Aromatic disulfides were found to inactivate truncated forms of the SHP-1 and PTP1B phosphatases by reaction with the essential active site cysteine residue. For truncated SHP-1 at pH 5.0, the reaction proceeded through an initial burst phase followed by a slower secondary phase. Our experiments demonstrated that the burst phase corresponded to the reaction of the aromatic disulfide with the active site cysteine. The magnitude of the burst phase was found to measure the active enzyme concentration, and the rate of the burst reflected the reactivity of the active site cysteine. The data were consistent with a mechanism in which an intramolecular disulfide is formed between the active site cysteine and a proximal cysteine during the burst reaction. Aromatic disulfides were found to react with the active site cysteines of full-length SHP-1 and truncated PTP1B also. Using vanadate to mask the active site cysteine, the active enzyme concentration could be assayed by comparing product yields for the reaction with aromatic disulfides in the presence and absence of vanadate at pH 8.0. These findings demonstrate the utility of aromatic disulfides as active site titrants and reactivity probes for tyrosine phosphatases.

The phosphorylation of tyrosine residues of specific proteins is an important element of the signal transduction mechanisms that control cell growth, proliferation, and differentiation (1–3). Tyrosine phosphorylation is controlled by the competing actions of tyrosine kinases and tyrosine phosphatases. Protein tyrosine phosphatases (PTPases,\(^1\)) EC 3.1.3.48 remove the phosphate group from the phosphotyrosyl residues of proteins and contain a signature sequence (I/V)HCXAGXXR(S/T)G which includes an essential cysteine residue that is the active site nucleophile (1). The active site cysteine has an unusually low \(p_K^e\) value (4, 5), and these enzymes have maximal turnover at low pH (6–8). PTPases may be grouped into two classes: receptor-like, transmembrane enzymes having an extracellular domain, a transmembrane region, and one or two intracellular

phosphatase domains; and soluble, non-transmembrane enzymes with a single catalytic domain.

PTP1B was the first tyrosine phosphatase to be identified and belongs to non-transmembrane family of PTPases (9). A subgroup of this family contains two src homology 2 (SH2) domains in addition to a catalytic domain. SH2 domains allow for protein-protein interactions based on tyrosine phosphorylation, and in proteins with catalytic activity SH2 domains can have regulatory roles (6, 8, 11). SHP-1 (also known as PTP1C, SH-PTP1, HCP, and SHP) is a tyrosine phosphatase containing two SH2 domains that regulate the catalytic domain (6, 12–14).

We studied the reaction of full-length and truncated forms of the SHP-1 phosphatase as well as a truncated form of PTP1B with two aromatic disulfides: 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman’s reagent) and 2,2'-dipyridyl disulfide (2-PDS). The truncated form of SHP-1 lacked the regulatory NH\(_2\)-terminal SH2 domain (SHP1ΔNSH2), whereas the truncated form of PTP1B lacked the COOH-terminal domain responsible for membrane localization (PTP1BΔCterm).

Aromatic disulfides (ArSSAr) react selectively with the conjugate base of a thiol (RS\(^-\)) giving a mixed disulfide (RSSAr) and an aromatic thiolate (ArS\(^-\)) whose release may be followed by UV-visible spectrophotometry. The rate of reaction varies with the pH and \(p_K^e\) of the thiol (15). Under acidic conditions, cysteines having “normal” \(p_K^e\) values of 8–9.5 react slowly because only a very small proportion are in the reactive thiolate form. In contrast, because of its low \(p_K^e\) value the essential cysteine residue at the phosphatase active site is ionized to a significant extent at low pH and would be predicted to react rapidly with aromatic disulfides. Thus, we expected that the active site and non-active site cysteine residues of tyrosine phosphatases might be distinguished on the basis of their differential reactivities with aromatic disulfides at low pH.

