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2,3-Diphosphoglycerate Phosphatase Activity of Phosphoglycerate Mutase: Stimulation by Vanadate and Phosphate†

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ABSTRACT: The binding of inorganic vanadate (V_i) to rabbit muscle phosphoglycerate mutase (PGM), studied by using ^{51}V nuclear magnetic resonance spectroscopy, shows a sigmoidal dependence on vanadate concentration with a stoichiometry of four vanadium atoms per PGM molecule at saturating [V_i]. The data are consistent with binding of one divanadate ion to each of the two subunits of PGM in a noncooperative manner with an intrinsic dissociation constant of 4×10^{-6} M. The relevance of this result to other studies which have shown that the V_i-stimulated 2,3-diphosphoglycerate (2,3-DPG) phosphatase activity of PGM has a sigmoidal dependence on [V_i] with a Hill coefficient of 2.0 is discussed. At pH 7.0, inorganic phosphate has little effect on the 2,3-DPG phosphatase activity of PGM, even at concentrations as high as 50 mM. Similarly, 25 μM V_i has little effect on the phosphatase activity. However, in the presence of 25 μM V_i, a phosphate concentration of 20 mM increases the phosphatase activity by more than 3-fold. This behavior is rationalized in terms of activation of the phosphatase activity by a phosphate/vanadate mixed anhydride. This interpretation is supported by the observation of strong activation of the phosphatase activity by inorganic pyrophosphate. A molecular mechanism for the observed effects of vanadate is proposed, and the relevance of this study to the possible use of vanadate as a therapeutic agent for the treatment of sickle cell anemia is discussed.

The 2,3-diphosphoglycerate (2,3-DPG)[1] phosphatase activity of phosphoglycerate mutase (PGM) is a well-studied phenomenon (Sasaki et al., 1971; Rose & Dube, 1978), and it has been known for some time that this activity is enhanced by inorganic vanadate, V_i (Carreras et al., 1982). This phosphatase activity has been invoked to rationalize the decrease in 2,3-DPG concentration inside erythrocytes incubated with

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[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; PGM, phosphoglycerate mutase; V_i, inorganic vanadate; V_2, inorganic divanadate; V_4, inorganic tetravanadate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid.

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Table I: Distribution of Vanadate among Free and Bound Forms in the Presence of PGM[a]

$[V_t]$	$[V_{vis}]$	$[V_i]$	$[V_2]$	$[V_4]$	$[V_b]$	$[V_{b,vis}]$	$[V_{b,invis}]$
1.50	1.25	0.61	0.23	0.17	0.50	0.24	0.25
1.00	0.74	0.37	0.09	0.02	0.52	0.25	0.26
0.75	0.55	0.26	0.05		0.44	0.24	0.20
0.50	0.34	0.13	0.01		0.35	0.19	0.16
0.25	0.21	0.08			0.17	0.13	0.04
0.10	0.08	0.04			0.06	0.04	0.09

[a] The data were obtained from the spectra shown in Figure 1 as described in the text. Concentrations are vanadium atom concentrations in units of millimoler.

V_i (Ninfali et al., 1983), and this lowered concentration may be the explanation for the increased oxygen affinity of whole human blood after incubation with V_i (Vives-Corrons et al., 1981). A therapeutic agent which leads to an increase in the oxygen affinity of blood may prove valuable in the treatment of the symptoms of sickle cell anemia since sickling is caused by a too high concentration of deoxyhemoglobin S (Dean & Schechter, 1978a–c). Vanadate may therefore be useful in treating this disease, especially in view of the elevated 2,3-DPG levels inside SS red cells (Poillon et al., 1985).

Vanadium, when administered in large amounts, is toxic but in small amounts can be tolerated. Reports that it causes cardiovascular disease have been questioned (Jandhyala & Hom, 1983), and human trials have been conducted in which vanadate was administered orally without harmful effects (Dimond et al., 1963). Diabetic rats have been successfully treated with orally administered vanadate over extended periods of time with no apparent harmful effects (Heyliger et al., 1985).

