Problem Set 2 (**Due January 28th**)

1. The KEGG Pathways website can also be used to investigate signal transduction. For example, we can use this website to learn about the p53 (a central protein in regulating the cell cycle) signaling network. Navigate to the p53 Signaling Pathway page from the KEGG Pathway homepage.
    a. What extracellular stress signals can induce a response from p53?
    b. P53 can induce apoptosis in a mechanism that is dependent on communication to the mitochondria AND a mechanism that is independent of the mitochondria.
        i. The mitochondria-dependent pathway can be stimulated by multiple pathways, but they all converge on one mitochondrial protein (which is actually ejected from the mitochondria). What is this protein?
            1. This protein is specifically implicated in one disease (this is in the disease box, NOT the Pathways box). Name this disease and briefly describe what it is.
            2. The next protein in the signal cascade is CASP9. In the definition box, you can learn what kind of enzyme this is. What chemical reaction does this protein catalyze?
        ii. What two proteins are involved in the mitochondria-independent pathway?
            1. IGF-1 can stimulate PKB (Akt). Please rationalize this observation (the full name of IGF might help you make the connection).

2. GPCR:
    a. Clearly describe how GPCRs work. In your description, make sure to comment on the different types of alpha subunits (i.e. G_s, G_i, G_q).
    b. Discuss the parallel role of PLC and AC in GPCR nucleated signal transduction cascades.

3. Adaptor proteins:
    a. What is the role of adaptor proteins in signal transduction?
    b. Name the two adaptor proteins that were discussed in lecture and identify what important interaction each make.
    c. If either of these proteins have important structural domains, identify them and discuss their role.
4. Calcium as a secondary messenger:
    a. Calcium is released by an IP3 gated Calcium Channel in the ER. Clearly explain what this means and why it is relevant.
    b. Inspect the structures of the IP3 binding domain in the unbound (pdbID 3UJ4) and bound (pdbID 3UJ0) forms. Describe major differences that are important in calcium release. You may find it helpful to read the primary literature citation (available here).
    c. Describe how Ca^{+2} is able to activate kinases. In your answer, please discuss the relevant calcium binding motif.
5. Compare signaling in prokaryotes and eukaryotes. What are the similarities and how do they differ?

6. A binding study was carried out to determine the binding properties of an adrenergic agonist interacting with the epinephrine receptor. Using the following experimental data, determine the affinity that the receptor has for the agonist. Assume that no competing interactions occur.

$[Ligand]_{total}$ (nM)	$[Ligand]_{bound}$ (nM)
26	25.3
65	62.28
103	99.26
142	136.24
181	173.22
221	210.2
261	247.18
302	284.16
344	321.14
388	358.12
435	395.1
486	432.08
547	469.06
633	506.04
815	543.02

7. Familiarize yourself with the following article (Huyer, G., et al. *J. Biol. Chem*. **272**, 843-851).
    a. What reaction does PTP1B catalyze? Noting that a phosphocysteine is formed in the active site as an intermediate, propose a mechanism for this reaction.
    b. Describe the technique that was used PTP1B Activity assay.
    c. Figure 1 clearly shows that vanadate affects the enzyme activity. Discuss the purpose of the experiment show in panel B.
    d. Still referring to Figure 1, comment on the affect EDTA has on the reaction. Why is EDTA influencing the experiment?
    e. Refer back to the mechanism you proposed in 7a. Do the conclusions drawn from the mass spectrometry experiments support your mechanism? Please justify your claim.
    f. Figure 3 establishes that vanadate is a(n) _____ inhibitor of PTP1B.
    g. The article points out that Pervanadate has insulin-mimetic properties. Justify this claim based on what you learned in the article.

Enzymology:

Mechanism of Inhibition of Protein-tyrosine Phosphatases by Vanadate and Pervanadate

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Mechanism of Inhibition of Protein-tyrosine Phosphatases by Vanadate and Pervanadate*

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Vanadate and pervanadate (the complexes of vanadate with hydrogen peroxide) are two commonly used general protein-tyrosine phosphatase (PTP) inhibitors. These compounds also have insulin-mimetic properties, an observation that has generated a great deal of interest and study. Since a careful kinetic study of the two inhibitors has been lacking, we sought to analyze their mechanisms of inhibition. Our results show that vanadate is a competitive inhibitor for the protein-tyrosine phosphatase PTP1B, with a K_i of 0.38 ± 0.02 μM. EDTA, which is known to chelate vanadate, causes an immediate and complete reversal of the inhibition due to vanadate when added to an enzyme assay. Pervanadate, by contrast, inhibits by irreversibly oxidizing the catalytic cysteine of PTP1B, as determined by mass spectrometry. Reducing agents such as dithiothreitol that are used in PTP assays to keep the catalytic cysteine reduced and active were found to convert pervanadate rapidly to vanadate. Under certain conditions, slow time-dependent inactivation by vanadate was observed; since catalase blocked this inactivation, it was ascribed to *in situ* generation of hydrogen peroxide and subsequent formation of pervanadate. Implications for the use of these compounds as inhibitors and rationalization for some of their *in vivo* effects are considered.

Protein-tyrosine phosphorylation plays a central role in regulating a variety of fundamental cellular processes (1–3). The tyrosyl phosphorylation state of a protein in the cell reflects the balance between the competing activities of the protein-tyrosine kinases and the protein tyrosine phosphatases (PTPs).[1] Substantial progress has been made in understanding the function of protein-tyrosine kinases, while increased attention has only been recently focused on the PTPs. The identification of a large family of PTPs (4), comprising two distinct groups, arose from the purification and sequencing of human placental PTP1B (5–7). The first group of PTPs has been described as receptor-like with a single transmembrane domain, one or two intracellular catalytic domains, and a unique extracellular domain of variable length. Several PTPs in this group also contain regions representing putative ligand binding domain motifs. The second group consists of cytoplasmic enzymes, having a single catalytic domain and a variable amino- or carboxyl-terminal regulatory domain.

PTPs are specific for the dephosphorylation of phosphotyrosyl residues of proteins and peptides (reviewed in Refs. 8–11). The hallmark of PTPs is an essential cysteine residue at the catalytic site, which forms a thiol-phosphate intermediate during catalysis (12, 13). Since reducing conditions usually maintained by thiol reagents are necessary for keeping the enzyme active, thiol-oxidizing agents are potent PTP inhibitors. Vanadate (VO_4^{3-}) is a general PTP inhibitor (14), whose mechanisms of action have not been investigated in detail. Vanadate is a phosphate analog and is generally thought to bind as a transition state analog to the phosphoryl transfer enzymes that it inhibits, since it can easily adopt a trigonal bipyramidal structure (15). Vanadate has a wide variety of effects on biological systems (16). It is insulin-mimetic (17) and has been shown in human clinical trials to be potentially useful in treating both insulin- and noninsulin-dependent diabetes mellitus (18). It has been suggested that part of vanadate's insulin-mimetic effect may be due to its inhibition of PTPs (19). Another inhibitor on which considerable attention has been recently focused is pervanadate (a general term for the variety of complexes formed between vanadate and hydrogen peroxide). Pervanadate is also insulin-mimetic and appears to be more effective than vanadate in increasing the level of cellular tyrosine phosphorylation (20–25). Peroxovanadium complexes containing one or more chelating ligands in addition to the oxo and peroxo ligands are generally more stable and exhibit even more potent insulin-mimetic effects (26–28).

