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Nonreductive Interaction of Vanadate with an Enzyme Containing a Thiol Group in the Active Site: Glycerol-3-phosphate Dehydrogenase†

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ABSTRACT: The inhibitory effects of vanadium(V) were determined on the oxidation of glycerol 3-phosphate (G3P) catalyzed by glycerol-3-phosphate dehydrogenase (G3PDH), an enzyme with a thiol group in the active site. G3PDH from rabbit muscle was inhibited by vanadate, and the active inhibiting species were found to be the vanadate dimer and/or tetramer. The dimer was a sufficiently weak inhibitor at pH 7.4 with respect to G3P; the tetramer could account for all the observed inhibition. The tetramer was a competitive inhibitor with respect to G3P with a K_i of 0.12 mM. Both the dimer and tetramer were noncompetitive inhibitors at pH 7.4 with respect to NAD with K_i's of 0.36 mM and 0.67 mM. G3PDH inhibited by vanadate was reactivated when EDTA complexed the vanadate. The reactivation occurred even after extended periods of incubation of G3PDH and vanadate, suggesting that the inhibition is reversible despite the thiol group in the active site. Analogous reactivation is also observed with glyceraldehyde-3-phosphate dehydrogenase (Gly3PDH). Gly3PDH is an enzyme that previously had been reported to undergo redox chemistry with vanadate. The work described in this paper suggests vanadate will not necessarily undergo redox chemistry with enzymes containing thiol groups exposed on the surface of the protein.

The in vivo and in vitro mechanisms by which the trace element vanadium interacts in biological systems are poorly understood in part due to its complex chemistry (Nechay et al., 1986; Gresser et al., 1987). Aqueous solutions of vanadate in the presence of biological materials will form many vanadium compounds, and several of these are likely to exhibit biological activities. Several mechanisms have recently emerged demonstrating how vanadate can affect various en-

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zymes in vitro. One mechanism involves deactivation of the enzyme through, for example, oxidation of an essential sulfhydryl group on the enzyme (Benabe et al., 1987). A second mechanism involves binding of vanadate monomer at a phosphate binding site, thus inhibiting the enzyme by acting as a phosphate analogue (Chasteen, 1983). A third mechanism facilitates the inhibition through generation of organic–vanadate dead-end inhibitor complexes (Stankiewicz et al., 1987; Percival et al., 1990; Ray et al., 1990; Ray & Post, 1990; Ray & Purvathingal, 1990). A fourth mechanism involves inhibition by vanadate oligomers (Boyd et al., 1985; Crans et al., 1989a, 1990a; Crans & Schelble, 1990; Crans & Willsky, 1990). A fifth mechanism focuses on the conversion of spontaneously formed organic vanadate derivatives (organic phosphate analogues) by enzymes converting organic phosphates (Nour-Eldeen et al., 1985; Crans et al., 1987, 1990b; Drueckhammer et al., 1989). Other mechanisms can be suggested. Since vanadate is increasingly used as a reagent in many biological systems, the need to explore the affinity and activity of various vanadate derivatives with different enzyme systems is increasing. This work describes the effect of vanadate on an enzyme that has the possibility of undergoing redox chemistry.

Aqueous vanadium(V) solutions in the physiological pH range contain a complex mixture of vanadate oligomers including vanadate monomer, dimer, tetramer, and pentamer (Pope, 1983). These vanadate derivatives exchange rapidly with each other, and since the exchange rates are on the order of milliseconds, none of the vanadate derivatives can be isolated and separated from each other (Crans et al., 1990c). It is therefore not possible to examine the biological activities of one of the oligomers without including the others. The distribution of vanadium derivatives is very sensitive to vanadate concentration, pH, ionic strength, temperature, buffers, and other compounds present during the biological studies (Heath & Howarth, 1981; Crans et al., 1989b). In the presence of various assay components additional vanadate derivatives form, and some of these derivatives may also influence biological activities. The vanadium(V) species that form in aqueous solutions, that is, the speciation of vanadate solutions, must be determined for each biological system.

Thiols and oxidizing alcohols react with vanadate to generate reduced vanadium species, and several mechanisms for such reduction have been reported (Kustin & Toppen, 1973). The reduction of vanadate to oxovanadium(IV) by cysteine generates cystine and a vanadium(IV) complex (Cohen et al., 1987). Although the details of the reaction were not explored, the formation of cystine suggests that the reduction of vanadate may require two accessible sulfhydryl groups. An enzyme with an isolated thiol group may not undergo the expected redox reactions for thiols free in solution. The redox properties of vanadate derivatives depend on protonation and the structure of the derivative. The vanadium(V) derivative that affects the enzyme activity most is likely to interact strongly with the enzyme and therefore provides the greatest opportunity for redox chemistry between the vanadium(V) and the enzyme. The most active vanadium species in the interaction of vanadate with glycerol-3-phosphate dehydrogenase will be identified.

