The Single Sulfur to Oxygen Substitution in the Active Site Nucleophile of the *Yersinia* Protein-Tyrosine Phosphatase Leads to Substantial Structural and Functional Perturbations†

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Received September 23, 1996; Revised Manuscript Received December 3, 1996⊗

ABSTRACT: Protein-tyrosine phosphatases (PTPases) feature an essential nucleophilic thiol group which attacks the phosphorus atom in a substrate. A single S to O atom substitution in the nucleophile (via Cys to Ser mutation) renders PTPases catalytically inactive. We suggest that the lack of activity in the Cys to Ser mutant may be caused by structural and/or conformational perturbations in the active site. *Yersinia* PTPase contains a single tryptophan residue, Trp354, which is invariant among all PTPases and is located in the vicinity of the active site nucleophile Cys403. Thus, Trp354 serves as an intrinsic probe of the PTPase active site conformation. We show that although C403S displays a nearly identical circular dichroism spectrum to that of the wild type enzyme, its ultraviolet spectrum in the region attributed to Trp is significantly different from that of the wild-type enzyme. In addition, the intrinsic fluorescence intensity of C403S is enhanced 3-fold and exhibits different ionic strength dependency from that of the wild-type enzyme. Trp354 also has different accessibilities to quenchers in the wild-type and the C403S mutant PTPases. Furthermore, unfolding experiments demonstrate that the structure of C403S is significantly less stable than the wild-type PTPase and displays a different sensitivity to urea and guanidine hydrochloride. Finally, binding of tungstate enhances the fluorescence of the wild-type *Yersinia* PTPase with a K_d of 55 μM, whereas binding of tungstate quenches the fluorescence of the C403S mutant with a K_d of 690 μM. Collectively, these results indicate that the single sulfur to oxygen change in the active site nucleophile leads to substantial structural/conformational and functional alterations in the *Yersinia* PTPase.

Protein-tyrosine phosphatases (PTPases) catalyze the hydrolysis of phosphate monoesters (such as phosphotyrosine) via a two-step, double-displacement mechanism, involving a covalent phosphoenzyme (E−P) intermediate (Zhang, 1990; Guan & Dixon, 1991; Wo et al., 1992; Cho et al., 1992). Biochemical and site-directed mutagenesis experiments show that the invariant Cys residue present in the PTPase signature motif (H/V)**C**$(X)_5$**R**(S/T) (*e.g.*, Cys403 in the *Yersinia* PTPase and Cys215 in PTP1B) is absolutely required for PTPase activity (Streuli et al., 1990; Guan & Dixon, 1990). In fact, replacement of the catalytically essential Cys residue with a Ser residue not only results in a complete loss of activity but also eliminates the PTPase's ability to form an E−P intermediate, suggesting that the intermediate is a phosphocysteine (Guan & Dixon, 1991). Crystallographic studies of PTPases complexed with oxyanions (such as tungstate, phosphate, and sulfate) are consistent with the conclusion that the invariant Cys residue present in the PTPase signature motif is the catalytic nucleophile (Stuckey et al., 1994; Barford et al., 1994).

The invariant Arg residue in the PTPase signature motif plays a role both in substrate binding and in transition state stabilization (Zhang et al., 1994a). The step leading to cysteinyl phosphate formation is facilitated by the protonation of the ester oxygen atom in the leaving group, which is accomplished by the conserved Asp residue acting as a general acid (Zhang et al., 1994b). After the formation of the E−P intermediate, the same Asp residue functions as a general base by activating a water molecule that approaches from the just-vacated leaving group side for the hydrolysis of the E−P intermediate with subsequent release of inorganic phosphate (Wu & Zhang, 1996). As part of the phosphate-binding loop, the conserved Thr/Ser residue immediately following the invariant Arg residue functions to facilitate the breakdown of the E−P intermediate (Zhang et al., 1995; Zhao & Zhang, 1996).

The nature of the transition state of the PTPase-catalyzed reaction has been probed by heavy-atom kinetic isotope effects (Hengge et al., 1995). It is concluded that the enzymic transition state is highly dissociative and similar to that in the uncatalyzed solution reaction, which involves a metaphosphate-like dissociative transition state where bond formation to the incoming nucleophile is minimal and bond breaking between phosphorus and the leaving group is substantial (Cleland & Hengge, 1995). Thus, minimal activation is required for the nucleophile to attack the phosphorus. A major mechanism for transition state stabilization by PTPase is stabilization of the developing negative charge on the leaving group (Wu & Zhang, 1996; Zhao & Zhang, 1996). During the E−P formation step, the phenoxide leaving group is stabilized by the essential Asp residue, while during the E−P hydrolysis step, the active site thiolate

† This work was supported by National Institutes of Health Grant CA69202 to Z.-Y.Z.

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

S0006-2960(96)02404-X CCC: $14.00

leaving group is stabilized by the conserved hydroxyl group (Ser/Thr) in the PTPase signature motif.

Although mutation of the invariant Cys residue results in a catalytically inactive PTPase, the Cys to Ser mutant still binds phosphotyrosine-containing peptides/proteins (Guan & Dixon, 1991; Bliska et al., 1992; Milarski et al., 1993; Sun et al., 1993). Due to the widespread practice of site-directed mutagenesis in probing enzyme structure−function relationships, it is important to understand how subtle perturbations in the active site affect enzyme activity due to changes in intrinsic chemical reactivity, conformational flexibility, physical size, or relative positioning of critical residues. Detailed comparative spectroscopic and functional studies of the wild-type *Yersinia* PTPase and its Cys to Ser mutant demonstrate that although the overall secondary and tertiary structural elements are similar for both proteins, there are striking conformational differences in their active sites. Thus, the reason for the lack of catalytic activity is likely due to structural perturbations in the PTPase active site introduced by the Cys to Ser mutation.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were obtained from commercial suppliers and used without further purification. Buffers were prepared using deionized and distilled water. *p*-Nitrophenyl phosphate (*p*NPP) was from Fluka. Ultrapure grade acrylamide was from Fisher Scientific. Potassium iodide (KI), sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), and L-tryptophan were from Sigma. Mutagenesis kits were from Bio-Rad. DNA sequencing kit was from USB.

