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Vanadate Dimer and Tetramer Both Inhibit Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*†

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ABSTRACT: Vanadate dimer and tetramer inhibit glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. The inhibition by a vanadate mixture containing vanadate monomer, dimer, tetramer, and pentamer was determined by measuring the rates of glucose 6-phosphate oxidation and reduction of NAD (or NADP) catalyzed by glucose-6-phosphate dehydrogenase. The inhibition by vanadate is competitive with respect to NAD or NADP and noncompetitive (a mixed type) with respect to glucose 6-phosphate (G6P) when NAD or NADP are cofactors. This inhibition pattern varies from that observed with phosphate and thus suggests vanadate interacts differently than a phosphate analogue with the enzyme. ^{51}V NMR spectroscopy was used to directly correlate the inhibition of vanadate solutions to the vanadate dimer and/or tetramer, respectively. The activity of the vanadate oligomer varied depending on the cofactor and which substrate was being varied. The vanadate dimer was the major inhibiting species with respect to NADP. This is in contrast to the vanadate tetramer, which was the major inhibiting species with respect to G6P and with respect to NAD. The inhibition by vanadate when G6P was varied was weak. The competitive inhibition pattern with respect to NAD and NADP suggests the possibility that vanadate oligomers may also inhibit catalysis of other NAD- or NADP-requiring dehydrogenases. Significant concentrations of vanadate dimer and tetramer are only found at fairly high vanadate concentrations, so these species are not likely to represent vanadium species present under normal physiological conditions. It is however possible the vanadate dimer and/or tetramer represent toxic vanadate species.

Vanadium is an important dietary trace element that is beneficial at low concentrations (Nechay et al., 1986; Boyd & Kustin, 1984). At high concentrations vanadium becomes toxic. Vanadium is an insulin-mimetic agent and has cardiovascular activity (Gresser et al., 1987). It affects the cAMP levels, protein kinase activity, and protein phosphatase activity. The recent discovery of the vanadium-requiring nitrogenase is an example of the increasing interest in this element (Robson et al., 1986). The action of vanadium is likely to vary with the oxidation state of the metal although little is currently understood about the mechanisms of action of vanadium in mammals or plants. Vanadate [vanadium(V)] is the most stable form of vanadium in the presence of oxygen at neutral pH although reducing environments readily convert it to vanadium(IV) (Chasteen, 1983; Boyd & Kustin, 1984). Vanadium is generally believed to exist intracellularly in oxidation state IV, and vanadate is gradually reduced by endogenous reducing agents after being injected in the blood stream. However, in view of the presence of some free oxygen in the blood it seems possible that vanadium(IV) will reoxidize to vanadate. Recently, both vanadium(IV) and vanadium(V) were found bound to transferrin and albumin in blood serum (Chasteen et al., 1986). The nature of the oxidation state of vanadium (V(III) or V(IV)) in tunicate blood has been controversial and is now believed to be species dependent (Lee et al., 1988). In-depth understanding of the biological activities of vanadium is likely to require an understanding of the chemistry and biochemistry of both vanadium(IV) and vanadium(V).

The variety of effects of vanadium when normalizing the glucose levels in mammals may not be explained completely by the action of vanadium as an insulin-mimetic agent (Gresser et al., 1987). Recent studies show several biological effects of vanadate that are not observed with insulin (Bernier et al., 1988). Vanadium stimulates hexose transport, and this stim-

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ulation is approximately of the same magnitude as insulin stimulatation of hexose transport. Other studies with intact cells specifically probing the mechanism of insulin activation show vanadate stimulates glucose metabolism and glycogen synthase in a manner that is different than insulin stimulation (Gresser et al., 1987; Bernier et al., 1988). Several mechanisms have recently emerged based on how vanadate in vitro can affect glucose and glycogen metabolism. Three of these mechanisms focus on the inhibition of enzymes involved in glucose metabolism. One mechanism focuses on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by oxidation (Benabe et al., 1987), and another focuses on the inhibition of phosphoglycerate mutase by generation of a dead-end inhibitor complex (Stankiewicz et al., 1987). A third mechanism involves inhibition of glycolytic enzymes by the vanadate tetramer (Crans et al., 1990a). A fourth 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; Druckhammer et al., 1989). The effect of vanadate on enzymes in alternative glucose pathways (for example, the pentose phosphate shunt) is relatively unexplored (Nour-Eldeen et al., 1986; Cohen et al. 1987; Crans et al., 1990a). It is possible the effect of vanadate on such pathways may also be important for understanding how vanadate affects glucose metabolism.

Monomeric vanadate acts in biological systems as a phosphate analogue but also has the potential to exhibit biological activities through oligomeric vanadate species or other vanadate derivatives. Most of the reported biological effects of vanadium are attributed to monomeric vanadate (V(V)) or vanadyl cation (V(IV)) (Chasteen, 1983). Although many researchers do not specify the active vanadate species, their studies are often conducted at such low vanadate concentrations that the major vanadate species in solution is monomeric vanadate. The oligomeric vanadate species that have been shown to have enzymatic activity include the decamer, dimer, and recently the tetramer. The vanadate decamer inhibits Ca^{2+} ATPase (Csermely, 1985), muscle phosphorylase (Soman, 1983), adenylate cyclase (DeMaster & Michell, 1973), hexokinase, and phosphofructokinase (Boyd & Kustin, 1985). The vanadate dimer has been shown to inhibit phosphoglycerate mutase (Stankiewicz et al., 1987) and acid phosphatase from human seminal fluid (Crans et al., 1989). The vanadate tetramer has been shown to inhibit 6-phosphogluconate dehydrogenase from a variety of sources including yeast, bacteria, and mammals (including human) (Crans et al., 1990a).

Glucose-6-phosphate dehydrogenase (G6PDH)[1] catalyzes the oxidation of glucose 6-phosphate (G6P) to D-glucono-δ-lactone 6-phosphate under the production of NADPH or NADH (Levy, 1986). D-Glucono-δ-lactone 6-phosphate rapidly hydrolyzes to 6-phosphogluconate (6GP) at neutral pH, or the hydrolysis can be catalyzed enzymatically by lactonase. G6PDH is located in the cytoplasm of the cells and is the first enzyme in the pentose phosphate pathway. The pentose phosphate pathway is a multifunctional pathway specialized to carry out four main activities, depending on the organism and the metabolic state of the cell. One function is the conversion of hexoses into pentoses, particularly D-ribose 5-phosphate, required in the synthesis of nucleic acids. A second function occurs in tissues such as liver and cells such as erythrocytes and involves the generation of NADPH in the extramitochondrial cytoplasm. G6PDH from yeast was previously found to be inhibited by vanadate, although the inhibiting vanadate species was not identified (Cohen et al., 1987). In this work we examine G6PDH from *Leuconostoc mesenteroides*; since this enzyme is active with both NAD and NADP, it allows comparison of the vanadate-induced activities when either cofactor is used (Olive & Levy, 1967).

