Journal of Bioscience and Bioengineering
VOL. 112 No. 1, 8 – 13, 2011

www.elsevier.com/locate/jbiosc

Isoleucine 259 and isoleucine 260 residues in *Streptococcus gordonii* soluble inorganic pyrophosphatase play an important role in enzyme activity

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Received 17 August 2010; accepted 21 February 2011
Available online 29 March 2011

The hinge region of the family II soluble inorganic pyrophosphatase (PPase) found in *Streptococcus gordonii* DL1 (Challis) has previously been shown to play an important role in opening and closing of the active site between the N- and C-terminal domains of PPase. Amino acid residues isoleucine 259 (I259) and isoleucine 260 (I260) are highly conserved among catalytically active family II PPases and are located very close to the hinge region. Substitution of either I259 or I260 with a hydrophilic acidic amino acid (glutamate or aspartate) resulted in adverse effects on the kinetic properties of the enzyme. The I259/E and I259/D variants were nearly catalytically inactive (k_{cat}/K_m <0.2% of the wild type), whereas both I260/E and I260/D variants showed less than 15% of the catalytic efficiency of the wild type *S. gordonii* PPase. Conservative substitution of both residues to valine (I259/V, I260/V) showed no significant effect on the catalytic activity. The solvent accessibility data for I259 and I260 and the proximity of these amino acids to the hinge region suggest that occlusion of these residues may stabilise the closed and open conformations of the protein, respectively, thus aiding the catalytic activity of the enzyme.

[**Key words:** Inorganic pyrophosphatase family II; *Streptococcus gordonii*; Interdomain region]

Inorganic pyrophosphatases (PPases; EC 3.6.1.1) are key enzymes in cellular metabolism that catalyse the hydrolysis of inorganic pyrophosphate (PPi) to orthophosphate. The X-ray crystallographic structures of four family II PPases derived from *Bacillus subtilis*, *Streptococcus gordonii*, *Streptococcus mutans* and *Streptococcus agalactiae* (RCSB Protein Data Bank accession codes 1K23, 1K20, 1I74 and 2ENXB, respectively) have shown them to be homodimers (1–3). In the crystal structure, each monomer comprises an N-terminal and a C-terminal domain joined by an interdomain linker. The active site (one per subunit) is located at the interface between the N- and C-terminal domains. In the *S. gordonii* PPase (S.g-PPase) active site His9, Asp13, Asp15, Asp77, His99 and Asp151 of the N-terminal domain interact with two Mn^{2+} ions and Lys207, Arg297, Lys298 of the C-terminal domain and His100 of the N-terminal domain bind with one of the two phosphate sites of PPi (1). The active site in *B. subtilis* PPase (B.s-PPase) structure is wide open due to approximately 90° rotation of the C-terminal domain relative to the position seen in the S.g-PPase structure (1). This conformational change in the quaternary structure between the open (B.s-PPase) and closed (S.g-PPase) conformations is presumably in response to the binding of PPi or substrate analogues to the active site. The catalytic mechanism of family II PPases has been studied in detail (4,5), and the interdomain region of *S. gordonii* PPase is known to play an important role with respect to the activity of this enzyme (6).

Analysis of the sequence alignment of the Ten-residue-long linker/hinge region between the N- and C-terminal domains of family II PPases reveals that amino acid residues 188 to 191 (KAGT; *S. gordonii*-PPase numbering) are the most conserved residues in the region (Fig. 1). The T191/G substitution variant of *S. gordonii* PPase was found to have a k_{cat}/K_m value of only 26% of that of the wild type enzyme (6). Interdomain variants of S.g-PPase produced by deletion of amino acids all have a threonine residue displaced from position 191, and these variants have a substantial loss of catalytic efficiency (6). Analysis of the S.g-PPase structures shows that the side chain of residue T191 can make two non-covalent interactions. One interaction is hydrophobic between the methyl group of T191 and the side chain of I259; the other interaction involves a hydrogen bond from the hydroxyl group of T191 to the carbonyl oxygen of K188 (Fig. 2). In all of the active family II PPases cloned, sequenced and overexpressed so far, I259 has been conserved (Fig. 1). Inspection of the S.g-PPase structure also shows that I259, as well as I260 (also highly conserved), are surface-exposed. Both I259 and I260 are in close proximity to T191, a residue determined to be part of the mechanical hinge by DynDom analysis (6). To investigate the roles of I259 and I260 in the catalytic activity of S.g-PPase, site-directed mutagenesis was

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1389-1723/$ - see front matter.
doi:10.1016/j.jbiosc.2011.02.011

