Active Site Labeling of the *Yersinia* Protein Tyrosine Phosphatase: The Determination of the pK_a of the Active Site Cysteine and the Function of the Conserved Histidine 402†

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ABSTRACT: In this report, we demonstrated that the *Yersinia* protein tyrosine phosphatase (PTPase) could be inactivated by the alkylating agent iodoacetate. The enzyme modification was selective, and the covalent attachment was stoichiometric. The residue that was labeled by iodoacetate was shown to be Cys403, which was the same catalytically essential residue identified by site-directed utagenesis [Guan, K. L., & Dixon, J. E. (1990) *Science 249*, 553–556]. The rate of iodoacetate modification decreased as the ionic strength of the media increased. There was no significant D_2O solvent isotope effect associated with the inactivation of the enzyme, suggesting that thiol anion of Cys403 reacted as a nucleophile. The *Yersinia* PTPase also displayed differential reactivity (940-fold) toward iodoacetate over iodoacetamide. This indicates that residues within the active site of the enzyme are positively charged. The pK_a of the active site thiol group was determined to be 4.67. The low pK_a value suggests that ionic interactions are important in stabilizing the thiolate anion. One candidate residue for this stabilization is the invariant histidine (His402) found in all PTPases. Substitutions of His402 with Asn or Ala altered the active site thiol pK_a to 5.99 and 7.35, respectively. Interestingly, the active site thiol in the mutants also showed enhanced reactivity toward iodoacetate. The second-order rate constants for the inactivation of the wild-type enzyme, H402N, and H402A were 59.7, 3305, and 1763 M^{-1} min^{-1}, respectively.

Protein tyrosine phosphorylation and dephosphorylation are key components of the signal transduction pathways that control the growth and differentiation of eukaryotic cells (Hunter, 1989). The concerted and regulated action of protein tyrosine kinases and protein tyrosine phosphatases (PTPases) is required to modify the phosphorylation status of a cell. An understanding of the structure–functional properties of both kinases and phosphatases is essential in developing a coherent picture of the mechanism of signal transduction.

One common feature exhibited by all PTPases characterized to date is their high sensitivity toward sulfhydryl reacting compounds. For example, the PTPases are irreversibly inhibited by alkylating agents such as iodoacetic acid, suggesting the presence of at least one reactive sulfhydryl that is essential for catalysis (Tonks et al., 1988, 1990). Site-directed mutagenesis experiments have demonstrated that a conserved Cys residue, which resides in the PTPase signature motif Ile/Val-His-Cys-X-Ala-Gly-X-Gly-Arg-X-Gly, is absolutely required for PTPase activity (Streuli et al., 1989; Guan & Dixon, 1990). It has also been demonstrated that the active site Cys residue is utilized to form a thiophosphoenzyme intermediate during the hydrolytic turnover (Guan & Dixon, 1991; Cho et al., 1992).

Recent work from our laboratory has shown that [^{14}C]-iodoacetate reacted with only one residue in the cytoplasmic domain of the receptor PTPase, LAR. The labeled residue was identified to be the active site Cys1522 (Pot & Dixon, 1992). In addition, the active site cysteine was shown to be more reactive toward iodoacetate than iodoacetamide even at pH 5 (Pot et al., 1991). The enhanced reactivity of iodoacetate with respect to that of iodoacetamide suggests the influence of a positive charge or a positive electrostatic field in the active site of PTPase. It is likely that destabilization of the thiol group is achieved by positioning it in a positively charged electrostatic field in the active site of the enzyme. Thus, the identity of contributors to this electrostatic field is an important issue in the elucidation of the catalytic mechanism of the enzyme. Obvious candidates are positively charged side chains of Arg, Lys, and His or secondary structural elements such as α-helices, which have dipolar character.

There is considerable evidence for the involvement of histidine residues in the activation (ionization) of cysteinyl sulfhydryl groups in the active sites of several enzymes, most notably papain (Lewis et al., 1976, 1981). In this instance an imidazolium cation appears to stabilize the thiolate anion of a neighboring cysteine by formation of a zwitterionic pair. Amino acid sequence alignment of the catalytic domains of both receptor and nonreceptor PTPases showed that the only histidine residue conserved among all of the PTPases was located in the signature motif (Ile/Val-His-Cys-X-Ala-GlyX-Gly-Arg-X-Gly) adjacent to the active site Cys residue (Guan & Dixon, 1990). Since the catalytic domains of all PTPases from bacteria to mammals have a highly invariant sequence of amino acids at their active site, it is likely that some of the conserved residues play critical roles in the structure or catalytic mechanism of PTPases.

