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Hypothesis

Shigella apyrase – a novel variant of bacterial acid phosphatases?

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Abstract A virulence-associated ATP diphosphohydrolase activity in the periplasm of *Shigella*, identified as apyrase, was found to be markedly similar to bacterial non-specific acid phosphatases in primary structure. When the *Shigella* apyrase sequence was threaded in to the recently published 3D structure of the highly similar (73%) *Escherichia blattae* acid phosphatase it was found to have a highly overlapping 3D structure. Our analysis, which included assays for phosphatase, haloperoxidase and catalase activities, led us to hypothesize that *Shigella* apyrase might belong to a new class of pyrophosphatase originating as one more variant in the family of bacterial non-specific acid phosphatases. It revealed interesting structure–function relationships and probable roles relevant to pathogenesis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Pyrophosphatase; Acid phosphatase; Actin binding; *Shigella* apyrase; *Shigella* pathogenesis

1. Introduction

The presence of a nucleoside triphosphate hydrolyzing activity in the periplasm of all four species of *Shigella* and enteroinvasive *Escherichia coli* was reported in 1995 [1]. The enzyme catalyzing the activity was called apyrase because of its ability to sequentially hydrolyze nucleoside triphosphates to corresponding diphosphates and then monophosphates. It lacked the ability to further hydrolyze the monophosphates, a distinguishing feature from normal phosphatases. An interesting observation was that this hydrolysis of tri- and diphosphates did not require metal ions and the enzyme was active even in the presence of EDTA [1]. This property was exploited in identifying the gene for the enzyme from the megaplasmid library of *Shigella flexneri*. The sequence comparison, however, clearly brought out the striking similarity between *Shigella* apyrase and other known bacterial non-specific acid phosphatases, in spite of basic functional dissimilarity with phosphatases. It was also pointed out that *Shigella* apyrase did not exhibit acid phosphatase activity but evidence for the presence of an independent acid phosphatase was provided [1]. Subsequently a separate acid phosphatase (SFPase) from *Shigella* was reported [2] and both apyrase and the new phosphatase were shown to share significant sequence homology and origin [3].

With widespread efforts in isolating new members of the family and improved tools in structure analysis, research in the past 5 years has revealed a common architecture and design of the active site in a new class of phosphatases, referred to as acid phosphatases from bacteria. Detailed sequence analyses [4] have brought to light the phylogenetic relatedness among acid phosphatases, integral membrane phosphatases and soluble haloperoxidases [3,5]. The most striking feature was the conservation of residues that make contacts with vanadium, a cofactor of haloperoxidases and an inhibitor of acid phosphatases [5,6]. It is now becoming clearer that subtle variations in the active site fold have given rise to different subclasses of acid phosphatase (A, B and C and subclasses A1, A2 and A3), membrane phosphatases like glucose 6-phosphatase and haloperoxidases like fungal *Curuvularia inaequalis*'s vanadium chloroperoxidase.

Recently the crystal structures of different members of the group have confirmed the inferences from sequence comparison and clearly revealed tertiary structure identity between the active sites. All of them are predominantly α-helical. Fungal *C. inaequalis*'s vanadium chloroperoxidase has two four helix bundles and the vanadium binding pocket is rather rigid and is formed by side chains that are conserved in bromoperoxidases and phosphatases [5,7]. Interestingly, the chloroperoxidases have been shown to possess phosphatase activity confirming the common design of the two active sites. Recently, the crystal structure of acid phosphatase (EBPase) from *Escherichia blattae* [6] was reported, highlighting the active site design common to itself and haloperoxidases. Taken together, it is an impressive divergent evolution seen in this class of phosphatases. Pathogens like *Shigella* might be exploiting the plasticity and versatility of the design in acid phosphatases (*Shigella* already has an acid phosphatase with about 85% identity and 92% similarity with EBPase, Fig. 1) to create novel virulence factors like apyrase. This report suggests a possible 3D structure for *Shigella* apyrase and some interesting structure–function relationships worth studying from an enzyme mechanism point of view as well as a pathogenesis point of view.

