Mechanistic Studies on Full Length and the Catalytic Domain of the Tandem SH2 Domain-Containing Protein Tyrosine Phosphatase: Analysis of Phosphoenzyme Levels and V_{max} Stimulatory Effects of Glycerol and of a Phosphotyrosyl Peptide Ligand†

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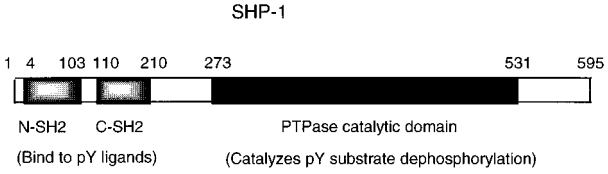
ABSTRACT: SHP-1, a protein tyrosine phosphatase containing two tandem SH2 domains, is autoinhibited at rest by its N-terminal SH2 (N-SH2) domain. Relief from autoinhibition and a subsequent 10−60-fold increase in V_{max} have been observed upon N-SH2 domain engagement by a specific phosphotyrosyl ligand or upon deletion of the SH2 domains to yield the catalytic PTPase domain. In this study, we observed that glycerol and propane-1,2-diols, at concentrations of 4−6 M, accelerated the k_{cat} of the full length enzyme by 47-fold and of the PTPase domain by 8-fold. Glycerol also increases the rate of proteolytic cleavage between the SH2 and catalytic PTPase domains. In stopped-flow studies using *p*-nitrophenyl phosphate (*p*NPP) as a substrate, a burst of *p*-nitrophenolate in the full length enzyme was not observed; however, a 50−70% stoichiometric burst was observed with the PTPase domain. Rapid quench studies using [^{32}P]*p*NPP as a substrate showed a very low level of covalent [^{32}P]phosphocysteinyl enzyme intermediate accumulation: 0.06% in the full length enzyme and 1% in the PTP domain. Stimulation by glycerol reduced the accumulating levels of phosphocysteinyl enzyme in both cases of full length SHP-1 and the PTPase domain; however, glycerol is not acting as a cosubstrate since no glycerophosphate product was detectable. It is likely that, for full length SHP-1, with *p*NPP as a model substrate, enzyme−substrate complex (ES) accumulates in its basal autoinhibited state, whereas enzyme−product complex (EP_i) accumulates in its pY ligand-bound activated state. Glycerol probably relaxes the compact structure of SHP-1 and the PTP domain, thereby accelerating the catalytic rates in both cases by increasing forward reaction rates of ES and EP_i.

Among the families of protein tyrosine phosphatases (PTPases)[1] (Barford *et al.*, 1995) that hydrolyze protein tyrosyl phosphoryl groups, the subfamily containing two tandem SH2 domains upstream of the catalytic domain has received much attention in the study of signal transduction pathways (Parson *et al.*, 1992). To date, this subfamily includes SHP-1 (Shen *et al.*, 1991; Plutzky *et al.*, 1992; Yi *et al.*, 1992; Pei *et al.*, 1993), expressed mainly in hematopoietic cells, and SHP-2 (Freeman *et al.*, 1992; Feng *et al.*, 1993; Vogel *et al.*, 1993; Ahmad *et al.*, 1993), expressed more widely in eukaryotic tissues. These phosphatases are recruited to phosphotyrosyl sequences on the cytoplasmic domains of growth factor receptors [EGFR (Vogel *et al.*, 1993; Bennet *et al.*, 1996; Lechleider *et al.*, 1993; Tomic *et al.*, 1995), IR (Kuhne *et al.*, 1993), and PDGFR (Vogel *et al.*, 1993; Lechleider *et al.*, 1993; Klinghoffer *et al.*, 1995)] and cytokine receptors [*e.g.* IL3R (Yi *et al.*, 1993), EpoR (Klingmüller *et al.*, 1995), and FcγR (D'ambrosio *et al.*, 1995)] by recognition of their SH2 domains, structures of *ca.* 100 amino acids that are able to bind phosphotyrosine (pY) residues [the nomenclature of SHP-1 has been recommended for that previously known as SHP, SH-PTP1, HCP, and PTP1C and that of SHP-2 for Syp, SH-PTP2, SH-PTP3, PTP2C, and PTP1D (Adachi *et al.*, 1996)]. It has been reported that at rest both SHP-1 and SHP-2 have low basal phosphatase activity due to intramolecular autoinhibition by both the SH2 domains and a C-terminal region downstream of the catalytic domain (Pei *et al.*, 1994; Pluskey *et al.*, 1995). Relief from the autoinhibition by specific binding of high-affinity phosphotyrosyl (pY) peptides to the SH2 domains serves as an "off" to "on" switch associated with recruitment of SHP-1 and SHP-2 to their specific target proteins (Pei *et al.*, 1994; Pluskey *et al.*, 1995). The activated enzyme exhibits a k_{cat} increasd by 10−60-fold. Equivalent levels of activation may be achieved by deleting the SH2 domains, consistent with the proposed model that these domains are involved in autoinhibition (Pei *et al.*, 1994; Pluskey *et al.*, 1995).

Scheme 1



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[1] Abbreviations: SH2, Src homology 2; PTPase, protein tyrosine phosphatase; SHP-1, Src homology 2 protein tyrosine phosphatase-1; Epo, erythropoietin; *p*NPP, *p*-nitrophenyl phosphate; pNP^-, *p*-nitrophenolate; pY, phosphotyrosine; P_i, inorganic phosphate.

