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Inhibition of Phosphatase and Sulfatase by Transition-State Analogues†

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ABSTRACT: The inhibition constants for vanadate, chromate, molybdate, and tungstate have been determined with *Escherichia coli* alkaline phosphatase, potato acid phosphatase, and *Helix pomatia* aryl sulfatase. Vanadate was a potent inhibitor of all three enzymes. Inhibition of both phosphatases followed the order $WO_4^{2-} > MoO_4^{2-} > CrO_4^{2-}$. The K_i values for potato acid phosphatase were about 3 orders of magnitude lower than those for alkaline phosphatase. Aryl sulfatase followed the reverse order of inhibition by group VI oxyanions. Phenol enhanced inhibition of alkaline phosphatase by vanadate and chromate but did not affect inhibition of acid phosphatase. Phenol enhanced inhibition of aryl sulfatase by metal oxyanions in all cases following the order $H_2VO_4^- > CrO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$, and *N*-acetyltyrosine ethyl ester enhanced inhibition of aryl sulfatase by $H_2VO_4^-$ and CrO_4^{2-} more strongly than did phenol. It is apparent that the effectiveness of metal oxyanions as inhibitors of phosphatases and sulfatases can be selectively enhanced in the presence of other solutes. The relevance of these observations to the effects of transition metal oxyanions on protein phosphatases in vivo is discussed.

Phosphotyrosine protein kinase activity has been linked to the action of insulin (Rosen et al., 1983), growth factors (Ushiro & Cohen, 1980), and oncogenes (Sefton et al., 1982). Control of phosphotyrosine protein phosphatases, which inactivate the kinases and oppose their action, is very likely important in these biological processes (Gresser et al., 1987). Both mammalian alkaline (Swarup et al., 1981) and acid phosphatases (Lau et al., 1985) have been implicated in the control of protein phosphotyrosine content, but the specificity of cellular protein phosphatases is poorly understood (Sparks & Brautigan, 1985, 1986).

Vanadate mimics the effects of insulin (Heiliger et al., 1985; Tamura et al., 1984) and epidermal growth factor (Dubyak & Kleinzeller, 1980; Smith, 1983; Carpenter, 1981) and affects various other physiological processes in which phosphorylation of tyrosine is thought to be important (Nechay et al., 1986). There have been some reports that vanadate selectively inhibits phosphotyrosine phosphatase relative to phosphoserine or phosphothreonine phosphatases (Leis & Kaplan, 1982; Leis et al., 1985; Swarup et al., 1982a,b; Nelson & Branton, 1984; Klarlund, 1985), but is also appears that vanadate is not an equally potent inhibitor for all phosphotyrosine phosphatases (Brunati & Pinna, 1985). The mechanism for the selective inhibition of protein phosphatases by vanadate is not clear, and no explanation for it has been advanced. The work reported here was undertaken in an effort to test one hypothesis which provides a rationalization of this behavior. The hypothesis being tested is that a vanadate ester which resembles the substrate should, in some cases, be a more potent inhibitor of the phosphatase than vanadate alone. In cases for which this is true, inhibition of a given phosphatase by vanadate will be stronger in the presence of the dephosphorylated substrate of the phosphatase if it forms a vanadate ester. Proteins which, when phosphorylated, are the best substrates for a phosphatase

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would most strongly enhance inhibition by vanadate. This behavior, if observed, would thus provide a method to screen proteins as potential substrates for a given phosphatase. This approach could also be used to screen protein kinase substrates by running the kinase reaction in reverse, using any convenient phosphorylated substrate. Added proteins which strongly enhance inhibition of this reaction by vanadate would be good potential substrates for the protein kinase.

Vanadate inhibits a variety of enzymes which catalyze phosphoryl transfer reactions, presumably by virtue of the ease with which it can expand its coordination shell to adopt a structure resembling the transition state for phosphoryl transfer reactions (Knowles, 1980; Chasteen, 1983). The inhibition of phosphatases by vanadate and other oxyanions has been reviewed elsewhere (Van Etten et al., 1974).

Although the type of behavior postulated above has not been reported for vanadate inhibition of phosphatases, a related phenomenon has been observed with ribonuclease (Lindquist et al., 1973). The uridine vanadate complex is a much stronger inhibitor of ribonuclease than is vanadate alone. Similarly, an ADP–vanadate complex binds very strongly to myosin (Goodno & Taylor, 1982), and ribonucleoside–vanadyl complexes inhibit a number of enzymes whose substrate is RNA (Puskas et al., 1982).