In this paper, we report the results of experiments designed to aid in elucidating the molecular mechanism of the vanadate-activated 2,3-DPG phosphatase activity of phosphoglycerate mutase.

Experimental Procedures

Materials. Crystalline phosphoglycerate mutase from rabbit muscle (EC 2.7.5.3) was purchased from Boehringer Mannheim Canada Ltd. Vanadium(V) oxide (Gold Label, 99.999%) was from Aldrich Chemical Co. All other chemicals were purchased from Sigma, Aldrich, Boehringer Mannheim, or Fisher and were used without further purification.

Preparation of Stock Solutions. Stock solutions of 0.1 M NaH_2VO_4 were prepared by dissolving vanadium(V) oxide in 1 molar equiv per vanadium atom of 1.0 M aqueous NaOH. The resulting orange solution was allowed to stand overnight during which time it became colorless. It was then diluted to 0.1 M vanadium atom concentration with H_2O. For the binding studies, phosphoglycerate mutase as a solid suspension in aqueous ammonium sulfate was centrifuged, the supernatant was discarded, and the pellet was dissolved in sufficient buffer containing 20 mM HEPES, 4.0 mM KCl, and 1.0 mM glycolate 2-phosphate to give a concentration of PGM of about 10 mg/mL. The resulting solution was then dialyzed for 4 h at 0 °C against 100 mL of the same buffer and then overnight at 0 °C against 2.0 L of a similar buffer containing no glycolate 2-phosphate. Aliquots of 2.0 mL of this solution were used in the NMR studies. For the kinetic studies, the PGM suspension was centrifuged, and the pellet was dissolved in the buffer used in the experiments as indicated in the figure legends. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Sodium orthovanadate from Fisher was used for the kinetic studies.

Determination of Free and Bound Vanadium Concentrations by Nuclear Magnetic Resonance Spectroscopy. Each NMR spectrum was the average of 2×10^4 scans. The spectra were transformed to the frequency domain and scaled identically so that a change in the integrated peak area of a given resonance from one spectrum to the next gave an accurate measure of the change in concentration of the species which gave rise to the resonance being observed. Absolute vanadium atom concentrations were obtained by comparing the integrated peak areas with those from spectra of solutions containing only buffer and sodium vanadate at known total vanadium atom concentrations. Integrated peak areas increased linearly with vanadium atom concentration. The equilibrium constants for formation of divanadate (V_2) and tetravanadate (V_4) from V_i under the conditions of the binding experiments are $(3.1 \pm 0.2) \times 10^2$ M^{-1} and $(3.0 \pm 0.2) \times 10^8$ M^{-3}, respectively. These values were determined by obtaining spectra over a range of vanadium atom concentrations and plotting $[V_2]/[V_i]^2$ and $[V_4]/[V_i]^4$. The concentrations were obtained from the integrated peak areas of the resonances corresponding to the indicated species, using methods which have been described elsewhere (Gresser & Tracey, 1985). These values were used to calculate the values for $[V_2]$ and $[V_4]$ shown in Table I.

Kinetic Studies. Rates of the 2,3-DPG phosphatase reaction were measured as described elsewhere (Carreras et al., 1982) using a coupled assay containing enolase, pyruvate kinase, lactate dehydrogenase, and the appropriate substrates. Concentrations and conditions are given in the figure legends. Rates were found to be unaffected by increasing the concentrations of coupling enzymes by 2-fold. In the presence of pyrophosphate, it was necessary to use a high ratio of enolase to PGM in order to avoid low rates. This is probably due to strong chelation by pyrophosphate causing the Mg^{2+}-requiring enolase to be a poorer catalyst.

NMR Spectroscopy. ^{51}V NMR spectra were obtained at 105 MHz by using the broad-band accessory of a Bruker WM-400 NMR spectrometer. Sweep widths of 40 kHz, 0.025-s acquisition times, 2K data sets, and 60° pulse angles were used for all spectra. Doubling the acquisition time was found to have no observable effect on signal intensities. A total of 20 000 transients were acquired for each spectrum. A line-broadening factor of 40 Hz was applied to all spectra before zero filling to 8 K and transforming to the frequency domain. Transformation was done in the absolute intensity mode so that signal intensities were directly comparable between spectra.