2. Materials and methods

2.1. Model building

BLAST [8] search against the NRDB with an *E*-value cut-off of 10

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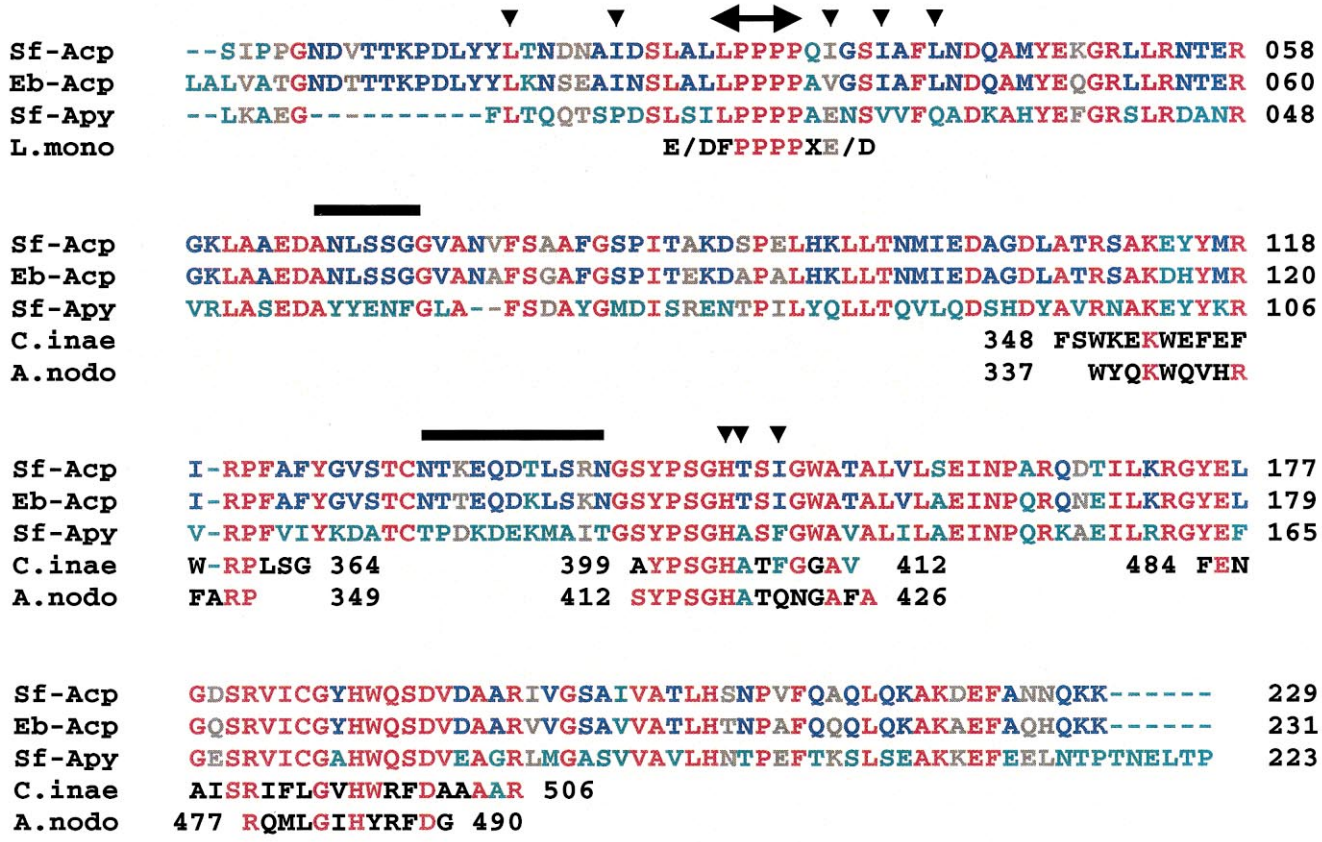

Fig. 1. Sequence alignment of *S. flexneri* apyrase (Sf-Apy) with acid phosphatases of *E. blattae* (Eb-Acp) and *S. flexneri* (Sf-Acp). Partial sequence alignment with chloroperoxidase of *C. inaequalis* (C.inae) and bromoperoxidase of *Ascophyllum nodosum* (A.nodo) has been shown to highlight conservation of active site residues. The poly-proline region, an actin-binding motif, has been indicated with a double-headed arrow. Identical residues in all three sequences are in red and blue represents identical residues in Sf-Acp and Eb-Acp. The bars and the ▼ represent regions of structure–function importance discussed in the text.

was used to obtain *Shigella* apyrase homologues. This resulted in the meaningful comparisons with the family of non-specific acid phosphatases and the vanadium-dependent haloperoxidases. The *E. blattae* crystal structure 1D2T was retrieved from PDB [9]. Pairwise sequence comparison with *E. blattae* using CLUSTALX [10] showed 72% similarity and therefore this was used as the PDB template in Swiss-Model [11,12] to thread the sequence of *Shigella* apyrase. A WHAT IF [13] verification of the modeled structure showed a *Z*-value of 0.890 for bond lengths, 1.852 for bond angles and 1.003 for ω angle restraint, which indicates a good prediction (*Z*-value is expected to be close to 1 for a good prediction). However, certain apparent differences relevant to the structure–function relationship were also noted. EBPase structure has already been compared with vanadium-dependent chloroperoxidase and the striking similarity between the two active sites has been noted [4] and in similar lines, we also compared the apyrase structure with that of chloroperoxidase.