Spectrophotometric observation of the reaction of aromatic disulfides with the essential cysteine in the phosphatase active site would permit easy measurement of active enzyme concentration, giving information important in kinetic studies. In cases where enzyme activity is lost with time, an active site titration is necessary to determine accurately the active enzyme concentration that is necessary to calculate \(k_{cat}\). Direct observation of the reaction between aromatic disulfides and the essential cysteine would allow these reagents to be used to assess the reactivity of the active site cysteine by measurement of the reaction rate, making them useful tools in kinetic and mechanistic studies of tyrosine phosphatases.

**EXPERIMENTAL PROCEDURES**

Reagents, Enzyme Expression, and Purification—All chemicals were reagent grade and were used without further purification. Full-length SHP-1, SHP1ΔNSH2 (amino acids 107–597), was expressed and purified as described elsewhere (6). PTP1BΔCterm (amino acids 1–321) was...
Active Site Titration of Tyrosine Phosphatases

obtained from Dr. C. Ramachandran (Merek-Frosst Center for Therapeutic Research, Montreal) (16).

Measurement of the Burst in the Reaction of SHP1NSH2 with Aromatic Disulfides—The buffers used were as follows: pH 4.5 and 5.0: 0.5 M NaOAc/HCl, 1 mM EDTA; pH 5.5: 0.5 M succinic acid/NaOH, 1 mM EDTA; pH 6.0: 0.5 M 3,3-dimethylglutaric acid/NaOH, 1 mM EDTA; pH 7.0: 0.2 M bisTrispropane/HCl, 1 mM EDTA; pH 8.0: 0.2 M Tris/HCl, 1 mM EDTA. The DTNB stock solution (10 mM) was prepared by dissolving solid DTNB in pH 5 buffer; 2-PDS stock solution (1 mM) was prepared by adding an aliquot of 2-PDS in methanol (0.1 m) to rapidly stirred pH 5 buffer.

With 1 ml of buffer in the reference cuvette and x ml of enzyme solution and (1 − x) ml of buffer in the sample cuvette, the spectrophotometer (Cary 3B) was zeroed at 410 nm (DTNB) or 342 nm (2-PDS). Aliquots of DTNB or 2-PDS stock solution were added to reference and sample; both cuvettes were well mixed. Data collection was started upon addition of aromatic disulfide to the sample cuvette. For experiments with phenylarsine oxide (PAO), PAO was added to the enzyme stock solution several minutes before reaction with DTNB. For experiments with sodium orthovanadate, pH 5.0 buffer without EDTA containing about 2 mM vanadate was prepared. The differences in the extinction coefficients between DTNB and the TNB product are: Δε410 = 11,860 m−1 cm−1 at pH 5.0 and 13,860 m−1 cm−1 at pH 8.0. When replicate burst measurements were fitted to Equation 1 (see below), the magnitude of the burst was reproducible to within 10% (1σ) and the burst size was reproducible to within 5% (1σ).

Measurements of Activity and Protein Concentration—During the reaction of the PTPases with aromatic disulfides at pH 5.0, activity was assayed using 10 mM pNPP in 50 mM NaOAc/HCl pH 5.0 buffer containing 1 mM EDTA, 1 = 0.15 M (NaCl), at 22 °C by following absorbance changes at 400 nm. Protein concentrations were measured by UV absorption using calculated extinction coefficients based on the numbers of Tyr and Trp residues in the proteins together with average extinction coefficients for these residues (17).

Titration of SHP1NSH2 with DTNB at pH 5.0—A series of samples was prepared containing enzyme (about 40 μM), pH 5.0 buffer, and DTNB (0–10 μM) in septum-sealed vials. The vials were well mixed, flushed with nitrogen gas, and stored in the dark. Residual activity was assayed periodically against pNPP as described above; aliquots (20 μl) from the reaction vials were added to 1 ml of phosphatase assay buffer. For each sample, activity was plotted as a function of time, and the plateau region where activity loss was slow was extrapolated back to zero time to estimate residual activity at the end of the burst reaction with DTNB. Activities were normalized with respect to the activity of a control sample without DTNB at zero time.

