Effect of Oxyanions of the Early Transition Metals on Rabbit Skeletal Muscle Phosphorylase†

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ABSTRACT: The differential effects of the oxyanions of the early transition metals ions V(V), W(VI), and Mo(VI) on the catalytic activity and coenzyme binding of rabbit skeletal muscle phosphorylase are studied. The oligoanions of V(V), W(VI), and Mo(VI) are potent inhibitors of phosphorylase. Kinetic studies revealed that oligovanadates inhibit pyridoxal-reconstituted phosphorylase *b* by competing with both the substrate, glucose 1-phosphate, and the activator, phosphite, with K_I values of 4 μM and 6 μM, respectively. Oligovanadates in the millimolar concentration range inhibit phosphorylases *a* and *b* by competing with glucose 1-phosphate binding. The polymeric decavanadate and paratungstates caused time-dependent inactivation of phosphorylase. Spectral studies with tungstate and phosphorylase *b* revealed that the inactivation is due to deformation of the coenzyme site. Kinetic studies and the protective effects of substrate and effectors on inactivation and deformation by tungstate or vanadate suggest that deformation and inactivation is caused by a primary binding of the oligoanions at the glucose 1-phosphate site. Nuclear magnetic resonance (NMR) studies of vanadate–phosphorylase complexes and vanadate solutions under different conditions were carried out to ascertain the nature of vanadate ions interacting with functional groups in phosphorylase. The results suggest that decavanadate is the major protein-bound species. NMR studies also showed that guanidino groups react with decavanadate and suggest that arginine residues in phosphorylase are potential functional groups that can interact with decavanadate. The effects of the oligoanions on the catalytic activity and coenzyme binding of phosphorylase are explained in terms of preferential binding of these ions at the catalytic site encompassing the glucose 1-phosphate and pyridoxal 5′-phosphate binding sites.

X-ray crystallography studies of glycogen phosphorylase show that the phosphoryl group of the coenzyme, pyridoxal 5′-phosphate, is in proximity to the phosphoryl group of glucose-1-P[1] (Sygusch et al., 1977; Weber et al., 1978). The inhibitory effects of pyrophosphate on pyridoxal phosphorylase and the binding and reactions of the pyridoxal analogue suggest that pyrophosphate or a pyrophosphate-like adduct could span the two sites (Parrish et al., 1977). The experiments of Withers et al. (1981) and Takagi et al. (1982) led them to suggest that the phosphoryl group of the coenzyme is distorted to a trigonal-bypyramidal form during catalysis. Because vanadate and other oxyanions of the early transition metals, e.g., tungstate and molybdate, are potent inhibitors of acid and alkaline phosphatases (Van Etten et al., 1974; Lopez et al., 1976; Macara, 1980) and inhibition was explained by imitating the trigonal-bypyramidal form of phosphoryl group, we initiated studies of the effects of oxyanions on the pyridoxal 5′-phosphate binding site in phosphorylase. Chang et al. (1983) observed that vanadate, molybdate, and tungstate are potent inhibitors of pyridoxal-reconstituted phosphorylase. The very strong inhibitory effects of vanadate and tungstate on the pyridoxal enzyme prompted us to carry out a detailed investigation of the effect of these oxyanions on native phosphorylase. In this paper, we report the effects of the oxyanions of V(V), W(VI), and Mo(VI) on the catalytic activity and coenzyme function of rabbit muscle glycogen phosphorylase.

Materials and Methods

Rabbit muscle glycogen phosphorylase *b* was prepared according to Fischer & Krebs (1962) and converted to phosphorylase *a* by the method of Krebs (1966). Apophosphorylase *b* was prepared according to the method of Shaltiel et al. (1966) and reduced phosphorylase *b* by the method of Graves et al. (1975). Pyridoxal-reconstituted phosphorylase *b* was prepared according to Parrish et al. (1977). Phosphorylase activity in the direction of glycogen synthesis was measured either by the estimation of liberated inorganic phosphate (Fiske & Subbarow, 1925) or by the measurement of the incorporation of [U-^{14}C]glucose into glycogen by the filter paper assay (Thomas et al., 1968).

Protein concentration of phosphorylase was measured by using an extinction coefficient $E^{1\%}_{1cm}$ of 13.2 at 280 nm (Krebs, 1966). [U-^{14}C]Glucose 1-phosphate was obtained from Amersham. All other chemicals were of the highest quality commercially available.

Vanadate solutions were prepared from sodium orthovanadate. A 0.2 M solution was prepared and the pH adjusted to 6.8 with 3 N HCl. The solution was diluted to 0.1 M and kept at room temperature for at least 24 h before use. Studies with vanadate were performed with this solution unless otherwise specified. Vanadate solutions free of the orange-yellow decavanadate were prepared by boiling a vanadate solution at pH 10.5. The boiled vanadate was carefully diluted in buffers at pH 6.8 at the time of the experiment. This solution still contains oligomeric species such as di-, tri-, and tetravanadates, along with monomers. But no significant decavanadate is present (Pope & Dale, 1968). Tungstate and molybdate solutions (0.1 M) were prepared from sodium tungstate or sodium molybdate, and the pH was adjusted with 3 N HCl.