In this work ^{51}V NMR and enzyme kinetics were employed to demonstrate that the vanadate dimer and tetramer inhibit G6PDH-catalyzed oxidation of G6P. The activity of the vanadate dimer and tetramer varies significantly depending on the cofactor and which substrate is being used. We find that the vanadate dimer and tetramer inhibit by noncompetitive (mixed-type) inhibition with respect to G6P and competitive inhibition with respect to NADP or NAD for G6PDH from *L. mesenteroides*. It is therefore possible the inhibiting vanadate species binds in the cofactor site in glucose-6-phosphate dehydrogenase. Since many enzymes require NAD or NADP as cofactors, it is possible this type of vanadate inhibition will occur with other NAD/NADP-requiring enzymes. Vanadate is toxic for mammals at high concentrations, and since the vanadate dimer and tetramer are only present in significant amounts at high vanadate concentrations, it is conceivable that these oligomeric vanadate ions are toxic forms of vanadate.

Experimental Procedures

General Methods

Reagents and Enzymes. The reagents used in this work were all reagent grade. The water was distilled and then deionized on an anion-exchange column. Vanadium pentoxide was purchased from Fisher Scientific Co. The enzymes and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The source of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was *L. mesenteroides*, and this enzyme was assayed by using both NAD and NADP.

Kinetic Measurements. Spectrophotometric determinations of rates of glucose 6-phosphate (G6P) oxidation were measured at 340 nm on a Lambda 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. All initial rates were measured at 25 °C and followed for 2 min. The reactions were started by adding enzyme to each preincubated assay solution 60 s after the inhibitor was added. Controls were run before and after each series of varying G6P concentration (at constant inhibitor concentration), and rate adjustments were made when necessary. At low substrate concentrations the rates were determined in duplicate or triplicate. The protein concentrations were quantified by the method of Lowry.

^{51}V NMR Spectroscopy. Vanadium-51 is an NMR active nucleus of 99.75% natural abundance. Although its spin is $^7/_2$, its line widths are relatively narrow and are easily resolved in the vanadium window. ^{51}V NMR is therefore a convenient and informative tool for the determination of vanadium(V) species in solution (Heath & Howarth, 1981; Gresser & Tracey 1985). The ^{51}V NMR spectra were recorded at 53 MHz on a ^1H 200-MHz Bruker WPSY (4.7-T) spectrometer and at 131.5 MHz on an AM ^1H 500-MHz Bruker (11.7-T) spectrometer. Typical conditions include a spectrum width of 8064 Hz, a 90° pulse angle, an accumulation time of 0.2

[1] Abbreviations: G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; NAD, β-nicotinamide adenine dinucleotide; NADP, β-nicotinamide adenine dinucleotide phosphate; T_1, relaxation time; ^{51}V NMR, vanadium-51 nuclear magnetic resonance; MHz, megahertz; V_1, monomeric vanadate; V_2, dimeric vanadate; V_4, tetrameric vanadate; K_{i2}, inhibition constant for vanadate dimer; K_{i4}, inhibition constant for vanadate tetramer; K_{i5}, inhibition constant for vanadate pentamer; K_{12} (K_{14}, K_{15}, or K_{24}), equilibrium constants relating to vanadate oligomerization reactions.

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 at pH 8.8 containing the vanadate–diethanolamine complex 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 assay with the exception that G6P, NAD or NADP, and enzyme were omitted. It was shown experimentally that omission of substrate and cofactors did not noticeably affect the distribution of vanadate species since the concentration of vanadate derivatives was reproducible within 5% with or without G6P, NAD, or NADP.

Vanadate solutions contain complex mixtures of mono- and oligovanadates that vary with ionic strength, temperature, pH, and vanadate concentration. The ^{51}V NMR spectra contain signals for the vanadate monomer (−555 ppm), dimer (−569 ppm), tetramer (−574 ppm), and pentamer (−582 ppm). Thus ^{51}V NMR was used to determine the concentration of vanadate species in the assay solutions containing total vanadate concentrations used in the enzyme assays. The ^{51}V NMR spectra allowed the calculation of the concentration of each oligomer from the integrated spectra and the known total vanadate concentrations. The oligomer concentrations determined in this manner follow the relationships shown by eqs 1–3 (the

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

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

$$2V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (3)$$

correlation coefficients were 0.99 or above) (Crans et al., 1988). The H^+-dependent equilibrium constants were determined to be $K_{12} = 2.9 \times 10^2\ M^{-1}$, $K_{14} = 1.9 \times 10^9\ M^{-3}$, and $K_{24} = 2.2 \times 10^4\ M^{-1}$ and correspond to those published previously in the literature (Crans et al., 1988).

Data Analysis. The rates were measured from linear portions of the rate profile, and doubling the enzyme concentration produced doubling of rates. The kinetic data were analyzed by using Cricket Graph, a program for statistical manipulations on the Apple computer. Both Lineweaver–Burk and Eisenthal–Cornish–Bowden types of plots were used to determine Michaelis–Menten parameters. Lineweaver–Burk and Dixon plots are used to illustrate the inhibition patterns. The K_i's and the nature of inhibition were determined from plots of the Lineweaver–Burk slopes or reciprocal intercepts as a function of the concentration of vanadium atoms found as monomeric, dimeric, tetrameric, and pentameric vanadate. The fits of the experimental points were determined by using Cricket Graph. The Michaelis–Menten constants were reproducible within 5% and were in agreement with previous determinations. The fits determining the K_i's through polynomials were reproducible within 20% for the slope and reciprocal intercept plots. We estimate the accuracy on these to be within 40%. The experimental uncertainty of the rates is on the order of 5% for the high substrate concentrations and 10% for the low substrate concentrations. At the high vanadate concentrations the uncertainty increases to about 30%. To decrease experimental scatter, experiments with higher uncertainties were carried out in duplicates (or triplicates), and the larger uncertainty was considered when analyzing the data. The correlation coefficients with the best fit were always 0.99 or above.

Specific Assays

G6PDH Assay Using NAD (Olive & Levy, 1967). (*A*) *Varying G6P Concentration.* All assays contained 100 mM imidazole (pH 7.1), 2.6 mM magnesium chloride, 6.9 mM NAD, and approximately 0.0013 mg of G6PDH. The rates were measured in solutions where the G6P concentration varied from 0.032 to 0.38 mM. The rates were measured in the presence of 0.0–6.0 mM vanadate. A corresponding assay was used when examining the inhibition of phosphate and pyrophosphate, with the exception of 200 mM KCl that was added at 0 mM inhibitor concentration such that a constant ionic strength could be maintained. The phosphate concentration was varied from 0.0 to 7.0 mM, and the pyrophosphate concentration was varied from 0.0 to 5.0 mM.