Yersinia PTPase Constructs. The coding sequence corresponding to the PTPase from *Yersinia enterocolitica* (the yop51 gene) was originally isolated using PCR and cloned behind the bacterial expression vector utilizing the T-7 promotor (pYop51/pT7) and transformed into an *E. coli* strain carrying an inducible copy of the T7 polymerase (Zhang et al., 1992). Similarly, a truncated version of Yop51 (Yop51/Δ162) in which the first 162 residues of the NH_2-terminal segment were deleted was also made for structural studies. The truncated molecule starts with Met163 instead of Pro163 in the full-length Yop51, and retains the complete PTPase domain. The crystal structure of Yop51/Δ162 has been determined (Stuckey et al., 1994).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using the Muta-Gene *in vitro* mutagenesis kit from Bio-Rad. Mutagenesis of residue Cys403 of the *Yersinia* PTPase was performed as described previously (Zhang et al., 1994b). The plasmid pYop51/pT7 was used to make single-stranded DNA for site-directed mutagenesis. The oligonucleotide primers used for the Cys403 to Ser and to Ala mutations were 5′-TAATACATTCCCGTGCGGG-3′ and 5′-GTAATACATGCCCGTGCGGGTGT-3′, respectively. The underlined bases indicate the change from the naturally occurring nucleotides. Similar procedures were used to generate the corresponding mutants in the NH_2-terminal (162 residues) truncated version of the *Yersinia* PTPases (Zhang et al., 1992). All mutations were confirmed by DNA sequencing.

Expression and Purification of the Recombinant Enzymes. Several *Yersinia* proteins were prepared for spectroscopic studies. These include the wild-type *Yersinia* PTPase (Yop51), the Cys403 to Ser mutant (Yop51/C403S), the Cys403 to Ala mutant (Yop51/C403A), the truncated version of the *Yersinia* PTPase Yop51/Δ162, and its Cys403 to Ser mutant (Yop51/Δ162/C403S). The truncated version retains the PTPase domain and has been very useful to structural studies (Stuckey et al., 1994; Schubert et al., 1995). Detailed physicochemical characterization of the purified homogeneous recombinant Yop51 and Yop51Δ162 suggests that this truncation does not alter the catalytic and fluorescence properties of the PTPase (Zhang et al., 1992). The wild-type and the mutant *Yersinia* PTPases were expressed in *E. coli* and purified to homogeneity as described previously (Zhang et al., 1992). All mutants had chromatographic properties similar to those of the wild type, suggesting that there were no major changes in the global structure of the mutants. Protein concentration was determined by quantitative amino acid analysis of all residues in the *Yersinia* PTPase using an Applied Biosystem Model 420H amino acid analyzer. Equal amounts of wild-type *Yersinia* PTPase and the Cys403Ser mutant (as determined by amino acid analysis) gave identical absorbance readings in the Lowry assay (Lowry et al., 1951).

Enzyme Assay. The *Yersinia* PTPase activity was usually assayed at 30 °C in a reaction mixture (0.2 mL) containing 10 mM *p*NPP as substrate and 100 mM acetate, 1 mM EDTA, pH 5.5, buffer; the ionic strength of the buffer was adjusted using NaCl to $I = 0.15$ M. The reaction was initiated by addition of enzyme and quenched after 2−3 min by addition of 1 mL of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18 000 M^{-1} cm^{-1}.

Spectroscopic Measurements. Ultraviolet absorption spectra of the *Yersinia* PTPases were recorded using a Perkin-Elmer Lambda 14 UV/VIS spectrometer. The spectra were corrected by subtracting the background absorbance of the same buffer solution used for the PTPase samples. Circular dichroism spectra were recorded with a Jasco 710 spectropolarimeter calibrated with ammonium *d*-camphor-10-sulfonate. Each spectrum was obtained as the average of three scans to optimize the signal to noise ratio and was corrected for background using the buffer solution over the range of 260−180 nm using a 0.5 mm path length cell at 25 °C. Fluorescence experiments were performed on a Perkin Elmer LS50B fluorometer. Spectral measurements were made with 295 nm excitation (slit width 3.5 nm), and emission spectra were recorded from 300 to 400 nm (slit width 5 nm). Emission spectra were corrected for solvent background and Raman scattering.

Fluorescence Quenching. Quenching experiments were performed using steady-state fluorescence emission techniques. *Yersinia* PTPases (1−3 μM) were dissolved in 10 mM succinate buffer, 1 mM EDTA, pH 6.0, with an ionic strength of either 0.2 M or 1.5 M (maintained by adding NaCl or KCl). The excitation wavelength was 295 nm (slit width 3.5 nm), and the fluorescence intensity was measured at 340 nm (slit width 5 nm). The quenching reagent acrylamide was added from stock solution freshly prepared in the same buffers as the enzyme. When the ionic quencher KI was used, the ionic strength was held constant using the method described earlier (McClure & Cook, 1994). In this procedure, PTPase and buffer concentrations were identical in the cuvette, which contained a high concentration of KCl (176.2 mM or 1.476 M), and in a test tube, which contained

the same high concentration of KI in the same buffer. Stock solutions also contained 0.1 mM sodium thiosulfate to prevent formation of I_3^-. Aliquots from the test tube were then added sequentially to the cuvette to achieve the indicated concentration of the quencher. The fluorescence quenching data were analyzed according to the Stern−Volmer equation, which assumes that all quenching is collisional (Eftink & Ghiron, 1981):

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence or the presence of quencher, K_{sv} is the collisional Stern−Volmer constant, and [Q] is the quencher concentration.