The stoichiometry of vanadate bound to phosphorylase *b* was determined by analyzing the protein-bound vanadate, with the metallochrome dye 4-(2-pyridylazo)resorcinol as described by Goodno (1979). Phosphorylase *b* (10 mg/mL) in 40 mM β-glycerophosphate–30 mM 2-mercaptoethanol was incubated

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[1] Abbreviations: glucose-1-P, glucose 1-phosphate; AMP, adenosine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

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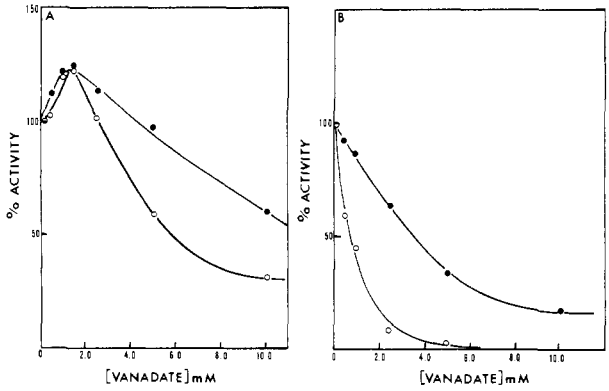

FIGURE 1: Effect of vanadate on catalytic activity of rabbit muscle phosphorylase. (A) Phosphorylase *b* activity as a function of vanadate concentrations. Phosphorylase *b* activities are measured in the direction of glycogen synthesis by measuring the incorporation of [^{14}C]glucose into glycogen by the filter paper assay. The reaction system contained 1% glycogen, 16 mM [U-^{14}C]glucose-1-P, (○) 1 mM AMP or (●) 5 mM AMP, and indicated amounts of vanadate at pH 6.8, 20 mM β-glycerophosphate, 1 mM EDTA, and 16 μg/mL phosphorylase *b*. The activity in the absence of vanadate is taken as 100%. (B) Phosphorylase *a* activity as a function of vanadate concentration. The reactions conditions are the same as in (A): (○) no AMP; (●) 1 mM AMP.

with vanadate at pH 6.8 for 90 min and then passed through a 2 × 45 cm column of Sephadex G-25 equilibrated in the same buffer to separate the free vanadate fromprotein-bound vanadate. Protein fractions were pooled, the pH was adjusted to 10.5 with 1 N NaOH, and the solution was boiled to dissociate the oligomers. An aliquot of the sample was analyzed for vanadium. A sample of vanadate was also adjusted to pH 10.5, boiled, and used as a reference standard.

For NMR analysis, phosphorylase *b* (10 mg/mL) was incubated with 10 mM vanadate at pH 6.8 for 90 min, and the free vanadate was removed from protein-bound vanadate by passage through a Sephadex G-25 column. Protein fractions were pooled and concentrated by filtration through a PM 30 membrane, in Amicon cells.

Vanadium-51 nuclear magnetic resonance measurements were made at 78.91 MHz on a Bruker MW300 spectrometer at 293 K. The ^{51}V NMR signal of $VOCl_3$ was used as a standard. D_2O (5% v/v) was included in the sample and used as the field/frequency lock. A 10-mm NMR tube was used in each ^{51}V NMR measurement. A spectral width of 41 666 Hz was employed, with a pulse width of 25 μs and a repetition time of 0.3 s. Exponential line broadening used before Fourier transformation of different spectrum is indicated in the figure legends.

Results and Discussion

Effect of Oxyanions on Phosphorylase Activity. The response of phosphorylases *b* and *a* to varying concentrations of V(V) oxyanions at pH 6.8 is given in Figure 1. At low concentration of vanadate, there is a slight stimulation of phosphorylase *b* activity, but higher concentrations of vanadate are inhibitory. Increasing the AMP concentration from 1 to 5 mM raises the activation level of vanadate and reduces the inhibition. Similar effects of activation and inhibition are seen with tungstate. Molybdate does not inhibit the enzyme but shows a slight activation (5–10%) wiith concentrations up to 15 mM (results not illustrated). Phosphorylase *a* is inhibited at all concentrations of vanadate, and the enzyme is more sensitive to inhibition by vanadate in the absence of AMP. The activity ratio (−AMP/+AMP) decreases with increasing vanadate concentrations, and above 4 mM vanadate, the enzyme shows an almost complete requirement for AMP for activity.

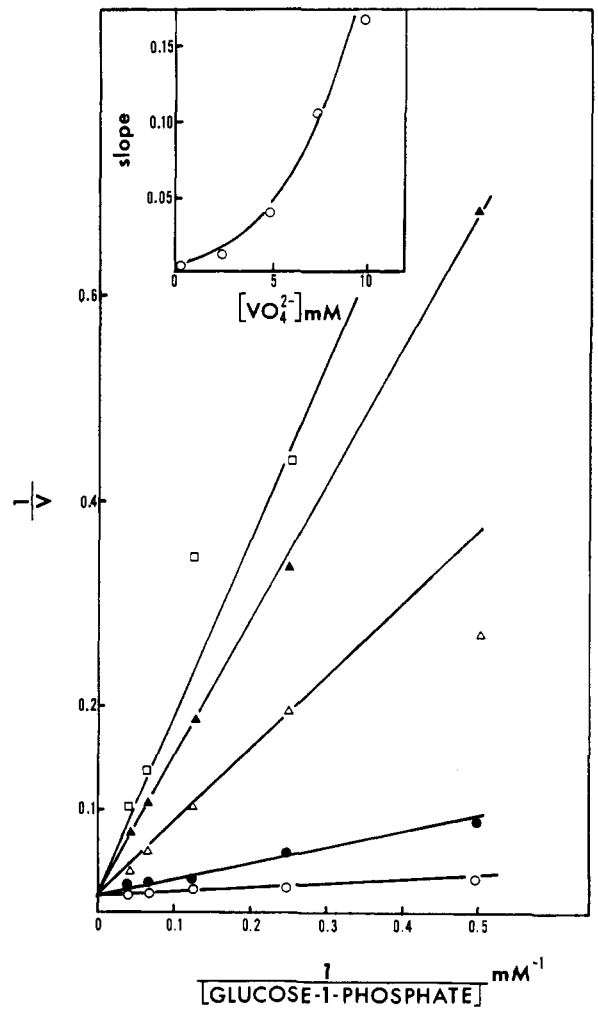

FIGURE 2: Kinetics of inhibition of phosphorylase *a* by vanadate. Phosphorylase *a* activity is followed in the direction of glycogen synthesis by measuring the incorporations of [^{14}C]glucose into glycogen. The reactions systems contained 1% glycogen, 2 mM AMP, and indicated amounts of [U-^{14}C]glucose-1-P, 20 mM β-glycerophosphate, 1 mM EDTA, and 10 μg/mL phosphorylase *a* at pH 6.8: (○) no vanadate; (●) 2.5, (△) 5, (▲) 7.5, and (□) 10 mM vanadate. (Inset) Secondary plot of slope vs. vanadate concentrations.