While vanadate and pervanadate have been widely studied for their insulin-mimetic effects and are commonly used as general inhibitors of PTPs, their mechanisms of inhibition have not been carefully studied. Here we show that vanadate and pervanadate inhibit PTPs by completely different mechanisms. Furthermore, common buffer components such as EDTA and reducing agents can interact with these inhibitors, greatly affecting their potencies. These medium effects and the different modes of inhibition of vanadate and pervanadate have important implications for their use and provide a rationalization for their different effects both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Vanadium(V) oxide (99.99%) and hydrogen peroxide (30%) were from Aldrich. Bovine liver catalase was from Sigma, and

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[1] The abbreviations used are: PTP, protein tyrosine phosphatase; DTT, dithiothreitol; FDP, fluorescein diphosphate; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; capillary LC-MS, capillary HPLC-electrospray ionization mass spectrometry; VL, monoperoxovanadate; VL_2, diperoxovanadate.

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sequencing grade trypsin was obtained from Boehringer Mannheim. All other chemicals were of reagent grade from Sigma.

Expression and Purification of PTP1B—The catalytic domain of PTP1B, consisting of amino acids 1–321, was inserted into the *Eco*RI site of the pFLAG-2 vector (Kodak). Bacterial cultures were grown in Terrific Broth (29) containing 100 μg/ml ampicillin and 0.4% glucose at 37 °C, 225 rpm, for 2–3 h (A_{600} = 0.4–0.8). The culture was then induced with the addition of isopropylthio-β-D-galactoside to 0.5 mM and grown overnight at 27 °C, 225 rpm. The induced culture (500 ml) was centrifuged at 4 °C for 20 min at 6000 × *g*. All subsequent steps were carried out at 4 °C. The cell pellet was resuspended in 25 ml of buffer A (10 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, 0.67 mg/ml lysozyme, 2 mg/ml each pepstatin and aprotinin, 1 mM DTT), and then 5 ml of buffer B (1.5 M NaCl, 0.1 M $MgCl_2$, 0.1 M $CaCl_2$, 250 mg/ml DNase I) were added. The resuspended cell pellet was sonicated (5 × 10-s bursts), incubated on ice 10–15 min or until no longer viscous, and centrifuged for 15 min at 43,000 × *g*. The supernatant was then loaded onto a 17.5-ml M2 FLAG monoclonal antibody affinity column (Interscience), the column was washed with phosphate-buffered saline (10 column volumes), and the FLAG-PTP1B fusion protein was eluted in phosphate-buffered saline by competition with the FLAG peptide as described by the manufacturer. Approximately 5–7 mg of PTP1B (>95% pure as estimated by SDS-polyacrylamide gel electrophoresis) were recovered from a 500-ml culture.

Preparation of Vanadate and Pervanadate—Vanadate stock solution was prepared by dissolving vanadium(V) oxide in 2.1 molar equivalents of 1 N NaOH, stirring the solution until the yellow color had essentially disappeared (2–3 days), and then diluting with water to a final concentration of 100 mM (30). Pervanadate stock solution (1 mM) was prepared by adding 10 μl of 100 mM vanadate and 50 μl of 100 mM hydrogen peroxide (diluted from a 30% stock in 20 mM HEPES, pH 7.3) to 940 μl of H_2O. Excess hydrogen peroxide was removed by adding catalase (100 μg/ml final concentration = 260 units/ml) 5 min after mixing the vanadate and hydrogen peroxide. The pervanadate solutions were used within 5 min to minimize decomposition of the vanadate-hydrogen peroxide complex (31).

FDP Assay for PTP1B Activity—PTP1B was assayed in a buffer containing final concentrations of 25 mM HEPES, pH 7.3, 5 mM DTT, and 10 μg/ml bovine serum albumin using FDP as a substrate. Activity was measured by following the increase in absorbance at 450 nm due to fluorescein monophosphate production[2] using a Hewlett Packard 8452A diode array spectrophotometer (ϵ_{450} for fluorescein monophosphate = 27,500 M^{-1} cm^{-1}).

Mass Spectrometric Analysis—PTP1B samples were prepared for MS by incubating 9.4 μM enzyme on ice in 25 mM HEPES, pH 7.3, 5 mM NaH_2PO_4, and 75 mM NaCl in the presence and absence of 47.6 μM pervanadate. The reaction with pervanadate was quenched after 15 min with the addition of DTT to a final concentration of 10 mM. The protein samples were analyzed by capillary HPLC-electrospray ionization mass spectrometry (capillary LC-MS). The capillary column used was a 254-μm inner diameter × 120-mm length of PEEK tubing packed with 10-μm POROS® R2 stationary phase (PerSeptive Biosystems Inc., Framingham, MA). A Waters 600-MS HPLC pump (Waters, Milford, MA) was used to supply a mobile phase gradient (10–80% aqueous acetonitrile, 0.5% glacial acetic acid in 20 min). The capillary column was attached directly to the exit port of a Valco 0.5-μl internal loop injector (Valco Instrument Co. Inc., Houston, TX). A precolumn splitter was installed between the pump and the injector to adjust the flow through the column to 15 μl/min (from a pump flow of 900 μl/min). The column eluate was fed directly to the electrospray ionization source of a TSQ 7000 triple quadrupole mass spectrometer (Finnigan Mat, San Jose, CA). The electrospray ionization voltage was +4.2 kV, the nebulization gas pressure was 30 p.s.i., and the inlet capillary temperature was 200 °C. Full scan mass spectra (*m/z* 600-2000) were acquired in profile mode using the third quadrupole (Q3) as the mass analyzer. The protein mass spectra were analyzed and deconvoluted using the Bioworks™ application software (Finnigan Mat).

Tryptic peptides were prepared by digesting the samples with approximately 1:20 (w/w) sequencing-grade trypsin for 18 h at 37 °C. The digests were analyzed by capillary LC-MS using the same instrument arrangement as above. In this case, a 300-μm inner diameter × 150-mm Hypersil C_{18} (3-μm particle size) capillary column was used (Keystone Scientific Inc., Bellefonte, PA). This column was fitted with slip-free fittings, which allowed direct attachment to the exit port of the injector. Tryptic peptides were resolved using a gradient of aqueous isopropyl alcohol, 0.5% glacial acetic acid (2–10% in 2 min, 10–80% in 28 min) at 6 μl/min. The column eluate was fed directly to the electrospray ionization source, which was operated under the same conditions as described above. Full scan mass spectra (*m/z* 300-2000) were acquired in centroid mode using Q3 as the mass analyzer.

