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^1H and ^{51}V NMR Studies of the Interaction of Vanadate and 2-Vanadio-3-phosphoglycerate with Phosphoglycerate Mutase†

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ABSTRACT: The formation of complexes of vanadate with 2-phosphoglycerate and 3-phosphoglycerate have been studied using ^{51}V nuclear magnetic resonance spectroscopy. Signals attributed to two 2,3-diphosphoglycerate analogues, 2-vanadio-3-phosphoglycerate and 2-phospho-3-vanadioglycerate, were detected but were not fully resolved from signals of inorganic vanadate and the anhydride formed between vanadate and the phosphate ester moieties of the individual phosphoglycerates. Equilibrium constants for formation of the two 2,3-bisphosphate analogues were estimated as 2.5 M^{-1} for 2-vanadio-3-phosphoglycerate and 0.2 M^{-1} for 2-phospho-3-vanadioglycerate. The results of the binding study are fully consistent with noncooperativity in the binding of vanadiophosphoglycerate to the two active sites of phosphoglycerate mutase (PGM). 2-Vanadio-3-phosphoglycerate was found to bind to the dephospho form of phosphoglycerate mutase with a dissociation constant of about 1×10^{-11} M at pH 7 and 7×10^{-11} M at pH 8. Three signals attributed to histidine residues were observed in the ^1H NMR spectrum of phosphoglycerate mutase. Two of these signals and also an additional signal, tentatively attributed to a tryptophan, underwent a chemical shift change when the vanadiophosphoglycerate complex was bound to the enzyme. The results obtained here are in accord with these vanadate–phosphoglycerate complexes being much more potent inhibitors of phosphoglycerate mutase than either monomeric or dimeric vanadate. The dissociation constant of 10^{-11} M for 2-vanadio-3-phosphoglycerate is about 4 orders of magnitude smaller than the K_m for PGM, a result in accordance with the vanadiophosphoglycerates being transition state analogues for the phosphorylation of PGM by 2,3-diphosphoglycerate. These results strongly support the view that phosphoryl transfer in this enzyme involves a pentacoordinate phosphate intermediate and suggests that the two active sites operate independently of each other.

Phosphoglycerate mutase (PGM) (EC 5.4.2.1) catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. The mammalian muscle enzyme is a dimer of about 55 000 molecular weight that requires 2,3-diphosphoglycerate for full activity. It also catalyzes, at much lower rates than the above interconversion, the hydrolysis of 2,3-diphosphoglycerate to inorganic phosphate and phosphoglycerate as well as the conversion of 1,3-diphosphoglycerate to 2,3-diphosphoglycerate. Information concerning this and related enzymes, which are involved in phosphoglycerate and diphosphoglycerate metabolism, has been reviewed recently (Fothergill-Gilmore & Watson, 1989).

There has been a number of reports on the inhibition of PGM by inorganic vanadate (V_i) (Carreras et al., 1980, 1982; Climent et al., 1981; Ninfali et al., 1983). It has been proposed that V_i inhibits PGM by destablizing the phosphoenzyme, which is the catalytically active form (Carreras et al., 1982; Ninfali et al., 1983). Vanadate does activate the 2,3-diphosphoglycerate phosphatase activity of PGM. Kinetic studies of this activity have been rationalized in terms of divanadate (V_2) binding at the catalytic site of the phosphoenzyme as a substrate analogue and activating the hydrolysis of the phosphoenzyme to inorganic phosphate and dephosphoenzyme (Stankiewicz et al., 1987). The dephosphoenzyme is then rephosphorylated by 2,3-diphosphoglycerate to complete the phosphatase cycle. Additional support for this mechanism was provided by the observation that V_2 binds noncooperatively to the two subunits of PGM with an intrinsic dissociation constant of 4×10^{-6} M (Stankiewicz et al., 1987). No binding of the vanadate monomer (V_1) was detected. It seems probable that low-affinity binding of V_1 does occur and could have been detected if the much higher affinity binding of V_2 did not compete so effectively.

More recently, it was reported that 2-phosphoglycerate or 3-phosphoglycerate potentiates inhibition of PGM by V_i and that development of the inhibition was time-dependent and required the presence of both V_i and phosphoglycerate (Stankiewicz & Hass, 1986a,b). This behavior is consistent with the inhibitor being a complex of phosphoglycerate and vanadate binding at the catalytic site of the dephosphoenzyme as a transition-state analogue for the transfer of a phosphoryl group between the enzyme and phosphoglycerate. An anal-

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ogous mechanism has been advanced, on the basis of detailed kinetic studies, for the inhibition of phosphoglucomutase by a complex of vanadate and glucose 1-phosphate, for which a K_i value of 2×10^{-12} M was determined (Percival et al., 1990). It thus appeared that phosphoglycerate mutase might be one of the group of phosphoryl transfer enzymes which is inhibited much more strongly by specific vanadate complexes than by V_i itself (Gresser & Tracey, 1990). These include ribonuclease A, phosphoglucomutase, alkaline phosphatase, aryl sulfatase, myosin, and dynein.

Since ^{51}V nuclear magnetic resonance spectroscopy had proved to be a useful technique for studying binding of V_2 to PGM (Stankiewicz et al., 1987), the present investigation was undertaken to study the formation of vanadate–phosphoglycerate (V–PG) complexes and their binding to PGM. The vanadium-51 nucleus has a spin of $^7/_2$ and consequently is a quadrupolar nucleus. The ^{51}V quadrupolar moment is moderate in magnitude so that quadrupole broadening generally is not excessive and NMR spectra often are well resolved. The situation changes, however, when the vanadium nucleus is attached to a slow tumbling molecule such as an enzyme. The long correlation time leads to efficient quadrupolar relaxation and, except in special cases, to broad NMR signals (Westlund & Wennerstrom, 1982; Rehder et al., 1989; Butler & Eckert, 1989). The loss of signal intensity to the broad, often unobservable, signals can readily be quantitated and used to characterize enzyme systems as has been done for PGM (Stankiewicz et al., 1987). In the present paper, the process has been reversed, and the release of enzyme-bound vanadium from phosphoglycerate mutase by the 2-vanadio-3-phosphoglycerate has been quantitated. Proton NMR spectroscopy was also used to test for possible interactions between PGM ligands and the histidine residues which are known to be present in the catalytic site of PGM.

Materials and Methods

All reagents were reagent grade chemicals and were used without further purification. Rabbit muscle phosphoglycerate mutase, 2- and 3-phosphoglyceric acid pentacyclohexylammonium salt, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES buffer) were purchased from Boehringer Mannheim. Glycolic acid, NaOD (30% in D_2O), and DCl (20% in D_2O) were purchased from Sigma Chemical Co., while vanadium(V) oxide and ethylenediamine dihydrochloride were obtained from Aldrich Chemical Co.

Preparation of Vanadate 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 until it became colorless, generally overnight. It was then diluted to 0.1 M vanadium atom concentration with distilled H_2O.