In this study, a nonspecific acid phosphatase which shows very little selectivity among substrates and an alkaline phosphatase which is selective were examined. In order to extend the study to sulfuryl transfer reactions, an aryl sulfatase was also tested.

Experimental Procedures

Materials. *Escherichia coli* alkaline phosphatase, type III-R (42 units/mg), *Helix pomatia* aryl sulfatase, type H-5 (25 units/mg), 4-nitrophenyl sulfate, and *N*-acetyl-1-tyrosine ethyl ester were from Sigma. Potato acid phosphatase (6 units/mg) was from Boehringer Mannheim Canada Ltd. Sodium orthovanadate was from Fisher. Sodium chromate and sodium molybdate were from Baker, and sodium tungstate was from Anachemia Montreal. All other chemicals were reagent grade.

Assay Conditions. All enzymatic reactions were carried out at 23 °C. Both phosphatases and sulfatase were assayed spectrophotometrically by the continuous production of *p*-nitrophenol using extinction coefficients obtained under the following conditions: 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)[1] and 0.1 M KCl, pH 6.0 ($\epsilon_{400} = 1.64 \times 10^3$ M^{-1} cm^{-1}); 20 mM Tris and 0.1 M KCl, pH 7.0 ($\epsilon_{400} = 9.43 \times 10^3$ M^{-1} cm^{-1}) and pH 7.4 ($\epsilon_{400} = 13.7 \times 10^3$ M^{-1} cm^{-1}). The observed values are consistent with the literature value, $\epsilon_{400} = 18.4 \times 10^3$ M^{-1} cm^{-1} (fully ionized form), and a pK of 7.0 (Lopez et al., 1976).

Rate measurements for alkaline phosphatase were carried out in 20 mM Tris, 0.1 M KCl, and 0.1 μM $ZnCl_2$ at pH 7.0–8.0 using 4-nitrophenyl phosphate as substrate and 0.05–0.20 μg/mL enzyme. Acid phosphatase activity was determined in 50 mM MES/0.1 M KCl, pH 6.0 and 7.0, using 4-nitrophenyl phosphate as substrate and 2.0–4.0 μg/mL enzyme, and aryl sulfatase activity was determined in 50 mM MES/0.1 M KCl, pH 6.0 and 7.0, using 4-nitrophenyl sulfate as substrate and 15–30 μg/mL enzyme. Reaction conditions were similar to those used by other investigators to assay alkaline (Lopez et al., 1976) or acid (Lynn et al., 1981) phosphatases or aryl sulfatase (Bolognani et al., 1984). Rates were found to increase linearly with enzyme concentrations.

Initial reaction velocities were measured after the addition of enzyme except in the case of chromate inhibition of aryl sulfatase. In this case, the development of inhibition required several minutes to reach steady state, but not more than 5% of substrate was utilized.

Inhibition of alkaline phosphatase was carried out at pH 7.0, 7.4, and 8.0 using phenol or TEE alone and in the presence of oxyanion inhibitors. Phenol was used in the concentration range of 20–80 mM, and TEE was used in the range of 0.5–1.0 mM. Metal oxyanion inhibition was carried out in the presence and absence of phenol or TEE. Vanadate was used in the concentration range of 40–100 μM at pH 7.0 and 7.4, and 1.0–10.0 μM at pH 8.0. Chromate concentration was 0.5 mM. Molybdate concentration ranged from 0.5 to 1.0 mM over the pH range tested, and tungstate concentration ranged from 15.0 to 30.0 μM over the pH range (higher concentrations were not used, in order to avoid complications due to molybdate or tungstate oligomerization). Inhibition by phenol was examined in the presence of 7.0 μM phosphate.

Inhibition of acid phosphatase was studied at pH 6.0 and 7.0 in the presence of phenol or metal oxyanion alone or in combination. Phenol concentration was 50 mM at both pH conditions. Vanadate concentration varied from 0.20 to 0.8 μM, chromate concentration varied from 1.25 to 2.5 μM, molybdate concentration varied from 0.1 to 0.3 μM, and tungstate concentration varied from 0.02 to 0.05 μM.

At pH 6.0, aryl sulfatase inhibition was examined with 10–30 mM phenol alone or with 2.0 mM TEE alone. Inhibition by vanadate alone was examined within the concentration range of 10–20 μM. At 10 μM vanadate, inhibition enhancement was examined using 2.0–5.0 mM phenol or 125 μM TEE. Inhibition by 0.2 mM chromate or 0.25 mM molybdate was examined alone or in the presence of 10 mM phenol or 2.0 mM TEE.

