Structural Comparisons among the Central Complexes in the Phosphoglucomutase System by Means of Spectral Techniques†

Chong Ma and William J. Ray, Jr.*

ABSTRACT: In the phosphoglucomutase system, the interconversion of the substrate (glucose 1-phosphate, Glc-1-P) and product (glucose 6-phosphate, Glc-6-P) complexes of the Mg^{2+} form of the phosphoenzyme, viz., E_P·Mg·Glc-1-P and E_P·Mg·Glc-6-P, respectively, is too fast to allow an assessment of possible differences in their physical properties. In the present paper, three different strategies are used to slow or prevent the interconversion of such complexes so that their spectral properties can be examined, separately: removal of the bivalent metal ion; substitution of bound Li^+ for bound Mg^{2+}; removal of the acceptor hydroxyl groups of the substrate and product, viz., using 6-deoxyglucose 1-phosphate and 1-deoxyglucose 6-phosphate. In *all three* cases, essentially the same marked spectral differences were observed between these "frozen" complexes of the 1- and 6-phosphates with the enzyme; hence, analogous spectral differences between the rapidly interconverting E_P·Mg·Glc-1-P and Ep·Mg·Glc-6-P complexes are inferred. Solvent perturbation difference spectroscopy, using dimethyl sulfoxide, shows that when glucose 1- or 6-phosphate binds to the enzyme to give a frozen complex, an essentially identical net shielding of tryptophan residues from the solvent is produced with little or no net shielding of tyrosine residues. However, the major spectral *difference* between the frozen complexes of glucose 1- and 6-phosphates with the enzyme strongly resembles a tyrosine difference spectrum with a $\Delta\epsilon_{287}$ of $\sim$1000. The size of this difference is sufficiently large to suggest that bound glucose 6-phosphate perturbs at least one more residue equivalent of tyrosine than does bound glucose 1-phosphate. Solvent perturbation studies indicate that the differentially perturbed tyrosine(s) is an *internal* residue(s), which is taken as an indication that the enzyme adopts a different conformation in its complexes with glucose 1- and 6-phosphates. Spectral comparisons involving complexes of the phosphoenzyme with the α-1-phosphates of glucose, mannose, galactose, and ribose as well as comparisons of the corresponding complexes with the isomeric ω-phosphates indicate that it is primarily the 2-, 3-, and 4-hydroxyl groups of the sugar ring that control the above spectral differences and thus the presumed differences in the conformation of the enzyme in these complexes. A procedure for making a direct spectral comparison of the monophosphate complexes of the phosphoenzyme with the bisphosphate complex of the dephosphoenzyme, e.g., E_P·Li·Glc-1-P with E_D·Li·Glc-P_2, was developed. These comparisons suggest that two complexes of E_D·Li·Glc-P_2 actually are present at equilibrium, that the more stable complex is structurally similar to E_P·Li·Glc-1-P, and that the less stable one is similar to E_P·Li·Glc-6-P. Thus, the rapid interconversion of (at least) four, different, central complexes with at least one accompanying conformational change appears to be required for completion of a single catalytic cycle in the phosphoglucomutase reaction. The results seem to be more nearly in accord with an exchange mechanism for this enzyme than a minimal motion mechanism [cf. Ray, W. J., Jr., Mildvan, A. S., & Long, J. W. (1973) *Biochemistry 12*, 3724].

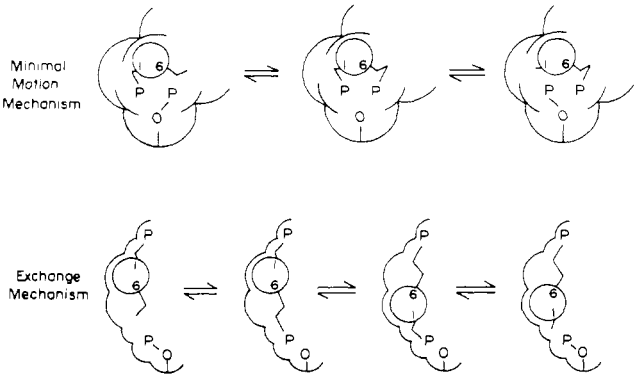
Kinetic studies of the catalytic cycle in the phosphoglucomutase reaction show that $-PO_3$ groups become covalently bonded to the enzyme at a single site and are detached from the enzyme at the same site (Ray & Roscelli, 1964b; Britton & Clark, 1968). On the basis of structural studies [cf. Ray & Peck (1972) and Ray et al. (1977)], this site must be the oxygen of the active-site serine residue. Since the catalytic cycle also includes the complex(es) of the dephosphoenzyme and glucose 1,6-bisphosphate, E_D·Glc-P_2,[1] and since this complex can react to give two different products, viz., to give either the complex of the phosphoenzyme with glucose 1-phosphate or that with glucose 6-phosphate, E_P·Glc-1-P or E_P·Glc-6-P, *without intervening dissociation steps* (Ray & Roscelli, 1964a), the structure of the bisphosphate complex must be such that *either* of its $-PO_3$ groups can be transferred to the hydroxyl group of the active-site serine. One way of achieving an alternative intramolecular $-PO_3$ transfer such as this involves a *unique* binding site for the glucose ring that is (essentially) unchanged during the catalytic cycle; the 1- and 6-phosphate groups of the bisphosphate occupy two different positions adjacent to the hydroxyl group of the acceptor serine residue which are so structured that $-PO_3$ transfer to the enzyme can occur from either. This reaction sequence has been referred to (Ray et al., 1976) as a "minimal motion" mechanism (see Scheme I). In a second type of mechanism, an "exchange" mechanism, two different E_D·Glc-P_2 complexes occur successively in the catalytic cycle and involve alternative binding interactions for the glucose ring. From an a priori standpoint, both mechanisms have their respective merits. Thus, the minimal motion mechanism simplifies the (observed) requirement that *both* Glc-1-P and Glc-6-P bind efficiently to the enzyme and that *both* bind much more tenaciously than other sugar phosphates (Lowry & Passonneau, 1969) but imposes a structural requirement that the *direction* of nucleophilic attack by the serine hydroxyl group on the 1- and 6-phosphate groups be different. On the other hand, a unidirectional attack of the serine hydroxyl group on either the 1- or 6-phosphate groups of bisphosphate is possible in an