Preparation of Phosphoglycerate Mutase for ^1H NMR Studies. A suspension of PGM in aqueous ammonium sulfate was centrifuged, the supernatant was discarded, and the pellet was dissolved in 0.5 mL of buffer containing 20 mM ethylenediamine dihydrochloride (EDA) and 1.0 mM glycolate 2-phosphate. Several changes of dialysate over a period of 3 days served to replace the exchangeable protons by deuterium. Protein concentration was determined by either absorbance index (1.0 mg/mL at 280 nm) of 1.48/cm or by the method of Lowry. The enzymes were assayed before and after the NMR experiments. The enzyme concentrations for all samples were close to 0.20 mM.

Preparation of PGM for ^{51}V NMR Studies. A suspension of PGM in aqueous ammonium sulfate was centrifuged, the supernatant was discarded, and the pellet was dissolved in sufficient buffer solution containing 20 mM HEPES, 6.0 mM KCl, and 1.0 mM glycolate 2-phosphate to give a concentration of PGM of about 10 mg/mL. The resulting solution was dialyzed for 4 h at 4 °C against 100 mL of the same buffer and then subsequently dialyzed overnight at 4 °C against 100 mL of a similar buffer containing no glycolate 2-phosphate. Aliquots of 2 mL of this solution were used in the NMR studies.

pH Titration. pH measurements of PGM solutions were made at room temperature using an Accumet 910 pH meter from Fisher Scientific with an extra long calomel microcombination electrode. The meter was calibrated with two freshly opened pH standards bracketing the pH reading. The pH values given are the actual meter readings and have not been corrected for the deuterium isotope effect at the glass electrode. pH adjustments were made by adding 0.05–0.1 N NaOD or 0.05–0.1 N DCl delivered from a micrometer syringe. The pH was measured both immediately before and immediately after obtaining the NMR spectra. If the difference in pH reading of a sample before and after recording a spectrum was greater than 0.05 pH unit, the results were not accepted. In general, the agreement was better than 0.04 pH unit.

Ligand Binding Studies. Solutions were made by introducing the appropriate amount of a freshly made ligand stock solution in buffer (D_2O) into a 10 mg/mL PGM solution by a micrometer syringe. The pH of these solutions were adjusted carefully to the desired values, and the mixtures were stirred for 45 min before the NMR spectra were obtained.

Binding of Inorganic Vanadate (V_i) to PGM. With reference to methods as described by Stankiewicz et al. (1987), solutions were prepared by successive additions of small volumes of 0.1 M NaH_2VO_4 to 2.0 mL of a solution containing 0.12–0.2 mM PGM.

Binding of Vanadate Ester to PGM. Solutions were prepared by successive additions of small volumes of substrate or substrate analogues to 2.0 mL of a solution containing 0.17–0.22 mM PGM, 1 mM vanadate in 20 mM HEPES buffer, and 6 mM KCl.

The pH adjustments were made, as required, by adding 0.05–0.1 N NaOH or 0.05–0.1 N HCl. The pH was measured both immediately before and immediately after obtaining the NMR spectra. If the difference in pH readings of a sample before and after recording a spectrum was greater than 0.1 pH units, the results were not accepted. In general, the agreement was better than 0.05 pH units.

Spectroscopy. ^1H NMR spectra were obtained with a Bruker WM-400 NMR spectrometer. Sweep widths of 4000 Hz, 60° pulse angles, 256 scans, and 32K data sets were used for all spectra. The acquisition time was 4.1 s, and no further recycle delay was used. The strong signal from residual HDO was removed by using a gated decoupling technique.

Samples were contained in Wilmad 5-mm precision bore nuclear magnetic resonance tubes with coaxial inserts containing the external standard DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) in D_2O. All chemical shifts are given in parts per million from this external DSS reference. All spectra were obtained at ambient room temperature, 22 ± 1 °C.

^{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, 0.05-s acquisition times, 2K data sets, and 60° pulse angles were used for all spectra. Doubling the acquisition times was found to have no observable effect on signal intensities. A total of 20 000 transients were

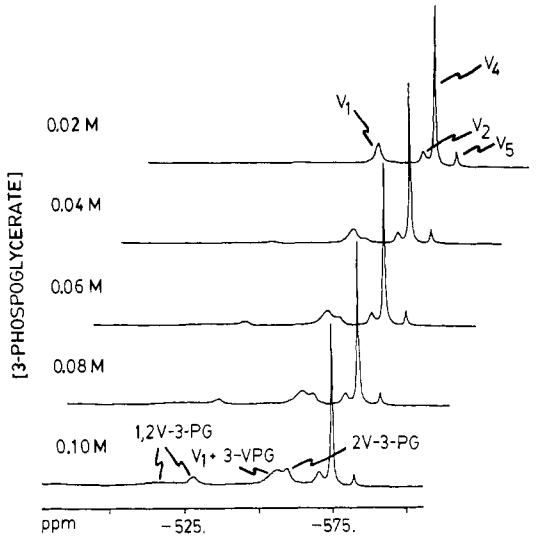


FIGURE 1: ^{51}V NMR spectra showing the progressive formation of 3-phosphoglycerate complexes of vanadate as the concentration of 3-phosphoglycerate is increased. The conditions of the experiments were 2.0 mM total vanadate, 20 mM Tris buffer, pH 7.5, μ = 1.0 M KCl, and the indicated concentrations of 3-phosphoglycerate. V_1 represents monovanadate and V_2, V_4, and V_5 its dimer, tetramer, and pentamer, respectively. All spectra are scaled to a constant amplitude for V_4.

acquired for each spectrum. A line-broadening factor of 40 Hz was applied to all spectra before zero-filling to 8K data points and transforming to the frequency domain. The Fourier transforms for any particular series of compounds were done in the absolute intensity mode so that signal intensities were directly comparable between spectra. Chemical shifts reported are relative to the external reference standard $VOCl_3$, which has been assigned to 0 ppm. All spectra were obtained at ambient temperature. Baseline roll was removed from all spectra before signal intensities were measured. The signal intensities were measured with the instrument manufacturer's software. No effort was made to obtain more accurate integrals as comparison of integrals obtained for reference samples covering a wide range of concentrations showed the integration routine performed well.

Results

Phosphoglycerate plus Vanadate. The 2- or 3-phosphoglycerate offers several possibilities for condensation with vanadate, including anhydride formation at the phosphate group, ester formation with the hydroxy group, and, in the case of the 3-phosphate, 1,2-cyclic vanadate products formed between the carboxylate and hydroxyl groups. The formation of a variety of products is demonstrated in Figure 1. The major products give rise to signals at −518 ppm (a broad signal almost lost in the baseline), −528.9 ppm, −560.2 ppm and a composite signal ranging from −555 to −559 ppm.

