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Structural Similarities and Differences among Metal Ion Complexes of Phosphoglucomutase by Solvent Perturbation and Ultraviolet Difference Spectroscopy†

William J. Ray, Jr.

ABSTRACT: Although the binding of bivalent metal-ion activators to phosphoglucomutase produces substantial changes in the near ultraviolet spectrum of the enzyme, the extent to which aromatic residues are exposed to the aqueous environment, as assessed by means of solvent perturbation spectroscopy (using D_2O), does not appear to be significantly altered by the binding process. Other ways in which the spectral effects induced by activation might arise are considered by making comparisons with those changes induced by various nonactivating monovalent and bivalent cations. The observed differences are most easily interpreted in terms of an electrostatic perturbation of (at least) two different tryptophan residues. This interpretation is supported by using cationic vs. neutral (zwitterionic) tryptophan in various solvent systems to generate difference spectra that are similar either to the observed metal-ion induced spectral differences or to the differences in the spectral changes produced by various pairs of metal ions. Although a rationale for the striking similarity in the spectral changes produced by Mg^{2+} and by Li^+ (which elicits less than 2×10^{-8} of the enzymic activity induced by Mg^{2+}) cannot be ascribed to a simple electrostatic effect, alone, the involvement of an additional, negatively charged group in the binding of Mg^{2+} (but not Li^+) could reduce the effective charge of bound Mg^{2+} to a value close to that of bound Li^+.

An earlier paper (Ray et al., 1978) describes the unusually tight binding of Li^+ to phosphoglucomutase in the presence of its substrates (glucose phosphates). In fact, bound substrates increase the binding of Li^+ by some 900-fold, although the binding of Mg^{2+}, the normal activator, scarcely is altered under analogous conditions. These observations indicate that there are important structural differences between the E_P·Li and E_P·Mg complexes,[1] a conclusion that is supported by the more than 5×10^7-fold difference in their catalytic activities (in the presence of bound substrates). Hence, it was surprising to find that the spectral change induced by the binding of Li^+ to the enzyme is quite similar to that induced by Mg^{2+}, although other monovalent cations produce spectral changes that are quite different in appearance. The similarity in the spectral change induced by Mg^{2+} and Li^+ is not consistent with the earlier suggestion (Peck & Ray, 1969) that such spectral differences are produced by the conformational changes upon which the activation process depends—changes which were thought to alter the exposure of aromatic residues to the aqueous environment. The present paper suggests, instead, that metal-ion induced spectral changes in this system arise primarily from electrostatic effects, not from changes in exposure to the solvent, and that a charge-compensating effect may accompany the binding of metal ion activators such as Mg^{2+}.

Experimental Section

Phosphoglucomutase was isolated, assayed, freed of contaminating bivalent metal ions, and stored according to previously described procedures (cf., Ray et al., 1978). UltraPure sodium, potassium, and cesium chlorides, and UltraPure magnesium nitrate were obtained from Ventron; lithium chloride was crystallized once from 20 mM EDTA and twice from water and was dried under vacuum over P_2O_5 before use. Reagent grade tetramethylammonium chloride was used,

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[1] Abbreviations are: E_P and E_D, the phospho and dephospho forms of phosphoglucomutase; E_P·M, the metal-ion complex in which M^{2+} is bound at the metal-ion activating site.

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without purification, in the presence of 1 mol % EDTA, disodium salt. Metal ions were removed from D_2O by passage over a mixed-bed column of ion-change resin (Dowex 501), after flushing the column to remove H_2O. Tris-Cl buffers were treated with Chelex, previously titrated to the same apparent pH as the buffer. Laboratory grade "deionized" water was distilled and passed through a mixed-bed column of ion-exchange resin before use.