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All PTPases identified to date have in their active sites a conserved and catalytically required cysteine residue which has been shown by X-ray (Jia *et al.*, 1995; Barford *et al.*, 1994; Stuckey *et al.*, 1994; Su *et al.*, 1994) and by chemical (Cho *et al.*, 1992; Denu *et al.*, 1996) studies to be properly positioned for nucleophilic attack on the substrate phosphate to produce an *S*-phosphocysteinyl covalent enzyme intermediate. This intermediate is subsequently hydrolyzed by water to produce inorganic phosphate and the free enzyme.

In this work, we examined whether the 10−60-fold increase in V_{max} of SHP-1 in the case of N-SH2 deletion and N-SH2 engagement by pY ligands could arise from a detectable rate change of the enzyme phosphorylation or dephosphorylation. In the course of these studies, we have noted that high concentrations of glycerol can also cause *ca*. 50-fold activation of SHP-1 (but not SHP-2) by changing the conformation of the enzyme.

EXPERIMENTAL PROCEDURES

Ultra-pure glycerol was from J. T. Baker. All other chemicals were from Aldrich or Sigma with at least 98% purity. Stopped-flow experiments were performed on an Applied Photophysics instrument; rapid quench experiments were performed on a Kintech rapid quench instrument, and NMR spectra were taken on a Varian 400 MHz instrument with a ^{31}P probe.

[^{32}P]*p*NPP was synthesized according to the procedure described by Wo *et al*. (1992).

Full length SHP-1 (63 kDa), the catalytic domain (residues 273−595), and mutants (R30K, R136K, and ΔCSH2) were overexpressed and purified to near homogeneity as previously described (Pei *et al.*, 1993, 1994, 1996). SHP-2 was purified as described (Pluskey *et al.*, 1995). Enzyme concentrations were determined by the Bradford assay with BSA as the standard.

Activity assays were carried out at room temperature in 50 or 100 μL of HBS buffer (100 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 1 mM mercaptoethanol at pH 7.4) containing 0.01−0.005 mg/mL enzyme, 10 mM or otherwise indicated amount of *p*NPP or pY, and, where applicable, 70−100 μM EpoR pY429 (DPPHLKpYLYLVVSDSK) (Pei *et al.*, 1996) and/or the indicated amount of glycerol. For steady state studies, reactions were stopped at 30 min (or the indicated period of time) after all reactants had been mixed by addition of 1 N NaOH followed by OD measurement at 405 nm. For conversion of A_{405} to initial reaction rates, an ϵ_{405} of 18 500 M^{-1} cm^{-1} was used.

In rapid quench studies, 10 μL of enzyme (80−200 μM) and 10 μL of [^{32}P]*p*NPP (concentrations corresponding to one to two turnovers in single turnover experiments and 50 turnovers in multiple-turnover experiments) were mixed, and the reactions were quenched with 1 N HCl after short time delays. The quenched mixtures were added to 2 × sample buffer and analyzed by SDS−PAGE. (*E*)-*cys*-PO_3^{2-} bands were visualized and quantitated on phosphoimage film. ^{32}P samples of known activity were codeveloped for quantitation.

In stopped-flow studies, enzyme (concentrations varied from 5 to 20 μM) and *p*NPP (50 mM) were mixed and the hydrolysis of *p*NPP was monitored by the release of pNP^- by UV abosorption at 405 nm.

