nm (β-band). Native full length protein binds with CO, and new peaks appear at 419

(Soret), 573 (α-band), and 535nm (β-band), suggesting that the native form of HemAC-Lm is in the ferrous state. Upon addition of dithionite into the native protein, the oxygenated full length form of the HemAC-Lm protein shifted the Soret band from 414 to 423 nm, and simultaneously, the αβ-band appeared at 560 and 532 nm. This spectral characteristic is similar to deoxyneuroglobin/deoxycytoglobin, indicating the formation of ferrous six-coordinate low-spin heme state. Although the oxygen bound absorption spectra of both full length HemAC-Lm and globin-A domain deleted proteins (Δ209) showed similar low-spin heme state, but the deoxy Δ209 protein displayed the Soret band at 428 nm and the αβ-band at 556 nm (like deoxyhemoglobin/deoxymyoglobin), indicating that the globin-A domain deleted proteins show ferrous five-coordinate high-spin heme state. Δ209 proteins bind with CO, indicating that the native form of globin B is in the ferrous-O_2 state (Roy *et al.*, 2015; Sen Santara *et al.*, 2013). Heme content studies of the H161A (heme binding residue of GlobinA domain) and H311A (heme binding residue of GlobinB domain) mutants in full length protein showed that only His 161 residue is responsible for heme binding (Roy *et al.*, 2015).

The size exclusion chromatography of purified full-length protein and localization studies suggest that HemAC-Lm forms a dimer and localize in the cytosol of *Leishmania major* promastigotes (SenSantara *et al.*, 2013). HemAC-Lm catalyzed only the conversion of ATP to cAMP not GTP to cGMP, suggesting that the enzyme utilizes only ATP as substrate. The adenylate cyclase activity of HemAC-Lm is induced by O_2 or CO-binding indicating that activation is due to binding of the molecule with heme. O_2-dependent spectral shift of deoxy enzyme and AC activity by sequential addition of O_2 demonstrate that adenylate cyclase activity is directly proportional to the conversion of the oxy form of full length enzyme. ATP dependent initial velocity of reactions was fitted into the Michaelis–Menten equation with the k_{cat} value 27±1.1 mim^{-1} and K_m value ~1.9 mM (SenSantara *et al.*, 2013). The rate of cAMP synthesis for full length ferric, apo (heme free) forms and truncated catalytic domain (Δ360) are 10-fold lower than wild type enzyme, whereas the catalytic activities of Δ209 and H311A-Δ209 (apo protein) have no detectable activities (Roy *et al.*, 2015; Roy *et al.*, 2014). Comparative activity studies among full length wild type, Δ209 and Δ360 proteins suggest that the globin-B domain inhibits catalytic activity of the AC domain, whereas globin-A domain may change the protein conformation of the HemAC-Lm and relieve the globin-B domain induced suppression. Interestingly, these findings were validated by *in vivo* complementation experiments in *E. coli* TP610 (Roy *et al.*, 2015), which carries a defect in the adenylyl cyclase gene. As only complemented strain can produce active β-galactosidase enzymes, it forms blue colonies on X-gal plates (Taylor *et al.*, 1999).

How the suppressive interactions between globin B and adenylate cyclase domain are relieved in a single subunit can be best explained by the domain swapping model shown in Figure 1B. According to this model, O_2-dependent conformational changes in the globinA domain that sequentially stimulate catalytic activity of the AC domain located on adjacent subunits. This arrangement may circumvent a physical barrier for interaction between globin B domain and the AC domain located on same subunit. Domain swapping is a comparatively common characteristic among heme proteins, like cytochrome c, myoglobin(Lin *et al.*, 2015), NO synthase (Siddhanta *et al.*, 1998), nitrite reductase (Heiss *et al.*, 1995), hemophore HasA (Czjzek *et al.*, 2007) and methyl accepting chemotaxis protein (Silva *et al.*, 2012), which allows to adopt different functional state of the protein. In evolutionary context, the inter-subunit interactions are preferred over, interaction between domains (intrasubunit) of a multidomain protein, because the intra-subunit interaction does not create any selective pressure to be preserved after

oligomerization (Bennett *et al.*, 1995). Thus, the multi domain structure of HemAC-Lm probably evolved first, followed by its inter-subunit interactions between adjacent AC and globin-A domains for regulation.