Ultraviolet difference spectra were obtained at 24 °C by use of a Cary-15 spectrophotometer equipped with a 0 to 0.1 OD slide wire and water-jacketed cell holders. Matched absorption cells with a 1.00-cm light path or matched, sectored cells with a light path of 0.438 cm per compartment were employed. In most cases, protein concentrations of about 3 mg per mL were used (OD at 280 $\approx$ 2.3). The operating procedure described by Herskovitz & Sorensen (1968) was observed, so that the maximum half-band width was maintained at less than 1 nm throughout the scan. A scanning rate of 5 nm per min, or less, was used above 250 nm, and half that, or less, below 250 nm. In addition, the spectrophotometer was operated in conjunction with a Sorensen ARC 3000 voltage regulator. Two successive scans of both the baseline and the spectrum were made in all cases; a third scan was employed if the first two deviated by more than 0.0005 OD over a significant wavelength interval. Difference spectra involving Mg^{2+} usually were produced by adding 0.005 mL of 0.1 M $MgCl_2$ to 1.00 mL of metal-free enzyme in the sample cell and an equal volume of water to the reference cell; those involving Li^+ were similarly produced by addition of 0.01–0.02-mL aliquots of 7.5 M LiCl and water. Difference spectra involving Na^+ were obtained after dilution of a stock solution of the enzyme in either water or NaCl solution. Difference spectra also were produced by means of the sectored-cell technique used previously (Peck & Ray, 1969). Solutions for D_2O difference spectroscopy were made up by weight from concentrated stock solutions, so that the molar concentrations of enzyme in H_2O and 90% D_2O were the same to within ±0.03%. Prior to preparing and using solutions for D_2O perturbation spectra, *all* surfaces that were to come in contact with the protein, those of transfer pipets, weighing vessels, centrifuge tubes, and absorption cells, were exposed for several minutes to essentially identical protein solutions and allowed to drain in a closed container. For less critical comparisons, only the transfer pipets were treated in this manner. Most spectra were obtained in the presence of 0.05 mM EDTA. Minor baseline corrections for the absorbance of EDTA in the 250–300-nm range were made on the basis of spectra obtained in the absence of protein. In the 220–250-nm range, matched, sectored cells were used to make the subtraction, spectrally, by use of the following arrangement, where 1 and 2 refer to the compartments of the sectored cells: sample 1 and reference 1, protein plus EDTA; sample 2 and reference 2, EDTA. Difference spectra were generated by adding the metal ion to sample 1 and reference 2 plus an equal volume of water to sample 2 and reference 1. The spectral difference produced by changing from bound Li^+ to "$\frac{1}{2}$ Be^{2+}" (see Results), with an internal correction for ancillary binding, was obtained with the following arrangement: sample 1 and reference 1, metal-free protein; sample 2 and reference 2, metal-free protein at half the concentration. The difference spectrum was generated by making the following additions: sample 1 and reference 2, 10 μL of LiCl and 10 μL of water; reference 2, 10 μL of Be^{2+} followed by 10 μL of LiCl; sample 2, same as reference 2, but with one-half of the Be^{2+} concentration. Baselines for D_2O perturbation spectra were obtained with 1-mL aliquots of water solution of metal-free protein in absorption cells that had been rinsed as above. The D_2O perturbation spectrum was generated by replacing the contents of the sample and reference cells with solutions of the protein in H_2O and D_2O, respectively. Subsequently, the appropriate metal ion was added to both sample and reference cells; alternatively, the contents of both cells again were replaced, this time with solutions to which the appropriate metal salts had been added (so that a brief centrifugation could be employed after adding the salt). Absorbance cells were kept tightly closed while spectra were being recorded. All spectra were reproduced by tracing, after subtraction of the appropriate baseline, so that the composite of the pen excursions for two successive scans is represented.

Results

Ultraviolet Difference Spectra Produced by the Binding of Cations to Phosphoglucomutase. Each of the monovalent cations, Li^+, Na^+, K^+, Cs^+, and $N(CH_3)_4^+$, induces a change in the ultraviolet spectrum of phosphoglucomutase, although binding is much too weak to be observed except in the presence of a large excess of the ion. However, the use of high purity salts in some cases (heavy metals less than 0.1 ppm; metal ion/enzyme ratio $\leq 10^4$), and the independence of the metal-ion induced difference spectra on EDTA concentration (in the range 0.05 to 1 mM) in all cases suggests that the observed difference spectra were not produced by the binding of bivalent metal ion contaminants. The greatly reduced size of the difference spectrum when either saturating Mg^{2+} or one equivalent of the more tightly bound Zn^{2+} (Ray, 1969; Ray & Peck, 1972) is present in both sample and reference cells indicates that the monovalent cations produce *most* of their spectral effect by binding at the metal-ion activating site. (This conclusion is supported, in the case of Li^+, by the similarity in the value of its dissociation constant, as measured spectrally, and as assessed by competition of Li^+ and Mg^{2+} for the metal-ion activating site of the enzyme (Ray et al., 1978).) However, part of the spectral changes that are produced at concentrations of monovalent cations sufficiently high to saturate the activating site involve ancillary sites, as is indicated by the spectral change in experiments where the activating site was *saturated* with Mg^{2+} or Zn^{2+} in the absence and presence of a monovalent metal-ion salt (at a concentration of 0.1 M or higher—spectra not shown). The sum of all spectral effects, *exclusive of that produced by binding at the activating site,* are referred to as the "ancillary binding effect". In the case of Li^+ and of Na^+, an attempt was made to eliminate or at least reduce the ancillary binding effect by subtracting the difference spectrum of the Mg^{2+} enzyme, in the presence and absence of LiCl or NaCl, from the difference spectrum obtained with metal-free enzyme in the presence and absence of these salts.

Figure 1, spectrum a, is the difference spectrum of phosphoglucomutase induced by 0.5 M Na^+ (a less than saturating concentration), after correction for the ancillary binding. The same *overall* difference spectrum, including ancillary binding effects (not shown), is produced by 0.5 M K^+ or Cs^+ (i.e., the Na^+ difference spectrum can be abolished by addition of either Na^+ or Cs^+ to the reference cell); $N(CH_3)_4^+$ (0.5 M) induces a similar difference spectrum. Spectrum b is the half-intensity difference spectrum (see figure legend) that is induced by the binding of one equivalent of Be^{2+} (see also the following section), presumably at the metal-ion activating site (cf., Hashimoto et al., 1967), while spectrum c is the difference spectrum produced by 0.075 M (essentially saturating) LiCl, after correction for very small ancillary binding effects. The difference spectrum produced by the binding of Mg^{2+}, under the same conditions, spectrum d, also is shown for comparison (cf., Peck & Ray, 1969). (The shapes of these difference spectra