Vanadate can bind as monomer or as oligomeric vanadate derivatives. The oligomeric vanadate species that have previously influenced enzymatic activity include the vanadate dimer, tetramer, and decamer. The vanadate dimer inhibits seminal fluid acid phosphatase (Crans et al., 1989a), and glucose-6-phosphate dehydrogenase (Crans & Schelble, 1990). The tetramer inhibits 6-phosphogluconate dehydrogenase (Crans et al., 1990a) and glucose-6-phosphate dehydrogenase (Crans & Schelble, 1990). The tetramer also binds to enzymes such as myosin and adenylate kinase as judged by the line broadening of the resonance in the ^{51}V NMR spectra (Cremo et al., 1990; Cremo & Wilcott, 1990). Decameric vanadate inhibits Ca^{2+}-ATPase (Csermely et al., 1985), muscle phosphorylase (Soman et al., 1983), adenylate cyclase (DeMaster & Mitchell, 1973), and hexokinase and phosphofructokinase (Boyd et al. 1985).

Glycerol-3-phosphate dehydrogenase (G3PDH)[1] is the enzyme that links glucose metabolism with lipid metabolism. It reversibly catalyzes the conversion of glycerol 3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP) with NAD as a cofactor (Young & Pace, 1958). G3PDH from rabbit muscle contains a sulfhydryl group in the cofactor site, which has been modified selectively by nucleotide analogues. Modification of this sulfhydryl group leads to enzyme inactivation. Various cofactor analogues bind to the enzyme near or at the sulfhydryl group. It has been shown that the pyrophosphate linkage in NAD is important for tight binding of the cofactor (Anderson et al., 1970; Kim & Anderson, 1969). The kinetic mechanism for G3PDH from rabbit muscle is ordered where the coenzymes bind before the substrates (Bentley & Dickinson, 1974). However, in the extreme pH range modifications to this mechanism have been suggested. G3PDH accepts a series of substrate analogues such as glycerol 3-sulfate (Grazi et al., 1974), *S*-(+)-3,4-dihydroxybutyl phosphoric acid (Cheng et al., 1974), glycerol 3-arsenate (Drueckhammer et al., 1989; Jaffé & Apitz-Castro, 1977), and glycerol 3-vanadate (Craig, 1986; Drueckhammer et al., 1989), which suggests that the active site is fairly flexible in binding substrates.

We are interested in examining how vanadate affects G3PDH for two reasons. First of all, the G3PDH from rabbit muscle contains a sulfhydryl group in the cofactor binding site. Vanadate [vanadium(V)] is reduced to vanadyl cation [vanadium(IV)] in the presence of thiols and other reducing reagents. It is therefore of interest to determine whether or not vanadate can interact with an enzyme with exposed sulfhydryl groups without undergoing redox chemistry. Second, G3PDH has been found to convert spontaneously formed glycerol 3-vanadate and dihydroxyacetone vanadate to corresponding products. In order to study the details of such a conversion, it is necessary to examine alternative mechanisms by which vanadate may interact with G3PDH. This work is therefore carried out to examine if vanadate can interact and coexist with an enzyme that contains chemically incompatible functionalities.

Experimental Procedures

General

Reagents and Enzymes. The reagents used in this work were all reagent grade and were purchased from Aldrich unless otherwise noted. The water was distilled and further deionized by using an anion-exchange column. Vanadium pentoxide was purchased from Fisher. In practice, stock solutions of 250 mM vanadate were used; after 6 months of storage in the refrigerator, the absorbance at 260 nm remained unchanged. The enzymes and biochemicals were purchased from Sigma

[1] Abbreviations: G3P, glycerol 3-phosphate; G3PDH, glycerol-3-phosphate dehydrogenase, Gly3PDH, glyceraldehyde-3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; PP, pyrophosphate; V_i, total vanadate; V_1, vanadate monomer; V_2, vanadate dimer; V_4, vanadate tetramer; V_5, vanadate pentamer.

Chemical Co. (St. Louis, MO). Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from rabbit muscle (type I) was dialyzed against 200 volumes of 1 mM EDTA solution at pH 5.65 and 4 °C for 20 h before use in the assays. The enzyme stock solution was diluted to the appropriate concentration with 1 mM EDTA.

Kinetic Measurements. Spectrophotometric determinations of the rates of glycerol 3-phosphate (G3P) oxidation coupled with semicarbazone formation were measured at 340 nm on a Lambda 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. Vanadate was added to the preincubated assay solutions 60 s before the reaction was begun by the addition of enzyme, and the initial rates at 25 °C were followed for 45 s. All rates were determined in duplicate or triplicate, and controls were run before and after each series of assays; rate adjustments were made when necessary.

^{51}V NMR Spectroscopy (Heath & Howarth, 1981; Gresser & Tracey, 1985; Crans et al., 1990b). The ^{51}V NMR spectra were recorded at 53 MHz on a ^{1}H 200 Bruker WPSY (4.7 T) spectrometer. Typical conditions include a spectrum width of 8064 Hz, a 90° pulse angle, an accumulation time of 0.2 s, and no relaxation delay. No change in integration of various peaks was observed if the relaxation delay was increased; the T_1's were approximately 10–15 ms and were measured by using the saturation–recovery method. The chemical shifts are reported relative to the external reference standard $VOCl_3$ (0 ppm). In practice, a solution containing vanadate–diethanolamine complex at pH = 8.8 was used as a reference (−490 ppm) (Crans & Shin, 1988). The ^{51}V NMR spectra were recorded by using external lock. The NMR samples were prepared as described for the assays with the exception that G3P, NAD, and enzyme were omitted; the small amount of EDTA from the enzyme solution was included in the NMR samples. The omission of substrate and cofactors did not measurably affect the distribution of vanadate species, since spectra with and without G3P and NAD resulted in vanadate anion distributions within 5%.