Sealock & Graves (1967) reported a similar result from using high concentration of perchlorate.

Because phosphorylase *a* was only inhibited by vanadate and tungstate, further kinetic studies were carried out to evaluate the nature of inhibition. In the absence of AMP, nonlinear double-reciprocal plots for glucose-1-P are obtained in the presence of vanadate, but in the presence of 2 mM AMP, vanadate exhibited competitive inhibition with glucose-1-P (Figure 2). But, the secondary plots of slopes (inset in Figure 2) are nonlinear. Because the vanadate solution at pH 6.8 contains several polyanions in varying proportion, the nonlinearity in the secondary plots may be due to the presence of more than one inhibitory species. Alternatively, inhibition could be explained by binding of the inhibitor ion at a site distinct from the glucose-1-P site (partial competitive inhibition) or by binding of the inhibitor at more than one site. The inhibition with phosphorylase *b* also is similar to that with phosphorylase *a* (results not illustrated).

Because of the complexity of the solution chemistry of the oxyanions of V(V), W(VI), and Mo(VI), the exact nature of the molecular species causing the activation and/or inhibition cannot be ascertained precisely. The nature and extent of polymerization of these oxyanions depend largely on concen-

Table I: Effect of Vanadate, Tungstate, and Molybdate on Catalytic Activity of Rabbit Muscle Phosphorylase *b* at Different pH Values[a]

ligand concn (mM)	% activity at different pH values[b]								
	pH 6.0			pH 6.8			pH 7.5		
	vanadate	tungstate	molybdate	vanadate	tungstate	molybdate	vanadate[c]	tungstate	molybdate
0.5	10.0	11.0	98.0	108	107	100	97	100	100
2.5	2.0	2.5	84.0	96	98	100	67	100	102
5.0	0.0	0.0	45.0	87	90	102	46	102	105
10.0	0.0	0.0	3.0	42	80	106	30	115	115

[a] Phosphorylase activity is determined by measuring the [^{14}C]glucose incorporation into glycogen. Reaction system contained 16 mM [U-^{14}C]glucose-1-P, 1 mM AMP, 1% glycogen, 20 mM sodium β-glycerophosphate, 1 mM EDTA at the respective pH, and 11 μg/mL phosphorylase *b*. [b] Activity in the absence of the oxyanions is taken as 100%. 0.1 M solutions of the oxyanions at the respective pH are prepared and diluted in the assay system at the time of experiment. [c] Vanadate solution at pH 7.5 was prepared by diluting a 0.1 M solution of vanadate boiled at pH 10.5 in the assay buffer at pH 7.5.

tration and pH. Hence, the effect of these ions on the activity of phosphorylase *b* at different pH values was compared. Table I shows the effect of vanadate, tungstate, and molybdate on phosphorylase activity at different pH values. At pH 6.0, vanadate, tungstate, and molybdate are all inhibitory. Vanadate or tungstate (0.5 mM) causes 90% inhibition, but molybdate at 0.5 mM and other concentrations is much less inhibitory. In 0.1 M solution at pH 6.0, polymerization to the decavanadate and paratungstate is almost complete (Pope & Dale, 1968; Kepert, 1962), and dilution to lower concentrations in the assay system may not cause any appreciable dissociation during the assay time. Hence, the inhibition at pH 6.0 is presumed to be due to the isopolyanions decavanadate or paratungstate. The molybdate solution at pH 6.0 contains the oligomeric paramolybdate and the monomeric species (Cannon, 1959; Sasaki et al., 1959). Studies at pH 6.8 show that vanadate and tungstate at low concentrations slightly enhance the phosphorylase *b* activity as measured with 1 mM AMP but higher concentrations are inhibitory. Molybdate has little effect under these conditions. Under these conditions, the vanadate solution contains the decavanadate with other oligomers, and tungstate solutions contain the polymeric paratungstates and monomers (Kepert, 1962, 1973). But, molybdate is essentially in the monomeric form (Sasaki et al., 1959). Thus the activation and inhibition observed with vanadate and tungstate at pH 6.8 are due to the oligomers. The lack of inhibition at pH 6.8 with molybdate further supports the presumption that the polyanion is the inhibitory species. At pH 7.5, both molybdate and tungstate slightly enhance the phosphorylase *b* activity measured with 1 mM AMP. Because there is no evidence for the polymerization of tungstate and molybdate at pH 7.5, the activation at pH 7.5 is presumably due to monomeric tungstate or molybdate. Solutions of vanadate boiled at pH 10.5 to remove the orange-yellow decavanadate and subsequently diluted in buffers at pH 6.8 or 7.5 are still inhibitory, but no activation is seen. The vanadate solution at pH 7.5 still contains the oligomeric di-, tri-, or tetravanadates along with monomeric forms. The inhibitory effect under these conditions is very likely due to the oligomeric vanadates.

At pH 6.8, oligomeric vanadate competes with both glucose-1-P and the activator phosphite sites of pyridoxal-reconstituted phosphorylase *b* with K_I values of 4 μM and 6 μM, respectively (results not illustrated). Molybdate, which exists in the monomeric state under similar conditions, also showed competitive inhibition with both glucose-1-P and phosphite, but the K_I values were much higher (Chang et al., 1983). A solution of vanadate boiled at pH 10 to remove the orange-yellow decavanadate and subsequently diluted in buffers at pH 7.2 and kept for several days to complete dissociation of oligomers is much less inhibitory than the unboiled vanadate at pH 6.8 and is competitive with glucose-1-P and noncompetitive with phosphite (Figure 3). The results suggest that the polyanion of vanadate can interact with both glucose-1-P and phosphoryl binding sites of pyridoxal enzyme possibly by extending over the two sites as suggested earlier for the inhibition by pyrophosphate (Parrish et al., 1977).

