Comparison of Vibrational Frequencies of Critical Bonds in Ground-State Complexes and in a Vanadate-Based Transition-State Analog Complex of Muscle Phosphoglucomutase. Mechanistic Implications†

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Received May 7, 1993; Revised Manuscript Received August 20, 1993®

ABSTRACT: The symmetric stretching frequency of the P–O bonds of the enzymic phosphate group in muscle phosphoglucomutase was measured via $^{16}O/^{18}O$ Raman difference spectroscopy. This frequency, and its shift on isotopic substitution, is characteristic of a dianionic phosphate ester. The P–O stretching frequency is not detectably altered by the binding of the metal ion activators Mg^{2+}, Zn^{2+}, or Cd^{2+} nor by the subsequent binding of glucose phosphate. Hence, a binding-induced distortion/polarization of the enzymic phosphate group in the ground state, or enzyme–substrate complex, cannot serve as a rationale for the large value of k_{cat} in the phosphoglucomutase reaction. By contrast, the stretching frequency of the V–O bonds within a vanadate group bound at the same site in the transition-state analog complex involving glucose 1-phosphate 6-vanadate is much lower than for a normal dianionic vanadate. This low V–O stretching frequency is best rationalized in terms of the extensive polarization of *all three* nonbridging oxygens of the vanadate ester dianion plus the formation of a weak, fifth bond to the vanadium atom. This distortion/polarization of the VO_3^{2-} group depends on the metal ion activator, since it is largely abolished, and the involvement of the fifth ligand eliminated, by substitution of Li^+ for Mg^{2+} at the metal activation site. To the extent that the vanadate–inhibitor complex mimics the transition state for the normal phosphoglucomutase reaction, as has been suggested [Ray, W. J., Jr., & Puvathingal, J. M. (1990) *Biochemistry 29*, 2790], the normal PO_3^- transfer is best described as a process with S_N2-like or associative character and thus is quite different from the process by which model phosphate ester dianions normally react in aqueous solution.

Phosphoglucomutase catalyzes the reversible transfer of the PO_3^- fragment of dianionic phosphate ester (Rhyu *et al.*, 1985) between the 1- and 6-oxygens of a glucose molecule in a process that involves two binding steps, two transfer steps, and a fifth step where the initially formed intermediate complex rearranges. The overall process, which involves both the phospho and dephospho forms of the enzyme, E_P and E_D,[1] respectively, is illustrated in Scheme I [*cf.* Ray *et al.* (1990)]. The present study involves an evaluation of the internal bonding in the phosphate group bound at the proximal or transfer subsite. This bonding is evaluated in the metal-free enzyme, in various binary enzyme metal–activator complexes, Ep·M, and in mixtures of ternary complexes involving primarily the reaction intermediate or reaction product, *viz.*, in E_D·M·P-6-Glc-1-P or Ep·M·Glc-6-P complexes, respectively. The results are compared with those for the vanadate group that replaces the proximal phosphate group in the transition-state analog complex, E_D·M·V-6-Glc-1-P (Percival & Gresser, 1990; Ray *et al.*, 1990; Ray & Post, 1990; Ray & Puvathingal, 1990). Note that, in representing such complexes, the phosphate to the left of –Glc– designates the one bound at the proximal subsite, where it interacts directly with the activating metal ion, M^{2+}. [This metal ion normally is Mg^{2+}, but Mg^{2+} can be replaced by other bivalent metal ions such as Zn^{2+} or Cd^{2+} (Rhyu *et al.*, 1984; Ray *et al.*, 1993b).] The phosphate group to the right of –Glc– represents the one bound at the distal subsite, some distance removed from the metal ion [*cf.* Ray *et al.* (1993a)], as is illustrated in Scheme I.

Although there are four structurally different ternary complexes in the phosphoglucomutase system (Scheme I), at equilibrium the intermediate complex, II, predominates over III because binding interactions are significantly stronger in II than in III (Ray *et al.*, 1993a). This difference, plus an equilibrium that favors bound Glc-6-P over bound Glc-1-P (Ray & Long, 1976a), effectively reduces the four-complex equilibrium mixture to a two-complex system:

$$\underset{\text{II}}{E_D\cdot M\cdot P\text{-}6\text{-}Glc\text{-}1\text{-}P} \rightleftharpoons \underset{\text{IV}}{Ep\cdot M\cdot Glc\text{-}6\text{-}P} \qquad (1)$$

In some cases, the relative contributions of the bisphosphate complex, II, and the monophosphate complex, IV, to the physical/chemical properties of the equilibrium mixture can be assessed by changing the identity of the activating metal ion, M. Thus, a change from Zn^{2+} to Cd^{2+} shifts the

† This research was supported by grants from the U.S. Public Health Service (GM-08963 to W.J.R. and GM-35183 to R.C.) and by a grant from the National Science Foundation (MCB-8912322 to R.C.).

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® Abstract published in *Advance ACS Abstracts*, November 15, 1993.

[1] Abbreviations: E_P and E_D, the phospho and dephospho forms of phosphoglucomutase, respectively; M, metal ion bound at the activation site of the enzyme, *e.g.*, E_P·M, otherwise, M^{n+}; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, in solution, the equilibrium mixture of (α + β)-D-glucose 6-phosphate, but in a complex with the enzyme, only the α-anomer is referred to, as in E_P·M·Glc-6-P; Glc-P, in solution, an equilibrium mixture of α-Glc-1-P and (α + β)-Glc-6-P, but in complexes with the enzyme, only the α-anomer of both is referred to, as in the monophosphate complexes of the phosphoenzyme E_P·M·Glc-P; Glc-1, 6-P_2 or Glc-P_2, α-D-glucose 1,6-bisphosphate; E·P·M·Glc-P, the equilibrium mixture of central complexes (*cf.* Scheme I); Glc-1-P-6-V and Glc-6-P-1-V, and 6-vanadate or 1-vanadate esters of Glc-1-P or Glc-6-P, respectively; V_i, inorganic vanadate.

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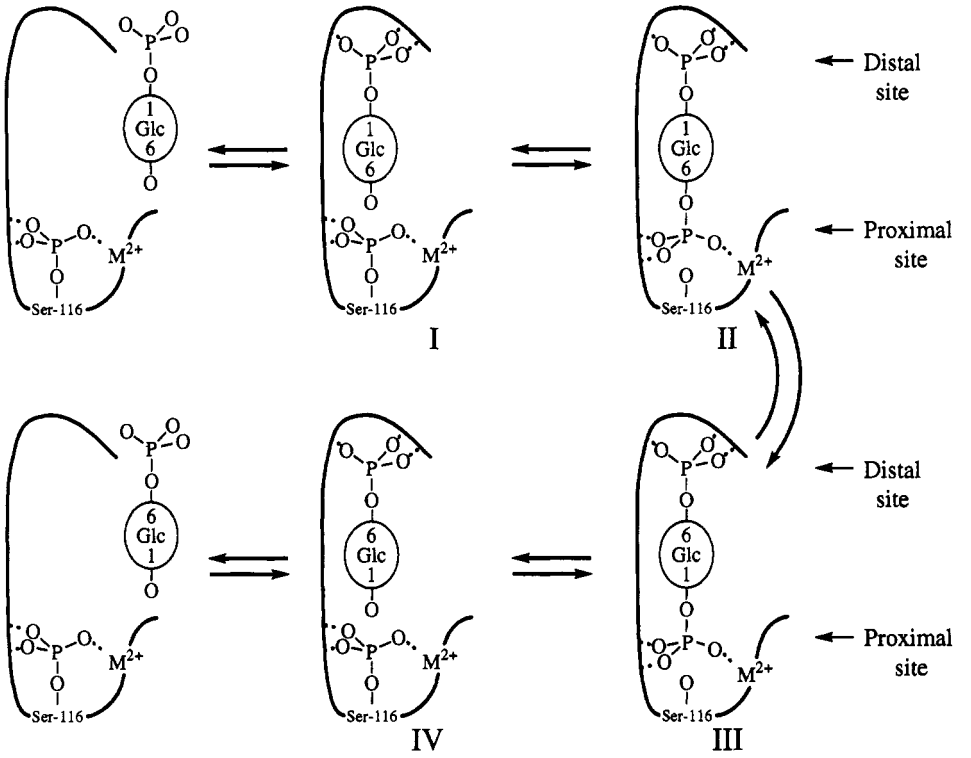
Scheme I: Schematic Showing the Gross Structural Relationships among the Four Ternary Enzyme–Metal Ion Complexes That Are Involved in the Phosphoglucomutase Reaction



equilibrium from predominately Ep·M·Glc-6-P to almost exclusively E_D·M·P-6-Glc-1-P [Ray & Long, 1976b; see also Ray *et al.* (1993b)].