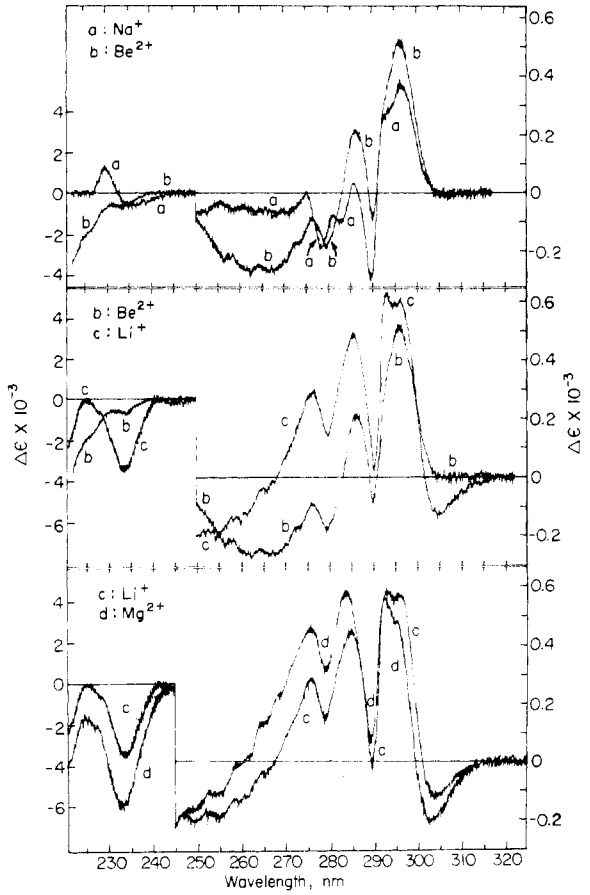

FIGURE 1: Difference spectra produced by the binding of metal ions to phosphoglucomutase. For difference spectra above 250 nm, the (initially metal free) enzyme was present at a concentration of 0.045 mM (2.9 mg per mL) in 20 mM Tris-Cl, pH 7.5, containing 0.05 mM EDTA, except for spectrum b, which was obtained at half this enzyme concentration. (Values of $\Delta\epsilon$ for spectrum b are twice those shown.) Except for NaCl, a saturating or nearly saturating concentration of metal ion was used (see Experimental Section). A numerical correction for ancillary binding effects was made for the Na^+ and Li^+ complexes of the enzyme (see Results); an additional minor correction for changes in EDTA absorbance also was made in conjunction with spectra c and d (see Experimental Section). Difference spectra below 250 nm were obtained by use of sectored cells (to allow an internal correction for absorbance changes due to EDTA; see Experimental Section); a protein concentration 0.16 of that used for the near ultraviolet spectra was used; a numerical correction for ancillary binding effects was made in the case of Na^+ and Li^+. In all cases, a composite of two successive scans is shown.

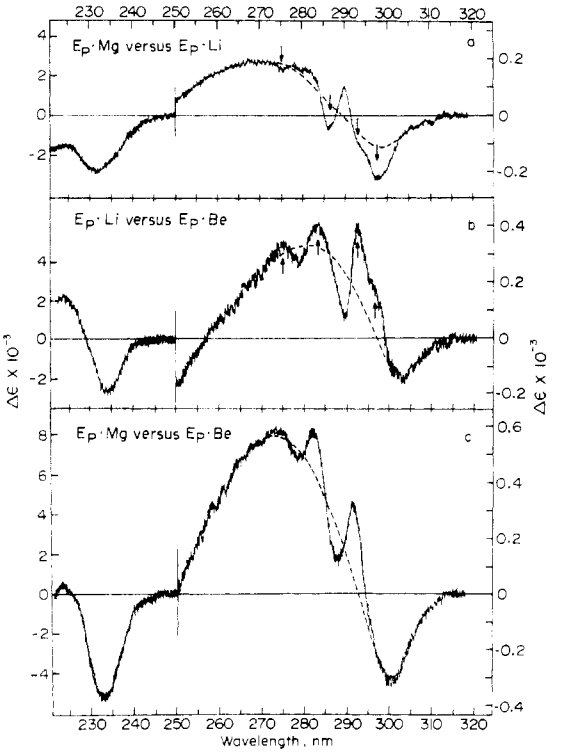

FIGURE 2: A direct comparison of difference spectra produced by the cations of lithium, magnesium, and beryllium. Conditions were similar to those described in the Figure 1 legend. (a) The difference between spectral changes induced by Mg^{2+} (15 mM $MgCl_2$, sample cell) and Li^+ (0.15 M LiCl, reference cell); differences due to ancillary binding were eliminated by including 0.15 M LiCl along with the $MgCl_2$ in the sample cell. (b) The difference between the spectral change induced by Li^+ (0.075 M, sample cell) and one-half of the spectral change induced by Be^{2+} (reference cell). Sectored cells were used and the procedure described in Experimental Section was employed to correct for the absorbance of half of the protein, and for ancillary binding effects. (c) The difference between the spectral change induced by Mg^{2+} (sample cell) and one-half of the spectral change induced by Be^{2+} (reference cell). A procedure analogous to that in b above was employed, except that $MgCl_2$ was used at a final concentration of 0.5 mM. The portion of the spectrum below 250 nm was obtained by direct subtraction of the corresponding Figure 1 spectra.

were essentially constant even when a reduced sensitivity and increased spectral width (twofold) were used.)