Isolation of the Products of the Reaction of SHP1NSH2 with DTNB at pH 5.0—Reaction mixtures containing enzyme and DTNB were assayed periodically against pNPP as described above; aliquots (20 μl) from the reaction vials were added to 1 ml of phosphatase assay buffer. For each sample, activity was plotted as a function of time, and the plateau region where activity loss was slow was extrapolated back to zero time to estimate residual activity at the end of the burst reaction with DTNB. Activities were normalized with respect to the activity of a control sample without DTNB at zero time.

Molecular-Graphical Analysis of the Crystal Structure of PTP1B—The atomic coordinates of the crystal structure of PTP1B (18) were provided by Dr. D. Barford (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and displayed using Insight (BioSym Inc.). To identify non-active site cysteine residues close to the active site cysteine of SHP-1, cysteine residues in the sequence of the SHP-1 catalytic domain were placed at the corresponding positions (based on sequence alignment) in the PTP1B structure. Residues 87, 141, and 242 were changed to Cys; the corresponding positions (based on sequence alignment) in the residues in the sequence of the SHP-1 catalytic domain were placed at the corresponding positions.

RESULTS

Reaction of SHP1NSH2 with DTNB and 2-PDS

Concentration Dependence of the Reaction—The reaction of DTNB with thiols liberates 2-thio-5-nitrobenzoic acid (TNB) (10, 19). Fig. 1A shows a trace of the concentration of TNB released as a function of time for the reaction of SHP1NSH2 with excess DTNB at pH 5.0. The TNB release is biphasic, with a rapid initial phase followed by a slower secondary phase. The reaction of SHP1NSH2 with 2-PDS releasing 2-pyridinedithione displayed similar behavior (data not shown). Reaction of enzyme with aromatic disulfide gave inactive enzyme, but activity was regained upon treatment with dithiothreitol.

To measure the size and rate of the burst, absorbance versus time data, e.g. the 0–5 min portion of the pH 5.0 trace in Fig. 1A, were fitted to an equation having an exponential term (to describe the burst phase) and a linear term (to describe the initial part of the secondary phase) (Equation 1). Both phases were assumed to be pseudo-first-order processes since DTNB was in large excess over enzyme. Data were collected so as to include the entire burst phase but only the initial part of the secondary phase, allowing the secondary phase to be approximated by a linear term. The adjustable parameters were b, the size of the burst; k_b, the rate constant for the burst phase; and k_s, the initial slope of the secondary phase.

\[ A(t) = b(1 - \exp(-k_b t)) + k_s t \]  \hspace{1cm} (Eq. 1)

In the presence of excess DTNB, there was a linear relationship between the size of the burst at pH 5.0 and the concentration of added enzyme. When different enzyme samples were used, a linear relationship between burst size and enzyme activity was found (data not shown), suggesting that DTNB reacted at the active site. At a fixed enzyme concentration, the size of the burst remained constant, whereas the rate of the burst phase increased linearly with DTNB concentration up to a DTNB concentration of about 0.25 mM (data not shown). When the magnitudes of the bursts in the TNB and 2-PDS reactions at pH 5.0 were compared, the difference (≤5%) was smaller than the experimental error.

Effects of a Competitive Inhibitor and a Substrate—In the burst phase at pH 5.0, the reaction of enzyme with DTNB was found to be competitive with both a substrate and a product of the phosphatase reaction. The burst rate was reduced about 5-fold in the presence of 0.37 M sodium phosphate, a product of the phosphatase reaction. When the magnitude of the burst remained essentially unchanged (Fig. 1B). Similarly, phosphotyrosine, a PTPase substrate, slowed the reaction in a concentration-dependent manner but also caused the enzyme to precipitate (data not shown). When the enzyme was denatured with 0.1% sodium dodecyl sulfate before reaction with DTNB, no burst was seen, only a slow reaction.
with a rate similar to that of the secondary phase in the reaction of untreated enzyme (Fig. 1B). Of the amino acid residues in proteins, aromatic disulfides react exclusively with cysteines. Because the burst reaction of DTNB with the PTPase is competitive with both a substrate and a substrate-competitive inhibitor of the phosphatase reaction, we concluded that the burst reaction must involve a cysteine residue at the active site.