At pH 7.0, inhibition of aryl sulfatase was examined using 25–50 mM phenol. Inhibition by 50 μM vanadate was examined in the presence and absence of 5.0 mM phenol. Inhibition by 0.5 mM chromate, 0.5 mM molybdate, and 0.5 mM tungstate was examined alone and in the presence of 50 mM phenol.

Analysis of Data. The K_i for competitive inhibition by a single inhibitor was estimated by classical analysis of slopes of Lineweaver–Burk plots. When inhibition was examined in the presence of a metal oxyanion and phenol, an additional component of inhibition arose in some cases, which is ascribed to ester formation from the alcohol and the metal oxyanion. The extent of this inhibition depends upon the equilibrium constant for spontaneous ester formation (K_{eq}) and the K_i for the ester according to

$$\text{slope} = (K_m/V_m)(1 + [M]/K_{i,M} + [ROM]/K_{i,ROM})$$

where M is the metal oxyanion and ROM is the ester. Since the concentration of ROM is unknown in general, it is substituted with the term derived from the equilibrium expression $K_{eq} = [ROM]/([ROH][M])$ where ROH is either phenol or TEE. The equation still contains two unknowns: $K_{i,ROM}$ and K_{eq}. It is therefore rearranged to the form

$$\text{slope} = (K_m/V_m)\left(1 + [M]/K_{i,M} + \frac{[ROH][M]}{K_{i,ROM}/K_{eq}}\right)$$

giving a single observable inhibition constant, $K_{i,ROM}/K_{eq}$. The K_{eq} value for formation of phenyl vanadate at pH 7.5 has been determined to be 0.97 M^{-1} (Tracey & Gresser, 1986). Formation of chromate esters in aqueous solution is a well-doc-

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TEE, *N*-acetyltyrosine ethyl ester.

Table I: Inhibition Constants for Inhibitors of *E. coli* Alkaline Phosphatase at Different pH Values[a]

inhibitor	K_i (M) (7.0)	phenyl ester[b] K_i/K_{eq} (M^2) (7.0)	K_i (M) (7.4)	phenyl ester[b] K_i/K_{eq} (M^2) (7.4)	K_i (M) (8.0)	phenyl ester[b] K_i/K_{eq} (M^2) (8.0)
vanadate	2.2×10^{-5} ±0.4	2.0×10^{-6} ±0.4	3.5×10^{-6} ±0.1	2.0×10^{-7} ±0.1	2.3×10^{-6} ±0.2	1.7×10^{-7} ±0.2
chromate	6.8×10^{-4} ±0.7	4.2×10^{-5} ±0.8	1.6×10^{-3} ±0.2	7.4×10^{-5} ±1.3	5.4×10^{-3} ±1.7	N
molybdate	4.4×10^{-4} ±1.1	N	1.04×10^{-4} ±0.05	N	3.2×10^{-4} ±0.3	N
tungstate	6.8×10^{-6} ±1.4	N	2.8×10^{-6} ±0.1	N	5.9×10^{-6} ±0.5	N

[a] pH values are indicated in parentheses. The ± values below the K_i or K_i/K_{eq} values are the standard deviations in the mantissa. N indicates no observable enhancement of inhibition. [b] Although the inhibition is ascribed to binding of phenyl esters of the metallooxyanions, the calculation of the K_i/K_{eq} values does not depend on this assumption (see Experimental Procedures).

umented phenomenon (Westheimer, 1949; Wiberg, 1965; Espenson, 1970), but no K_{eq} values appear to be available. Equilibrium constants for formation of chromate esters from alcohols and acetochromate in 97% acetic acid have been reported (Wiberg & Mukherjee, 1974a,b), but in the absence of an equilibrium constant for formation of acetochromate, these values cannot be used to obtain equilibrium constants for chromate ester formation in water.

It is possible that the inhibition due to phenol or TEE and metal oxyanion in combination is not due to binding of an ester at the catalytic site but rather to some other effect of phenol or TEE which enhances the inhibition by metal oxyanion. In this case, the values of K_i/K_{eq} reported in Tables I and III simply represent inhibition constants for inhibition by the unknown mechanism.

The estimates of K_i and K_i/K_{eq} values obtained from analysis of the Lineweaver–Burk plots were used as first approximations in fitting the Michaelis–Menten form of the rate equation to the data using the nonlinear least-squares curve fitting program BMDP on the mainframe computer at the computing center at Simon Fraser University. The standard deviations reported in Tables I–III are those provided by the program BMDP.