Studies in which either total vanadate or total ligand concentrations were varied showed that both the −518 and −528 ppm products were mononuclear complexes containing only one ligand. They were formed from their precursors with formation constants of 4.3 ± 0.9 M^{-1} and 4.5 ± 0.5 M^{-1}, respectively. It is not clear what product the −528 ppm signal corresponds to as there are a number of possibilities because of the presence of a phosphate group. One possibility is that the product is octahedral with PGA acting as a bi- or tridentate ligand which utilizes the phosphate group for complex formation (Gresser et al., 1986).

Vanadate in the presence of lactate or glycerate gives rise to an NMR signal at −517 ppm. This has been assigned to a pentacoordinate vanadate complex formed between the carboxyl and α-hydroxyl group of the ligand (Tracey et al., 1987; Gil, 1989). The −518 ppm signal observed here is similarly assigned. An additional broad signal, not readily observable in Figure 1, is centered at about −540 ppm. The product giving rise to this signal presumably is similar to the octahedral products observed in the presence of lactate and glycerate.

The resonances of major interest for this study are the two signals centered near −558 ppm. One of these signals ranges from −555 to −557 ppm and shifts to high field with incremental amounts of 3-phosphoglycerate. It is assigned to a composite signal deriving from vanadate, V_1, and its anhydride 3-VPG ($HO_3VOPO_2OCH_2CHOHCO_2$). Phosphate/vanadate anhydrides tend to be kinetically labile at room temperature, resulting in a time-averaged vanadium NMR signal which alters its position as the concentration of the phosphate ligand is varied (Gresser et al., 1986). The remaining ^{51}V signal at −558.9 ppm is assigned to 2-vanado-3-phosphoglcyerate. This is a vanadate analogue of 2,3-diphosphoglycerate, the cofactor for phosphoglycerate mutase, and it is thought to be this compound that inhibits the function of phosphoglycerate mutase.

The formation of the major vanadate oligomers, V_2 and V_4, the phosphovanadate anhydride, 3-VPG, and the diester, 2-V-3-PG, can be represented as in eqs 1–4. Combination of eqs 1, 3, and 4, followed by rearrangement, gives eq 5. In this equation the measured concentration of V_4 is utilized to specify $[V_1]$ using the formation constant, K_0.

$$4V_1 \overset{K_0}{\rightleftharpoons} V_4 \qquad [V_1]^4K_0 = [V_4] \tag{1}$$

$$2V_1 \overset{K_1}{\rightleftharpoons} V_2 \qquad [V_1]^2K_1 = [V_2] \tag{2}$$

$$V_1 + \text{3-PG} \overset{K_2}{\rightleftharpoons} \text{3-VPG} \qquad [V_1][\text{3-PG}]K_2 = [\text{3-VPG}] \tag{3}$$

$$V_1 + \text{3-PG} \overset{K_3}{\rightleftharpoons} \text{2-V-3-PG} \qquad [V_1][\text{3-PG}]K_3 = [\text{2-V-3-PG}] \tag{4}$$

$$\frac{[V_1] + [\text{3-VPG}] + [\text{2-V-3-PG}]}{[V_4]^{1/4}} = K_0^{-1/4} + K_0^{-1/4}(K_2 + K_3)[\text{3-PG}] \tag{5}$$

If the stoichiometry of the products, as specified in eqs 3 and 4, is correct, then a plot of the ratio on the left of eq 5 (obtained from the ^{51}V NMR integrals of the signals centered near −558 and the sharp V_4 signal at −576 ppm) versus the known concentration of 3-phosphoglycerate in solution will give a straight line of intercept $K_0^{-1/4}$ and slope $K_0^{-1/4}(K_2 + K_3)$. In agreement with the proposed structures, a good linear relationship was obtained as a function of added 3-PGA. From the slope and intercept, the values $K_0 = (7.6 \pm 0.6) \times 10^9$ M^{-3} and $K_2 + K_3 = 5.8 \pm 0.2$ M^{-1} were determined. This value of K_0 is in good agreement with other measurements for pH 7.5 and 1.0 M KCl (Tracey et al., 1988a). The constants K_2 and K_3 can be separated by curve resolving the −558 ppm signals into the two components and applying eqs 3 and 4 along with the conservation equation

$$[V_1] + [\text{3-VPG}] = [-557] \tag{6}$$

where −557 is used to identify the variable chemical shift component of the −558 ppm signals. From these considerations the values obtained were $K_2 = 3.3 \pm 0.4$ M^{-1} and $K_3 = 2.5 \pm 0.4$ M^{-1}. The value determined for the formation of the anhydride ester is about a factor of 3 smaller than that for a somewhat similar product with adenosine monophosphate

also determined at pH 7.5 (Tracey et al., 1988a). The formation constant for the vanadate ester, 2.5 M^{-1}, is much larger than that generally observed for ester formation, about 0.2 M^{-1} (Tracey et al., 1988b). In this case, the adjacent groups apparently affect product formation.

For the case of 2-phosphoglycerate, it did not prove possible to separate K_2 and K_3. The overall value observed was $K_2 + K_3 = 1.4 \pm 0.2$ M^{-1}. Under similar conditions, 2-phosphoglycolate gave a value of 1.6 ± 0.2 M^{-1} for K_2 while 2-phosphoglycerol gave the sum $K_2 + K_3 = 2.6 \pm 0.3$ M^{-1}. These latter results indicate that the value of 1.4 M^{-1} measured for 2-phosphoglycerate represents, almost completely, anhydride formation and that K_3 is relatively small, possibly similar to that for other alcohols, ~0.2 M^{-1} (Tracey et al., 1988b).

Phosphoglycerate Mutase plus Vanadate. It has previously been demonstrated that divanadate binds to the two active sites for phosphoglycerate mutase in a noncooperative manner (Stankiewicz et al., 1987). This binding is represented in eq 7 where E is the dephosphoenzyme, PGM. Since binding is

$$E \underset{K_4}{\overset{V_2}{\rightleftharpoons}} EV_2 \underset{K_5}{\overset{V_2}{\rightleftharpoons}} E(V_2)_2 \qquad (7)$$

noncooperative, $K_5 = 4K_4$.

Studies of V_2 binding were carried out at pH 6.0, 7.0, and 8.0 in order to obtain information concerning hydrogen ion requirements. At pH 7 and 8, noncooperativity of binding was observed, as reported previously. At pH 6.0, binding of vanadate occurred as for the other pH values. It was observed that the binding curve increased rapidly for the initial parts of the curve but it did not level off at a stoichiometry of four bound vanadates but rather it continued to increase with incremental additions of vanadate. The excess binding over four vanadates was taken to indicate that nonspecific binding was important at the lower pH. Recent studies have shown that vanadate readily forms anhydrides with carboxylic acids (Tracey et al., 1990). Significant proportions of many carboxylate groups are protonated at pH 6, and these may be one source of the nonspecific binding.