For tandem mass spectrometric analysis of tryptic peptides, the digests were first resolved by capillary LC-MS as described above. Fragmentation of the selected precursor ions was achieved by collisional activation with argon in the RF-only quadrupole (Q2). The collision gas pressure was 3.0 millitorr, and the collision energy was 75 V (laboratory frame of reference). The fragment ion spectra were acquired in profile mode at an acquisition rate of 3.5 s/scan.

RESULTS

Inhibition of PTP1B by Vanadate and Pervanadate—When assaying inhibition of PTPs by vanadate or pervanadate, the choice of assay conditions is very important because vanadate is known to interact with many buffer salts and organic compounds. Since HEPES is one of the few buffers that does not complex with vanadate (34, 35), it was used here. A reducing agent is essential under aerobic conditions to prevent oxidation of the catalytic Cys residue, which would inactivate the enzyme. DTT was chosen for these studies, and while it is known that DTT does complex with vanadate, these complexes are binuclear in vanadium and are not formed to a significant extent at the vanadium concentrations used in this study.[3] A small amount of bovine serum albumin was also included as a carrier protein, and while bovine serum albumin will complex with vanadate, the formation constant is ~1 × 10^3 M^{-1} (34), making the amount of complexation negligible at these concentrations. EDTA is commonly included in PTP assays; however, it is known to form a complex with vanadate (32–35), with a K_{eff} at pH 8.0 of 1.4 × 10^4 M^{-1} (34), and it was thus excluded here.

In a side-by-side comparison of vanadate and pervanadate inhibition, the order of the addition of enzyme and inhibitor was observed to be very important. When vanadate or pervanadate was added to the assay mixture before initiating the reaction with enzyme, no difference in inhibition was observed (Fig. 1*A*), with 500 nM vanadate or pervanadate causing approximately 50% inhibition. Furthermore, the inhibition by both was completely reversible upon the addition of EDTA to the assay (Fig. 1*A*) or by dilution (data not shown). When vanadate or pervanadate was added to the assay mixture after the enzyme, clear differences between the two were observed (Fig. 1*B*). The extent of inhibition due to vanadate was the same as in Fig. 1*A*, while for pervanadate a greater amount of inhibition was observed, and it was only partially reversible with EDTA.

Mode of Vanadate Inhibition—Vanadate is generally considered as a phosphate analog, since it can adopt a similar structure to inorganic phosphate (Fig. 2) as well as a five-coordinate trigonal bipyramidal structure that resembles the transition state of many phosphoryl transfer reactions (15). The x-ray crystal structure of the *Yersinia* PTP complexed with vanadate shows that the vanadium molecule occupies the active site within covalent distance of the thiol of the catalytic Cys residue, forming a thiol-vanadyl ester linkage that resembles the covalent thiol-phosphate linkage formed during catalysis (37). These data suggest that vanadate is a competitive inhibitor of PTPs. A Lineweaver-Burk analysis of vanadate inhibition was carried out (Fig. 3), and the pattern of inhibition corresponds to that expected for competitive inhibition, with a calculated K_i of 0.38 ± 0.02 μM. The calculated K_m and k_{cat} for PTP1B of 21.7 ± 1.1 μM and 4.85 ± 0.07 s^{-1}, respectively, are in good agreement with previously determined values.[2]

[2] Huang, Z., Wang, Q., Ly, H., Govindarajan, A., Scheigetz, I., Zamboni, R., and Ramachandran, C., manuscript in preparation.

[3] A. Tracey, personal communication.

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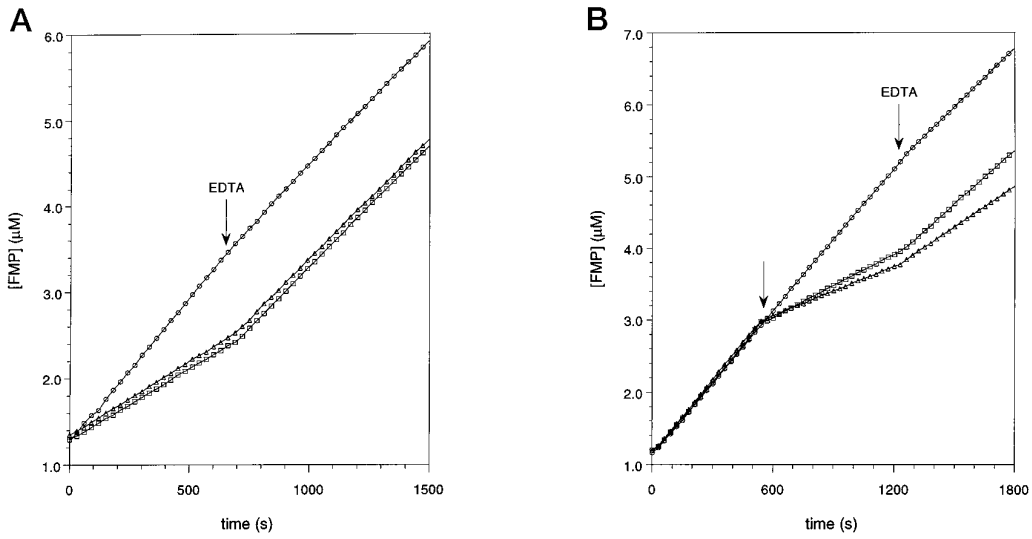

FIG. 1. **Inhibition of PTP1B by vanadate and pervanadate.** PTP1B (200 ng/ml final assay concentration) was assayed with 20 μM FDP as described under "Experimental Procedures." The time courses show the inhibition by 500 nM vanadate (*squares*) or pervanadate (*triangles*) relative to the control (*circles*). The inhibitors were added from 100 μM stock solutions 5 min before (*A*) or 9 min after (*B*, *first arrow*) enzyme. EDTA was added from a 0.5 M stock solution to a final concentration of 1 mM as indicated by the *arrow*.

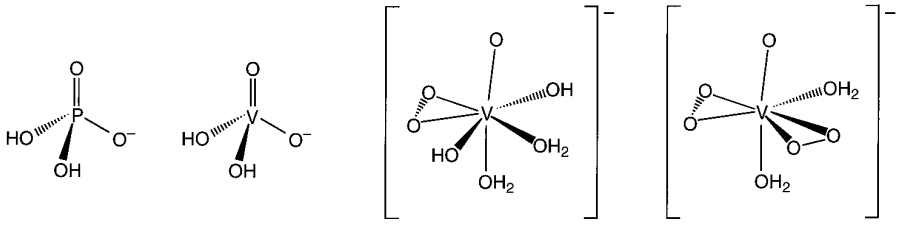

FIG. 2. **Structures of phosphate, vanadate, and pervanadate.** Vanadate in the +5 oxidation state adopts a tetrahedral structure similar to that of phosphate. Pervanadate exists as a number of complexes and is shown here in the monoperoxo (VL) and diperoxo (VL_2) forms, the major species present at the concentrations of vanadate and hydrogen peroxide used in these experiments. The pervanadate species are shown as heptacoordinate structures with two water molecule ligands. However, the exact coordination geometry and the number and arrangement of water ligands is uncertain, and other structures may exist in solution (for further details, see Refs. 31 and 36).