Results

Alkaline Phosphatase. All inhibition described in Table I was competitive. Phenol was a weak competitive inhibitor with a K_i of 180 mM over the pH range tested. TEE alone had no effect up to 2.0 mM.

The K_i value for vanadate progressively decreased at higher pH. This is consistent with previously published observations (Lopez et al., 1976) and follows the same pattern seen for phosphate and arsenate (Lopez et al., 1976; Lazdunski & Lazdunski, 1966). This increased inhibition occurs in the pH range of the second pK_a of H_3VO_4, consistent with the enzyme specifically binding the dianion.

Chromate, molybdate, and tungstate did not show decreasing K_i values at higher pH. This is consistent with the greater acidity of these compounds (pK_2 = 5.8 for chromate and pK_2 = 3.8 for both molybdate and tungstate; Baes & Mesmer, 1976). These compounds are nearly fully ionized over the pH range tested, and thus no substantial change in the fraction of the dianion form occurs.

The enhancement of vanadate inhibition by phenol is shown in Figure 1. This enhancement can be accounted for by formation of a phenyl vanadate ester which has greater affinity for the enzyme than the metal ion alone. Using a value of 0.97 M^{-1} for the K_{eq} for phenyl vanadate formation at pH 7.4 (Tracey & Gresser, 1986), we calculated a K_i of 2×10^{-7} M for phenyl vanadate. This represents about 1 order of magnitude greater affinity than vanadate alone.

Phenol at 80 mM did not enhance inhibition by molybdate or tungstate. This is consistent with the low pK_a of these

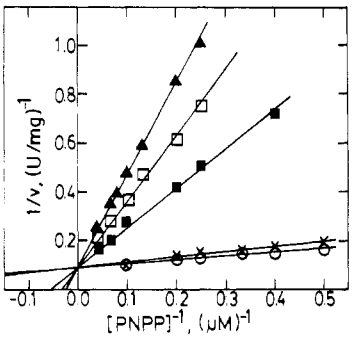

FIGURE 1: Effect of phenol on vanadate inhibition of alkaline phosphatase at pH 7.4. (○) No inhibition; (×) 80 mM phenol; (■) 40 μM vanadate; (□) 40 μM vanadate + 40 mM phenol; (▲) 40 μM vanadate + 80 mM phenol.

compounds and the absence of a significant amount of phenyl ester formation with the fully ionized species of the oxyanion. At pH 7.0, chromate exists as about 6% in the monoanion form. This should provide for a small amount of phenyl chromate formation and thus account for the detectable enhancement of inhibition by phenol. Thermodynamically less favorable ester formation is a reasonable explanation for the larger K_i/K_{eq} for phenyl chromate at higher pH. It is reasonable to expect formation of phenyl chromate to be less favorable than formation of phenyl vanadate at pH 7.0, because most of the chromate exists as the dianion. Also, the K_{eq} for formation of phosphochromate dianion (Frennesson et al., 1968) from chromate and phosphate monoanions is about 10-fold smaller than the K_{eq} for formation of phosphovanadate dianion from vanadate and phosphate monoanions (Gresser et al., 1986).

No inhibition enhancement was observed with TEE at the concentrations employed, which were limited by the solubility of TEE. No enhancement of phosphate inhibition by phenol was observed. This is consistent with the very low K_{eq} for formation of phenyl phosphate (Tracey & Gresser, 1986) and the very slow rate of spontaneous formation of phosphate esters (Lagunas, 1980). This observation strongly supports the hypothesis that the enhancement of metal oxyanion inhibition reported here is due to ester formation and not due simply to phenol and oxyanion being together at the catalytic site.

Acid Phosphatase. Phenol alone at 50 mM had no observable effect on activity at pH 6.0 or 7.0.

Metal oxyanion inhibition was strictly competitive, and Table II shows very little change in K_i over the pH range tested. The observed K_i values are similar to those previously reported for potato acid phosphatase (Uehara et al., 1974) and other acid phosphatases (Van Etten et al., 1974). In another study of metal oxyanion inhibition of potato acid phosphatase (Lora-Tamayo et al., 1969), larger K_i values than found in this study were reported, but the order of effectiveness of the

Table II: Inhibition Constants for Inhibitors of Potato Acid Phosphatase at Different pH Values[a]

inhibitor	K_i (M) (6.0)	phenyl ester K_i/K_{eq} (M^2) (6.0)	K_i (M) (7.0)	phenyl ester K_i/K_{eq} (M^2) (7.0)
vanadate	2.1×10^{-7} ±0.2	N	2.2×10^{-7} ±0.2	N
chromate	5.9×10^{-7} ±0.5	N	1.8×10^{-6} ±0.2	N
molybdate	4.1×10^{-8} ±0.3	N	1.1×10^{-7} ±0.1	N
tungstate	9.2×10^{-9} ±0.8	N	1.0×10^{-8} ±0.9	N

[a] pH values are indicated in parentheses. The ± values below the K_i values are the standard deviations in the mantissa. N indicates no observable enhancement of inhibition.

different anions was the same as that reported here.