Results

Various ^{51}V nuclear magnetic resonance (NMR) spectra which were obtained from solutions containing a fixed concentration of PGM and varying concentrations of vanadium (as sodium vanadate) are shown in Figure 1. The concentrations indicated are total vanadium atom concentrations. The resonance occurring at −560 ppm is from monomeric tetrahedral vanadate V_i, while those at −573 and −576 ppm arise from divanadate (V_2) and tetravanadate (V_4), respectively (Rehder, 1982). The integrated areas of the various signals,

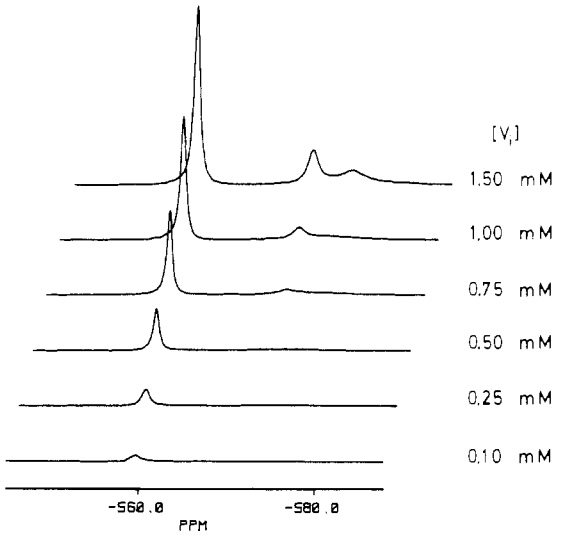


FIGURE 1: ^{51}V NMR spectra of V_i at various concentrations in the presence of PGM. Solutions were prepared by successive additions of small volumes of 0.1 M NaH_2VO_4 to 2.0 mL of a solution containing 8.1 mg/mL PGM. Using a molecular weight of 54000 for the PGM dimer, this corresponds to 0.15 mM PGM or a concentration of 0.30 mM catalytic sites. The PGM solution contained 20 mM HEPES, pH 7.0, 6.0 mM KCl, and vanadate as indicated (total vanadium atom concentrations).

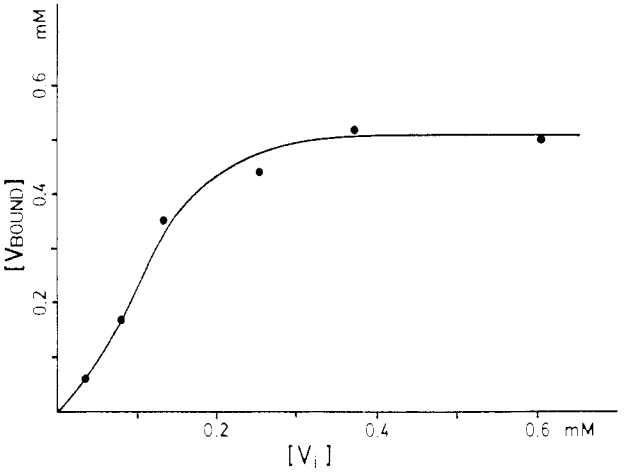


FIGURE 2: Dependence of the concentration of bound vanadium atoms on V_i concentration. The data were taken from Table I and were calculated from the spectra shown in Figure 1 as described in the text. The sigmoidal dependence of $[V_{bound}]$ on $[V_i]$ is apparent.

when compared to those of standard solutions containing no PGM, provide the concentrations of the species of interest, in particular that of V_i, from which the concentrations of the other species can be calculated.