**pH Dependence of the Reaction**—The kinetic behavior for the reaction of SHP1\(\Delta\)NSH2 with a large excess of DTNB at pH 5.0 is compared with the behavior at pH 8.0 in Fig. 1A. At pH 5.0, release of TNB was biphasic, whereas at pH 8.0, the release appeared to be monophasic. When allowed to proceed to completion, reactions at both pH values gave the same total amount of TNB release, whereas the extent of reaction at pH 8.0 was 3.4-fold larger than the burst at pH 5.0. The reaction of SHP1\(\Delta\)NSH2 with 2-PDS displayed similar behavior (data not shown). SHP1\(\Delta\)NSH2 has a total of six cysteine residues in its sequence. The process at pH 8.0 was assumed to reflect reaction of all free cysteines in the protein. If all six react with DTNB, this suggests that two cysteines may be involved in the burst reaction at pH 5.0.

Experiments were also performed at various other pH values between 4.5 and 8.0, and the traces of absorbance versus time were fitted to Equation 1. The pH dependence of the burst rate \(k_{\text{cat/K_m}}\) in reactions with DTNB and 2-PDS are shown in Fig. 2. Both pH profiles show high reactivity at pH 8.0 as well as maxima at pH 5.0. At pH \(\leq 6\) the rate represents the burst phase only, whereas at pH > 6 the primary and secondary phases of the reaction could not be separated, and the fitted rate constant was a composite of the rates of the primary and secondary phases. These profiles are reminiscent of the reactivity of the active site cysteine of papain toward 2-PDS under acidic conditions, which gives a bell-shaped pH profile centered at pH 3.75 (20), as well as the pH profile for the reaction of DTNB with iodoacetamide-treated cysteolic phospholipase A\(_2\), which showed a maximum at pH 6.5 as well as high reactivity at pH 9.5 (21).

The pH profile of \(k_{\text{cat/K_m}}\) for the reaction of SHP1\(\Delta\)NSH2 with pNPP substrate also has a maximum at low pH (pH 5.5) (Fig. 2) (6). Although the conditions under which the pH profiles were measured were not identical, the similarity of the profiles strongly suggests that the burst in the DTNB and 2-PDS reactions at low pH, like the reaction with substrate, involved the essential active site cysteine residue. The ionic strength was 0.15 M throughout the pNPP pH profile; higher ionic strength conditions were used for the DTNB and 2-PDS pH profiles, and the ionic strength was not constant across the profiles (for details, see “Experimental Procedures”).

**Correlation between Product Release and Activity Loss at pH 5.0**—To test whether DTNB reacts at the active site, we monitored TNB release and enzyme activity simultaneously during a slow reaction between SHP1\(\Delta\)NSH2 and DTNB at pH 5.0 (Fig. 3). As TNB was released, enzyme activity decreased in a mirror-image fashion. The inset of Fig. 3 is a Tsou plot of the residual activity versus the number of equivalents of TNB released (relative to the size of the burst in reaction of enzyme with a large excess of DTNB at pH 5.0, measured in a separate experiment) (22). The inset shows that activity loss is directly proportional to TNB release and that the enzyme was inactivated completely upon release of 0.95 eq of TNB, i.e. at the end of the burst phase. Control experiments showed that activity loss under the conditions of this experiment but in the absence of DTNB was \(\leq 5\%\) over 2 h. The relationship between the release of 2-pyridinethione and loss of activity in the reaction of 2-PDS with SHP1\(\Delta\)NSH2 at pH 5.0 was also investigated, and similar behavior was observed (data not shown).

These results show that the initial reaction between the PTPase and DTNB or 2-PDS is stoichiometric and results in enzyme that is completely inactive. Thus, the burst reaction at pH 5.0 must involve either the active site cysteine or another cysteine whose reaction completely blocks the active site. From the similarity between the pH profiles of the DTNB reaction and the substrate reaction, the former possibility appears most likely. Site-directed mutagenesis to prepare enzyme in which the active site cysteine was replaced by serine to test this hypothesis directly failed to yield sufficient quantities of protein for reaction with aromatic disulfides.