Fluorescence Titration by Tungstate. The binding of tungstate was followed by steady-state fluorescence measurements as described for the quenching experiments. The binding of tungstate to the wild-type *Yersinia* PTPase enhanced its fluorescence while the binding of tungstate to the Cys to Ser mutant quenched its fluorescence. In both cases, the binding was saturable. Since the dissociation constants of tungstate for the PTPases were much larger than the concentrations of PTPases (which were typically around 1 μM), the dissociation constants were determined using eq 2 (Ward, 1985):

$$\Delta F = \Delta F_{max}[WO_4^{2-}]/(K_d + [WO_4^{2-}]) \quad (2)$$

where ΔF is the difference in fluorescence between the free and the tungstate-bound PTPase, ΔF_{max} is the maximum difference in fluorescence between the free and the tungstate-saturated PTPase, K_d is the dissociation constant, and $[WO_4^{2-}]$ is tungstate concentration.

Guanidine Hydrochloride and Urea Denaturation. Guanidine hydrochloride denaturation was studied by monitoring the change in fluorescence at 340 nm (slit width 5 nm) with an excitation wavelength at 295 nm (slit width 3.5 nm). Typically, PTPase was added to appropriate guanidine hydrochloride solution prepared in pH 6.0 buffer with a final succinate concentration of 50 mM, 1 mM EDTA, and I = 0.15 M. After incubation at 25 °C for at least 6 h, the fluorescence of the sample was measured. Urea denaturation was monitored as described (Zhang et al., 1992).

RESULTS AND DISCUSSION

Why the Cys to Ser Mutant Is Inactive. Substitution of the active site nucleophile Cys residue by a Ser residue abolishes the PTPase activity. Since the Cys to Ser mutation is confined to a single atom change, *i.e.*, a sulfur to an oxygen, it has generally been assumed that the Cys to Ser mutant has a similar structure to that of the wild-type enzyme. Since substrate binding by PTPases requires the presence of phosphate on the tyrosine residue within peptide substrates (Zhang et al., 1993), the fact that the Cys to Ser mutant still binds phosphotyrosine-containing peptides/proteins suggests that the structure of the active site, especially the phosphate-binding loop, in the cysteine mutant is not grossly perturbed. Crystallographic studies seem to support this conclusion. There are no noticeable differences between structures of the *Yersinia* PTPase−tungstate complex and the C403S−sulfate complex (Stuckey et al., 1994; Schubert et al., 1995). There are also no noticeable differences between structures of the PTP1B−tungstate complex and the C215S−substrate complexes (Barford et al., 1994; Jia et al., 1995). In addition, the structures of unliganded wild type and C215S PTP1B are essentially identical (Jia et al., 1995).

Considering the dissociative nature of the transition states in PTPase-catalyzed reactions, it is intriguing that replacing the Cys residue with a Ser residue completely abolishes the PTPase activity. In the *Yersinia* PTPase, the pK_a of the active site thiol group, Cys403, is 4.7 (Zhang & Dixon, 1993), suggesting that it exists as a thiolate anion at physiological pH. One possibility for the lack of phosphatase activity may be the lower nucleophilic reactivity of the hydroxyl group in a Ser residue (pK_a 13.4; Metzler, 1971). Although in the absence of a nearby basic residue for efficient activation, the hydroxyl group of a Ser residue is intrinsically a much weaker nucleophile than the thiolate group of a Cys residue near neutral pH, one would still expect the Cys to Ser mutant to retain some catalytic activity. This is due to the minimal bond formation that is required between the attacking group and the phosphorus in a dissociative transition state so that the serine hydroxyl group, protonated or not, would not have a major impact on the E−P formation step. This should especially be true if the mutation does not alter the structure of the active site and other major mechanisms for catalysis (*e.g.*, protonation of the leaving group by the general acid Asp residue) are still in place. Indeed, the general acid Asp181 in the Cys215 to Ser mutant PTP1B can still form a hydrogen bond with the phenolic oxygen of phosphotyrosine (Jia et al., 1995). An analogous precedent is the hydrolysis of phosphate monoanions by water (pK_a 15.4) which attacks the substrate that is activated by protonation of the ester bond (Kirby & Varvoglis, 1967). When a high concentration (0.1 mM) of C403S was incubated with 10 mM *p*NPP for 12 h, no PTPase activity was observed even at pH 9.5.

Another possibility may be that in the absence of adequate means for the Ser alkoxide stabilization, the Cys to Ser mutation may prevent the E−P hydrolysis once it is formed. If this were the case, one would observe a stoichiometric amount of *p*-nitrophenol and E−P formation in the presence of high concentrations of the Cys to Ser mutant. None of these were observed. In fact, replacement of the catalytically essential Cys residue with a Ser residue not only results in a complete loss of activity but also eliminates the PTPase's ability to form a phosphoenzyme intermediate (Guan & Dixon, 1991).

While the possibility that the explanation may lie in the structural perturbation introduced by the mutation has yet to be completely excluded, there is no direct evidence for it. We would like to seek molecular explanations for the lack of catalytic activity of the Cys to Ser mutant and believe the reason for this may lie in subtle structural perturbations in the active site. Although crystallographic techniques provide the majority of information about the tertiary structure of proteins, they seldom allow the determination of local minute structural disturbances caused by mutagenesis. Good protein structures derived from X-ray diffraction have an average uncertainty of a few tenths of an angstrom for non-hydrogen atoms (Kuntz et al., 1994). However, such minute deviations from the optimal geometric alignments in the active site of an enzyme could have a profound effect on the enzyme-catalyzed reaction. Furthermore, a conformation observable in a crystal structure may be selected by crystal packing and have limited relevance to those existing in solution. Spectroscopic techniques are generally more sensitive to small changes (*e.g.*, a fraction of an angstrom)

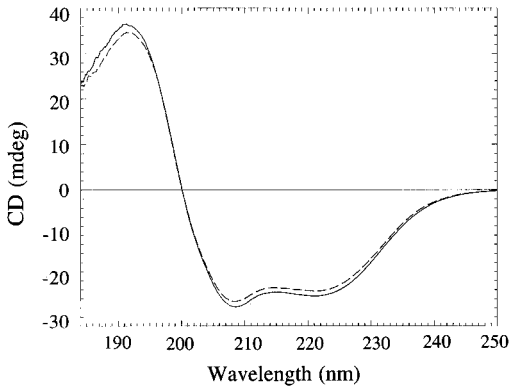

FIGURE 1: Comparison of circular dichroism spectra of the *Yersinia* PTPases. The spectra were recorded at a protein concentration of 0.577 mg/mL (wild-type Yop51) and 0.557 mg/mL (Yop51/C403S) at pH 6.0, 10 mM Tris−acetate buffer, and 25 °C. Solid line, Yop51; dashed line, Yop51/C403S.

in structure and conformation than X-ray crystallography and should be useful in studying the structural features of active sites of PTPases. The most useful optical probe for detecting conformational dynamic changes in proteins is the tryptophan residue because its spectroscopic properties are sensitive to changes of the environment.