The group VI oxyanions showed K_i values almost 3 orders of magnitude lower for acid phosphatase than for alkaline phosphatase. However, the order of potency remained the same: $WO_4^{2-} > MoO_4^{2-} > CrO_4^{2-}$.

Phenol at 50 mM produced no enhancement of inhibition with any of the metal oxyanions tested.

Aryl Sulfatase. Phenol alone had very little effect on activity at pH 6.0 (K_i = 100 mM). At pH 7.0, phenol increased both V_m(app) and K_m(app). V_m(app) increased by a factor of 1.83 at 25 mM phenol and by a factor of 2.44 at 50 mM phenol. At both concentrations, the K_i for phenol calculated from the slopes was 12.5 mM. The values of K_i/K_{eq} calculated for the phenyl esters and shown in Table III were corrected for the effect of phenol alone. TEE alone at 2.0 mM had no effect on the reaction rate.

Group VI oxyanion inhibition was weaker for this enzyme than for the phosphatases. In contrast to the phosphatases, the order of potency is $CrO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$.

The order of K_i values of the metal oxyanions is consistent with the specificity of aryl sulfatase for the monoanion (Dodgson & Powell, 1959; O'Fagain et al., 1983). Vanadate, the most potent inhibitor, exists entirely as the monoanion (pK_2 = 8.4; Tracey & Gresser, 1986) over the pH range. The weaker affinity of chromate at pH 6.0 compared to vanadate is consistent with the fact that chromate exists as only 50% monoanion at pH 6.0, and the still weaker inhibition by molybdate and tungstate is in accord with their lower pK_a values and low affinity of the sulfatase for the dianion.

The weaker inhibition of all metal oxyanions at pH 7.0 compared to 6.0 may be explained in part by an increased fraction of the dianion form of the metal oxyanion but must also reflect a change in ionization of the enzyme, since even at pH 7.0 vanadate exists almost entirely as the monoanion.

Aryl sulfatase was very sensitive to inhibition enhancement by phenol. The values of K_i/K_{eq} reflect the same order of potency as the metal oxyanions alone. The decreased affinity of the phenyl esters at higher pH may be explained by the lower value expected for K_{eq} as well as a change in ionization of the enzyme.

TEE provided approximately 8-fold greater inhibition enhancement than did phenol. This can be explained by the increased favorable hydrophobic interactions provided by TEE. It was surprising that no inhibition enhancement was observed with molybdate and tungstate in the presence of TEE. This may be due to a more favorable interaction of these compounds with the nitrogen atom of TEE (Spence & Lee, 1965) over ester formation.

Discussion

E. coli alkaline phosphatase has long been known to form a stable phosphoseryl enzyme intermediate (Reid & Wilson, 1971). Acid phosphatases from human prostate, wheat germ, and other sources (Van Etten et al., 1975) have been isolated as phosphohistidyl enzymes. Indirect evidence indicates that potato acid phosphatase also contains an active-site histidine (Alverez, 1962). Inhibition by vanadate of enzymes which catalyze phosphoryl transfer reactions is thought to occur via formation of a pentacoordinate structure which is a transition state analogue for the phosphoryl transfer reaction (Knowles, 1980). Because of this special property, vanadate is usually a more potent inhibitor than tetrahedral phosphate. It is therefore surprising that the K_i value for phosphate with alkaline phosphatase (1.5 μM) is approximately equal to that for vanadate. This may result in part from the greater affinity of phosphate for the active-site zinc atoms (Sowadski et al., 1985) over that of vanadate or the group VI oxyanions.