(*B*) *Varying NAD Concentration.* The assay solutions contained from 0.070 to 0.70 mM NAD, in the presence of 2.1 mM G6P, 100 mM imidazole (pH 7.1), 2.6 mM magnesium chloride, and approximately 0.0013 mg of G6PDH. The rates were measured from 0.0 to 5.5 mM vanadate.

G6PDH Assay Using NADP (Olive & Levy, 1967; Nour-Eldeen et al., 1987). (*A*) *Varying G6P Concentration.* The assay solutions contained from 0.032 to 0.19 mM G6P in the presence of 2.8 mM NADP, 100 mM imidazole (pH 7.1), 2.6 mM magnesium chloride, and approximately 0.013 mg of G6PDH. The rates were determined in the presence of 0.0–4.0 mM vanadate.

(*B*) *Varying NADP Concentration.* The assay solutions contained from 0.014 to 0.070 mM NADP in the presence of 3.2 mM G6P, 100 mM imidazole (pH 7.1), 2.6 mM magnesium chloride, and approximately 0.0013 mg of G6PDH. The rates were determined in the presence of 0.0–5.0 mM vanadate.

Results

G6P Oxidation Catalyzed by G6PDH from L. mesenteroides. The G6PDH-catalyzed oxidation of G6P to 6-phosphogluconate (6PG) reduces NAD to NADH (or NADP to NADPH), which was measured by UV spectroscopy. The enzyme reaction rates were monitored at 340 nm at 25 °C. The assay conditions were similar to those used previously except for substitution of the buffer with imidazole. Imidazole was chosen because fewer interferences of redox reactions and addition reactions of vanadate are observed in this buffer (Vyskocil et al., 1980; Crans & Shin, 1988). The reaction rates were determined at various G6P concentrations from 0.063 to 0.38 mM in the presence of 100 mM imidazole, 6.9 mM NAD (or 0.28 mM NADP), and 2.6 mM $MgCl_2$ at pH 7.1. The K_m for G6P was 0.21 mM (or 0.073 mM). The reaction rates were determined at various NAD concentrations from 0.070 to 0.70 mM in the presence of 2.1 mM G6P, 100 mM imidazole, and 2.6 mM $MgCl_2$ at pH 7.1. The K_m for NAD was determined to 0.35 mM. The reaction rates were determined at various NADP concentrations from 0.014 to 0.070 mM in the presence of 3.2 mM G6P, 100 mM imidazole, and 2.6 mM $MgCl_2$ at pH 7.1. The K_m for NADP was 0.022 mM. The Michaelis–Menten parameters vary considerably with assay conditions; however, these results are in agreement with those published previously (Levy, 1979).

Inhibition by Phosphate, Pyrophosphate, and Vanadate of G6P Oxidation Catalyzed by G6PDH. The G6PDH-catalyzed oxidation of G6P using NAD as a cofactor was determined at G6P concentrations from 0.063 to 0.25 mM in the presence of various phosphate and pyrophosphate concentrations up to 70 mM. The ionic strength was kept constant by adding varying concentrations of KCl. Plotting the reciprocal rates as a function of reciprocal G6P concentrations yielded a Lineweaver–Burk plot (data not shown) showing the expected

competitive inhibition pattern for phosphate. The K_i was about 70 mM and suggests that phosphate only binds weakly to the enzyme. These results are in accord with the observations that bicarbonate, phosphate, and sulfate all inhibit the oxidation of G6P catalyzed by G6PDH from yeast (Rutter, 1957; Anderson et al., 1968; Horne et al., 1970). These three anions were also found to stimulate the oxidation of glucose catalyzed by G6PDH from yeast (Melzger et al., 1972). Detailed kinetic studies with the above anions, nucleotides, and G6P suggested that all these anions bind to the active site in the yeast enzyme. Although correspondingly complete studies have not been carried out with the *L. mesenteroides* enzyme, our inhibition studies with phosphate are in accord with the above observations and interpretations.

Similar inhibition patterns were not observed with pyrophosphate. Plotting the reciprocal rates as a function of reciprocal G6P concentrations yielded a Lineweaver–Burk plot (data not shown) with a noncompetitive inhibition pattern (K_i = 30 mM). Measuring the rates of oxidation as the NAD concentration was varied from 0.014 to 0.070 mM in the presence of pyrophosphate only weak inhibition with an ill-defined inhibition pattern was observed. A corresponding experiment with NADP showed no inhibition up to 200 mM pyrophosphate. These results suggest that pyrophosphate interacts significantly differently with G6PDH than phosphate.

Inhibition by Vanadate of G6P Oxidation Catalyzed by G6PDH from L. mesenteroides Using NAD as Cofactor. The rates of G6P oxidation catalyzed by G6PDH were determined from 0.032 to 0.38 mM G6P with NAD as a cofactor in the presence of 0.0–6.0 mM vanadate concentrations. A plot of the reciprocal rates as a function of the reciprocal G6P concentrations for various vanadate concentrations gives linear curves of increasing slopes with increasing vanadate concentrations. A Lineweaver–Burk plot (data not shown) suggests vanadate behaves as a noncompetitive (mixed-type) inhibitor with respect to G6P. Vanadate is structurally and electronically an analogue of phosphate and behaves as a phosphate analogue in many biological systems (Nechay et al., 1986; Chasteen, 1983). It would therefore have been reasonable to expect that vanadate would bind to G6PDH at the same binding site as phosphate and the other anions. However, since we observe a noncompetitive (mixed-type) inhibition pattern (contrary to a competitive inhibition pattern for phosphate), vanadate appears to interact differently with the enzyme. This conclusion is supported by the fact that vanadate is found to be a significantly more potent inhibitor than phosphate. It was therefore of interest to examine the inhibition of vanadate in further detail. In order to probe the inhibition pattern with respect to NAD, a series of experiments were conducted by varying the NAD concentration from 0.070 to 0.70 mM in the presence of 2.1 mM G6P and from 0.0 to 7.0 mM vanadate. Plotting the reciprocal rates as a function of the reciprocal NAD concentrations gave Lineweaver–Burk curves showing a competitive inhibition pattern. This is in accord with the interpretation that vanadate prevents binding of the cofactor by the enzyme (data not shown).