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received July 16, 1979.* This work was supported in part by a grant from the National Institute of General Medical Sciences, U.S. Public Health Service (GM-08936).

[1] Abbreviations used: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; Glc-P, an equilibrium mixture of Glc-6-P and Glc-1-P; Glc-P_2, α-D-glucose 1,6-bisphosphate; 6-deoxy-Glc-1-P, 6-deoxy-α-D-glucose 1-phosphate; 1-deoxy-Glc-6-P, 1,5-anhydro-D-glucitol 6-phosphate; Man-1-P, α-D-mannose 1-phosphate; Man-6-P, D-mannose 6-phosphate; Gal-1-P, α-D-galactose 1-phosphate; Gal-6-P, D-galactose 6-phosphate; 3-PGA, D-glycerate 3-phosphate; 1,3-P_2GA, D-glycerate 1,3-bisphosphate; E_P and E_D, the phospho and dephospho forms of phosphoglucomutase.

0006-2960/80/0419-0751$01.00/0

Scheme I: Minimal Motion and Exchange Mechanisms for Phosphoglucomutase[a]



[a] The minimal motion mechanism involves only one E_D·Glc-P_2 complex, so structured that when the 6-PO_3 group is in position for transfer to the enzyme, only a minimal structural change is required in order to bring the 1-PO_3 group into position for transfer, but from a different site. The exchange mechanism involves two substantially different E_D·Glc-P_2 complexes that can be interconverted intramolecularly: one with the 6-PO_3 group in position for transfer to the enzyme and the other with the 1-PO_3 group in an equivalent position.

exchange mechanism. However, alternative binding interactions for the glucose ring are required in which the 6-phosphate group of one E_D·Glc-P_2 complex and the 1-phosphate of the alternative E_D·Glc-P_2 complex occupy equivalent positions for transfer to the enzyme.

In previous studies, the binding patterns of inorganic phosphate and its analogue methyl phosphonate were examined, together with the binding patterns for mono- and bisphosphate esters, and bulk nuclear relaxation in some of these that was produced by the Mn^{2+} complexes of the phospho and dephospho forms of the enzyme was assessed (Ray & Mildvan, 1973; Ray et al., 1973). The results indicated two functionally nonequivalent binding sites for phosphates in E_D, in accord with the exchange mechanism, where one phosphate site is the $-PO_3$ transfer site and the other functions solely as a binding site. The results are difficult to rationalize by means of the minimal motion mechanism, since this mechanism requires that $-PO_3$ transfer occur from *either* of two different phosphate binding sites. The present study was undertaken in an attempt to extend these conclusions by demonstrating that Glc-1-P and Glc-6-P do indeed interact with the enzyme in a different manner. Since numerous enzymic reactions involve reaction sequences in which the enzyme·product complex produced in the first step must rearrange before a second catalytic step occurs, such a demonstration would validate the phosphoglucomutase system as a model for such reactions.

Experimental Section

The phospho form of phosphoglucomutase was isolated from rabbit muscle, purified, and concentrated to ~40 mg/mL in 42% ammonium sulfate according to a procedure that will be published in a subsequent paper. The enzymic assay has been described previously (Ray et al., 1978). Metal-free phosphoglucomutase was obtained by dialysis first against EDTA in 20 mM Tris–SO_4 buffer, pH 7.5, and later by dialysis against the buffer alone [cf. Ray & Mildvan (1970)]. Subsequently, the solution was concentrated via pressure filtration to 50–100 mg/mL enzyme and was stored as frozen pellets in liquid nitrogen.

The metal-free, dephospho form of phosphoglucomutase was prepared as follows. To the phosphoenzyme at 20 mg/mL in 20% $(NH_4)_2SO_4$, containing 15 mM Mg^{2+}, 1 mM EDTA, and 0.1 M Tris–SO_4, pH 7.5, in an open-end, collodion, dialysis sac, was added 0.05 volume of 0.2 M Glc-1-P, and the enzyme was dialyzed at room temperature against the above solution (without the Glc-1-P) for 1 h. The addition and subsequent dialysis were repeated four more times using fresh dialysis solution each time. The solution then was dialyzed against two changes of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA, and four changes of 20 mM Tris-HCl buffer, pH 7.5, 0.1 mM in EDTA, 3 h apart. The final solution was concentrated by pressure filtration and stored as above. Conversion to the dephosphoenzyme was assessed by a modification of the procedure used by Lowry & Passonneau (1969) except that the change in optical density instead of fluorescence was used. On the basis of $\epsilon_{278} = 0.77$ (Najjar, 1955) and a molecular weight of 64 000 [cf. Ray & Peck (1972)], more than 90% of E_P was converted to E_D by this treatment.