Inactivation of Phosphorylase by the Oxyanions. As vanadate and tungstate at pH 6.8 inhibited phosphorylases *a* and *b*, the effect of incubation of phosphorylases *a* and *b* with these oxyanions was studied to determine if a time-dependent inactivation process could occur. Both vanadate and tungstate (5–10 mM) caused time-dependent inactivation of the enzyme, but molybdate (10 mM) has no significant effect. With 10 mM vanadate or tungstate, complete inactivation occurs in 75 min. A boiled solution of vanadate free of decavanadate did not inactivate. Solutions of vanadate and tungstate prepared at pH 6.0 are several fold more effective in inactivating the enzyme than those prepared at pH 6.8, which is consistent with inhibitory effects shown in Table I. These results suggest that the inactivation is effected by the polyanions decavanadate and paratungstate. Phosphorylase *a* is also inactivated under these conditions.

Incubation of phosphorylase *b* with tungstate results in distinct spectral changes at 330, 415, and 390 nm. Figure 4A shows the spectral changes accompanying the incubation of phosphorylase *b* with tungstate. The inactivation of phosphorylase *b* with tungstate follows the same profile as the absorbance changes at 415 nm. Both inactivation and increase in absorbance at 415 nm are complete within 75–90 min of incubation with 10 mM tungstate, while the release of coenzyme as judged by the appearance of a free PLP band at 390 nm is much slower. The results are consistent with distortion of the pyridoxal 5′-phosphate binding site followed by removal of coenzyme from native enzyme. Figure 4B shows results obtained with $NaBH_4$-reduced phosphorylase *b* and tungstate. No coenzyme is released from reduced phosphorylase because it is attached covalently to a lysyl residue (Fischer et al., 1958). Yet, it is found that tungstate influences the spectral properties of reduced enzyme and causes a time-dependent inactivation. The increase in absorbance at 340 nm and the inactivation of reduced enzyme with tungstate follows a similar profile (results not illustrated). Figure 4C shows the spectral changes with pyridoxal-reconstituted phosphorylase *b* and tungstate. With 5 mM tungstate, the spectral shift at 415 nm was maximum within 5 min of incubation, and the hydrolysis of the pyridoxal from the distorted enzyme was complete within 1 h as judged by the disappearance of 415-nm band. Thus, it is obvious that the distortion of the coenzyme site and subsequent resolutions are much faster with pyridoxal phosphorylase *b*. The relatively high affinity of the oxyanions for the pyridoxal-reconstituted

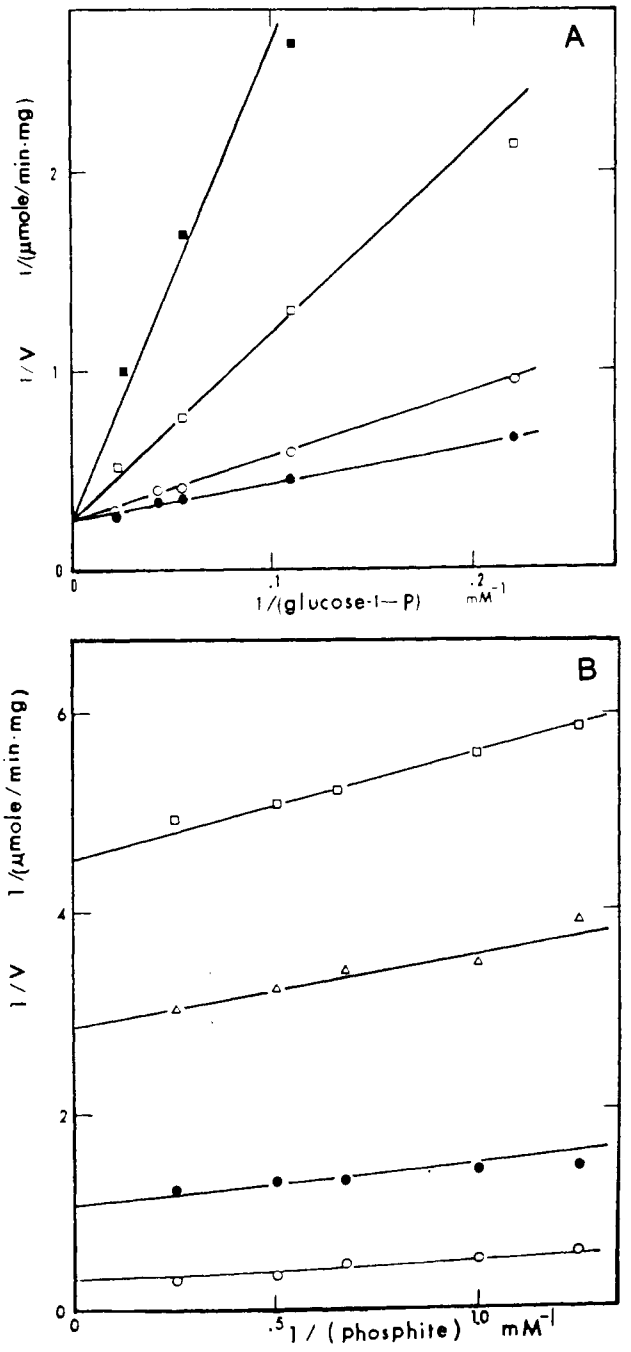

FIGURE 3: Kinetics of inhibition of pyridoxal-reconstituted phosphorylase *b*. Double-reciprocal plots for vanadate inhibition of pyridoxal-reconstituted phosphorylase *b*. Reaction mixture at pH 7.2 and 30 °C consisted of 0.06 M Tris, 0.004 M 2-mercapthoethanol, 0.001 M AMP, 1% glycogen, and 250 μg/mL pyridoxal-reconstituted phosphorylase. (A) Phosphite, 0.002 M, [U-^{14}C]glucose-1-P at the indicated concentrations, and vanadate at 0 (●), 0.4 (○), 0.8 (□), and 1.25 mM (■). (B) [U-^{14}C]Glucose-1-P, 0.016 M, phosphite at the indicated concentrations, and vanadate at 0 (○), 1.25 (●), 3.0 (△), and 5 mM (□). The vanadate solution used in this study was boiled at pH 10.5 to remove the polymeric vanadate before use.

phosphorylase *b* compared with that of native phosphorylase *b* (pyridoxal enzyme is inhibited by micromolar concentrations of vanadate or tungstate, whereas native enzyme is inhibited only at millimolar concentrations) and the relative easiness of detachment of coenzyme from pyridoxal enzyme as compared to native enzyme suggests that the phosphate moiety of coenzyme can influenze the binding of these oxyanions. Either this covalently bound phosphate of enzyme competes with the oligoanions binding at the active site or the proper fitting of the oligoanions requires the phosphate site of coenzyme.