A PTPase from *Yersinia enterocolitica*, has recently been expressed in *Escherichia coli*, purified in high yield, and physicochemically characterized (Zhang et al., 1992). The *Yersinia* PTPase has also been crystallized, and its structure determination is in progress. Thus, it serves as a model for structure–function studies of the PTPases. The utility of employing the *E. coli* alkaline phosphatase as a model for all

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alkaline phosphatases has been proven to be most insightful (Kim & Wyckoff, 1989).

The objectives of this study are (1) to determine if the active site sulfhydryl (Cys403) in the *Yersinia* PTPase is modified by iodoacetate in a fashion similar to that noted for the mammalian receptor PTPases, (2) to determine the pK_a of the active site thiol residue from the pH dependence of iodoacetate inactivation, and (3) to assess the effect of the only invariant His residue (His402) on the ionization and reactivity of the active site thiol group.

EXPERIMENTAL PROCEDURES

Materials

Iodoacetate, iodoacetamide and *p*-nitrophenyl phosphate were obtained from Sigma. D_2O (99.9%) was from Aldrich. ^{14}C-labeled iodoacetate was purchased from Amersham and diluted with unlabeled iodoacetate to obtain the noted specific activities. Sequencing grade endoproteinase Lys-C, Quick Spin G-25, and G-50 columns were from Boehringer-Mannheim.

Methods

Isolation and Purification of the Yersinia PTPase. The recombinant *Yersinia* PTPase was expressed in *E. coli* and purified to homogeneity as described previously (Zhang et al., 1992). The concentration of the purified protein was determined by amino acid analysis.

Oligonucleotide-Directed Mutagenesis. The oligonucleotide directed mutagenesis procedure of Eckstein (Sayers et al., 1988; Potter & Eckstein, 1984) was employed using an Amersham *in vitro* Mutagenesis system (RPN.1523) to create the desired mutations. The oligonucleotide primer used for His 402 → Asn (H402N) was 5′-GCCGGTAATAAAT-TGCCGTGC, and, for His 402 → Ala (H402A), 5′-GGCCGGTAATAGCTTGCCGTGCGG was used. All mutations were verified by sequencing using the dideoxy method (Sanger et al., 1977).

Kinetics and pH Dependence of Iodoacetate Modification. The experimental procedures for the inactivation of the wild-type *Yersinia* PTPase, the H402N mutant, and the H402A mutant, using iodoacetate employed the following buffers. From pH 4 to 5.6, 100 mM acetate; from pH 5.8 to 6.5, 50 mM succinate; from pH 6.6 to 7.3, 50 mM 3,3-dimethyl glutarate, and from pH 7.5 to 8.4, 100 mM glycinamide. In all the buffer systems, 1 mM EDTA was included, and the ionic strength was kept at 0.15 M (adjusted by additions of NaCl). The D_2O solvent isotope effects on the alkylation of *Yersinia* PTPase were measured at several pL values in the same buffer systems described above. The modification reaction was started by adding 2 μL of PTPase stock to the reaction mixture (total volume of 200 μL) containing an appropriate amount of iodoacetate at 25 °C. A control sample was prepared in the same way, except that iodoacetate was omitted. At intervals of time, 10-μL aliquots were withdrawn, and the enzyme activity was assayed at 30 °C and pH 5.5, 100 mM acetate, 1 mM EDTA, I = 0.15 M buffer containing 8 mM pNPP. The percentage of residual activity of the modified enzyme was calculated relative to the control, and the pseudo-first-order rate constant for the inactivation was determined by the nonlinear regression program GraFit (Leatherbarrow, 1989).