2.2. Enzyme activity assays

2.2.1. Apyrase. Apyrase activity was measured according to the method described by Bhargava et al. [1]. In a microplate assay format, 150 μl of assay volume contained 50 mM Tris–HCl, 5 mM EDTA and 4 mM ATP, pH 7.5 and the enzyme preparation. After incubation for 15 min at 37°C, 100 μl of Chen's reagent was added. The blue color due to phosphomolybdate complex was read at 630 nm after standing at room temperature for 15 min.

2.2.2. Pyrophosphatase. Pyrophosphatase assay was done with the new phosphate reagent developed (Geetha and Sankaran, 1998, unpublished) in the lab. Chen's reagent gives a deep blue-colored product with pyrophosphate and therefore interferes with phosphate estimation. The assay mix contained 50 mM Tris–HCl, 5 mM EDTA, 4 mM sodium pyrophosphate, pH 7.5 and the enzyme preparation in a volume of 150 μl. After 15 min incubation at 37°C, 100 μl of the new reagent (10% ammonium molybdate, 2.5 mM copper sulfate and 100 mM ferrous sulfate in 1.25 M sulfuric acid, final) was added. The blue color was read at 630 nm after standing at room temperature for 15 min.

2.2.3. Chloroperoxidase. A microplate assay volume of 240 μl contained 5–50 mM sodium chloride, 4 mM H_2O_2, 25 mM Tris–HCl, pH 7.5 and the enzyme preparation [5]. After 15 min incubation at 37°C, 10 μl of *ortho*-toluidine was added. Previously a standard graph for chlorine under the assay conditions was constructed using different concentrations of chlorax and *o*-toluidine. The assay was able to detect above 1 nmol of chlorine in solution. This assay was also done with sodium *ortho*-vanadate varying from 5 μM to 1 mM (final) and it was repeated at lower pH using 60 mM citrate buffer, pH 5.0.

2.2.4. Peroxidase. Peroxidase activity was assayed using either *o*-dianisidine or 4-aminoantipyrine and *p*-hydroxybenzene sulfonate as oxygen acceptors. The microplate assay mixture contained 10 mM H_2O_2, 40 mM Tris–HCl, 5 mM EDTA, pH 7.5 and enzyme preparation. To this mixture, 150 μl color reagent was added and incubated for 15 min at 37°C. After standing for 15 min at room temperature, absorbance readings were taken at 495 nm. A standard graph was made with varying concentrations of H_2O_2 and externally added horseradish peroxidase.

2.2.5. Catalase. Catalase activity was assayed by measuring unreacted hydrogen peroxide using commercial glucose reagent kit from Sigma (Trinder, Sigma Cat. No. 315-100,2674). The horseradish peroxidase in the reagent was used to measure the unreacted peroxide. The microplate assay mixture contained 1 mM H_2O_2, 40 mM Tris–HCl, pH 7.5 and enzyme preparation. The mixture was incubated for 15 min at 37°C and then 150 μl of Trinder reagent was added. The pink color was read at 495 nm after standing at room temperature for 15 min.

2.3. Enzyme preparations

Periplasmic enzyme preparation and further purification (ion-exchange followed by electroelution from native PAGE) was done according to the published procedure by Bhargava et al. [1] using an over expressing *E. coli* clone containing plasmid pARC251 with *apy* ORF [1]. Crude as well as pure electroeluted protein preparations were assayed for the above activities.

3. Results and discussion

3.1. The 3D structure of Shigella apyrase resembles acid phosphatase of E. blattae

The overall identity between the primary structures of apyrase and EBPase was 50% and similarity was as high as 73%. In the C-terminal half that contains the active site, the identity over a stretch of 120 amino acids was 56.7% and similarity was 78.3%. All the residues reported to be involved in binding sulfate, vanadium and the His that forms phospho-histidine as covalent enzyme intermediate [6] are highly conserved and in places indicate a highly overlapping active site design (Fig. 2). However, unlike *Shigella* apyrase, EBPase is predominantly a phosphatase and its pyrophosphatase activity, as judged from the hydrolysis rates of ATP and pyrophosphate, was 23% and 36% respectively relative to *p*-nitrophenylphosphate [6]. *Shigella* apyrase on the other hand has negligible phosphatase activity.