RESULTS

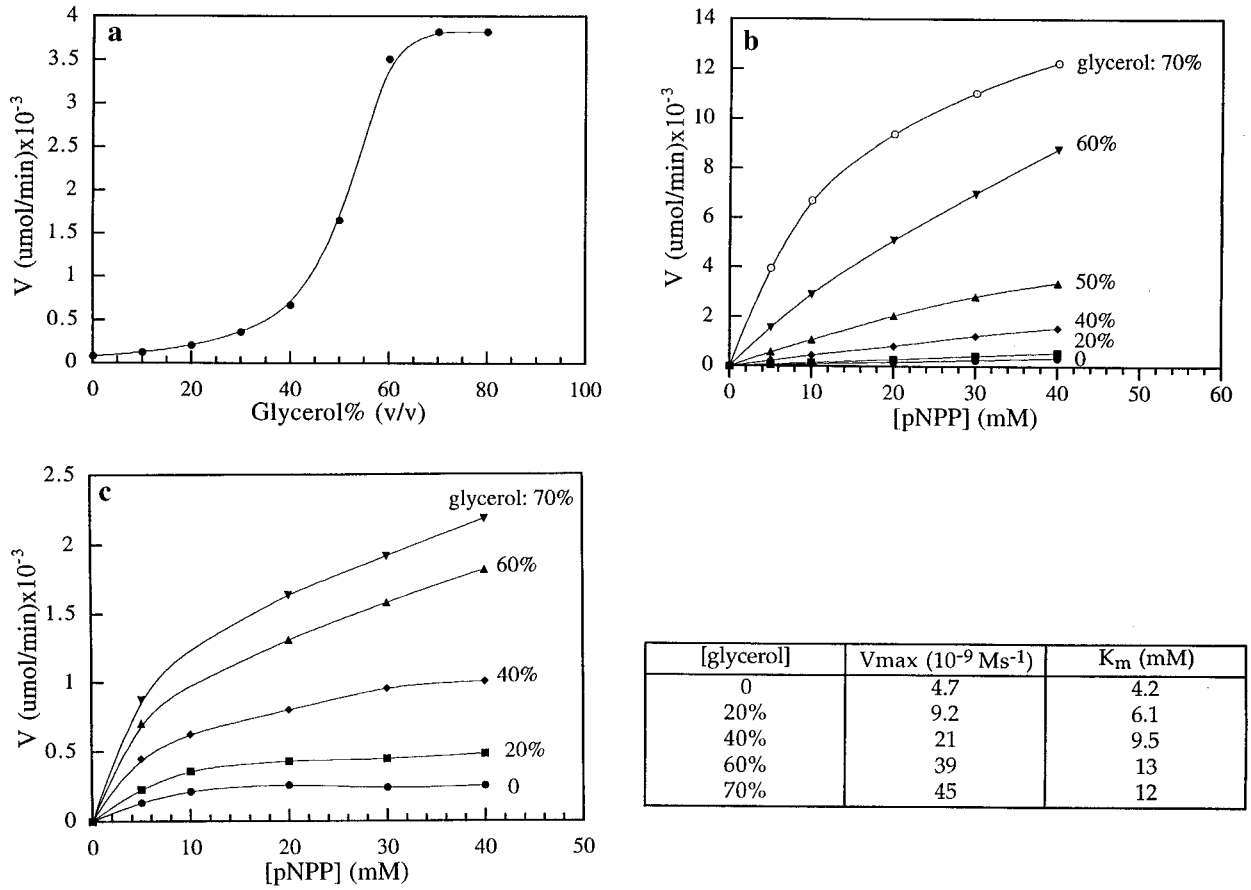
Glycerol Stimulation of SHP-1 Activity. During the course of examination of the accumulation of (*E*)-*cys*-$^{32}PO_3^{2-}$ from the catalytic turnover of *p*-nitrophenol phosphate ([^{32}P]-*p*NPP), discussed below, it was observed that glycerol caused the covalent phosphoenzyme species to dissipate. The data in Figure 1a demonstrate that glycerol, with low affinity (40−50%, or 5−7 M to achieve half-maximal activation), results in a dramatic activation of pure, full length SHP-1, with a 47-fold increase in k_{cat} for processing *p*NPP. When the 322-amino acid catalytic PTPase domain of SHP-1 was assayed (Figure 1c), an 8-fold stimulation was observed. Furthermore, Figure 1b shows that glycerol has both k_{cat} and K_m effects on the full length enzyme, while Figure 1c shows only a k_{cat} effect on the catalytic domain. No such effect was observed with sucrose (up to 35%), an alternate viscogen, arguing against a general viscosity effect. In addition, when pure SHP-2 was assayed, stimulation was not observed; rather, full inhibition of activity by 10% glycerol was observed (data not shown), despite the high homology among these related SH-PTPases [55% overall (Freeman *et al*., 1992)]. Thus, the glycerol effect is specific for SHP-1. On dilution of glycerol from 50 to 10%, the activation effect is fully reversible as activity returns to the basal level.

We have in previous studies disabled the N-SH2 or the C-SH2 domains by R-to-K point mutation (R30K and R136K, respectively) or by deleting the C-SH2 domain to disrupt pY recognition in order to prove that the N-terminal SH2 domain is sufficient to provide the tight basal autoinhibition of PTPase activity (Pei *et al*., 1994, 1996). As shown in Figure 3, glycerol stimulation was observed for all of these mutants, consistent with an activation mechanism that does not require SH2 involvement.

The ability to markedly relieve autoinhibition and to fully activate SHP-1 is not unique to glycerol. As demonstrated in Figure 4, 1,2-diols such as ethylene glycol and (*R*)- and (*S*)-1,2-propanediol substitute for glycerol in the activation of SHP1. However, 1- and 2-propanol do not, nor does the three-carbon 1,3-diol regioisomer, propane-1,3-diol, approach the effectiveness of the 1,2-diols. This pattern argues against merely a solvent or a dielectric effect as an explanation for the observed SHP-1 stimulation by 1,2-diols.

A comparison of the stimulatory effects of glycerol and a pY peptide ligand specific to the N-SH2 domain [DPPHLK-pYLYLVVSDSK, an EpoR pY429 peptide encompassing residues 423−438 around pY429 of erythropoietin (Epo) receptor (Pei *et al.*, 1996)] is shown *vs* increasing *p*NPP concentrations (Figure 2a) and in a time course (Figure 2b). The activity of autoinhibited full length SHP-1 (Figure 2a, line 1) is almost negligible on this scale. When EpoR pY429 is added to the system, the activation curve of line 2 is observed. For comparison, line 3 shows the stimulatory effect of glycerol. It is clear from the time course in Figure 2b that glycerol relief of autoinhibition was more rapid than that elicited by pY ligand engagement of the SH2 domains. When glycerol and EpoR pY429 are added together (Figure 2b), no stimulation above that caused by glycerol is achieved, consistent with our previous interpretation of relief from autoinhibition.

Glycerol Is Not an Alternate Nucleophilic Substrate for SHP-1. Given the dramatic increase in k_{cat} caused by



[glycerol]	Vmax (10^{-9} Ms^{-1})	K_m (mM)
0	4.7	4.2
20%	9.2	6.1
40%	21	9.5
60%	39	13
70%	45	12

FIGURE 1: *p*NPP assay shows activation of SHP-1 by glycerol. Conditions were as follows: SHP-1, 0.16 μM; catalytic domain, 0.13 μM; *p*NPP, 10 mM or as indicated in the figure; and pH 7.4. (a) Initial velocity of SHP-1 is fully activated by 47-fold in 80% glycerol, while half-maximal activation is reached at 50% glycerol. Glycerol (b) affects both k_{cat} and K_m of wild type SHP-1 and (c) affects mainly k_{cat} of the catalytic domain.