Speciation Analysis. Vanadate solutions contain a complex mixture of species that varies with ionic strength, temperature, pH, and vanadate concentration. The ^{51}V NMR spectra contain signals for the vanadate monomer (−555 ppm at pH 7.4 to −538 ppm at pH 9.0), dimer (−570 ppm at pH 7.4 to −563 ppm at pH 9.0), tetramer (−574 ppm), and pentamer (−581 ppm). The concentration of each vanadate derivative was calculated from the total vanadium concentration and the integrations of the ^{51}V NMR spectra. Thus ^{51}V NMR was used to determine the concentrations of the vanadate species in the assay solutions containing the total vanadate concentrations specified in the enzyme assays. The oligomer concentrations determined in this manner follow the relationships shown by eqs 1–4 with correlation coefficients of 0.99 or above. The H^+-dependent equilibrium constants were determined and are shown in Table I both at pH 7.4 and at pH 9.0.

$$2V_1 \underset{}{\overset{K_{12}}{\rightleftharpoons}} V_2 \quad (1)$$

$$4V_1 \overset{K_{14}}{\rightleftharpoons} V_4 \quad (2)$$

$$5V_1 \overset{K_{15}}{\rightleftharpoons} V_5 \quad (3)$$

$$2V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (4)$$

Data Analysis. The rates were measured from the linear portions of the rate profile, and doubling the enzyme concentration produced a doubling of rates. The kinetic data were analyzed with Cricket Graph, a program for graphical analysis on the Apple Macintosh computer. Both Lineweaver–Burk and Eisenthal–Cornish–Bowden types of plots were used to determine Michaelis–Menten parameters. The K_i's and the nature of inhibition were determined from plots of the Lineweaver–Burk slopes as a function of the concentration of vanadium monomer, dimer, tetramer, and pentamer. Fits of the experimental points were determined by using Cricket Graph. We estimate that the errors on our concentration determinations range from 2 to 10% and that the K_i's determined in the fits are accurate within 50%.

Specific Assay

G3PDH Assay at pH 7.4. Assays were carried out in 50 mM imidazole with 0.20 M KCl added to provide a constant ionic strength. The assay solutions contained 5 mM semicarbazide and 10.5 mM G3P, and NAD was varied from 0.15 to 0.75 mM. The inhibition of several vanadate concentrations from 0.0 to 2.5 mM was determined. When varying the G3P concentration from 0.5 to 2.0 mM, the NAD concentration was 4.0 mM. Corresponding assays were used in examining the inhibition by phosphate and pyrophosphate, with the exception that the KCl concentration was varied to provide a constant ionic strength. The phosphate concentration was varied from 0.0 to 30 mM, and the pyrophosphate concentration was varied from 0.0 to 20 mM. The assays were carried out at 25 °C.

G3PDH Assays at pH 9.0. Assays were carried out in 50 mM TAPS with 0.20 M KCl added to provide a constant ionic strength. The assay solutions contained 5 mM semicarbazide and 10.5 mM G3P, and NAD was varied from 0.15 to 0.75 mM. Rates at this pH were faster than those observed at lower pH, so vanadate concentrations from 0.0 to 4.0 mM were examined. When varying the G3P concentration from 0.5 to 2.0 mM, the NAD concentration was 4.0 mM. Corresponding assays were used in examining the inhibition by phosphate and pyrophosphate, with the exception that the KCl concentration was varied to provide a constant ionic strength. The phosphate concentration was varied from 0.0 to 30 mM, and the pyrophosphate concentration was varied from 0.0 to 20 mM. The assays were carried out at 25 °C.

Results

Oxidation of Glycerol 3-Phosphate Catalyzed by Glycerol-3-phosphate Dehydrogenase. Interactions between semicarbazide and vanadate were carefully examined by using both ^{51}V NMR and UV spectroscopy such that assay conditions were chosen where vanadium(V) was not reduced. ^{51}V NMR spectra with and without 10 mM semicarbazide were similar even after a 1-h incubation with 10 mM vanadate at both pH 7.4 and pH 9.0. Reduction of vanadium(V) to vanadium(IV) is therefore not observable for up to 10 mM semicarbazide at pH 7.4 and 9.0 in this time period. This is in contrast to a solution of 25 mM hydrazine and 2 mM vanadate where reduction occurred within 200 s as evidenced by the decrease in absorbance at 340 nm. The reaction rates were measured in solutions containing from 0.15 to 0.75 mM NAD in the presence of 50 mM imidazole at pH 7.4, 0.20 M KCl, 5 mM semicarbazide, 10.5 mM G3P, and approximately 0.0017 mg/mL G3PDH. $K_{m,NAD}$ was 0.28 mM. The reaction rates were measured in solutions containing G3P concentrations from 0.50 to 2.0 mM in the presence of 50 mM imidazole, 0.20 M KCl, 5 mM semicarbazide, 4.0 mM NAD, and approximately 0.0017 mg/mL G3PDH. K_{mG3P} was 2.1 mM. These observations correspond to those reported in the literature (Bentley & Dickinson, 1974; Young & Pace, 1958).