Although a previous study suggests that several, second-row, bivalent metal ions produce the same difference spectrum as does Mg^{2+} (Peck & Ray, 1969), a direct comparison of the spectral shifts induced by Zn^{2+} (radius $\approx$ 0.66 Å) and Cd^{2+} (radius $\approx$ 0.97 Å) was made at the higher protein concentrations used in Figure 1 (not shown). In fact, these results show that the molar $\Delta\epsilon$ for both metal ions is the same, ± 10 cm^{-1} M^{-1}, at all wavelengths between 250 and 310 nm. Ca^{2+} (radius $\approx$ 1 Å) also produces what appears to be the same difference spectrum. However, 10 mM Ca^{2+} causes a slow denaturation of the enzyme at room temperature and pH 7.5 (even when Mg^{2+} is bound at the metal-ion binding site) and it is necessary to obtain the Ca^{2+}-induced spectrum at a reduced temperature (10 °C). The difference spectrum, thus obtained, only is about 65% as intense as that induced by Mg^{2+} (at 24 °C), presumably because of incomplete binding. In such a case the dissociation constant for Ca^{2+} is about 5 mM, or about 500-fold larger than that of Mg^{2+} (at 30 °C; Ray et al., 1978).

A Direct Comparison of Metal-Ion Induced Difference Spectra. Differences between the spectra of the Ep·Mg and Ep·Li complexes, the Ep·Li and Ep·Be complexes, and the Ep·Mg and Ep·Be complexes are shown in Figure 2. In all of these difference spectra, ancillary binding effects presumably cancel, since, for example, the spectrum of Ep·Mg (sample cell) was obtained in the presence of the same concentration of Li^+ that was used in the reference cell (which is possible because of the large differences in binding constants for Li^+ and Mg^{2+}: Ray et al., 1978). These spectra were obtained partly in an attempt to decide whether a rationale of the differences among the Figure 1 spectra would require that perturbation of tyrosyl residues be considered, and partly in an attempt to determine whether there is a correlation between the 233-nm trough in the difference spectra induced by Li^+ and Mg^{2+} and features of the near ultraviolet difference spectrum induced by these metal ions. Figure 2a shows the spectral change on substituting Mg^{2+} for bound Li^+ (and is equivalent to the difference between Figures 1d and 1c). Figure 2b is a comparison of the Li^+-induced difference spectrum with a half-intensity, Be^{2+}-induced difference spectrum, i.e., the difference between Figures 1c and 1b, and thus represents the spectral change produced by replacing bound "$\frac{1}{2}$ Be^{2+}" by Li^+. (A half-intensity spectrum was used because Be^{2+} is expected to produce

twice the charge perturbation as a similarly-bound monovalent cation—see Discussion; the use of a sectored-cell technique allowed ancillary binding effects to be minimized by inclusion of LiCl in the solution of the Be^{2+}-inhibited enzyme.) Figure 2c shows a comparison of the Mg^{2+}-induced difference spectrum with a half-intensity Be^{2+}-induced difference spectrum (see the Discussion for a rationale of this comparison), which is equivalent to the sum of the spectra in Figures 2a and 2b or the difference in those of Figures 1d and 1b. The rationale for the arrows and dashed lines in the Figure 2 spectrum is noted in the following section.

The above comparisons indicate that (a) the apparent prominance of the difference peaks at shorter wavelengths in the E_P·Mg vs. E_P spectra is caused primarily by a broad difference band in the 350–390-nm region and (b) that *most* of the features in the Figure 1 difference spectra and the Figure 2 double difference spectra can be rationalized without invoking the perturbation of tyrosines (see below). However, the possibility that tyrosine perturbation makes a contribution to the above difference spectra cannot be ruled out.

Model Systems. In order to determine whether the general characteristics of the various difference spectra in Figures 1 and 2 could be mimicked in a model system involving only the indole chromophore, difference spectra arising from electrostatic perturbation (Trp^+ vs. $Trp^{\pm}$) and solvent perturbation ($Trp^{\pm}_{solvent}$ vs. $Trp^{\pm}_{water}$), as well as combinations of these ($Trp^{+}_{solvent}$ vs. $Trp^{\pm}_{water}$), were generated. Figure 3 shows the results obtained by using 20% and 40% glycerol. Figure 3b is similar in overall appearance to Figure 2c (the difference spectrum induced by replacing bound Mg^{2+}), while spectrum 3c resembles Figures 1c and 1d (the Li^+- and Mg^{2+}-induced difference spectra) as well as Figure 2b (the E_P·Li vs. $\frac{1}{2}$ E_P·Be difference spectrum), except that in the enzymic systems two classes of tryptophans that produce noncoincident difference peaks are involved. With the latter reservation, the Figure 3a difference spectrum resembles Figure 2a (the E_P·Mg vs. E_P·Li difference spectrum), the reciprocal of the Figure 3a spectrum resembles Figure 1b (the Be^{2+}-induced difference spectrum), and the Figure 3d spectrum resembles Figure 1a (the Na^+-induced difference spectrum). Hence, reasonable models involving only the indole chromophore can be specified for all of the difference spectra in Figures 1 and 2.

Although the Figure 3 difference spectra involve both charge and solvent effects, it does not necessarily follow that both effects must be involved in the metal-ion induced difference spectra of the enzyme (Figures 1 and 2). Thus, the near-UV absorption spectrum of indole is a composite of contributions from two different types of transitions (Strickland et al., 1970) usually designated as ^1L_a and ^1L_b (Klevins & Plant, 1949; Jaffé & Orchin, 1952) by comparison with other aromatic systems. (In the case of tryptophan, shifts in ^1L_b transitions produce the narrow difference peaks that appear at about 292, 284, 275 nm in solvent perturbation studies (cf., Herskovitz & Sorenson, 1968), while shifts in ^1L_a transitions, that are more common in charge-perturbation spectra (Andrews & Forster, 1972), produce a broad, essentially featureless peak and accompanying valley that can extend from 310 to below 250 nm.) The conditions in Figure 3 were chosen in an attempt to generate difference spectra of indole in which differential contributions from ^1L_a and ^1L_b (broad and narrow) transitions could be more or less systematically varied. Judging from the spectral assignments of Strickland et al. (1970), Figure 3 shows, to a first approximation, indole difference spectra that arise from a blue shift in both ^1L_a and ^1L_b transitions (spectrum a); a red shift in ^1L_b transitions (spectrum d); a blue shift in ^1L_a transitions (spectrum b); and a blue shift in ^1L_a transitions plus a red shift in ^1L_b transitions (spectrum c).