As tetrahedral species in solution (Busey & Keller, 1964; Gonzalez-Vilchez & Griffith, 1972), the group VI dianions are nearly isostructural with sulfate, which does not inhibit alkaline phosphatase observably (Georgatsos, 1967). However, these anions are capable of accepting two additional ligands such as an active-site serine hydroxyl and a water molecule to form octahedral structures. Such structures can be viewed as transition state analogues for hydrolysis of the phosphoenzyme. Formation of this type of structure has been proposed to rationalize molybdate and tungstate inhibition of acid phosphatase (Van Etten et al., 1975), and this is in accord with the fact that periodate, the most potent known inhibitor of alkaline phosphatase (Ohlsson & Wilson, 1974), is known to form the octahedral dihydrate structure $H_3IO_6^{2-}$ in slightly alkaline solution (Crouthamel et al., 1951; Kustin & Lieberman, 1964) and could form a similar structure as a transition state analogue by replacing one water molecule with the serine hydroxyl at the active site for alkaline phosphatase. It is noteworthy that the K_i values of the group VI anions decrease with atomic number, but this may be related to the relative stabilities of octahedral structures. This order for

Table III: Inhibition Constants for Inhibitors of *H. pomatia* Aryl Sulfatase at Different pH Values[a]

inhibitor	K_i (M) (6.0)	phenyl ester[b] K_i/K_{eq} (M^2) (6.0)	TEE[b] K_i/K_{eq} (M^2) (6.0)	K_i (M) (7.0)	phenyl ester[b] K_i/K_{eq} (M^2) (7.0)
vanadate	6.0×10^{-6} ±0.4	5.5×10^{-9} ±0.4	5.0×10^{-10} ±0.6	6.5×10^{-5} ±0.1	5.3×10^{-8} ±0.5
chromate	4.8×10^{-5} ±0.4	1.9×10^{-7} ±0.2	2.9×10^{-8} ±0.3	2.2×10^{-3} ±0.6	1.3×10^{-6} ±0.2
molybdate	1.3×10^{-4} ±0.1	8.8×10^{-7} ±1.1	N	NI	N
tungstate	6.4×10^{-4} ±0.8	1.1×10^{-5} ±0.4	N	NI	N

[a] pH values are indicated in parentheses. The ± values below the K_i or K_i/K_{eq} values are the standard deviations in the mantissa. NI indicates no observable inhibition. N indicates no observable enhancement of inhibition. [b] Although the inhibition is ascribed to binding of phenyl or tyrosine esters of the metallooxyanions, the calculation of the K_i/K_{eq} values does not depend on this assumption (see Experimental Procedures).

increased stability of octahedral structure is supported by the fact that formation of octahedral isopolytungstates in water is more favorable than formation of isopolymolybdates (Rollison, 1973), and such structures for chromate are unknown in aqueous solution.

A study of *E. coli* alkaline phosphatase (Williams & Nayler, 1971) demonstrated that the K_m of synthetic substrates decreased as a function of increased electron-withdrawing character of substituents. Electron withdrawal is thought to activate phosphate for attack by the active-site serine nucleophile. Similarly, the increased affinity of phenyl vanadate or phenyl chromate over the metal ion alone could be due in part to the electron-withdrawing character of phenol which can facilitate formation of a 5- or 6-coordinate structure resembling the transition state for the phosphatase reaction.

Another possible explanation for the enhanced inhibition of alkaline phosphatase by phenyl vanadate derives from a change in the ionization state of the inhibitor. The value of the second K_a for phenyl vanadate is 0.4 pH unit lower than that for H_3VO_4 (Tracey & Gresser, 1986). at any pH over the range studied, there will be a larger fraction of phenyl vanadate in the dianion form compared to vanadate, and alkaline phosphatase is thought to bind the dianion form.

In agreement with previous work, the potent inhibition of acid phosphatase by group VI oxyanions is attributed to the formation of pentacoordinate or hexacoordinate structures which are transition state analogues. The order of inhibition, $WO_4^{2-} > MoO_4^{2-} > CrO_4^{2-}$, is the same as for alkaline phosphatase, and an explanation for this order is again based on the relative stability of octahedral complexes. The thousandfold greater affinity of these inhibitors for acid phosphatase over alkaline phosphatase may result from their greater affinity for imidazole nitrogen over groups at the active site of alkaline phosphatase. This is supported by NMR data which show that a stable complex is formed between molybdate and histidine (Spence & Lee, 1965).

A study of potato acid phosphatase revealed no change in K_m or V_m over a wide range of electron-withdrawing substituents in a series of substituted phenyl phosphates (Lynn et al., 1981). Human prostatic acid phosphatase showed no change in V_m over an even wider range of substrates (Tsuboi & Hudson, 1955; Kilsheimer & Axelrod, 1957). This is consistent with the absence of a noticeable inhibition enhancement by phenol. Milk acid phosphatase did show a positive substituent effect, so different acid phosphatases may give different inhibition patterns.

