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Supervisor: Dr. P.K. Chakraborti Acc. No. : Th-230

SUMMARY OF THE THESIS

Intracellular pathogens invade, survive and replicate in mammalian cells by modulating the membrane trafficking and cytoskeleton dynamics of the host-cell. Macrophages, a frequent target of microbial infections, have been known to respond by producing agents as reactive nitrogen and oxygen intermediates with strong microbicidal activity. In order to survive, pathogens on the other hand, have devised different strategies to avoid the destruction by macrophages. *Mycobacterium tuberculosis*, the causative agent of devastating disease tuberculosis inhabits a compartment whose endocytic maturation is delayed. *Salmonella typhimurium* on the other hand lives in a special vacuole called *Salmonella* containing vacuole (SCV) and blocks lysosome-phagosome fusion. Unlike *M. tuberculosis*, the protozoan parasite *Leishmania amazonensis* thrives in a parasitophorous vacuole that has an acidic pH and high hydrolytic activity and *Trypanosoma cruzi* induces its uptake into lysosome like host cell vacuoles and then escapes into cytosol. The release of these intracellular pathogens into the host cell triggers macrophage death and induces local inflammation accompanied by the release of ATP. Therefore, extracellular ATP is considered to be a danger signal which can be used by neighbouring macrophages to inhibit infection. In order to survive, these pathogens have evolved enzyme system(s) which either degrades or consumes the extracellular ATP and help in their survival. For *M. tuberculosis*, *L. amazonensis* and *T. cruzi*, enzymes that degrade or consume or produce ATP have been considered as virulence factors.

Nucleoside diphosphate kinase (NdK), one of the key enzymes which is involved in the consumption of ATP and has also been reported to be secretory in nature in several pathogens. This secretory enzyme in certain bacteria has also been shown to prevent apoptosis of host cells. These intriguing findings emphasize the need to study NdK in intracellular pathogens (e.g. *M. tuberculosis* and *S. typhimurium*). The work presented here aims to understand the biochemical and functional aspects of NdK in intracellular pathogens with primary focus on *S. typhimurium*.

The study on *S. typhimurium* NdK (sNdK) embodied in this thesis is divided [illegible] introduces the topic of research and reviews various

aspects and functions of NdK protein. It depicts the role of NdK in different pathogens and also defines the main objectives of the study.

Chapter 2 describes the various techniques applied and the procedures/protocols followed during the course of this study. The study employed various recombinant DNA techniques and a series of protein purification and characterization procedures.

Chapter 3 deals with the detailed biochemical characterization of sNdK. With the use of [γ^{32}P]-ATP, the property of autophosphorylation and its dependence on divalent cation, pH and temperature has been described. Transfer of phosphate (phosphotransfer) from NTP to NDP including the kinetics was studied with the help of an enzyme coupled assay system. The mutational analysis pinpointed the active site His residue indispensible for the activity of the enzyme and also the residues important for the optimum activity of sNdK. The noticeable observation of this chapter is the ability of sNdK to autophosphorylate within broad pH range.

NdK, well known as a phosphoprotein leads to phosphotransfer reaction from a NTP/dNTP to a NDP through a ping-pong mechanism involving a high-energy phosphorylated intermediate. However, the detailed mechanism of this reaction is not known as yet. Chapter 4 deals with this aspect of sNdK. Taking a cue from earlier chapter, the acid/base sensitivity assay of sNdK demonstrated the presence of O- and N- linked phosphorylations. Mutational studies identified the residues accountable for acid and base sensitive phosphorylation of sNdK. Through both *in vitro* and *in vivo* experiments, the participation of serine in addition to histidine at the enzyme active site was demonstrated to be essential for the efficient catalytic activity of sNdK. This chapter therefore reveals the intermolecular phosphotransfer from His-117 to Ser-119 as the integral part of the enzyme for its functionality. Since sequence at the active site of different NdKs is conserved, such an observation seems to be not limited to sNdK only.