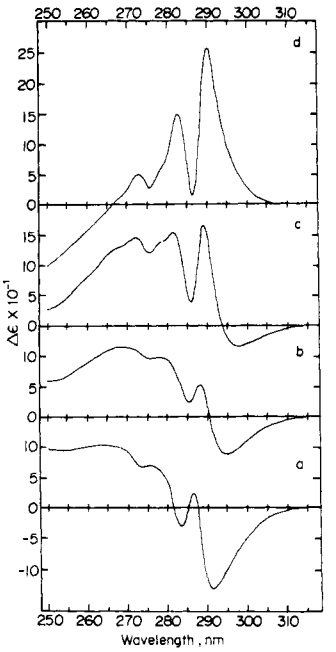

FIGURE 3: Tryptophan difference spectra produced by direct electrostatic effects, by solvent effects, and by a combination of these. Sectored cells were used, and base lines were obtained with 1.00 mL of 1 mM tryptophan, 20 mM NaCl, 10 mM sodium phosphate buffer, pH 7, in one compartment and 1.00 mL (spectra a and b) or 1.01 mL (spectra c and d) of glycerol–water–hydrochloric acid mixtures in the other compartment of both sample and reference cells. Difference spectra were generated by mixing the contents of the sample cell. Final concentrations: (a) 0.1 M HCl; (b) 0.1 M HCl, 20% glycerol; (c) 0.1 M HCl, 50% glycerol; (d) 40% glycerol.

Clearly the above type of approach could provide a rationale for the difference spectra of enzyme–metal complexes. Thus, the dashed lines in Figure 2 suggest the peaks and valleys that might arise from a shift in a broad spectral transition and the arrows show features that could arise from shifts in more narrow transitions, provided that two different tryptophans or classes of tryptophans in the enzyme are perturbed.

Titration of Phosphoglucomutase with Divalent Beryllium. When Be^{2+} is added to a Tris-Cl buffer, pH 7.5, followed by 1 equiv of phosphoglucomutase (at a final concentration of 40 μM), only a small spectral change in the enzyme occurs, presumably because Be^{2+} hydrolyzes fairly rapidly under such conditions (cf., Cotton & Wilkerson, 1966). However, when a stock solution of 0.25 M $BeSO_4$ (pH <2) is diluted in water, and an aliquot containing 1 equiv of Be^{2+} rapidly mixed with the enzyme, a relatively large spectral change is produced. A titration curve generated by means of analogous, but sequential additions of Be^{2+} is shown in Figure 4; the difference peak at 297 nm is used to follow the titration. Although the addition of about 2 equiv of Be^{2+} is required to complete the titration ($\Delta\epsilon_{max} \approx 11\,000$), the final difference spectrum likely involves the binding of only 1 equiv of this metal ion (cf., Hashimoto et al., 1967). Thus, the apparent nonstoichiometric nature of the titration curve arises from the competitive effect of binding and hydrolysis of added Be^{2+}, considering that bound Be^{2+} is not readily removed from the enzyme (see above reference).

Spectral Changes Induced by the Binding of Protons at the Metal-Ion Activating Site. Figure 5 shows difference spectra produced by the binding of protons at the metal-ion binding site. In this figure the pH of the reference solution (at 24 °C) is 8.6 and that of the sample solutions is a, pH 7.5; b, pH 6.6; and c, pH 5.3. Absorbance changes that are induced by proton binding at sites other than the metal ion activating site were eliminated, or at least substantially reduced by using sectored

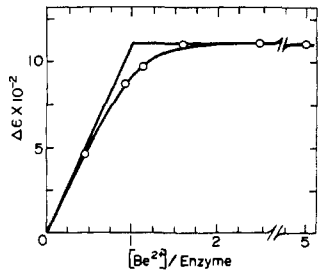

FIGURE 4: Titration of phosphoglucomutase with beryllium sulfate. A baseline was obtained under conditions analogous to those in the Figure 1 legend, except for the protein, which was used at one-half the concentration. A 0.25 M stock solution of $BeSO_4$ was diluted in water and a 0.01-mL aliquot quickly added to the sample cell; an equal aliquot of water was added to the reference cells and the spectrum was scanned twice from 320 to 280 nm. The entire procedure was repeated five times; $\Delta\epsilon_{297}$ is shown as a function of the $[Be^{2+}]/[enzyme]$ ratio after making a minor correction for dilution. The straight lines show the expected titration curve if one molecular equivalent of Be^{2+} binds to phosphoglucomutase and none of the added Be^{2+} hydrolyzes.