The presence of a hydrophobic region on alkaline phosphatase near the catalytic site could explain the enhancement by phenol of inhibition by vanadate. A representation of the putative bound phenyl vanadate is shown in Figure 2. The vanadate is represented as a five-coordinate, transition state analogue structure, and close contact between the hydrophobic region of the enzyme and the phenyl group is shown. The existence of such a hydrophobic region on *E. coli* alkaline phosphatase is supported by the more than 100-fold greater k_{cat}/K_m ratio for aryl phosphates compared with alkyl phosphates having similar thermodynamic stability (Hall & Williams, 1986), although the crystal structure of the enzyme does not show an obvious hydrophobic "pocket" near the active-site serine residue (Sowadski et al., 1985). It is possible that the presence of this hydrophobic region, which may make the site of phosphorylation somewhat less accessible to water, is also responsible for the higher K_i values of all of the metal oxyanions for *E. coli* alkaline phosphatase than for potato acid phosphatase. In order for the metal oxyanion to act as an

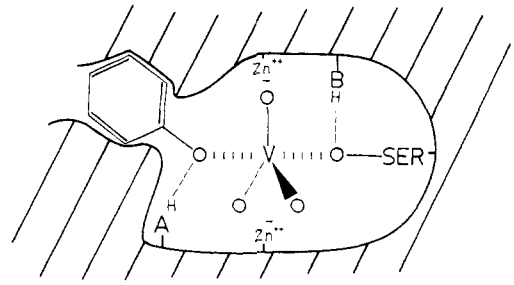

FIGURE 2: Representation of the putative phenyl vanadate bound at the catalytic site of alkaline phosphatase. The vanadate moiety is shown in a five-coordinate transition state analogue structure, with stabilization provided by binding of the oxygens to zinc ions and interaction of the phenyl group with a hydrophobic region near the catalytic site.

transition state analogue for the dephosphorylation reaction, it must accept one or more additional water ligands. This is likely to be less favorable near a hydrophobic region.

Aryl sulfatase is an enzyme which is thought to catalyze the hydrolysis of sulfate esters by an S_N1 elimination mechanism with protonation of the oxygen atom of the leaving group (O'Fagain et al., 1983; Benkovic & Danikoski, 1970). The resulting sulfur trioxide is solvated in a second step to produce the product, HSO_4^-. Group VI oxyanion inhibition of this enzyme follows a pattern different than that observed for either phosphatase. If the elimination mechanism is the catalytic mechanism for aryl sulfatase, then as transition state analogues group VI oxyanions must adopt a structure intermediate between trigonal-planar sulfur trioxide and tetrahedral sulfate monoanion. Group VI oxyanions tend to form octahedral structures which are apparently poorly recognized by aryl sulfatase. Aryl sulfatase would then be expected to have greater affinity for tetrahedral ligands such as phosphate or sulfate. This would account for the reverse order ($CrO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$) of inhibition by the group VI series. This same order of inhibition might also be expected to occur in phosphatases whose catalytic mechanisms do not involve formation of a pentacoordinate transition state.

The potent inhibition of aryl sulfatase by vanadate may be explained by its adopting a structure resembling metavanadate, which can be viewed as an analogue of sulfur trioxide. Vanadate may also inhibit as a tetrahedral species.

The large inhibition enhancement of aryl sulfatase provided by phenol or TEE may be explained by their electron-withdrawing ability or by the existence of a hydrophobic region in the catalytic site. The aryl sulfatase of *Alcaligenes metalcaligenes* showed a large decrease in K_m and an increase in V_m as a function of increased electron-withdrawing character in a series of substituted phenyl sulfates (Dodgson et al., 1956). The much greater enhancement of inhibition by TEE compared with phenol supports a rationalization based on an interaction of tyrosine or phenol with the enzyme, since the phenol group of tyrosine is similar to phenol in electron-withdrawing ability.

The hypothesis which was tested in these studies is that in some cases a vanadate complex which resembles the substrate should be a more potent inhibitor of a phosphatase or sulfatase than vanadate alone. The results with alkaline phosphatase and aryl sulfatase support this hypothesis and indicate that the effectiveness of vanadate as an inhibitor of a given phosphatase might vary considerably depending on the concentration of dephosphorylated product present. If the phosphatase is fairly nonspecific, then the presence of dephosphorylated substrate would not be expected to enhance vanadate inhibition, because the dephosphorylated substrate

has no strong specific interactions with the enzyme. This is the case with the acid phosphatase studied here, and the serine and threonine protein phosphatases which have been studied also appear to have fairly broad specificity (Sparks & Brautigan, 1986).