3.2. Shigella apyrase is a pyrophosphatase that has emerged from bacterial acid phosphatases

In their report on acid phosphatase from *Prevotella intermedia* having phosphotyrosyl phosphatase activity, Chen et al. [3] argued that because *Shigella* apyrase is a non-metallo monomeric enzyme inhibited by millimolar concentrations of sodium fluoride, vanadate and other metal ions, it should be classified as a class A3 acid phosphatase. The rate of release of phosphate from AMP, glycerolphosphate and *p*-nitrophenylphosphate under the assay conditions reported for apyrase was either nil or negligible [1]. Its ability to hydrolyze phosphates like phosphotyrosine is not known. On the other hand, it efficiently hydrolyzed inorganic (540 μmol/min/mg for sodium pyrophosphate) and organic exo-pyrophosphates like ATP. It does not hydrolyze nicotinamide adenine dinucleotide and its phosphate derivative (results not shown). Among the NTPs, GTP (630 μmol/min/mg protein) was the most preferred substrate compared to ATP, UTP and CTP (420, 420 and 340 μmol/min/mg protein respectively) [1]. Moreover, the pH optimum was found to lie between 7.0 and 7.5 with practically no activity below pH 5 [1]. These distinguishing functional properties would justify consideration of *Shigella* apyrase as an exo-pyrophosphatase rather than a phosphatase as suggested.

Since the active site architecture of apyrase is highly overlapping with that of vanadium-dependent haloperoxidase and the latter has been shown to possess phosphatase activity, we assayed for haloperoxidase, peroxidase and catalase activities