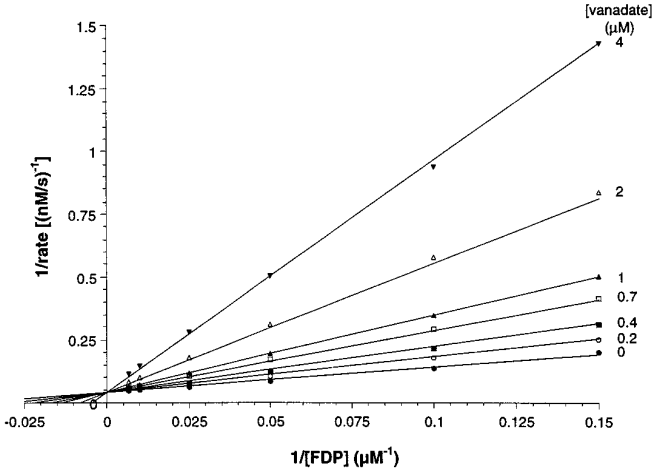

FIG. 3. **Lineweaver-Burk analysis of vanadate inhibition.** PTP1B (400 ng/ml final assay concentration) was assayed in the presence of the indicated vanadate concentrations while varying the FDP concentration, as described under "Experimental Procedures." Catalase (10 μg/ml) was included in the assay buffer as a precaution against the *in situ* formation of pervanadate (see "Results"). All of the data points were fit simultaneously to the Lineweaver-Burk equation for competitive inhibition using the program GraFit. The values reported under "Results" for K_m, k_{cat}, and K_i were calculated from a nonlinear fit of the data to the Michaelis-Menten equation for competitive inhibition using the same program.

Mode of Pervanadate Inhibition—From Fig. 1*B*, it is apparent that pervanadate is inhibiting PTP1B by a different mechanism than vanadate. When pervanadate is added to the enzyme assay last, the inhibition is only partially reversible with EDTA (Fig. 1*B*) or by dilution (data not shown), and the amount of irreversible inhibition increases with increasing pervanadate. The fact that no difference was observed between vanadate and pervanadate when the two are added to the assay mixture before enzyme suggested that perhaps the pervanadate is being converted to vanadate. In fact, if pervanadate is preincubated in DTT alone before addition to the PTP assay solution, it behaves exactly like vanadate (data not shown), consistent with pervanadate being converted to vanadate by the presence of excess DTT; this was confirmed by NMR studies.[3] However, when pervanadate is added to the assay solution after enzyme, there is a portion of the inhibition that cannot be reversed with EDTA or by dilution, suggesting that the pervanadate is modifying or inactivating PTP1B before it is converted to vanadate by the DTT.

Vanadium peroxide complexes are known to be potent oxidizing agents (38), and the irreversible inhibition described above would be consistent with oxidation of the enzyme. In fact, Shaver *et al.* (39) have speculated that peroxovanadium compounds may be inhibiting PTPs by oxidizing the catalytic Cys residue. There are four oxidation states of cysteine that can be generated: disulfide (–S-S–), sulfenic acid (–SOH), sulfinic acid ($-SO_2H$), and sulfonic (or cysteic) acid ($-SO_3H$). Failure of DTT (or other reducing agents; data not shown) to reactivate the pervanadate-inactivated enzyme is consistent with one of the higher oxidation states. The latter three oxidations would result in a change in mass of PTP1B that could be measured by mass spectrometry. To test for this, PTP1B was incubated on ice for 15 min with an approximately 5-fold molar excess of

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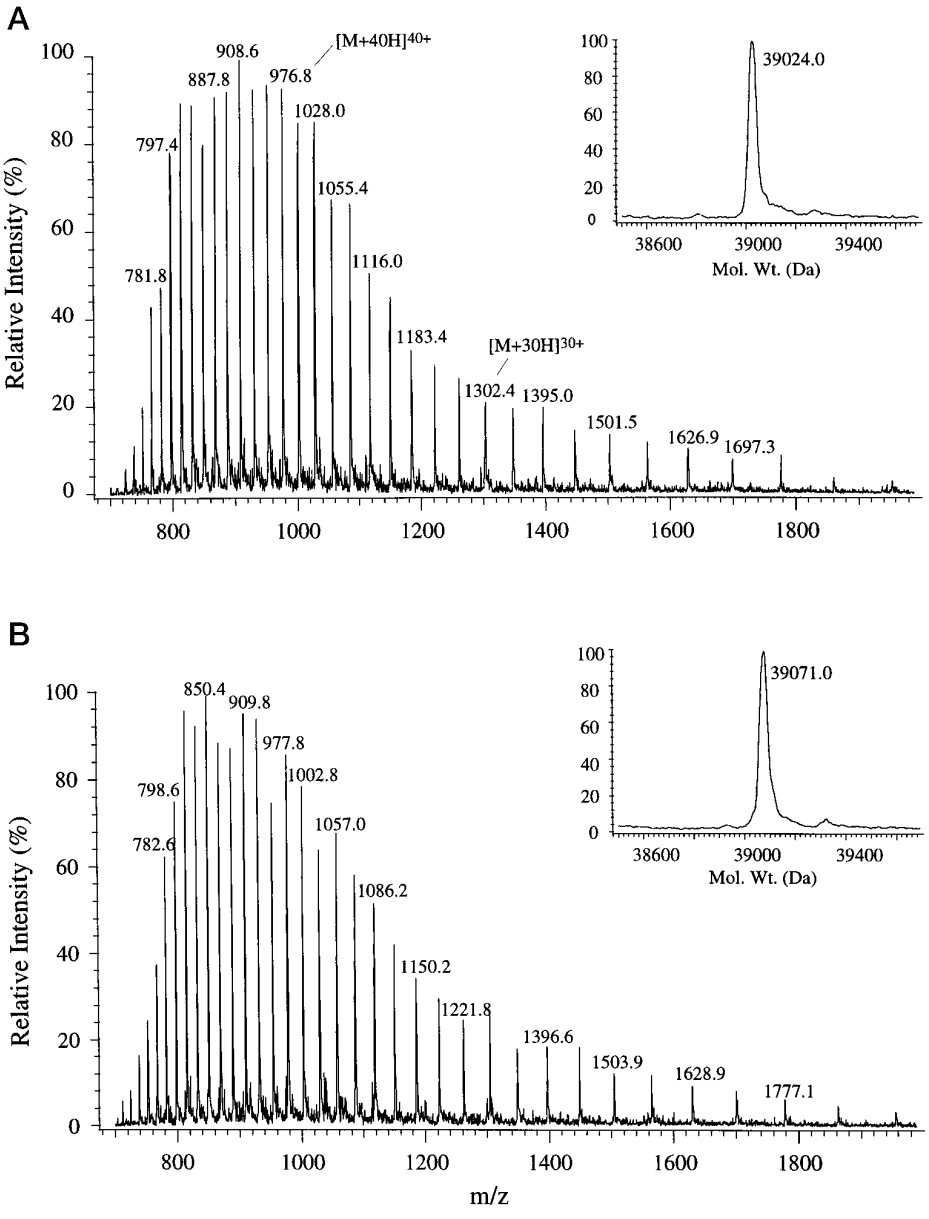