Aqueous vanadate solutions at neutral pH contain complex mixtures of mono- and oligovanadates, and the speciation distribution varies with ionic strength, concentration, and temperature. Monomer, dimer, tetramer, and pentameric vanadate species are rapidly exchanging at neutral pH; however, these exchange rates are on a millisecond time scale (Crans et al., 1990b) and are thus significantly slower than the enzyme-catalyzed reaction under examination. G6PDH will therefore interact with different vanadate species, and it is likely each species will interact differently with the enzyme. Thus a nonlinear relationship between $1/v$ as a function of total vanadate concentration may be a result of the combined interactions of all the vanadate species. In order to examine the affects of the individual vanadate species, the concentration of various vanadate species was determined by ^{51}V NMR spectroscopy.

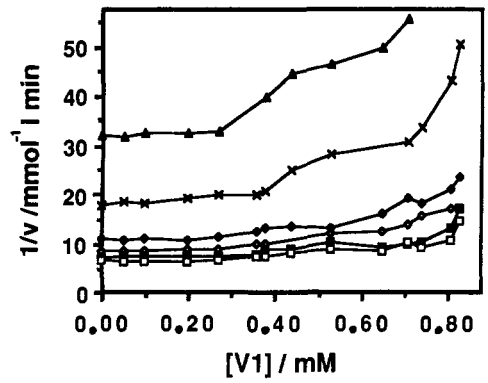

FIGURE 1: Reciprocal rates are plotted as a function of monomeric vanadate concentrations. The rates were determined in 100 mM imidazole (pH 7.1), 2.1 mM G6P, 2.6 mM $MgCl_2$, approximately 0.0013 mg of G6PDH, and various NAD concentrations. The NAD concentrations were as follows: 0.070 mM (▲), 0.14 mM (×), 0.28 mM (◆), 0.42 mM (◇), 0.56 mM (■), and 0.70 mM (□).

Concentration of Vanadate Mono- and Polyanions As Determined by ^{51}V NMR Spectroscopy. ^{51}V NMR was used to measure the distribution of vanadate species at various vanadate concentrations in 100 mM imidazole and 2.6 mM $MgCl_2$ and at pH 7.1 under the assay conditions used for monitoring the G6P oxidation catalyzed by G6PDH from *L. mesenteroides*. At vanadate concentrations below 0.2 mM the monomeric vanadate species is the only form of vanadate observed in solutions by using ^{51}V NMR. As the total vanadate concentration increases above 0.2 mM, both the vanadate dimer and tetramer emerge. Above 0.5 mM total vanadate the tetramer concentration is rapidly increasing, and at 1.0 mM it has become the major vanadate species in solution. At 3.0 mM the vanadate pentamer emerges and its concentration slowly increases. The concentration of monomer, dimer, and tetramer are related as shown in eqs 1, 2, and 3.

Inhibition by Monomeric Vanadate of G6PDH with Respect to NAD. Combining kinetic data with the vanadate speciation determined by ^{51}V NMR spectroscopy, the effects of various vanadate species can be examined. Plotting the reciprocal rate as a function of monomeric vanadate concentration (a Dixon plot) gives a nonlinear relationship (Figure 1). Figure 1 shows that no vanadate inhibition is observed below 0.2 mM vanadate monomer ($[V_1]$) whereas a rate decrease is observed above 0.2 mM vanadate monomer. If monomeric vanadate significantly inhibited the G6P oxidation, the inhibition would have been observable below 0.2 mM monomer where the monomer is the major vanadate form. Accordingly, these results suggest that *the inhibition due to the monomeric vanadate is low and within the experimental uncertainty.* In contrast, the reaction rates observed in the presence of 0.4 mM monomer (or above) were considerably decreased. At these monomer concentrations significant concentrations of dimer and tetramer are present in the solutions, and it is likely these other vanadate species are responsible for the competitive inhibition pattern observed by the vanadate solutions.

Our results thus far are unexpected on three counts. First, vanadate inhibits the oxidation of G6P catalyzed by G6PDH with a noncompetitive (mixed-type) inhibition pattern with respect to G6P, contrary to phosphate, which inhibits the

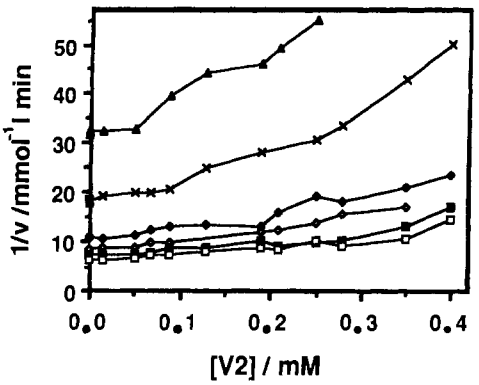

FIGURE 2: Reciprocal rates are plotted as a function of dimeric vanadate concentration (expressed as millimolar vanadium atoms). The rates were determined as described in the caption of Figure 1. The NAD concentrations were as follows: 0.070 mM (▲), 0.14 mM (×), 0.28 mM (◆), 0.42 mM (◇), 0.56 mM (■), and 0.70 mM (□).

oxidation of G6P catalyzed by G6PDH in a competitive inhibition pattern. Second, vanadate inhibits the oxidation of G6P with respect to NAD with a competitive inhibition pattern. Accordingly, vanadate (contrary to phosphate) may bind in the cofactor site of the enzyme. Third, most of the known biological effects of vanadate have been attributed to monomeric vanadate whereas the inhibition by vanadate of G6PDH appears to be due to higher oligomeric vanadate anions.

Inhibition by Vanadate Dimer, Tetramer, and Pentamer of G6PDH with Respect to NAD. Since monomeric vanadate did not inhibit the reaction catalyzed by G6PDH, we examined which other vanadate species could be responsible for the rate inhibition. Plotting the reciprocal rate as a function of the dimeric vanadate concentration shows that the oxidation rate decreased as the dimeric vanadate concentrations increased (Figure 2). The relationship shown in Figure 2 appears to be linear until dimer concentrations of about 0.35 mM are reached, and from 0.35 mM an upward curve is followed. It is possible that the vanadate dimer and an additional species in solution inhibit G6PDH. The latter species becomes more important at higher vanadate concentrations and therefore results in the upward curvature in Figure 2. Alternatively, the vanadate dimer concentration is directly related to the vanadate tetramer or pentamer concentrations, and the relationship shown in Figure 2 may reflect inhibition by such a higher oligomer alone. Further analysis is therefore necessary to determine if the dimer indeed inhibits the G6PDH from *L. mesenteroides*.

