Characterization of Vanadate-Based Transition-State-Analogue Complexes of Phosphoglucomutase by Spectral and NMR Techniques†

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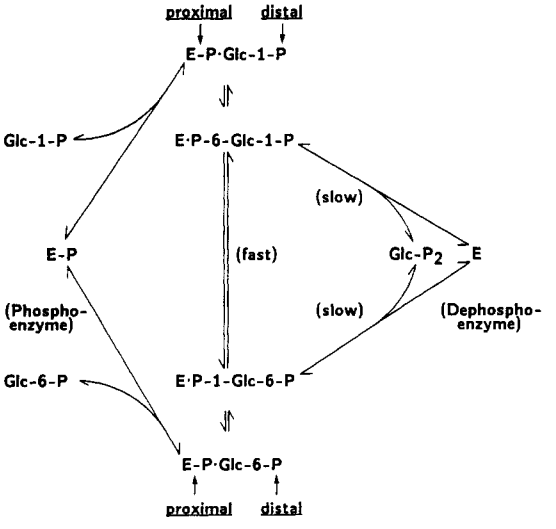
ABSTRACT: Near ultraviolet spectral studies were conducted on two inhibitor complexes obtained by treating the *dephospho* form of the phosphoglucomutase·Mg^{2+} complex with inorganic vanadate in the presence of either glucose 1-phosphate [cf. Percival, M. D., Doherty, K., & Gresser, M. J. (1990) *Biochemistry* (first of four papers in this issue)] or glucose 6-phosphate. Part of the spectral differences between the two inhibitor complexes arises because the glucose phosphate moiety in the complex derived from glucose 1-phosphate binds to the enzyme in a different way from the glucose phosphate moiety in the complex derived from glucose 6-phosphate and because these alternative binding modes produce different environmental effects on the aromatic chromophores of the *dephospho* enzyme. These spectral differences are strikingly similar to those induced by the binding of glucose 1-phosphate and glucose 6-phosphate to the *phospho* enzyme—which shows that the glucose 1-phosphate and glucose 6-phosphate moieties occupy positions in the inhibitor complexes closely related to those that they occupy in their respective catalytically competent complexes. This binding congruity indicates that in the inhibitor complexes the oxyvanadium grouping is bound at the site where (PO_3^-) transfer normally occurs. ^{31}P NMR studies of the phosphate group in these complexes also provide support for this binding pattern. A number of other systems based on compounds with altered structures, such as the deoxysugar phosphates, or systems with different compositions, as in the case of the metal-free enzyme or of the glucose phosphates plus nitrate, also were examined for evidence that complexes analogous to the inhibitor complexes were formed, but none was found. Difference spectroscopy was used to resolve the spectrum of both inhibitor complexes to obtain the absorbance of their oxyvanadium chromophores. The resolved spectrum, which is unlike that of any known vanadate ester, consists of a broad, nearly symmetrical peak with $\epsilon \approx 5 \times 10^3$ mol cm^{-2}, $\lambda_{max} \approx 312$ nm, and a width at half-height of about 5000 cm^{-1}. A spectrum more nearly like that of a normal vanadate ester is observed for the oxyvanadium chromophore in the corresponding complex involving glucose 1-phosphate and Li^+ instead of Mg^{2+}. A comparison of both spectra with that of vanadate and a number of vanadate esters in several different solvent systems is described in the third paper of this series [Ray, W. J., Jr., & Post, C. B. (1990) *Biochemistry* (third of four papers in this issue)]. Kinetic and thermodynamic studies that support the suggestion that the glucose phosphate/vanadate complexes of the Mg^{2+} enzyme should be considered as transition-state-analogue complexes are described in a fourth paper [Ray, W. J., Jr., & Puvathingal, J. M. (1990) *Biochemistry* (fourth of four papers in this issue)].

Phosphoglucomutase is one of a number of enzymes that facilitates multiple bond-breaking/bond-making steps, interspersed by internal reorientation, so that the product of one set of bond-breaking/bond-making steps can serve as the reactant for a subsequent set (Ray & Peck, 1972), without an intervening dissociation step. Thus, after transfer of a (PO_3^-) group[1] from the phospho enzyme to the 6-position of Glc-1-P,[2] the Glc-1,6-P_2 produced must be reoriented to bring its 1-phosphate into position for return to the enzyme to complete the catalytic cycle. This reorientation occurs at a much faster rate than that at which Glc-1,6-P_2 dissociates from the enzyme ($k_d \approx 10$ s^{-1}; Ray et al., 1989), since it has been shown (Ray & Roscelli, 1964) that the reorientation rate must be substantially faster than the turnover rate, which is 630 s^{-1} at 25 °C. We thus include two bisphosphate complexes, E·P-6-Glc-1-P and E·P-1-Glc-6-P, in the catalytic cycle: in the first, the 6-phosphate of the bisphosphate occupies the "(PO_3^-) transfer" or proximal site while the 1-phosphate is at the "phosphate-binding" or distal site; in the second, the

Scheme I: Reaction Sequence of Phosphoglucomutase, Including the Interconversion of the Two Bisphosphate Complexes[a]



[a] The phosphate groups at the proximal and distal sites of the enzyme are indicated by vertical arrows.

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positions of the phosphates of the bound bisphosphate are reversed (see Scheme I). A recognition of this binding pattern is necessary to interpret the manner in which inorganic vanadate, together with the glucose phosphates, forms tightly bound enzyme–inhibitor complexes.

Although several studies of vanadate inhibition of phosphoglucomutase have appeared, the mechanism by which inhibition is produced was worked out first by Gresser and colleagues in a study that is described in an accompanying paper (Percival et al., 1990). These workers show that the inhibitor competes with Glc-1,6-P_2 for the dephospho enzyme, E. They also show that in initial velocity studies with Glc-1-P the extent of inhibition is related to the *product* of the concentrations of Glc-1-P and inorganic vanadate and suggest that the 6-vanadate ester of Glc-1-P, V-6-Glc-1-P, which undoubtedly is present in such solutions, acts as the inhibiting species. (We concur with this suggestion for reasons detailed under Discussion.) In addition, because of the unusual thermodynamic stability of the complex between E and the vanadate-based inhibitor, and because of the propensity of vanadium(V) to expand its valence shell (Pope & Dale, 1968; Kepert, 1973), Percival et al. also suggest that the active-site serine hydroxyl group forms a pentacoordinate adduct with the vanadate group of bound V-6-Glc-1-P. Such an adduct might resemble the transition state for the normal enzymic reaction and thus bind unusually well.

In spite of the attractiveness of the above arguments, it seems desirable to provide a direct demonstration that the vanadate group in these inhibitor complexes actually occupies the (PO_3^-) transfer or proximal phosphate site as opposed to the distal or phosphate-binding site. Thus, the inhibitor might bind to give E·V-6-Glc-1-P, analogous to E-P·Glc-1-P, or it might bind to give E·P-1-Glc-6-V, analogous to E-P·Glc-6-P (see Scheme I).