FIG. 4. **Mass spectrometry of native and pervanadate-inhibited PTP1B.** Capillary LC-MS spectra of native (*A*) and pervanadate-treated (*B*) PTP1B protein are shown. Samples were prepared and analyzed as described under "Experimental Procedures." Approximately 250 ng (6.5 pmol) of protein sample were injected on the column in both cases. Each spectrum is the average of four scans taken across the protein LC peak (*m/z* 600-2000, 4 s/scan). The *insets* represent the reconstructed molecular weight profiles obtained from these spectra.

catalase-treated pervanadate, after which DTT was added to a final concentration of 10 mM to destroy the remaining pervanadate. Under these conditions essentially all of the enzyme was inactivated, as determined by assaying the enzyme with FDP and comparing with a control incubation of PTP1B without pervanadate. The deconvoluted mass spectrum obtained for the control protein by capillary LC-MS (Fig. 4*A*, *inset*) demonstrated that this sample consists primarily of a protein with a mass of 39,024 ± 1.3 Da. The reconstructed molecular weight profile of the pervanadate-treated protein (Fig. 4*B*, *inset*) indicated that the mass of the protein had increased by 47 Da, to 39,071 ± 0.6 Da. This could correspond to the addition of three oxygen atoms to the protein. As further controls, PTP1B was also incubated under the same conditions with the same concentrations of either vanadate or hydrogen peroxide used in the preparation of the pervanadate, resulting in no loss of activity in either case (data not shown). Thus, the inactivation of PTP1B by pervanadate and the corresponding increase in mass is clearly not due to the presence of vanadate or free hydrogen peroxide.

Further examination of the trypsin-digested enzymes by capillary LC-MS indicated that the mass of the tryptic peptide containing the active-site cysteine (ESGSLSPEHGPVVVHC-SAGIGR; mass = 2174.1 Da) had increased by 48 mass units after pervanadate treatment relative to the native digest (data not shown). In several replicate control digests, no modified active-site peptides were observed. Other Cys- and Met-containing peptides in the pervanadate-treated PTP1B tryptic digest were examined, and none was found to be modified. The predicted mass of FLAG-PTP1B from the sequence is 38,713.8, which is ~310 mass units less than the MS mass. Peptides corresponding to the entire protein sequence could be identified in the native tryptic digest, except for the extreme C-terminal 28 residues. Perhaps some post-translational modification on this portion of the enzyme gives rise to the increased mass, a hypothesis currently under investigation.

The active-site-containing peptide (in both native and modified form) was observed principally as a triply protonated ion. The MS/MS spectrum of the triply protonated modified active-site peptide (Fig. 5*B*) is dominated by a series of singly and doubly charged Y ions (fragment ions containing the C-terminal residue) (40, 41). Although the ion intensity of the singly charged Y ions decreases with increasing mass, it is evident that the extra 48 mass units were added to the Cys residue,

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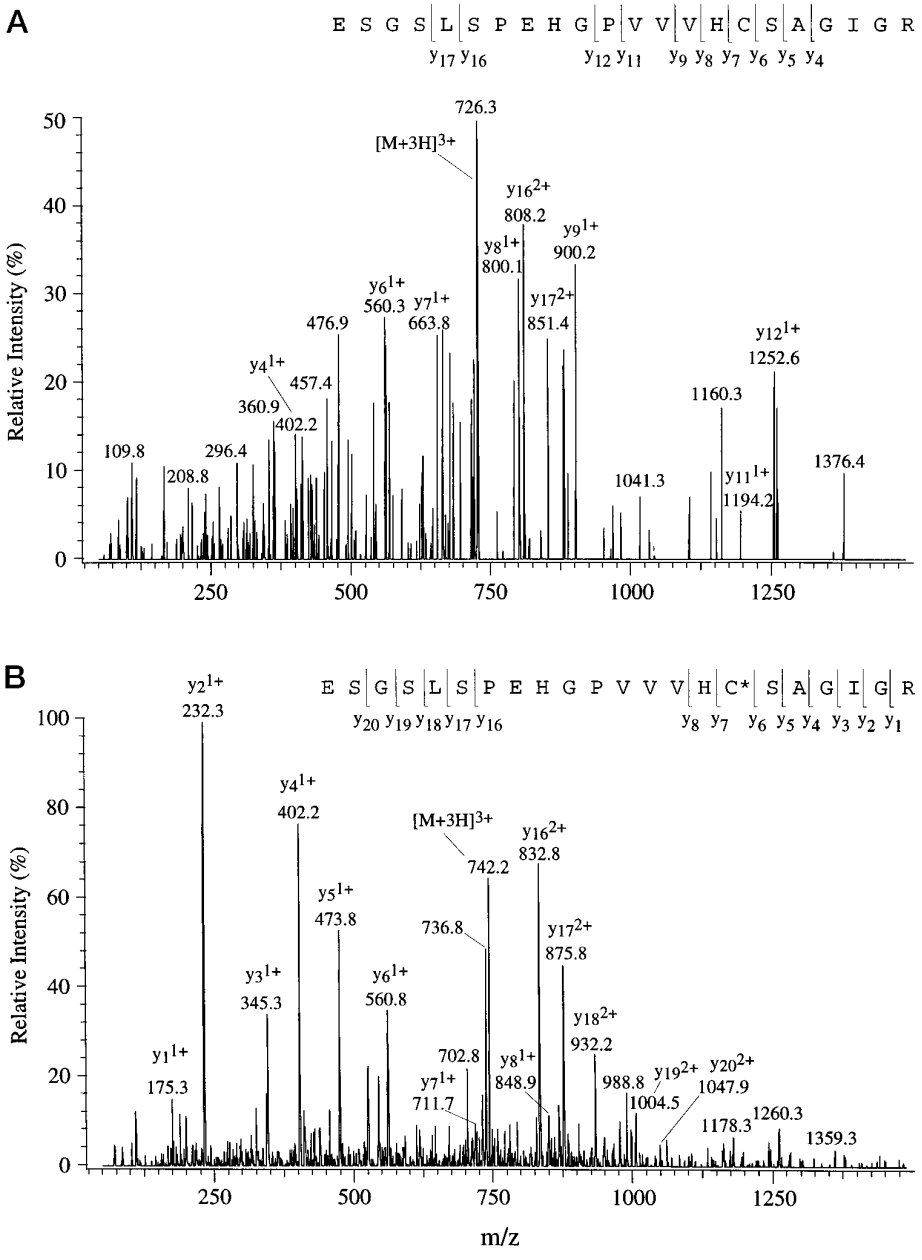

