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 $0.38 \pm 0.02 \mu M$, 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 maintaining by thiol reagents are necessary for keeping the enzyme active, thiol-oxidizing agents are potent PTP inhibitors. Vanadate ($\text{VO}_4^{3-}$) 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 o xo and p eroxo 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.
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 EcoRI site of the pFLAG-2 vector (Kodak). Bacterial cultures were grown in Tryptic Soy Broth containing 100 μg/ml ampicillin at 37°C, 225 rpm, for 1–2 h (A_{600} = 0.4–0.8). The culture was then induced with the addition of isopropyl-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 NaH₂PO₄, pH 7.3, 5 mM NaCl, 0.67 mg/ml lysozyme, 2 mg/ml each peptatin and aprotinin, 1 mM DTT), and then 5 ml of buffer B (1.5 mM NaCl, 0.1 mM MgCl₂, 0.1 mM CaCl₂, 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 (Intercescence), 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.

Expression and Purification of Pavanadate 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). Pavanadate 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 H₂O. 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 production² using a Hewlett Packard 8452A diode array spectrophotometer (ε₄₅₀ 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 NaH₂PO₄, 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 25-μm inner diameter × 120-mm length of PEEK tubing packed with 100-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 psi, and the inlet capillary temperature was 250°C. Full scan mass spectra (m/z 300-2000) 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 described above. In this case, a 300-μm inner diameter × 30-cm Hypersil C₁₈ (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.

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

³ A. Tracey, personal communication.

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 Q1 and Q3 as the mass analyzers.

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. 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 ~10⁻⁸ 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_{eq} at pH 8.0 of 1.4 × 10⁴ 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. 1A), 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. 1A) 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. 1B). The extent of inhibition due to vanadate was the same as in Fig. 1A, 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 solvent 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_{eq} 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
Mode of Pervanadate Inhibition—From Fig. 1, 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. 1B) 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. 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), sulfenic acid (–SO2H), and sulfonic (or cysteic) acid (–SO3H). 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...
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. 4A, 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. 4B, 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 (ESGSLPEHGPVVHCSAGIGR; 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. 5B) 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,
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. 5A), 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 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
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$_2$L$_4$ 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,
Tyrosine Phosphatase Inhibition by Vanadate and Pervanadate

The percentage of inhibition relative to a control behaves as a competitive inhibitor of PTP1B (Fig. 3), with a Ki of 0.38 ± 0.02 μM. This is not unexpected, considering the fact that vanadate is a structural phosphate analog (15), and the crystal structure of the Yersinia PTP complexed with vanadate (34) and may therefore be a practical alternative when divalent cations must be chelated.

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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 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 of excess pervanadate. No other sites on the enzyme were observed to be oxidized.
Tyrosine Phosphatase Inhibition by Vanadate and Pervanadate

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