The present paper shows that the suggestion of Percival et al. (1990) is correct in that the inhibitors produced from vanadate plus Glc-1-P or vanadate plus Glc-6-P bind with the vanadate group at the (PO_3^-) transfer site. Our approach in this paper takes advantage of earlier observations that when bound Mg^{2+} either is removed (Ray et al., 1965) or replaced by Li^+ (Ray et al., 1978), the binding of glucose phosphates is relatively unaffected, while the subsequent (PO_3^-) transfer step is slowed to the point where E-P·Glc-1-P or E-P·Glc-1-P(Li^+) and E-P·Glc-6-P or E-P·Glc-6-P(Li^+) can be studied separately, by both ultraviolet and NMR spectroscopies (Ma & Ray, 1980; Rhyu et al., 1984). The former studies show that the effect of bound Glc-1-P on the spectral properties of aromatic chromophores in the enzyme is characteristically different from that of bound Glc-6-P; the latter studies show that the ^{31}P resonances of both bound Glc-1-P and Glc-6-P occur at characteristic chemical shifts. Here, both UV and NMR spectroscopies are used to characterize the inhibitor complexes that the dephospho enzyme makes with inorganic vanadate and Glc-1-P or Glc-6-P, which in subsequent papers are referred to as transition-state-analogue complexes on the basis of a number of their properties [cf. the fourth paper in this series: Ray and Puvathingal (1990)].

Attempts to characterize the bonding of the oxyvanadium constellation in the inhibitor complexes via spectral comparisons with the vanadate chromophore in compounds whose structure is at least approximately known are described in the following paper (Ray & Post, 1990). On the basis of this comparison we do not consider the suggestion of Percival et al. (1990) with regard to a pentacoordinate adduct as entirely attractive. Hence, for reasons described in the Ray and Post paper (1990) the inhibitor complexes derived from Glc-1-P and Glc-6-P are formulated as E*V*6-Glc-1-P and E*V*1-Glc-6-P, respectively, in the case of the Mg^{2+} enzyme. This representation emphasizes their structural relationship to the normal enzymic complexes, E·P-6-Glc-1-P and E·P-1-Glc-6-P, as is verified in the present paper, and serves to indicate a degree of uncertainty about the actual bonding within these complexes as is described in the following paper. By contrast, for reasons noted in both subsequent papers, the corresponding complex involving Glc-1-P and the Li^+ enzyme is represented as E·V-6-Glc-1-P(Li^+).

A thermodynamic comparison of the binding of V-6-Glc-1-P and of P-6-Glc-1-P to the Mg^{2+} dephospho enzyme is described in the fourth paper of this series (Ray & Puvathingal, 1990). That paper also considers differences between the complexes formed when Li^+, an exceedingly poor activator, occupies the active site instead of the normal activator, Mg^{2+}.

Experimental Procedures

Materials. The phospho and dephospho forms of phosphoglucomutase were prepared, demetalated, and stored as was described previously (Magneson et al., 1987). Glc-6-P (Sigma), which contains no Glc-P_2, was used without further purification. Glc-1-P (Sigma) was freed of contaminating Glc-P_2, 10 g at a time, by chromatography on a column, 5 × 7 cm, of Dowex 1–HCO_3, 8% cross-link, 200–400 mesh. A gradient of 0–0.3 M $NaHCO_3$ in a total of 3.6 L was used. Fractions in which a precipitate was induced on treatment with 3 volumes of absolute ethanol were combined. After standing overnight, the disodium salt was isolated by filtration and subsequently dried under vacuum. Solutions of approximately 0.1 M were adjusted to pH 8 and passed through a column of Chelex resin (Bio-Rad), 1 × 5 cm, that had been freshly regenerated, equilibrated with 0.1 M Na_2HPO_4, and briefly flushed with water. The material in the effluent plus wash was isolated by lyophilization and subsequently dried under vacuum over P_2O_5. Xylose-1-P was from Sigma; the preparation of 6-deoxyglucose-1-P and 1-deoxyglucose-6-P was described previously (Ma & Ray, 1980). Before use, solutions of LiCl (Alfa, high purity) were passed through a column that contained the Li^+ form of Chelex resin. Imidazole (Sigma) was crystallized from toluene and dried under vacuum. Subsequently, it was dissolved in water, passed first through a mixed-bed ion-exchange resin and subsequently through the imidazolium form of Dowex 50, and isolated by lyophilization. An aqueous stock solution of the product, 0.2 M, pH 8, was passed through the imidazolium form of Chelex resin after the column was equilibrated with this buffer. A 0.5 M stock solution of inorganic vanadate was obtained by dissolving V_2O_5 (Alfa) in a solution that contained 4 equiv of NaOH.

Procedures. Ultraviolet spectra of phosphoglucomutase and its complexes were recorded with a Perkin-Elmer Lambda 6

[1] "(PO_3^-) transfer" is used herein to designate the process $R_1OPO_3^{2-} + R_2OH \rightarrow R_1OH + R_2OPO_3^{2-}$ and refers to the identity of the group transferred, without inference regarding the mechanism of transfer.

[2] Abbreviations: E-P and E, the phospho and dephospho forms of phosphoglucomutase; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate—equilibrium mixture of α and β anomers unless otherwise specified; Glc-P_2, P-6-Glc-1-P, or P-1-Glc-6-P, α-D-glucose 1,6-bisphosphate; P_i and V_i, inorganic phosphate and vanadate, respectively, V-6-Glc-1-P and V-1-Glc-6-P, the 6-vanadate ester of Glc-1-P and the α-1-vanadate ester of Glc-6-P; E*V*6-Glc-1-P and E*V*1-Glc-6-P, with or without the suffix Mg^{2+}, the inhibitor complexes produced by treating the dephospho enzyme, Mg^{2+} form, with V_i plus Glc-1-P or with V_i plus Glc-6-P, respectively; E·V-6-Glc-1-P(Li^+) the corresponding complex involving the Li^+ form of the enzyme; CHES, (*N*-cyclohexyl-2-amino)ethanesulfonic acid; CAPS, (*N*-cyclohexyl-3-amino)propanesulfonic acid.

spectrophotometer by using a one-cell procedure that employed a sectored cell with a light path of 0.442 cm/compartment. Solutions of 0.800 mL were delivered to both compartments of the cell with the same micropipet. Spectra were scanned repetitively at a rate of 60 nm/min, both before and after mixing (slit = 2 mm; response = 2). In an alternative procedure, additions of 2 μL of vanadate were made to 0.8 mL of a protein solution in a cell with a 1-cm light path. Spectra were recorded both before and after the addition, and a minor correction for dilution was made before the spectral difference was obtained digitally. In both procedures the concentration of enzyme was about 2 mg/mL. Spectra of concentrated solutions of the enzyme (24.6 mg/mL, or 0.4 mM) were collected by using 0.45-mL samples in a cell with a quartz insert (Helma) that reduced the light path to 0.094 cm. Difference spectra were obtained in such cases by digitally subtracting spectra obtained from solutions mixed in a 1:1 ratio by using the same micropipette.

^{31}P NMR spectra were measured at room temperature with a Varian XL-200A spectrometer that was operated at 4.7 T (phosphorous frequency, 81.0 MHz). Sample volumes of 1.6 mL were placed in a spherical microcell inserted in a 16-mm tube. Chemical shifts are relative to trimethyl phosphate and were measured from the internal reference, tetraphenylphosphonium chloride, whose chemical shift is 19.62 ppm. Other experimental details are given in the figure legend or have been described previously (Post et al., 1989).

Results

In all studies with the metal ion complexes of phosphoglucomutase reported here, the concentration of Mg^{2+} or Li^+ was saturating. Since in most cases the physiological activator, Mg^{2+}, was used [cf. Peck and Ray (1971)], the identity of the bound metal ion frequently is omitted from representations of the various enzymic complexes, except where a distinction between the Mg^{2+} and Li^+ complexes is made.