HemAC-Lm mediated Signalling in *Leishmania*

cAMP signaling has been implicated as one of the major environmental sensing machineries in many unicellular eukaryotes like *Trypanosoma*, *Toxoplasma*, *Plasmodium* and others (Tagoe *et al.*, 2015). Overexpression and knock down of heme-containing AC (HemAC-Lm) in *Leishmania major* promastigotes under normoxic conditions, lead to alterations in cell shape and slower growth rate without compromising cell survival. These data strongly suggest that the typical level of cAMP plays a positive role, whereas an excess or shortage of cAMP plays a negative role in the initiation of cell proliferation under normoxia (Pawelek *et al.*, 1975). However, under hypoxic condition HemAC-Lm overexpression and knock down cells are less viable than control cells. These results suggest that HemAC-Lm is an essential gene but excessive cAMP is toxic to the cell (Bronstad *et al.*, 1983). For validation of the above results, we found similar percentage of cell death in the presence or absence of different concentrations of CPT-cAMP and PKA inhibitors (H89 and PKI). The results of PKA inhibitor suggest that cAMP signaling occurred through protein kinase A. It is also shown that O_2-dependent cAMP generation by HemAC-Lm is a key factor that maintains the optimum level of anti-oxidant enzymes, including glutathione peroxidase, peroxidoxin and superoxide dismutase through the activation of PKA (Sen Santara *et al.*, 2013). However, it is really unknown what triggers the expression of these antioxidant genes in *Leishmania* because the parasite is lacking transcriptional regulation. Studies from another group showed that ACs from kinetoplastida acts as an environmental sensors and controllers of host innate immune response (Saada *et al.*, 2014; Salmon *et al.*, 2012). Parasites that are pre-exposed to environmental stress (pH 5.5 and temperature 37◦C) have been shown to induce resistance against oxidative damage (Miller *et al.*, 2000; Zarley *et al.*, 1991). Earlier results also showed that disruption of anti-oxidant genes in parasites make them more susceptible to ROS inducing agents or intracellular killing in the macrophages (Barr and Gedamu, 2001; Ghosh *et al.*, 2003; Tovar *et al.*, 1998). Overexpressed and knock down cells show more pronounced oxidative stress compared to control parasite during hypoxia indicating that excess or shortage of cAMP produced excessive ROS, which is toxic to the parasite at limited oxygen levels. The attenuation of ROS by using N-acetyl-L-cysteine (antioxidant) abolished the acceleration of cell death seen during hypoxia, indicating that O_2-dependent cAMP regulation in control cells is required for the protection of cells against the accelerated rates of cell death. Altogether, the schematic diagram (Figure 2) shows a possible role of leishmanial HemAC in the adaptation of parasites at various concentrations of environmental oxygen.

Concluding Remarks and Perspectives

Like in other *Trypanosomes*, leishmanial receptor adenylatecyclases (LdRACs) are known for their differential expression, localization and diversity of function. HemAC-Lm which is the most recently discovered source of cAMP in *Leishmania* parasite, acts as a sensor for the external gaseous ligand like oxygen. HemAC-Lm is found diffusely distributed in the cytoplasm of promastigotes, although it's localization in amastigotes or during hypoxia is not yet known. The mammalian cytoplasmic sAC translocates to the mitochondria during acidosis/ischemia to promote the mitochondrial apoptotic pathway (Acin-Perez *et al.*, 2009). The unique structural