Whereas the distal subsite is structured to optimize the binding of a tetrahedral phosphate ester dianion, or analogs thereof, the proximal subsite, which is vacant in the dephospho enzyme, may not be so structured (Ray *et al.*, 1993a). This possibility, together with the nearly 10^6-fold greater stability of E_D·Mg·V-6-Glc-1-P relative to the corresponding P-6-Glc-1-P complex (Ray & Puvathingal, 1990), plus a consideration of the structure of the probable transition state for the PO_3^--transfer process (see Discussion), suggests that the proximal subsite is designed to optimally bind a phosphate ester dianion that is substantially distorted/polarized in the ground and/or transition state, either geometrically or electronically (Ray *et al.*, 1993a). The activating metal ion apparently is intimately involved in producing this distortion/polarization since the replacement of the bivalent metal ion at the metal ion activating site by monovalent Li^+ dramatically decreases both enzymic activity (Ray *et al.*, 1989) and the stability of a transition-state analog complex (Ray & Puvathingal, 1990).

The present paper describes the use of classical Raman difference spectra to determine whether part of the distortion/polarization of the proximal-site phosphate group that should characterize the transition state for PO_3^- transfer might be produced in the ground state by the binding of the metal ion activator or the subsequent binding of the substrate. In fact, part of the significance of our results lies in the demonstration that Raman spectroscopy of single phosphate or vanadate groups at the active site of an enzyme with a molecular mass in the range of 60 KDa can furnish such information when the sensitive difference techniques, developed in the laboratory of one of the authors [*cf.* Yue *et al.* (1989)], are used. In the present study, the phosphate band, which arises from the symmetrical P–O stretching mode of the phosphate group [*cf.* Ray *et al.* (1993c)], is very weak relative to many of the protein bands and is identified by isotopic editing with ^{18}O [*cf.* Manor *et al.* (1991)]. Fortunately, the corresponding vanadate band is much stronger, and its identification does not require isotopic labeling. By comparison of the frequencies of these bands in the apoenzyme, and in complexes thereof, with the solution frequencies of model compounds, changes in internal bonding of phosphate and vanadate groups can be assessed on the basis of the studies described in the accompanying paper (Ray *et al.*, 1993c).

EXPERIMENTAL PROCEDURES

In the interest of brevity a number of experimental procedures are presented here in outline form. A detailed version of these is available as supplementary material.

Materials. Phosphoglucomutase, phospho form, was prepared from mature rabbit muscle (Pel Freeze) via an unpublished procedure, available on request [see Ray *et al.* (1983) for an outline of this procedure]. Extinction coefficients at 278 nm of 7.0 for 1 mg/mL or 43 100 for 1 M [*cf.* Ray *et al.* (1983)] were used in calculating enzyme concentrations. In solutions where the enzyme was present as its complex with Glc-1-P and Na_2HVO_4, enzyme concentration was measured by using a modified version of the Bradford dye-binding assay designed to provide a more reproducible, linear response, especially at low protein concentration. The dephosphoenzyme (Mg^{2+} complex) was prepared from the phosphoenzyme by multiple treatments with excess Glc-1-P in the presence of 30–65% $(NH_4)_2SO_4$, interspersed by protein precipitation steps, until an assay for the phosphoenzyme (see below) showed that <0.2% remained. Unless otherwise indicated, Glc-1-P (Sigma) was used without further purification; when necessary, essentially all contaminating Glc-P_2 was eliminated via batchwise chromatography (Ray *et al.*, 1990). A stock solution of 1 M "Na_2HVO_4" was prepared by the reaction of V_2O_5 (Aldrich) with 4.0 equiv of NaOH.

[$^{18}O_4$]inorganic phosphate was prepared from PCl_5 and [^{18}O]H_2O (Ray, 1992). Glc-1-[$^{18}O_4$]P was prepared from [$^{18}O_4$]P_i via the sucrose phosphorylase reaction [*cf.* Ray *et al.* (1989)].

A number of solutions with which the enzyme was treated were "demetallated" by passing a stock solution through an appropriately equilibrated column of Chelex resin (Bio-Rad). In the remainder of this section components marked with an asterisk were so treated.

[$^{18}O_3$]Phosphate-labeled phosphoglucomutase was prepared by treating the Zn^{2+} form of the phosphoenzyme three successive times with 3 equiv each of Glc-1-[$^{18}O_4$]P in the presence of excess NAD^+ and Glc-6-P dehydrogenase and chromatographically separating the labeled phosphoenzyme from any dephosphoenzyme formed in the process. The phosphoenzyme thus obtained was precipitated with ammonium sulfate and dissolved in a minimal volume of Tris buffer*, pH 7.5, containing EDTA. The enzyme solution was concentrated and demetallated by pressure dialysis against EDTA at 4 °C. The EDTA subsequently was eliminated and the sample further concentrated (to 250–380 mg/mL) by additional pressure dialysis. A second identical sample of the enzyme was treated in the same way, but with natural abundance Glc-1-P, prepared in the same way as Glc-1-[$^{18}O_4$]P.

Enzyme samples for Raman spectroscopy were made in 0.5-mL plastic centriguge tubes by mixing in the required additives and dilutent so that the protein concentrations in ^{18}O- and ^{16}O-labeled samples were as closely matched as possible. When Glc-1-P was added to pairs of samples, Glc-1-[$^{18}O_4$]P* was added to the [$^{18}O_3$]P-phosphoenzyme and Glc-1-[$^{16}O_4$]P* to the [$^{16}O_3$]P-phosphoenzyme.

A concentrated solution of the E_D·Mg·V-6-Glc-1-P complex was prepared in three different ways. The direct procedure, procedure A, involved treatment of a concentrated solution of E_D·Mg with 0.8 equiv of Na_2HVO_4 in the presence of excess Glc-P [*cf.* Ray and Post (1990)]. To eliminate the substantial fraction of the normal E·P·Mg·Glc-P complexes present in that mixture, the vanadate complex also was prepared from an $(NH_4)_2SO_4$ precipitate of the phosphoenzyme by successively suspending the precipitate in $(NH_4)_2SO_4$ solutions (65% of saturation) that contained both Glc-1-P and V_i (procedure B). At the end of the treatment, the protein precipitate was dissolved and pressure dialysis at 4 °C employed to desalt and concentrate the protein. Partial equilibration during this step converted 15–20% of the E_D·Mg·V-6-Glc-1-P to E_D·Mg·V-1-Glc-6-P (see Results). To produce a product that contained less than 2% of the alternative E_D·Mg·V-1-Glc-6-P complex, as well as essentially no E·P·Mg·Glc-P, treatment with Glc-1-P plus V_i was conducted in solution, with a subsequent $(NH_4)_2SO_4$ precipitation step (procedure C). After the protein was dissolved in a small volume of buffer, the excess Glc-1-P and V_i were eliminated by centrifugal gel filtration at 4 °C on a column of Sepharose (Spectrogel), and the effluent was concentrated by pressure dialysis. This product contained a small amount of inorganic vanadate bound to the enzyme in excess of that bound at the proximal subsite; see Results. Assays for the E_D·Mg·V-6-Glc-1-P and E_D·Mg·V-1-Glc-6-P complexes have been described (Ray & Puvathingal, 1990).