**Titration of SHP1\(\Delta\)NSH2 with DTNB at pH 5.0**—To learn more about DTNB inactivation of the phosphatase, we titrated PTPase activity against DTNB to determine the minimum concentration of DTNB required to cause complete inactivation. A series of samples at pH 5.0 having identical enzyme concentrations but increasing DTNB concentrations was prepared. The decrease in enzyme activity for each sample was monitored as a function of time, and the plateau region where the activity decrease was very slow was extrapolated back to zero time, thus giving a measurement of the residual activity at the end of the burst phase. Residual activity was normalized with respect to the activity of control sample without added DTNB at zero time and then plotted against the initial DTNB concentration (Fig. 4). The zero activity intercept in the plot of residual activity against DTNB concentration was calculated, giving the minimal concentration of DTNB required to inacti-
active toward pNPP, but activity was regained upon treatment with dithiothreitol. Dithiothreitol treatment of protein isolated after complete reaction resulted in release of TNB. In contrast, protein isolated after the burst phase gave no TNB release, indicating that this protein was not an enzyme-TNB mixed disulfide (ESSAr), a species that would release TNB under these conditions. Both forms of isolated protein behaved as monomers in sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments under nonreducing conditions, so these products were not intermolecular disulfides (ESSE).

Mechanism of Reaction of SHP1ΔNSH2 with DTNB—The stoichiometry and product isolation results presented above are consistent with formation of an intramolecular disulfide during the reaction of the PTPase with DTNB as shown in Fig. 5. In the first step, the active site cysteine would react with DTNB to form an enzyme-TNB mixed disulfide and release 1 eq of TNB. Subsequent rapid attack of another (proximal) cysteine on the mixed disulfide would displace a second equivalent of TNB and form an intramolecular disulfide bond between the active site cysteine and the proximal cysteine. Together, these steps would make up the burst of TNB release and yield 2 eq of TNB. The initial reaction of the active site cysteine would be the rate-limiting step. Other cysteine residues would react slowly with DTNB to form mixed disulfides and release 1 eq of TNB each, giving a ratio of 3:1 in released TNB for complete reaction (six Cys) relative to the burst reaction at pH 5.0 (two Cys). Complete reaction with DTNB at pH 5.0 and 8.0 resulted in the observed release of 3.4 and 3.5 eq of TNB relative to the burst, respectively, and the burst was found to be 1.8-fold greater than the protein concentration determined by UV absorption (Fig. 1A). This could arise if the enzyme sample contained a small proportion of enzyme oxidized at the active site, which would cause TNB release in the burst at pH 5.0 to be low, resulting in a ratio greater than the expected value of 3. Similarly, the TNB release during the burst reaction was found to be 1.6-fold greater than the minimal concentration of DTNB required for complete inactivation, less than the expected ratio of 2. This may also be caused by oxidation of the enzyme sample if the proximal cysteine became partially oxidized, which would reduce the size of the burst more than the concentration of DTNB required to inactivate the enzyme.

Effect of Pretreatment of SHP1ΔNSH2 with Phenylarsine Oxide on the Reaction with DTNB—To obtain evidence for or against the existence of a cysteine residue proximal to the active site as proposed in Fig. 5, we investigated the effect of pretreatment of the enzyme with PAO on the DTNB reaction. PAO is known to react selectively with vicinal dithiols to form stable dithiaarsenane adducts in many cases (23, 24), although an exception has been reported (25). Pretreatment with PAO at pH 8.0 was found to inhibit SHP1ΔNSH2 with IC50 = 5 μM.
Stoichiometry of the Reaction of SHP-1 with DTNB—Like SHP1\Delta NSH2, full-length SHP-1 gives biphasic kinetics in its reaction with DTNB at pH 5.0, and the reaction results in inactive enzyme. The stoichiometry of the burst reaction with respect to enzyme concentration was determined by measuring the size of the burst in the DTNB reaction at pH 5.0 for a freshly purified sample of full-length SHP-1 and comparing with the protein concentration determined by UV absorption. As for SHP1\Delta NSH2, the burst (14.4 ± 1.4 μM) was twice the enzyme concentration determined directly by UV absorption (7.3 μM), confirming that the intramolecular disulfide is formed for full-length SHP-1 also.