Spectral Studies Involving Glucose Phosphates Bound to Phosphoglucomutase in the Presence of Bound Vanadate. The spectral changes produced by the binding of Glc-1-P plus V_i and of Glc-6-P plus V_i to the dephospho enzyme are shown in Figure 1a, spectra 1 and 2, respectively ([Glc-P] $\gg K_D$; [V_i] > [E]). The overall spectral change is the sum of spectral changes that can be attributed to perturbation of aromatic residues in the enzyme by the different phosphoglucosyl moieties, Glc-6-P and Glc-1-P [cf. Ma and Ray (1980)], plus changes in the oxyvanadium chromophore that are produced by the binding process. The procedures used to disentangle these spectral changes are described below.

Figure 1b shows the difference between the two difference spectra in Figure 1a, which is the spectral difference between the complex of the enzyme, V_i, and Glc-6-P, on one hand, and of the enzyme, V_i, and Glc-1-P on the other. Discounting the broad, base-line absorbance difference (see below), the "fine structure" of the difference spectrum in Figure 1b is similar to the spectral difference between E-P·Glc-6-P and E-P·Glc-1-P reported previously under somewhat different conditions (Ma & Ray, 1980). Thus, Figure 1c shows the spectral changes in the reaction-blocked E-P(Li^+) complex that are produced by binding of Glc-6-P, spectrum 4, or Glc-1-P, spectrum 5, under the current conditions. The primary differences between parts a and c of Figure 1 thus must be produced by differences in the response of the oxyvanadium chromophore in Figure 1a to its incorporation into the inhibitor complexes. [Earlier studies (Ma & Ray, 1980) showed that the spectral differences in Figure 1c are essentially identical with those produced by the binding of 1-deoxyglucose-6-P and 6-deoxyglucose-1-P, respectively, to the Mg^{2+} enzyme, and therefore that the identity of the bound metal ion does not affect the spectral changes induced by the binding of the Glc-1-P and Glc-6-P groupings to the enzyme.]

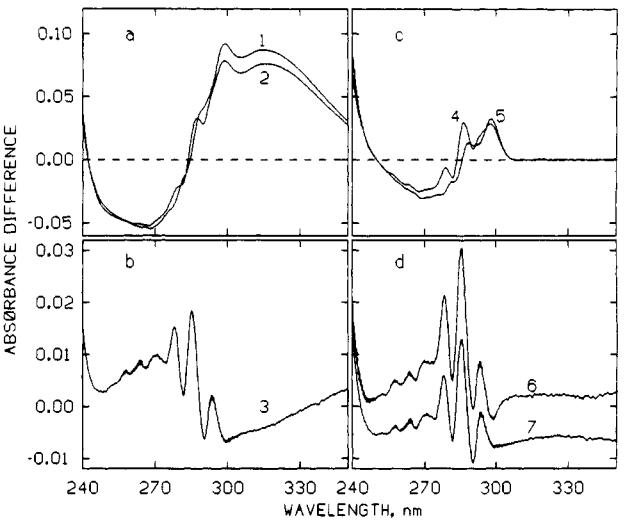

FIGURE 1: Difference spectra produced by the binding of glucose 1- and 6-phosphates to the dephospho enzyme in the presence of inorganic vanadate and related difference spectra. All difference spectra were obtained with 28–30 μM enzyme in 40 mM imidazolium chloride buffer, pH 8. The solution also contained 8 mM Mg^{2+} (dephospho enzyme) or 25 mM LiCl (phospho enzyme) plus 1 mM EDTA. The sectored-cell procedure is described under Experimental Procedures. All concentrations reported are those produced after mixing. (a) Difference spectra produced by the binding of Glc-6-P plus V_i or Glc-1-P plus V_i to the Mg^{2+} complex of the dephospho enzyme: 20 mM Glc-6-P and 125 μM (excess) V_i, spectrum 1, or 20 mM Glc-1-P and 125 μM V_i, spectrum 2, were used. (b) Spectral difference between the inhibitor complexes obtained by binding Glc-6-P plus V_i or Glc-1-P plus V_i to the Mg^{2+} form of the dephospho enzyme, obtained as the difference between difference spectrum 1 and difference spectrum 2 in (a). (c) Difference spectra produced by binding Glc-6-P, spectrum 4, or Glc-1-P, spectrum 5, to the Li^+ complex of the phospho enzyme, at a glucose phosphate concentration of 20 mM. (d) Spectral difference between E-P·Glc-6-P(Li^+) and E-P·Glc-1-P(Li^+), spectrum 6, obtained as the difference between difference spectra 4 and 5 in (c). Also shown, spectrum 7, is the spectral difference between the inhibitor complexes involving Glc-6-P and Glc-1-P, spectrum 3 in (b), after the spectral difference between the oxyvanadium constellations in these two complexes is corrected for (see text). (The latter spectrum is displaced downward on the y axis for presentation.)

Figure 1d, spectrum 6, shows the spectral difference between the E-P·Glc-6-P(Li^+) and E-P·Glc-1-P(Li^+) complexes obtained from spectra 4 and 5. The similarity of the fine structure in difference spectrum 6 to that in difference spectrum 3 of Figure 1b, where the enzymic complexes of V-1-Glc-6-P and V-6-Glc-1-P are compared, is obvious. In fact, in spectrum 7, essentially all differences between spectra 3 and 6 are eliminated by subtracting from spectrum 3 a broad peak with no fine structure equal to 12% of the average spectrum of the oxyvanadium chromophore in the inhibitor complexes (see next section). This subtraction accounts for the less intense absorbance of the oxyvanadium constellation in E*V*1-Glc-6-P than in E*V*6-Glc-1-P that is apparent in Figure 1a at wavelengths greater than about 300 nm. Thus, the 1-phosphoglucosyl moiety of the inhibitor produced from Glc-1-P plus V_i binds to and affects aromatic chromophores of the dephospho enzyme in a manner analogous to the way that Glc-1-P binds to and affects the aromatic chromophores of the phospho enzyme. An analogous binding pattern also characterizes the inhibitor complex produced from Glc-6-P plus V_i. Such binding modes require that in the inhibitor complexes the oxyvanadium constellation occupy the proximal

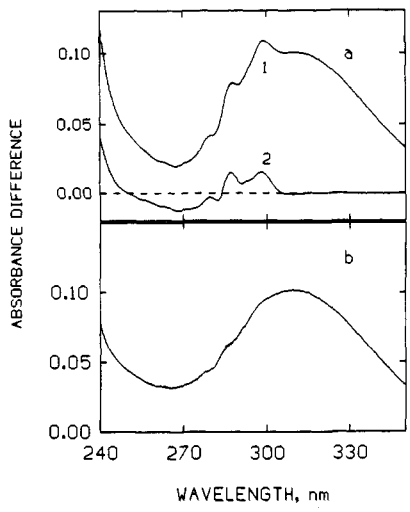

FIGURE 2: Spectra of the oxyvanadium chromophore in complexes of glucose 1- and 6-phosphates with the dephospho enzyme in the presence of inorganic vanadate. The conditions and general procedure described in Figure 1 were used. (a) (Spectrum 1) Difference spectrum produced by addition of a limiting amount of inorganic vanadate (0.7 equiv) to the Mg^+ dephospho enzyme (43 μM) plus an equilibrium mixture of Glc-1-P plus Glc-6-P: total glucose phosphates, 10 mM. (Spectrum 2) Estimated contribution of the phosphoglucosyl/enzyme fine structure spectrum to spectrum 1 (see Results and footnote 3). (b) Spectrum of the oxyvanadium chromophore in the inhibitor complex: the difference between the difference spectra 1 and 2 in (a).