The Li^+ form of dephosphophosphoglucomutase was prepared from the Mg^{2+} form by dialyzing (and concentrating) the protein (under pressure) at 4 °C versus 30 mM LiCl (Alpha, high purity)*, 10 mM EDTA, 20 mM Tris buffer*. After the Mg^{2+} was eliminated, EDTA was removed by continuing the dialysis against the same mixture but with a suspension of Chelex resin, Li^+ form, substituted for the EDTA.

The E_D·Li·V-6-Glc-1-P complex was produced by addition of Na_2HVO_4 to a solution that contained 20 mM Glc-1-P*, 20 mM Tris buffer*, pH 7.4, and 30 mM LiCl* plus E_D·Li. The corresponding complex involving Glc-P_2 was obtained by substituting a slight excess of the bisphosphate* for the Glc-1-P plus Na_2HVO_4 in the above solution.

Procedures. The concentration of the phosphoenzyme, in the presence of a much higher concentration of the dephosphoenzyme, was determined by quantifying the amount of Glc-6-P produced by 0.2 mg of the Mg^{2+} enzyme after addition to a 2-mL reaction mixture that contained 0.15 mM purified Glc-1-P (no Glc-P_2) and excess EDTA, 5 mM. The reaction mixture, in a stirred spectrophotometer cell (light path = 1 cm) at 25 °C, also contained 10 mM Tris/Tris-HCl*, pH 7.4, and 5 μg of Glc-6-P dehydrogenase [from a 5 mg/mL stock solution in 10 mM $(NH_4)_2SO_4$]. [Only the Ep·Mg present in the added sample is active in the assay, and that form of the enzyme is active only for as long as Mg^{2+} remains bound to the enzyme; *cf.* Magneson *et al.* (1987).] The assay was calibrated by including known amounts of the phosphoenzyme in the 20-μL sample of enzyme that was added to the assay. The maximal optical density was obtained in 3–5 min; the assay response was linear to 4% Ep: $\Delta OD_{340nm} = 0.52$.

The procedures and controls used in obtaining Raman difference spectra of the enzyme and its complexes with various ligands, which involve "isotopic editing", have been discussed (Yue *et al.*, 1989; Deng *et al.*, 1991; Manor *et al.*, 1991). In essence, a specially designed cuvette with a split chamber, the sections of which hold the "sample" and the "reference" solutions, is mounted on a translator stage attached to a stepping motor and placed in the laser beam of the spectrometer. Spectra from one side of the cuvette and the other are collected alternatively by using an optical monochrometer with a multichannel detector system. Difference spectra are obtained by direct subtraction of the individual spectra, after appropriate scaling. The spectrometer was calibrated with a toluene reference, and reported band positions are accurate to within ±3 cm^{-1}; slits were set to achieve a resolution of 8 cm^{-1}. However, wavelength mismatches caused by small differences in how scattered light from the sample and reference cells impinges on the detector sometimes produced subtraction artifacts [*cf.* Yue *et al.* (1989)] that were comparable in intensity to the relatively weak phosphate band. The strong, sharp 1004-cm^{-1} phenylalanine band was particularly troublesome in this respect. To reduce the size of subtraction artifacts in isotope-edited difference spectra, and thus improve the signal to noise ratio, the frequency of the reference spectrum usually was shifted before the subtraction was conducted. The assumption that is made in utilizing a shifted reference spectrum, which was confirmed in those runs where no shift was necessary, is that ^{18}O labeling of the phosphate group of the enzyme and its substrate does not affect the 1004-cm^{-1} Raman band of the protein; *viz.*, the protein/phosphate ^{16}O–^{18}O difference spectrum should not contain a sharp difference pattern at 1004 cm^{-1}. If a difference pattern is observed, a frequency shift that will null the false pattern can be calculated from the peak/valley distance in that pattern plus the band width and intensity of the peak in the primary spectrum that produces the false pattern (Rouseau, 1981; Yue *et al.*, 1989). In all cases, the calculated shift was in the range of 0–0.2 cm^{-1} and in no case was the broad 977/935-cm^{-1} peak/trough pattern produced by bound phosphate (see Results) substantially affected in either position or intensity. Not only was the false difference pattern at about 1004 cm^{-1} largely eliminated by utilizing of the shift calculated with this band but false difference patterns arising from other protein bands also were minimized. In addition, this procedure produced sufficiently flat background signals that averaging of difference spectra obtained at different times

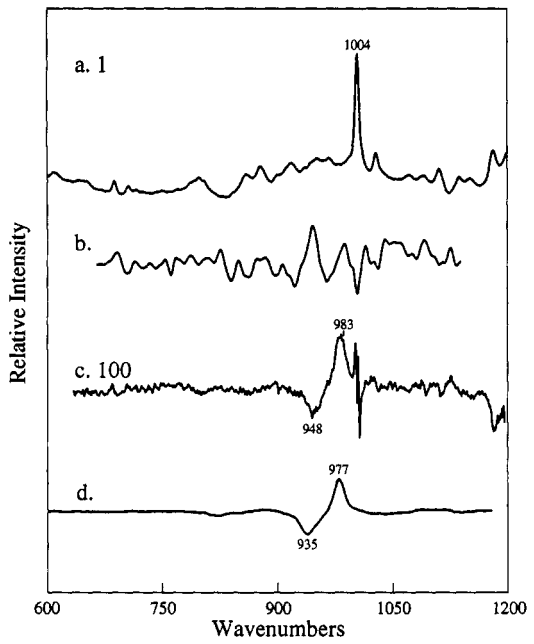


FIGURE 1: Raman spectra of phosphoglucomutase and Glc-6-P in the phosphate region of the spectrum. The pH was 7.4, and the temperature of the water circulated through the jacketed sample cells was 4 °C. Approximately 100 scans each for both sample and reference cells were accumulated during a time interval of about 2 h when spectra of the enzyme were measured; in the case of Glc-6-P, 20 scans each were obtained. A 514.5-nm laser was used, and spectra involving the enzyme were scaled to the 1004-cm^{-1} phenylalanine peak; relative scaling factors for panels a and c are 1 and 100, respectively. Panels: (a) metal-free phosphoenzyme; (b) the $E_P - E_D$ difference spectrum; (c) average of three $[^{16}O_3]E_P - [^{18}O_3]E_P$ difference spectra, after frequency corrections based on the size of the 1004-cm^{-1} difference peak were applied (see Experimental Procedures); (d) Glc-6-$[^{18}O_3]$P – Glc-6-$[^{18}O_3]$P difference spectrum.

with different samples was possible. This not only improved the final signal to noise ratio but provided a check on reproducibility. A quantitative discussion of this procedure will be published elsewhere (J. W. Burgner, II, and R. Callender, manuscript in preparation). Bond strengths, in valence units (vu), were calculated as in the accompanying paper.