In support of this, carboxylate-containing compounds such as lactate and other α-hydroxycarboxylates (Tracey et al., 1987; Caldeira et al., 1987) and the amino acid *N*-[tris(hydroxymethyl)methyl]glycine (Crans et al., 1991) undergo increasingly favorable reactions with vanadate as the pH is lowered to moderate levels, pH ~4.5. At lower pH, the products tend to be destabilized in favor of other vanadate products such as decavanadate or cationic VO_2^+.

Studies of nonspecific binding of vanadate to bovine serum albumin (Arora et al., 1983; Crans et al., 1989; Chasteen et al., 1986) showed that vanadate binds with a binding constant in the order of 10^3 at near neutral pH. This constant was found to increase steadily with a moderate decrease in pH (Arora et al., 1983), in agreement with the above proposal.

The values determined at pH 7.0 and 8.0 for K_4 of eq 7 were $(4.0 \pm 2.0) \times 10^{-6}$ M and $(3.0 \pm 2.0) \times 10^{-7}$ M, respectively. The increased stability of the PGM/V_2 complexes with increase in pH, reflected by these constants, indicates that the trianionic species, $O_3VOVO_3H^{3-}$, binds more tightly than the dianionic form. The protonation state of the active site histidines will not vary significantly from pH 7 to 8 because these histidines all have pK_a values below 7.0. The pK_a of $V_2O_7H_2^{2-}$ is about 7.7, dependent to an extent on the experimental conditions (Heath & Howarth, 1981; Pettersson et al., 1983, 1985), and consequently it will undergo significant deprotonation between pH 7 and 8.

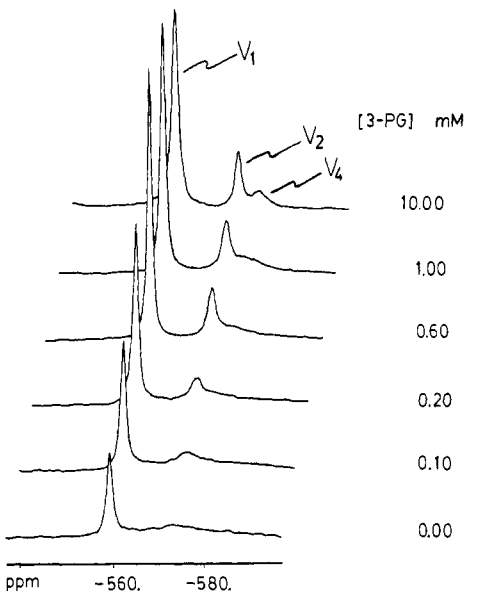
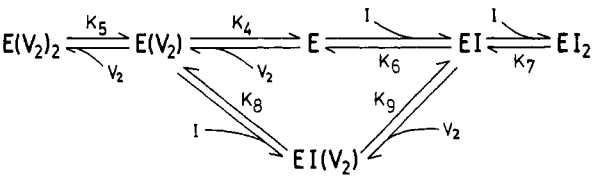


FIGURE 2: ^{51}V NMR spectra showing the release of phosphoglycerate-bound vanadate as a function of increasing proportions of 3-phosphoglycerate. The conditions of the experiments were 0.21 mM phosphoglycerate mutase, 1 mM vanadate, 20 mM HEPES buffer, pH 7.0, 6.0 mM KCl, and the indicated concentrations of 3-phosphoglycerate. V_2 and V_4 represents the dimer and tetramer, respectively, of monovanadate, V_1. All spectra are scaled identically.

Scheme I



Phosphoglycerate Mutase plus Vanadate and 3-Phosphoglycerate. 2,3-Diphosphoglycerate is the specific cofactor for phosphoglycerate mutase. The interaction of 2,3-DPG with PGM produces as products the phosphorylated enzyme and 2- or 3-PG. The K_m for this reaction is about 3×10^{-7} M (Stankiewicz & Hass, 1986; Fothergill-Gilmore & Watson, 1989). In view of the tight binding observed for V_2 and the similarity in structure of 2-V-3-PG to 2,3-DPG, it seems reasonable to expect that 2-V-3-DPG will function well as a 2,3-DPG analogue, as of course should 3-V-2-PG.

This assumption may be tested by adding incremental proportions of 3-PG to a solution of PGM and vanadate [at concentrations that provide $E(V_2)_2$] and then testing for the release of vanadate from the enzyme as the 3-PG is added. Figure 2 shows the effect of the addition of 3-PG on the ^{51}V NMR spectrum of vanadate in such a solution at pH 7.0, where initially the major proportion of the vanadate is complexed by PGM. Vanadate very clearly is released from the enzyme. The results are quantitatively presented in Table I from which it is evident that there is release of half of the vanadate from the enzyme.

The results of the titrations are readily interpreted by the rather simple processes encompassed by Scheme I. In this scheme, I refers to the inhibitor, 2-vanadio-3-phosphoglycerate, and E the free enzyme.

In Scheme I, K_4 and K_5 can be determined from studies of the binding of vanadate. The observation of noncooperativity in this binding (Stankiewicz et al., 1987; and this study) means that $K_5 = 4K_4$. If this noncooperativity extends to the binding of the transition state analogue 2-V-3-PG, then on a statistical

Table I: Distribution of Vanadate between Free and Bound Forms When in the Presence of Dephospho(Phosphoglycerate Mutase) and 3-Phosphoglycerate[a,b]

$[3\text{-PG}]_T$	$[V_f]_T$	$[V_B]$	$[V_B]/[E_T]$
pH 7.0			
0.00	0.19	0.81	3.8
0.05	0.27	0.73	3.4
0.10	0.33	0.67	3.1
0.15	0.41	0.59	2.8
0.20	0.49	0.51	2.4
0.40	0.57	0.43	2.0
0.60	0.57	0.43	2.0
1.00	0.57	0.43	2.0
2.00	0.62	0.38	1.8
10.00	0.64	0.36	1.7
pH 8.0			
0.00	0.22	0.78	4.0
0.10	0.31	0.69	3.5
0.20	0.46	0.54	2.8
0.50	0.57	0.43	2.2
1.00	0.61	0.39	2.0
4.00	0.64	0.36	1.9
10.00	0.58	0.42	2.2

[a] All concentrations are millimolar and were measured under conditions of 1 mM total vanadate, 20 mM HEPES buffer, 6 mM KCl, and the indicated pH values and the corresponding concentrations of 3-PGA. [b] Abbreviations: $[3\text{-PG}]_T$, total concentration of added 3-phosphoglyceric acid; $[V_f]_T$, measured total concentration of free vanadate; $[V_B]$, concentration of bound vanadate [given by 1.0 mM (total vanadate) − $[V_f]$; $[E_T]$, total concentration of phosphoglycerate mutase.

basis $K_5 = 4K_4 = 2K_9$ and $K_7 = 4K_6 = 2K_8$.