The nature of NdK in *S. typhimurium* has been addressed in chapter 5. The NTP synthesis assay and the generation of *ndk* deleted strain demonstrated that NdK is the major protein in *S. typhimurium*. Lactate dehydrogenase (LDH) assay [illegible] NdK of intracellular pathogens is involved in the modulation of

ATP induced cell death. This chapter also describes the ability of NdK from an extracellular pathogen to complement the function of NdK in intracellular pathogen.

Chapter 6 summarizes the findings presented in the thesis and also highlights the implications of this study. Since NdKs are highly conserved throughout the phylogeny, the outcome of the work presented here is not *Salmonella* specific and certainly opens up new vistas which may aid in explaining other properties of this multifunctional protein.

SUMMARY

NdK is well known as a phosphoprotein and autophosphorylaion of the protein occurs as a part of its catalytic mechanism. This leads to phosphotransfer reaction from a NTP/dNTP to a NDP through a ping-pong mechanism involving a high-energy phosphorylated intermediate. Thus it is involved in the maintenance of NTP pools in an organism, which is crucial for its survival. NdK is universally conserved across all domains of life; archaea, eubacteria and eukaryotes including plants. Availability of genome sequences indicates conservation of its amino acid sequences throughout the genra. NdKs from different sources have been characterized, even their crystal structures have been solved. However, detailed mechanism of autophosphorylation and phosphotransfer reactions has not yet been elucidated in greater detail. In this context, our lab is focused on two intracellular pathogens, *M. tuberculosis* and *S. typhimurium*. Previously, NdK from a gram positive intracellular pathogen *M. tuberculosis* has been characterized and it has been shown that the amino acids involved in the process of autophosphorylation and phosphotransfer are distinct. The work embodied in this thesis deals with *S. typhimurium,* which is a gram negative intracellular pathogenic bacterium, where NdK is reported to be the only enzyme that catalyzes the synthesis of triphosphates from diphosphates and ATP. However, no detailed study on the *Salmonella* NdK protein has been carried out as such.

The *ndk* gene from *Salmonella* was PCR amplified, cloned in pET-28c and after transformation in *E. coli* strain BL21(DE3), expression of the His-tagged protein (sNdK) was monitored following IPTG induction. Cell lysates resolved in SDS-PAGE yielded an expected induced band of 17.9 ± 0.24 kDa (Mean ± SD, n = 4) and the expressed protein purified through Ni-NTA column was recognized by anti-His antibody. This recombinant protein (sNdK) is used throughout the study.

In vitro autophosphorylation assays revealed that sNdK was capable of phosphorylating itself in a concentration dependent manner and the presence of divalent cations is essential for this activity. In fact, it has been reported that NTP/NDP-Mg^{2+} is the actual substrate of the enzyme. In addition to Mg^{2+}, other divalent cations like Ca^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} also supported the

autophosphorylation of sNdK. Among different known inhibitors, ADP and UDP, when analyzed on SDS-PAGE appeared to act as inhibitors of the autophosphorylation reaction catalyzed by sNdK. However, same samples when resolved in TLC clearly demonstrated that both ADP and UDP (NDPs) accept phosphate from [γ^{32}P]-ATP and are converted to cognate NTPs (ATP or UTP). Thus, in contrast to earlier reports, the results presented in this thesis establish that NDPs are not true inhibitors of autophosphorylation of NdKs. To evaluate the phosphotransfer activity of sNdK, an enzyme coupled assay was used, where dGTP and ADP are utilized as phosphate donor and acceptor molecules respectively. The assay clearly demonstrated that the recombinant sNdK exhibited the phosphotransfer activity and as reported in the literature, has the ability to utilize different substrates. Intrestingly, in contrast to autophosphorylation, the optimum transfer activity of sNdK was found in the presence of Mg^{2+} and Mn^{2+}.