RESULTS

Raman Difference Spectra. Phosphate Group of the Phosphoenzyme. In the direct procedure for obtaining Raman difference spectra, where the spectrum of the ligand-free protein is subtracted from that of the protein–ligand complex, the difference spectrum usually contains vibrational difference bands caused by ligand-induced changes in the protein, in addition to the vibrational bands of the bound ligand. In such cases, the spectrum of the protein, *cf.* Figure 1a, can aid in identifying vibrational bands of the protein that are altered by ligand binding. In the present case, even a small change in the sharp prominent peak at 1004 cm^{-1} in the protein spectrum, which arises from an aromatic ring mode involving the 31 phenylalanines in phosphoglucomutase (Ray *et al.*, 1983), would produce a sizable peak/trough pattern in a difference spectrum. Thus, the intensity of this band (signal to noise ratio > 1000/1) is some 140-fold greater than the P=O stretching band of the phosphoenzyme, which is expected near 977 cm^{-1}, on the basis of solution studies of dianionic inorganic phosphate and its methyl ester (Ray *et al.*, 1993c).

Figure 1b shows the spectral difference between the phospho and dephospho forms of the enzyme, $E_P - E_D$, on an amplified scale. Unfortunately, in Figure 1b the number and intensity of protein difference bands in the 900–1000-cm^{-1} spectral region largely obscure the phosphate band of Ep, and significant conformational differences between the phospho and dephospho forms of the enzyme seem likely. But, as in other cases (Manor *et al.*, 1991), the alternative approach to Raman difference spectroscopy, which involves isotopic editing, can be employed to isolate the vibrational mode or modes of the enzymic phosphate group. This is accomplished by obtaining a difference spectrum of unlabeled and labeled enzyme, where the labeled enzyme contains ^{18}O in all three nonbridging oxygens of its phosphate group. In such a spectrum, only those normal modes that are affected by the isotopic substitution are observed; *i.e.*, vibrational bands due to structural differences between Ep and E_D, which are prominent in Figure 1b, no longer appear.

Figure 1c shows the spectral difference between the ^{16}O- and ^{18}O-labeled phosphoenzyme, *i.e.*, $[^{16}O_3]E_P - [^{18}O_3]E_P$. In this spectrum, a positive peak at 983 cm^{-1} and a negative trough at 948 cm^{-1} now are obvious. The noisy part of the difference spectrum near 1004 cm^{-1} arises from a combination of an incomplete nulling of the phenylalanine band (see above) and of larger shot noise inherent in this strong band. For comparison, the related $^{16}O - {}^{18}O$ phosphate difference spectrum of Glc-6-P is shown in Figure 1d, where the 977/935-cm^{-1} peak/trough pattern is more distinct because of the higher concentration of phosphate used and hence the reduced noise. From its position and shift upon ^{18}O labeling, the 983-cm^{-1} band in Figure 1c is assigned to the symmetrical stretching frequency of the enzymic $-PO_3^{2-}$ group [*cf.* the accompanying paper: Ray *et al.* (1993c)]. Although there appears to be a change in frequency of the enzymatic phosphate band from its position in a solution model, Glc-6-P^{2-}, *i.e.*, from 977 to 983 cm^{-1}, this difference is close to our error limits of about ±3 cm^{-1}. On the other hand, a significant shift in band position can be produced by environmental effects, as is indicated by the effect of various other solvents on the stretching frequency of the $-PO_3^{2-}$ group of $((CH_3)_4N)_2(CH_3OPO_3)$. Thus, solvents that are less polar than water, or solvents that cannot serve as H-bond donors, *e.g.*, dimethylformamide or $CHCl_3$, can produce frequency shifts of up to 20 cm^{-1} (H. Deng, unpublished observations). However, a large change in solvent polarity or hydrogen-bonding capacity is required to produce a substantial environmental effect in the above model system, since changing the solvent from water to CH_3OH produces a frequency change of only about 5 cm^{-1}. In any case, bond strength within the $-PO_3^{2-}$ group, which can be estimated in terms of P=O stretching frequency [see accompanying paper: Ray *et al.* (1993a)], is practically unaffected by its incorporation into the enzyme.

Effect of Metal Ion Binding on the Raman Difference Spectrum of the Enzymic Phosphate Group. Figure 2 shows the effect on the symmetrical P=O stretching mode of the enzymic phosphate produced by the binding of bivalent metal ions at the activation site of the phosphoenzyme. The addition of neither Zn^{2+}, Mg^{2+}, nor Cd^{2+} produces a significant effect in the respective $[^{16}O_3]E_P{\cdot}M - [^{18}O_3]E_P{\cdot}M$ difference spectra, shown as overlays in panel a, relative to $[^{16}O_3]E_P - [^{18}O_3]E_P$ shown in panel b, which is repeated as a reference from Figure 1d, after scaling as in panel a. Similarly, the binding of Li^+, which produces an essentially inactive binary complex (Ray *et al.*, 1989), also fails to produce a significant effect on the frequency of the phosphate stretching mode (not shown). This lack of sensitivity to direct metal ion coordination (Ray *et al.*, 1993b) is not particularly surprising since the replacement of hydrogen-bonded water by Mg^{2+} does not produce a substantial change in the P=O stretching frequency of Glc-6-P^{2-}. Thus

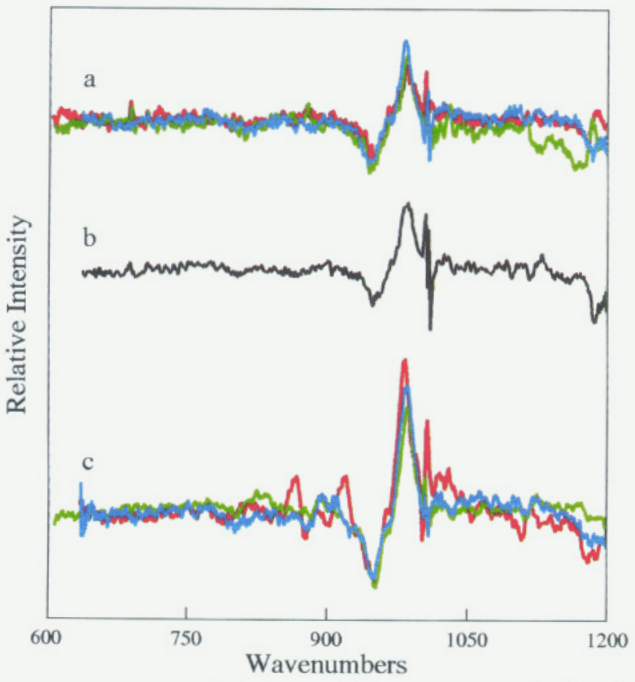


FIGURE 2: Isotope-edited Raman difference spectra showing the effect of the metal ion binding step and the substrate binding plus PO_3^- transfer steps on the stretching frequency of the proximal phosphate group. Conditions were as in Figure 1. (a) Overlays of the $[^{18}O_3]E_P{\cdot}M - [^{16}O_3]E_P{\cdot}M$ Raman difference spectrum produced when $M^{2+} = Mg^{2+}$ (red), Cd^{2+} (blue), and Zn^{2+} (green). (b) Isotopic difference spectrum obtained with the metal-free enzyme (repeated from Figure 1c for reference). (c) Isotopic difference spectrum after the addition of 1 equiv of Glc-P to each of the three metal ion complexes of the enzyme in panel a. All difference spectra were scaled to the 1004-cm^{-1} peak in the parent spectrum.

when the Mg^{2+} concentration (100 mM at $\mu = 0.36$) is high enough to ensure that most of the Glc-6-P in a solution is present as its Mg^{2+} complex,[2] the above frequency increases only by about 5 cm^{-1} (as deduced from the relevant Raman difference spectrum; not shown). [A similar change in stretching frequency is observed when Mg^{2+} binds to ADP or ATP (Takeuchi *et al.*, 1988); in addition, there is no significant ^{18}O isotope effect on Mg^{2+} coordination to inorganic phosphate (dianion: Jones *et al.*, 1991.] By contrast, conversion of one of the P=O groups in $(CH_3OPO_3^{2-})_{aq}$ to P=OH increases the stretching frequency of the remaining P=O groups by some 95 cm^{-1} (Ray *et al.*, 1993c). In addition to the failure of coordination Mg^{2+} to alter the P=O frequency of Glc-6-P in solution, P=O bond distances in crystalline phosphate ester anions are not systematically altered to a substantial extent by differences in $P{=}O{\cdot\cdot}M^{2+}$ interactions [*cf.* Corbridge (1967, 1990)].