The Yersinia PTPase Is an Ideal System for Spectroscopic Investigation. The *Yersinia* PTPase possesses a desirable property for spectroscopic investigation since it contains only one Trp residue in a polypeptide of 51 kDa. More importantly, the singular tryptophan residue, Trp354, is invariant among all PTPases (Zhang et al., 1994b), and is located on the same flexible loop as is the general acid Asp356 that undergoes a major conformational change when tungstate or sulfate is bound to the enzyme (Stuckey et al., 1994; Schubert et al., 1995). This loop movement brings Asp356 within 3.5 Å of an oxygen atom of the tungstate bound to the active site which is structurally homologous to the scissile oxygen of a phosphotyrosine substrate. Similarly, binding of either phosphotyrosine or a phosphotyrosine-containing peptide to the Cys215 to Ser mutant PTP1B causes a conformational change of an equivalent surface loop that brings the corresponding Asp181 into the catalytic site, which forms a hydrogen bond with the phenolic oxygen of phosphotyrosine (Jia et al., 1995). These results are consistent with the role of the conserved Asp residue acting as a general acid to facilitate the departure of the leaving group. Thus, the *Yersinia* PTPase provides a unique and ideal system to study the active site conformational properties of the wild-type as well as the Cys to Ser mutant PTPases.

Circular Dichroism. Circular dichroism spectroscopy, which is responsive to the contribution of various secondary structural elements, was used in order to evaluate the overall conformation of the *Yersinia* PTPases. To examine the structures of the purified homogeneous recombinant *Yersinia* PTPase, Yop51, and the Cys to Ser mutant, Yop51/C403S, circular dichroism spectra were recorded from 250 to 184 nm (Figure 1). The circular dichroism spectra of Yop51 and Yop51/C403S are nearly superimposable, taking into account the different protein concentrations used. This indicates that Yop51/C403S has the same secondary structure as that of the wild-type enzyme, and that the C403S mutation does not induce gross differences in the overall secondary structure of the protein, in accord with results from crystallographical studies.

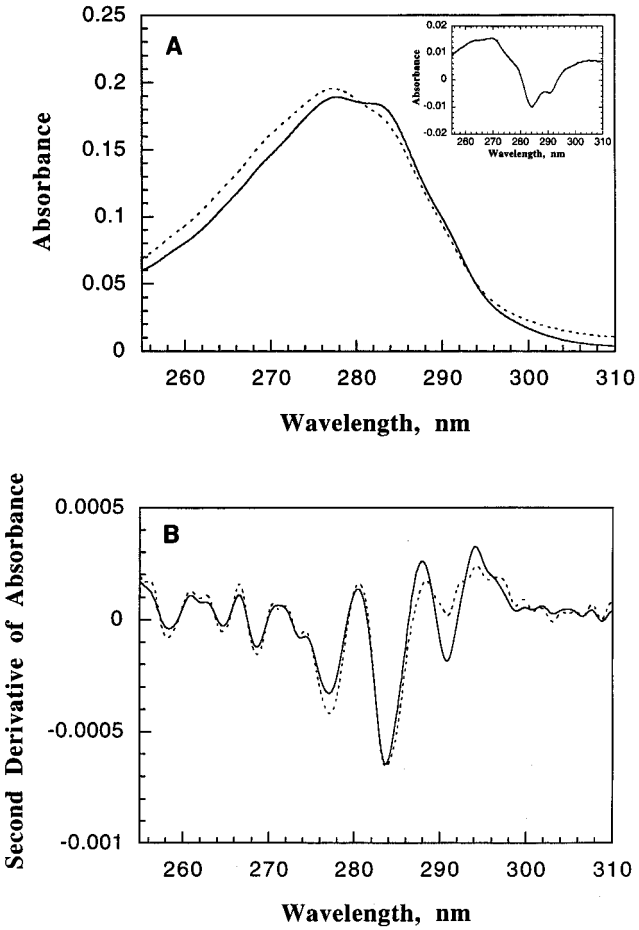

FIGURE 2: (A) Ultraviolet absorption spectra of the *Yersinia* PTPases. Spectra were taken at 25 °C, 50 mM succinate, 1 mM EDTA, $I = 0.15$ M, pH 6.0 buffer. The protein concentration was 0.53 mg/mL for both Yop51 (solid line) and Yop51/C403S (dashed line). Inset: difference spectrum for Yop51/C403S − Yop51. (B) Second-derivative absorbance spectra of Yop51 (solid line) and Yop51/C403S (dashed line).

Ultraviolet Spectra. The ultraviolet spectra of Yop51 and Yop51/C403S are shown in Figure 2A. Unlike a typical protein whose ultraviolet spectrum is usually dominated by contributions from Trp residues, the contributions from Tyr residues to the absorbance in the *Yersinia* PTPases are actually greater than that from Trp354. This is because there are nine Tyr residues and only one Trp residue in the molecule. In fact, the ultraviolet spectra of Yop51 and Yop51/C403S are characterized by two absorption maxima, one corresponding to the Tyr residues (277 nm) and the other corresponding to the Trp residue (283 nm) (Figure 2A). The spectrum of C403S displays different characteristics from that of the wild-type enzyme. Interestingly, although the Cys403 to Ala mutation is more dramatic than the Cys to Ser change, the spectra of C403S and C403A are quite similar (data not shown). The ultraviolet absorption spectrum of the indole ring of tryptophan consists of three or more electronic transitions in the wavelength region 240−290 nm (Cantor & Schimmel, 1980). It is clear that mutations at residue Cys403 perturb the local environment of Trp354 and its electronic interactions with nearby active site residues. This is evident from the decrease in relative contributions from the Trp residue at 283 and 290 nm and the striking increase in absorbance at wavelengths higher than 300 nm in the mutants (Figure 2A, inset). In addition, secondary derivative analysis indicates that the wild-type Yop51 spectrum contains three components at 277, 283, and 290