The relationship between the concentration of V_i and that of V_2 and V_4 is given by eq 1 and 2 where K_1 and K_2 are the

$$[V_i]^2K_1 = [V_2] \quad (1)$$

$$[V_i]^4K_2 = [V_4] \quad (2)$$

formation constants for V_2 and V_4, respectively. These two equilibrium constants were determined from a set of independent experiments carried out in solution containing no PGM but otherwise unchanged from the enzyme-containing solution. Under the conditions of these experiments, the concentrations of other vanadate oligomers are negligible so that the sum of the vanadium atom concentrations of V_i, V_2, and V_4 is equal to the total vanadate concentration, $[V_t]$. In the enzyme-containing solutions, the difference between $[V_t]$ and the observed sum ($[V_i] + [V_2] + [V_4]$) corresponds to the

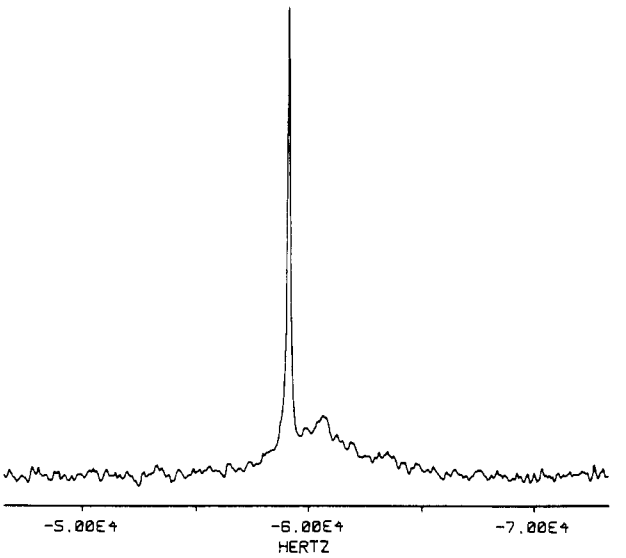


FIGURE 3: ^{51}V NMR spectrum of 0.5 mM vanadate in the presence of 8.1 mg/mL PGM. This spectrum is the same as the one shown in Figure 1 at 0.5 mM vanadate. Here it is replotted with a contracted horizontal scale and an expanded vertical scale to show the broad resonance due to the bound vanadium which is NMR visible.

amount of enzyme-bound vanadate, $[V_b]$. Table I gives the observed concentrations of the various vanadate species obtained as a function of total vanadate in solution.

Information concerning the stoichiometry of binding was obtained by plotting the various $[V_i]$ against the corresponding $[V_b]$. This plot is shown in Figure 2. The figure shows a sigmoidal dependence of $[V_b]$ on $[V_i]$. A double-reciprocal plot of the data used for Figure 2 provides a line with upward curvature. However, if $1/[V_b]$ is plotted against the reciprocal of vanadate concentration squared, a linear plot is obtained. From the vertical intercept of this plot, one obtains a limiting stoichiometry of 3.74 ± 0.05 mol of vanadium atoms per mole of PGM which corresponds to 4 atoms per PGM if the PGM is 94% active. It is not immediately obvious how to rationalize this linear plot with the stoichiometry of 4 bound vanadium atoms per PGM molecule. The simplest means to do so appears to be in terms of noncooperative binding of V_2 to each of the two subunits of PGM as depicted in eq 3. Equations 4 and 5 define the two dissociation constants, K_3 and K_4. In

$$E \underset{K_3}{\overset{V_2}{\rightleftharpoons}} E(V_2) \underset{K_4}{\overset{V_2}{\rightleftharpoons}} E(V_2)_2 \quad (3)$$

$$K_3 = [E][V_2]/[E(V_2)] \quad (4)$$

$$K_4 = [E(V_2)][V_2]/[E(V_2)_2] \quad (5)$$

terms of this model, the equation for the horizontal intercept of the $1/[V_b]$ vs. $1/[V_i]^2$ plot can be combined with eq 1 to provide an equation for the intrinsic dissociation constant of V_2 bound to PGM. From the value measured for this intercept (-80 mM^{-2}), an intrinsic dissociation constant of 4×10^{-6} M was obtained. This corresponds to values for the experimental dissociation constants K_3 and K_4 of 2×10^{-6} M and 8×10^{-6} M, respectively. These values differ from each other because of the statistical factor deriving from the occurrence of two identical binding sites.