P=O Stretching Frequency of the Proximal-Site Phosphate in Enzyme–Glucose Phosphate Complexes. The subsequent binding of glucose phosphate to the phosphoenzyme produces an equilibrium mixture of substrate/intermediate/product complexes (Scheme I and introduction) that is represented as E·P·M·Glc-P. One of the two phosphate groups present in each of these complexes occupies the proximal site, where the attachment of phosphate may be to the enzyme or to the glucose moiety, whereas the other occupies the distal site, where its attachment always is to the glucose moiety; see Scheme I. As in the case of metal ion binding, the binding/transfer steps that produce this mixture also do not substantially alter the P=O stretching frequency of the phosphate group at the proximal site. This is shown in Figure 2c, which displays overlaid $^{16}O - ^{18}O$ difference spectra of the equilibrium mixture of the above complexes, *viz.*, $E{\cdot}[^{16}O_3]P{\cdot}M{\cdot}Glc[^{16}O_3]P - E{\cdot}[^{18}O_3]P{\cdot}M{\cdot}Glc[^{18}O_3]P$, where M is Zn^{2+}, Mg^{2+}, or Cd^{2+}. Here, only a single phosphate difference band is observed—but at a substantially increased intensity relative to $[^{16}O_3]E_P - [^{18}O_3]E_P$ and the corresponding M^{2+} complexes, since now two phosphate groups instead of one are present. *Cf.* panels a and b of Figure 2, which are scaled in the same way (see figure legend). To provide a guide for evaluating the spectra in Figure 2c, model $^{16}O - ^{18}O$ difference spectra were constructed by using the observed isotopic spectra for Glc-1-P and Glc-6-P, according to the known proportions of the various complexes present in these equilibrium mixtures (Ray & Long, 1976b) with the following assumptions: the P=O frequencies of the proximal-site phosphate, regardless of its attachment, and of the distal-site phosphate when attached to the 6-position of glucose are the same as that of free Glc-6-P; the P=O frequency of the distal-site phosphate when attached to the 1-position of glucose is 10 cm^{-1} lower than that of Glc-6-P, as it is in solution. In each case, the stretching frequency of the proximal-site phosphate subsequently was changed systematically by 10, 15, and 20 cm^{-1} to simulate changes in that group that might occur in the equilibrium mixture of bound substrate/intermediate/product complexes to prepare the phosphate for PO_3^- transfer. Although the pattern of the simulated $^{16}O - ^{18}O$ difference spectrum produced by a 10-cm^{-1} frequency change was easy to recognize in these essentially noise-free spectra, we can claim only that the binding-transfer steps that produce the above equilibrium mixture must change the frequency of the proximal-site phosphate by less than 15 cm^{-1}, which is equivalent to a change in bond strength of less than 0.02 vu, if at all (see below). This limit stands in contrast with the 95-cm^{-1} frequency change noted above that is produced by the conversion of one P=OH group in $-PO_3^{2-}$ to P=OH. Hence, we conclude that the internal bonding of the PO_3^- fragment whose transfer interconverts substrate, intermediate, and product complexes is nearly the same before and after the transfer step and is similar to that found in a normal phosphate ester dianion in aqueous solution.

Raman Spectrum of the Vanadate-Based Transition-State Analog Complex. Figure 3a shows the Raman difference spectrum of the vanadate-based transition-state analog complex and the related ternary complexes, where a phosphate instead of a vanadate group is present, *viz.*, $E_D{\cdot}Mg{\cdot}V\text{-}6\text{-}Glc\text{-}1\text{-}P - E{\cdot}P{\cdot}Mg{\cdot}Glc\text{-}P$. Here, bands (two) due to V=O stretching modes are more intense than protein bands with similar frequencies, and the vanadate bands clearly dominate the difference spectrum. Hence, it was unnecessary to resort to ^{18}O editing to evaluate the frequency of the V=O band. The enhanced Raman intensity of bound V=O groups, relative to the corresponding P=O groups, is caused in part by the unusual spectral properties of vanadate in this complex, where the ligand to metal electron-transfer spectrum is red-shifted by approximately 5000 cm^{-1} relative to $CH_3OVO_3^{2-}$ and where the UV absorbance peak at about 310 nm "tails" almost into the visible region (Ray & Post, 1990). A preresonance enhancement for the $-VO_3^{2-}$ group of this complex produced by this tailing is demonstrated by the observation that the intensity of the vanadate peak, relative to that of the breathing mode of the enzymic phenylalanines (at 1004 cm^{-1}), varies from about 8% to 5% to 4% to 3% (±0.5%) as the wavelength of the irradiating laser is centered successively at 457.9, 514.5, 528.7, and 647.1 nm. The intensity of the vanadate band thus

[2] Clark *et al.* (1954) report a dissociation constant for Mg·Glc-6-P of about 20 mM at $\mu = 0.4$; Ray and Roscelli (1964) report a similar value for Mg·Glc-1-P.

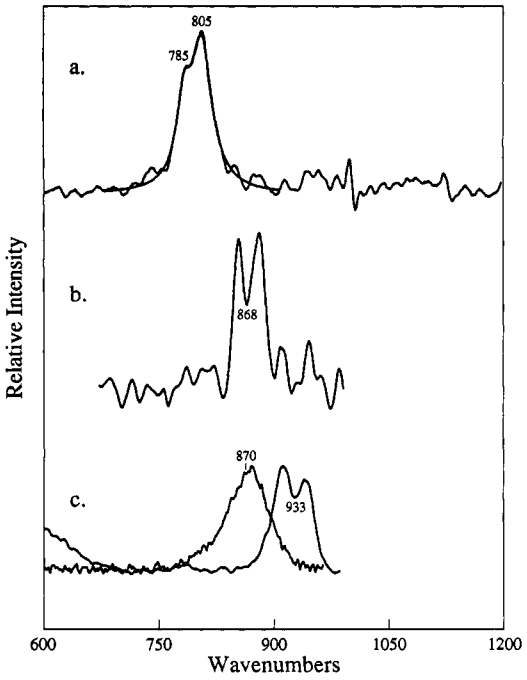


FIGURE 3: Raman difference spectra of the V-6-Glc-1-P complex of the Mg^{2+} and Li^+ enzymes and of model vanadates. The same conditions were used as in Figure 1, except that the laser excitation frequency was different in two cases. (a) E_D·Mg·V-6-Glc-1-P complex with 457.9-nm laser excitation. The smoothed curve shows the reconstructed peak obtained as the sum of two resolved Gaussian peaks (not shown). (b) Same complex as in (a) but with Li^+ replacing Mg^{2+}. (c) 25 mM $LiCH_3OVO_3$ in 94% methanol (457.9-nm laser excitation) (less noisy line with higher frequency band) and 25 mM $Li(CH_3O)_2VO_2$ in 98% methanol (528.7-nm laser excitation) (more noisy line with lower frequency band).

varies from about 11-fold greater than that of the corresponding phosphate band (which is about 0.7% that of the 1004-cm^{-1} protein peak) to about 4-fold greater, depending on the frequency of the irradiating laser.