To further ensure that inactivation of native phosphorylase is due to deformation followed by the removal of PLP, an aliquot of phosphorylase *b* completely inactivated in 5 mM vanadate was diluted in 0.04 M β-glycerophosphate–0.03 M L-cysteine at pH 6.8 to a vanadate concentration <50 μM and incubated at 30 °C with a 20-fold excess of pyridoxal 5′-phosphate for 20 min and then assayed for activity. The enzyme could be reactivated to approximately 40% of the original activity by this process. In the absence of L-cysteine, the recovery was only 10%, and in the absence of pyridoxal 5′-phosphate, it was nil.

Figure 5 shows the effect of substrate and AMP on the rate of inactivation of phosphorylase *b* by vanadate. Neither the substrate glucose-1-P (20 mM) nor the activator AMP (1 mM) alone can afford much protection against inactivation. But, when both glucose-1-P and AMP are present together, significant protection was observed. The protective effect is more pronounced at the initial period. During the initial 5 min in presence of the substrate and AMP, there is no inactivation, whereas in their absence, the enzyme is 40% inactivated. Various other ligands and conditions were also tested for their effect on deformation or inactivation by the oxyanions by following spectral changes or activity. Imidazole buffer enhanced the rate of inactivation and deformation, and citrate (50 mM), EDTA (50 mM), pyrophosphate (50 mM), or gluconolactone (20 mM) reduced inactivation caused by vanadate or tungstate. Complete inactivation of phosphorylase *b* could be effected by incubating the enzyme with 1 mM vanadate or tungstate in 0.4 M imidazole–HCl at pH 6.8 for 20–30 min. Similar results were obtained with reduced phosphorylase *b*. The results suggest a common mechanism by which the oxyanions can cause inactivation of native, reduced, and pyridoxal-reconstituted phosphorylase *b*. The loss of activity can be explained by a distortion of the coenzyme site and not by the loss of coenzyme. A similar conclusion was reached earlier by Ford & Mason (1968) in their study of the effects of anionic steroids on native phosphorylase *b*.

The kinetics of inhibition and the protective effects of substrates and inhibitors like pyrophosphate and gluconolactone against deformation of the coenzyme site and inactivation suggest that the inactivation is effected by a primary binding of the oligoanions at the glucose-1-P site. Because of their large size, these oligoanions bound at the glucose-1-P site may extend over to the coenzyme site and cause its distortion. The close proximity of the phosphate sites of PLP and glucose-1-P has been established from X-ray crystallographic studies (Johnson et al., 1980). Withers et al. (1981) and Takagi et al. (1982) recently suggested the possibility of direct interaction of the phosphate of the substrates glucose-1-P and PLP. The active site encompassing the glucose-1-P and PLP site also contains a number of positively charged residues. A few of these are presumed to be involved in interactions with PLP. The highly negatively charged decavanadate or paratungstate ions bound at the active site can neutralize these positive charges and thereby displace the interactions of PLP.

Binding of Vanadate with Phosphorylase. The protein-bound vanadate can be separated from free vanadate by filtration on a Sephadex G-25 column. Dialysis of the protein fractions in several buffers and in the presence of chelating agents does not remove the protein-bound decavanadate. The results suggest that the orange-yellow decavanadate is firmly bound to the protein. However, repeated precipitation of the protein in 50% ammonium sulfate and extensive dialysis can