Analysis of the titration results according to Scheme I requires utilization of the conservation equations for total vanadate, total bound vanadate, total enzyme, and total phosphoglycerate. These in combination with eqs 1–4 and the equilibrium equations corresponding to Scheme I lead to a rather complicated equation from which the ratio $[V_B]/[E_T]$ can be obtained as a function of the 3-phosphoglycerate added (see the Appendix for a complete derivation). $[V_B]$ of the above ratio corresponds to the total bound vanadium atom concentration, while $[E_T]$ is the total concentration of PGM in the solutions.

With the known dissociation constant of V_2 from $E(V_2)$ and the formation constant of 2-V-3-PG from V_1 and 3-PGA (eq 4), binding curves can be calculated according to Scheme I by assuming various values for the dissociation constant K_6 corresponding to the dissociation of 2-V-3-PG from PGM. The results of the calculations are shown in Figure 3 for the pH 7 results. It is evident from this figure that K_6 could well be smaller than 1×10^{-11} M, but, unfortunately, a lower limit cannot be placed on it. The value of 1×10^{-11} M, however, probably is quite close to the actual value, since at pH 8 the value of K_6 of 7×10^{-11} M fits the experimental results very well while a value of 1×10^{-11} M is too small, as is evident in Figure 4.

It is interesting that the $E(V_2)$ and $E(V_2)_2$ complexes are favoured by higher pH while the E(2-V-3-PG) complex is not. Unlike V_2, the 2-V-3-PG is trianionic at pH 7 and probably tending toward its tetranionic form at pH 8 since vanadate esters tend to have pK_a values just above 8 (Tracey et al., 1988b). A general observation which is relevant to the decrease in binding affinity with increase of pH is that when 2,3-DPG, 2-V-3-PG, or 3-V-2-PG (buffered at pH 7.7 with 20 mM ethylenediamine) was added to PGM (similarly buffered at pH 7.7), the pH increased to a maximum of 8.5, dependent on the amount of substrate added. ^1H NMR studies do not indicate that active site histidines are protonated so the proton uptake may be involved with conformational changes in the enzyme when binding occurs. Crystalline yeast PGM shatters when exposed to 2,3-DPG indicating a conformational change (Winn et al., 1977). Similarly, a conformational change might be expected for rabbit muscle PGM.

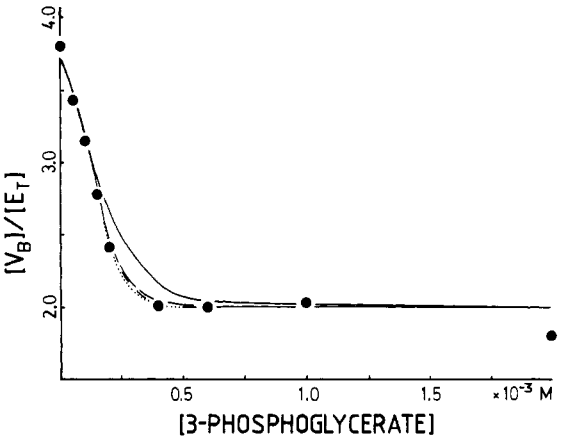


FIGURE 3: Experimental results and calculated curves showing the dependence of $[V_B]/[E_T]$ on the 3-phosphoglycerate concentration at pH 7.0. The calculations were based on Scheme I, and the experimental conditions were as for Figure 2. The solid circles represent the experimental points while the calculated lines are for different assumed values of K_6: (—) $K_6 = 1 \times 10^{-10}$ M; (– – –) $K_6 = 1 \times 10^{-11}$ M; (- - -) $K_6 = 1 \times 10^{-12}$ M; (···) $K_6 = 1 \times 10^{-13}$ M.

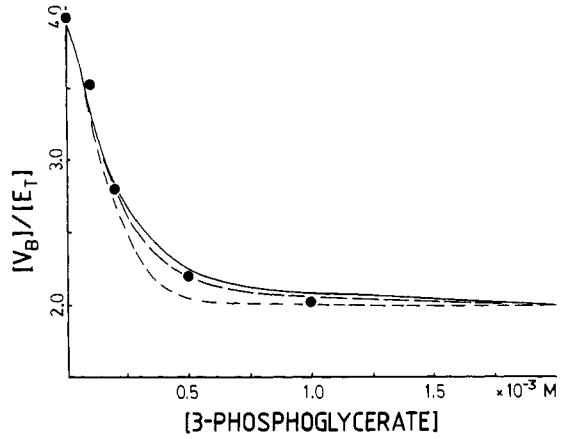


FIGURE 4: Experimental results and calculated curves showing the dependence of $[V_B]/[E_T]$ on the 3-phosphoglycerate concentration at pH 8.0. The calculations were based on Scheme I, and the experimental conditions, except for the pH, were as for Figure 2. The solid circles represent the experimental data while the calculated lines are for different assumed values of K_6: (—) $K_6 = 1 \times 10^{-10}$ M; (– – –) $K_6 = 7 \times 10^{-11}$ M; (- - -) $K_6 = 1 \times 10^{-11}$ M.

^1H Studies of Phosphoglycerate Mutase. It is possible to obtain more detail concerning this PGM system by investigating the ^1H signals of the histidine residues of the PGM. Histidines give rise to NMR signals near 8 ppm and generally have pK_a values near 6. Tryptophan can also give an NMR signal near 8 ppm, while under special conditions other aromatic protons might also occur in this region of the spectrum. pH titration provides a potential means of distinguishing between the two types of signals; although, of course, if a histidine does not come into contact with the solvent, this method will fail. From the results of the pH titration, it could be seen that three of the four signals (arbitrarily labeled as A, B, C, and D) that occur in the appropriate region of the spectrum shifted position with change in pH. A plot of the data showing the chemical shifts as a function of pH is displayed in Figure 5, from which it is evident that the pK_a values are as given in Table II [see Gresser et al. (1986) for details of the calculations]. Since only three histidines have been identified, this suggests that there are only three histidines per subunit in the

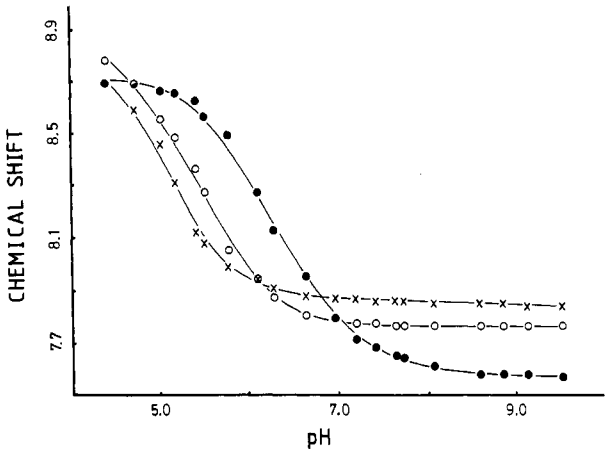


FIGURE 5: ^{1}H NMR titration curves of the histidine C-2 protons of phosphoglycerate mutase. The solid lines represents the curves calculated according to the parameters of Table 2. The experimental points correspond to (●) histidine A, pK_a = 6.34; (○) hisitidine B, pK_a = 5.25; (×) histidine C, pK_a = 5.20.