The two Raman bands produced by the enzyme-bound vanadate group were resolved by a curve-fitting procedure that utilizes Gaussian line shapes. The resolved peaks are centered at 805 and 785 cm^{-1}, with relative intensities of about 4/1, respectively (see the reconstructed spectrum in the figure). Both parallel and perpendicularly polarized spectra also were obtained (data not shown). The depolarization ratio for the major 805-cm^{-1} band is about 0.15; thus, it is a polarized band that arises from a symmetrical stretching mode. The depolarization ratio for the minor 785-cm^{-1} band is substantially larger, about 0.6 (±0.2), based on an intensity ratio of 4/1 for the two bands. This depolarization ratio suggests that the 785-cm^{-1} band also may be a symmetrical stretching mode. But one of the above error limits does overlap the ratio, 0.75, that characterizes an unpolarized band that would be produced by an asymmetric mode.

There are several possible explanations for the 785-cm^{-1} band. As noted above, it could be an asymmetric stretching mode, although these modes generally exhibit substantially lower intensities in Raman spectra, relative to symmetric modes, than in the present case. It also could be a mode with a low degree of symmetry. Another possibility is that the sample contains two different vanadate complexes. However, if so, it is unlikely that the second complex is the alternative one involving V-1-Glc-6-P instead of V-6-Glc-1-P, although all samples of the Mg^{2+} enzyme that were studied contained at least minor amounts of this alternative complex. However, the general shape of the composite peak at about 800 cm^{-1} did not change significantly as the ratio of V-6-Glc-1-P/V-1-Glc-6-P complexes changed from about 8/1 to 1.4/1 for samples prepared in three different ways (see Experimental Procedures),[3] nor with increasing irradiation time. But the best rationale for this band is that the nonbridging oxygens of the vanadate reside in sufficiently different environments to produce two energetically similar but unequal V⎓O stretching modes. This suggestion is in accord with crystallographic studies showing that one of the nonbridging V⎓O groups interacts with the bound metal ion whereas the other two oxygens do not (Y. Liu and W. J. Ray, Jr., unpublished results).

V⎓O Bond Strength in the Transition-State Analog Complex. The bond strength of vanadium in the E_D·Mg·V-6-Glc-1-P complex is unusually low for a tetrahedral vanadate ester. Thus, the V⎓O stretching frequency for the monoanionic and dianionic forms of methyl vanadate are about 933 and 870 cm^{-1} (whereas that in the analog complex is about 800 cm^{-1}); see Figure 3. Although the vanadate of V-6-Glc-1-P is predominantly monoanionic in solution at the pH of these studies, 7.5 ($pK_a \approx 8.3$ at an ionic strength similar to that used here; Ray & Puvathingal, 1990), phosphate esters bound at the proximal subsite are dianionic, whether the PO_3^- group remains attached to the 6-oxygen of Glc-1-P or is transferred to $Ser^{116}O\gamma$ (Rhyu *et al.*, 1985; see also the first section of Results). Thus, the vanadate of *bound* V-6-Glc-1-P likely is dianionic, also. We assume that it is (below) to simplify our presentation, but this assumption is not critical to the conclusion and represents the more conservative assumption, in terms of the size of the binding-induced change in stretching frequency (see above).

On the basis of the relationship in the accompanying paper that links stretching frequency and bond strength for vanadates (eq 3: Ray *et al.*, 1993c), the strengths of the three V⎓O bonds of the vanadate in the inhibitor complex are only about 1.20 vu (calculated by using a frequency of 805 cm^{-1}) and actually are significantly lower even than those in VO_4^{3-} [ν_s = 823 cm^{-1}; bond strength = 1.25 vu (Ray *et al.*, 1993c)]. Three such bonds thus would account for a summed bond strength only of about 3.6 vu. Since the bond strength of a normal RO–V ester bond in a stable, tetrahedral vanadate cannot make up the difference between 3.6 and 5.0 vu (see the accompanying paper: Ray *et al.*, 1993) some type of pentacoordinate complex with a summed bond strength for the apical bonds of about 1.4–1.6 vu is indicated.[4] If the strength of one of these apical bonds is close to 1 vu, as expected for a normal RO–V ester bond, *e.g.*, as in V-6-Glc-1-P, the other apical RO(H)··V bond should have strength of about 0.4–0.6 vu, a length of about 2.2–2.0 Å [*cf.* Brown and Wu (1976)], and a stretching frequency of about 600–660 cm^{-1} [*cf.* Ray *et al.* (1993c)]. Such a bond could be provided by $Ser^{116}O\gamma$; see Discussion. Although no Raman band in the range of 600–660 cm^{-1} that could be assigned to a vanadate stretching mode was identified, it is unlikely that the stretching mode for a single, low-frequency bond of this type could be detected because of a low Raman cross section. Of course, other combinations of apical bond strengths that sum to 5 also are possible.

Effect of the Active Site Metal Ion on the Raman Spectrum of the Glucose 1-Phosphate 6-Vanadate Complex. Figure

[3] The complex prepared by procedure C, Experimental Procedures, was almost completely in the V-6-Glc-1-P form, initially, but was partially converted to the alternative V-1-Glc-6-P complex during Raman spectroscopy, presumably via the processes described previously (Ray & Puvathingal, 1990).

[4] Although the data base is limited, the summed bond strengths for vanadate esters seem to run slightly high and can be as high as 5.2 vu (W. J. Ray, Jr., unpublished calculations based on crystallographic data).

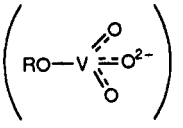
3b, in comparison with Figure 3a, shows how the substitution of Li^+ for Mg^{2+} in the E_D·M·V-6-Glc-1-P complex affects the Raman spectrum of the vanadate group. As in Figure 3a, this spectrum was obtained as E_D·Li·V-6-Glc-1-P – E_D·Li·Glc-P_2, where E_D·Li·Glc-P_2 is predominantly E_D·Li·P-6-Glc-1-P (Ray *et al.*, 1993b). In addition to large differences in spectral position, relative to the corresponding complex involving Mg^{2+}, the peaks are sharper than those for the Mg^{2+} enzyme. Since none of the alternative V-1-Glc-6-P complex was present in this sample,[5] the observation of dual peaks cannot be rationalized in terms of V-6-Glc-1-P and V-1-Glc-6-P complexes (see above). Hence, these peaks also likely arise from stretching modes of V=O groups in somewhat different environments. But, in any case, the bond strength of V··O groups within the Li^+ complex is substantially higher than within the Mg^{2+} complex. Using an average frequency of 868 cm^{-1} for the V=O bonds in this complex, the summed bond strengths for vanadium would be $3 \times 1.35 + 1.0$ or 5.1 vu. Hence, no additional fifth bond is required, as it is in the case of the Mg^{2+} complex.