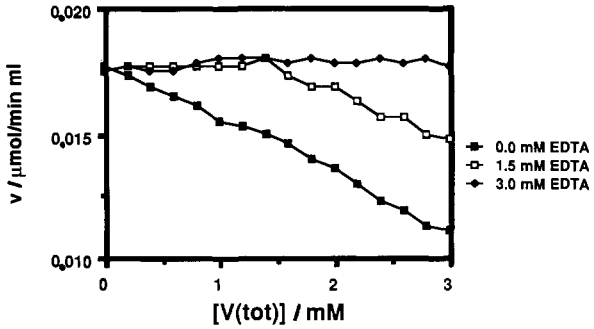

FIGURE 1: Rates of G3PDH-catalyzed oxidation of G3P were measured as a function of vanadate concentration in the presence of no EDTA (■), 1.5 mM EDTA [□], and 3.0 mM EDTA (◆) in solutions containing 50 mM imidazole, 5 mM semicarbazide, 10.5 mM G3P, 3 mM NAD, 200 mM KCL, and G3PDH at pH 7.4.

Glycine is commonly the buffer of choice when assaying G3PDH at high pH. Since glycine interacts significantly with vanadate, we have replaced glycine with TAPS (Crans et al., 1989b). Reaction rates at pH 9.0 were therefore measured by using NAD concentrations from 0.15 to 0.75 mM in 50 mM TAPS, 0.20 M KCl, 5 mM semicarbazide, 10.5 mM G3P, and approximately 0.0017 mg/mL G3PDH. $K_{m,NAD}$ was 0.26 mM. Reaction rates were measured by using G3P concentrations ranging from 0.50 to 2.0 mM in the presence of 50 mM TAPS at pH 9.0, 0.20 M KCl, 5 mM semicarbazide, 4.0 mM NAD, and approximately 0.0017 mg/mL G3PDH. $K_{m,G3P}$ was 1.5 mM. These Michaelis–Menten parameters correspond to those reported in the literature (Bentley & Dickinson, 1974; Young & Pace, 1958).

Inhibition by Phosphate and Pyrophosphate. The inhibition by phosphate was determined by using assay solutions described in detail under Experimental Procedures. Even at 30.0 mM phosphate, a potential inhibition by phosphate is not distinguishable from experimental error. This is in accord with the fact that phosphate has been used as a buffer for assaying G3PDH (Bentley & Dickinson, 1974; Young & Pace, 1958). Pyrophosphate did not inhibit the G3PDH reaction either. The effects of phosphate (from 0.0 to 30.0 mM) and pyrophosphate (from 0.0 to 20.0 mM) were also determined at pH 9.0, and phosphate and pyrophosphate were not significant inhibitors at this pH. The inhibition by phosphate and pyrophosphate is therefore negligible.

Inhibition of G3PDH by Vanadate. The inhibition of glycerol-3-phosphate dehydrogenase by vanadate was measured at both low and high pH. Plotting the reciprocal rates as a function of the reciprocal NAD concentrations generated a Lineweaver–Burk plot with a noncompetitive inhibition pattern (data available in the supplementary material). Since phosphate and pyrophosphate interact little with G3PDH, it is likely that neither vanadate monomer nor dimer will be the main vanadium derivative responsible for the inhibition. The possibility that redox chemistry may be occurring between vanadate and the enzyme in these studies warrants examination.

G3PDH was incubated from 1 to 15 min in the assay solution with and without various concentrations of vanadate before enzyme rates were determined. The enzyme activity was then measured after adding substrate and 3.0 mM EDTA; no differences in rates with various incubation times of vanadate and G3PDH were observed. Representative results carried out at pH 7.4 are shown in Figure 1. At vanadate concentrations below 3.0 mM the addition of EDTA regenerated the original enzyme activity (as measured by the incubation without vanadate). An analogous experiment was carried out with up to 3.0 mM vanadate using 1.5 mM EDTA to reactivate G3PDH. As shown in Figure 1, the EDTA reactivated the samples containing less than 1.5 mM vanadate, but above 1.5 mM vanadate decreased rates were observed. EDTA forms a vanadate complex with a stoichiometry of 1:1 (Scheidt et al., 1971). The vanadate in solutions containing 1.5 mM is not completely complexed when the total vanadium concentration is higher than that of EDTA; oligomers form, and inhibition of G3PDH is observed. Since inhibition is reversible, the inhibition of G3PDH by vanadate is not likely to involve redox chemistry of the thiol group.

A Lineweaver–Burk plot shows that vanadate is a noncompetitive inhibitor with respect to NAD. When plotting the Lineweaver–Burk slopes as a function of total vanadate concentration, a nonlinear curve is observed, presumably because several vanadium derivatives exist in the solutions. The various vanadate anions were quantified by using ^{51}V NMR spectroscopy, and the effect of each anion determined as follows. The plot of the Lineweaver–Burk slopes (or reciprocal rates) against the monomer concentration gave nonlinear curves with a flat, horizontal portion at low monomer concentrations at both high and low pH (data available in supplementary material). At the low concentrations, monomeric vanadate is for all practical purposes the only vanadate oligomer present in solutions. The fact that samples containing low monomer concentrations show no significant inhibition suggests that inhibition by monomer is negligible. At higher monomeric vanadate concentrations where the enzyme is inhibited, significant concentrations of oligomers are present in the assay solutions. Plots examining whether one species (i.e., monomer, dimer, tetramer, or pentamer) could explain the observed inhibition each showed nonlinear curves. This suggests that more than one species is likely to inhibit the enzyme reaction. We will examine whether two noncompetitive inhibitors can explain the observed enzyme inhibition using an analogous approach to that described previously for two competitive inhibitors (Crans & Schelble, 1990).