6-Deoxyglucose 1-phosphate was synthesized from 1-*O*-methyl 6-deoxy-β-D-glucoside by (a) acetylating to give 6-deoxy-α-D-glucose tetraacetate (Richtmeyer, 1962), (b) converting to 6-deoxy-β-D-glucose tetraacetate (Wolfrom & Thompson, 1963), and (c) phosphorylating with anhydrous phosphoric acid (MacDonald, 1962). 1-Deoxyglucose 6-phosphate (1,5-anhydro-D-glucitol 6-phosphate) was synthesized from β-D-glucose pentaacetate by brominolysis of the 1-acetoxy group followed by reduction with $LiAlH_4$ to give 1,5-anhydro-D-glucitol (Gletcher, 1963); an enzymatic phosphorylation with yeast hexokinase (type IV, Sigma) gave the desired product (Slein, 1957). Both phosphates were further purified by chromatography on columns of Dowex-1-HCO_3, 8%, 200–400 mesh, with a gradient of 0–0.25 N $Et_3NH\cdot HCO_3$. The final products were converted to their potassium salts and characterized by their NMR spectra in D_2O (not shown).

1,3-Bisphosphoglyceric acid was synthesized from glyceraldehyde 3-phosphate by a modification of the procedure of Rose (1968)[2] and, after chromatography, was stored as frozen pellets in liquid nitrogen. Aliquots of 1,3-P_2GA were heated in boiling water for 1 h or at 30 °C overnight in sealed vials for complete hydrolysis. Glucose 1-phosphate was purified on Dowex-1-HCO_3, 8%, 200–400 mesh, with an elution gradient of 10–200 mM $KHCO_3$. The potassium salt was recrystallized from hot, 75% alcohol. α-D-Glucose 1,6-phosphate was prepared from Glc-6-P by phosphorylating the tetraacetate with anhydrous phosphoric acid in a manner similar to that described by Hanna & Mendicino (1970) and was chromatographed in a manner analogous to that used for Glc-1-P. Other sugar phosphates were obtained commercially and treated with dithizone in carbon tetrachloride to remove heavy metal ion contaminants (Sandell, 1950). Heavy metal ion contaminants were removed from solutions of Tris-HCl, pH 7.5, by passing the 0.5 M buffer over Chelex-100 and adjusting the pH of the effluent with a solution of ultrapure HCl (Alfa). Ultrapure grade LiCl (Alfa) was used in the presence of 1 mM EDTA. EDTA was crystallized from a boiling, 1 N HCl solution, washed with distilled water, and dried under vacuum. Other chemicals were reagent grade and were used without further purification. Laboratory grade deionized water was distilled and passed through a column of mixed-bed resin before use.

Ultraviolet spectra were obtained at 25 °C by use of a Perkin-Elmer 575 spectrophotometer equipped with a constant-temperature cell holder and a background corrector and

[2] The main difference was in the use of 0.05 volume of 30% hydrogen peroxide to stop the enzymic reaction. This was followed, 10 min later, by excess catalase to decompose the remaining peroxide.

operated in conjunction with a Sorenson ARC 3000 voltage regulator. A slit width of 1 nm and a scanning rate of 12 nm/min were used. Unless otherwise specified, spectra were obtained in the presence of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Solutions of the enzyme for spectral studies were prepared from concentrated, metal-free stock solutions (~40 mg/mL) in plastic test tubes that had been soaked in 2 N HNO_3 overnight and thoroughly rinsed with water before use. Noncritical amounts of enzyme solution were transferred with mechanical pipets employing freshly rinsed plastic tips; critical amounts were transferred with volumetric micropipets that had been rinsed with the same enzyme solution and then with water and air-dried just prior to use. Solutions used in solvent perturbation studies were made up by weight with a precision of at least 0.03% [cf. Ray (1978)]. Solutions were centrifuged prior to use in all spectral studies. All reported spectra were stable for hours and unless otherwise specified were reproduced by tracing a single scan. Difference spectra usually were obtained by using matched absorption cells with a 1-cm light path and solutions of enzyme at ~2.1 mg/mL (33 μM; OD_{280} = 1.62). After temperature equilibration and base line adjustment with a background corrector, difference spectra were obtained on the 0.1-OD scale, after making identical additions to both sample and reference cuvettes (no more than 0.02 mL/addition). Cuvettes were kept tightly closed except when making additions. The final concentrations of substrates, metal ions, or both were in excess of 20 times the dissociation constant. In cases where the additive, itself, made a contribution to absorbance differences, matched sectored cells with a 0.438-cm light path per compartment were used to obtain an internal correction for this contribution, and spectra were obtained on the 0.05-OD scale.

In comparisons involving the phospho and dephospho forms of phosphoglucomutase, the phospho form was generated in situ from the dephospho form, which was present, initially, in both sample and reference cuvettes. A fivefold excess of 1,3-bisphosphoglycerate (10 μL of a 15 mM solution) was added to one cuvette and the mixture was allowed to stand for 0.5 h to accomplish this. The same amount of hydrolyzed 1,3-bisphosphoglycerate was added to the other cuvette.

The solvent perturbation difference spectrum of the phosphoenzyme was obtained after first setting and recording the base line with 20% perturbant and water in separate compartments of sectored sample and reference cells (see above); all solutions contained 40 mM Tris–SO_4, pH 7.5, and 2 mM EDTA. Subsequently, the perturbant solution in the sample cell and the water solution in the reference cell were replaced with 1.000 to 10.000 dilutions of a concentrated stock solution of the enzyme in 22% perturbant or water, respectively. Solvent perturbation difference spectra of complexes of the enzyme subsequently were obtained with the same solutions by adding 10-μL aliquots containing an excess of either the required materials or water to each compartment and mixing with a bent Teflon rod. Differences between the solvent perturbation difference spectrum of the enzyme and that of the enzyme·substrate complexes were obtained by placing solutions containing identical concentrations of the enzyme, in either 20% perturbant or water, in both compartments of sectored sample and reference cells. Complexes of the enzyme were formed in the appropriate compartments by mixing in 10-μL aliquots containing an excess of the required materials (see figure legends) after obtaining a base line.