Figure 3 shows a spectrum from Figure 1 replotted with an expanded vertical scale and a contracted horizontal scale. In addition to the signal from V_i, a broad resonance is apparent. This resonance is not present in solutions lacking PGM. It derives from a vanadate species which is interacting strongly with the enzyme. Any contributions to the intensity of this

signal which derive from V_2 and V_4 can readily be calculated from the known concentration of V_i using eq 1 and 2 and the values for K_1 and K_2 independently determined. The sum of the vanadium atom concentrations in the species V_i, V_2, and V_4 gives the total free vanadium concentration. The difference between the total free vanadium concentration and the total observed vanadium concentration gives the bound "visible" vanadium concentration which corresponds to the vanadium giving rise to the broad signal in the NMR spectrum. The difference between the total bound and visible bound vanadium concentrations gives the "invisible" bound vanadium concentration. The visible bound and invisible bound vanadium concentrations are tabulated in Table I under $[V_{b,vis}]$ and $[V_{b,invis}]$, respectively. At the lower concentrations of vanadate, the integration of the broad resonance is not reliable since the signal intensity is comparable to those arising from instrumental artifacts. At the higher concentrations of vanadate, more reliable integrations are obtained, and it is apparent that $[V_{b,vis}]$ and $[V_{b,invis}]$ are approximately equal. This division of $[V_b]$ into $[V_{b,vis}]$ and $[V_{b,invis}]$ is much less certain than the measurement of $[V_b]$ since $[V_b]$ is determined by the integration of a "sharp" signal (from V_i) rather than the broad signal deriving from $V_{b,vis}$. The approximately equal division of V_b between visible and invisible forms is significant and is consistent with the existence of two types of vanadium bound in equal amounts.

The bound vanadium was quantitatively released from the PGM by addition of sufficient 2,3-DPG to form a solution 10 mM in 2,3-DPG. Addition of the 2,3-DPG resulted in a negligible change in concentration of the other reagents. Under the conditions of these experiments, inactivated PGM was found not to bind vanadium. These results indicate very strongly that the observed binding of vanadium occurred at the catalytic site of PGM and that the binding neither was nonspecific nor was due to an impurity in the enzyme preparation. The enzyme preparation is highly purified, according to the supplier, with low levels of contaminants. When assayed for mutase activity, it was found to have a specific activity equal to the highest values reported in the literature.

The effect of phosphate, together with vanadate, on the rate of hydrolysis of 2,3-DPG by PGM has also been studied. The results are shown in Figure 4. As seen from this figure, phosphate in the absence of vanadate had only a slight inhibitory effect on the phosphatase reaction. Vanadate at a concentration of 25 μM had a small activating effect; however, added phosphate increased the rate of phosphatase activity 3-fold. In the presence of 50 μM vanadate, but without added phosphate, a considerable activation of the phosphatase activity was observed. This enhanced rate was again almost tripled by the addition of phosphate. This result is in accord with activation of the phosphatase activity by a phosphate/vanadate anhydride and is consistent with the known activation of this activity by inorganic pyrophosphate (Zancan et al., 1965). The K_m value for inorganic pyrophosphate as an activator of the 2,3-DPG phosphatase activity of PGM was determined to be 20 mM at pH 7.0, in agreement with the published value (Zancan et al., 1965).

The effect of vanadate concentration on the phosphatase activity was also studied. The sigmoidal dependence of hydrolysis rate on vanadate concentration was clearly observed, in confirmation of published work (Stankiweicz & Hass, 1986a,b). A double-reciprocal plot of 1/rate vs. $1/[V_i]$ shows upward curvature while a replot of the data as 1/rate vs. $1/[V_i]^2$ shows a linear relationship between the two quantities. The equation defining the horizontal intercept of the latter