Stoichiometry of Iodoacetate Labeling. In a final reaction volume of 100 μL, 21 μM *Yersinia* PTPase was incubated with 2 mM ^{14}C-labeled iodoacetate (18.6 mCi/mmol of iodoacetate) in 50 mM 3,3-dimethyl glutarate, 1 mM EDTA, I = 0.15 mM and pH 7.0 buffer at 25 °C. At each time point, a 3-μL aliquot was withdrawn to assay for residual activity. At the same time 10-μL aliquot was also removed and quenched into 25 μL of 200 mM DTT in 100 mM Tris at pH 8.5. The DTT quenched reaction sample was then applied to a Quick Spin G-50 column. Incorporation of iodoacetate into the enzyme was determined by measuring the radioactivity of the flow-through of the spin column.

Isolation and Sequencing of the Iodoacetate-Radiolabeled Peptide. Homogeneous *Yersinia* PTPase (0.689 mg, 13.5 nmol) was incubated with 2 mM ^{14}C-labeled iodoacetate (18.6 mCi/mmol iodoacetate) in 50 mM 3,3-dimethyl glutarate, 1 mM EDTA, I = 0.15 mM, pH 7.0, buffer in a total volume of 200 μL. The labeling reaction was terminated by passing the mixture through a G-25 Quick Spin column when there was only 30% original activity left. The ^{14}C-labeled *Yersinia* PTPase was then reduced with 1.67 mM DTT in 1 M guanidine hydrogen chloride and 250 mM Tris, pH 8.5, at 25 °C for 30 min. The reduced PTPase was carboxymethylated in 10 mM iodoacetate in the dark for 30 min at 25 °C. The labeled, carboxymethylated *Yersinia* PTPase was isolated by a G-25 desalting column preequilibrated in the Lys-C digest buffer (25 mM Tris, 1 mM EDTA, pH 8.5). Finally, the PTPase was digested with 5 μg of Lys-C (50:1 ratio of PTPase to protease) in the presence of 1 M guanidine hydrogen chloride at 37 °C for 4 h.

The resulting peptide fragments were resolved on an Applied Biosystems Microbore 130 HPLC system equipped with a Vydac C18 reversed-phase column (2.1 × 250 mm). One-fifth of the digests was run over the column equilibrated in solvent A (0.08% trifluoroacetic acid in water) at a flow rate of 150 μL/min. The separation conditions were as follows: 0–5 min, 5% solvent B (0.07% trifluoroacetic acid in 80% acetonitrile); 5–50 min, 5–60% solvent B; and 50–70 min, 60–75% solvent B. Peaks were collected manually and analyzed for radioactivity. The radiolabeled peptide was subjected to gas phase amino acid sequencing using an Applied Biosystems model 470. Half of the product from each cycle of the sequencer was counted for radioactivity, and the other half of the sample was used for amino acid analysis. The radiolabeled peptide was also subjected to electrospray ionization mass spectrometry analysis. Electrospray ionization mass spectra were obtained using a Vestec electrospray source and model 201 single quadrupole mass spectrometer (Vestec Corp. Houston, TX) fitted with a 2000 m/z range (Allen & Vestal, 1992; Andrews et al., 1992). Samples were delivered to the source in a 10-μL injection loop at 5 μL/min in 4% acetic acid and 50% acetonitrile.

RESULTS

Kinetics of Inactivation by Iodoacetate. The *Yersinia* PTPase can be inactivated by iodoacetate in a pseudo-first-order kinetic manner. Iodoacetate also shows striking reactivity over iodoacetamide toward the *Yersinia* PTPase. For example, at pH 6.0 and 25 °C, the inactivation of *Yersinia* PTPase by iodoacetate and iodoacetamide displays a second-order rate constant k_2 = 59.7 M^{-1} min^{-1} and 0.0633 M^{-1} min^{-1}, respectively. Interestingly, the iodoacetate modification of the *Yersinia* PTPase is strongly ionic strength dependent (Figure 1). The apparent first-order rate constant of inactivation at pH 5.5 and 25 °C, in acetate buffer using 2 mM iodoacetate, decreases from k_1 = 0.457 min^{-1} at I = 0.0423 M to k_1 = 0.0130 min^{-1} at I = 0.80 M, a 35-fold reduction. The inactivation of the enzyme by iodoacetate was also studied

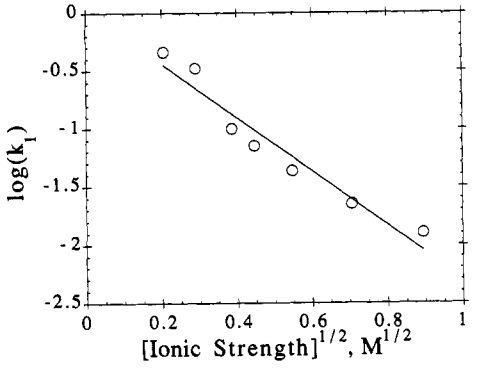

FIGURE 1: Ionic strength dependence of iodoacetate inactivation. The inactivation reaction was done at pH 5.5 and 25 °C in acetate buffer using 2 mM iodoacetate.