Effect of Vanadate on the Reaction of SHP-1 with DTNB—In a complementary experiment, we employed sodium orthovanadate, an active site-directed covalent inhibitor of PTPases known to interact exclusively with the active site cysteine residue (26). Vanadate was found to be ineffective as a SHP-1 inhibitor at low pH, so the effect of pretreatment of SHP-1 with vanadate was investigated at pH 8.0. Fig. 7 shows a comparison of ΔAbs values for complete reaction of DTNB with SHP-1 with and without added vanadate. Relative to the protein concentration determined by UV absorption, 6.1 eq of TNB product were released in the absence of vanadate, and 5.2 eq were released in its presence. We conclude that six cysteines react with DTNB (the SHP-1 sequence contains seven Cys), and one cysteine, the essential cysteine at the active site, is protected from reacting by vanadate. Thus, the concentration of active enzyme can be assayed by comparing the amount of TNB release for reaction with DTNB in the presence and absence of vanadate at pH 8.0.

Reactor PTP1B\Delta Cterm with DTNB

Stoichiometry of the Reaction of PTP1B\Delta Cterm with DTNB; Effect of Vanadate—The reaction of DTNB with another member of the tyrosine phosphatase family, a truncated form of PTP1B, was also investigated. Because of insolubility of this enzyme at low pH, the reaction with DTNB at pH 5.0 could not be investigated. In reactions at pH 8.0, DTNB was found to inactivate the enzyme. As above, sodium orthovanadate was used to mask the active site cysteine residue of PTP1B\Delta Cterm before reaction with DTNB. Fig. 7 shows a comparison of ΔAbs values for complete reaction with and without added vanadate. The protein concentration was determined by UV absorption, as above. Comparison of the concentrations of TNB released in the two reactions revealed that 3.1 eq of TNB (relative to the protein concentration) were released in the absence of vanadate, whereas 2.3 eq were released in its presence. Thus, three cysteines out of a total of six in the PTP1B\Delta Cterm sequence react with DTNB, and vanadate masks the reaction of a single cysteine residue (0.8 eq of TNB released). The amount of released TNB may be lower than the expected value (1.0 eq) because of partial air oxidation of the enzyme sample.

DISCUSSION

Aromatic Disulfides as Active Site Titrants for PTPases

Accurate measurement of active enzyme concentration by active site titration is an important element of kinetic mechanistic studies (27). Since the enzyme solution may contain small amounts of protein impurities or inactive enzyme, a determination of protein concentration is often not satisfactory for this purpose (27). This is particularly true in the case of PTPases where activity is sensitive to air oxidation, and enzyme samples usually contain mixtures of active and inactive enzyme (6). We have demonstrated the measurement of active

We have found that chromatography using PAO covalently bound to agarose (ThioBond Resin, Invitrogen) is an effective final purification step for full-length and truncated forms of SHP-1.
enzyme concentration in three ways: (a) by measurement of the size of the burst phase in the reaction of SHP1ΔNSH2 with DTNB at low pH; (b) by assaying for the minimal concentration of disulfide which results in zero residual activity after reaction at low pH; and (c) by comparison of the extent of reaction of SHP-1 or PTP1BΔCterm with DTNB at high pH in the presence and absence of vanadate.

Measurement of enzyme concentration from the burst of released aromatic thiol at low pH is a rapid technique applicable to relatively concentrated enzyme samples and should be calibrated to determine the stoichiometry of the reaction. This method requires the enzyme to be soluble and relatively stable at pH 5.0. For SHP1ΔNSH2, it was seen that DTNB is approximately 3-fold more reactive than 2-PDS and that the DTNB assay is 1.6-fold more sensitive than the 2-PDS assay while the measured enzyme concentrations agree to within about 5%.