Results

Ultraviolet Difference Spectra Induced by Substrate Binding. For examination of the difference spectrum induced in phosphoglucomutase by the binding of *individual* substrates, viz., Glc-1-P, Glc-6-P, or Glc-1,6-P_2, transfer of $-PO_3$ groups catalyzed by the enzyme must be essentially stopped. Two basically different ways of doing this are deactivating the enzyme and using chemically altered pseudosubstrates. Deactivating the enzyme can be achieved by removing the activating metal ion. Thus, the activity of metal-free phosphoglucomutase is no more than 10^{-7} that of the Mg^{2+} enzyme (Ray et al., 1978) and the rate constant for $-PO_3$ transfer to bound substrates in the absence of bound metals thus is no greater than 10^{-4} s^{-1}. Moreover, the metal-free enzyme is expected to bind substrates in the same manner as its Mg^{2+} complex since the binding of substrates and Mg^{2+} is noncompulsory and essentially independent, both kinetically and thermodynamically [cf. Ray & Peck (1972)]. In addition, the essentially inactive Li^+ complex ($k_{cat} \leq 2 \times 10^{-5}$ s^{-1}) can be used as a deactivated form of the enzyme. [Li^+ binds competitively with Mg^{2+} and induces a similar spectral change in E_P as Mg^{2+} (Ray, 1978).] Alternatively, transfer of the $-PO_3$ group within complexes of the Mg^{2+} enzyme also can be blocked by using the pseudosubstrates 1-deoxy-Glc-6-P or 6-deoxy-Glc-1-P (Ray et al., 1976).

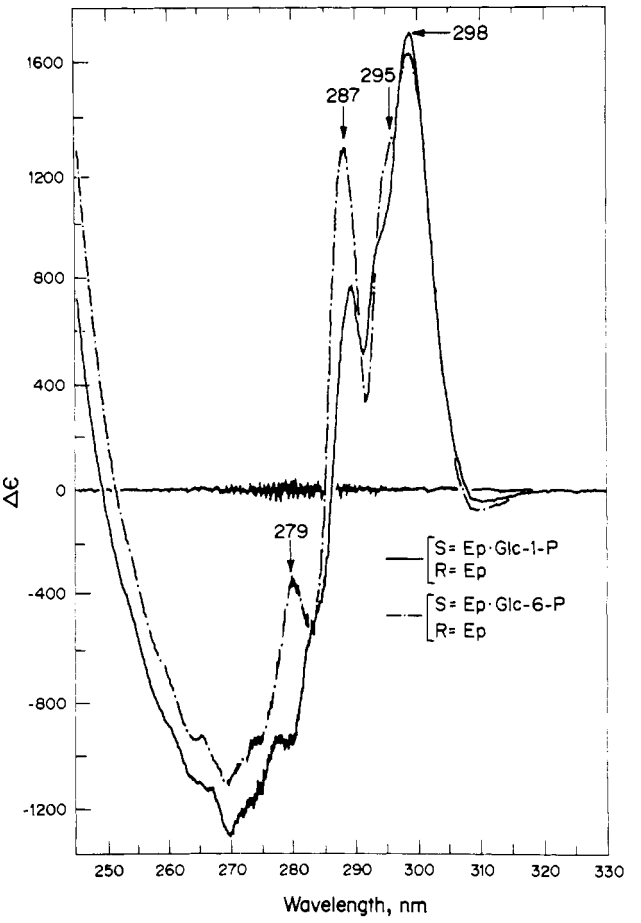


FIGURE 1: Substrate-induced difference spectra produced by the binding of glucose 1-phosphate and glucose 6-phosphate to metal-free phosphoglucomutase. The enzyme was present at a concentration of 2.1 mg/mL; a saturating concentration of 2 mM Glc-1-P or 4 mM Glc-6-P was used. Spectra were obtained in the manner described under Experimental Section; the important components in the sample and reference cells, S and R, respectively, are shown. An experimental base line is reproduced which shows the decreased signal-to-noise ratio in the region of maximum protein absorbance.

Figure 1 shows the spectral differences induced in metal-free phosphoglucomutase by the binding of Glc-1-P (—) and Glc-6-P (-·-). Successive scans of these spectra, as well as activity measurements at high enzyme concentrations, showed

that no significant interconversion of the complexes (via $-PO_3$ transfer) occurred within a 1-h time period. The subsequent addition of Li^+ to either E_P·Glc-1-P or E_P·Glc-6-P induced only minor changes ($\Delta\epsilon \leq 200$) in the 280–300-nm region (spectra not shown). The difference spectra induced by the binding of 1-deoxy-Glc-6-P and 6-deoxy-Glc-1-P to the metal-free enzyme were essentially indistinguishable from those induced by the corresponding glucose phosphates, and subsequent addition of Mg^{2+} to the E_P·deoxy-Glc-P system induced only minor spectral changes (spectra not shown). Since it is unlikely that all three of the transfer-blocking procedures would produce *anomalous* spectral effects that are the *same*, both qualitatively and quantitatively, we conclude that the difference spectra in Figure 1 are not significantly affected by any of them and that these spectral differences are quite similar to those produced by the binding of Glc-1-P or Glc-6-P to E_P·Mg to give catalytically active complexes. In subsequent studies, where possible, the Li^+ complex of the enzyme is used in place of the metal-free enzyme, since enzymic action, presumably caused by residual free Mg^{2+}, produces spectral changes over a period of several hours when "metal-free" enzyme is used in the presence of the normal substrates and 1 mM EDTA.[3]