properties and biochemical characteristics of HemAC-Lm makes it an ideal target for therapeutic approaches. HemAC-Lm is distantly related to the mammalian sAC and it is also found to be insensitive to the mammalian sAC inhibitor KH7. Different component of the cAMP pathway (like phosphodiesterase) of *Leishmania* have already been tested for safe and effective anti-leishmanial drug target with little success. Therefore, HemAC-Lm provides an alternative drug target for effective anti-leishmanial therapy. Mammalian tmACs, as well as, sAC are thought to function through a signaling microdomain that comprises A-kinase anchoring proteins (AKAPs), which tether PKA and PDEs to regulate cAMP diffusion or cross-communication. Several AKAP-like proteins are reported in unicellular parasites like PfAKAL from *P. falciparum* merozoites and gametocytes (Bandje *et al.*, 2016). Although HemAC-Lm is known to activate PKA, involvement of any putative AKAP is not characterized yet. Several studies on different human cell lines showed direct involvement of mitochondrial oxidative phosphorylation (OXPHOS) complexes in hypoxia induced ROS production that lead to the activation of the hypoxia-inducible factor (HIF) pathway which ultimately modulates gene expression (Movafagh *et al.*, 2015). Very little is known about the role of cAMP in hypoxia as well as the exact function of neuroglobin and cytoglobin in oxygen homeostasis and hypoxia protection. HemAC-Lm might provide an important clue for the unknown function of neuroglobin and cytoglobin which could interact with different sAC or tmAC that lead to O_2 regulated cAMP production. Further structure function and cell biological studies are needed to validate HemAC-Lm's candidature as an anti leishmanial drug target as well as its implication in human biology.

References

1. Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin L R, *et al.* (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation *Cell Metab* **9** 265-276
2. Bandje K, Naissant B, Bigey P, Lohezic M, Vayssieres M, *et al.* (2016) Characterization of an A-kinase anchoring protein-like suggests an alternative way of PKA anchoring in Plasmodium falciparum *Malar J* **15** 248
3. Barr S D, Gedamu L (2001) Cloning and characterization of three differentially expressed peroxidoxin genes from Leishmania chagasi. Evidence for an enzymatic detoxification of hydroxyl radicals *J Biol Chem* **276** 34279-34287
4. Bennett M J, Schlunegger M P, Eisenberg D (1995) 3D domain swapping: a mechanism for oligomer assembly *Protein Sci* **4** 2455-2468
5. Bronstad G O, Sand T E, Christoffersen T (1983) Bidirectional concentration-dependent effects of glucagon and dibutyryl cyclic AMP on DNA synthesis in cultured adult rat hepatocytes *Biochim Biophys Acta* **763** 58-63
6. Czjzek M, Letoffe S, Wandersman C, Delepierre M, Lecroisey A, *et al.* (2007) The crystal structure of the secreted dimeric form of the hemophore HasA reveals a domain swapping with an exchanged heme ligand *J Mol Biol* **365** 1176-1186
7. Farhana A, Saini V, Kumar A, Lancaster J R, Jr., Steyn A J (2012) Environmental heme-based sensor proteins: implications for understanding bacterial pathogenesis *Antioxid Redox Signal* **17** 1232-1245
8. Ghosh S, Goswami S, Adhya S (2003) Role of superoxide dismutase in survival of Leishmania within the macrophage *Biochem J* **369** 447-452
9. Girvan H M, Munro A W (2013) Heme sensor proteins *J Biol Chem* **288** 13194-13203
10. Hanoune J, Defer N (2001) Regulation and role of adenylyl cyclase isoforms *Annu Rev Pharmacol Toxicol* **41** 145-174