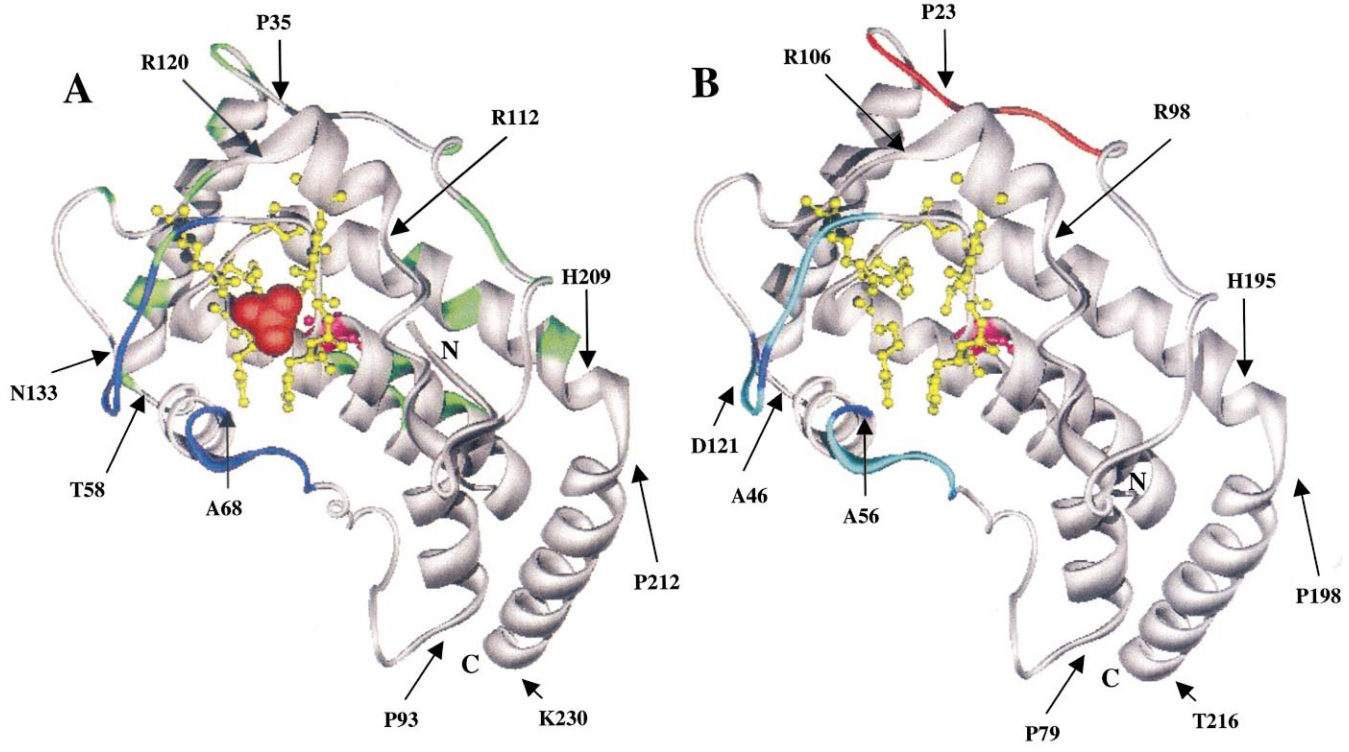

Fig. 2. The 3D structure of *Shigella* apyrase (B) obtained by threading using Swiss-Model with EBPase (A) as PDB template (1D2T). EBPase is shown as a monomer complexed with sulfate. All the active site residues are conserved in both structures and are represented as ball and stick structures in yellow. The dimer and trimer contact residues are highlighted in green in A. *Shigella* apyrase is probably a monomer (see Section 3). Two sequences, ^{68}ANLSSGG^{74} and ^{133}NTTEQDKLSKN^{143}, implicated in the movement upon substrate binding are shown in blue in A; the corresponding regions AYYENFG and TPDKDEKMAIT in B are quite different in sequence and this has been highlighted in cyan. Thr151 next to the active His150 (involved in forming the phospho-intermediate) in A and the corresponding Ala137 in B are shown in magenta. The poly-proline region, recognized as an actin-binding motif in *Listeria*, is colored red in B. Arrows indicate certain amino acid positions for easy recognition of regions referred to in the literature.

using crude as well as purified preparations of apyrase. Presence of orthovanadate up to 1 mM in the assay mix did not help to detect the above activities and it did not affect apyrase or pyrophosphatase activities. In fact, H_2O_2 in the presence of high concentrations of NaCl or NaI did not affect apyrase or pyrophosphatase activity. From this evidence, it appears that *Shigella* apyrase has most probably only exo-pyrophosphatase activity and has no significant phosphatase, haloperoxidase, peroxidase, or catalase activities. Therefore, it may be classified as an exo-pyrophosphatase and considered a new variant in the bacterial acid phosphatase family.

Even as a pyrophosphatase *Shigella* apyrase is distinct from known pyrophosphatases. Unlike the known bacterial pyrophosphatases, which act exclusively on either organic or inorganic pyrophosphates and require metal ions, *Shigella* apyrase acts on both and does not require metal ions [14]. The pyrophosphatases are exclusively β proteins [15] whereas *Shigella* apyrase is predominantly α-helical. It therefore appears that the structural design of acid phosphatases could have been exploited by *Shigella* to generate a novel pyrophosphatase. This adaptation perhaps has made apyrase a novel cytotoxin that indiscriminately hydrolyzes cellular NTPs and helps *Shigella* take control of the host cells. Drastic reductions in cellular NTPs subsequent to *Shigella* invasion have been confirmed recently [16] and its expression is under the control of HNS, like other important virulence factors.

3.3. Some interesting structure–function relationships

Shigella apyrase has been reported to act as a monomer as revealed by gel filtration data [1], whereas EBPase is a homohexamer, as inferred from the crystal structure and gel filtration data [6]. The large flat surface area of interaction in dimeric and trimeric association between the dimers in the crystal structure is quite impressive [5]. In Fig. 2, the residues reported to be important for dimer and hexamer formation in EBPase are highlighted in green. Among the hydrophobic residues that are found buried in these interactions in EBPase, Ile25, Val37, and Leu43 have been replaced by Pro, Glu and Gln respectively in apyrase. Ile40 and Leu43 have been pointed out as critical residues in the subunit interactions in EBPase. Their replacement with the less hydrophobic and polar residues Val and Gln in apyrase and the change of Val37 to the charged residue, Glu, might contribute to preventing oligomerization of apyrase. Also the hydrogen bonding pairs in the subunit interactions are considerably different between the two molecules. This might further help apyrase to remain a monomer.