The shapes and positions of the difference spectra in Figure 1 show that Glc-1-P and Glc-6-P induce nearly the same spectral shifts (red) in some tryptophan absorption bands since $\Delta\epsilon_{298}$, the maximum of the difference peaks, is essentially the same in both cases. Since this maximum is at longer wavelengths than usually is observed for solvent-induced perturbation of the narrow absorption bands of tryptophan (Ananthanarayanan & Bigelow, 1969), the tryptophan absorption bands that produce this difference peak probably occur at unusually long wavelengths prior to substrate binding. The shoulder at ~295 nm appears at a more normal wavelength for tryptophan perturbation spectra and probably represents a different tryptophanyl residue or residues since the *relative* intensities of the difference peaks at 295 and 298 nm vary markedly with conditions (see below). A significant difference between the spectral changes induced by Glc-6-P and Glc-1-P is observed in the peaks at ~287 nm, which, at least in the case of bound Glc-6-P, arises partly from the perturbation of tyrosine residues (see below).

Direct Comparison of Substrate-Induced Difference Spectra. Differences between the spectral *changes* in the enzyme induced by the formation of complexes with its normal substrates are equivalent to the spectral differences between the complexes themselves. These are shown in Figure 2. Figure 2a shows the differences in the spectra of the Glc-6-P and Glc-1-P complexes of E_P (—). Closely similar differences were observed between the corresponding complexes involving E_P·Li (-·-) as well as between the complexes of 1-deoxy-Glc-6-P and 6-deoxy-Glc-1-P with E_P·Mg (not shown), as was indicated in the previous section. The difference peaks at 279 and 287 nm suggest that the environment of a tyrosine residue or residues is substantially different in the Glc-1-P and Glc-6-P complexes. Also, there is a small difference in the environments of a tryptophan residue or residues in these complexes: 295-nm peak and long-wavelength valley at ~300 nm.

A spectral comparison of the phosphoenzyme·monophosphate complexes with the dephosphoenzyme·bisphosphate complex can be made by utilizing the procedure outlined in Scheme II. The use of this procedure is based on the following observations: no quantitative in situ conversion of E_P to E_D is known which can be employed at concentrations of enzyme that are useful for difference spectroscopy (~30 μM); an essentially quantitative in situ conversion of E_D to E_P can be achieved by treatment with excess 1,3-P_2GA [cf. Alpers & Lam (1969)]. After addition of *hydrolyzed* 1,3-P_2GA to an untreated sample of E_D, a direct spectral comparison of E_P and E_D in identical environments is possible. Only small differences in absorbance between E_P and E_D were detected in the range of 250–300 nm, and the largest difference ($\Delta\epsilon_{292}$ = 440) is not much larger than the largest absorbance difference between E_D·P_i and E_D ($\Delta\epsilon_{292}$ = 250), at least in com-

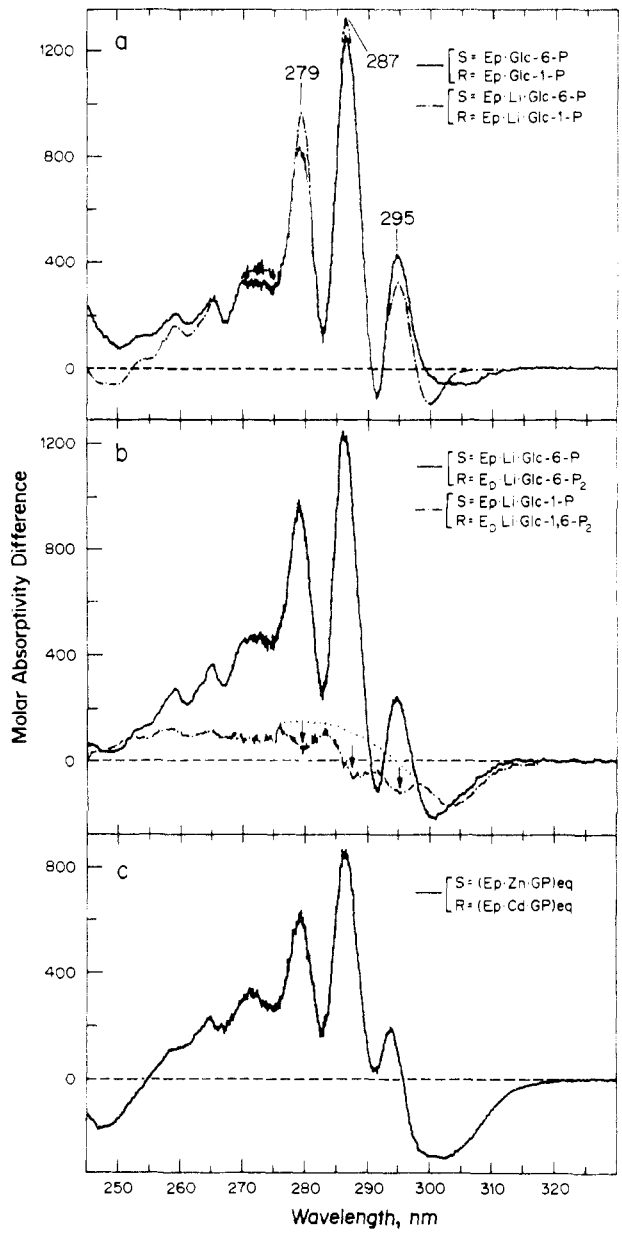
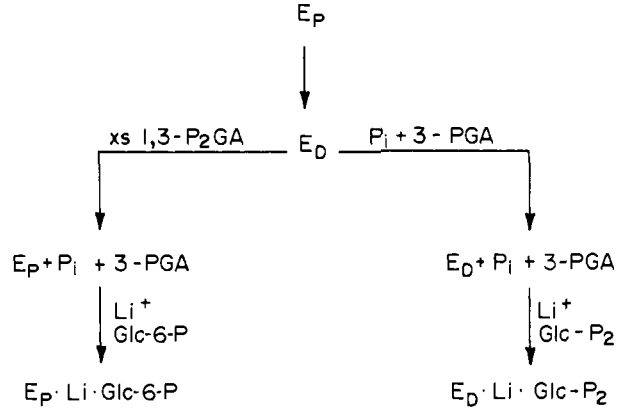