NdK is a histidine kinase, known to be phosphorylated on a conserved histidine residue at the active site of the protein. Sequence analysis of sNdK revealed the presence of four histidines at positions 40, 53, 85 and 117. To identify the residue(s) involved in autophosphorylation and phosphotransfer activities, all four histidine residues of sNdK were mutated (one at a time) and analysed for autophosphorylation as well as phosphotransfer activities. His-117 of sNdK was found to be the active site residue involved in both autophosphorylation and phosphotransfer activities, since its substitution with either Ala or Gln completely eliminated both the activities (autophosphorylation and phosphotransfer) of sNdK. Mutation of His-40 and His-85, on the other hand, did not affect either autophosphorylation or phosphotransfer activity of the enzyme. The mutation of His-53 to Ala or Gln, although had no effect on the autophosphorylating ability of sNdK, its phosphotransfer ability was decreased by 50 % in comparision to that of the wild type. This finding was at par to the behaviour displayed by *M. tuberculosis* NdK.

Phosphoryl-protein linkage is a pH dependent phenomenon. To monitor the effect of pH on the autophosphorylating ability of sNdK, the reaction was carried out using buffers of different pH ranging from 1 to 14. Surprisingly, sNdK displayed

autophosphorylation activity within wide range (3-12), suggesting phosphorylating sNdK is resistant to both acidic and alkaline conditions. To have an insight on this aspect, when autophosphorylated sNdK was tested with either acid or alkali, it displayed sensitivity to both of them. Available literature suggested that distinct amino acid residues are responsible for acid stable (Ser/Thr) and alkaline stable (His) phosphorylation of a protein. Phospho-His residues, governed by P-N bonding, is susceptible to acid treatment but resistant to alkaline conditions. On the other hand, phospho-Ser/Thr bonding is a typical example of P-O linkage, which is impervious to acid but vulnerable to treatment with alkali. Therefore, this observation leads to the logical postulation that at least two different types of amino acids are phosphorylated in sNdK. Interestingly, NdK from *M. tuberculosis* yielded same result suggesting that such an event is not sNdK specific.

After demonstrating the presence of both acid as well as base sensitive phosphorylations in sNdK, it was tempting to identify the residues involved in the process. In identifying phosphorylated amino acids of sNdK, out of the four histidines present, only His-117 did not exhibit any autophosphorylation indicating the predominant involvement of this histidine in the base resistant phosphorylation. For the detection of O-linked phosphorylation, Ser/Thr residues which are either conserved in different NdKs or present in the active site of sNdK (S69, S119, S121, T05, T92, T102 and T116) were mutated to Ala, expressed as his-tagged proteins and used for autophosphorylation assay. Intriguingly, phosphorylating ability of S119A was significantly compromised compared to the wild-type or other mutants emphasizing that Ser-119 might be involved in exhibiting acid stable phosphorylation of sNdK. To substantiate that Ser-119 was responsible for the acid stable phosphorylation of sNdK, acid-base sensititvity assay of S119A mutant along protein was performed and the results were compared with that of wild type and S121A. γ^{32}P-labeled-S119A (where His-117 is intact) exhibited acid sensitive but alkali resistant behaviour giving further support to the notion that Ser-119, in addition to His-117, could be a phosphorylating residue in sNdK. Not only in autophosphorylation, the NTP synthesizing ability of S119A mutant also displayed

significant decrease in the enzyme turnover rates indicating that Ser-119 is essential for the efficient catalytic activity of sNdK. Further, the CD and gel filtration experiments indicated that there was no significant change in the structure of the S119A mutant which highlighted the fact that position of Ser-119 is important for the activity of sNdK.