Plotting the reciprocal rate as a function of the tetrameric vanadate concentration gave a seemingly linear relationship. It is therefore likely that the tetrameric vanadate species is a major inhibitor under these conditions. On the basis of these considerations, we will examine if a vanadate species in addition to the tetramer inhibits the enzyme reaction. The various possibilities are explored as follows.

The inhibition of G6PDH by one competitive inhibitor V_4 would have Lineweaver–Burk slopes and intercepts on the x axis (x intercepts) as defined in

$$\text{slope} = \frac{K_{m,app}}{V_{max}} = \frac{K_m}{V_{max}}\left(1 + \frac{[V_4]}{K_{i4}}\right) \quad (4)$$

$$\frac{-1}{x \text{ intercept}} = K_m\left(1 + \frac{[V_4]}{K_{i4}}\right) \quad (5)$$

The Lineweaver–Burk slopes and reciprocal x intercepts plotted as a function of tetramer concentration should therefore

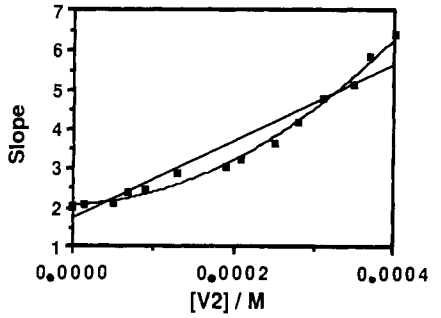

FIGURE 3: Lineweaver–Burk slopes were plotted as a function of dimeric vanadate concentration (expressed as millimolar vanadium atoms) and fitted with a line (correlation coefficient 0.96) or a polynomial (correlation coefficient 0.99). The rates were determined as described in the caption of Figure 1.

give linear relationships if the tetramer is the only vanadate species exhibiting the observed level of inhibition. If the vanadate dimer were the competitive inhibitor in place of the tetramer, the term $[V_4]/K_{i4}$ would be substituted with $[V_2]/K_{i2}$.

The inhibition of G6PDH by two competitive inhibitors V_2 and V_4 has Lineweaver-Burk slopes and x intercepts as defined in

$$\text{slope} = \frac{K_{m,app}}{V_{max}} = \frac{K_m}{V_{max}}\left(1 + \frac{[V_2]}{K_{i2}} + \frac{[V_4]}{K_{i4}}\right) \quad (6)$$

$$\frac{-1}{x \text{ intercept}} = K_m\left(1 + \frac{[V_2]}{K_{i2}} + \frac{[V_4]}{K_{i4}}\right) \quad (7)$$

However, as seen from eq 3, the tetramer concentration can be expressed as the dimer concentration squared ($[V_4] = K_{24}[V_2]^2$). Consequently, eqs 6 and 7 become

$$\text{slope} = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}K_{i2}}[V_2] + \frac{K_mK_{24}}{V_{max}K_{i4}}[V_2]^2 \quad (8)$$

$$\frac{-1}{x \text{ intercept}} = K_m + \frac{K_m}{K_{i2}}[V_2] + \frac{K_mK_{24}}{K_{i2}}[V_2]^2 \quad (9)$$

If the two competitive inhibitors effecting G6PDH were V_2 and V_5, one can obtain relationships analogous to those shown in eqs 6 and 7. When the pentamer concentration ($[V_5] = K_{15}[V_1]^5$) and dimer concentration ($[V_2] = K_{12}[V_1]^2$) are expressed in terms of the vanadate monomer concentration, the derived relationships are

$$\text{slope} = \frac{K_m}{V_{max}} + \frac{K_mK_{12}}{V_{max}K_{i2}}[V_1]^2 + \frac{K_mK_{15}}{V_{max}K_{i5}}[V_1]^5 \quad (10)$$

$$\frac{-1}{x \text{ intercept}} = K_m + \frac{K_mK_{12}}{K_{i2}}[V_1]^2 + \frac{K_mK_{15}}{K_{i5}}[V_1]^5 \quad (11)$$

Alternatively, the two competitive inhibitors affecting G6PDH could be V_4 and V_5, and an analogous relationship to eqs 10 and 11 would substitute the term $K_mK_{12}[V_1]^2/V_{max}K_{i2}$ with $K_mK_{14}[V_1]^4/V_{max}K_{i4}$.

These five possibilities are tested by examining the type of relationship that fits the experimental data. The three best fits for the experimental data at varying NAD concentrations are shown in Figures 3 and 4. The linear relationships eqs 4 and 5 that suggested the vanadate dimer is the only inhibiting species gave a poor fit at high vanadate concentrations. A much better fit was obtained with a polynomial of the type $y = a + bx + cx^2$ (where $b \geq 0$ and $c \geq 0$). Since the vanadate

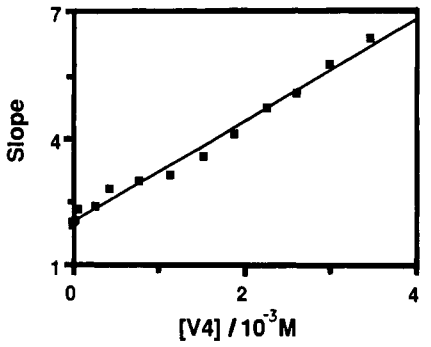

FIGURE 4: Lineweaver–Burk slopes were plotted as a function of tetrameric vanadate concentration (expressed as millimolar vanadium atoms) and fitted with a line (correlation coefficient 1.00). The rates were determined as described in the caption of Figure 1.

Table I: Summary of Michaelis–Menten Constants and Inhibition Constants for Glucose-6-phosphate Dehydrogenase from *L. mesenteroides*

NAD as cofactor		NADP as cofactor	
constant	mM	constant	mM
$K_{m,G6P}$	0.21	$K_{m,G6P}$	0.073
$K_{m,NAD}$	0.35	$K_{m,NADP}$	0.022
inhibitors with respect to G6P		inhibitors with respect to G6P	
K_{i2}, linear fit		K_{i2}, linear fit	
K_{i4}, linear fit	0.45	K_{i4}, linear fit	0.53
inhibitors with respect to NAD		inhibitors with respect to NADP	
K_{i2}, linear fit		K_{i2}, linear fit	0.090
K_{i2}, polynomial	15	K_{i2}, polynomial	0.16
K_{i4}, linear fit	0.25	K_{i4}, linear fit	
K_{i4}, polynomial	0.25	K_{i4}, polynomial	1.2