FIGURE 2: Spectral differences between complexes of phosphoglucomutase. Procedures and conditions are described under Experimental Section, and the important components in the sample and reference cells, S and R, respectively, are shown. The enzyme concentration was 2.1 mg/mL, and the final concentrations of substrates (or pseudosubstrates) and metals, when present, were as follows: Glc-1-P, 2 mM; Glc-6-P, 4 mM; Glc-P, 4 mM; Glc-1,6-P_2, 0.1 mM; 1-deoxy-Glc-6-P, 3.5 mM; 6-deoxy-Glc-1-P, 4.5 mM; Mg^{2+}, 2 mM; Li^+, 2 mM; Zn^{2+}, 1.1 mM; Cd^{2+}, 1.1 mM. (a) E_P·Glc-6-P vs. E_P·Glc-1-P (—); E_P·Li·Glc-6-P vs. E_P·Li·Glc-1-P (-·-). (b) E_P·Li·Glc-6-P vs. E_D·Li·Glc-1,6-P_2 (—); E_P·Li·Glc-1-P vs. E_D·Li·Glc-1,6-P_2 (-·-); the origin of (···) is described under Results. (c) $(E_P$·Zn·Glc-P$)_{eq}$ vs. $(E_P$·Cd·Glc-P$)_{eq}$ (—). The solutions used in (c) were equilibrated for 20 min before use; initial concentration of Glc-1-P was 4 mM.

[3] A concentration of $\sim 10^{-12}$ M free Mg^{2+} would be sufficient to cause the slow changes that were observed.

Scheme II: Schematic Showing the Procedure for Comparisons of Phospho and Dephospho Forms of Phosphoglucomutase and Their Complexes



parison with the larger spectral differences produced on substrate binding (Figure 1).

Figure 2b shows the difference in absorbance between one complex of the phosphoenzyme, E_P·Li·Glc-6-P, and the corresponding complex of the dephosphoenzyme, E_D·Li·Glc-P_2 (—). Since this difference spectrum closely resembles that of E_P·Li·Glc-6-P vs. E_P·Li·Glc-1-P (–·–, Figure 2a), the spectrum of E_D·Li·Glc-P_2 must resemble that of E_P·Li·Glc-1-P. That this is indeed the case also is shown in Figure 2b (–·–).

The spectral differences between the E_P·Li·Glc-1-P and E_P·Li·Glc-6-P complexes suggest that the glucose ring is interacting differently with the enzyme in these complexes (see Discussion). In such a case two different forms of the E_D·Li·Glc-P_2 complex are expected. These are designated as $(E_D$·Li·Glc-$P_2)_1$ and $(E_D$·Li·Glc-$P_2)_6$ to indicate enzyme–glucose ring interactions that are analogous to those observed in E_P·Li·Glc-1-P and E_P·Li·Glc-6-P, respectively. Since the equilibrium constants for binding of Glc-1-P to E_P or E_P·Mg (to give E_P·Glc-1-P or E_P·Mg·Glc-1-P) are substantially larger than those for the binding of Glc-6-P (to give E_P·Glc-6-P or E_P·Mg·Glc-6-P; Ray & Long, 1976a) and since these differences probably reflect differences in the enzyme–glucose ring interactions within these complexes, the $(E_D$·Li·Glc-$P_2)_1$ complex would be expected to exhibit the strongest interactions with the enzyme and thus to predominate over the $(E_D$·Li·Glc-$P_2)_6$ complex. Because differences in the enzyme–glucose ring interactions appear to be responsible for the spectral differences in Figure 2a (see Discussion), a similarity in the spectra of E_P·Li·Glc-1-P and $(E_D$·Li·Glc-$P_2)_1$ might be expected, as well as a similarity in E_P·Li·Glc-6-P and $(E_D$·Li·Glc-$P_2)_6$. The spectra in Figure 2b are in accord with these expectations; viz., the mixture of E_D·Li·Glc-P_2 complexes is similar, spectrally, to E_P·Li·Glc-1-P (–·–) but differs from the latter spectrum at wavelengths that suggest the presence of a minor component (see arrows) that is similar, spectrally, to E_P·Li·Glc-6-P. Actually, the characteristic peaks for the presumed minor component of the E_D·Li·Glc-P_2 mixture are superimposed on relatively common features of difference spectra in the phosphoglucomutase system: a long-wavelength valley at 300–305 nm and a broad maximum at shorter wavelengths, both of which are apparently related to perturbation of broad tryptophan bands (Ray, 1978; see also below). The outlines of part of these features in the lower spectrum of Figure 2b are *suggested* by the dotted line.