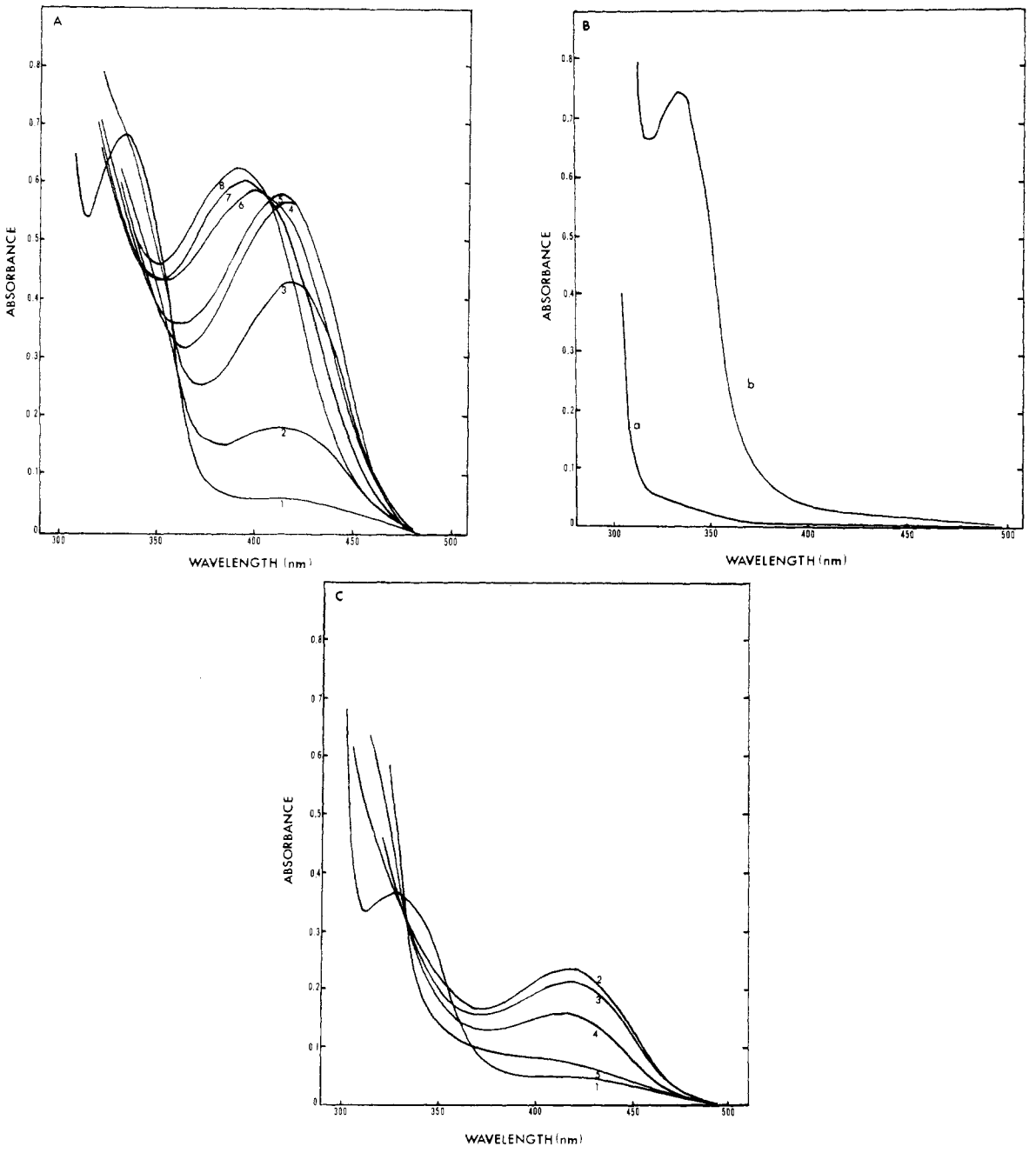

FIGURE 4: Spectral changes accompanying incubation of phosphorylase *b*, reduced phosphorylase *b* and pyridoxal-reconstituted phosphorylase *b* with tungstate. (A) Phosphorylase *b* (10 mg/mL) in 40 mM β-glycerophosphate–2 mM EDTA at pH 6.8 was incubated with 10 mM tungstate (pH 6.8) and the spectrum was recorded at (1) 0, (2) 0.5, (3) 5, (4) 20, (5) 33, (6) 150, (7) 180, and (8) 420 min of incubation. (B) (a) Spectrum of reduced phosphorylase *b* in 40 mM sodium glycerophosphate–30 mM 2-mercaptoethanol, pH 6.8. (b) Spectrum of reduced phosphorylase *b* (9.0 mg/mL) in the same buffer recorded after 1 h of incubation with 5 mM sodium tungstate. The reference cell contained 5 mM tungstate in the same buffer. (C) Pyridoxal-reconstituted phosphorylase *b* (9.0 mg/mL) in 40 mM glycerophosphate–30 mM 2-mercaptoethanol was incubated with 5 mM tungstate at pH 6.8 and spectrum recorded at different time intervals: (1) zero time; (2) 0.5, (3) 5, (4) 25, and (5) 60 min of incubation.

remove most of the orange-yellow species from the protein. At this stage, the enzyme can be reactivated to 40–45% of its original activity by incubation with L-cysteine and PLP. Analysis for vanadate in protein fractions (see Materials and Methods) gave a stoichiometry of 20–30 vanadium molecules/phosphorylase subunit. If one presumes that the decavanadate is the species bound tightly to the protein, this gives rise to a protein to vanadate ratio of 1:2 to 1:3. It seems that more than one decavanadate can interact with the protein subunit. This is consistent with the fact that vanadate can cause some activation as well as inhibition of the enzyme (Figure 1, Table I). It is also possible that inactivation by vanadate exposes new binding sites in the protein.

^{51}V NMR Studies of Vanadate and Its Protein Complex. ^{51}V NMR spectral studies were performed to ascertain the nature of the vanadate species interacting with phosphorylase and the functional groups involved in the interactions. NMR results revealed that in vanadate solution boiled at pH 10.5, the major species is monomeric (−532 ppm) but appreciable dimers (−562 ppm) and trimers (−577 ppm) are also present. In a vanadate solution directly adjusted to pH 6.8, the major species is decavanadate (−516, −498, and −423 ppm), but some dimers and trimers are also present. The results are in good agreement with the reports of Howarth & Richards (1965)

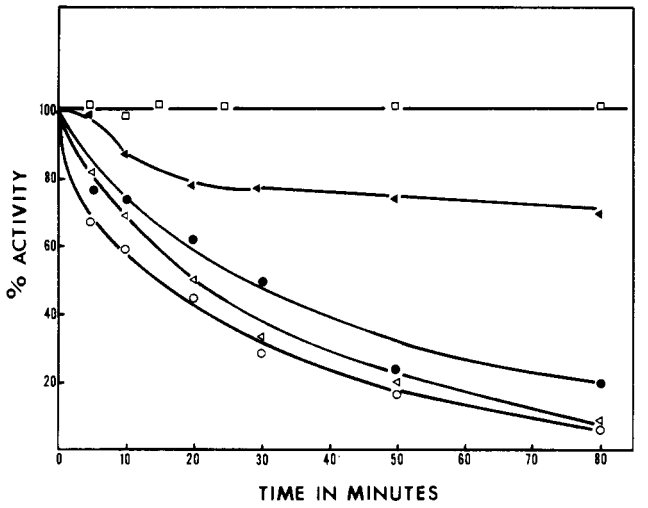

FIGURE 5: Effect of glucose-1-P and AMP on inactivation of phosphorylase *b* by vanadate. Phosphorylase *b* (2.5 mg/mL) in 40 mM β-glycerophosphate–2 mM EDTA, pH 6.8, was incubated at 30 °C: (□) control in the absence of vanadate; (○) in presence of 5 mM vanadate; (△) 20 mM glucose-1-P + 5 mM vanadate; (●) 1 mM AMP + 5 mM vanadate; (▲) 20 mM glucose-1-P + 1 mM AMP + 5 mM vanadate. At various time intervals, aliquots were removed and diluted 100-fold in 40 mM β-glycerophosphate–30 mM mercaptoethanol, pH 6.8, and assayed for phosphorylase activity.