phosphate site where (PO_3^-) transfer to and from the enzyme normally occurs, as opposed to the distal phosphate site that serves as the normal binding site for the phosphate group of Glc-1-P or Glc-6-P in the phospho enzyme. (The fine structure of difference spectrum 7 of Figure 1b would have been inverted relative to that in spectrum 6 if, for example, the phosphoglucosyl moiety of P-1-Glc-6-V were bound to the dephospho enzyme in the manner that Glc-6-P binds to the phospho enzyme, viz., with the vanadate group of the inhibitor at the distal phosphate site.) This conclusion thus verifies the postulate of Percival et al. (1990) regarding inhibitor binding modes that was based on the relative affinities of P-6-Glc-1-P and V-6-Glc-1-P for the dephospho enzyme. But because the nature of the chemical bonding of the vanadate ester group in the inhibitor complexes is not as yet defined, these complexes subsequently are represented as E*V*6-Glc-1-P and E*V*1-Glc-6-P. The corresponding bisphosphate complexes are E·P-6-Glc-1-P and E·P-1-Glc-6-P, while the related complexes involving the phospho enzyme are E-P·Glc-1-P and E-P·Glc-6-P, respectively.

Spectral Studies Involving Vanadate Bound to Phosphoglucomutase in the Presence of Glucose Phosphates. To obtain the spectrum of the oxyvanadium chromophore in the complexes of vanadate with the dephospho enzyme and Glc-1-P or Glc-6-P, a difference spectrum was recorded after V_i was added to an equilibrium mixture of (E·Glc-1-P + E·Glc-6-P) to give (E*V*6-Glc-1-P + E*V*1-Glc-6-P) under conditions of excess enzyme, where, according to the binding studies described in the fourth paper of this series, all of the added vanadate is converted to inhibitor complexes (spectrum 1, Figure 2a). This spectrum consists of a broad absorbance band: $\epsilon \approx 5 \times 10^3$ mol cm^{-2}, $\lambda_{max} \approx 312$ nm, and a width at half-height of about 5000 cm^{-1}, with superimposed fine structure, produced by differences between the interaction of the 1-phosphoglucosyl and 6-phosphoglucosyl moieties with aromatic chromophores of the enzyme in (E·Glc-1-P + E·Glc-6-P), before the addition of vanadate, and in (E*V-6-Glc-1-P + E*V*1-Glc-6-P), afterward. This fine structure can be eliminated to a large extent by subtracting the spectral change that would accompany the process (E·Glc-1-P + E·Glc-6-P)$_{eq}$ + P_i → (E·P-6-Glc-1-P + E·P-1-Glc-6-P)$_{eq}$. [Because the spectrum of the oxyvanadium chromophore is an electron-transfer spectrum [see the third paper in this series (Ray & Post, 1990)], it is not expected to exhibit fine structure in aqueous solution.] The spectrum subtracted, spectrum 2, is shown in Figure 2a;[3] the result is shown in Figure 2b. The spectrum in the latter figure should represent a reasonably accurate *average* spectrum for the oxyvanadium constellation in both inhibitor complexes,[4] although there is a minor, reproducible, intensity difference (about 12%) in the absorbance bands due to the oxyvanadium chromophore in the E*V*6-Glc-1-P and E*V*1-Glc-6-P complexes.[5] Two similar procedures for obtaining the spectrum of the oxyvanadium constellation in the E*V*6-Glc-1-P and E*V*1-Glc-6-P complexes produced spectra closely related to those above: treatment of the dephospho enzyme with Glc-1-P or Glc-6-P plus 50% excess V_i (after correction for excess V_i, assuming a 1:1 stoichiometry), or with Glc-1-P plus Glc-6-P plus limiting V_i (20% excess enzyme) (results not shown).

Attempt To Detect the Vanadate Analogue of the Phospho Enzyme, Spectrally. An attempt was made to obtain the spectrum of the vanadate ester of the active-site serine residue of the dephospho enzyme, E-V, analogous to the phospho enzyme, E-P, by adding V_i to a solution that contained excess dephospho enzyme, Mg^{2+} form, at pH 7.5. The spectrum obtained is reproduced in Figure 3a, spectrum 2. Since the extent of complex formation is unknown, it is not possible to subtract the contribution of unbound V_i to this spectrum to obtain the spectrum of whatever E-V is present, if any. Hence, spectrum 2 is shown in conjunction with the spectrum obtained in the same way but in the absence of enzyme (spectrum 4). As an added control, the same operation was conducted in the presence of sufficient Glc-1-P plus Glc-P_2 to saturate the enzyme and thus block the binding of V-6-Glc-1-P at the active site (spectrum 3). While the increased absorbance of spectrum 2 relative to the second control (spectrum 3) suggests that E-V might be formed in this way, the results are equivocal since whatever species is formed neither gives rise to an inactive complex on subsequent addition of Glc-1-P (Ray & Puvath-

[3] Spectrum 2 provides an estimate of the spectral change that should accompany the reaction (E·Glc-1-P + E·Glc-6-P)$_{eq}$ + P_i → (E-P·Glc-1-P + E-P·Glc-6-P)$_{eq}$ if this reaction could be conducted under the conditions used. The expected spectral change is obtained as the difference between two spectra: simulated spectrum *X* and observed spectrum *Y*, neither of which is shown. The phosphoglucosyl–enzyme interactions in (E*V*6-Glc-1-P + E*V*1-Glc-6-P)$_{eq}$ were simulated, spectrum *X*, by using spectra of E-P·Glc-1-P(Li^+) + E-P·Glc-6-P(Li^+) (see the introduction). From simulated spectrum *X* was subtracted observed spectrum *Y* for [E·Glc-1-P(Mg^{2+}) + E·Glc-6-P(Mg^{2+})]$_{eq}$. The ratio of the two spectra used in simulated spectrum X was varied so that when the difference spectrum, *X* − *Y*, was subtracted from spectrum 1, Figure 2a, most of the fine structure was eliminated. Difference spectrum *X* − *Y*, when simulated spectrum *X* involved a 40:60 ratio of E-P·Glc-1-P(Li^+):E-P·Glc-6-P(Li^+), is spectrum 2. This ratio is reasonably close to that estimated for the equilibrium mixture of E*V*1-Glc-6-P(Mg^{2+}) and E*V*6-Glc-1-P(Mg^{2+}), 60:40 (see last section under Results).

[4] The average spectrum of the oxyvanadium constellation in Figure 2b is used for that of the corresponding constellation in E*V*6-Glc-1-P and E*V*1-Glc-6-P because spectral differences between the oxyvanadium constellation these two complexes are not large and because it is easier to eliminate enzymic contributions to the required difference spectra when an equilibrium mixture of glucose phosphates is used.

[5] A spectral difference between the oxyvanadium constellation in the E*V*6-Glc-1-P and E*V*1-Glc-6-P complexes is not particularly surprising since the environment of the enzymic phosphate in E-P·Glc-1-P-(Li^+) is somewhat different from that of E-P·Glc-6-P(Li^+) as is indicated by the difference in ^{31}P NMR chemical shift (Rhyu et al., 1984).