DISCUSSION

In order to assess how phosphoglucomutase promotes transfer of the PO_3^- fragment of its phosphate group to a suitable acceptor, we have studied the phosphate moiety as it exists in the metal-free phosphoenzyme, in its binary complex with metal ion activators, and in its ternary complexes with Glc-6-P and Glc-P_2 (the latter after the first transfer step). The results allow us to contrast the properties of the relatively "rigid" – OPO_3^{2-} group with those of a similarly bound but less rigid vanadate group. We believe this comparison is particularly meaningful because nature has endowed vanadate with sufficiently "plastic" properties to make it an attractive transition-state analog for the transfer step in this and other enzymes [*cf.* Lindquist *et al.* (1973) and comments in the accompanying paper: Ray *et al.*, 1993c). Thus V–O bond lengths and O–V–O bond angles vary widely in crystals (Holloway & Melnik, 1986), and vanadates are much easier to polarize than phosphates. (Compare the slopes of the Figure 1 plots for phosphates and vanadates in the accompanying paper: Ray *et al.*, 1993.) In addition, the vanadium in tetracoordinate vanadates can interact with a fifth ligand much more readily than phosphate [*cf.* Crans *et al.* (1991) and Rehder (1991)]. In fact, the properties of V=O bonds in various ester/salts of vanadic acid to some extent exhibit the variations (plasticity) that separate coordination complexes from covalent compounds. Our approach thus differs from most other studies of transition-state analog complexes where attempts are made to deduce properties of the transition state by comparing the relative affinity of various molecules of *known* structure for the active site of the enzyme.[6] By contrast, in the present study we assess the *unknown* structure of a somewhat plastic probe, bound at the active site of the enzyme, by using classical Raman spectroscopy to evaluate its internal bonding. Phosphoglucomutase provides a particularly attractive system in which to do this since, in the dephosphoenzyme–vanadate–glucose phosphate complex, the vanadate group is known to bind in a manner closely similar to that of the phosphate group in the reactive glucose bisphosphate complex. In addition, the remainder of the glucose phosphate moiety appears to be bound in the same way in both complexes (Ray *et al.*, 1990). However, we first consider the enzymic phosphate group.

The Raman spectrum of the metal-free phosphoenzyme shows that the internal bonding of the enzymic phosphate group closely approximates that of an ordinary dianionic phosphate ester in aqueous solution. Moreover, the binding of bivalent metal ion activators produces only minor changes, if any, in the internal bonding of that group. In fact, the formation of the normal substrate complexes also fails to produce a substantial change in the enzymic phosphate group, whether Mg^{2+}, other divalent metal ions, Li^+, or no metal ion is bound at the adjacent metal ion activation site or whether the alternative attachment of that phosphate group, to O(6) of Glc-1-P, has been produced by the transfer process. Thus, the large values of *both* k_{cat} and k_{cat}/K_m for the normal phosphoglucomutase reaction must be due almost exclusively to the ability of the enzyme to utilize a transition state where bond making is more extensive and bond breaking less so than in the uncatalyzed reaction—as opposed to a distortion within the enzyme·substrate complex [*cf.* Fersht (1985)].

By contrast, the emerging picture of vanadate bound at the proximal subsite is one where the polarization of all three nonbridging V=O bonds of the monoester dianion is increased substantially by the binding process. This polarization can be visualized in terms of a resonance composite where the importance of the contribution from a more polar, single bond arrangement is enhanced, as in V=O ↔ V^+—O^-, where the right-hand form contributes more heavily, thus producing a net decrease in bond strength. In Results, we estimate that the bond strengths of all three nonbridging V=O groups are about 1.20 vu each, *i.e.*, less than for the three V=O groups of



by about 0.17 vu per V=O group. Thus, the sum of the strengths of the nonbridging V=O bonds, scaled as in the accompanying paper (Ray *et al.*, 1993c), is 3.6 vu. This sum is sufficiently less than expected for vanadium(V), 5 vu [*cf.* Brown (1978, 1992)], that accounting for the difference

[5] All attempts to produce the alternative E_D·Li·V-1-Glc-6-P complex in a manner analogous to that used for producing E_D·Li·V-6-Glc-1-P failed, presumably because of weak binding. Thus, the only V=O stretching mode observed when E_D·Li is treated with excess Glc-6-P plus Na_2HVO_4 (at about 945 cm^{-1}) also is observed when Glc-6-P is omitted, *i.e.*, is produced by the nonspecific binding of vanadate. A much smaller peak at this same position, in addition to the composite 800-cm^{-1} peak, also is formed when E_D·Mg·V-6-Glc-1-P is obtained via procedure C, which involves the highest concentration of free vanadate and the shortest time period for the separation of excess reagent. Again, this peak likely is caused by nonspecific binding of vanadate.

[6] These approaches should be complementary, since with rigid probes there is a limit to the extent that bond angles/distances can be varied; there also is a limit to the plasticity of any chemical probe.

[7] This model of VO bonding within the E_D·Mg·V-6-Glc-1-P complex differs from that posed earlier to rationalize the unusual electron-transfer spectrum of V($\bar{V}$) in this complex (Ray & Post, 1990). According to the analysis by Lever (1974) of electron-transfer energetics in terms of ligand field theory, a red shift in such spectra should occur within the series MX_6, MX_4, and MX_2, when the M–X bonds are of the same type. This red shift is caused by the decreased number of ligands and thus the reduced effect of the remaining ligands on vacant orbitals primarily associated with the metal ion. In the proposed model of the E_D·Mg·V-6-Glc-1-P complex, where V=O bond strength is substantially decreased and bond length increased relative to $ROVO_3^{2-}$, Lever's analysis suggests that the observed spectral red shift is caused by lengthening the three V=O bonds of $ROVO_3^{2-}$, even though a fifth V··O bond is formed in the process. Presumably the long, weak, fifth V··O bond that is formed increases the energy of the orbitals in question to a smaller extent than the decrease in energy produced by lengthening the three relatively short V=O bonds initially present.

requires an additional weak bond to vanadium. Put another way, it is unlikely that binding of a vanadate ester at the proximal subsite of the dephosphoenzyme could produce the bond polarization required to decrease the summed bond strengths for the four original V=O groups from 5.0 to 4.6 vu without involving what likely is a long, weak, fifth bond, presumably involving $Ser^{116}O\gamma$.[8]

Whereas the coordination of vanadium(V) can involve five (or more) ligands, both in the crystal phase (Holloway & Melnick, 1986) and in nonpolar solutions (*cf.* Priebsch and Rehder (1990)], a common denominator for these pentacoordinate vanadates, in contrast with the present system, is that formation of the fifth V–O bond does not produce a true phosphorane analog; *viz.*, the adduct differs substantially from that produced by the addition of a nucleophile to a phosphate triester. Thus, pentacoordinate complexes of vanadium exhibit a substantial tendency to retain at least one and frequently two oxygens whose bond strength exceeds 1.5 vu [calculations performed as in the accompanying paper (Ray *et al.*, 1993c) using data from Caughlan *et al.* (1966), Priebsch and Rehder (1990), and Crans *et al.* (1991).] By contrast, in the only phosphorane whose crystal structure is known, the greatest bond strength for P=O bond is 1.12 vu (Hamilton *et al.*, 1967).[9] But in the vanadate adduct involving the Mg^{2+} form of phosphoglucomutase and glucose-1-P, the present Raman results show that no V=O group with a bond strength greater than about 1.2 vu remains. Hence, the fifth weak bond which, as noted above, presumably involves $Ser^{116}O\gamma$ must be formed at the expense of *all three* V=O bonds of the dianion, rather than at the expense of the GlcO–V ester bond, as would be expected on the basis of model compounds in aqueous solution (W. J. Ray, Jr., J. Zheng, H. Deng, J. W. Burgner, II, manuscript in preparation).

Formation of an enzymic adduct of the type described above would be favored by two factors that are not present in model systems: an active site crevice designed so that partial bonding occurs between $Ser^{116}O\gamma$ and vanadium when the –Glc-1-P portion of V-6-Glc-1-P is optimally bound[8] and an electrostatic environment that stabilizes an increased polarization of *all three* V=O bonds of the ester dianion as the weak, fifth bond is formed.