The tyrosine protein phosphatases, on the other hand, appear from available information to be quite selective, even among proteins and peptides phosphorylated on tyrosine residues (Sparks & Brautigan, 1985, 1986). In order to ascertain quantitatively the relative selectivity of phosphoserine or phosphothreonine and phosphotyrosine phosphatases, it will be necessary to determine k_{cat}/K_m values for a range of phosphoprotein substrates. In view of the present study, and the information cited above about protein phosphatases, it is reasonable to expect that if a serine or threonine phosphatase is assayed in the presence of a concentration of its substrate well above its K_m, then vanadate at around its K_i value will cause little inhibition even in the presence of relatively high concentrations of dephosphorylated substrate. Tyrosine phosphatases, on the other hand, would be expected to be inhibited by vanadate under similar conditions. These conditions are likely to be those which exist physiologically, since any protein whose activity is regulated by phosphorylation/dephosphorylation will be present at significant concentration in its dephosphorylated form when the phosphatase which dephosphorylates it is active. Thus, the hypothesis provides a rationalization for the effects of vanadate on processes which are regulated by phosphorylation of tyrosine.

The relevance of the hypothesis developed in this study to protein phosphatases cannot be ascertained until additional detailed kinetic studies have been done using purified protein phosphatases.

The work reported here on aryl sulfatase extends application of the hypothesis to the still poorly understood phenomenon of tyrosine sulfation (Hortin et al., 1986; Baeuerle & Huttner, 1985; Liu & Lipmann, 1985; Hille et al., 1984; Liu & Baenziger, 1986).

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Registry No. TEE, 840-97-1; VO_4^{3-}, 14333-18-7; CrO_4^{2-}, 13907-45-4; MoO_4^{2-}, 14259-85-9; WO_4^{2-}, 14311-52-5; phenol, 108-95-2; alkaline phosphatase, 9001-78-9; acid phosphatase, 9001-77-8; aryl phosphatase, 9016-17-5; tyrosine protein phosphatase, 80449-02-1.

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Isotope Partitioning for NAD-Malic Enzyme from *Ascaris suum* Confirms a Steady-State Random Kinetic Mechanism†

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ABSTRACT: Isotope partitioning studies beginning with E·[^{14}C]NAD, E·[^{14}C]malate, E·[^{14}C]NAD·Mg^{2+}, and E·Mg·[^{14}C]malate suggest a steady-state random mechanism for the NAD-malic enzyme. Isotope trapping beginning with E·[^{14}C]NAD and with varying concentrations of Mg^{2+} and malate in the chase solution indicates that Mg^{2+} is added in rapid equilibrium and must be added prior to malate for productive ternary complex formation. Equal percentage trapping from E·[^{14}C]NAD·Mg and E·Mg·[^{14}C]malate indicates the mechanism is steady-state random with equal off-rates for NAD and malate from E·NAD·Mg·malate. The off-rates for both do not change significantly in the ternary E·Mg·malate and E·NAD·Mg complexes, nor does the off-rate change for NAD from E·NAD. No trapping of malate was obtained from E·[^{14}C]malate, suggesting that this complex is nonproductive. A quantitative analysis of the data allows an estimation of values for a number of the rate constants along the reaction pathway.

The NAD-malic enzyme from *Ascaris suum* is proposed to have a steady-state random mechanism in the direction of oxidative decarboxylation of malate. The mechanism is based on initial velocity studies varying substrate and metal ion concentrations over a wide range (Park et al., 1984), determination of enzyme-reactant dissociation constants (Kiick et al., 1984), and deuterium isotope effects (Kiick et al., 1986). Further, it has been proposed that two of the transitory enzyme-substrate complexes, E·malate and E·NAD·malate, are nonproductive.

Recently, studies on the malic enzyme (Kiick et al., 1986) have shown small deuterium isotope effects of 1.45 on V/K_{malate}, V/K_{NAD}, and V_{max}. This suggests that hydride transfer may not be the primary rate-limiting step. $^{D}(V/K_{malate})$ and $^{D}(V/K_{NAD})$ remained constant over the pH range of 5-10, but $^{D}V_{max}$ decreases to a value of 1 at low pH values. The change in $^{D}V_{max}$ with pH gave a pK about 4.9 in agreement with that obtained for the pH dependence of V_{max} and suggests that NADH release limits the rate of the reaction partially at

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