and Pope & O'Donnell (1976). Since studies with phosphorylase were carried out in β-glycerophosphophosphate–EDTA or 2-mercaptoethanol buffers, the effect of these buffer components on ^{51}V NMR was studied. Glycerophosphate buffer (40 mM) has no effect on the vanadate signals. EDTA at 2 mM (Figure 6 A) does not cause any significant effect, but in 50 mM EDTA, the resonance signals of dimers and trimers disappears and a new signal appears at −520 ppm (Figure 6C). In the presence of 30 mM 2-mercaptoethanol, all the three resonance signals of decavanadate gradually disappear, and a new intense signal appears at −363 ppm, but the signals due to dimers and trimers do not change (Figure 6B).

In 40 mM β-glycerophosphate–30 mM 2-mercaptoethanol, the NMR spectrum of vanadate bound to phosphorylase *b* revealed an intense signal at −363 ppm, corresponding to the decavanadate signal in presence of 30 mM 2-mercaptoethanol, and another signal at −562 ppm (Figure 6D). Because the free vanadate is removed by gel filtration before the NMR experiment, the observed signals must arise from protein-bound vanadate. The results can be explained if the protein-bound vanadate is slowly released by interaction with mercaptoethanol, which was in large excess during the long run of the NMR experiment (~12 h). Because the signal at −363 ppm arose solely from the interaction of decavanadate and mercaptoethanol, this result strongly suggests that decavanadate is the major protein-bound species. In 40 mM β-glycerophosphate–2 mM EDTA, the NMR spectrum of protein-bound vanadate revealed a broad resonance signal at −520 ppm. The EDTA-complexed dimers and trimers also give resonance signals at the same position. It is likely that tightly complexed vanadate may have a characteristic resonance signal at −520 ppm.

Among the various ligands tested for their ability to interact with vanadate species in solution, only guanidine was found to have any significant effect. In 0.2 M guanidine at pH 6.8, the decavanadate is precipitated. The supernatant solution after centrifugation showed resonance signals due to only dimers and trimers (Figure 6F). The precipitate is insoluble in water but can be dissolved in 40 mM β-glycerophosphate–30 mM 2-mercaptoethanol. The resulting solution after 90 min

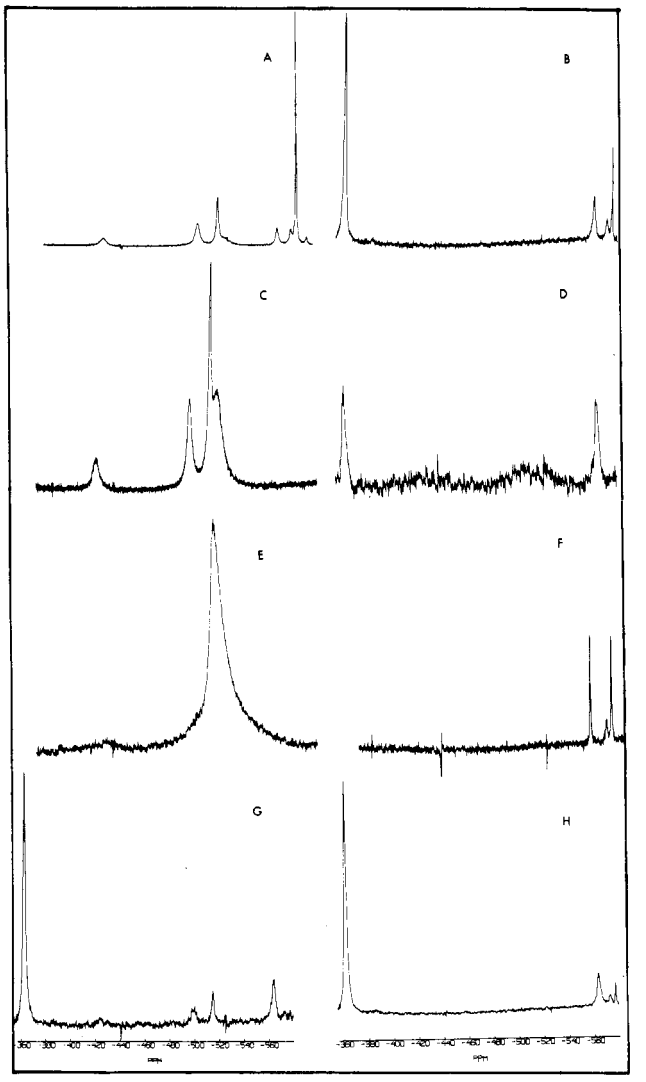

FIGURE 6: ^{51}V NMR spectra of vanadate–phosphorylase complex and vanadate solutions under different conditions. (A) Vanadate (10 mM) in 40 mM β-glycerophosphate–2 mM EDTA, pH 6.8, buffer; 3504 acquisitions; line broadening (LB) = 20 Hz. (B) Vanadate (10 mM) in 30 mM 2-mercaptoethanol–40 mM β-glycerophosphate, pH 6.8, buffer; 1802 acquisitions; LB = 5 Hz. (C) Vanadate (10 mM) in 50 mM EDTA, pH 6.8, buffer; 5840 acquisitions; LB = 2 Hz. (D) Vanadate–phosphorylase *b* complex isolated from the reaction mixture in 30 mM 2-mercaptoethanol–40 mM β-glycerophosphate, pH 6.8, buffer; 36 999 acquisitions; LB = 20 Hz. (E) Vanadate–phosphorylase *b* complex made in 2 mM EDTA–40 mM β-glycerophosphate, pH 6.8, buffer; 117 503 acquisitions; LB = 0 Hz. (F) Supernatant solution of 10 mM vanadate and 0.2 M guanidine–HCl mixture; 2972 acquisitions; LB = 10 Hz. (G) Precipitate from 10 mM vanadate and 0.2 M guanidine–HCl mixture, washed twice by water, and redissolved in 30 mM 2-mercaptoethanol–40 mM β-glycerophosphate, pH 6.8, buffer; spectrum taken 2 h after the precipitate had been redissolved. (H) Spectrum of the same sample of (G) taken 7 h after the precipitate had been redissolved.