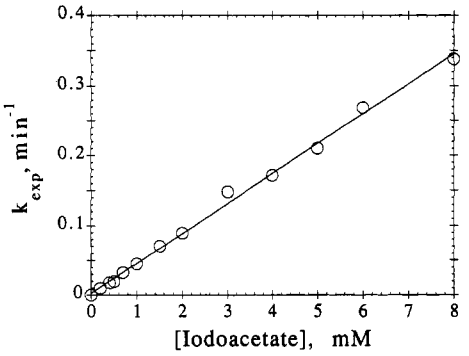

FIGURE 2: Concentration dependence of iodoacetate inactivation. The reaction was performed at pH 6.0 and 25 °C in 50 mM succinate, 1 mM EDTA, I = 0.15 M buffer.

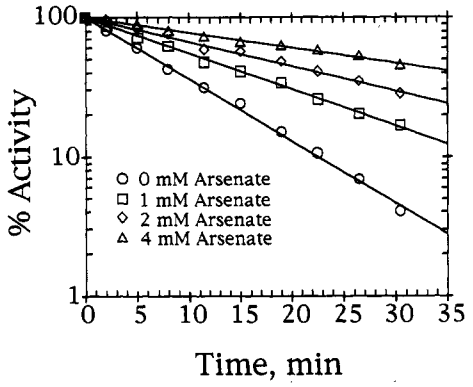

FIGURE 3: Effect of arsenate on the inactivation of *Yersinia* PTPase by iodoacetate.

in D_2O at several pL values from 5 to 7.5. No significant D_2O kinetic solvent isotope effect was observed. For example, $k_1^H/k_1^D = 1.02 \pm 0.08$ at pL 5.5, and $k_1^H/k_1^D = 0.98 \pm 0.05$ at pL 7.0. Iodoacetate concentration dependence of the inactivation at pH 6.0 and 25 °C, in 50 mM succinate, 1 mM EDTA, I = 0.15 M buffer indicates that the kinetic order is one with respect to the modifier (Figure 2). Arsenate, a competitive inhibitor of the *Yersinia* PTPase (K_i = 1.4 mM at pH 6), provides protection against the inactivation (Figure 3), suggesting that iodoacetate interacts with the active site of the enzyme.

Stoichiometry of Iodoacetate Labeling. Since it appears that iodoacetate reacts with the active site of the enzyme, the selectivity of the modification was evaluated by determining the stoichiometry of labeling using radiolabeled iodoacetate. As shown in Figure 4, the loss of phosphatase activity upon incubation with ^{14}C-labeled iodoacetate coincides with the incorporation of radiolabel with a stoichiometry approaching

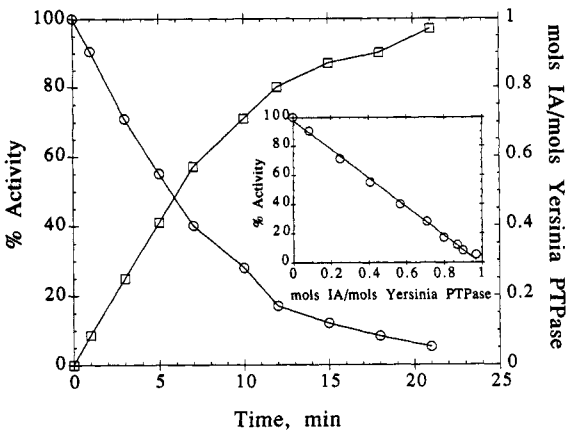

FIGURE 4: Time course of *Yersinia* PTPase inactivation by iodoacetate. The reaction was followed by incorporation of ^{14}C label per mol of enzyme (□) or loss of PTPase activity (○). (Inset) Correlation of incorporation of [^{14}C]iodoacetate with the extent of enzyme inactivation.