glycerol and the 1,2-propanediols, it was plausible that at high concentrations (5−7 M) these diols could compete, as alternate acceptor substrates, with 55 M water in the active site for attack on the *S*-phosphocysteinyl enzyme covalent intermediate to yield in the case of glycerol either α- or β-glycerophosphate coproduct and thereby bypass a rate-limiting enzyme dephosphorylation step. A similar mechanism has been described for the *O*-phosphoseryl enzyme intermediate in alkaline phosphatase catalysis (Neumann, 1969; Wilson *et al.*, 1964). However, ^{31}P-NMR analysis (Figure 5) demonstrated that only inorganic phosphate (P_i) was a product for glycerol concentrations that produce 40-fold V_{max} stimulation, where a rerouting of flux by phosphate transfer to glycerol should have made α- or β-glycerophosphate as the major product (40/41−98%). Neither glycerophosphate isomer is detectable, arguing against a substrate role for glycerol. Further, addition of either α- or β-glycerophosphate to SHP-1 resulted in no stimulation, but rather inhibition that reduced the activity of SHP-1 to less than 10% at 100−200 mM glycerophosphate concentrations (data not shown). Finally, the rate of P_i production paralleled the rate of *p*-nitrophenolate production during glycerol stimulation of SHP-1 (data not shown), consistent with 100% flux to P_i.

Limited proteolysis of SHP-1 by elastase yielded four major proteolytic products (27, 33, 37, and 40 kDa) (Figure 6). N-Terminal sequencing of these fragments indicates that the cleavage sites are between amino acids 300 and 380 which lie in the N-terminal region of the PTPase catalytic domain (Scheme 1). [Among the four products, only the 27 kDa fragment has an N-terminal residue (S378) other than Met 1.] As shown in lanes 3 and 4 of Figure 6, in 50% glycerol, the rate of proteolysis is 5-fold faster than that in water, suggesting that glycerol affects both k_{cat} and K_m of SHP-1 perhaps by relaxing a compact structure of the enzyme. Limited proteolysis with trypsin showed similar results (data not shown). These experiments provide evidence supporting our previously proposed model in which physical contact between the N-SH2 domain and the catalytic domain inhibits the enzyme activity (Pei *et al.*, 1994, 1996).

Detection of Covalent [^{32}P]Phosphoryl Enzyme during SHP-1 Hydrolysis of [^{32}P]pNPP. In rapid quench experiments, [^{32}P]*p*NPP was mixed with the full length or the catalytic domain of SHP-1, and the reaction was quenched by 1 N HCl 1−30 ms later. [^{32}P]*p*NPP, $^{32}P_i$, and [^{32}P]enzyme were separated by SDS gel electrophoresis, and the ^{32}P-labeled enzyme bands were visualized by autoradiography. It should be noted that the intermediate is stable in HCl, but not in NaOH or SDS. As shown in lane 1 of Figure 7, there is a detectable but faint band of radioactive enzyme. The label persists through steady state and disappears as all of the substrate is consumed, as expected for an intermediate. When the PTPase catalytic domain was treated similarly, a substantially more intense radioactive enzyme band was detected (lane 3). When corrections for enzyme concentrations were made, the steady state (*E*)-*cys*-PO_3^{2-} level of activated PTPase catalytic domain is 17-fold higher than that of full length enzyme. This 17-fold increase in the (*E*)-*cys*-PO_3^{2-} concentration during turnover is comparable with the 60-fold higher ratios in V_{max} of PTPase domain/full length

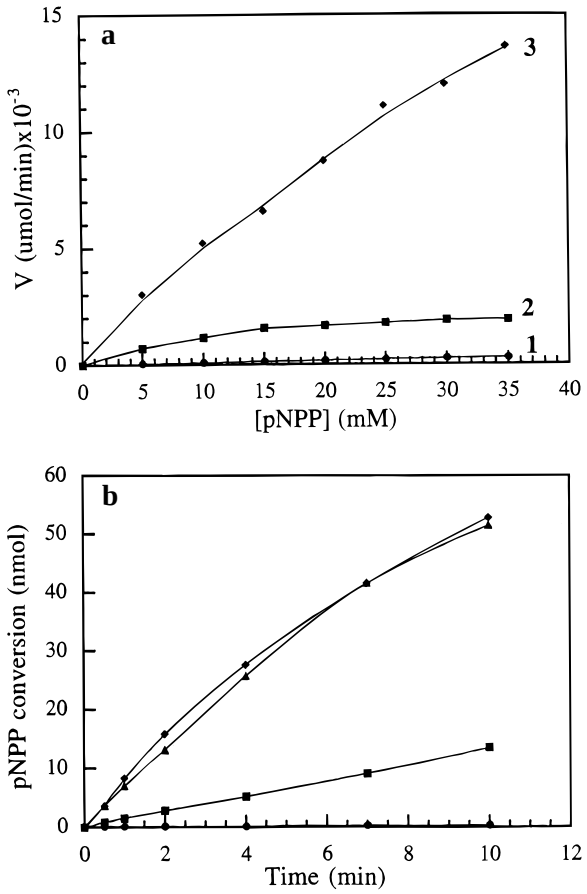


FIGURE 2: Comparison of activation of SHP-1 by glycerol and by EpoR pY429 peptide (without preincubation of the peptide with SHP-1). (a) Rate *vs* *p*NPP concentrations (assays for 30 min at room temperature): full length SHP-1 (●), with 100 μM EpoR pY429 (■), and with 65% glycerol (◆). SHP-1 was at 0.13 μM and pH 7.4. (b) Time course of product (pNP$^-$) formation: full length SHP-1 (●), with 100 μM EpoR pY429 (■), with 70% glycerol (◆), and with 100 μM EpoR pY429 and 70% glycerol (▲). SHP-1 was at 0.13 μM and *p*NPP at 10 mM and pH 7.4.