Table II: ^{1}H Chemical Shifts and pK_a Values for the Histidine Residues of Dephospho(Phosphoglycerate Mutase)[a]

histidine[b]	δ_{HA}	δ_A	pK_a
His A	8.69	7.57	6.54 ± 0.04
His B	9.00	7.77	5.24 ± 0.04
His C	8.80	7.85	5.20 ± 0.05

[a] Conditions of the experiments were 0.2 mM PGM, 20 mM ethylenediamine, 99.8% D_2O, ambient (~22 °C) temperature, and varying pH values, where pH is the direct meter reading uncorrected for D_2O. [b] Chemical shifts are for the C-2 protons. δ_{HA} and δ_A represent the chemical shifts for the protonated and deprotonated forms of the histidine residue, respectively.

PGM which are accessible to water. The fourth signal, signal D, does not shift with pH. As will be seen, this signal is affected by the presence of 2-vanadio-3-phosphoglycerate and therefore presumably is accessible to water although changes in protein conformation may be responsible for the effects observed. A number of phosphoglycerate mutases and the related bisphosphoglycerate mutases have been sequenced, are highly homologous, and seem to have a minimum of six histidines per subunit [see Sakoda et al. (1988) for a compilation of sequences]. It therefore seems that more than three or four NMR signals attributable to histidine should be observable. The absence of these signals cannot unequivocally be taken to mean that there are not more than three histidines in rabbit muscle PGM since histidine NMR signals can be quite broad and therefore difficult to observe.

The effect of incremental amounts of vanadium on the proton signals was surprisingly small at pH 7.7. There was only a small differential high field shift in the positions of signals A, B, and D. The differences were real and the changes were much more obvious at pH 8.5. Addition of up to 10 mM 3-phosphoglyceric acid to a PGM solution similarly had only minor effects on the histidine signals, the major effect being a decrease in intensity of the His B signal, accompanied by the emergence of a broadened signal close to but slightly to low field of resonance D. There was, however, a marked change in the proton spectrum when 3-PG was added to a solution of 0.3 mM PGM with 1.0 mM vanadate. The effects of the titration are shown in Figure 6. A similar result was obtained if vanadate was added to a PGM/3-PGA solution. Figure 6 clearly shows that the signal attributed to His C is unaffected by incorporation of the 2-V-3-PG into the mutase. The signal assigned as His B is progressively eliminated from the spectrum, indicating the involvement of this residue in

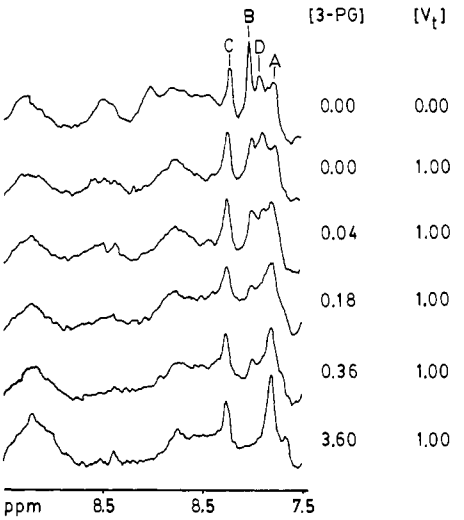


FIGURE 6: ^{1}H NMR spectra of the phosphoglycerate mutase histidine C-2 protons shown as a function of successive additions of vanadate and 3-phosphoglycerate at pH 7.70 ± 0.05. Conditions of the experiments were 0.18 mM phosphoglycerate mutase, 20 mM ethylenediamine buffer, pH 7.7 ± 0.05, and the indicated proportions of added vanadate and 3-phosphoglycerate.

binding. Interestingly enough, signal D, here attributed to a buried histidine or a tryptophan, also is eliminated from the spectrum. This may indicate that there is a tryptophan in the active site of PGM. Recent studies of fluorescence decay in PGM have suggested the presence of a unique tryptophan in or near the active site of rabbit muscle PGM (Schauerte & Gafni, 1989). The present results lend support to this suggestion. Interestingly enough, if it has been properly assigned, then only one tryptophan is observed. If there are other tryptophans in rabbit muscle PGM monomer (there are seven in human muscle PGM), they must be in very different environments from that of the one observed. Yeast PGM has five tryptophans, but the unique fluorescence decay of the rabbit muscle PGM tryptophan is not observed.

Evidently from Figure 6 it is not possible to determine if His A is affected by the binding of 2-V-3-PG because NMR signals of the product–enzyme complex occur as superimposed signals at the chemical shift of His A. It is possible to resolve this problem by varying the pH.

The results of such a titration at pH 8.5 are shown in Figure 7, from which it is clear that His A also is strongly affected by the binding process. The intense product signal occurs at 7.65 ppm both at pH 7.7 and 8.5, while a much less intense product signal occurs at 7.60 ppm at pH 7.7 and 7.56 ppm at pH 8.5. It is not clear what this latter signal corresponds to since it is not intense enough to correspond to a single proton which might arise from one of the units of the dimer.

Recent kinetic studies have shown that the dephospho-PGM has mutase activity even in the absence of 2,3-DPG (Stankiewicz & Hass, 1986). At the high concentrations of this study, such activity should be significant. This expectation was confirmed when it was found that the proton spectra were indistinguishable when either 3-PG or 2-PG were used in the titration of PGM with vanadate. It might be speculated that in the presence of vanadate the mutase activity is slowed enough that ^{1}H signals are observed that correspond to the two forms of the transition state analogue on the enzyme, 2-V-3-PG or 3-V-2-PG. This could explain the occurrence of the lower intensity signal at 7.60 ppm. Interestingly enough, no signal at 7.60 ppm was observed for PGM in the presence of 10 mM 2,3-DPG. However, in this case rapid interconversion between orientations seems likely. The resultant

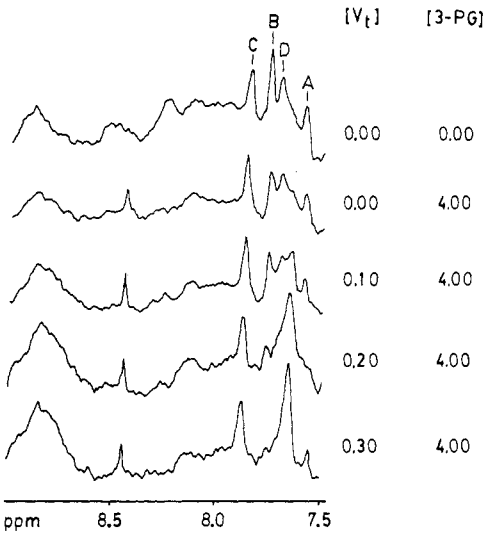


FIGURE 7: ^{1}H NMR spectra of the phosphoglycerate mutase histidine C-2 protons shown as a function of added 3-phosphoglycerate and then successive additions of vanadate at pH 8.50 ± 0.06. Conditions of the experiments were as for Figure 6 except for the change in pH. The signal at 8.44 ppm derives from a small amount of formate contaminate.

spectrum in its other details is virtually identical to that of the bottom spectra of Figures 6 and 7. A similar result was obtained if 3-phosphonomethylglycerate was used in the enzyme titration instead of 3-phosphoglycerate. From experiments similar to those whose results are shown in Figures 2–4, it was concluded that replacement of the $-CH_2OPO_3$ by $-CH_2CH_2PO_3$ diminished the binding affinity by about a factor of 500, $K_6 = 1 \times 10^{-9}$ M if $K_3 = 2.5$, the value for 3-PG. The relationship between K_3 and K_6 is such that if K_3 is halved then K_6 also is approximately halved, a reflection of the fact that K_3 defines the amount of vanadium ester in solution.