FIG. 5. **MS/MS analysis of active-site peptides.** Capillary LC-MS/MS spectra of the active-site peptide for the native (*A*) and pervanadate-treated (*B*) PTP1B protein are shown. Approximately 250 ng of protein digest were injected on the column in each case. The precursor ions were *m/z* 726.3 (*A*) and *m/z* 742.3 (*B*). Each spectrum represents the average of two scans (*m/z* 50–2200, 3.5 s/scan) taken across the apex of the peptide LC peak. The sequence of the active-site-containing tryptic peptide is shown, with the oxidized cysteine (cysteic acid) marked by an *asterisk* (*C**) and the sites of the Y ion fragmentation indicated.

confirming that the catalytic Cys is oxidized to the cysteic acid. Interestingly, the Y fragment ions are not as prominent in the MS/MS spectrum of the unoxidized active-site peptide (Fig. 5*A*), but they do confirm that the cysteine residue is not oxidized. A similar phenomenon was observed by Burlet *et al.* (42, 43) in the fast atom bombardment-MS/MS spectra of peptides containing a cysteic acid close to a C-terminal arginine. They attributed this phenomenon to a gas phase interaction between these two residues leading to delocalization of the site of protonation and thus an increased yield of Y series ions.

Kinetics of Pervanadate Inhibition—The irreversible inhibition observed when pervanadate was added to the enzyme assay last (after PTP1B) is consistent with the oxidation of the catalytic Cys residue by pervanadate. Time-dependent inhibition is normally anticipated in the case of an irreversible inactivator; however, pervanadate has a short half-life in the DTT-containing buffer. Upon the addition of pervanadate, then, there are at least two competing reactions going on: conversion of pervanadate to vanadate by DTT, and oxidation of the catalytic Cys by pervanadate. Thus, once the pervanadate is destroyed by the DTT (which appears to occur too quickly to be measured on our kinetic time scale), there is no inhibitor available for any further inactivation. Increasing the concentration of DTT in the enzyme assay was predicted to decrease the amount of inhibition observed upon the addition of pervanadate to the enzyme assay by favoring the reaction of pervanadate with DTT over that with the enzyme; this was in fact observed (Fig. 6). Similar results were observed with the reducing agents β-mercaptoethanol and reduced glutathione (data not shown), indicating that this effect is not exclusive to DTT. The assay mixtures in Fig. 6 contained EDTA to chelate vanadate and catalase to consume hydrogen peroxide, ensuring that the observed inhibition was due exclusively to inactivation by pervanadate.

The overall reaction scheme proposed for the inhibition by vanadate and pervanadate and the effects of the thiol reductant on pervanadate and EDTA on vanadate is outlined in Fig. 7. Since similar inhibition kinetics for vanadate and pervanadate were observed with the PTP CD45 (data not shown), this scheme may be applicable to PTPs in general. A pathway by which pervanadate can reform in the assay reaction is also shown in Fig. 7. Under certain assay conditions (*e.g.* when

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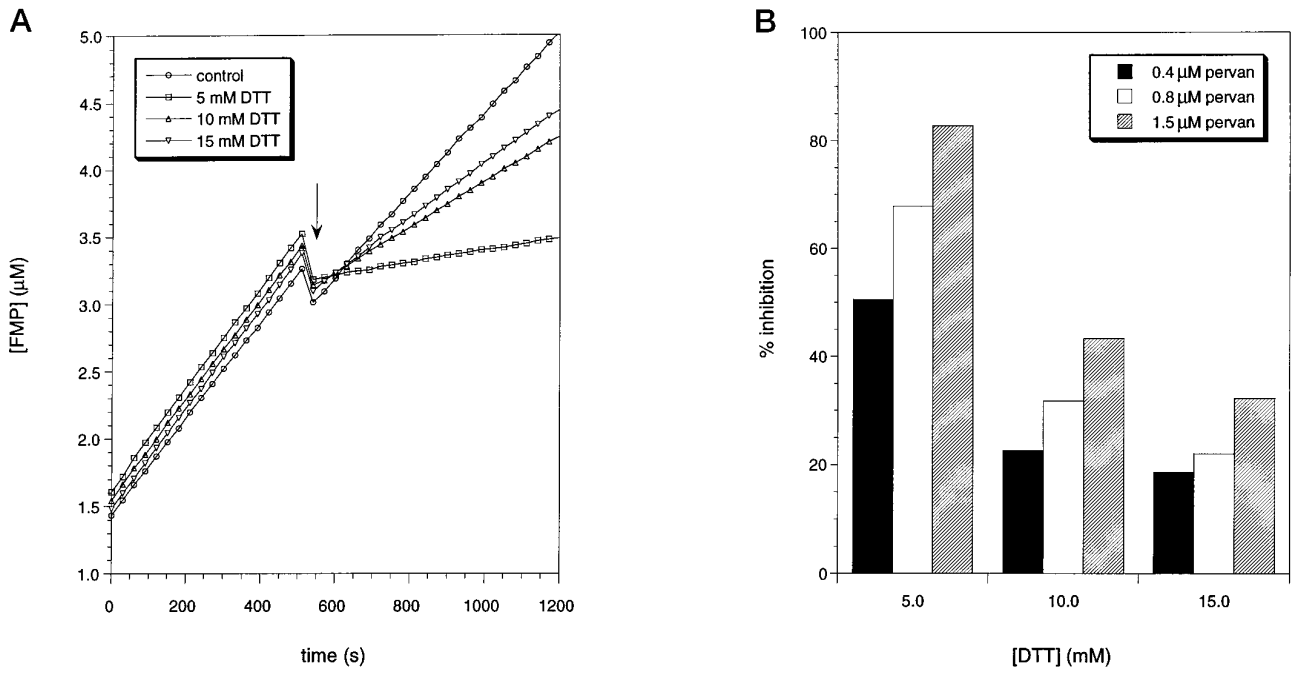

FIG. 6. **Inhibition of PTP1B by pervanadate.** PTP1B (200 ng/ml final assay concentration) was assayed with 20 μM FDP as described under "Experimental Procedures," with 1 mM EDTA and 10 μg/ml catalase included in the assay buffer. *A*, time courses showing the addition of 100 μl of 15 μM pervanadate to a 900-μl assay containing enzyme and DTT at the indicated concentrations. For the control, H_2O was added, and the rate was the same at all DTT concentrations used. *B*, *bar graph* showing the percentage of inactivation relative to the control calculated from the time courses in *A* and at additional pervanadate concentrations as indicated.