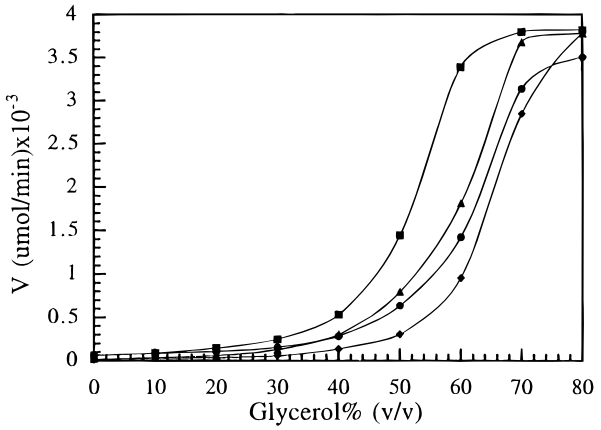


FIGURE 3: Full length mutants [R30K (●), ΔCSH2 (◆), and R136K (■)], in glycerol, and full length wild type (▲) SHP-1s show a similar activation pattern. Concentrations of full length and mutant enzymes were ~0.13 μM; *p*NPP was at 10 mM and pH 7.4.

SHP-1. This suggests that (*E*)-*cys*-PO_3^{2-} dephosphorylation is relatively rate-limiting in the higher turnover conditions of the PTPase domain and also suggests that autoinhibition in full length SHP-1 is due to the slower formation of (*E*)-*cys*-PO_3^{2-}. Quench studies with EpoR pY429 as the activation ligand for full length SHP-1 with [^{32}P]*p*NPP as substrate yielded the data of Figure 8. We observed no

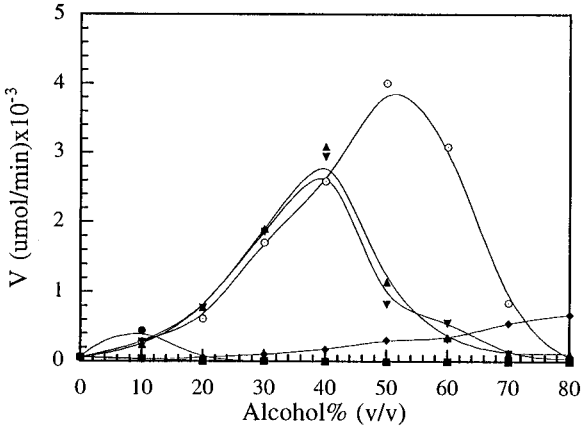


FIGURE 4: 1,2-Diols activate SHP-1: 2-propanol (●), 1-propanol (■), 1,3-propanediol (◆), 1,2-propanediol (△), (*S*)-1,2-propanediol (▼), and ethylene glycol (○). SHP-1 was at 0.13 μM and *p*NPP at 10 mM and pH 7.4.

significant effect in the first 60 s but subsequently observed a 3−4-fold rise in (*E*)-*cys*-PO_3^{2-} levels induced by EpoR pY429. Two aspects are of note. First, the SH2 domain-engaging pY peptide ligand indeed increases the phosphoryl enzyme intermediate concentration, consistent with selective acceleration of a slow enzyme phosphorylation step. Second, the activation by pY ligand has its effect on a time scale that is so slow that there is no way to measure an activating effect on the first turnover. Rather, it is tens of turnovers before full activation is achieved, vitiating possibilities of quenched-flow analysis on the first turnover. The mechanism of this slow activation is not known. When the effect of glycerol stimulation was examined on the steady state level of (*E*)-*cys*-PO_3^{2-} of the catalytic domain, it is clear, from lane 4 of Figure 7, that glycerol acts to lower amounts of (*E*)-*cys*-PO_3^{2-}. The 8−10-fold stimulation of k_{cat} by glycerol does not yield 8−10-fold higher (*E*)-*cys*-PO_3^{2-} levels but rather a lower accumulation. Finally, the absolute calibration of the mole fraction of (*E*)-*cys*-PO_3^{2-} accumulation (peak value, single-turnover) indicated that in the catalytic domain (lane 3, Figure 7) about 1% of the enzyme molecules was present as (*E*)-*cys*-PO_3^{2-}; the full length enzyme (lane 1) accumulates to only 0.06% as phosphoryl enzyme. This low stoichiometry of the intermediate is not ascribable to only a small fraction of the recombinant enzyme [human SHP-1 from *Escherichia coli* (Pei *et al.*, 1993)] being active, given a 50−70% burst of pNP$^-$ in the stopped-flow studies described below. Thus, only a small fraction of enzyme is distributed as (*E*)-*cys*-PO_3^{2-} in turnover even when autoinhibition has been fully relieved by glycerol, by pY peptide ligands engaging the N-SH2 domain, or by proteolysis to yield the catalytic domain.

Stopped-Flow Analysis of SHP-1, Full Length and Catalytic Domain, with pNPP as Substrate. The rate of the production of the colored product, *p*-nitrophenolate anion, can be monitored in stopped-flow experiments to measure the rate of formation of the first mole of product per mole of enzyme as well as the rate of subsequent turnovers. When full length SHP-1 was assayed, no burst was detected (data not shown), with only the slow linear rate of autoinhibited enzyme seen ($k = 8.2 \times 10^{-2}$ s^{-1}). When the PTPase catalytic domain was assayed, there was a burst detected as seen in Figure 9. In multiple experiments, the burst corresponded to 50−70% of the amount of PTPase domain.