In an earlier study, Ray & Long (1976b) showed that in an equilibrium mixture of enzyme·substrate complexes in the presence of Zn^{2+} the monophosphate complex, E_P·Zn·Glc-6-P, predominates, while in the presence of Cd^{2+} complexes the other bisphosphate complex(es), E_D·Cd·Glc-P_2, is essentially absent. They suggested that the primary spectral difference between these mixtures of complexes (in the 280–290-nm region) does not originate in the difference in identity of the bound metal ion but is produced by the differences in the attachment of the transferable $-PO_3$ group. The negative aspect of this suggestion is now verified by the observation that there are only small spectral differences between the 6-deoxy-Glc-1-P (or 1-deoxy-Glc-6-P) complexes of E_P·Zn and E_P·Cd (maximum $\Delta\epsilon \leq 150$; difference spectrum not shown). Hence, in accord with expectation, Figure 2c shows that the same general features characterizing the spectral difference between Ep·Li·Glc-6-P and E_D·Li·Glc-P_2 also are found between the equilibrium mixtures of central complexes involving Zn^{2+} and Cd^{2+}, respectively. The smaller *size* of the difference peaks in the latter case is caused by the substantial amounts of E_P·Zn·Glc-1-P and E_D·Zn·Glc-P_2 that are present in the former equilibrium mixture. [The valley at around 300 nm is much larger but presumably is analogous to one of the background features that was noted in Figure 2b (···); see above.] However, the present results do not support the positive aspect of the suggestion in the earlier study about the *origin* of the spectral difference in the 280–290-nm region. This suggestion was based on the assumption that E_P·Glc-6-P and E_P·Glc-1-P were more likely to be similar, spectrally, than E_P·Glc-6-P and E_D·Glc-P_2, an assumption that is invalidated by the present results (see Discussion).

Solvent Perturbation Studies. Solvent perturbation difference spectra of the phosphoenzyme and its complexes with Glc-1-P and Glc-6-P were examined by using 20% Me_2SO or 20% sucrose as perturbants. The Me_2SO perturbation spectrum of the phosphoenzyme is shown in Figure 3a (—). It is similar to previously published spectra (Peck & Ray, 1969) and indicates the presence of tyrosine and tryptophan residues that are accessible to the perturbant, although the long-wavelength difference peak for tryptophan is substantially red-shifted with respect to the corresponding difference peak of model compounds under analogous conditions, e.g., *N*-acetyltryptophan ethyl ester (Herskovits & Sorenson, 1968). The binding of Glc-1-P causes a decrease in the intensity of this long-wavelength peak (–·–, Figure 3a), which, together with intensity changes in other parts of the spectrum, indicates that substrate binding produces a shielded or less accessible tryptophan(s), with little change in the accessibility of tyrosines. Thus, the difference between the two solvent perturbation difference spectra in Figure 3a (—, Figure 3b)[4] is similar to an Me_2SO perturbation spectrum of *N*-acetyltryptophan ethyl ester (Herskovits & Sorenson, 1968) (- - -, Figure 3b), which has been reduced in intensity by one-half (on a molar basis) and shifted to longer wavelengths by ~2.5 nm.

The solvent perturbation difference spectrum for E_P·Glc-6-P (not shown) was visually indistinguishable from that of E_P·Glc-1-P (–·–, Figure 3a); viz., the solvent perturbation difference spectrum of E_P (—, Figure 3a) and thus the *exposure* of aromatic residues were similarly altered by the binding of Glc-6-P and Glc-1-P. A direct comparison of these alterations was made by obtaining the difference between the solvent perturbation spectra of the respective substrate complexes in sectored absorption cells. However, in this study the Li^+ form of the enzyme was used to eliminate possible interconversion

[4] The plot (—, Figure 3b) was obtained from a point-by-point subtraction of the two spectra in Figure 3a; a direct measurement of the difference between the two difference spectra, obtained by using sectored sample cells, was quite similar in appearance (not shown).