The importance of electrostatic effects on formation of the fifth V–O bond is in accord with how replacement of the activating metal ion, Mg^{2+}, by Li^+ affects both binding and catalysis in the phosphoglucomutase system. Whereas this replacement does not substantially affect the binding of P-6-Glc-1-P (Ray *et al.*, 1989), it has a dramatic effect on the rate of the phosphoglucomutase reaction [rate reduction ($\sim 2.5 \times 10^{-9}$)-fold; Ray *et al.*, 1989] and on the binding of V-6-Glc-1-P [reduction ($\sim 7 \times 10^{-4}$)-fold; Ray & Puvathingal, 1990]. In fact, an earlier approximation based on the transition-state binding paradigm suggests that E_D·Li·V-6-Glc-1-P likely mimics a state relatively close to the *ground* state for the normal PO_3^--transfer process, whereas the corresponding Mg^{2+} adduct mimics a state where bond breaking/bond making has proceeded about half-way toward the transition state (where binding differences between the Mg^{2+} and Li^+ forms of the enzyme are enormous; Ray *et al.*, 1989). The present Raman studies extend these observations by showing that replacement of Mg^{2+} by Li^+ shifts the V=O stretching frequency from an average position of *ca.* 800 cm^{-1} to an average position of *ca.* 868 cm^{-1} (Figure 3, panels a and c). This shift returns the V=O stretching frequency to the same range as that observed for $(CH_3OVO_3^{2-})_{aq}$: 870 cm^{-1}. In other words, the enzyme with Li^+ bound at the activation site is unable to significantly polarize the bound vanadate, and the properties of the E_D·Li·V-6-Glc-1-P complex do indeed appear to be like those of a ground-state complex. In fact, in view of the present results, the earlier estimate of how closely the E_D·Mg·V-6-Glc-1-P complex approximates the transition state for the normal enzymic reaction actually may be somewhat low, since that approximation does not consider the apparent reluctance of vanadium(V) to adopt a pentacoordinate structure in which the bond order of no V=O bond substantially exceeds 1.2 vu, as it does in the transition-state analog complex.

Mechanistic Implications. It is not clear whether the metal ion interacts primarily with the nonbridging oxygens or with $Ser^{116}O\gamma$ *during* the normal PO_3^--transfer process. In the two alternative forms of the "resting" Cd^{2+} enzyme, E_P·Cd and E_D·Cd, the metal ion interaction is with a nonbridging phosphate oxygen in E_P·Cd (Ray *et al.*, 1993b; see also Scheme I) and, in E_D·Cd, with what previously was the bridging oxygen in E_P·Cd: $Ser^{116}O\gamma$ (Dai *et al.*, 1992). In contrast with the latter observation, when a phosphate group occupies the proximal subsite, the manner in which different metal ions affect the relative stabilities of substrate (glucose monophosphate) complexes and intermediate (glucose biophosphate) complex (Ray & Long, 1986b) can be interpreted in terms of a metal–phosphate interaction that involves a nonbridging oxygen. The maintenance of a direct interaction between the metal ion and a nonbridging oxygen in such complexes also is in accord with more recent work, including X-ray diffraction studies on the crystalline E_D·Mg·V-6-Glc-1-P complex and spectral studies of the E_D·Co·V-6-Glc-1-P complex (W. J. Ray, Jr., unpublished results). Although there is a real possibility that conformational changes accompany the transfer step and thus exacerbate the problem of metal ion coordination *during* transfer, our working hypothesis is that the metal ion interacts with a nonbridging phosphate oxygen or oxygens in the transition state.

Although coordination of a bivalent metal ion with nonbridging oxygens of phosphate monoester dianions *per se* produces only modest rate enhancements in model reactions [*cf.* Herschlag and Jencks (1990)], such reactions differ from the enzymic process, here, in terms of the presence of water, which undoubtedly forms a structured environment about the phosphate dianion. In model reactions, the failure to provide a simultaneous electrostatic interaction with *all three* nonbridging oxygens of the type suggested by the V=O stretching frequency in the V-6-Glc-1-P complex likely represents a second critical difference. However, transition-state stabilization in the phosphoglucomutase system probably involves more than simply the stabilization of the $-PO_3^-$ group in its progress toward the transition state.

As for the enzymic PO_3^--transfer step, the substantial formation of a second bridging PO bond before the breaking of the original bridging bond is essentially complete would represent a process exhibiting more associative character than the dissociative process that characterizes the reactions of all model phosphate ester dianions studied to date (Herschlag &

[8] If the long, weak, apical bond in the E_D·Mg·Glc-1-P complex involves the oxygen bridge between the VO_3^{2-} and Glc-1-P moieties, with the stronger apical bond to Ser^{116}, breaking of the weaker apical bond should allow Glc-1-P to dissociate from the analog complex independent of the VO_3^{2-} moiety. If so, one should be able to trap the VO_3^{2-} moiety bonded to $Ser^{116}O\gamma$ by using high concentrations of Glc-1-P, thereby re-forming the analog complex. However, an attempt to trap such a vanadoenzyme was unsuccessful, even at a very high Glc-1-P concentration (Ray & Puvathingal, 1990).

[9] The bond strengths of the P–OR groups of the phosphorane, phenanthrenequinone–triisopropyl phosphite 1:1 adduct (Hamilton *et al.*, 1967), calculated with the relationship of Brown and Wu (1976), are 1.12, 1.06, and 1.00 for equatorial oxygens and 0.98 and 0.70 for apical oxygens.

Jencks, 1990, and references therein). But, as noted above, an enzymic PO_3^--transfer process may differ substantially from its nonenzymic counterpart, and in spite of the generalizations drawn from model reactions, PO_3^--transfer processes with associative character frequently are posed for enzyme-catalyzed reactions. On the other hand, no unequivocal evidence for a truly "nondissociative" enzymic PO_3^- transfer involving a dianionic phosphate has been published (*cf.* the above reference), although we believe the current studies provide unusually strong evidence for the operation of such a process in the present enzymic reaction. But even here this conclusion rests on the assumption that the E_D·Mg·V-6-Glc-1-P complex provides reliable clues about changes in bonding on the way to the transition state for PO_3^- transfer. Thus, one might contend that the enzyme distorts a vanadate ester in a manner that differs from the way a phosphate ester is distorted in the transition state for PO_3^- transfer. We discount this possibility on the basis that the transition-state binding paradigm [*cf.* Page (1987) and Fersht (1985)] seems to require sufficiently unique properties of the active site of this enzyme that a spurious polarization/distortion of a more plastic group is unlikely. In addition, increased bond formation in the transition state, coupled with decreased bond breaking, provides one of the few general strategies available for producing very large increases in the rate of PO_3^- transfer in *both* directions when the leaving group is a poor one, *e.g.*, the oxyanion group of Glc-P. In fact, this is precisely what the present results with the vanadate analog suggest, when taken at face value: that in the PO_3^--transfer process substantial bond formation precedes extensive bond breaking and that formation of the fifth P–O bond occurs more at the expense of the nonbridging phosphate oxygens than the bridging P–O bond. In the extreme, such a transfer would become a two-step process involving a phosphorane-like intermediate, as opposed to an S_N2-like process where the fifth bond forms primarily at the expense of the bridging P–O bond (Ray *et al.*, 1993c). However, such an extreme seems unlikely. In any case, since there is no evidence for significant formation of a fifth P–O bond in the ground-state enzyme–substrate complex, the above interpretation can (and will) be subjected to a critical test: measurement of the secondary ^{18}O kinetic isotope effect on the transfer catalytic process. If the present interpretation is correct, *viz.*, that the transition state is characterized by a decreased strength of nonbridging bonds that is more or less balanced by an increased strength of bridging bonds, a direct isotopic effect is expected; if not, an inverse effect, as in the alkaline phosphatase reaction (Weiss & Cleland, 1989), will be observed, provided of course the transfer process selected for study involves a rate-limiting bond-breaking/bond-making process.

SUPPLEMENTARY MATERIAL AVAILABLE

A detailed version of the synthesis of [$^{18}O_3$]phosphoglucomutase and the Li^+ form of dephosphophosphoglucomutase and formation of the E_D·Mg·V-6-Glc-1-P complex (5 pages). Ordering information is given on any current masthead page.

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