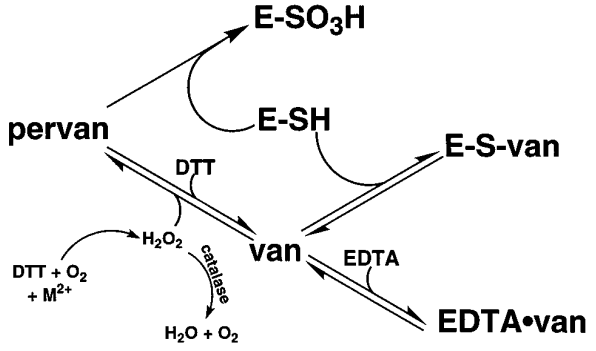

FIG. 7. **Scheme for vanadate and pervanadate inhibition of PTPs.** See "Results" for details. *Pervan*, pervanadate; *van*, vanadate; *E-SH*, catalytically active PTP (reduced catalytic Cys); *E-SO$_3$H*, PTP oxidized at the catalytic Cys residue.

imidazole buffer was used for the enzyme assays), a slow time-dependent inactivation of the enzyme that increased with increasing vanadate concentration was observed (data not shown). It is known that DTT autoxidation is catalyzed by transition metals, and superoxide is formed upon the reduction of O_2 by a DTT thiol radical or by the reduced transition metal; the superoxide can then disproportionate to hydrogen peroxide and O_2 (44–46). Hence, the slow inactivation observed with vanadate is consistent with the generation of pervanadate *in situ* through the complexation of hydrogen peroxide with vanadate and the subsequent oxidation of the catalytic Cys of the enzyme. The time-dependent inhibition was blocked by catalase and stimulated by superoxide dismutase and Cu^{2+} (data not shown), supporting the mechanism in Fig. 7. Hydrogen peroxide itself is a much poorer inhibitor than pervanadate, since concentrations >100 μM were required for inhibition in our standard conditions (data not shown). However, the presence of vanadate in the enzyme assay greatly potentiates the effect of hydrogen peroxide (data not shown), consistent with the *in situ* formation of pervanadate as described above.

It is important to note that pervanadate is a mixture of several different complexes of vanadate with hydrogen peroxide (Fig. 2). At the concentrations of vanadate and hydrogen peroxide used here, the predominant species are monoperoxovanadate (VL) and diperoxovanadate (VL_2), as calculated from the equilibrium constants determined by Jaswal and Tracey (31). The literature equilibrium constants were obtained in 1.0 M ionic strength maintained with KCl, so the actual values here may be different but should follow the same trend. In order to determine the species active in oxidizing the enzyme, pervanadate solutions were prepared with different ratios of VL and VL_2 but a constant amount of VL + VL_2 (Table I). These mixtures were added to PTP1B assays at a final concentration of VL + VL_2 = 400 nM, and the extent of inhibition was determined. As shown in Fig. 8, the amount of inhibition increases as the ratio of VL to VL_2 decreases, suggesting that VL_2 is a more active or potent inhibitor than VL. The concentrations of VL_3 and V_2L_4 present in the assay solution were estimated to be 2–3 orders of magnitude less than the enzyme concentration, and for all practical purposes they can be ignored. Little or no (≤5%) inhibition was observed when vanadate or free hydrogen peroxide was added separately to the PTP1B assay at the same concentrations used in the preparation of the pervanadate solutions. This result was expected, since EDTA and catalase were present in the assay solutions to, respectively, chelate the vanadate and remove the hydrogen peroxide.

DISCUSSION

Enzyme inhibitors are very important as research tools and for their pharmacological potential. Few inhibitors are known for PTPs, and inhibitors that are selective for a particular PTP have been elusive. Two general PTP inhibitors, vanadate and pervanadate, are commonly used, and their insulin-mimetic properties have been the subject of much research. Despite their routine use, a clear understanding of their inhibition mechanisms is still lacking. Furthermore, many researchers often seem to treat vanadate as a simple phosphate analog without considering its rich redox and coordination chemistry,

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TABLE I

Pervanadate species present in mixtures prepared for Fig. 8

Vanadate (V) and hydrogen peroxide (L) were mixed in distilled water at the indicated concentrations ($[V]_t$ and $[L]_t$) and allowed to equilibrate for 5 min before use. The equilibrium concentrations of vanadate, hydrogen peroxide, and the various pervanadate species (VL, VL_2, VL_3, and V_2L_4) are indicated, as calculated from the published equilibrium constants (31).

$[V]_t$	$[L]_t$	[V]	[L]	[VL]	$[VL_2]$	$[VL_3]$	$[V_2L_4]$	[VL] + $[VL_2]$	[VL]/$[VL_2]$
μM	μM	μM	μM	μM	μM	nM	nM	μM	
25	22	21.0	15.3	1.32	2.70	0.68	0.47	4.03	0.49
100	11.5	96.0	5.73	2.27	1.75	0.17	0.19	4.02	1.30
225	8.3	221.0	3.11	2.83	1.18	0.060	0.089	4.01	2.40
360	7.0	356.0	2.12	3.11	0.88	0.031	0.050	4.00	3.52
500	6.3	496.0	1.60	3.28	0.71	0.019	0.032	3.99	4.65

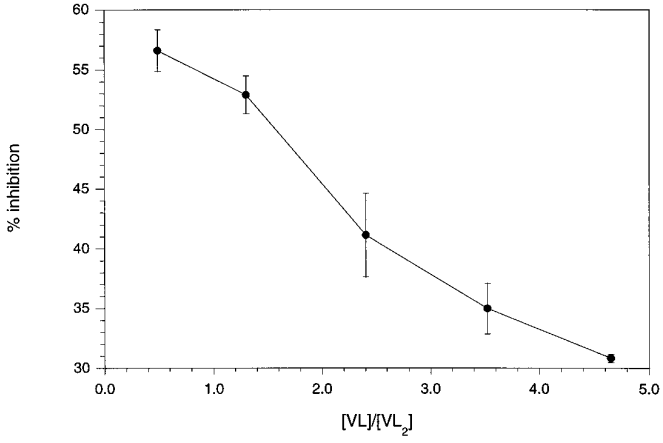

FIG. 8. **Dependence of pervanadate inhibition on the relative proportion of pervanadate species.** Pervanadate solutions were prepared with varying proportions of vanadate (*V*) and hydrogen peroxide (*L*) such that the major species generated were VL and VL_2 and the total concentration of VL + VL_2 remained constant at 4.0 μM (see Table I). PTP1B (200 ng/ml final assay concentration) was assayed with 20 μM FDP as described under "Experimental Procedures" with 2 mM EDTA and 10 μg/ml catalase present, and 100 μl of the pervanadate solutions were added after 9 min (final concentration of VL + VL_2 = 400 nM) as in Fig. 6. The percentage of inhibition relative to a control (addition of 100 μl of water) was determined and plotted *versus* the ratio of VL to VL_2 added. The plot shows the average of data from two separate experiments.

including varied oxidation states and coordination with many reagents. In this study, we set out to elucidate the mechanisms of PTP inhibition by vanadate and pervanadate and also to establish the reactivity of these inhibitors under various conditions relevant to their general use.