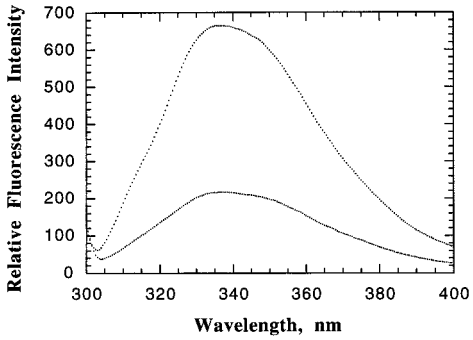

FIGURE 3: Fluorescence emission spectra of the *Yersinia* PTPases. Full-length *Yersinia* PTPases Yop51 (2.00 μM), bottom spectrum, and Yop51/C403S (2.00 μM), top spectrum. All spectra were recorded at 25 °C, pH 6.0, 50 mM succinate, 1 mM EDTA, I = 0.15 M buffer.

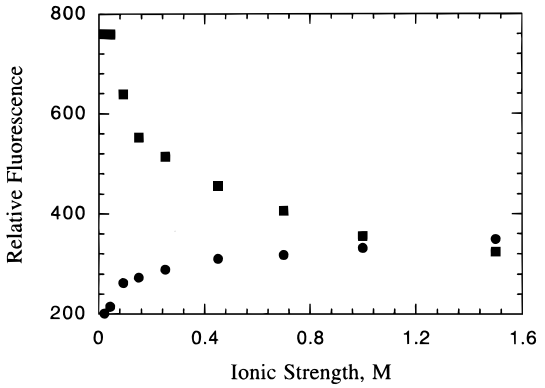

FIGURE 4: Ionic strength dependency of the intrinsic fluorescence of the *Yersinia* PTPases. (●) Wild-type Yop51 (2.32 μM) and (■) Yop51/C403S (2.34 μM). The experiment was performed in 10 mM acetate buffer at pH 5.5 and 25 °C, containing 1 mM EDTA.

nm, while the C403S and the C403A mutants spectra contain only two components at 277 and 283 nm (Figure 2B). The large increase in the 270 nm region of the difference spectrum may also indicate effects on tyrosine residue(s).

Fluorescence Emission. The single tryptophan residue, located at position 354 in the *Yersinia* PTPases, can be selectively excited at 295 nm. The fluorescence emission spectra of the full-length *Yersinia* PTPases Yop51 and Yop51/C403S are shown in Figure 3. Yop51/C403A exhibits a fluorescence emission spectrum similar to that of Yop51/C403S (data not shown). The fluorescence emission spectra of the NH_2-terminal truncated *Yersinia* PTPase Yop51/Δ162 and Yop51/Δ162/C403S are superimposible to those of Yop51 and Yop51/C403S, respectively (data not shown). Excitation at 295 nm results in an emission maximum at 338 nm for both the full-length and the truncated *Yersinia* PTPases. Since under the same conditions the emission maximum for free L-tryptophan is 355 nm, Trp354 in the wild-type and the mutant *Yersinia* PTPases is situated in a more hydrophobic environment and is not completely solvent accessible (Lakowicz, 1983). Interestingly, although alterations at residue Cys403 do not change the emission maximum for the mutants, the fluorescence intensities of Yop51/C403S and Yop51/C403A are enhanced 310% and 340%, respectively, relative to the wild-type Yop51. Similarly, the fluorescence intensity of Yop51/Δ162/C403S is enhanced 310%, relative to Yop51/Δ162. To our knowledge, this corresponds to the largest fluorescence change due to a single atom substitution in a protein. Since tryptophan fluorescence is sensitive to the polarity and the microenvironment surrounding the aromatic amino acid, these results suggest that the local structure surrounding Trp354 in the wild-type *Yersinia* PTPase is different from those of the Cys403 mutants. Furthermore, since the fluorescence (excitation at 295 nm) of the *Yersinia* PTPase arises solely from the single invariant Trp354, the changes in fluorescence in C403S and C403A also suggest that Cys403 and Trp354 exist in close proximity. The average distance between the sulfur of Cys403 and the indole ring of Trp354 is 8.42 Å in the unliganded *Yersinia* PTPase[1] (Stuckey et al., 1994). One could postulate that the *Yersinia* PTPase and the C403S mutant have similar active site structure but the thiolate anion in the wild-type enzyme has a specific quenching effect on the fluorescence of the Trp354 residue. Replacement of the thiolate by a hydroxyl group[2] can thus produce a large increase in Trp354 fluorescence. Although we cannot exclude this possibility, evidence presented in this paper suggests that the altered fluorescence property of the C403S mutant is due to subtle conformational differences between the active sites of the two proteins.

Ionic Strength Dependency. If Trp354 in the C403S mutant adapts the same conformational environment as in the wild-type enzyme, similar ionic strength dependencies of the intrinsic fluorescence should be observed. The fluorescence of the C403S mutant exhibits different ionic strength dependence from that of the wild-type enzyme (Figure 4). The fluorescence of the C403S mutant decreases as the ionic strength increases while the fluorescence of the wild-type enzyme increases as the ionic strength increases. It is worthwhile to point out that in the *Yersinia* PTPase the negative charge of the thiolate anion of Cys403 is stabilized by a number of factors including hydrogen bonds from the main chain NH groups of the active site phosphate-binding loop, an extensive network of hydrogen bonds radiating out from the phosphate-binding loop, and the presence of nearby invariant Arg residues (Stuckey et al., 1994). The reduced negative charge at the active site in the C403S mutant has left these thiolate-stabilizing partial positive charges uncompensated for. Thus, it is understandable that salt would have different effects on the intrinsic fluorescence of the *Yersinia* PTPase and the C403S mutant, since different Coulombic screens are experienced by the Trp354 in the two proteins. However, the detailed mechanism by which the salt exerts its effect is not clear. It is interesting that the fluorescence of the C403S mutant approaches that of the wild-type enzyme at 1 M salt concentration, suggesting that the environment surrounding Trp354 in the two proteins may be similar at high salt concentrations.