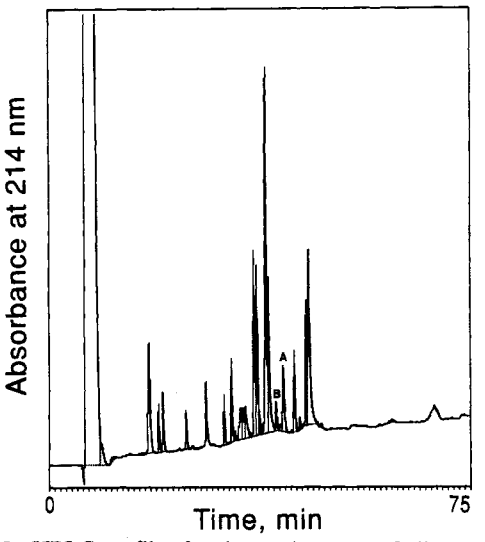

FIGURE 5: HPLC profile of endoproteinase Lys-C digest of [^{14}C]-iodoacetate labeled *Yersinia* PTPase. The Lys-C digests (10 μL) were chromatographed on a Vydac microbore reverse-phase column (2.1 × 250 mm) using a buffer system consisting of 0.08% trifluoroacetic acid in water (buffer A) and 0.07% trifluoroacetic acid in 80% acetonitrile (buffer B). The flow rate was 150 μL/min. The radiolabeled peptides are identified as A and B in the profile.

0.96 mol of iodoacetate/mol of enzyme. Therefore, the evidence supports that the inactivation of *Yersinia* PTPase by iodoacetate involves its covalent attachment to a single residue.

Identification of the Site of Iodoacetate Attachment. To determine the location of iodoacetate attachment within the *Yersinia* PTPase, the enzyme was inactivated with ^{14}C-labeled iodoacetate (specific activity 18.6 mCi/mmol). The radiolabeled *Yersinia* PTPase was then reduced with DTT, and all free thiol residues were completely carboxymethylated with iodoacetate. The protein was then digested with endoproteinase Lys-C. Figure 5 shows the separation of the resulting peptides by microbore reverse-phase HPLC. Each peak was collected and the radioactivity in each sample determined. Only two peaks, indicated by A and B in the HPLC profile (Figure 5), contained radioactivity. Peak A contains 70% of the radioactivity while peak B contains 30% of the total radioactivity injected onto the column. The relative amount of peptide A and peptide B as judged by their optical density at 214 nm correlated with the amount of radioactivity associated with each peptide. Both radioactive peptides were sequenced by gas phase Edman degradation for 10 cycles.

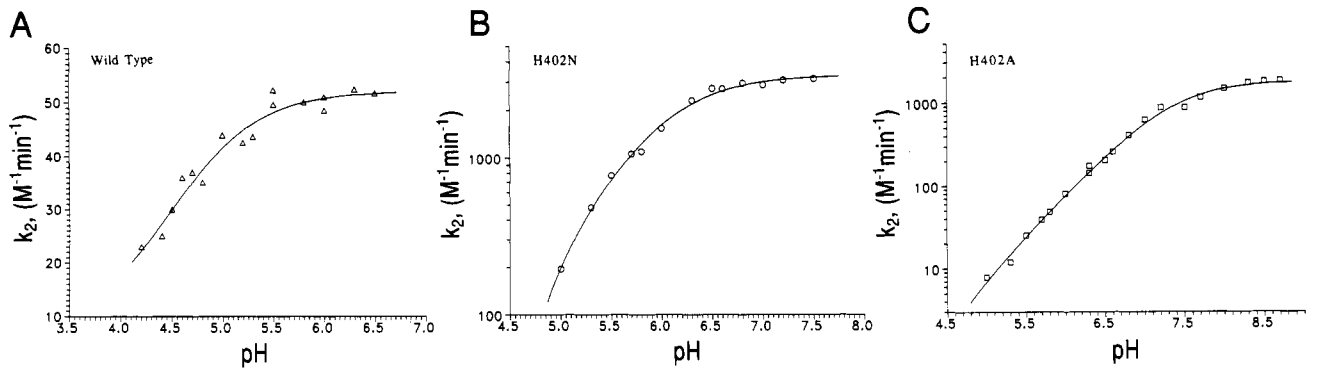

FIGURE 6: pH dependence of *Yersinia* PTPase by iodoacetate. The experimental conditions were specified under Experimental Procedures. (A) Wild-type *Yersinia* PTPase; (B) H402N mutant; and (C) H402A mutant.