Discussion

Proton NMR studies of dephospho(phosphoglycerate mutase) have led to the identification of three NMR signals which can be assigned to histidine residues. An additional signal has tentatively been attributed to a tryptophan (Trp A). Variation of the pH provided the pK_a values for the histidines, which in order of increasing chemical shifts at pH 7 are labeled as His A, His B, and His C. The pK_a values measured were 6.34, 5.24, and 5.20, respectively.

Titration of PGM with 2-vanadio-3-phosphoglycerate had a negligible effect on the chemical shift of His C while significantly affecting the shifts of His A, His B, and Trp A. This suggests that the tryptophan and the A and B histidines are in the active site of PGM. It has been suggested that a histidine in the active site of PGM plays a role as a general acid–base catalyst during phosphorus transfer (Rose, 1980; Fothergill-Gilmore & Watson, 1989), consistent with the pH optimum for PGM that has been reported to be 5.9 (Rodwill et al., 1957). The pK_a values measured for the histidines lend support to this above hypothesis and are in accord with the reported pH optimum.

The possibility that the ^{1}H NMR signal assigned to a tryptophan actually derives from a histidine which is not accessible to water cannot be ignored. The change in chemical shift as binding occurs may then represent a conformational change in the enzyme. However, fluorescence decay studies (Schauerte & Gafni, 1989) have indicated the presence of a unique tryptophan in rabbit muscle PGM that is accessible to halide ions which quench the fluorescence. Tryptophan possibly acts as a hydrogen bond donor and may help orient the phosphoglycerate molecule for reaction.

When titrated with vanadate, phosphoglycerate mutase incorporated a pair of ions per active site in a noncooperative manner. Addition of 3-phosphoglycerate to the PGM–vanadium complex resulted in release of two of the four vanadium atoms from the enzyme. The analysis of the results of this second titration also revealed noncooperativity in the binding of 2-V-3-PG. These results suggest that the two active sites of PGM function independently of each other. This view is supported by the proton spectrum of the histidine region, which shows a total of four nonsuperimposed signals for PGM. Titration with vanadium or 2-V-3-PG does not give rise to extra signals in the spectrum; rather there is a loss of one set of signals and generation of a second set. If there were cooperative behavior between the two active sites, then extra, to a maxiumum of eight, ^{1}H signals might have been observed from the intermediate region of the complete titration range. Only two sets of signals were observed for all titrations of this study, including the titrations with vanadium, with vanadium plus 3-PG, with 3-PG plus vanadium, and also with a 2-V-3-PG solution.

There is an intrinsic difference between the binding of divanadate and of 2-vanadio-3-phosphoglycerate which was revealed by the pH studies. Divanadate binds increasingly tightly to PGM with increase in pH, about a factor of 10 more tightly on going from pH 7.0 to 8.0. This contrasts with the binding of 2-V-3-PG, which is much less favored at the higher pH, apparently requiring protons for binding to occur. The ionization states of V_2 and 2-V-3-PG are quite different. V_2 at pH 7.0 is mostly doubly anionic but has a pK_a of about 7.7, so it is mostly triply anionic at pH 8. The vanadiophosphoglycerate on the other hand will carry between three and four negative charges at both pH 7.0 and 8.0 since the second pK_a of phosphate is below 7 while the second pK_a of vanadate is above 8. Apparently at least one negative charge is neutralized as binding occurs. However, it is not certain that a proton is actually incorporated into the active site. The ^{1}H spectrum of the PGM–2-V-3-PG complex does not indicate that a histidine becomes protonated when complexation occurs since there is only a small effect on the chemical shifts, less than 0.1 ppm, as compared to 1–2 ppm observed during the ^{1}H titration of PGM. However, since the coordination about the vanadium nucleus changes as the enzyme complex is formed, the proton may be required for this reaction.

There is no evidence that there is any cooperativity in the binding of divanadate (this study; Stankiewicz et al., 1987) or also, as shown here, in the binding of vanadiophosphoglycerate to the two active sites of PGM. This strongly suggests that the two active sites behave independently of each other during the normal function of this enzyme. In its reaction with phosphoglycerate mutase, a phosphate group is transferred from 2,3-DPG to PGM and 2- or 3-phosphoglycerate is released from the enzyme. It has been proposed that the transfer step involves a pentacoordinate phosphorus in the transition state complex (Knowles, 1980), while the transition state involved in PGM phosphorylation is probably identical to that involved in the mutase activity of this enzyme (Fothergill-Gilmore & Watson, 1989).

NMR studies of the interactions of divanadate with PGM (Stankiewicz et al., 1987) have suggested strongly that, when V_2 is bound to PGM, one vanadate moiety retains its tetrahedral coordination but not the other. It was proposed that the tetrahedral vanadate was occupying a site normally occupied by a carboxylate group while the second vanadium of

V_2 was in a location normally filled by a pentacoordinate phosphate group. In the present study, all interaction possibilities normally available for 2,3-DPG are present, except that a vanadate replaces a phosphate group. Again, the evidence is in favor of a pentacoordinate vanadate, and the tight binding of 2-V-3-PG is consistent with this. In fact, a similar argument has been advanced for the formation of a vanadate derived transition state analogue for ribonuclease A (Lindquist et al., 1973). In this case the product has been characterized by X-ray and neutron diffraction studies (Borah et al., 1985) which clearly revealed the pentacoordinate geometry about vanadium. It is the relative ease of changing the coordination geometry about the vanadium (Butler, 1990; Pope & Dale, 1968) which leads to the strong binding of 2-V-3-PG. The dissociation constant of 2-V-3-PG from PGM is at least 4 orders of magnitude smaller than the K_m for 2,3-DPG. This supports the view that 2-V-3-PG is a transition state analogue for phosphorylation of dephospho-PGM by 2,3-DPG.