The inhibition of G3PDH by two noncompetitive inhibitors (in this case, dimer and tetramer) has Lineweaver–Burk slopes as defined in eq 5 (Crans & Schelble, 1990; Segel, 1975). As

$$\text{slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[V_2]}{K_{i2}} + \frac{[V_4]}{K_{i4}}\right) \quad (5)$$

seen in eq 4, the tetramer concentration can be expressed in terms of dimer concentration squared ($[V_4] = K_{24}[V_2]^2$). Corresponding relationships can be shown for both dimer and pentamer and for both tetramer and pentamer (Crans & Schelble, 1990).

Although the present relationships could be distinguished using polynomial fits, we have chosen to modify these relationships to eqs 6 and 7 so that linear fits can be tested.

$$\frac{\left(\frac{\text{slope}}{\text{slope}_{[V_{tot}]=0}} - 1\right)}{[V_2]} = \frac{1}{K_{i2}} + \frac{K_{24}}{K_{i4}}[V_2] \quad (6)$$

$$\frac{\left(\frac{\text{slope}}{\text{slope}_{[V_{tot}]=0}} - 1\right)}{[V_1]^4} = \frac{K_{14}}{K_{i4}} + \frac{K_{15}}{K_{i5}}[V_1] \quad (7)$$

Plotting the ratio of the Lineweaver–Burk slopes minus one divided by $[V_2]$ as a function of $[V_2]$ yields a graph with a slope larger than 0 and shows that the tetramer is an inhibiting species. If the y-axis intercept is greater than zero, the va-

Table I: Various Michaelis–Menten Parameters for G3PDH from Rabbit Muscle and Speciation Parameters for Aqueous Vanadate Solutions at pH 7.4 and pH 9.0[a]

pH 7.4				pH 9.0			
$K_{m,G3P}$ (mM)	2.1	$K_{m,NAD}$	0.28	$K_{m,G3P}$	1.5	$K_{m,NAD}$	0.26
K_{i2} (mM)			0.36		0.65		3.3
$K_{i,H_2V_2O_7^{2-}}$ (mM)			0.27				
$K_{i,HV_2O_7^{3-}}$ (mM)			1.5				1.5
K_{i4} (mM)	0.12		0.67		0.38		1.2
K_{12} (M^{-1})	4.0×10^2				5.0×10^1		
K_{14} (M^{-3})	2.3×10^9				3.8×10^6		
K_{15} (M^{-4})	3.1×10^{11}				2.1×10^8		
K_{24} (M^{-1})	1.1×10^4				1.4×10^3		
$K_{a,H_2V_2O_7^{2-}}$	1.6×10^{-8}			$K_{a,HV_4O_{12}^{3-}}$	1.3×10^{-10}		
$K_{a,HV_2O_7^{3-}}$	1.3×10^{-9}						

[a] The K_m's are determined with 20% accuracy. The K_i's determined by linear and polynomial fits are reproducible (from different data) within 30%. The equilibrium constants are within 10%.

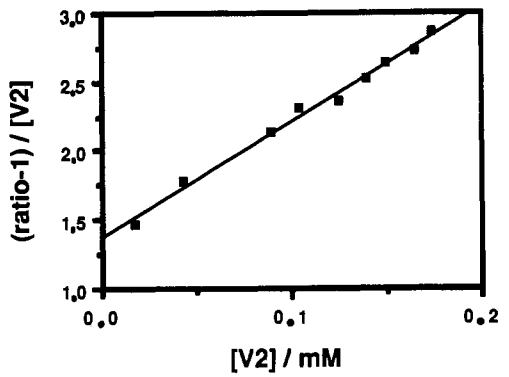

FIGURE 2: The ratio of the Lineweaver–Burk slopes determined from vanadate inhibition studies (against varying NAD concentrations) minus one divided by $[V_2]$ was plotted as a function of $[V_2]$. The kinetic studies were conducted as described under Experimental Procedures for the assay at pH 9.0.

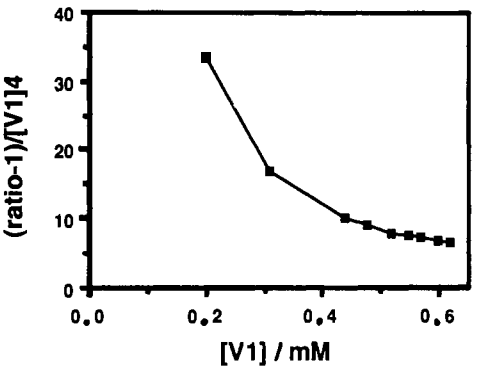

FIGURE 3: The ratio of the Lineweaver–Burk slopes determined from vanadate inhibition studies (against varying NAD concentrations) minus one divided by $[V_1]^4$ was plotted as a function of $[V_1]$. The kinetic studies were conducted as described under Experimental Procedures for the assay at pH 9.0.