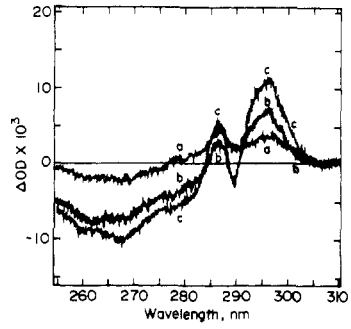

FIGURE 5: The difference spectrum induced by proton binding at the metal-ion activating site of phosphoglucomutase. The metal-free protein and the Zn^{2+} complex of the protein initially were present, respectively, in compartments 1 and 2 of sectored sample and reference cells at a concentration of 0.052 mM, in 5 mM NaCl, 1 mM Tris-Cl, pH 8.6. To generate difference spectra, aliquots (10 μL) of concentrated buffer solutions were added to sample compartment 1 and reference compartment 2, while water (10 μL) was added to sample compartment 2 and reference compartment 1, so that the difference in the absorbance change of the metal-free enzyme and of the Zn^{2+} enzyme produced by changing the pH was recorded directly. The buffers used are given and the final pH is enclosed in parentheses: (a) 1 M Tris, pH 7.3 (7.5); (b) 1 M acetate buffer, pH 6.0 (6.6); 5 M acetate buffer, pH 5.0 (5.3). All spectra were obtained at 10 °C, but pH values refer to 25 °C.

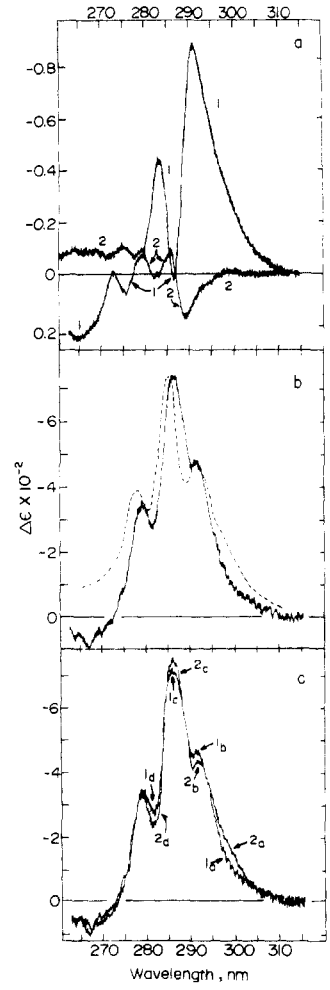

FIGURE 6: D_2O perturbation spectra. (a) The D_2O perturbation spectrum of zwitterionic tryptophan, 1, and the *difference* in the D_2O perturbation spectrum of the protonated and zwitterionic forms of tryptophan, 2. Sectored sample and reference cells were used; initially these contained 1.00 mL of 0.4 mM tryptophan, 20 mM NaCl, and 10 mM sodium phosphate, pH 7, in each compartment. Spectrum 1 was generated by replacing the solution in one compartment of the cell with an identical solution made up in 90% D_2O. After making a similar replacement in the reference cell, spectrum 2 was generated by adding 0.01 mL of concentrated HCl to the D_2O solution in the sample cell and to the H_2O solution in the reference cell; equal volumes of water were aded to the other two compartments. (b) The observed D_2O perturbation spectrum for metal-free enzyme (—) and a calculated perturbation spectrum for fully exposed tryptophans, 1.9 residues, and tyrosine, 7.5 residues; see Results section for details. (c) A comparison of the D_2O perturbation for the metal-free enzyme (spectrum 1) and its Mg^{2+} complex (spectrum 2); see Experimental Section for details.

absorption cells and placing the E_P·Zn complex at pH 8.6 in one section of the sample cell and the same complex at the lower pH in the reference cell. Because of the tendency of the metal-free enzyme to denature at pH values lower than about 7 at room temperature, this study was conducted at 10 °C. A generally increased intensity of the difference spectrum was observed with an increase in the pH difference between the sample and reference. Moreover, the profile of the difference spectrum produced by comparing enzyme at pH 7.5 with that at pH 5.3 (not shown) resembles the profile of the Be^{2+}-induced difference spectrum in Figure 1b, but the spectrum is less intense: $\Delta\epsilon \approx 350$ at 297 nm.

Solvent Perturbation Studies Using D_2O. D_2O-perturbation difference spectra of the metal-free enzyme and its metal-ion complexes were obtained in an attempt to determine whether the metal-ion induced difference spectra (Figure 1) were the result of conformational changes that alter the solvation of tryptophan side chains by changing their accessibility to the aqueous medium, or other environmental perturbations. If metal ion binding produces spectral changes by altering the exposure of chromophores to the aqueous medium, the largest changes in the D_2O perturbation spectrum will occur near the maxima or minima of the metal-ion induced difference spectrum. By contrast, if metal-ion binding produces other types of spectral shifts different changes will be observed. Since difference spectra produced by small changes in band position resemble derivative spectra (Brandts & Kaplan, 1973), the *change* in the D_2O-induced difference spectrum produced by an environmentally induced spectral shift would constitute a second derivative spectrum, i.e., a derivative spectrum of a derivative spectrum. For truly Gaussian absorption bands, the largest absorbance differences in the second derivative spectrum would occur in regions of maximum slope in the (first) derivative spectrum, would be opposite in sign on either side of first derivative maxima and minima, and be zero at those maxima and minima. However, such idealized behavior will be adversely affected both by the possible perturbation of

multiple chromophores in a protein and by the extent to which perturbation spectra are not true derivative spectra (e.g., because of the changes in band intensity). The latter problem is illustrated in Figure 6a, which shows the D_2O perturbation spectrum of zwitterionic tryptophan, spectrum 1, and the *change* in the D_2O perturbation spectrum produced by protonation of the carboxyl group, spectrum 2. However, spectrum 2 is quite different from the type of spectral change that would be produced by removing part of the tryptophan from solution, or decreasing the exposure of tryptophan to the aqueous medium, in the case of a protein.