Like the burst measurement, titration of the PTPase activity against aromatic disulfide under acidic conditions is a technique that should be applicable to other PTPases provided they are stable and soluble at pH 5.0. Measurement of active enzyme concentration by comparison of the extent of reaction with DTNB at high pH in the presence and absence of vanadate makes use of the selectivity of vanadate for the PTPase active site (it is a transition state analog) as well as the selectivity of aromatic disulfides for cysteine residues in proteins. This assay and the titration against DTNB give the concentration of active enzyme directly, regardless of the stoichiometry of aromatic thiol release, and their sensitivity is in principle limited only by the sensitivity of the activity assay or DTNB assay and the stabilities of the enzyme. Neither the titration nor the burst measurement is likely to be useful for crude preparations containing significant concentrations of thiols (including the cysteine residues of other proteins) other than the PTPase because the reactivity difference at low pH between the active site cysteine and thiols having normal $pK_a$ values is large (about 150-fold) but not enormous. Nevertheless, because an active site cysteine residue that is reactive under acidic conditions and is subject to inhibition by vanadate is an essential feature of the PTPase family, active site titrations using DTNB or 2-PDS are expected to be widely applicable in this family of enzymes and should facilitate detailed kinetic studies of PTPases.

Aromatic Disulfides as Reactivity Probes for PTPases

In addition to measuring the extent of reaction between the active site cysteine of a PTPase with aromatic disulfides, we have also directly measured the rate of this reaction, thus probing the reactivity of the active site cysteine directly. The pH profile for reaction of the active site cysteine with the aromatic disulfide was obtained at low pH where the primary and secondary phases of the reaction were separated mathematically, and the rate-limiting step of the burst reaction was nucleophilic attack of the essential cysteine residue on the aromatic disulfide. In addition to the similarity between the substrate and DTNB pH profiles, our preliminary data show that SHP1ΔNSH2 gives a greater burst rate than SHP-1 in the reaction with DTNB at pH 5.0, consistent with the 1.8-fold greater maximal $k_{cat}/K_m$ value for pNPP substrate of SHP1ΔNSH2 relative to SHP-1 (6). This suggests that the different reactivities of the two enzymes arise at least in part from differences in the intrinsic reactivities of their active site cysteine residues.

pH profiles for the aromatic disulfide reaction may be used to gain information on the $pK_a$ values of residues involved in catalysis without the complications inherent in determination of $pK_a$ values from substrate pH profiles, as has been demonstrated in research on cysteine proteinases (20). For example, reaction of a PTPase with an aromatic phosphate ester may proceed by a two-step addition-elimination mechanism in which either step may be rate-limiting. In contrast, reaction with aromatic disulfides involves a single-step covalence change, simplifying interpretation of the results.

**Formation of an Intramolecular Disulfide at the Active Site of SHP-1**

Role of the Proximal Cysteine Residue—The data presented above provide chemical evidence for the presence of one or more cysteine residues close to the active site cysteine of SHP-1 which can react with the active site cysteine to form an intramolecular disulfide. Similar stoichiometry for the release of TNB was observed for full-length SHP-1, SHP1ΔNSH2, and SHP1ΔNSH2ΔCSH2, indicating that the proximal cysteine is part of the catalytic domain of SHP-1 and not part of the SH2 domains. Regarding the detailed mechanism of intramolecular disulfide bond formation, our data are consistent with initial, rate-limiting reaction of DTNB with the active site cysteine rather than with the proximal cysteine (as shown in Fig. 5) since the pH dependences of reaction with DTNB and reaction with pNPP substrate are similar at low pH. Subsequent rapid reaction of the proximal cysteine with the active site cysteine/TNB mixed disulfide is likely the result of its proximity to the mixed disulfide rather than to any perturbation of the $pK_a$ of this residue. Vanadate pretreatment of SHP-1 protects only one cysteine (the active site nucleophile) from reaction with DTNB, indicating that the proximal cysteine is able to react with DTNB independently of the active site cysteine at pH 8.0. Because vanadate masks only the active site cysteine for PTP1BΔCterm also, our data do not allow us to speculate on the involvement of a proximal cysteine of this enzyme in the reaction of with DTNB.