nadate dimer is also an inhibiting species. If the slope of the graph is zero, the vanadate dimer is the only inhibiting species of the enzyme. Figure 2 shows a straight line with a slope greater than zero ($R = 1.00$), suggesting the tetramer is an inhibiting species in the rate data when NAD is varied at pH 9.0. The intercept suggests that the dimer is a significant inhibitor at this pH (Table I). The analogous equations were also tested for other combinations of active vanadium derivatives, but none of these other combinations showed an acceptable fit. Figure 3 shows one of these plots of the ratio of the Lineweaver–Burk slopes minus one divided by $[V_1]^4$ (testing eq 7), confirming that this combination does not yield a straight line. This result suggests the vanadate pentamer is not a significant inhibitor for G3PDH under these conditions. Only Figure 2 yielded a fit in agreement with our analysis. We therefore conclude that V_2 and V_4 are the major inhibiting species against NAD at both pH 7.4 and pH 9.0. Table I summarizes the inhibition constants determined by this analysis. The inhibition constants in Table I also show differences that can be attributed to changes in protonation state in the vanadate anions.

The inhibition by vanadate with respect to G3P was also determined. The rates of oxidation were measured and generated a Lineweaver–Burk plot with a competitive inhibition pattern. Using the analysis described previously (Crans & Schelble, 1990), we find that the best fit is observed using the vanadate tetramer as the only inhibitor. This K_{i4} was calculated to be 0.12 mM at pH 7.4.

The proposed interpretation was further supported by the following dilution experiments. The enzyme was incubated with 1.5 mM vanadate for 1 min, and a fraction of the incubated sample was diluted to 0.15 mM vanadate. The rates were measured in both samples. The dilution decreased the concentration of the vanadate oligomers below detection, and the rate in the diluted sample was the same as that of a diluted control sample containing no vanadium. The original incubated sample still showed the expected inhibition compared to a control sample.

Dependence of Inhibition Constants on Protonation. The vanadate dimer had a K_i of 0.36 mM at pH 7.4 and a K_i of 3.3 mM at pH 9.0. Since the dimer changes protonation state in this pH range, we examined these observations in more detail. The pK_a's of the vanadate dimer under these assay conditions were determined by measuring the ^{51}V NMR chemical shifts of the dimer. Plotting the chemical shift (in ppm) of the dimer against the pH of the samples yielded a titration curve from which the pK_a's could be determined. The fourth pK_a of the dimer is 8.9, and the third pK_a is 7.8. These pK_a values are somewhat in agreement with those previously observed under different conditions (Pettersson et al., 1985). Using these data, it is possible to estimate the relative amounts of $H_2V_2O_7^{2-}$, $HV_2O_7^{3-}$, and $V_2O_7^{4-}$ from pH 7.0 to 9.5 because the total dimer concentration was determined in the series of ^{51}V NMR spectra recorded as a function of pH. Since K_{i2} is so much larger at pH 9.0 than it is at pH 7.4, this suggests the dimer is less potent as an inhibitor upon deprotonation of $HV_2O_7^{3-}$ to $V_2O_7^{4-}$. Below pH 7.8 the diprotonated dimer becomes the major species, and since the vanadate inhibition is largest in this range, it is possible the diprotonated vanadate dimer is the most potent inhibitor and that $V_2O_7^{4-}$ has little or no effect on the enzyme reaction. At pH 9.0, only $HV_2O_7^{3-}$ and $V_2O_7^{4-}$ are present in solution, and less inhibition is observed at this pH.

Assuming only protonated forms of the dimer inhibit the enzyme reaction at pH 9.0, only $HV_2O_7^{3-}$ is inhibiting the enzyme, and the dimer present as $V_2O_7^{4-}$ does not significantly contribute to the enzyme inhibition. The real inhibition constant for $HV_2O_7^{3-}$ can then be derived by using the experimental data to the modified eq 6, giving a $K_{i,HV_2O_7^{3-}}$ of 1.5 mM and a K_{i4} of 1.2 mM, compared to a K_{i2} of 3.3 mM and a K_{i4} of 1.2 mM. At pH 7.4 the two major dimeric vanadium species in solution are $HV_2O_7^{3-}$ and $H_2V_2O_7^{2-}$. By use of $pK_{a,H_2V_2O_7^{2-}} = 7.8$, the relative amounts of the dimeric protonated species can correspondingly be calculated from the $K_{a,H_2V_2O_7^{2-}}$ (by $[HV_2O_7^{3-}][H^+] = K_{a,H_2V_2O_7^{2-}}[H_2V_2O_7^{2-}]$). Accordingly, by use of a $K_{i,HV_2O_7^{3-}}$ of 1.5 mM as determined at pH 9.0, $K_{i,H_2V_2O_7^{2-}}$ was calculated to be 0.27 mM and K_{i4} was calculated to be 0.67 mM.