In the present system, the fact that the apparent D_2O-perturbation spectrum below 290 nm was critically dependent on the manner in which solutions of the enzyme were prepared further complicates the problem. For example, a mismatch in protein concentration of 0.1% produces a change of 50 in the value of the molar $\Delta\epsilon$ at 275 nm, where the molar $\Delta\epsilon \approx 350$. However, values of the molar $\Delta\epsilon$ that were constant to within the ± 30 could be obtained by use of stock solutions of the enzyme, and changes in the molar $\Delta\epsilon$ produced by the subsequent addition of metal ions to both sample and reference cells were reproducible to within about ± 10 above 290 nm and about ± 20 between 275 and 290 nm. Below 275 nm reproducibility was substantially decreased, presumably because of the increased importance of light-scattering effects, coupled with increased distance from the region in which the baseline was established (325 to 340 nm).

An additional problem is the nonideality of the D_2O perturbation spectrum of the metal free enzyme (—, Figure 6b). Also shown (- - -), is the spectrum calculated on the basis of data from model studies involving the ethyl esters of *N*-acetyltryptophan and *N*-acetyltyrosine (Herskovitz & Sorensen, 1968), if an average of 1.9 residues of tryptophan and 7.5 residues of tyrosine are fully exposed to water. Although this combination represents the best fit among several such combinations, these estimates of exposure must be considered only as approximations.

Figure 6c compares the D_2O perturbation spectrum of the metal-free enzyme, spectrum 1, with that of Mg^{2+}-enzyme, spectrum 2. At wavelengths longer than 275 nm, these spectra differ in a reproducible manner in four regions, designated by a through d; maximum differences occur at about 298, 291, 287, and 282 nm, respectively, but the differences are quite small. All four of these regions correspond with regions of maximum slope in Figure 1d, and the observed differences alternate between positive and negative values. Although, as noted above, there is no reason to expect such an idealized behavior, on the basis of these results it seems much more reasonable to suggest that Mg^{2+} induces the Figure 1d spectrum not by producing a change in the solvation of tryptophan side chains by water but by producing a spectral shift via some other mechanism.

The change in the D_2O spectrum that is produced by the binding of Li^+ is not detectably different from that produced by the binding of Mg^{2+}, as might be expected from the similarity in the Figures 1c and 1d difference spectra. On the other hand, even though the Be^{2+}-induced difference spectrum (Figure 1b) is about twice as intense as the Mg^{2+}-induced spectrum, the change in the D_2O perturbation spectrum produced by Be^{2+} (not shown) was much smaller. (Although the change in region b, Figure 6, was approximately the same as that induced by Mg^{2+}, the changes in regions a, c, and d were of only marginal significance.)

A Comparison of the Circular Dichroism Spectrum of the Phospho-Enzyme and Its Magnesium(II) Complex. The circular dichroism of the (metal-free) phosphoenzyme was measured in the 260–285-nm range. Although the spectrum was noisy, because of the low relative value of the dichroic ratio to the overall absorbance, no significant difference between the dichroism of the metal-free phospho-enzyme and its Mg^{2+} complex was observed (not shown); viz., the profiles of both spectra were similar and the values of the measured dichroism were the same, at all wavelengths studied, within an error of about $\pm 10\%$. Hence, no *major* conformational change *involving tryptophan residues* occurs upon Mg^{2+} binding.

Discussion

Although efficient catalytic activity in many enzymic systems requires a bivalent metal ion activator, there are few if any other enzymic systems in which substitution of a monovalent metal ion (here, Li^+) for a bivalent metal ion (here, Mg^{2+}) is known to reduce activity as drastically as in the phosphoglucomutase system: to less than 2×10^{-8} of the normal activity (Ray et al., 1978). This huge reduction is particularly striking because the two metal ions bind completely, exhibit similar affinities for the enzyme (in the presence of substrate), and produce similar changes in the near ultraviolet spectrum on binding. Hence, identifying the structural basis for the difference between the reactivities of the Li^+ and Mg^{2+} forms of the enzyme could provide clues as to how Mg^{2+} (and other metal-ion activators) normally produce activation in this system.

Previous studies (Peck & Ray, 1969) have shown that metal ion activators, all of which are bivalent, induce essentially identical red shifts in the ultraviolet spectrum of the enzyme. On the basis of these and other spectral measurements it was suggested that (a) a minor conformational change accompanies the binding process, (b) the accompanying spectral change is produced by a reduction in the accessibility of tyrosine and tryptophan residues to the aqueous environment, and (c) the spectral change and the activation process are linked, mechanistically (Peck & Ray, 1969). The present results undercut the basis for one of these suggestions, and invalidate the other two. Thus, the perturbation of tyrosine residues was invoked originally in rationalizing the difference spectra induced by activating metal ions partly because of the prominence of the difference peaks at about 286 and 278 nm (Peck & Ray, 1969). However, it now appears that this prominence is caused primarily by a broad, underlying difference band that spans the region between 250 and 290 nm (see Results). In addition, differences in exposure of chromophores to the aqueous environment in the metal-free enzyme and the enzyme–metal complexes were suggested on the basis of solvent perturbation studies with D_2O, although no significant differences in exposure were detected with the perturbants, ethylene glycol, propanediol, and dimethyl sulfoxide (Figure 5 and Table I of Peck & Ray, 1969). In the present studies the previous results with D_2O were traced to metal ion contamination of the D_2O that was used in those studies; in fact, metal-free D_2O produces a closely similar perturbation spectrum with metal-free enzyme and the enzyme–metal complexes (Figure 6c). This observation is more easily rationalized, since it is difficult to visualize the type of conformational change required by the earlier results: one that substantially alters the exposure of aromatic residues to water (and D_2O) but not to small perturbants, such as ethylene glycol. Finally, the spectral change induced by metal-ion activators is not directly coupled with the activation process, itself, since Li^+ (a nonactivator; see above) produces a nearly equivalent spectral change (Figure 1, spectra c and d).