Interestingly, the first 10 residues in both peptides were identical, having the amino acid sequence LRPVIHXRAG (Leu397 to Gly406 in the protein sequence). The identity of X was determined to be the PTH-carboxymethylated cysteine from its retention time on the sequencer. Furthermore, this was the only Edman cycle which contained significant amount of radioactivity. The summation of the radioactivity from this cycle plus the carry over from the next cycle constituted 90% of the total radioactivity applied to the sequencer. Thus, the site of modification on the *Yersinia* PTPase by iodoacetate is Cys403, i.e., the active site Cys residue in the PTPase signature motif. The identity of the radioactive peptide from the Lys-C digestion was further revealed by electrospray ionization mass spectrometry analysis. The radiolabeled peptide A was shown to have a mass of 6856.8 ± 8.7. The mass data together with the 10-cycle Edman degradation data suggest the active site peptide has the following sequence: LRPVIHCRAGVGRTAQLIGAMCMNDSRN-SQLSVEDMVSQMRVQRNGIMVQKDEQLDVLIK (Leu397–Lys456 in the protein sequence). Since the enzyme was reduced and completely carboxymethylated after the ^{14}C iodoacetate labeling, it is reasonable to assume that, in addition to the active site Cys403, the only other cysteine residue in the peptide, Cys418, was also carboxymethylated. The theoretical value (6857.05) was calculated from the known amino acid sequence starting at Leu397–Lys456 (6740.98) plus [two-CH_2COOH (2 × 59.045) groups] minus [two H (2 × 1.00797) atoms]. Thus, the experimentally determined mass value (6856.8 ± 8.7) is in excellent agreement with the theoretical value (6857.05). Similarly, the radiolabeled peptide B was shown to have a mass of 5803.4 ± 9.5, again in excellent agreement with the theoretical value of 5802.78, calculated from the amino sequence LRPVIHCRAGVGR-TAQLIGAMCMNDSRNSQLSVEDMVSQM-RVQRNFIMVQK (Leu397–Lys447 in the protein sequence). Apparently, peptide B was derived from peptide A through further Lys-C cleavage at Lys447.

The pK_a of the Active Site Cysteine and the Effect of His402 Mutation. The only residue modified by iodoacetate both in the catalytic domain of the receptor PTPase (Pot & Dixon, 1992) and in the *Yersinia* PTPase corresponds to the active site cysteine in the PTPase signature motif. This supports the idea that both mammalian receptor-like PTPase and the bacterial PTPase are using similar catalytic strategies employing an unusually reactive Cys residue which is selectively labeled by iodoacetate. The next question which we addressed is why is this Cys residue so reactive in the two proteins. All PTPases contain an invariant His residue adjacent to the reactive Cys. We set out to test the effects of the invariant histidine residue, His402, on the reactivity of Cys403 toward iodoacetate. Two mutant *Yersinia* PTPases, H402N and H402A, were prepared using site-directed mutagenesis (Sayers et al., 1988; Potter & Eckstein, 1984). Both mutants were purified to homogeneity following the procedure previously described (Zhang et al., 1992). Apparently, the point mutation did not perturb their tertiary structures in a major way, since the cation exchange chromatographic behavior of the mutants were identical to that of the wild-type enzyme. In addition, UV, CD, and fluorescence spectra of the mutants were almost superimposable to that of the wild-type enzyme (data not shown). In fact, both H402N and H402A retained residual catalytical activities with k_{cat} values 1.2% and 0.03% of the wild-type enzyme when compared at their respective pH optima (Zhang et al., unpublished results). As shown in Figure 6A–C and Table I, the *Yersinia* PTPase mutants H402N and H402A displayed altered pH-activation profiles toward iodoacetate compared with the wild-type enzyme. Thus, the thiol group of Cys403 in the wild-type *Yersinia* PTPase exhibited a pK_a of 4.67, whereas the corresponding pK_as in H402N and H402A were 5.99 and 7.35, respectively (Table I). Interestingly, the active site thiol also showed enhanced reactivity toward iodoacetate. The second-order rate constants for the inactivation of the wild-type enzyme, H402N, and H402A were 59.7, 3305, and 1763 M^{-1} min^{-1}, respectively (Table I).