Urea and Guanidine Hydrochloride Denaturation. The conformational stability of the *Yersinia* PTPases was studied using both urea and guanidine hydrochloride. Typical urea unfolding curves are shown in Figure 5A. The conformation of the *Yersinia* PTPase is sensitive to perturbation by

[1] The average distance between S^- of Cys403 and the indole ring in the tungstate-bound conformation is 8.41 Å (Fauman et al., 1996). When an oxyanion is bound, the indole ring of W354 optimizes the van der Waals contact with the side chain of R409 by sliding 1−2 Å into the hydrophobic core of the protein and altering the W354 side chain $\chi 2$ torsion angle (Schubert et al., 1995).

[2] We do not know whether the hydroxyl group in Ser403 is ionized at neutral pH. Preliminary experiments show that the pH dependency of C403S fluorescence is not significantly different from that of the wild-type enzyme from pH 3 to 11.

denaturant. For both the wild type and the C403S mutant, the fluorescence intensities of the native forms are higher than those of the unfolded forms. To analyze the urea denaturation curves, a two-state folding mechanism was assumed. This allows the calculation of ΔG as a function of urea concentration from the points in the transition region using the equation: $\Delta G = -RT \ln K = -RT \ln [(f_F - f)/(f - f_U)]$ (Pace, 1986), where K is the equilibrium constant, f is the observed fluorescence intensity, and f_F and f_U are the values of the fluorescence intensities characteristic of the folded and unfolded conformations, which are obtained by extrapolation of the pre- and post-transition base lines into the transition region. For both Yop51 and Yop51/C403S, ΔG was found to vary linearly with urea concentration, as described by the equation: $\Delta G = \Delta G^{(H_2O)} - m$[urea], where $\Delta G^{(H_2O)}$ is the value of ΔG in the absence of urea and m is a measure of the dependence of ΔG on urea concentration. The free energy of unfolding ($\Delta G^{(H_2O)}$) is the most frequently used parameter for quantitating protein stability and comparing stabilities of closely related proteins. For the wild-type enzyme, $\Delta G^{(H_2O)}$ and m were found to be 6.14 ± 0.41 kcal mol^{-1} and 4.80 ± 0.32 kcal mol^{-1} M^{-1}, respectively, while for the C403S mutant, $\Delta G^{(H_2O)}$ and m were found to be 4.02 ± 0.20 kcal mol^{-1} and 4.22 ± 0.18 kcal mol^{-1} M^{-1}, respectively. Thus, Yop51 is 2.1 kcal/mol more stable and shows different sensitivity toward urea than Yop51/C403S. In addition, the urea concentration required to unfold 50% of the protein, $[urea]_{1/2}$, was 1.3 M for the wild-type enzyme and 1.0 M for the C403S mutant, consistent with the wild type being more stable than the C403S mutant.

Guanidine hydrochloride (Gn·Cl) is another often used denaturant, but unlike urea, it is ionic in nature. Thus, as the Gn·Cl concentration varies, so does the ionic strength of the unfolding solution. Since the fluorescence intensities of Yop51 and Yop51/C403S show different ionic strength dependencies, one might expect different Gn·Cl denaturation curves for the two proteins, which is exactly what was observed (Figure 5B). As the concentration of the Gn·Cl increased, the fluorescence emission of the wild-type *Yersinia* PTPase initially increased, reaching a maximum at 0.2−0.3 M Gn·Cl, and then decreased to a final plateau when the protein was fully unfolded. As shown above, when Yop51 is still in its native conformation, increasing the ionic strength enhances the protein fluorescence which leads to the initial rise in fluorescence. The denaturation curve of the truncated *Yersinia* PTPase was identical to those of the full-length molecule as probed by the sensitivity of the tryptophan fluorescence to Gn·Cl (Figure 5B) and urea (Zhang et al., 1992), suggesting that the stability of the PTPase domain is not altered by the elimination of the NH_2-terminal 162 amino acid residues. On the contrary, since raising the ionic strength suppresses the fluorescence of the C403S mutant, a plateau corresponding to the native state (as seen in the urea-induced denaturation) was not observed for the mutant (Figure 5B). Thus, due to the interfering ionic strength effects to the Gn·Cl-induced denaturation, a similar analysis of the unfolding curves is not possible. However, it is apparent that the C403S mutant is more prone to denaturation by Gn·Cl ($[Gn·Cl]_{1/2} = 0.22$ M) than the wild-type enzyme ($[Gn·Cl]_{1/2} = 0.48$ M). Collectively, urea and Gn·Cl unfolding experiments show that the structure of the C403S mutant is significantly less stable than the wild-type PTPase and displays a different sensitivity to urea and Gn·Cl. The abnormal appearances of the Gn·Cl-induced unfolding curves