dimer is related to the tetramer through the equation $[V_2]^2K_{24} = [V_4]$, the exclusive inhibition by the tetramer will require that $[V_4]$ or $[V_2]^2$ is directly proportional to the slopes. From eq 4 we see the slope in Figure 4 equals $K_m/(V_{max}K_{i4})$ and the calculated K_{i4} was 0.25 mM (see Table I). The fit with this polynomial allows us to calculate the inhibition constants when *both* the dimer ($b > 0$) and the tetramer ($c > 0$) inhibit the enzyme. As seen from eq 8, $b = K_m/(V_{max}K_{i2})$ and $c = K_mK_{i24}/(V_{max}V_{i4})$. By use of this K_{i2} was calculated to be 15 mM and K_{i4} was calculated to be 0.25 mM (see Table I). A linear relationship as a function of tetramer concentration does give an excellent fit and is shown in Figure 4 ($R = 1.00$). This observation is in agreement with the above result that the tetramer indeed is the major vanadate inhibitor under these conditions. A linear relationship of the slopes was also found with the squared dimer concentration ($R = 0.99$). Polynomials of the type $y = a + bx^2 + cx^5$ or $y = a + bx^4 + cx^5$, on the other hand, agreed very poorly with the experimental result. Agreement of the experimental data with these types of polynomials would have suggested that the dimer and pentamer were the inhibitors ($y = a + bx^2 + cx^5$) or that the tetramer and pentamer were the inhibitors ($y = a + bx^4 + c^5$). The analysis suggests that the vanadate tetramer and possibly the dimer inhibit G6PDH. These two species were sufficient to account for all the observed inhibition. If the vanadate tetramer were the only inhibiting species the K_{i4} would be 0.25 mM (1.0 mM vanadium atoms as tetramer) ($R = 0.99$). By use of eqs 8 and 9 K_{i2} and K_{i4} (with respect to NAD) were calculated as 15 mM (30 mM vanadium atoms as dimer) and 0.25 mM (1.0 mM vanadium atoms as tetramer) ($R = 0.99$). Since the tetramer is present in vanadate solutions in much higher concentrations than the dimer and the $K_{i2} \gg K_{i4}$, the vanadate tetramer is the most potent inhibitor in this system. The inhibition constants determined by using either fit are summarized in Table I.

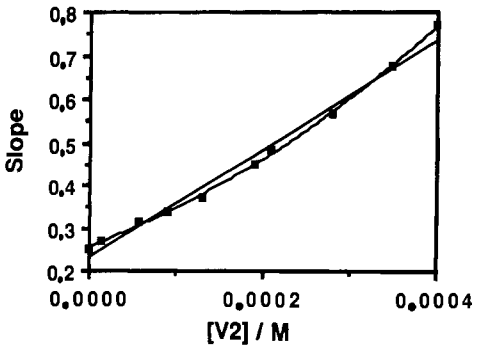

FIGURE 5: Lineweaver–Burk slopes are plotted as a function of vanadate dimer concentration (expressed as millimolar vanadium atoms). The rates were determined from 0.014 to 0.070 mM NADP in the presence of 3.2 mM G6P, 100 mM imidazole (pH 7.1), 2.6 mM magnesium chloride, and approximately 0.0013 mg of G6PDH. The linear relationship had a correlation coefficient of 0.99, and the polynomial fit had a correlation coefficient of 1.00.

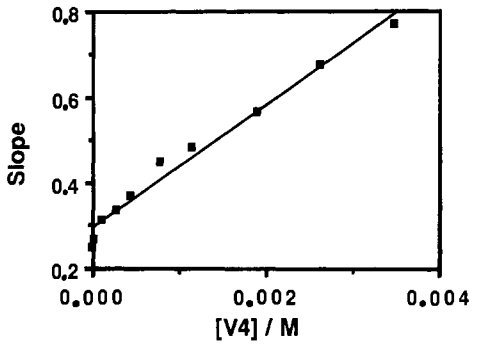

FIGURE 6: Lineweaver–Burk slopes were plotted as a function of vanadate tetramer concentration (expressed as millimolar vanadium atoms). The rates were determined as described in the caption of Figure 5.

Inhibition by Vanadate Monomer, Dimer, Tetramer, and Pentamer of G6PDH with Respect to NADP. The vanadate inhibition pattern observed with respect to NADP has similarities and differences with the NAD studies. First, the Lineweaver–Burk plot shows a competitive inhibition pattern of vanadate with respect to NADP analogous to the NAD system. Second, monomeric vanadate gives nonlinear Dixon plots where no inhibition is observed at low monomer concentrations. Accordingly, the inhibition due to the monomeric vanadate is low and is within experimental uncertainty. Third, the Dixon plots for the dimer and tetramer give linear or near-linear relationships, suggesting further analyses are also required for determination of the active inhibitor. Plotting the slopes and reciprocal intercepts from the Lineweaver–Burk as a function of the dimer concentration yields a linear or near-linear relationship, contrary to the NAD system (Figure 5). Analogous plots as a function of the tetramer concentration also appear to be linear when the entire vanadate concentration range is plotted (Figure 6). However, when the latter figure is examined at low tetramer concentrations, a rapid increase in slopes (corresponding to a rapid decrease in reaction rates) is observed at low tetramer concentrations. These changes are not satisfactorily described as inhibition by the tetramer (Figure 7). This rapid increase in slope at low tetrameric vanadate concentrations therefore suggests that a vanadate oligomer other than the tetramer is the real inhibiting species. Since the vanadate dimer is the other vanadate species present in this concentration range, it is likely the dimer is the actual inhibiting species.

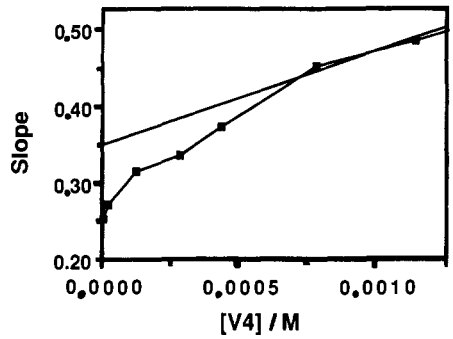

FIGURE 7: Lineweaver–Burk slopes were plotted as a function of vanadate tetramer concentration (expressed as millimolar vanadium atoms). The low tetramer concentration region was expanded, such that the initial nonlinearity is clearly illustrated.

When the dimer is subjected to the analysis described above for the NAD studies, the two best relationships for plots against dimer concentration were a line ($y = a + bx$) and a polynomial ($y = a + bx + cx^2$) (Figure 5). A good linear fit will suggest that the dimer is the exclusive inhibitor, and a good fit to the second-order polynomial suggests that both vanadate dimer and tetramer are inhibitors. In the former case the dimer is the exclusive inhibitor ($R = 0.99$) and the K_{i2} was found to be 0.090 mM (0.18 mM vanadium atoms as dimers). If both dimer and tetramer ($R = 1.00$) are inhibitors, K_{i2} was calculated from b to be 0.16 mM (0.32 mM vanadium atoms as dimer). The K_{i4} for the tetramer in the latter case was calculated from c and was found to be 1.2 mM (4.6 mM vanadium atoms as tetramer). These results are summarized in Table I.