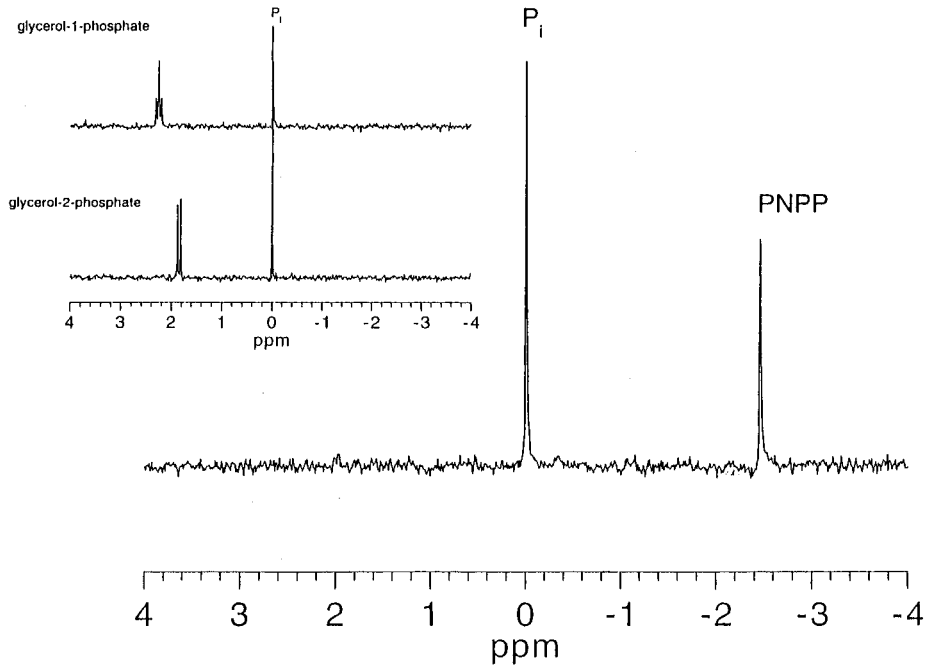


FIGURE 5: NMR spectroscopy indicates no formation of glycerophosphate during hydrolysis of *p*NPP by SHP-1 in 50% glycerol. SHP-1 (2 μM) and *p*NPP (50 mM) were reacted at 30 °C for 2 h before the spectrum was taken. The inset shows spectra of α- and β-glycerophosphate, with P_i at 0 ppm as a standard.

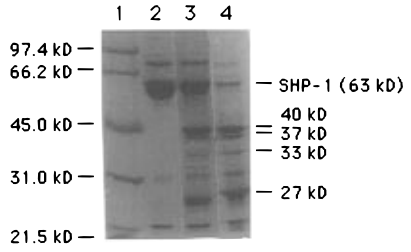


FIGURE 6: Limited proteolysis of SH-PTP1 by elastase in 50% glycerol (lane 4) is faster (*ca.* 5×) than that in HBS buffer (100 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 1 mM mercaptoethanol at pH 7.4) (lane 3). To 25 μg of SHP-1 in 20 μL of HBS or 50% glycerol was added 0.1 unit of elastase (Sigma), and it was digested at room temperature for 10 s. The reactions were stopped by adding 20 μL of 10% SDS. Digestion products were separated on a 12% acrylamide gel, blotted to a nitrocellulose film, and submitted for N-terminal amino acid sequencing. Lane 1, molecular marker; and lane 2, full length SHP-1.

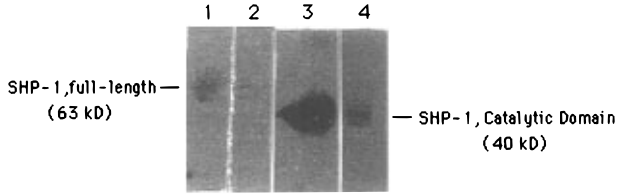


FIGURE 7: Autoradiography of the [^{32}P]phosphocysteinyl enzyme intermediate showing that glycerol, during multiple turnovers, activates both full length SHP-1 (lanes 1 and 2) and its catalytic domain (lanes 3 and 4) but decreases (*E*)-*cys*-PO_3^{2-} levels in both cases. (lane 1) SHP-1 (19 μM) and [^{32}P]*p*NPP (688 μM) (specific activity of 12 Ci/mol) were reacted for 30 s in pH 7.4 HBS buffer at room temperature followed by quenching with 1 N HCl. The mixture was then loaded to a 12% acrylamide−SDS gel and run under 200 V. The radioactive band was visualized on a phosphoimage film. (Lane 2) Same reaction condition as described for lane 1, but with 50% glycerol. (Lane 3) Same reaction condition as described for lane 1, but with 25.8 μM catalytic domain instead of full length SHP-1. (Lane 4) Same reaction condition as described for lane 3, but with 50% glycerol. Each lane contains *ca.* 30 μg of enzyme.