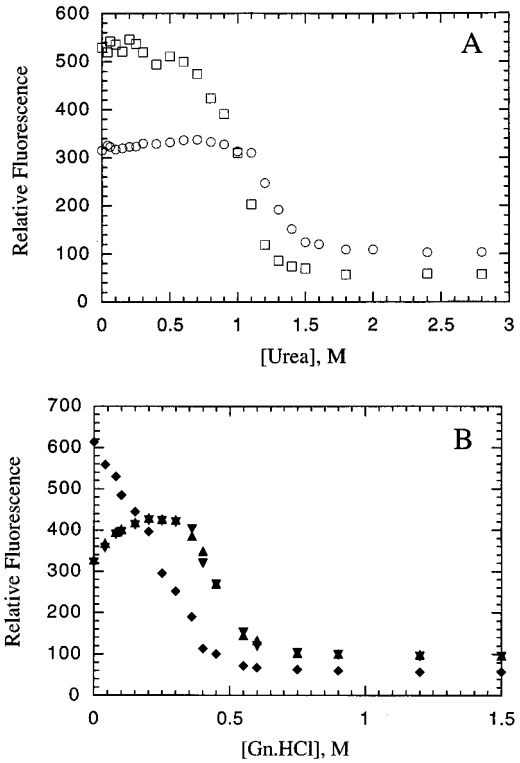

FIGURE 5: Urea and guanidine hydrochloride induced unfolding of the *Yersinia* PTPases. (A) Urea denaturation: (○) Yop51 (3.47 μM); (□) Yop51/C403S (1.87 μM). (B) Guanidine hydrochloride denaturation: (▲) Yop51 (3.47 μM); (▼) Yop51/Δ162 (3.45 μM); and (◆) Yop51/C403S (2.18 μM). Measurements of the relative fluorescence at 340 nm (slit width 5 nm) with an excitation wavelength at 295 nm (slit width 3.5 nm) were obtained as a function of urea or guanidine hydrochloride concentration at pH 6.0, 50 mM succinate, 1 mM EDTA, and $I = 0.15$ M buffer and 25 °C. Denaturation was studied by monitoring the change in fluorescence at 340 nm.

for the wild-type and the C403S mutant are consistent with the different ionic strength dependencies of the fluorescence of the two proteins described above.

Steady-State Quenching by Acrylamide and Iodide. Fluorescence quenching experiments are commonly performed to probe the degree of exposure of tryptophanyl residues to exterior solvent (Eftink & Ghiron, 1981). The *Yersinia* PTPase and the C403S mutant were analyzed for their sensitivity to commonly used quenchers such as iodide and acrylamide. Iodide is large, negatively charged, and hydrated, and it is considered to have access only to surface tryptophans. Its ability to quench also depends on the location of neighboring charged groups. Acrylamide is a polar nonionic quencher that has access to all but the most buried residues. In every case examined in this study, the Stern−Volmer plot showed a linear relationship between fluorescence intensity and quencher concentration. Identical results were obtained whether the full-length or the NH_2-terminal truncated PTPase was used. Typical Stern−Volmer plots for iodide quenching at 1.5 M ionic strength are shown in Figure 6 for Yop51/Δ162 and Yop51/Δ162/C403S. Stern−Volmer quenching constants (K_{sv}) for Yop51/Δ162 and Yop51/Δ162/C403S are summarized in Table 1.

It is clear from the magnitudes of K_{sv} for the *Yersinia* PTPase and the C403S mutant using iodide and acrylamide that Trp354 is fairly exposed to the solvent in both proteins. However, the extent to which it is accessible by the quencher is different. For example, at the ionic strength of 0.2 M, K_{sv}(iodide) for the wild-type enzyme is greater than the

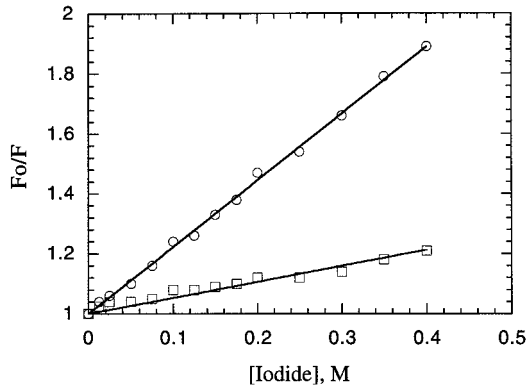

FIGURE 6: Stern−Volmer plots of quenching of *Yersinia* PTPase fluorescence by iodide. (○) Yop51/Δ162; (□) Yop51/Δ162/C403S. Measurements were performed at pH 6.0 in 10 mM succinate, 1 mM EDTA, $I = 1.5$ M.

Table 1: Stern−Volmer Quenching Constants for the Wild-Type and the Mutant *Yersinia* PTPases

K_{sv} (M^{-1})	wild type ($I = 0.2$ M)	wild type ($I = 1.5$ M)	C403S ($I = 0.2$ M)	C403S ($I = 1.5$ M)
acrylamide	1.29 ± 0.03	1.92 ± 0.02	2.14 ± 0.03	1.53 ± 0.03
iodide	5.36 ± 0.04	2.22 ± 0.02	4.34 ± 0.05	0.53 ± 0.03

C403S mutant, whereas the reverse is true when acrylamide is the quencher. Interestingly, when the quenching experiments were performed at an ionic strength of 1.5 M, a condition at which the wild type and the mutant enzyme exhibit similar fluorescence emission (Figure 4), the two proteins still showed different sensitivities toward these quenching reagents. Thus, although the efficiency of iodide as a quencher decreased for both proteins at high ionic strength, the effect was much greater for the C403S mutant than the wild-type enzyme. Further, in contrast to the result at low ionic strength, K_{sv}(acrylamide) for the wild-type enzyme increased at high salt concentration and was greater than for the C403S mutant. These results indicate that Trp354 has different accessibilities in the wild type and the C403S mutant.

Effects of Tungstate Binding. Tungstate is a competitive inhibitor of the *Yersinia* PTPase with a K_i of 61 μM (Zhang et al., 1994a). Trp354 is located on a surface-accessible loop (residues 351−359 between β7 and α4) in the vicinity of the active site region. Upon binding of tungstate, this loop swings like a "flap" to cover the active site. In contrast to the unliganded structure, the loop main-chain atoms in the tungstate-bound Yop51/Δ162 complex move as much as 7 Å toward the active site that positions the Asp356 carboxylate within 3.5 Å of the ester oxygen (Stuckey et al., 1994). One would predict that the spectroscopic properties of the proteins in the presence of tungstate will be different from those in the absence of tungstate, since the binding of the tungstate causes a conformational change that involves the invariant Trp354. Indeed, addition of tungstate to a solution containing Yop51/Δ162 enhanced Trp354 fluorescence (Figure 7A). This is in accord with the structural observation that the loop moves toward the active site upon binding of tungstate, which may reduce the solvent exposure to Trp354 and/or optimize its van der Waals contacts with adjacent nonpolar residues, and thus increase its fluorescence. Furthermore, the fluorescence enhancement was saturable, which allowed the determination of the dissociation constant, K_d, and the maximum difference in fluorescence between the free and the tungstate-saturated PTPase, ΔF_{max} (Experimental Procedures). The K_d was determined to be 55 ± 5 μM, which is similar to the K_i value (61 μM) determined by steady-state kinetics, and the maximum fluorescence enhancement by tungstate was 40%.