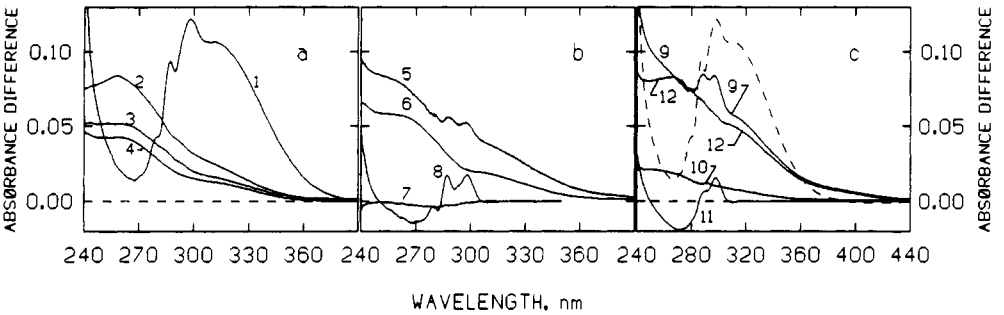

FIGURE 3: Spectral studies of complex formation in solutions related to those from which the vanadate-based inhibitor complexes of phosphoglucomutase are obtained. All spectra in (a) and (b) were obtained with a cell of 1-cm light path, except for the sectored-cell study involving NO_3^-. Solutions, 0.8 mL, contained 32 μM dephospho enzyme in 20 mM imidazole/imidazolium chloride, pH 7.5. The procedure is described under Experimental Procedures. Additions of 2 μL of 10 mM vanadate were employed (28% excess of enzyme). Enzyme solutions were prepared from a "demetalated" stock solution. When Mg^{2+} was present, its concentration was 8 mM, and 1 mM Mg·EDTA also was present. In each case, the spectral change produced by the addition of vanadate is shown, after a minor correction for dilution was made. (a) Attempt to produce an E–V complex. (Spectrum 2) Spectral change produced by adding vanadate to the Mg^{2+} complex of the dephospho enzyme. (Spectrum 4) Change produced by addition of vanadate to buffer only. (Spectrum 3) Change produced by addition of vanadate to the Mg^{2+} complex of the enzyme in the presence of saturating glucose phosphates (active site blocked): 1 mM Glc-6-P, 0.05 mM Glc-1-P, and 1 mM Glc-P_2. (Spectrum 1) Change observed on formation of the vanadate-based inhibitor complexes: conditions same as for the spectrum 3, except for the absence of Glc-P_2. (b) Spectral comparisons used to assess the possible formation of complexes analogous to the vanadate-based inhibitor complexes. (Spectrum 5) Change observed on addition of vanadate to the Li^+ complex of the dephospho enzyme in the presence of 5 mM Glc-1-P and 15 mM LiCl. (Spectrum 6) Change produced by addition of vanadate to the Mg^{2+} complex of the enzyme in the presence of 1 mM 1-deoxyglucose-6-P. (Spectrum 7) (base-line spectrum) Effect of nitrate on the absorbance of an equilibrium mixture of the Glc-1-P and Glc-6-P complexes of the Mg^{2+} enzyme. The recorded spectral change was obtained by using the sectored-cell procedure (see Experimental Procedures); absorbances were corrected for the reduced light path before plotting: 10 mM Glc-6-P and 20 mM NO_3^- were present after mixing. The aromatic difference spectrum produced by adding V_i to the same mixture (spectrum 2, Figure 2a) is reproduced for comparison (after appropriate scaling) as spectrum 8. (c) Spectrum of the oxyvanadium constellation in the E·V-6-Glc-1-P(Li^+) complex and the difference spectra used to generate this spectrum. Spectra were obtained by using a single cell with a light path of 0.094 cm (see Experimental Procedures) that contained 30 mM LiCl, 10 mM Glc-1-P, 0.4 mM phosphoglucomutase, dephospho form, and 20 mM imidazole, pH 7.5. (Spectrum 9) Spectral difference in the presence and absence of 0.2 mM V_i. (Spectrum 10) Spectral difference in the presence and absence of 0.03 mM V_i when the above solutions also contained 2 mM Glc-P_2. (Spectrum 11) 0.9 of the spectral change produced by conversion of 0.17 mM E·Glc-1-P(Li^+) to E·Glc-P_2(Li^+). (Spectrum 12) Spectrum of the oxyvanadium constellation in E·V-6-Glc-1-P (Li^+), i.e., spectrum 9 after spectra 10 and 11 are subtracted. The spectrum of the oxyvanadium constellation in the inhibitor complex, E*V*Glc-1-P(Mg^+) from Figure 2b, is shown for comparison (---).

ingal, 1990) nor exhibits the spectral characteristics of E*V*6-Glc-1-P [Figure 3a, spectrum 1 (which is analogous to the upper spectrum in Figure 2a and reproduced here for comparison)]. In fact, the difference between the spectrum obtained with the free dephospho enzyme (spectrum 2) and that obtained with the active site blocked (spectrum 3) may be caused by the binding of dimeric or tetrameric vanadate at the distal phosphate-binding site in the dephospho enzyme rather than by formation of E-V (M. J. Gresser, personal communication).

Spectra of Other Systems That Might Produce Complexes Structurally Related to the Inhibitor Complexes of Phosphoglucomutase. Figure 3b shows the spectrum obtained when 30 μM dephospho enzyme is treated with 25 μM vanadate in the presence of 1-deoxyglucose-6-P (spectrum 6). This spectrum is almost identical with that of the control, where the active site of the enzyme has been blocked by the binding of Glc-P_2 (spectrum 3, Figure 3a). Spectra closely similar to spectrum 3 also are obtained when 6-deoxyglucose-1-P (1 mM) or xylose-1-P (2.5 mM) is used instead (not shown). These spectra fail to exhibit either the prominent absorbance peak of the oxyvanadium chromophore in E*V*6-Glc-1-P and E*V*1-Glc-6-P (spectrum 1, Figure 3a) or the changes associated with structural differences between the monophosphate complexes of the dephospho and phospho enzymes, viz., between E·Glc-1-P and E-P·Glc-1-P or between E·Glc-6-P and E-P·Glc-6-P, the result of which appears as fine structure superimposed on the broad difference peak in spectrum 1 (Figure 3a, see also below).

An attempt also was made to detect binding of NO_3^- to an equilibrium mixture of (E·Glc-6-P + E·Glc-1-P) complexes, since from a structural standpoint NO_3^- should resemble VO_3^-, and VO_3^- may well be present under the conditions used to produce the inhibitor complexes—at least in trace amounts[6]—and might bind to E·Glc-1-P to give E*V*6-Glc-1-P. But even at concentrations of NO_3^- at least 10^6-fold larger than the concentration of free V_i required to produce half-saturation of the above mixture of complexes with V_i [see the fourth paper in this series (Ray & Puvathingal, 1990)], no hint of the spectral changes associated with treatment of these complexes with V_i was observed (spectrum 7, Figure 3b). Thus, spectrum 8 of Figure 3b shows the size of the change expected, on the basis of spectrum 2 of Figure 2a, if NO_3^- could bind to (E·Glc-1-P + E·Glc-6-P) to produce a mixture of complexes analogous to the inhibitor complexes obtained on treatment with V_i.