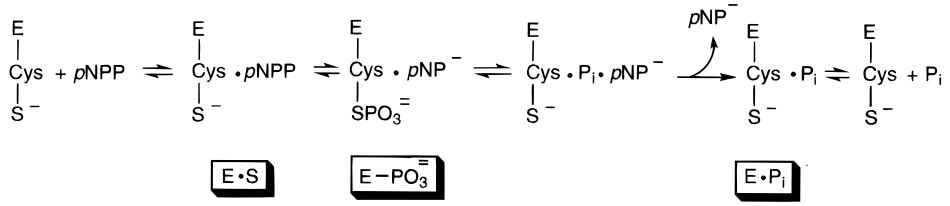
The subsequent turnovers occurred at 2.7 s^{-1}, consistent with the steady state turnover rate of the catalytic domain. The initial phase is fit to 8.1 s^{-1}, predicting a burst of 58%. Thus, it appears that essentially all the molecules of the SHP-1 catalytic domain are active, allowing interpretation of the 1% (*E*)-*cys*-PO_3^{2-} levels as an issue of distribution of enzyme species rather than inactive enzyme fraction. Given that the full length enzyme can be activated in the presence of EpoRpY429 to a k_{cat} value equivalent to that of the catalytic domain, it is likely that all of the full length SHP-1 molecules in the population of recombinant SHP-1 are also active.

DISCUSSION

SH2-containing SHP-1 and SHP-2 have a modular architecture (Scheme 1) reminiscent of many types of enzymes that participate in signal transduction pathways. These enzymes have a catalytic domain and one or more other domains that are involved in docking/regulation. In SHP-1, the SH2 domains serve both a recruiting (N-SH2 and C-SH2) (Vogel *et al.*, 1993; Bennet *et al.*, 1996; Lechleider *et al.*, 1993; Tomic *et al.*, 1995; Kuhne *et al.*, 1993; Klinghoffer *et al.*, 1995; Yi *et al.*, 1993; Klingmüller *et al.*, 1995; D'ambrosio *et al.*, 1995) and a regulatory role (N-SH2 domain of SHP-1) (Pei *et al.*, 1996). In particular, the SH2-containing PTPases, like the great majority of regulatable protein kinases, are off in the basal state by virtue of intramolecular autoinhibition by an adjacent domain (Shokat, 1995). We have shown, by deletion mutation which yields an activated catalytic domain, and by the use of specific pY peptides binding to the SH2 domains which induces full activation, that the SH2 domains serve that autoinhibitory function for both SHP-1 and SHP-2 (Pei *et al.*, 1994, 1996).

All PTPases investigated have conserved active site cysteines shown to be the catalytic nucleophiles such that transfer of pY phosphoryl groups to water proceeds through a covalent *S*-phosphocysteine intermediate in the PTPase active sites (Jia *et al.*, 1995; Barford *et al.*, 1994; Stuckey *et al.*, 1994; Su *et al.*, 1994; Cho *et al.*, 1992; Denu *et al.*, 1996;

Scheme 2



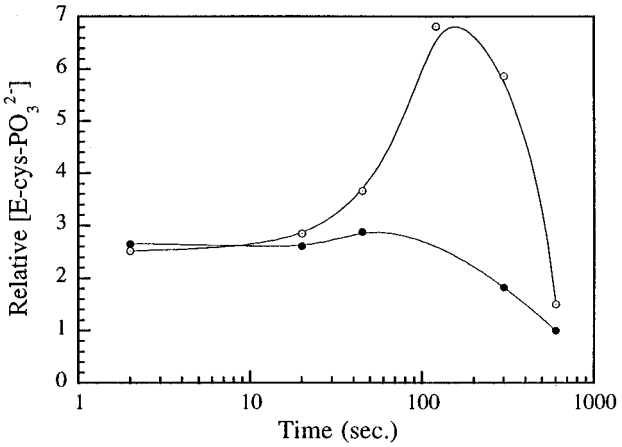


FIGURE 8: Slow activation of SHP-1 upon EpoR pY429 peptide engagement by the SH2 domain. The ○'s show the time course and relative amount of phoshoenzyme in the presence of 70 μM EpoR pY429, while the ●'s are without EpoR pY429 stimulation. The relative (*E*)-*cys*-PO_3^{2-} concentration is determined by the ratio [(*E*)-*cys*-PO_3^{2-}]/[(*E*)-*cys*-PO_3^{2-}]$_{10min,\ without\ pY}$. SHP-1 was at 63 μM and [^{32}P]*p*NPP at 1.1 mM (specific activity of 12 Ci/mol) and pH 7.4. Reactions were quenched at different time intervals with 1 N HCl, and the mixtures were separated on a 12% acrylamide gel. The [^{32}P]phosphocysteinyl enzyme intermediate was detected on a phosphoimage film. Each lane contains *ca.* 100 μg of enzyme.

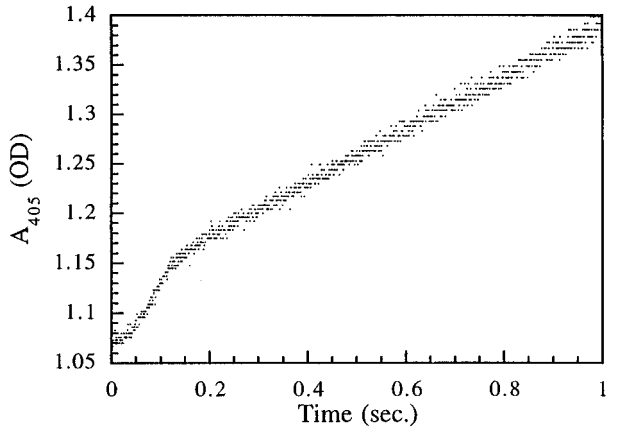


FIGURE 9: Stopped-flow experiments show a burst phase for the catalytic domain of SHP-1. The burst corresponds to 70% of total enzyme. Enzyme (5.3 μM) and *p*NPP (50 mM) in HBS buffer (100 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 1 mM mercaptoethanol at pH 7.4) at 25 °C.