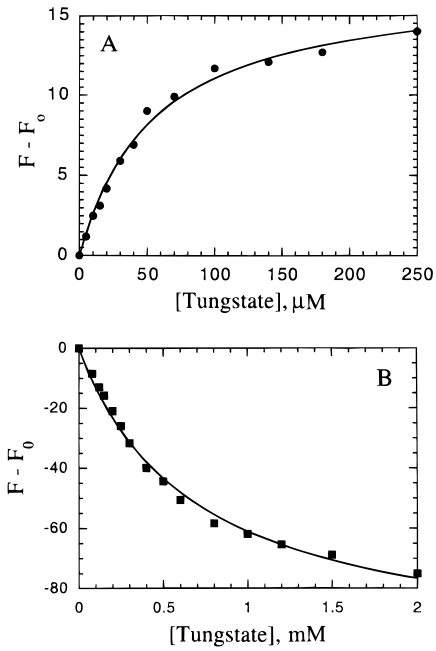

FIGURE 7: Effects of tungstate on the fluorescence of the *Yersinia* PTPases. (A) Yop51/Δ162. (B) Yop51/Δ162/C403S. Measurements of relative fluorescence at 340 nm were obtained as a function of tungstate concentration at pH 6.0, 50 mM succinate, 1 mM EDTA, $I = 0.15$ M buffer, and 25 °C.

Surprisingly, tungstate quenched Trp354 fluorescence in the C403S mutant (Figure 7B). This is surprising because it appears that a similar loop closure also occurs in the Yop51/Δ162/C403S−sulfate complex (Schubert et al., 1995) as well as in the PTP1B/C215S−substrate complex (Jia et al., 1995). The decrease in fluorescence is not due to a nonspecific ionic strength effect, since under the experimental condition ($I = 0.15$ M), increasing the salt concentration by 4 mM [which is similar in ionic strength to the highest sodium tungstate concentration (2 mM) used] did not appreciably change the fluorescence. The tungstate-induced fluorescence quenching of the C403S mutant was also saturable. Interestingly, Yop51/Δ162/C403S exhibited a K_d of 690 ± 50 μM toward tungstate which is 12.5-fold higher than for the wild-type PTPase. The maximum fluorescence decrease caused by tungstate binding was 90%.

The observation that tungstate binding has different effects on the fluorescence properties of the wild-type and the C403S mutant, *i.e.*, the ligand-free *Yersinia* PTPase is less fluorescent than the ligand-bound form while the ligand-free C403S mutant is more fluorescent than the ligand-bound form, further supports the conclusion that the immediate surroundings of Trp354 in the two proteins must be different. From the above results, it would appear that Trp354 in C403S resides in an environment that may differ not only from the wild-type ligand-free form but also from the ligand-bound form. It is noteworthy that the structure of the C403S in the ligand-free form has not been determined. It is possible that in solution, the Trp354-containing loop may prefer a conformation that is different from those observed in the crystal structures. Thus, the 12.5-fold reduced affinity of C403S toward tungstate is most likely due to structural and conformational alterations in the active site. For example,

it is known that Arg409 is involved in oxyanion binding (Zhang et al., 1994a). Changes in the Trp354 conformation may alter its interaction with the hydrophobic moiety of the side chain of Arg409 (Schubert et al., 1995) that could affect the tungstate-binding affinity.

Summary and Conclusion. PTPases feature an essential nucleophilic thiol group. When the sulfur of the active site Cys is converted to an oxygen atom, an inactive PTPase is produced. Since the *Yersinia* PTPase contains only one Trp residue which is invariant among all PTPases and is close to the active site, Trp354 serves as an intrinsic probe for the active site conformation and dynamics. Detailed comparative analyses of the wild-type *Yersinia* PTPase and its C403S mutant using a variety of spectroscopic techniques have led to the conclusion that the active site local conformation in C403S is different from that of the wild-type enzyme. The structural perturbations in the active site are responsible not only for the lack of catalytic activity of the mutant but also for its reduced affinity for tungstate. The negative charge of the thiolate anion may be responsible for holding the phosphate-binding loop in an active conformation. The structural alterations may be due to the inability of the hydroxyl group in Ser403 of the C403S mutant to ionize in the normal pH range and/or due to the fact that C−S and S−H bonds are 0.4 and 0.37 Å longer than C−O and O−H bonds, respectively (Streitwieser & Heathcock, 1985), causing disturbances to the critical alignment and relative positioning of the functional groups in the active site. Thus, structural and electronic factors that are important for the native PTPase activity may not be operational in the Cys to Ser mutant.

This work highlights the extreme susceptibility of the active site structure even to a very conservative mutation in a protein, such as Cys to Ser. Therefore, one cannot be too careful when applying site-directed mutagenesis to the study of structure−function relationship of proteins. Even if the crystal structure of a mutant enzyme appears very similar to that of the wild-type enzyme, significant structural and functional perturbations can still occur. Our results also raise the question whether it is appropriate to utilize the Cys to Ser mutant as a bait in a two-hybrid screen for *in vivo* PTPase substrates. Indeed, a recent biochemical study indicates that stable and high-affinity association between PTP−PEST and its physiological substrate $p130^{cas}$ requires the presence of the Cys residue, rather than a Ser, in the phosphatase active site (Garton et al., 1996). Further detailed NMR, time-resolved fluorescence, and resonance Raman spectroscopic studies of the wild-type and the C403S mutant PTPases should provide added insight into the molecular nature of the structural perturbations in the active site and address the precise conformation and environment of the invariant tryptophan.

ACKNOWLEDGMENT

We thank Drs. Dan Eads, Mark Chance, and Jack Peisach for reading and commenting on the manuscript. We also thank Dr. Mark Chance for help in analyzing the second-derivative spectra of the *Yersinia* PTPases.

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BI9624043