11. Heiss K F, Clark R H, Cornish J D, Stovroff M, Ricketts R, *et al.* (1995) Preferential use of venovenous extracorporeal membrane oxygenation for congenital diaphragmatic hernia *J Pediatr Surg* **30** 416-419
12. Lin Y W, Nagao S, Zhang M, Shomura Y, Higuchi Y, *et al.* (2015) Rational design of heterodimeric protein using domain swapping for myoglobin *Angew Chem Int Ed Engl* **54** 511-515
13. Miller M A, McGowan S E, Gantt K R, Champion M, Novick S L, *et al.* (2000) Inducible resistance to oxidant stress in the protozoan Leishmania chagasi *J Biol Chem* **275** 33883-33889
14. Movafagh S, Crook S, Vo K (2015) Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate *J Cell Biochem* **116** 696-703
15. Pawelek J, Halaban R, Christie G (1975) Melanoma cells which require cyclic AMP for growth *Nature* **258** 539-540
16. Roy J, Sen Santara S, Adhikari A, Mukherjee A, Adak S (2015) Control of catalysis in globin coupled adenylate cyclase by a globin-B domain *Arch Biochem Biophys* **579** 85-90
17. Roy J, Sen Santara S, Bose M, Mukherjee S, Saha R, *et al.* (2014) The ferrous-dioxy complex of Leishmania major globin coupled heme containing adenylate cyclase: the role of proximal histidine on its stability *Biochim Biophys Acta* **1844** 615-622
18. Saada E A, Kabututu Z P, Lopez M, Shimogawa M M, Langousis G, *et al.* (2014) Insect stage-specific receptor adenylate cyclases are localized to distinct subdomains of the Trypanosoma brucei Flagellar membrane *Eukaryot Cell* **13** 1064-1076
19. Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, *et al.* (2012) Adenylate cyclases of Trypanosoma brucei inhibit the innate immune response of the host *Science* **337** 463-466
20. Sen Santara S, Roy J, Mukherjee S, Bose M, Saha R, *et al.* (2013) Globin-coupled heme containing oxygen sensor soluble adenylate cyclase in Leishmania prevents cell death during hypoxia *Proc Natl Acad Sci U S A* **110** 16790-16795
21. Siddhanta U, Presta A, Fan B, Wolan D, Rousseau D L, *et al.* (1998) Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer *J Biol Chem* **273** 18950-18958
22. Silva M A, Lucas T G, Salgueiro C A, Gomes C M (2012) Protein folding modulates the swapped dimerization mechanism of methyl-accepting chemotaxis heme sensors *PLoS One* **7** e46328
23. Steegborn C (2014) Structure, mechanism, and regulation of soluble adenylyl cyclases - similarities and differences to transmembrane adenylyl cyclases *Biochim Biophys Acta* **1842** 2535-2547
24. Tagoe D N, Kalejaiye T D, de Koning H P (2015) The ever unfolding story of cAMP signaling in trypanosomatids: vive la difference! *Front Pharmacol* **6** 185
25. Taylor M C, Muhia D K, Baker D A, Mondragon A, Schaap P B, *et al.* (1999) Trypanosoma cruzi adenylyl cyclase is encoded by a complex multigene family *Mol Biochem Parasitol* **104** 205-217
26. Tovar J, Cunningham M L, Smith A C, Croft S L, Fairlamb A H (1998) Down-regulation of Leishmania donovani trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival *Proc Natl Acad Sci U S A* **95** 5311-5316

27. Zarley J H, Britigan B E, Wilson M E (1991) Hydrogen peroxide-mediated toxicity for Leishmania donovani chagasi promastigotes. Role of hydroxyl radical and protection by heat shock *J Clin Invest* **88** 1511-1521