By contrast with the above results, the addition of 25 μM vanadate to the E·Glc-1-P(Li^+) complex produces both a significantly increased absorbance relative to the control (active site blocked; spectrum 3, Figure 3a) and evidence for the perturbation of aromatic residues (spectrum 5, Figure 3b). However, these changes are far less dramatic than those produced under the same conditions when Mg^{2+} occupies the metal ion activating site (spectrum 1, Figure 3a). To determine whether this response is smaller, intrinsically, than that obtained with the Mg^{2+} enzyme requires the use of higher concentrations of enzyme or vanadate, or both, plus an independent estimate of vanadate binding—to correct for the change in the absorbance of the free vanadate that binds to

[6] At higher vanadate concentrations, polymers of VO_3^-, $(VO_3^-)_3$, or $V_3O_9^{3-}$ and $(VO_3^-)_4$ or $V_4O_{12}^{4-}$, etc., are present at near neutral pH (Habayeb & Hileman, 1980; Heath & Howrath, 1981). However, as with PO_3^- (Jencks, 1981; Herschlag & Jencks, 1986) free monomeric VO_3^- remains an elusive species. [VO_3^- in crystalline $NaVO_3$ is pentacoordinate and is present as a linear polymer (Bjornberg & Hedman, 1977).]

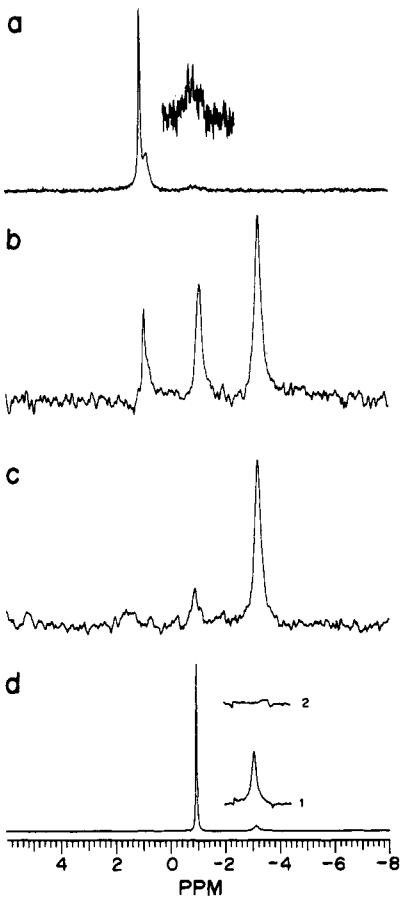

FIGURE 4: ^{31}P NMR spectra of complexes of the dephospho enzyme with glucose monophosphates in the absence or presence of inorganic vanadate. All spectra were obtained with solutions that contained 1.62 mM dephospho enzyme. The spectrometer recycle time was 5 s with a 60° pulse angle. Approximately 3600 transients were accumulated in each case, and an exponential line broadening of 1–5 Hz was applied before Fourier transformation. (a) Spectrum of 2.5 mM Glc-1-P plus Glc-6-P (equilibrium mixture) in the presence of dephospho enzyme and 40 mM imidazolium chloride buffer, pH 7.4, that contained 11 mM $MgCl_2$ and 1 mM EDTA. Resonances at about 1.0, 0.85, and −1.0 ppm are those of β-Glc-6-P, α-Glc-6-P, and α-Glc-1-P, respectively. (Inset) Segment of the same spectrum (between 0.5 and −22 ppm) on an expanded vertical but the same horizontal scale, with the base line displaced upward. (b) Spectrum of an equilibrium mixture of E*V*6-Glc-1-P(Mg^{2+}), −3.2 ppm, and E*V-1-Glc-6-P (Mg^{2+}), −1.05 ppm. A solution analogous to that in (a) but with only a slight excess of Glc-1-P was made 2 mM in V_i. The resonance at about 1.0 ppm is that of (α + β)Glc-6-P, due to the excess glucose phosphate that was present. (c) Spectrum of an equilibrium mixture of E*V*6-Glc-1-P(Cd^{2+}) and E*V*1-Glc-6-P (Cd^{2+}). Metal-free dephospho enzyme was treated with 1 equiv of Cd^{2+}, glucose phosphate, and V_i. (d) Spectrum of E·V-6-Glc-1-P(Li^+) obtained under forcing conditions. The metal-free dephospho enzyme was treated with 30 mM Li, 10 mM Glc-1-P, and 2.6 mM V_i. The resonance at about −1 ppm is that of the excess Glc-1-P. (Insets 1 and 2) Expanded vertical scale (10-fold) but same horizontal scale as (d). (Inset 1) Segment of the spectrum in (d); (inset 2) segment of a spectrum obtained under the same conditions except for the absence of V_i.

the enzyme. The fourth paper in this series (Ray & Puvathingal, 1990) describes a spectral titration of E·Glc-1-P-(Li^+) with V_i which shows that approximately 36 μM *free* V_i half-saturates the enzymic system under these conditions. Accordingly, a difference spectrum was obtained (in a cell with a light path of 0.094 cm) by making a solution of 0.4 mM E·Glc-1-P(Li^+) 0.2 mM in V_i (spectrum 9, Figure 3c). From this was subtracted the spectrum of 0.03 mM vanadate, the calculated concentration of "free vanadate", obtained in the presence of the same concentration of enzyme, but with the active site blocked (by Glc-P_2) (spectrum 10). The result (not shown) is a composite of the spectrum of the oxyvanadium constellation in E·V·6-Glc-1-P(Li^+) plus the spectral changes in the aromatic residues of the enzyme associated with the binding of V_i—as in spectrum 1 of Figure 2a. In contrast with the binding of V_i to the Mg^{2+} form of the enzyme, where binding involves an equilibrium mixture of E·Glc-1-P and E·Glc-6-P (because preparations of the dephospho enzyme always contain traces of catalytically active phospho enzyme) in the case of the Li^+ enzyme, it is binding to E·Glc-1-P, alone, that perturbs aromatic residues of the enzyme [because the Li^+ form of the contaminating phospho enzyme is such a poor catalyst (Ray et al., 1989)]. Hence, the binding-induced enzyme perturbation spectrum, though qualitatively similar to that in spectrum 8 of Figure 3b, is quantitatively somewhat different. However, as in Figure 2b, subtracting the appropriate binding-induced difference spectrum, spectrum 11 of Figure 3c, eliminates essentially all of the fine structure of the composite spectrum to give spectrum 12.[7]

The spectrum of the oxyvanadium constellation in the E·V-6-Glc-1-P(Li^+) complex, obtained as above, is quite different from the spectrum for the Mg^{2+} complex, which also is shown in Figure 3c (dashed-line plot) for reference. A spectrum for E·V-6-Glc-1-P(Li^+), similar to that obtained above with excess enzyme, also was obtained in the presence of a large excess of V_i at a saturating concentration (0.4 mM) after a spectral correction was made on the basis of stoichiometric binding of V_i to the E·Glc-1-P(Li^+) complex (not shown). [However, nonspecific binding effects are substantial at 0.4 mM V_i: see the fourth paper in this series (Ray & Puvathingal, 1990).]