**PAO Inactivation and Intramolecular Disulfide Formation**—A degree of selectivity has been noted in the inhibition of PTPases by phenylarsine oxide (2). Some enzymes, like SHP-1, SHP1ΔNSH2, and CD45 (29) are inactivated by micromolar concentrations, whereas others, such as rat leukocyte common antigen-related protein (LAR), are unaffected by millimolar levels of PAO (30). Because PAO reacts with vicinal dithiols, effective inhibition may arise from the presence of one or more cysteine residues proximal to the active site cysteine for enzymes that are inhibited strongly, and it is conceivable that the same paired cysteine residues could also be linked to form an intramolecular disulfide upon reaction with DTNB. These vicinal cysteines may provide an attractive target for the selective inhibition of certain PTPases.

**Formation of an Intramolecular Disulfide at the Active Site**—Formation of an intramolecular disulfide at or near the active site following treatment of an enzyme with aromatic disulfide has been observed previously for 5-aminolevulinic acid dehydratase (31), 6-phosphogluconate dehydrogenase (32), chymopapain (33), and pyruvate kinase (34). Several flavin-dependent reductases such as thioredoxin reductase, glutathione reductase, and dihydrolipoamide dehydrogenase have vicinal thiols at the active site which interconvert between dithiol and intramolecular disulfide states to allow electron transfer during the catalytic cycle (35).

Inactivation of a PTPase by formation of an intramolecular disulfide with the active site cysteine has been observed previously. Inactivation of the low molecular weight PTPase from bovine liver by nitric oxide arises from formation of an intramolecular disulfide between the active site nucleophile, Cys-12, and Cys-17 in the phosphate binding loop (36). Low molecular weight PTPases have only limited sequence homology and no
general structural homology to their higher molecular weight counterparts such as PTP1B and SHP-1 (36), but crystal structures of bovine low Mr phosphatase and PTP1B show local structural similarity (37). There is no counterpart to Cys-17 in the SHP-1 or PTP1B sequences and no cysteines close (in sequence) to the active site, so the intramolecular disulfide in SHP-1 must be formed in a different manner.

**Molecular Graphics Analysis of the PTP1B Structure—**Work is in progress to identify the proximal cysteine residue involved in intramolecular disulfide formation. In the absence of the final result, the crystal structure of PTP1B (18), a non-transmembrane PTPase homologous to SHP-1, may be used to identify the cysteine residues likely to be involved in disulfide with the active site cysteine. When the four non-active site Cys residues of the SHP-1 catalytic domain were placed at the corresponding positions (based on sequence alignment) in the PTP1B structure, two were found close to the active site. Cys-329 was found to be 9.6 Å away (Cα–Cα distance), whereas Cys-363 was 7.9 Å away, and Cys-329 and Cys-363 were separated by 6.1 Å (a Cα–Cα distance in the range of 4.4–6.8 Å would be expected for a disulfide (38)). Thus, if one assumes that the structure of SHP-1 is similar to that of PTP1B, then Cys-363 appears most likely to form a disulfide with the active site cysteine, and disulfide formation between Cys-329 and Cys-363 appears most likely to form a disulfide with the active site cysteine. If this residue is involved in disulfide (38). Thus, if one assumes that the structure of SHP-1 is similar to that of PTP1B, then Cys-363 appears most likely to form a disulfide with the active site cysteine, and disulfide formation between Cys-329 and Cys-363 also appears probable. Cys-363 (which corresponds to Cys-121 in the PTP1B sequence) is highly conserved in the eukaryotic PTPases (18). If this residue is involved in disulfide formation, then our results may have relevance to tyrosine phosphatases other than SHP-1. Some possible consequences of dithiol/intramolecular disulfide interconversion at the active site include regulation of enzyme activity by oxidation of the active dithiol form of the enzyme to an inactive disulfide (28, 35) as well as selective inhibition by bifunctional reagents or active site-directed oxidants.

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