shows the same spectrum as phosphorylase *b* bound vanadate in 30 mM 2-mercaptoethanol (Figure 6H). These results suggest arginyl groups in phosphorylase may function as potential functional groups capable of interactions with decavanadate. The chemical-modification studies of Dreyfus et al. (1980) and inhibition studies of Miller et al. (1981) suggested that Arg-568 was involved in the active site. Two Lys-Arg sequences around the active site were also revealed by the sequence analysis of phosphorylase (Titani et al., 1977). The possible interaction of pyridoxal 5′-phosphate with Arg-576 was suggested by Johnson et al. (1980). It is quite possible that the decavanadate or paratungstate bound at the active

site can interact with some arginine residues involved in the interaction with coenzyme and thereby loosen the coenzyme binding.

Recent X-ray diffraction studies on adenylate kinase crystals soaked in a decavanadate solution revealed that the oligovanadates are bound to a highly positively charged segment on that enzyme and fit across the phosphate binding sites of both AMP and ATP sites (Pai et al., 1977). Our results on phosphorylase and the results of DeMaster & Mitchell (1973) and Pai et al. (1977) on adenylate kinase reveal that the oligoanions can fit across two phosphate binding sites on these two enzymes. In both situations, the regions embodying the two sites contained a number of positive-charged residues. It is not certain whether any structural or conformational features of this region other than the clustering of the charged residues are important for the binding of these oxyanions.

Registry No. PLP, 54-47-7; glucose-1-P, 59-56-3; vanadate, 37353-31-4; tungstate, 12737-86-9; molybdate, 11116-47-5; decavanadate, 12397-12-5; phosphorylase *b*, 9012-69-5.

References

Cannon, P. (1959) *J. Inorg. Nucl. Chem. 9*, 252–266.

Chang, C. Y., McCalmont, T., & Graves, D. J. (1983) *Biochemistry* (preceding paper in this issue).

DeMaster, E. G., & Mitchell, R. E. (1973) *Biochemistry 12*, 3616–3621.

Dreyfus, M., Vandenbunder, B., & Buc, H. (1980) *Biochemistry 19*, 3636–3642.

Fischer, E. H., & Krebs, E. G. (1962) *Methods Enzymol. 5*, 369–373.

Fischer, E. H., Kent, A. B., Snyder, E. R., & Krebs, E. G. (1958) *J. Am. Chem. Soc. 80*, 2906.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Ford, J., & Mason, M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 980–987.

Goodno, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2620–2626.

Graves, D. J., Carlson, G. M., Skuster, J. R., Parrish, R. F., Carty, J. T., & Tessemer, G. W. (1975) *J. Biol. Chem. 250*, 2254–2258.

Howarth, O. W., & Richards, R. E. (1965) *J. Chem. Soc.*, 864–890.

Johnson, L. N., Jenkins, J. A., Wilson, K. S., Stura, E. A., & Zanotti, G. (1980) *J. Mol. Biol. 140*, 565–580.

Kepert, D. L. (1962) *Prog. Inorg. Chem. 4*, 196–236.

Kepert, D. L. (1973) *Comprehensive Inorganic Chemistry* (Bailer, J. C., Jr., Emelens, H. T., Ngholm, R. S., & Dickenson, A. F. T., Eds.) Vol. 5, pp 607–672, Pergamon Press, New York.

Krebs, E. G. (1966) *Methods Enzymol. 18*, 543–546.

Lopez, V., Stevens, T., & Lindquist, R. N. (1976) *Arch. Biochem. Biophys. 175*, 31–38.

Macara, I. G. (1980) *Curr. Trends Biochem. Sci. 5*, 92–96.

Miller, J. F., Seybold, M. C., & Graves, D. J. (1981) *Biochemistry 20*, 4579–4586.

Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schuk, G. E. (1977) *J. Mol. Biol. 114*, 37–45.

Parrish, R. F., Uhing, R. J., & Graves, D. J. (1977) *Biochemistry 16*, 4826–4831.

Pope, M. T., & Dale, B. W. (1968) *Q. Rev., Chem. Soc. 22*, 527–548.

Pope, M. T., & O'Donnell, S. E. (1976) *J. Chem. Soc., Dalton Trans.*, 2290–2291.

Sasaki, Y., Lindquist, I., & Sillen, L. G. (1959) *J. Inorg. Nucl. Chem. 9*, 93–94.

Sealock, R. W., & Graves, D. J. (1967) *Biochemistry 6*, 201–207.

Shaltiel, S., Hedrick, J. L., & Fischer, E. H. (1966) *Biochemistry 5*, 2108–2166.

Sygusch, J., Madsen, N. B., Kavinsky, P. J., & Fletterick, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4757–4761.

Takagi, M., Fuki, T., & Shimomura, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3716–3719.

Thomas, J. A., Schlender, K. K., & Larner, J. (1968) *Anal. Biochem. 25*, 486–499.

Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsch, K. A., Neurath, H., & Fischer, E. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4762–4766.

Van Etten, R. L., Waymack, P. P., & Rehkop, D. M. (1974) *J. Am. Chem. Soc. 96*, 6782–6785.

Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G. R., Wild, D. L., & Jenkins, J. A. (1978) *Nature (London) 274*, 433–437.

Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759–10762.