A few significant changes in the primary structure may also be relevant in accounting for the differences in the functioning of EBPase and apyrase. In all acid phosphatases the residue next to His that forms the phospho-intermediate is invariably Thr [3,5,6]. Its hydroxyl group is placed 5.34 Å to the closest oxygen of sulfate. However, the equivalent residue in *Shigella* apyrase is Ala. The fact that changes in the vicinity of phospho-intermediate-forming His could play a role in deciding the type of activity is clearly borne out in the mutation study of Mihara et al. [17] on another acid phosphatase (MMPase) from *Morganella morganii*, which shows a high degree of similarity (45% identity and 67% similarity) to *Shigella* apyrase. The transphosphorylation activity of the native enzyme is not appreciable owing to a high K_m for inosine [17]. When Ile at position 171 of MMPase (corresponding to Ile153 in EBPase and Phe in apyrase) is changed to Thr, the K_m for inosine reduces three-fold and the overall transphosphorylation reaction rate increases [17].

Another region reported to affect transphosphorylation and phosphatase reaction rates in MMPase is Gly92 [17]. When this is converted to Asp, the phosphatase activity reduces so that inosine phosphate formation progresses without considerable hydrolysis. The corresponding position in both EBPase and *Shigella* apyrase is also Gly, but in the latter the flanking amino acids are poorly conserved.

In their discussion on the conformational alterations upon molybdate binding to EBPase Ishikawa et al. [6] have implicated two regions, Ala68–Gly74 and Asn133–Asn143, being important in the catalytic mechanism. Leu70 of the former moves 6.7 Å from the surface to the protein interior causing a structural change in the region and stabilization of the enzyme–molybdate complex through a hydrogen bond involving the side chain of His150 (involved in the phospho-intermediate) and Ala68. The flexible region Asn133–Asn143 becomes an α-helix and covers the bound molybdate [6]. Leu140 moves 6.2 Å and is placed next to the oxyanion within 3.3 Å distance of the equatorial oxygen atom of molybdate. These structural alterations have been implicated in the prevention of accessibility of water to the site of the phospho-histidine intermediate, thereby making its hydrolysis the rate-limiting step in catalysis [6]. On the other hand, the efficient transphosphorylation activity exhibited by EBPase has been attributed to the free access of acceptor alcohol to phospho-histidine from the other side through a depression provided by Leu16, Ser71, Ser72 and Glu104 [6].

The uncharged region ^{68}ANLSSGG^{74} in EBPase is identical in SFPase but modified to AYYENFG in apyrase with a net negative charge. The region ^{133}NTTEQDKLSKN^{143} of EBPase has been modified to NTKEQDTLSRN in SFPase retaining the net positive charge, but changed to negatively charged TPDKDEKMAIT in apyrase. Leu16 is totally missing, and in the sequence alignment there is a gap in this region (Fig. 1). It is therefore apparent that the details of the structure around the phospho-histidine are different between EBPase and *Shigella* apyrase. These differences may be relevant in accounting for the structure–function relationship of *Shigella* apyrase.

3.4. Poly-proline region

There is a poly-proline sequence, ^{17}LLPPPPAE^{24}, at the N-terminal region (Fig. 1) of all acid phosphatases and *Shigella* apyrase. Interestingly such a peptide sequence has been reported in the actin binding protein, ActA, of *Listeria* [18]. In the case of EBPase the poly-proline region at the N-terminus is in an extended structure (between helices 1 and 2), apparently buried in subunit interactions during dimer and hexamer formation. In monomeric *Shigella* apyrase this region is probably exposed, as in the case of ActA (PDB code: 1QC6). Furthermore, this sequence in ActA has been shown to be involved in actin binding and to be important for virulence by mutation studies, using synthetic peptides [19] and specific antibodies to the sequence [20]. The actual significance of this region is not clear in *Shigella*. However, it may be worth noting that interactions with actin and very intense actin polymerization activities are central to *Shigella*'s pathogenesis, especially in its intracellular spread. In actin polymerization and growth of actin, a process called treadmilling, ATP hydrolysis

and actin-bound ADP are critical [21]. It is in this regard that the pyrophosphatase or the diphosphohydrolase activity of *Shigella* apyrase might be involved in modulating actin polymerization rates. Apyrases have been utilized in tubers like potatoes [22] to push the equilibrium of the reaction towards starch synthesis by consuming the uridine diphosphate, which is a product in carbohydrate polymer synthesis, and the role of *E. coli* pyrophosphatase in DNA polymerization is well known.

It is therefore tempting to conclude that *Shigella* apyrase, having the necessary attributes of a virulence factor, including its regulation of expression as seen in other important virulence factors [23], is perhaps a novel variant of bacterial phosphatase capable of indiscriminate hydrolysis of nucleoside di- and triphosphates and pyrophosphates and has a plausible role in *Shigella*-induced actin polymerization.

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