Figures

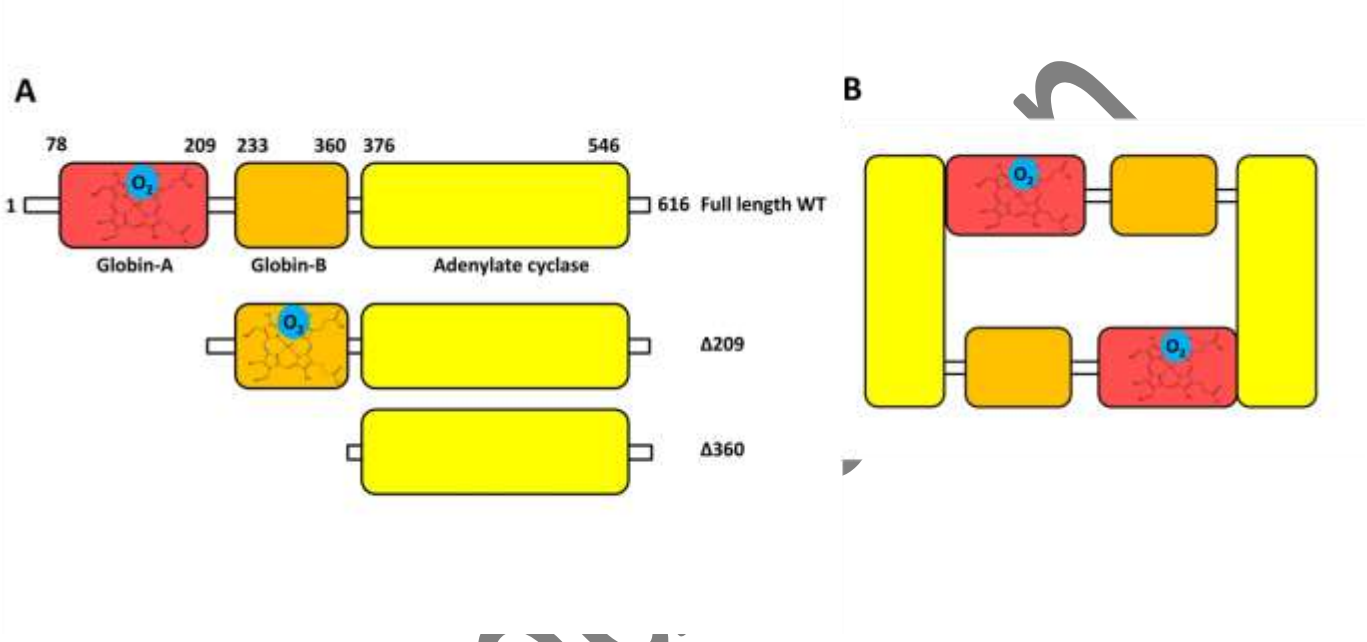

Figure 1: Schematic of domain architecture of HemAC-Lm (A) Proposed model for active dimeric form of HemAC-Lm (B)

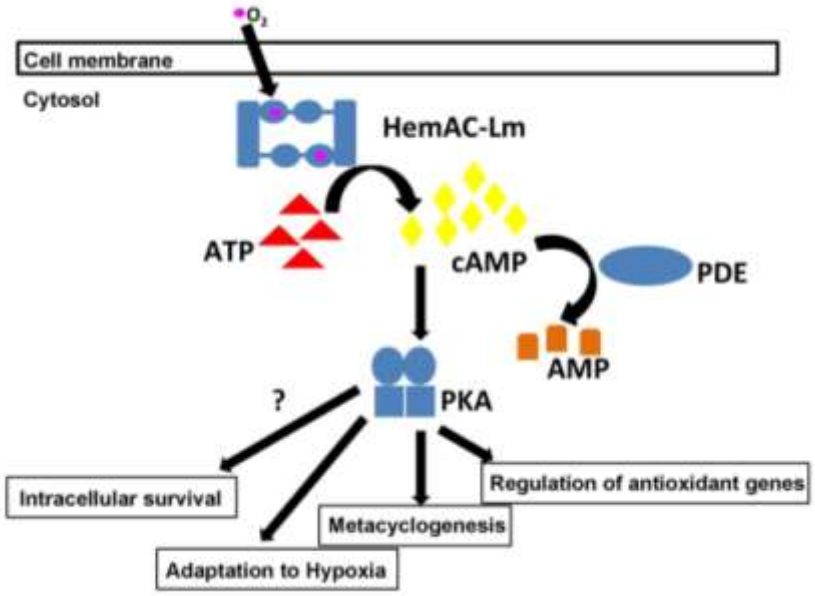

Figure 2: Schematic representation of cAMP signaling via HemAC-lm in *Leishmania* parasite. HemAC-Lm primarily localized in the cytosol of *Leishmania* and catalyses cAMP from ATP when it binds to oxygen. cAMP activates PKA enzyme which leads to the optimal expression of antioxidant genes like peroxidoxin, superoxide dismutase and tryparedoxin peroxidase, differentiation in infective metacyclic stage of the parasite, increased survival during hypoxia. HemAC-Lm generated cAMP may also have implication in intracellular survival in mammalian host. Intracellular cAMP pool is regulated by a stage specific phosphodiesterase enzymes that converts cAMP to AMP.