Discussion

Vanadium(V) reduces to vanadium(IV) in the presence of reducing agents such as thiols, reactive aldehydes or ketones, or other weakly reducing reagents (Chasteen, 1983). In addition, vanadate forms complexes with hydroxyl groups, amine groups, carboxylic acid groups, phosphate groups, and any combination of such moieties in a molecule (Gresser & Tracey, 1985; Gresser et al., 1986; Crans & Shin, 1988). The commonly used buffers for G3PDH assays from pH 7 to 8 include Tris and phosphate, but both these buffers interact with vanadate and facilitate nonenzymatic reduction of vanadium(V). The usual assay conditions reported for oxidation of G3P or reduction of DHAP are incompatible with vanadate and cause the reduction of vanadium(V) to vanadium(IV). It was therefore necessary to use assay conditions that would not form significant concentrations of vanadate–ligand complexes or facilitate reduction of vanadium(V) to vanadium(IV) on the time scale in which the experiment was carried out. Imidazole has previously been used as a buffer in enzymatic studies with vanadate because this buffer resists the buffer-induced redox chemistry of vanadate (Vyskocil et al., 1980).

G3PDH is commonly assayed by measuring the reduction of DHAP because the equilibrium and the reaction rates are more favorable for enzyme studies. Such an assay is not recommended for studies with millimolar concentrations of vanadate because vanadate is quickly reduced by dihydroxyacetone and DHAP (Drueckhammer et al., 1989). Because enzyme kinetic studies require varying vanadate and substrate concentrations, and the rates are strongly sensitive to substrate concentration, it is desirable to use a labile enzyme substrate with defined concentrations. Since DHAP reacts with vanadate, the extent of reaction would vary with vanadate concentrations, thus changing both the DHAP and vanadate concentrations. We have chosen to keep the DHAP concentration as low as possible, and consequently to study the G3PDH reaction in the direction of DHAP formation.

Using speciation studies, the vanadate tetramer was found to be the most inhibiting vanadate oligomer of G3PDH. The dimer also inhibited, although less than the tetramer. The vanadate anions may bind in the G3P binding site since a competitive inhibition pattern was observed against G3P. The low affinity for phosphate or pyrophosphate in either substrate or cofactor site suggests that the enzyme must bind the vanadate dimer and tetramer differently than the simple P_i and PP. G3PDH represents the third enzyme that is inhibited by the vanadate tetramer, and this observation suggests these vanadate oligomers may in general have some affinity for proteins. Cremo and Wilcott (1990) have correlated the affinity of vanadate tetramer with a specific 20 amino acid sequence in these proteins. It is possible that certain structural sequences in these proteins will recognize the vanadate tetramer or other cyclic polyanions (D. C. Crans, T. Stites, and S. M. Schelble, manuscript in preparation). The solution structure of the tetramer is generally believed to be cyclic (Pope, 1983) although an adamantane-like structure has been suggested (Gresser et al., 1988). Recent potentiometric studies (Pettersson et al., 1985) and structural characterization of the $V_4O_{12}^{4-}$ strongly support the cyclic structure of this anion (Day et al., 1990).

Examination of the vanadate inhibition as a function of pH suggested that protonated forms of the dimer exhibited the biological activity. The vanadate dimer has no activity as a tetranion. The monoprotonated dimer has a $K_{i,HV_2O_7^{3-}}$ of 1.5 mM, and the diprotonated dimer has a $K_{i,H_2V_2O_7^{2-}}$ of 0.27 mM. The lower K_i for the diprotonated vanadate dimer suggests that a smaller charge favors binding of the vanadate derivative. Pyrophosphate has pK_a's somewhat lower than the vanadate dimer. It is possible that pyrophosphate is a poor analogue for the vanadate dimer because the charge distribution does not favor binding monoprotonated pyrophosphate.

Despite the presence of a thiol cofactor site of G3PDH, we see no evidence for reduction of vanadium(V) to vanadium(IV). The G3PDH activity is completely regenerated when vanadate is complexed with EDTA. The thiol group is essential for enzyme activity as evidenced by the deactivation of the enzyme by treatment with reagents such as *N*-ethylmaleimide (Anderson et al., 1970). The sulfhydryl group is located in the cofactor site as suggested by the selective modification by 3-aminopyridine adenine dinucleotide (Anderson et al., 1978). The cofactor site is fairly hydrophobic although the pyrophosphate linkage in the cofactor is important for specific binding (Kim & Anderson, 1969). On the basis of the previous observations with G6PDH (Crans & Schelble, 1990) a high affinity of vanadate oligomers for the cofactor site in G3PDH was anticipated but not substantiated in this work. The interactions with vanadate oligomers with G3PDH are somewhat higher than interactions with simple anions such as phosphate and sulfate. The inhibition pattern suggests that vanadate oligomers may bind to the substrate site. Since the substrate site and the cofactor site must be spacially in the same area of the protein, it is surprising that reduction of the thiol is not observed.

Glyceraldehyde-3-phosphate dehydrogenase (Gly3PDH) has been suggested to undergo oxidation in the presence of vanadate (Benabe et al., 1987). These workers have carefully documented the effects of vanadate in red blood cells. The appearance of an EPR signal of solutions containing 10 000 units/mL Gly3PDH, 6 mM vanadate, and 9 mM oxalate in Tris buffer at pH 7.1 was interpreted as direct evidence that redox chemistry occurred between vanadate and Gly3PDH. The effects of vanadate on the activity of Gly3PDH was not examined in the previous work. In our hands (D. C. Crans and P. K. Shin, unpublished results) Gly3PDH incubated with vanadate is inhibited at concentrations above 0.5 mM vanadate. However, inhibiting concentrations of vanadate are reactivated in the presence of EDTA as described in this paper for G3PDH. It is therefore unlikely that the major mechanism of interaction of vanadate is based on redox chemistry with Gly3PDH. Our observations are supported by those of DeMaster and Mitchell (1973), who found vanadate could serve as a cosubstrate replacing phosphate in the reaction. Since oxalate is known to easily reduce vanadium(V) (Sathyanarayana & Patel, 1964), it is possible that the in vitro reduction of vanadate and the observed EPR signal is caused by the oxalate (Benabe et al., 1987). The in vivo reduction of va-

nadate observed in the red blood cells could be a result of the action of other compounds such as glutathione present in the cell.