To address how Ser-119 is phosphorylated in sNdK, a strategy was devised where a kinetically defective mutant (S119A) protein was mixed with an inactive mutant (H117A) protein in different molar ratios (1:0,1:1, 1:2 and 1:4) and this was followed by assessment of autophosphorylation as well as NTP synthesizing abilities. Interestingly, compared to the amount of active population/protein (S119A) present, the mixture of proteins (S119A + H117A) displayed enhanced activity in both autophosphorylation and phosphotransfer assays. This result was captivating and for the first time pointed towards the participation of the inactive mutant (H117A) in both the phosphorylation and phosphotransfer activities of any NdK reported so far. However, magnitude of the complementation was certainly lower than the wild-type. The participation of the inactive partner/mutant is only possible, if His-117 in S119A mutant is autophosphorylated and able to transfer the phosphate to Ser-119 of H117A protein, which is an intermolecular event. This was further confirmed by co-expressing both the mutants *in vivo* using two different tags (GST and His). After induction protein was purified through Ni-NTA column which exhibited co-purification of both the mutant proteins. Interestingly, incubation of the co-purified protein with [γ^{32}P]-ATP displayed autophosphorylation of both H117A and S119A proteins indicating the prevalence of intermolecular autophosphorylation of sNdK. To rule out the possibility that this is not an artifact, pre-incubation of the co-purified proteins with ADP exhibited a decrease in phosphorylation signals of both the mutant proteins, thereby suggesting their interdependence. Further, the γ^{32}P-labeled co-purified protein, when chased with different concentrations of ADP, displayed similar results. Thus these results, for the first time laid emphasis on the prevalence of intermolecular phosphotransfer in NdK for its proficient catalytic activity. Since the concept of intermolecular phosphotransfer was developed utilizing defective mutants,

this brought up the question of its occurrence in wild type.

To gain insight into the existence of inter-molecular phosphotransfer in the wild type for its functionality, the autophosphorylating ability of the mixture of wild-type and H117A proteins was further examined. Surprisingly, compared to the contribution of wild type protein alone, the mixture (wild type + H117A) demonstrated enhanced autophosphorylation activity, indicating that intermolecular transfer of phosphate between His-117 and Ser- 119 is critical for the functionality of sNdK.

To evaluate the feasibility of intermolecular phosphotransfer in sNdK, the structure of NdK from *E. coli* was used since amino acid sequence comparison revealed its ~96% identity with sNdK and both of them have been found to be active as tetramers (dimer of the dimer). Therefore, it seems that phosphotransfer from His-117 to Ser-119 in sNdK could occur either within a tetramer or between two tetramers. The likelihood of a phosphotransfer within the tetramer is energetically unfavorable since it may need a conformational change to perform the reaction. Alternatively, it may be envisaged that two tetramers come close together and perform the reaction in solution. In fact, crystal structure shows that His-117 of one tetramer faces Ser-119 of another making the transfer energetically feasible. Finally, our results unequivocally established that the phosphorylation of Ser-119 through intermolecular transfer of phosphate from autophosphorylated His-117 as an intrinsic property of sNdK and demonstrated its crucial role in NTP synthesis. It is worth mentioning here, that amino acids like 'HGSD' (residues 117-120 of sNdK), are fairly conserved at the active site throughout different NdKs. Therefore, these findings seem to have broader implications and definitely not restricted to sNdK only. Since NdKs show high homology at amino acid level throughout the phylogeny (*E. coli* to Human 40%), the contribution of the conserved residues (K10, Y50, R86, R104, N114) to the autophosphorylation and phosphotransfer activities of sNdK was also determined. The mutational study indicated that these conserved residues are important for the functionality of the enzyme. Thus, the biochemical studies suggested that NdKs from both the intracellular pathogens (*M. tuberculosis* and *S. typhimurium*) displayed akin

behaviour, despite the difference in their subunit association.