Zhou *et al*., 1994). For the highly active PTPases, of which the small *Yersinia* PTPase is a paradigm (Zhang *et al*., 1994), where the k_{cat} of 90 s^{-1} is more than 10^3 faster than that of full length SH-PTPases (8.2×10^{-2} s^{-1}), the breakdown of (*E*)-*cys*-PO_3^{2-} appears to be substantially rate-limiting in catalysis (Zhang *et al*., 1994). In this work, we have studied whether SHP-1 activation by pY ligand, by SH2 domain deletion, and by the glycerol effect correlated with chemical steps and with the mole fraction of the phosphoryl enzyme intermediate.

The initial stopped-flow spectrophotometric analyses on *p*-nitrophenolate production from the *p*NPP substrate confirmed qualitative expectations that the full length SHP-1 operating at a slow speed would not show a burst while the 40−60-fold more activated catalytic PTPase domain would. These data validated the stoichiometry, the population of active enzyme molecules, and gave rate constants indicating that $k_{phosphorylation}/k_{dephosphorylation}$ ratios of 4/1 were closely balanced in the on state. It is likely that $k_{phosphorylation}$ is much slower in the off state of full length SHP-1. Full length SHP-1 in reactions with saturating levels of EpoR pY429 peptide gave a hint of a burst (∼13% of full stoichiometry, data not shown).

When we turned to rapid quench analyses with [^{32}P]*p*NPP and SDS gel electrophoresis to monitor the kinetics of appearance and disappearance of (*E*)-*cys*-PO_3^{2-} and the mole fraction accumulating through the course of a catalytic turnover, we could detect low levels of [^{32}P]phosphoryl enzymes: very low (0.06%) for the full length and only about 1% for the catalytic PTPase domain. Consistent with the *p*NP^- burst, these data strongly imply that phosphoryl enzyme breakdown is not rate-limiting even in the on state, which is 50-fold more active than the off state. One possible explanation is that shown in Scheme 2 for *p*NPP hydrolysis by SHP-1. Given low (*E*)-*cys*-PO_3^{2-} levels and yet a sizable burst of *p*NP^- (50−70%), the catalytic domain of PTPase may distribute itself mostly as the EP_i product complex, limited by the P_i dissociation rate. This is the case for alkaline phosphatase with certain substrates where, even though a covalent phosphoenzyme forms in each cycle, it is the release of P_i product at 15−60 s^{-1} that limits turnover (Han *et al.*, 1995). In the full length off state, SHP-1 would most likely accumulate as the ES complex.

Attempts to observe the buildup of the [^{32}P]phosphoryl enzyme intermediate in rapid quench studies and *p*NP burst in the stopped-flow studies with full length SHP-1 and EpoR pY429 peptide were unsuccessful in the first or first few turnovers. Indeed, as shown in Figure 8, one had to observe for a good 60 s before (*E*)-*cys*-PO_3^{2-} levels build up in enzyme stimulated by EpoR pY429. The cause of this slow rise time is currently unknown.

The Ser-*O*-phosphoenzyme intermediate in alkaline phosphatase can be intercepted by a series of alcohols which compete with water at the enzyme active site when they are present in substantial mole fractions (*e.g.* 1−2 M) in the medium and yield alcohol−phosphate products (Neumann, 1969; Wilson *et al.*, 1964). Given the 50-fold stimulation of V_{max} in full length SHP-1 by glycerol and by 1,2 (but not 1,3-)-propanediols, it seemed a reasonable mechanistic premise that interception of the *S*-phosphocysteinyl enzyme intermediate at 5−7 M alcohol concentrations was similarly accounting for the stimulation. In the event, glycerol and by inference the 1,2-propanediols are *not* alternate substrates. The potential glycerol phosphate products are indeed inhibi-

tors of the catalytic reactions. Glycerol acts to accelerate k_{cat} for the catalytic PTPase domain as well and decreases the steady state (*E*)-*cys*-PO_3^{2-} level, perhaps accelerating both P_i production and release from the active site.

Given the low affinity of glycerol and 1,2-propanediols toward the full length SHP-1 and the catalytic domain, it may be difficult to ascribe a specific effect to these glycols. Clearly, they increase the susceptibility of enzyme to proteolysis. Also, they activate the enzyme above the level provided by relief from autoinhibition by the tandem SH2 domains since the catalytic domain is also activatable by glycerol. A precedent from Zhao and Fischer on the dramatic activation (up to 1000-fold) of SHP-1 by anionic phospholipids, such as phosphatidylserine and/or phosphatidic acid, could be relevant (Zhao *et al.*, 1993). These phospholipids in vesicles actually totally inhibit activity of SHP-1 toward *p*NPP and pY peptides (Zhao *et al*., 1993), presumably by phase incompatibility, so we cannot test the parallel to glycerol directly. For myelin basic protein and MAP kinase as dephosphorylation substrates, there was specific activation of k_{cat} by 1000-fold, presumably due to some interfacial activation phenomena. More recently, the observation was made that SHP-1 associated with the EGF receptor showed low phosphatase activity as judged by [^{32}P]pY levels in the EGFR substrate. Addition of phosphatidic acid vesicles activated the phosphatase (Tomic *et al*., 1995). Thus, it appears that SHP-1 is activatable in two ways. One attends the recruitment to pY sites on partner proteins where ligand engagement relieves autoinhibition. Second, for those protein substrates at membrane interfaces (*e.g.*, the cytoplasmic tails of both the cytokine receptors and transmembrane tyrosine kinase receptors), the presence of patches of acidic phospholipids will also drive an activating conformational change. This may be mimicked by glycerol. In the activated forms of SHP-1, product release, both of dephosphorylated protein and of inorganic phosphate, may limit catalysis.

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