^{31}P NMR Studies of the Inhibitor Complexes Produced by the Binding of Glucose Phosphates and Vanadate to the Mg(II) and Cd(II) Forms of the Dephospho Enzyme. Figure 4a shows the ^{31}P NMR spectrum of an equilibrium mixture of 2.5 mM glucose phosphates, Glc-1-P plus Glc-6-P, in the presence of 1.6 mM E(Mg^{2+}). The resonance of α-Glc-6-P, at about 1 ppm, is broadened slightly and shifted upfield somewhat by chemical exchange with bound Glc-6-P. The resonance of (α-)Glc-1-P at about −1 ppm, expanded scale, (inset), is broadened much more extensively by exchange, at least in part because the ratio of free to bound Glc-1-P is substantially smaller than that of free to bound α-Glc-6-P at chemical equilibrium [cf. data reported in Ray and Long (1976a)].

Figure 4b shows the NMR spectrum produced by a small excess of Glc-1-P plus Glc-6-P[8] and a larger excess of V_i (2 mM) relative to E(Mg^{2+}) (1.6 mM). In view of the spectral studies in the previous sections, the two resonances at about −3 and −1 ppm must be those of E*V*6-Glc-1-P(Mg^{2+}) and E*V*1-Glc-6-P(Mg^{2+}), respectively. Chemical shifts for ^{31}P

[7] According to the above calculations, the binding-induced difference spectrum for E_D·Glc-1-P(Li) → E_D·Glc-P_2(Li) involving 170 μM enzyme should have been subtracted. However, the elimination of fine structure from spectrum 9 was most effective when only 90% of the calculated value was employed. In view of the number of measurements and approximations required to obtain the estimate, 170 μM, using 90% of this value seems reasonable.

[8] With the relatively large ratio of enzyme:glucose phosphate used in the NMR studies, about 1:1, the same spectrum was observed whether the inhibitor complexes were produced from Glc-1-P or Glc-6-P, viz., equilibrium between the glucose phosphates is achieved rapidly relative to the time required to accumulate the spectrum. In absorbance studies, where the enzyme:glucose phosphate ratio was approximately 0.001, the complex obtained with Glc-1-P plus V_i initially was different from that obtained with Glc-6-P plus V_i (Figure 1a). However, after several hours, the same spectrum was observed with both sugar phosphates, as is expected from the NMR spectra.

in these complexes are assigned on the basis of a relatively close correspondence to those determined previously (Rhyu et al., 1983) for glucose phosphate in E-P·Glc-1-P(Li^+), −3.2 ppm, versus −3.2 ppm for E·V-6-Glc-1-P, and in E-P·Glc-6-P(Li^+), −0.2 ppm, versus −0.8 ppm for E·V-1-Glc-6-P. On the basis of previous studies with E·Glc-P_2(Cd^{2+}) (Post et al., 1989), T_1 for the enzyme-bound phosphates likely is about 5 s, which was the recycle time for successive transients in this study. If T_1 for the resonances of both bound phosphates is the same, from the ratio of their intensities the equilibrium mixture contains about 60% E*V*6-Glc-1-P and about 40% E*V*1-Glc-6-P.

Figure 4c shows the corresponding spectrum when Cd^{2+} instead of Mg^{2+} is bound at the metal ion activating site of the enzyme. The much larger ratio of E*V*6-Glc-1-P (−3.2 ppm) to E*V*1-Glc-6-P (−0.8 ppm) for the Cd^{2+} enzyme, relative to the same ratio for the Mg^{2+} enzyme (Figure 4b), is consistent with previous conclusions (Ray et al., 1989) that the Cd^{2+} enzyme is unable to facilitate (PO_3^-) transfer to and from the 1-position of Glc-6-P as efficiently as to and from the 6-position of Glc-1-P.

Figure 4d shows the results of similar studies with the Li^+ enzyme, but under "forcing" conditions: 10 mM Glc-1-P and 4 mM (total) V_i. The only broad resonance characteristic of enzyme-bound phosphate in this spectrum occurs at about −3 ppm, as above, and its intensity, relative to that of free Glc-1-P, is that expected if all of the enzyme is present as the E*V*6-Glc-1-P(Li^+) complex. That this resonance is dependent on the presence of V_i is shown by using the same expanded scale (insets of Figure 4d) to compare intensities of the resonance at about −3 ppm for the bound 1-phosphate: excess vanadate present (spectrum 1, inset); same conditions but in the absence of V_i (spectrum 2, inset). (Because the Li^+ form of the enzyme is a very poor catalyst, only Glc-1-P was present in these studies—as opposed to the other studies in this figure, where an equilibrium mixture of Glc-6-P and Glc-1-P was generated.) Hence, E·V-1-Glc-6-P(Li^+) is not present in Figure 4d.

Discussion

Inorganic vanadate, V(V), rapidly forms esters, anhydrides, and various adducts with the hydroxyl groups of a wide variety of compounds [cf. references by Gresser and co-workers and Tracey and co-workers in the third paper of this series (Ray & Post, 1990)]. In fact, the monoanion discriminates only marginally in the formation of esters from primary, secondary, and tertiary alcohols (Tracey et al., 1988), and both the *mono*anion, $(HO)_2VO_2^-$, and the *di*anion, $HOVO_3^{2-}$, form esters with most alcohols with comparable ease. In view of the variety of possible products that can form in solutions of vanadate, the simple observation that in the presence of a suitable hydroxyl compound vanadate inhibits an enzyme involved in the metabolism of phosphates is particularly difficult to interpret unequivocally.

In spite of such interpretational problems, interest in vanadates as phosphate analogues has remained, partly due to the tight binding of vanadate, itself, to some enzymes (VanEtten et al., 1974; Stankiewicz & Gresser, 1988) and partly due to the successful use of vanadate plus a hydroxyl compound to produce esters that in some cases are subject to catalytic reactions analogous to those of the corresponding phosphate ester, as in the case of glucose-6-phosphate dehydrogenase, which reduces glucose much more efficiently in the presence of vanadate (via Glc-6-V) than in its absence (Nour-Eldeen et al., 1985). But of even greater importance is the possibility that inorganic vanadate might form a pentacoordinate, enzyme-bound, diester anion and thus act as a type of transition-state analogue for enzyme-catalyzed reactions involving (PO_3^-) transfer. This possibility was posed first by Lindquist et al. (1973) for the ribonuclease system and subsequently for other enzymic systems [cf. VanEtten et al. (1974) and Stankiewicz and Gresser (1988)]. Later, Wlodawer et al. (1983) and Borah et al. (1985) verified the pentacoordinate nature of the ribonuclease/uridine/vanadate complex by analysis of neutron and X-ray diffraction data, respectively. Unfortunately, a comparable validation for the formation of a pentacoordinate complex thus far is lacking in other enzymic systems.

This paper, as well as the subsequent two papers in this series (Ray & Post, 1990; Ray & Puvathingal, 1990), describes attempts to use ultraviolet spectroscopy and binding studies to address various interpretational complexities associated with the many reactions in which vanadate may participate. Thus, to provide a unique rationale for the vanadate-induced inhibition of phosphoglucomutase (Percival et al., 1990), one first must consider the following questions: What is the actual inhibitor? How is it bound? What is the nature of the oxyvanadium constellation in the bound inhibitor?