When NADP is a cofactor, the dimer was clearly found to be the major inhibiting species in both the best fits. Even at 2–3 mM vanadate concentrations, the vanadate dimer is still the most potent inhibitor in the vanadate solution despite the fact the tetramer is present in higher concentrations. This observation is contrary to the inhibiton of the reaction with NAD as cofactor where the tetramer was the most potent inhibitor.

Inhibition by Vanadate of G6PDH with Respect to G6P Using NAD as a Cofactor. Plotting the data in a Dixon plot as a function of vanadate monomer allows convenient analysis of the data. No inhibition was observed below 0.3 mM monomeric vanadate, and above this concentration the rates decreased rapidly. We therefore conclude that inhibition due to monomeric vanadate is low and within experimental uncertainty when NAD is as a cofactor. The dimer gives a partially linear Dixon plot with an upward curvature, and the tetramer gives a Dixon plot that appears linear. Plotting the slopes from the Lineweaver–Burk plot against the dimer concentration yields a nonlinear plot that is fitted well by a polynomial of the type $y = a + bx^2$ ($R = 0.99$). Consequently, a linear plot is obtained from a plot of the Lineweaver–Burk slopes as a function of the dimer concentration squared ($R = 0.99$) or as a function of the tetramer concentration ($R = 0.99$). The tetramer was therefore found to be the most potent inhibitor in this system, and the K_{i4} was calculated to be 0.45 mM (1.8 mM vanadium atoms as tetramer).

Inhibition by Vanadate of G6PDH with Respect to G6P Using NADP as a Cofactor. The rates of G6P oxidation catalyzed by G6PDH were determined from 0.032 to 0.19 mM G6P with 2.8 mM NADP in the presence of 0.0–4.0 mM vanadate. A Lineweaver–Burk plot gives a mixed-type inhibition pattern as observed with NAD as a cofactor. A Dixon plot of the reciprocal rate as a function of monomeric vanadate gave nonlinear relationships. No inhibition was observed below 0.4 mM monomeric vanadate, and above this concentration the rates decreased rapidly. We therefore conclude that the inhibition due to monomeric vanadate is low and is within experimental uncertainty for G6PDH with NADP as a cofactor.

Dixon plots of the reciprocal rate as a function of either dimer or tetramer show that both these oligomers could be inhibiting species of G6PDH. The dimer shows a partially linear Dixon plot with an upward curvature, and the tetramer shows a plot that appears linear. Plotting the Lineweaver–Burk slope and reciprocal intercept as a function of the vanadate dimer concentration yields a fit with a polynomial of the type $y = a + bx^2$ ($R = 1.00$). The slopes as a function of the vanadate tetramer concentration (or the vanadate dimer concentration squared) give a linear relationship ($R = 0.99$). We therefore conclude the vanadate tetramer is the most potent inhibitor in this system, and the K_{i4} was calculated to be 0.53 mM (2.1 mM vanadium atoms as tetramer). At concentrations above 4.0 mM vanadate the observed inhibition became more effective than could be explained by the tetramer alone. The vanadate pentamer emerges at ~3.5 mM total vanadate concentrations. It is therefore posible that the pentamer also inhibits the enzyme reaction. This suggestion could be examined by more detailed inhibition studies at vanadate concentrations from 3.5 to 6.0 mM.

Discussion

Vanadate solutions containing monomeric, dimeric, tetrameric, and pentameric vanadate inhibit the oxidation of G6P catalyzed by G6PDH with respect to G6P with a noncompetitive (mixed-type) inhibition pattern when both NAD and NADP were cofactors. The former observation was surprising on several counts. Phosphate and monomeric vanadate were known to inhibit many enzymes by binding in the active site in place of the substrate. Glycolytic and pentose phosphate shunts are inhibited by phosphate, and it therefore seemed reasonable to expect vanadate as a phosphate analogue would show similar activity. However, the observed inhibition pattern was noncompetitive (mixed type) with respect to G6P. The kinetic inhibition patterns with respect to NAD or NADP were both found to be competitive and thus raised the possibility that vanadate may be binding in the cofactor site in G6PDH.

Both ^{51}V NMR and enzyme kinetics were used to probe the vanadate inhibition and led to the suggestion that the inhibition was caused mainly by the vanadate dimer and tetramer, depending on the specific conditions. The dimer appears to be the most potent inhibitor when NADP ($K_{i2} = 0.16$ mM) was the cofactor. Since the NADP cofactor site presumably is more charged than the NAD cofactor site because the extra phosphate group on the cofactor must be accommodated (Kurlandsky et al., 1988), it seems reasonable that the vanadate dimer is a more potent inhibitor for NADP than for NAD ($K_{i2} = 15$ mM). The binding of the dimer in the binding pocket for the phosphate group may not inhibit the binding of the NAD cofactor in analogy with previous observations by Boxsignore and Horecher (Boxsignore et al., 1960) on transketolase, in which phosphate bound competively against only fructose 6-phosphate and none of the other substrates. Vanadate oligomers have previously been found to inhibit enzymes such as phosphorylase (Soman et al., 1983), hexokinase (Boyd & Kustin, 1985), phosphoglycerate mutase (Stankiewicz et al., 1985), and 6-phosphogluconate dehydrogenase (Crans et al., 1990a). In most of these cases the vanadate oligomer presumably binds to the active site in the enzyme. However, it is unprecedented that the affinity for the vanadate oligomer is changing depending on the reaction

the enzyme is catalyzing. Since both the dimer and tetramer presumably bind to the cofactor site in G6PDH from *L. mesenteroides*, the effects of pyrophosphate as a model for the vanadate dimer on this enzyme were determined. Pyrophosphate was found to be a much weaker inhibitor than vanadate; however, it was a better inhibitor for the NAD reaction than for the NADP reaction. This observation does not correlate with the observed inhibition by the vanadate dimer, and thus pyrophosphate may not be a good model for the vanadate dimer. The vanadate dimer, at pH 7.1, is either a dianion ($H_2V_2O_7^{2-}$) or a trianion ($HV_2O_7^{3-}$). Pyrophosphate at pH 7.1 is mostly a trianion. The natural substrate and cofactors are either anionic or dianionic at the phosphate or diphosphate ester functionalities. It is therefore possible that the dianionic dimer but not the trianionic dimer fits into the binding site. Alternatively, the lability of the vanadate dimer may be important for interaction with the protein. The ability of both the dimer and tetramer to bind to G6PDH suggests the cofactor sites have considerable flexibility and/or that the two oligomers bind to different sections of these binding sites.