Organism	UniPortKB Accession		Hinge region			Interacting Ile259 region	
Streptococcus gordonii	P95765	188	**K**AG**T**NLASKS	197	257	TD**II**NSNSEIL	267
Streptococcus pneumoniae	P65755		**K**AG**T**NLASKS			TD**IV**NSNSEIL	
Streptococcus pyogenes	P65758		**K**AG**T**NLASKS			TD**IV**NSNSEIL	
Streptococcus mutans	O68579		**K**AG**T**NLASKT			TD**IL**NSNSEIL	
Streptococcus agalactiae	Q8DYS6		**K**AG**T**NLSSKT			TD**IV**NSNSEIL	
Lectococcus lactis	Q9CEM5		**K**AG**T**NLSTKT			TD**IV**NSNSEII	
Clostridium acetobutylicum	Q97H75		**K**AG**T**SLKGKT			TD**II**KEGSLII	
Staphylococcus aureus	Q6G813		**K**AGASTTDKS			TD**II**NSDSKIL	
Bacillus subtilis	P65754	186	**K**AGADLSKKT	195	255	TD**IL**ENDSLAL	265
			***: *:			***:: * :	
Vibrio cholerae	Q9KP34		IAK**T**DIEGLS			TD**I**TTAQTRLL	
Deinococcus radiodurans	Q9RRB7		AAKSDLGNTP			VD**IL**NETNRTL	
Archaeoglobus fulgidus	O29502		AKLSAVDDLT			TD**IM**KEGTELL	
Methanococcus jannaschii	Q58025		**K**AKSVVGKLK			TD**IM**KEGSEAL	
			: :			** *	

FIG. 1. Sequence alignments of the hinge region and the interacting isoleucine residues of family II PPases (prepared using ClustalW2). Residues fully conserved in all sequences are marked with *asterisks*. *Colons* denote residues of groups conserved including conservative replacements. K188, T191, I259 and I260 in S.g-PPase and their corresponding residues in other family II PPases are highlighted in bold.

employed to individually replace both residues with valine, aspartate or glutamate. The mutant PPase constructs were overexpressed in *Escherichia coli*.

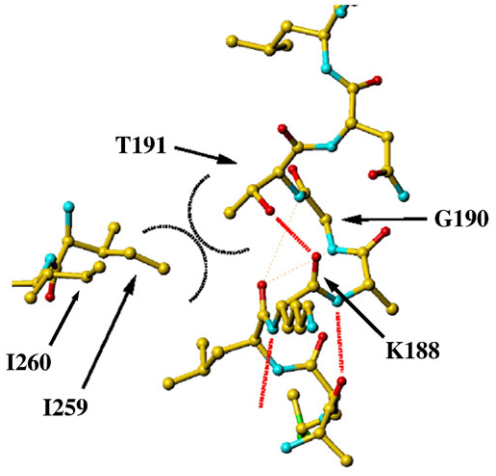

FIG. 2. Possible interaction of residue I259 with residue T191 of the interdomain region of *S. gordonii* PPase. The hydrophobic interaction is shown with black dotted half-circles and hydrogen bonds are shown as dotted lines. The diagram was prepared using Turbo-Frodo v OpenGl.1.

MATERIALS AND METHODS

Generation of site-specific mutants Site-specific mutants of I259 and I260 (I259/D, I259/E, I259/V, I260/D, I260/E and I260/V) were constructed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according the manufacturer's instructions. The reaction mixture included approximately 50 ng of *S. gordonii* PPase clone DNA (ORF1 of pCW1 in pET-11c; (6)), 125 ng (each) of the appropriate two 3′- and 5′- primers (Table 1), 0.5 mM of each deoxynucleoside triphosphate, 20 mM Tris–HCl pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA and 2.5 units of *Pfu Turbo* DNA polymerase in a total volume of 50 µl of sterile ultra high quality water (Elgastat UHQ Option 4 system, Elga, UK). The amplification reaction was carried out using a Hybaid PCR Express Thermal Cycler, and the reaction was cycled 18 times. The PCR produced mutant sequences (6577 bp) which were digested with *Dpn*I to remove the methylated, non-mutated parental DNA template. The mutated dsDNA was transformed into *E. coli* DH5α cells (Bethesda Research Laboratories) (7). Mutant DNA isolated from a DH5α clone was sequenced in both directions to confirm the modification in the coding sequence. Only after the desired mutation was confirmed was the mutant DNA transformed into *E. coli* BL21(DE3) (Novagen) (8) for overexpression.

Overexpression Overexpression and protein extraction from *E. coli* BL21(DE3) cultures were carried out following methods described in our previous paper (6). The protein expression was induced with 1 mM IPTG in the presence of 100 µg ampicillin/ml medium in cultures grown in 200 ml LB broth for 2 h at 37°C and with agitation at 200 rpm. Overexpressed cells were disrupted by sonication on ice, and the soluble fraction was separated from cell debris by centrifugation (30,000×*g*, 30 min). The supernatant was collected and stored at −20°C.

Protein purification Site-specific mutants of *S. gordonii* PPase were purified from crude cell extracts in two steps by FPLC using a Mono Q (HR5/5) anion exchange column and a Phenyl Superose (HR5/5) hydrophobic interaction column (Amersham Pharmacia Biotech). In the Mono Q column, a linear gradient of 0.14–0.6 M NaCl in 50 mM Tris–HCl and 1 mM EDTA (pH 7.5) was used for elution at a flow rate of 0.5 ml/min. In the second step of purification, the active fractions from the Mono Q column were diluted with an equal volume of buffer that contained 50 mM Tris–HCl, 1 mM EDTA and 2 M NaCl (pH 7.5); these diluted active fractions were applied to a Phenyl Superose column equilibrated