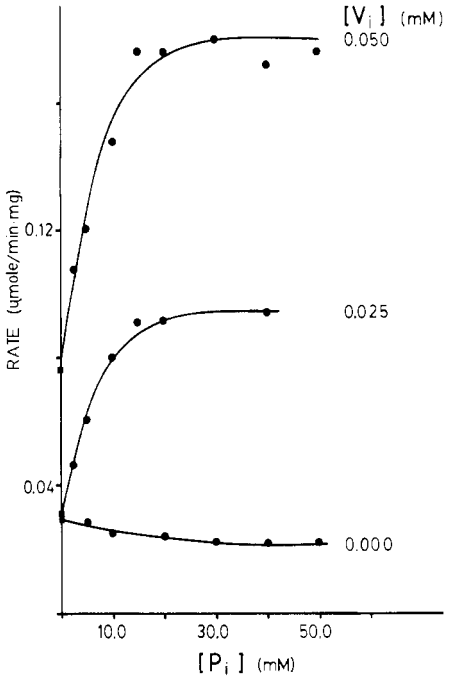


FIGURE 4: Effect of vanadate and phosphate on the 2,3-DPG phosphatase activity of PGM. Rates were measured at pH 7.0 and 37 °C as described under Experimental Procedures with the following concentrations of solutes: 20 mM imidazole, 5.0 mM $MgSO_4$, 5.0 mM KCl, 0.1 mg/mL enolase, 0.1 mg/mL pyruvate kinase, 0.05 mg/mL lactate dehydrogenase, 0.3 mM ADP, 0.1 mM NADH, 0.5 mM 2,3-DPG, 5.8 μg/mL PGM, and the indicated concentrations of phosphate and vanadate.

plot in combination with eq 1 provides a K_m of 3×10^{-5} M for the activation of the phosphatase activity by V_2. In all cases, the rates of the phosphatase reaction increased linearly with PGM concentration.

Discussion

The results of this study, encompassed by Table I and Figure 3, severely restrict the choice of models which can explain the observations. The noncooperative binding of one divanadate ion to the individual binding site of each of the two subunits which form the enzyme explains, in a logical and simple manner, both the limiting stoichiometry of four bound vanadium atoms per enzyme molecule and also the linear relationship between $1/[V_b]$ and $1/[V_i]^2$.

It is perhaps useful to examine this model a little more closely and to consider alternatives to it. In terms of eq 3, the fraction of saturation, Y, of the enzyme with vanadium is given by

$$Y = \frac{\frac{1}{2}[E(V_2)] + [E(V_2)_2]}{[E] + [E(V_2)] + [E(V_2)_2]} \quad (6)$$

Combination of this equation with eq 1, 4, and 5 gives

$$Y = \frac{\frac{1}{2}(K_1/K_3)[V_i]^2 + (K_1^2/K_3K_4)[V_i]^4}{1 + (K_1/K_3)[V_i]^2 + (K_1^2/K_3K_4)[V_i]^4} \quad (7)$$

Y is equal to $[V_b]$ divided by the total enzyme concentration (e_t) times the number of vanadium atom binding sites (n = 4). Therefore, the derivative of $1/Y$ with respect to $1/[V_i]^2$ is simply ne_t times the derivative of $1/[V_b]$ with respect to $1/[V_i]^2$. For the case in which there is no cooperativity in the binding of V_2 to the two binding sites of PGM (i.e., $K_3 = {}^1/_4K_4$), the intrinsic dissociation constant of V_2 is equal to $K_4/2$, and the derivative of $1/Y$ with respect to $1/[V_i]^2$ is equal to $K_4/2K_1$. Thus, the noncooperative binding of one divanadate

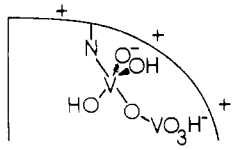


FIGURE 5: Representation of divanadate bound at the catalytic site of PGM. The three positive charges indicated on the representation of the catalytic site of the enzyme are used to indicate that the site is able to interact favorably with the three negatively charged groups of 2,3-DPG.

species to each of the two subunits of PGM is consistent with the observed linear plot of $1/[V_b]$ vs. $1/[V_i]^2$.