Table I: Summary of pH Dependence of Iodoacetate Inactivation

Yersinia PTPase	pK_a of active site thiol	k_2^{lim} (M^{-1} min^{-1})
wild type	4.67 ± 0.15	59.7 ± 1.3
H402N	5.99 ± 0.04	3305 ± 108
H402A	7.35 ± 0.04	1763 ● 119

DISCUSSION

In this study, we demonstrated that the *Yersinia* PTPase can be inactivated by iodoacetate and that the loss of enzymatic activity was due to the specific modification of the active site Cys403. This is consistent with early observation that substitution of either Ser or Ala at this position caused a complete loss of PTPase activity (Guan & Dixon, 1990). The inactivation rates decrease in the presence of a competitive inhibitor, and the amount of iodoacetate incorporation parallels the loss of enzyme activity. These results are similar to those obtained with the mammalian receptor-like PTPase LAR (Pot & Dixon, 1992), suggesting that chemical and structural features in PTPases from bacteria to mammals are conserved.

The reaction between the active site thiol in the *Yersinia* PTPase and iodoacetate did not exhibit a significant D_2O solvent isotope effect at several pL values examined. This is

consistent with the reaction being nucleophilic in nature. Interestingly, the iodoacetate inactivation of *Yersinia* PTPase was strongly ionic strength dependent. According to the Debye–Hückel theory, an increase in ionic strength will accelerate a reaction between like charges and decelerate a reaction between unlike charges in a dilute aqueous solution where interionic attractions are minimal. Although the ionic strength of most of our reaction systems is higher than the theoretical valid limiting values (Moore & Pearson, 1981), it is still striking to see that a plot of log k_1 (the apparent first-order rate constant of inactivation) vs the squared root of ionic strength gave a straight line with a slope of –2.5 (Figure 1). This would be in accord with the negatively charged iodoacetate interacting with a positively charged environment in the *Yersinia* PTPase active site. This is further supported by the 940-fold preferential reactivity of the *Yersinia* PTPase toward iodoacetate over iodoacetamide. Similarly, the rate of the *Yersinia* PTPase catalyzed hydrolysis of pNPP, which is also negatively charged, decreased as the ionic strength increased (Zhang et al., 1992). We cannot exclude the possibility that the observed ionic strength effect on enzyme reactivity may be due to a protein structural perturbation induced by changes of solvation of charged groups on the surface.

Model reactions with low molecular weight thiolates (Halasz & Polgar, 1977) have shown that their reactivity with iodoacetate is about an order of magnitude less than that of iodoacetamide. In contrast, the results with the *Yersinia* PTPase illustrated that iodoacetate was 940-fold more reactive than iodoacetamide at neutral pH. Similar differences in reactivity have also been noted with papain and other cysteine proteinases (Chaiken & Smith, 1969; Halasz & Polgar, 1977). Arguments have been put forward which support the idea that the reactivity of haloacetates is enhanced by the presence of an adjacent positively charged residue(s). The histidinium ion within the active site of the proteinases appears to form an ion pair with the reactive thiol anion (Wallenfels & Eisele, 1968; Chaiken & Smith, 1969; Polgar & Csoma, 1987). The similarity between the *Yersinia* PTPase and papain includes the differential reactivity of the thiol toward haloacetates, as well as an abnormal low pK_a of the Cys residue at the active site of the two respective proteins [i.e., PTPase pK_a 4.67 and papain pK_a 3.3–4 (Lewis et al., 1976, 1981)]. The pK_a of a Cys residue is generally 8.5 (Lundblad & Noyes, 1984), and a reduction in the pK_a of this magnitude for a thiol requires unusual stabilization by its surrounding residues.