An analysis of vanadate inhibition showed that vanadate behaves as a competitive inhibitor of PTP1B (Fig. 3), with a K_i of 0.38 ± 0.02 μM. This is not unexpected, considering the fact that vanadate is structurally a phosphate analog (Fig. 2) that mimics the transition state of phosphoryl transfer reactions (15), and the crystal structure of the *Yersinia* PTP complexed with vanadate shows that vanadate occupies the active site within covalent bonding distance (2.5 Å) of the thiol of the catalytic Cys residue (37). While the mechanism of vanadate inhibition may be simple, many researchers appear to neglect the interaction of buffers and assay components with vanadate (34, 35). Vanadate will reversibly coordinate free hydroxyl and thiol groups and is also chelated by many organic molecules. HEPES is one of the few buffers that does not interact appreciably with vanadate (34, 35); thus, it was chosen for the studies undertaken here. More important, though, is the interaction of vanadate with EDTA. This interaction has been well documented (32–35) but appears to be ignored by many researchers, both those studying PTPs (for examples, see Refs. 47–52) and those analyzing phosphotyrosine signaling pathways (for examples, see Refs. 53–61). The addition of EDTA in excess over vanadate to a PTP assay containing vanadate will immediately and completely reverse the inhibition due to vanadate (see Fig. 1). The presence of EDTA in an assay buffer can dramatically reduce the free concentration of vanadate; for example, 1 mM EDTA can decrease the potency of vanadate for PTP1B over 1000-fold (data not shown). This EDTA effect has important implications for the use of vanadate in cell lysis buffers or immunoprecipitations of phosphotyrosyl proteins in which endogenous cellular PTPs must be inhibited. For example, when 100 μM vanadate is used in the presence of 2 mM EDTA during cell lysis, a much lower level of total phosphotyrosyl proteins is observed as compared with a lysate in which EDTA is excluded (data not shown). The fact that EDTA and vanadate are often included together in cell lysis buffers suggests that in such procedures the endogenous PTPs are not being completely inhibited, which may affect the quality of the results obtained. EGTA, on the other hand, forms a weaker complex with vanadate (34) and may therefore be a practical alternative when divalent cations must be chelated.

Pervanadate (the complexes of vanadate with hydrogen peroxide) was found to be an irreversible inhibitor of PTPs, unlike vanadate, whose inhibition is completely reversible. Mass spectrometry showed that when PTP1B was incubated with 5 molar equivalents of pervanadate, an increase of 48 mass units relative to the untreated enzyme was observed, which was localized to the catalytic Cys (Figs. 4 and 5). This modification is consistent with the addition of three oxygen atoms. Only the triply oxidized cysteine (*i.e.* cysteic acid; $-SO_3H$) was observed. However, it is possible that intermediate oxidized forms exist (*i.e.* sulfenic and sulfinic acids; –SOH and $-SO_2H$, respectively) and that the cysteic acid represents the stable end point in the presence of excess pervanadate. No other sites on the enzyme were observed to be oxidized. This specificity may be because the catalytic Cys exists as a thiolate anion (8–11), making it more reactive and susceptible to oxidation; furthermore, pervanadate may have a strong affinity for the active site. Oxidation of the catalytic Cys of PTPs may actually be an *in vivo* mechanism of down-regulating PTP activity. Endogenous reactive oxygen intermediates, which may act as signaling molecules, have been observed to increase cellular phosphotyrosine levels, and it has been proposed that at least part of this effect may be due to inactivation of PTPs (62). For example, platelet-derived growth factor signal transduction has been reported to cause a transient increase in intracellular hydrogen peroxide concentration, which may prolong phosphotyrosine-dependent signaling by oxidizing and inactivating PTPs (63). The fact that the effect of reactive oxygen intermediates is potentiated by added vanadate (64, 65) would be consistent with the formation in the cell of pervanadate, which is a much more effective inhibitor than reactive oxygen intermediates alone. This is supported by the *in vitro* kinetic data showing a potentiation of hydrogen peroxide inhibition by vanadate (data not shown).

As was seen with vanadate, assay components can also interact with pervanadate and affect its inhibition. For example, it was observed that pervanadate is rapidly converted to van-

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adate by thiol reductants such as DTT that are included in PTP assay buffers to protect the catalytic Cys residue from oxidation. When pervanadate is added to an assay solution containing enzyme and DTT, there appears to be a partitioning of pervanadate between oxidation of the enzyme and oxidation of DTT (Figs. 6 and 7). Considering that the DTT concentration in Fig. 6 is 6 orders of magnitude greater than the PTP1B concentration (5–15 mM *versus* ~5 nM, respectively), pervanadate must have a much higher relative affinity for the enzyme active site. Although pervanadate is an irreversible inactivator, time-dependent inhibition was not observed. This is explained by the fact that when the pervanadate is added to the enzyme assay, there are at least two competing reactions: oxidation of the enzyme by pervanadate and reduction of pervanadate to vanadate by DTT. No further inactivation of the enzyme can occur after the destruction of the pervanadate, and since these reactions occur faster than the time scale of our measurements, no time-dependent inhibition is observed. It is impossible, therefore, to determine the intrinsic potency of pervanadate under aerobic conditions because of the need to include a thiol reductant to prevent inactivation of the enzyme in air. It is possible, though, to make qualitative conclusions about the relative potencies of the pervanadate species. Vanadate can form several different complexes with hydrogen peroxide (31), with the major species at the concentrations used here being the mono- and diperoxovanadates, or VL and VL_2 (Fig. 2). When the total amount of VL and VL_2 is kept constant but the ratio of VL to VL_2 is varied (Table I), the amount of inhibition observed increases with increasing VL_2 (Fig. 8). Thus, VL_2 appears to be a more potent inactivator of PTP1B than VL. It is possible that VL is not inhibitory at all; however, the individual potencies of VL and VL_2 cannot be assessed in this experiment, since both complexes are always present together.

The different modes of vanadate and pervanadate inhibition provide a possible rationale for the differences observed in their *in vivo* effects. Pervanadate has been reported to be more potent than vanadate both in increasing the level of phosphotyrosine-containing proteins in intact cells (23–25) and in insulin mimesis (21, 22). Vanadate can easily enter cells, after which it is reported to be reduced to vanadium(IV) (66–68). While the inhibition of PTPs by vanadium(IV) has not been determined, vanadium(IV) is likely to be a much less potent inhibitor than vanadium(V), since it is not a phosphate analog nor a PTP transition state mimic. In fact, kinetic studies suggest that vanadium(IV) is a much poorer inhibitor of the Na,K-ATPase than vanadium(V) (66, 68). Since any inhibition of intracellular PTPs by vanadate that does occur is reversible, it will be eliminated as the vanadate dissociates from the PTPs and is reduced. Pervanadate, by contrast, may partition in the cell between conversion to vanadate and oxidative inactivation of PTPs, analogous to that observed in the *in vitro* kinetic assays. Irreversible inactivation would result in a lower level of total intracellular PTP activity than in the case with vanadate, resulting in a greater overall level of protein tyrosine phosphorylation. Clearly, an understanding of the inhibition mechanisms of vanadate and pervanadate and their potential reactions and interactions are key to explaining their *in vivo* and *in vitro* effects and must be taken into consideration when these inhibitors are used.

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