Various explanations involving the protection of a thiol group against vanadate can be offered. Interactions between the G3PDH thiol and vanadate are likely to lead to redox chemistry if the electron transfer and appropriate reaction steps can occur. If, however, the protein sterically prevents the vanadate or other groups such as a second SH group from having appropriate access to the thiol group, reduction of vanadate may not occur. It is possible that the initial electron transfer takes place either directly or through a complex, but the resulting radical intermediate is not appropriately stabilized and the electron-transfer reaction reverses before the vanadate–protein complex cleaves. The vanadate tetramer has previously been found to oxidize a serine residue in myosin upon photolysis (Cremo & Wilcott, 1990), demonstrating that the vanadate tetramer is a redox-active vanadium species under these conditions. However, direct comparison cannot be made between the photolytic oxidation of myosin with our activity studies because it is not known how photolysis affects either myosin or vanadate or what the products of the photolysis treatment were. In the event that the vanadate tetramer is located in a binding site outside the range of the thiol group, one cannot exclude the possibility that the vanadate monomer can reach the thiol in the active site because modification reagents reach this thiol. If one vanadate derivative reaches the thiol, it is possible that the initial events in the redox reaction will occur. Since no redox chemistry was observed for G3PDH, and the initial oxidation reaction steps are likely to occur, the redox reaction must have been prevented because the latter steps could not occur. Our observations are thus suggesting that the G3PDH is protecting the thiol group from oxidation by the vanadate and that trace levels of vanadate may not affect enzymes such a G3PDH by redox chemistry.

Since vanadate does not appear to affect G3PDH by redox chemistry and has only weak effects through any vanadate oligomers, how will vanadate affect this enzyme? Glycerol 3-vanadate forms from aqueous glycerol and vanadate and is rapidly converted to dihydroxyacetone and vanadate by G3PDH (Craig, 1986; Drueckhammer et al., 1989). The k_{cat}/K_m for glycerol 3-vanadate (G3V) is 1.5 and for G3P 0.50. The corresponding ratio assayed in the other direction was measured as 84 for dihydroxyacetone vanadate (DHAV) and 7.6 for dihydroxyacetone phosphate (DHAP) (Craig, 1986). The latter measurement for DHAV is subject to many experimental problems due to the instability of vanadate in the presence of DHA and the product inhibition of DHAP. Nevertheless, both G3V and DHAV appear to be slightly better substrates than the natural substrates. Given the poor affinity of other vanadium(V) derivatives, G3PDH may be an example of an enzyme where the preferred mode of action of vanadium(V) would be through the spontaneously formed G3V or DHAV.

This paper has examined the mechanisms by which vanadate can interact with G3PDH in vitro and determined which mechanism is more likely to occur. Despite the fact that no redox chemistry occurred with this enzyme, the question of how the interactions of vanadium(IV) compare to those described for vanadium(V) still remains to be addressed. The aqueous chemistry of vanadium(IV) is also complex and very poorly understood. However, vanadium(IV) also can mimic the phosphate appendage (Chasteen, 1983; Drueckhammer et al., 1989). A complex between vanadium(IV) and dihydroxyacetone (DHA) has been found to have somewhat lower activity than DHAV tested at similar DHA concentrations (Drueckhammer et al., 1989). It is possible that the most potent effects of vanadium on G3PDH would be through the vanadium(V) or vanadium(IV) analogues of G3P and DHAP.

Conclusion

We found that G3PDH is reversibly inhibited by both vanadate dimer and tetramer in aqueous vanadate solutions despite the thiol in the active site. The inhibition observed is noncompetitive with respect to cofactor and competitive with respect to substrate. Our studies suggest the diprotonated vanadate dimer may be the form of the dimer that binds best to the enzyme. Although both rabbit muscle G3PDH and Gly3PDH contain thiols in the active site, the reduction of vanadium(V) to vanadium(IV) is not observed in the presence of either enzyme. These studies suggest that enzymes containing exposed sulfhydryl groups can interact with vanadate though nonredox mechanisms.

Acknowledgments

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Supplementary Material Available

Lineweaver–Burk plot of the inhibition by vanadate of G3PDH with respect to NAD (Figure 1) and Lineweaver–Burk slopes determined from vanadate inhibition studies, plotted as a function of monomeric vanadate concentrations (Figure 2) (3 pages). Ordering information is given on any current masthead page.

Registry No. G3PDH, 9075-65-4; V_1, 14333-18-7; V_2, 22466-30-4; V_4, 78197-79-2; NAD, 53-84-9; G3P, 57-03-4; $H_2V_2O_7^{2-}$, 103884-11-3; $HV_2O_7^{3-}$, 63643-82-3.

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