The expression for the fraction of saturation Y' for any scheme involving the binding of four separate V_i species to PGM will be of the form shown in eq 8 where the coefficients C_n, for the case of sequential binding of four V_i species, are as defined by eq 9 where the K_n' values are the dissociation

$$Y' = \frac{C_1[V_i] + 2C_2[V_i]^2 + 3C_3[V_i]^3 + 4C_4[V_i]^4}{4(1 + C_1[V_i] + C_2[V_i]^2 + C_3[V_i]^3 + C_4[V_i]^4)} \quad (8)$$

$$C_n = \prod_{n=1}^{n} K_n'^{-1} \quad (9)$$

constants for the individual reactions. Differentiation of $1/Y'$ with respect to $1/[V_i]^2$ gives

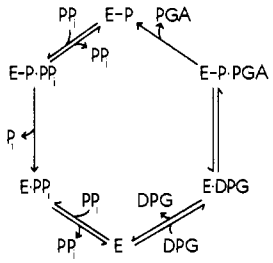
$$\frac{d(1/Y')}{d(1/[V_i]^2)} = 2[V_i]^3\{C_1 + 4C_2[V_i] + (9C_3 + C_1C_2)[V_i]^2 + (16C_4 + 4C_1C_3)[V_i]^3 + (9C_1C_4 + C_2C_3)[V_i]^4 + 4C_2C_4[V_i]^5 + C_3C_4[V_i]^6\}/\{C_1[V_i] + 2C_2[V_i]^2 + 3C_3[V_i]^3 + 4C_4[V_i]^4\}^2 \quad (10)$$

This generalized differential reduces to that for noncooperative binding of two V_2 ions, as shown in eq 3, when C_1 and C_3 are zero and $K_1'K_2' = K_3'K_4'/4$. The equation is then a constant, equal to $K_3'K_4'/2$. No other coefficients, which simultaneously satisfy the requirement of a total uptake of four vanadium atoms by the enzyme, can be assigned so that this equation is independent of vanadate concentration. If C_1 and C_3 are small compared to C_2 and C_4, then, within the error of the measurements, eq 10 may appear independent of vanadate concentration. It is likely that some binding of V_i to PGM does occur. However, this binding must be weak compared to that of V_2 in accord with relatively small C_1 and C_3.

The proposed structure of V_2 bound to one catalytic site of PGM is shown in Figure 5. The schematic representation of the catalytic site shows three positive charges which are intended only to indicate that the site can interact favorably with three different negatively charged groups, such as the carboxylate and two phosphates of 2,3-DPG. One of the two vanadate moieties in the bound V_2 represented in Figure 5 is tetrahedral; the other is trigonal bypyramidal and interacting with a nucleophile present in the catalytic site. This nucleophile is perhaps the nucleophile which is normally phosphorylated when 2,3-DPG transfers its 2-phosphate group to the dephosphorylated enzyme. The structure shown in Figure 5 is speculative, but it is supported by two pieces of evidence. One is the observation shown in Table I that the bound vanadium is divided approximately equally between NMR-visible and NMR-invisible forms. The bound vanadium tumbles at the same rate as the enzyme, which is considerably slower than the tumbling rate of free vanadium. Since the ^{51}V nucleus has a rather large quadrupole moment, slower tumbling is expected to cause broadening of the ^{51}V resonance. However, because the tetrahedral structure is more symmetrical than the trigonal bipyramidal structure, the broadening is expected to be greater for the resonance due to the trigonal bipyramidal vanadium. It is not surprising that this latter resonance is sufficiently broadened that it is lost in the base line. Similar behavior has been observed for a trigonal bipyramidal vanadate species bound to ribonuclease (Borah et al., 1985).