Appendix

Calculation of the Binding Curve. The definitions of the equilibrium constants required in the development of the proposed mechanism for vanadate ester inhibition of rabbit muscle PGM are listed in the following equations:

$$K_0 = \frac{[V_4]}{[V_1]^4} \quad \text{(A2.1)}$$

$$K_1 = \frac{[V_2]}{[V_1]^2} \quad \text{(A2.2)}$$

$$K_3 = \frac{[I]}{[PG][V_1]} \quad \text{(A2.3)}$$

$$K_4 = \frac{[E][V_2]}{[E(V_2)]} \quad \text{(A2.4)}$$

$$K_5 = \frac{[E(V_2)][V_2]}{[E(V_2)_2]} \quad \text{(A2.5)}$$

$$K_6 = \frac{[E][I]}{[EI]} \quad \text{(A2.6)}$$

$$K_7 = \frac{[EI][I]}{[EI_2]} \quad \text{(A2.7)}$$

$$K_8 = \frac{[E(V_2)][I]}{[EI(V_2)]} \quad \text{(A2.8)}$$

$$K_9 = \frac{[EI][V_2]}{[EI(V_2)]} \quad \text{(A2.9)}$$

The conservation equations are

$$[V_B] = 4[E(V_2)_2] + 2[E(V_2)] + [EI] + 2[EI_2] + 3[EI(V_2)] \quad \text{(A2.10)}$$

$$[E_T] = [E(V_2)_2] + [E(V_2)] + [E] + [EI] + [EI_2] + [EI(V_2)] \quad \text{(A2.11)}$$

$$V_T = [V_1] + 2[V_2] + 4[V_4] + [I] + 4[E(V_2)_2] + 2[E(V_2)] + [EI] + 2[EI_2] + 3[EI(V_2)] \quad \text{(A2.12)}$$

$$[PG_T] = [PG] + [EI] + [EI(V_2)] + [I] \quad \text{(A2.13)}$$

From the definitions of the equilibrium constants in eqs A2.1–A2.9, the following expressions were obtained for substitution into the conservation equations, eqs A2.10–A2.13:

$$[V_2] = K_1[V_1]^2 \quad \text{(A2.14)}$$

$$[V_4] = K_0[V_1]^4 \quad \text{(A2.15)}$$

$$[I] = K_3[PG][V_1] \quad \text{(A2.16)}$$

$$[E(V_2)] = \frac{[E][V_2]}{K_4} = \frac{K_1[E][V_1]^2}{K_4} \quad \text{(A2.17)}$$

$$[E(V_2)_2] = \frac{[E(V_2)][V_2]}{K_5} = \frac{K_1^2[E][V_1]^4}{K_4K_5} \quad \text{(A2.18)}$$

$$[EI] = \frac{[E][I]}{K_6} = \frac{K_3[E][PG][V_1]}{K_6} \quad \text{(A2.19)}$$

$$[EI_2] = \frac{[EI][I]}{K_7} = \frac{K_3^2[E][PGA]^2[V_1]^2}{K_6K_7} \quad \text{(A2.20)}$$

$$[EI(V_2)] = \frac{[E(V_2)][I]}{K_8} = \frac{K_3K_1[E][PG][V_1]^3}{K_4K_8} = \frac{[EI][V_2]}{K_9} = \frac{K_1K_3[E][PG][V_1]^3}{K_6K_9} \quad \text{(A2.21)}$$

By making the appropriate substitutions into eqs A2.11–A2.13 (including $K_5 = 4K_4$ and $K_7 = 4K_6$, which are required if the binding of V_2 and I are to be noncooperative) and rearranging, eq A2.22 can be obtained from eq A2.13, eq A2.23 from eq A2.11, and eq A2.24 from eq A2.12.

$$\frac{K_3^2[E][PG]^2[V_1]^2}{2K_6^2} + [PG] \times \left\{1 + K_3[V_1]\left(1 + \frac{[E]}{K_6} + \frac{[E][V_1]^2K_1}{K_4K_8}\right)\right\} - [PG_T] = 0 \quad \text{(A2.22)}$$

$$[E] = 4K_4^2K_6^2K_8[E_T]/\{4K_4^2K_6^2K_8 + 4K_4^2K_6K_8K_3[PG][V_1] + (4K_1K_4K_6^2K_8 + K_4^2K_8K_3^2[PG]^2)[V_1]^2 + 4K_4K_6^2K_1K_3[PG][V_1]^3 + K_1^2K_6^2K_8[V_1]^4\} \quad \text{(A2.23)}$$

$$4[V_1]^4\left(K_0 + \frac{K_1^2[E]}{4K_4^2}\right) + 3[V_1]^3\left(\frac{K_1K_3[E][PG]}{K_4K_8}\right) + 2[V_1]^2\left(K_1 + \frac{K_1[E]}{K_4} + \frac{K_3^2[E][PG]^2}{4K_6^2}\right) + [V_1]\left(1 + K_3[PG] + \frac{K[PG][E]}{K_6}\right) - [V_T] = 0 \quad \text{(A2.24)}$$

These equations can be used, given values for the equilibrium constants and $[V_T]$, $[E_T]$, and $[PG_T]$, to calculate equilibrium values of $[V_1]$ and $[PG]$. These can then be used in eq A2.25, which was obtained from eq A2.10, to calculate $[V_B]/[E_T]$.

$$\frac{[V_B]}{[E_T]} = \left[\frac{4K_1^2[V_1]^4}{4K_4^2} + \frac{2K_1[V_1]^2}{K_4} + \frac{K_3[PG][V_1]}{K_6} + \frac{2K_3^2[PG]^2[V_1]^2}{4K_6^2} + \frac{3K_1K_3[PG][V_1]^3}{K_4K_8}\right] \Big/ \left[1 + \frac{K_1^2[V_1]^4}{4K_4^2} + \frac{K_1[V_1]^2}{K_4} + \frac{K_3[PG][V_1]}{K_6} + \frac{K_3[PG]^2[V_1]^2}{4K_6^2} + \frac{K_1K_3[PG][V_1]^3}{K_4K_8}\right] \quad \text{(A2.25)}$$

The iterative procedure is as follows:

(Step 1) Assume a reasonable value of $[V_1]$ for a given value of $[PG_\tau]$. The experimental value can be used as a reasonable first approximation. [PG] can then be obtained from eq A2.22 because [E] is known since K_6 is assumed.

(Step 2) The value of [PG] obtained in step 1 and the value of $[V_1]$ used in step 1 will be used to calculate the left-hand side of eq A2.24. If zero, within ±0.001 mM, is not obtained from eq A2.24, the value $[V_1]$ has to be changed in the appropriate direction, and this value of $[V_1]$ will be used in the next execution of step 1.

(Step 3) The sequence of step 1, 2, 1, 2, ... will be continued until the values of [PG] and $[V_1]$, which satisfy both eqs A2.22 and A2.24 to within ±0.001 mM, are obtained. These [PG] and $[V_1]$ values will then be substituted into eq A2.25 for calculation of $[V_B]/[E_\tau]$.

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