The apparent thiol pK_a and its reactivity toward iodoacetate were altered when the adjacent invariant histidine residue (His402) was changed to Asn402 and Ala402. The pK_a of the active site thiol was elevated from 4.67 in the wild-type enzyme to 5.99 in H402N and 7.35 in H402A. This supports the conclusion that the invariant His402 plays a role in stabilizing the active site thiolate anion. If the imidazolium side chain of His402 and the thiol group of Cys403 interact directly, one would expect that ionization of Cys403 and His402 to be thermodynamically linked. The absence of such behavior in Figure 6A may reflect a pK_a for the second ionization that is out of the pH range studied or suggest that the two residues do not interact directly. In addition to the direct interaction between His402 and Cys403, it is also possible for the two residues to interact with one another *via* a water molecule. Alternatively, the interaction might take place though a "network" of other amino acid residues. Regardless of the specific nature of the interaction or the presence of other amino acids in the network, altering His402

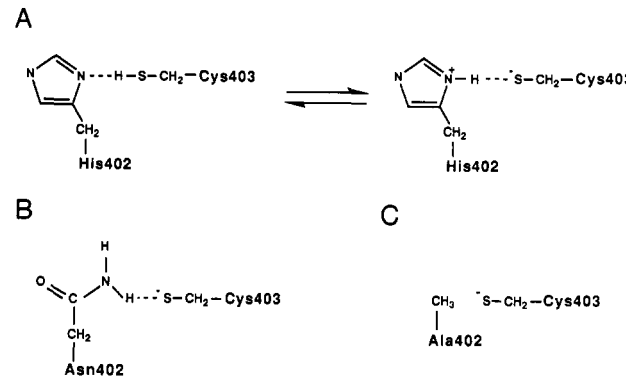

FIGURE 7: Suggested active site structure of the *Yersinia* PTPase.

has a dramatic effect on the apparent pK_a of the active thiol. A simplified model which explains the shift in pK_a value of the reactive thiol is shown in Figure 7. The imidazole side chain of His402 and the thiol side chain of Cys403 could interact favorably to form a zwitterionic ion pair (Figure 7A). In the H402N mutant, the side chain of Asn402 may form a hydrogen bond with the thiolate anion (Figure 7B). This interaction would be less favorable than the corresponding His402/Cys403 ion pair. Hydrogen bonding and a partial positive charge on the amide nitrogen arising from a resonance stabilization would provide a stabilizing effect compared with the Ala402 structure. No stabilization effect would be expected from the side chain of Ala402 in the H402A mutant (Figure 7C). It should be pointed out that, even in the case of H402A, the pK_a of the thiol (7.35) is still one pH unit lower than that of a free cysteine. This may indicate that, aside from His402, there may be other electrostatic interactions which are needed to stabilize the active site cysteine. Arg and/or Lys residues which are commonly found in phosphate binding proteins (Kim & Wyckoff, 1989; Luecke & Quiocho, 1990; Waksman et al., 1992) are good candidates. The Arg/Lys residues could function to bind the phosphate residue on the substrate and also function to stabilize the thiolate anion. Different Arg/Lys residues could be involved in each process. There are several invariant Arg residues in the PTPase catalytic domain (Guan & Dixon 1990) which are good candidates for this charge–charge interaction. In the *Yersinia* PTPase, Arg228, Arg409, Arg437, and Arg440 are potential residues which could interact with the negative charged phosphate or which could also stabilize the thiolate anion.

The specific consequence of the His402 mutation on the active site thiol group reactivity toward iodoacetate is very interesting. It is well known that, for certain reactions and in certain pK_a ranges, the nucleophilicity of a nucleophile is proportional to its basicity (Jencks, 1969). We observed that the limiting second-order rate constant (k_2^{lim}) of the inactivation by iodoacetate for the wild-type, H402N, and H402A were 59.7, 3305, and 1763 M^{-1} min^{-1}, respectively. Therefore, the substitution of His402 by Asn or Ala enhanced the nucleophilicity of the active site thiol. The reason that the active thiol in H402A was less reactive toward iodoacetate than that in H402N is not clear, although it is well known that enzymes frequently do not follow structure–reactivity correlations common for small molecules. It is likely that the difference between the enzymes could merely be the accessibility of the sulfhydryl. The same mutations showed that k_{cat} values at the pH optimal for H402N and H402A as measured with the artificial substrate *p*-nitrophenyl phosphate were only 1.2 and 0.03% of that of the wild-type enzyme, respectively (Zhang et al., unpublished results). Although the reactivity of the *Yersinia* PTPase with iodoacetate appears

to be nucleophilic in nature, the PTPase catalyzed phosphate monoester hydrolysis is more complex and will require further detailed kinetic analysis.

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