The other evidence in favor of the structure is the very tight binding of V_2 to PGM. The intrinsic dissociation constant is 4 μM. This is within about a factor of 10 of the K_m for 2,3-DPG, the specific cofactor for PGM. Another interesting comparison is that between V_2 and pyrophosphate as activators of the 2,3-DPG phosphatase activity of PGM. Pyrophosphate and V_2 cause similar maximum phosphatase rates, but the K_m for V_2 is 3×10^{-5} M while the K_m for pyrophosphate is 2×10^{-2} M. These K_m values possibly do not correspond to dissociation constants, but they probably do give a measure of the relative affinities of V_2 and pyrophosphate for PGM. The nearly 10^3-fold lower K_m for V_2 is consistent with the structure shown in Figure 5 because the catalytic site is designed to stabilize the transition state for phosphoryl transfer, which is thought to resemble the trigonal bipyramidal vanadate structure shown (Knowles, 1980). Vanadate adopts this structure much more readily than does phosphate, and this is the probable reason why vanadate is a strong inhibitor of many enzymes which catalyze phosphoryl transfer reactions (Chasteen, 1983).

In the mechanism for activation of the 2,3-DPG phosphatase activity by V_2 and pyrophosphate shown in eq 11, 2,3-DPG



(11)

phosphorylates the enzyme, and the monophosphoglycerate dissociates. In the phosphoglycerate mutase reaction, the next step would be for either 2-PGA or 3-PGA to bind to the phosphorylated enzyme and become phosphorylated on the available hydroxyl group. Pyrophosphate or V_2 might mimic 2-PGA by occupying the anion-stabilizing positions occupied by phosphate and carboxylate moieties when 2-PGA is bound. This presumably activates the enzyme to transfer its phosphate group to the nearest hydroxyl group, which in this case is furnished by a water molecule. The product is the dephosphorylated enzyme, which is then phosphorylated by 2,3-DPG. A similar mechanism can account for the activation of the 2,3-DPG phosphatase activity of PGM by 2-phosphoglycolate (Rose & Dube, 1978).

It is not surprising that the K_m for V_2 as a phosphatase activator is larger than the dissociation constant for V_2 determined by the ^{51}V NMR binding studies. Aside from the fact that K_m values determined from kinetic experiments do not necessarily correspond to dissociation constants, V_2 presumably activates the phosphatase activity by binding to the phosphorylated enzyme, while dephosphorylated PGM was used in the binding study. Binding of V_2 to dephosphorylated PGM should be favored by the possibility of two different modes of binding, and by the absence of the negatively charged phosphate group which is present on phosphorylated PGM.

Although activation of the phosphatase activity by V_2 and pyrophosphate is interesting and useful for mechanistic studies, the activation is probably negligible under normal physiological conditions because of the very low concentrations of these

species. Vanadate, however, has a potentially important role because of the synergistic effect of phosphate shown in Figure 4, and this could be of physiological significance. This provides a possible explanation for the decrease in 2,3-DPG concentration in erythrocytes incubated with vanadate (Ninfali et al., 1983). It is reasonable to interpret this effect in terms of activation of the phosphatase activity by a mixed phosphate/vanadate anhydride (P–V) acting by the mechanism shown in eq 11 for pyrophosphate. A detailed study of the spontaneous formation of P–V from P_i and V_i in aqueous solution has been published elsewhere (Gresser et al., 1986). From the equilibrium constant of 24 M^{-1} for formation of P–V at pH 7.0 (Gresser et al., 1986), and the observation from Figure 4 that half-maximal activation of the phosphatase activity at 25 μM V_i occurs at about 10 mM P_i, it can be calculated that if P–V is the activating species it causes half-maximal activation at a concentration of about 6 μM. It is thus obvious that both the apparent K_m and the apparent V_m for P–V as a phosphatase activator are considerably lower than the apparent K_m and the apparent V_m values for V_2 as a phosphatase activator. This could be accounted for by inhibition due to P_i binding to phosphorylated PGM, which is the likely explanation for the inhibitory effect of P_i on the glycolate 2-phosphate activated phosphatase (Rose & Dube, 1978).

Registry No. 2,3-Diphosphoglycerate phosphatase, 9033-04-9; phosphoglycerate mutase, 9032-62-6; vanadate, 14333-18-7; phosphate, 14265-44-2.

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