In general, difference spectra involving aromatic chromophores can arise from environmental perturbations that induce (a) differences in the nature of nearest neighbor interactions,

including those involved in H bonding, (b) differences in bond angles and distances within the chromophore, as well as (c) differences in the electrostatic field gradient to which the chromophore is exposed; however, these differences do not produce uniquely characteristic spectral effects. Hence, the origin of the difference spectra induced by metal-ion binding in the present system cannot be specified with certainty. However, changes in exposure to the solvent have been eliminated as a *major* contributing factor. In addition, several features of the observed difference spectra seem most reasonably rationalized in terms of direct electrostatic effects. Thus, changes in the average nearest neighbor interactions in solutions of free tryptophan in moderately to strongly polar solvents primarily alter the relatively narrow transitions within the long-wavelength portion of the near ultraviolet band; when the broader transitions at somewhat shorter wavelengths also are altered (usually to a much smaller extent) both types of transitions are shifted in the same direction (Andrews & Forster, 1972). By contrast, electrostatic perturbation not only can elicit substantially increased shifts of the broader indole transitions but can shift the broad and narrow transitions in opposite directions (see above reference). In fact, the metal-ion induced spectral changes observed here appear to exhibit both of the latter characteristics, although possible changes in band intensities (cf., Bailey et al., 1968) cloud the issue somewhat. In addition, changes in nearest neighbor interactions involving tryptophan in various solvents (Herskovitz & Sorensen, 1968) usually do not produce spectral differences with maxima at wavelengths as long as 297 nm (as in Figure 1), whereas differences in electrostatic field gradient can, even in aqueous solution (Andrews & Forster, 1972). Finally, the differential binding of protons (at different pH values) produces an enzymic difference spectrum that generally is similar to (but somewhat weaker than) that induced by metal-ion activators (see Results).[2]

If metal-ion binding elicits the observed difference spectra by virtue of a direct electrostatic effect primarily on enzymic tryptophans, the closely similar spectral changes elicited by Li^+ and all bivalent activators (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+}; Ray & Peck, 1972) must be the result of a decrease in the effective charge of the activator, relative to Li^+, during binding. Such a decrease would be easily rationalized if the binding of an activator, but not Li^+, were accompanied by the ligation of a carboxylate group that otherwise is some distance from the metal-ion binding site. The nearly double intensity of the difference spectrum in the 290–295-nm region that is produced by the binding of Be^{2+} also can be rationalized similarly, since Be^{2+} shows a substantially decreased tendency to coordinate with carboxylate groups relative to most of the above bivalent metal ions (cf., Sillen & Martel, 1969). Since activating metal ions such as Mg^{2+} are known to be bound at or very close to the substrate-binding site of the enzyme (Ray & Mildvan, 1973), the involvement or lack of involvement of a particular ligand in the binding of different metal ions would produce metal-ion dependent structural differences at or close to the substrate-binding site. Such differences in turn could provide a rationale for the much tighter binding of glucose 1-phosphate to the Li^+ than to the Mg^{2+} complex of the enzyme (Ray et al., 1978), as well as serve as a basis for the difference in activation produced by Mg^{2+} and by Li^+: less efficient utilization of the intrinsic binding energy of the substrate (cf., Jencks, 1975) in the latter case. Whatever explanation is used to rationalize the similarities between the shape and intensity of the difference spectrum induced by Mg^{2+} and Li^+, the dissimilarities between the shapes of these difference spectra and those induced (a) by all other monovalent cations and (b) by Be^{2+} would not follow directly. Size differences per se cannot be invoked since ions ranging in diameter from Li^+ (1.4 Å) to Ca^{2+} (2 Å) induce the same spectral changes, while closely similar changes are induced by monovalent cations ranging in size from Na^+ (2 Å) to $N(CH_3)_4^+$ (~4.4 Å). However, differences in coordination strengths and preferences again could be involved, if the three observed classes of cations (Be^{2+}, Li^+ and the bivalents except for Be^{2+}, and the monovalents except for Li^+) were bound to different sets of protein ligands in which some members of all sets are the same (cf., Banaszak et al., 1965). Thus, if the metal-ion induced difference spectra in the present system are produced by a direct electrostatic effect, positional differences of bound metal ions could modulate the overall spectral effect by altering the electrostatic field vector vis á vis the perturbed tryptophans.

[2] The possibility that distortion-induced spectral changes are involved in metal-ion binding cannot be eliminated, entirely; however, invoking such a mechanism to rationalize spectral differences in protein systems appears to be without precedent (cf., Brandts & Kaplan, 1973), and the approximately additive contribution to protein spectra of the contributions from its constituent amino acids (Herskovitz & Sorensen, 1968; Wetlaufer, 1962; Donovan, 1969) argues against the importance of this type of effect in several protein systems.

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