Besides, its function in the control of intracellular nucleotide homeostatis, NdKs from different microorganisms have also been found to be involved in multiple physiological and pathological cellular processes. Although known to be a cytoplasmic protein, in many pathogens like *M. tuberculosis*, *P. aeruginosa, L. amazonensis* and *V. cholera*, NdK has been found to be secretory in nature. Proteins secreted by pathogens have been known to play critical role in the host-pathogen interactions. In fact, some pathogens employ these secreted molecules (also called effectors) to modulate the functions of the host. To know the nature of NdK in *S. typhimurium*, the cell free filtrate of *Salmonella* was prepared and utilized for kinase assay. The ability of the concentrated culture filtrate of *S. typhimurium* to synthesize NTPs from ATP and NDPs categorically demonstrated that *Salmonella* NdK is secretory in the nature. The formation of NDP from NMP suggested that in addition to NdK, *Salmonella* was able to secrete other ATP utilizing enzyme (s). To confirm the secretion of NdK, a *ndk* deleted strain of *Salmonella* (*Δsndk*) was generated which as expected, did not show any NTP synthesizing ability in the concentrated cell free culture filtrate. Interestingly, the *Δsndk* strain synthesized ADP from AMP, implying that the deletion of *ndk* did not affect the secretion of other ATP utilizing enzymes. Furthermore, it also needs to be emphasized here that the other ATP utilizing enzymes present in the concentrated culture *Δsndk* strain were unable to take over or complement the function of sNdK.

NdK secreted by the pathogens has been reported to alter the eATP mediated effect of host cells. eATP is known to function as a 'danger signal' that alerts immune system to the presence of pathogen and serves as ammunition for neighboring macrophages to inhibit the infection. It has been reported that binding of eATP to P2Z receptors of macrophages results in cytolysis via necrotic or apoptotic pathways depending on the duration of exposure. Surprisingly, both intracellular and extracellular pathogenic bacteria have been reported to secrete ATP utilizing enzymes, which is very intriguing. Based on the distinct physiological conditions of these two types of pathogens, it is possible that the purpose of their secretion of NdK

may be different. Extracellular pathogenic bacteria like *P. aeruginosa*, *V. cholera* have developed strategies to avoid phagocytic activity of macrophages by releasing the enzymes which increase the level of eATP, hence eliciting apoptosis of these and other professional phagocytes. In contrast, intracellular pathogens like *M. tuberculosis* are expected to develop strategies in the opposite direction by releasing eATP utilizing enzymes, thereby prolonging the survival of their host cells. This interesting and exciting behavior of NdK has not been elucidated in greater detail so far.

In this context, role of NdK in ATP induced cell death in intracellular pathogens has been focussed. For this purpose, the macrophage cells (J774) were treated with ATP and concentrated culture filtrate of either intracellular (*S. typhimurium, M. tuberculosis*) or extracellular pathogens (*V. cholera*) and the cell death was monitored by LDH assay of the cell free supernatant. It was observed that concentrated culture filtrate of intracellular pathogens (*S. typhimurium, M. tuberculosis*) prevented ATP induced cell death while that of extracellular pathogen (*V. cholerae*) was unable to alter the effect of ATP. However, for the extracellular pathogen (*Vibrio*) cell death occurred irrespective of the induction by ATP. This was intriguing, given the fact that NdK proteins from the three pathogens displayed high homology (59.5 %) at amino acid level, similar secretory nature of the proteins and the concentrated culture filtrate of all three pathogens displayed NTP synthesizing ability. This suggested that in *V. cholera* culture filtrate, the presence of NdK could not prevent ATP induced cell death. This may only happen if the secretory NdK exhibits pathogen specific behavior. To answer this question, NdK from *V. cholera* (vNdK) was expressed in *Δsndk* strain, its concentrated culture filtrate was prepared and analyzed for its behavior by LDH assay. Surprisingly, it was found that vNdK expressed in *Δsndk* strain could modulate the effect of ATP on macrophage cells. This ruled-out the bifunctional nature of NdK and in fact, specified that the intrinsic property of NdK (to convert cell death inducing ATP into non-cytotoxic product) was same in both intracellular and extracellular pathogens. However, pathogens are known to develop strategies depending on the physiological conditions and the need

to survive. Thus, for its survival, *V. cholera* either modulates the function of NdK or its cell free supernatant contained some factor(s) which are cytotoxic and act at a brisk rate. The behaviour of concentrated culture supernatant of NdK deleted *V. cholera* strain towards J774 cells supported the later notion. Thus, all these lines of evidences argue that the intrinsic property of NdK protein from both intracellular and extracellular pathogens remained unchanged and not bifunctional, however, the modulation of its activity by some host factor(s) cannot be ruled out.