Percival et al. (1990) show that, in the steady state, inhibition by vanadate plus Glc-1-P develops at a rate that is proportional to the concentration *product* of the two and therefore suggest that inhibition is produced by a mixed vanadate/phosphate diester, V-6-Glc-1-P, rather than by the separate binding of Glc-1-P and V_i. Their observation that the onset of inhibition "saturates" supports their suggestion, as do several of our observations. Thus, neither 6-deoxyglucose-1-P nor xylose-1-P nor 1-deoxyglucose-6-P acts together with V_i to produce a spectrally comparable complex of the dephospho enzyme—even at a concentration product more than 100-fold larger than the half-saturation value for the E*V*6-Glc-1-P complex. In addition, trapping experiments with Glc-1-P fail to detect E-V [analogous to E-P; see the fourth paper in this series (Ray & Puvathingal, 1990)], and NO_3^- fails to bind to E·Glc-6-P, as might be expected if the inhibitor complex were formed by the binding of VO_3^- to the E·Glc-6-P complex[6] in the manner that NO_3^- binds to the E·ADP·creatine complex in the creatine kinase system (Reed et al., 1978).

How are the V-6-Glc-1-P and V-1-Glc-6-P inhibitors bound? Our approach, here, involves a study of near ultraviolet spectral differences between the inhibitor complexes formed by V-6-Glc-1-P and V-1-Glc-6-P that reflect either direct or indirect interactions of specific hydroxyl groups of the glucose ring with aromatic chromophores of the enzyme (Ma & Ray, 1980). Thus, spectral *differences* between the complexes of V-1-Glc-6-P and V-6-Glc-1-P with the dephospho enzyme are compared with the spectral *differences* between the complexes of Glc-6-P and Glc-1-P with the phospho enzyme, where, by definition, the phosphate group attached to the glucose ring is bound at the distal site (see the introducton and Scheme I). The two sets of differences were essentially the same. Thus, in both inhibitor complexes the phosphate group binds as it does in those complexes that Glc-1-P and Glc-6-P normally make with the phospho enzyme, viz., at the distal phosphate site (Scheme I). Hence, the vanadate ester grouping must bind at the proximal site, and we represent the V-6-Glc-1-P complex as E*V*6-Glc-1-P, by analogy with the normal complex, E-P·Glc-1-P, and the V-1-Glc-6-P complex as E*V*1-Glc-6-P, by analogy with E-P·Glc-6-P. As noted in the introduction, the asterisks are used to emphasize a degree of uncertainty about chemical bonding in the oxy-

vanadium constellation of these complexes.

The validity of the above conclusion about preferred binding modes is reinforced by the sensitivity of the pairwise spectral comparison. In fact, differences in the way in which even one hydroxyl group in a substrate/product pair interacts with the enzyme are readily detectable, as in complexes involving the E-P·Man-1-P/E-P·Man-6-P pair, as opposed to the E-P·Glc-1-P/E-P·Glc-6-P pair. Moreover, dissimilarities in the way that two sugar hydroxyl groups interact with the enzyme in a substrate/product pair, as in E-P·Gal-6-P/E-P·Gal-1-P, produce spectral differences quite unlike those observed for both the E-P·Glc-6-P/E-P·Glc-1-P and E*V*1-Glc-6-P/E*V*6-Glc-1-P pairs (Ma & Ray, 1980). Hence, in the inhibitor complexes of the dephospho enzyme the sugar ring with its attached vanadate ester grouping must interact with the enzyme in the same manner as the normal sugar phosphate complexes of the phospho enzyme.

The results of ^{31}P NMR studies reinforce the case. Thus, the chemical shift of the phosphate group in E*V*6-Glc-1-P(Mg^{2+}) is essentially the same as that for the *sugar phosphate* in E-P·Glc-1-P(Li^+). In addition, the chemical shift in E*V*1-Glc-6-P(Mg^{2+}) differs from that above by about 2 ppm and is similar to that for the sugar phosphate in E-P·Glc-6-P(Li^+) (see Results and Figure 4c).

Since the vanadate group of the mixed phosphate/vanadate esters of glucose binds at the proximal site where (PO_3^-) transfer normally occurs, it is particularly important to evaluate the structure of the oxyvanadium constellation in these complexes, to decide whether the greatly increased kinetic and thermodynamic stability of these complexes, relative to that of the normal E·Glc-P_2 complexes, is caused by the formation of a pentacoordinate adduct of the vanadate ester grouping and to determine how the structure of these complexes might relate to that of the transition state for (PO_3^-) transfer in the normal enzymic reaction. These are the subjects of the two following papers in this series (Ray & Post, 1990; Ray & Puvathingal, 1990).

But, first, a brief consideration of whether the spectrum of the oxyvanadium constellation in Figure 2b might be the result of an artifact seems in order. The possibility of an artifact is considered, even though active-site binding seems firmly established, because of differences in the chemistry of vanadates and phosphates. These differences might allow a spurious ligand to enter the coordination sphere of V(V) during formation of the E*V*6-Glc-1-P complex, viz., a ligand that would not be bonded to phosphorus during the normal enzymic reaction. Thus, the entry of groups, such as RS^- or ArO^-, could provide a rationale for the spectrum of V(V) in the enzymic complex since these ligands are more polarizable than HO^- or RO^- [cf. Ray and Post (1990)]. But such an entry does not provide a reasonable rationale for some of the properties of these complexes. Specifically, simple thiol/vanadate complexes undergo a rapid reaction (Ray & Post, 1990), which, in view of the disappearance of the $^{51}V(V)$ NMR spectrum in the presence of thiols (M. J. Gresser, personal communication), likely is caused by reduction of V(V) to V(IV). By contrast, the binding of the V_i/Glc-1-P inhibitor in the phosphoglucomutase system is completely reversible [see the fourth paper in this series (Ray & Puvathingal, 1990)]. Alternatively, the entry of an ArO^- ligand from a tyrosine hydroxyl group would have produced anomolous absorbance changes in the region of the near ultraviolet where tyrosine absorbs. In fact, all tyrosine absorbance changes that accompany formation of the inhibitor complex are readily accounted for in terms of binding of the phosphoglucosyl moiety to the enzyme. Other observations that make an artifact seem unlikely include the failure of the two deoxysugar phosphates or of xylose-1-P to form a complex with vanadate plus the dephospho enzyme that is comparable to the complex formed by Glc-1-P plus vanadate.

In addition, several observations suggest that the process generating the unusual absorbance spectrum which accompanies formation of the inhibitor complexes is mechanism-based. For example, although Glc-P_2 binds to the metal-free enzyme nearly as well as to E(Mg^{2+}) (Ray et al., 1965; Ray & Long, 1976b), V-6-Glc-1-P does not bind detectably to the catalytically inactive metal-free form of the dephospho enzyme on treatment with Glc-1-P and V_i at a concentration product much larger than that which produces half-saturation of the Mg^{2+} enzyme, as judged by the failure to form the characteristic spectrum of E*V*6-Glc-1-P(Mg^{2+}). The absorbance spectrum of the complex that eventually forms when the catalytically inactive Li^+ complex of the enzyme is treated with much higher concentrations of V_i and Glc-1-P than required to half-saturate the Mg^{2+} enzyme (spectrum 12, Figure 3c) also is rather different from that of the E*V*6-Glc-1-P(Mg^{2+}) complex (dashed-line spectrum, Figure 3c). Thus, the formation of the inhibitor complex depends on a bivalent metal ion in a manner related to the dependence of catalytic activity on metal ions [cf. the fourth paper in this series (Ray & Puvathingal, 1990)]. The present results thus legitimize the candicacy of bound V-6-Glc-1-P for designation as a transition-state analogue and place the more detailed analysis of spectral and thermodynamic observations described in the accompanying papers on a firm foundation.

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