G6PDH undergoes reaction through an ordered mechanism adding NADP before G6P and releasing NADPH last (Levy, 1986). The NAD-linked reaction in contrast is random. K_m for the NADP cofactor is smaller; however, the enzyme pays for such specificity and the V_{max} for NADP is slower than for NAD. The vanadate dimer was found to be a more potent inhibitor against NADP than the tetramer against NAD. Since the concentration of vanadate tetramer increases much more rapidly than the concentration of vanadate dimer, the total vanadate concentration window in which the dimer is the most active vanadate inhibitor is very small. At higher concentrations than 1.0 mM total vanadate the tetramer is the most active inhibitor. However, whether the dimer or the tetramer will inhibit the enzyme reaction will depend on which reaction is occurring. Since the physiological reaction involves NADP as cofactor and the cofactor is usually the limiting substrate reagent, one may expect the dimer to be the vanadate species of most biological significance. In vivo studies by Willsky et al. (1984) with yeast did indeed lead to vanadium-imposed growth inhibition, and on the basis careful EPR and NMR studies they proposed the vanadate tetramer (at −576 ppm) was a toxic vanadate derivative.

There is evidence that the adenine moiety of the NAD coenzyme binds more tightly to its subsite than the nicotinamide portion to its subsite (Levy, 1986). It is believed that the two subsites move apart upon binding, and the conformational changes upon cofactor binding are more extensive with NAD than with NADP (Levy, 1979). This observation may explain why the vanadate tetramer is a more potent inhibitor for the smaller cofactor. The tetramer is able to gain a significant stabilization by binding to the protein because the enzyme is rearranging its conformation in a manner similar to binding the NAD. A corresponding degree of stabilization is not obtained when the tetramer binds in place of NADP.

Although vanadate has shown many potent biological activities with a wide variety of enzymes, studies with vanadate are commonly carried out with enzymes that convert organic phosphates as substrates (ATPase, phosphatases, kinases, glycolytic enzymes). The observation that a vanadate species will bind to the cofactor site in the enzyme instead of the substrate site in the enzyme suggests that other enzymes using NAD or NADP as a cofactor may also have an affinity for vanadate. Since vanadium is a dietary trace element with no known function in mammals, such a suggestion represents an alternative possible mechanism of action of vanadate in biological systems. The inhibition by vanadate of the glucose-6-phosphate dehydrogenases from bakers' yeast and torula yeast is also complex and involves vanadate oligomers (Crans and Stites, unpublished results). Both of these dehydrogenases exclusively utilize NADP. Lactate dehydrogenase was also competitively (although weakly) inhibited by vanadate with respect to NADH (Crans and Simone, unpublished results). These preliminary studies therefore support the possibility for binding and inhibition by vanadate in enzyme cofactor sites. Such possibilities should particularly be considered in in vitro studies where high vanadium concentrations are common. If such vanadate interactions indeed occur in mammalian systems, they are likely to be found in organisms that contain toxic levels of vanadium. In in vitro studies, however, vanadate–enzyme interactions are likely to occur when vanadium is used as a reagent in assays.

The vanadate dimer was previously found as cell-associated vanadium in yeast cultures and was suggested to cause some of the toxic effects observed in growth-inhibited cells (Willsky et al., 1984). This suggestion was based on the observations that no growth inhibition was observed with cells containing only little cell-associated vanadate dimer. The tetramer has also been shown to have high affinity for binding to some proteins (Crans et al., 1990a). The presence of dimer in the yeast growth media is commonly accompanied by growth inhibition (Crans & Willsky, unpublished results), but only little vanadate tetramer has been observed as cell-associated vanadate. The vanadate inhibition studies with G6PDH suggest that both vanadate dimer and tetramer potentially could inhibit dehydrogenases in general. Since G6PDH from *L. mesenteroides* under physiological conditions utilizes NADP, the vanadate dimer is most likely to interfere with this enzyme under physiological conditions. Both the tetramer and dimer may be important for inhibition of growth in yeast. It is possible oligomeric vanadate species may be important in explaining some of the ample biological effects exhibited by millimolar concentrations of vanadate.

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Registry No. G6PDH, 9001-40-5; G6P, 56-73-5; NAD, 53-84-9; NADP, 53-59-8; phosphate, 14265-44-2; pyrophosphate, 14000-31-8.

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A Protein Engineering Study of the Role of Aspartate 158 in the Catalytic Mechanism of Papain†

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ABSTRACT: The controversy concerning the various suggested roles for the side chain of Asp158 in the active site of papain has been clarified by using site-directed mutagenesis. Both wild-type papain and an Asp158Asn variant were produced in a baculovirus–insect cell expression system, purified to homogeneity from the culture, and characterized kinetically. With CBZ-Phe-Arg-MCA as substrate, the k_{cat}/K_M and k_{cat} values obtained for the Asp158Asn papain are 20 000 $M^{-1}\cdot s^{-1}$ and 34 s^{-1}, respectively, as compared with values of 120 000 $M^{-1}\cdot s^{-1}$ and 51 s^{-1} obtained for the wild-type papain. In addition, the pH–(k_{cat}/K_M) profile for the Asp158Asn enzyme is shifted relative to that for the wild-type enzyme to lower values by approximately 0.3 pH unit. This shows clearly that Asp158 is not, as previously postulated, an essential catalytic residue. In addition, the pH dependency data are interpreted to indicate that, contrary to earlier suggestions, the negatively charged side chain of Asp158 does not significantly stabilize the active-site thiolate–imidazolium ion pair. However, its presence does influence the pK_a's associated with ion-pair formation in a manner compatible with electrostatic considerations.

Since papain was first described in 1879 (Wurtz & Bouchut, 1879), a large database of information on the enzyme has been gathered. In particular, evidence has accumulated for the formation of an ion pair at neutral pH between residues Cys25 and His159, and it has been proposed that this is the active form of the enzyme [e.g., Polgar (1974), Sluyterman and Wijdenes (1976), Halasz and Polgar (1977), Lewis et al. (1981), and Migliorini and Creighton (1986)]. For an in-depth review of cysteine proteases in general and papain in particular, see Brocklehurst et al. (1987). Mechanistically and kinetically, papain has been extensively characterized, and the structure of the native enzyme has been determined at 1.65-Å resolution (Kamphuis et al., 1984). In addition, the structures of several papain–inhibitor complexes are also available (Drenth et al., 1976; Varughese et al., 1989). These facts together with the recent construction of a synthetic gene coding for the papain precursor (Vernet et al., 1989) and its expression in a bacu-

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