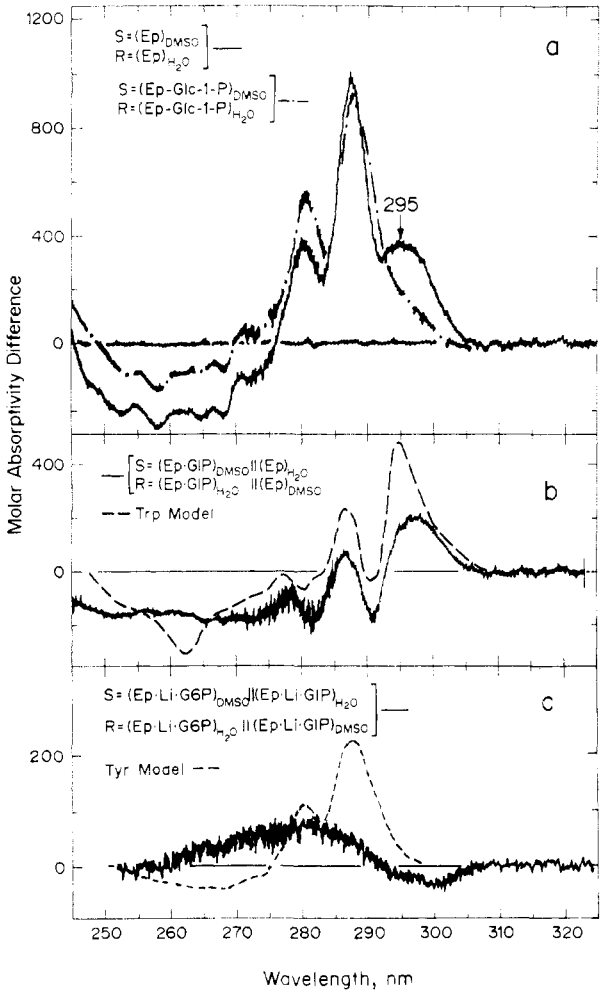


FIGURE 3: Me_2SO perturbation difference spectra and the differences between these. The important components in the sample and reference cells, S and R, respectively, or the compartments of S and R are shown in each case, and the procedure is described under Experimental Section, along with the concentrations of noncritical components. (a) The perturbation difference spectra induced by 20% Me_2SO for E_P (—) and for the complex E_P·Li·Glc-1-P (·–·). Concentrations were as follows: E_P, 2.1 mg/mL; LiCl, 2.0 mM; Glc-1-P, 2.0 mM. (b) The differences between the Me_2SO perturbation difference spectra of E_P and E_P·Li·Glc-1-P, viz., between the two difference spectra in part a.[4] The dashed line is a half-intensity Me_2SO (20%) perturbation difference spectrum of acetyltryptophan ethyl ester, constructed from data in Herskovits & Sorenson (1968) and shifted to longer wavelengths by 2.5 nm. (c) The difference between the Me_2SO perturbation difference spectra of the E_P·Li·Glc-1-P and E_P·Li·Glc-6-P complexes. Concentrations were the same as in part a, except for Glc-6-P, which was present at 4.0 mM. The average results from five successive scans of the base line (no glucose phosphates present) were subtracted from the average results of five successive scans in the presence of the indicated arrangement of glucose phosphate complexes.

of these complexes (see previous section), since the spectral difference between E_P·Glc-6-P and E_P·Glc-1-P is not significantly altered by added Li^+ (Figure 2a). The difference between the solvent perturbation spectra of E_P·Li·Glc-6-P and E_P·Li·Glc-1-P (—, Figure 3c) is broad and shallow, with an essentially featureless maximum in the 260–290-nm region and valley in the 290–305-nm region. This difference resembles the background spectral features noted in the previous section (cf. Figure 2b) that are relatively common in difference spectra of phosphoglucomutase and that probably arise from environmental effects on tryptophans. In the present case, the effect is relatively small ($\Delta\epsilon_{max} \approx 70$, on a molar basis) and, in fact, minor differences in the environments of tryptophans are expected (from the small differences in the spectra of the E_P·Li·Glc-6-P and E_P·Li·Glc-1-P complexes between 290 and 300 nm; Figure 2a). Also shown in Figure 3c (- - -) is the expected difference between the Me_2SO perturbation spectra of the E_P·Li·Glc-6-P and E_P·Li·Glc-1-P complexes if one less residue equivalent of tryosine were exposed to the aqueous environment in E_P·Li·Glc-6-P than in E_P·Li·Glc-1-P [data from Herskovits & Sorenson (1968)]. From this comparison, it seems unlikely that the difference in exposure of tyrosines between these two complexes exceeds a small fraction of one residue equivalent. Hence, it is difficult to account for the major spectral differences between these complexes (between 275 and 290 nm) (Figure 2a) in terms of a differential exposure of tyrosines. Thus, the size of the molar extinction difference at 288 nm due to tyrosine perturbation must be nearly 1000, if a reasonable assumption is made about possible contributions to this difference that might arise from a minor differential perturbation of tryptophan residues. In fact, the largest reported molar extinction difference for tyrosine of which we are aware is ~1000 (Bigelow, 1960), and this extinction difference involves the complete exposure (on denaturation) of completely buried tyrosines. Hence, in view of the small differences in residue equivalents of exposed tyrosine between the E_P·Li·Glc-6-P and E_P·Li·Glc-1-P complexes, neither a direct nor indirect differential shielding of tyrosines by Glc-6-P and Glc-1-P is likely to provide a rationale for the major spectral differences between these two complexes in the tyrosine difference region (Figure 2a). A differential electrostatic perturbation of tyrosine residues [cf. Anderson & Forster (1972)] that are equally accessible to the solvent before and after binding also is unlikely. Thus, the results in Figure 3c (—) require that the spectral differences between the E_P·Li·Glc-6-P and E_P·Li·Glc-1-P complexes in 20% Me_2SO be essentially identical with the observed differences in water (Figure 2a) to within a molar extinction difference of ~70. Thus, the spectral differences between the above complexes most likely arise from a differential perturbation of tyrosine residues that, even in E_P·Li, are either *inaccessible* or only *marginally accessible* to the solvent. A differential perturbation of such residues could be rationalized in terms of conformational changes that accompany the binding process and that are different for Glc-6-P and Glc-1-P.

Spectral Comparison of Sugar Phosphate Complexes of Phosphoglucomutase. The spectral differences between E_P·Li·Glc-6-P and E_P·Li·Glc-1-P complexes in the tyrosine difference region (275–290 nm) also are observed in comparisons of the corresponding complexes with 1- and ω-phosphates of mannose, galactose, and ribose (Figure 4a) but to successively smaller extents. A similar comparison of complexes involving the corresponding 1-phosphates with the E_P·Li·Glc-1-P complex (Figure 4b) indicates a perturbation equal to or greater than that in the latter complex: plateau or peaks (↑) in the tyrosine difference region. [The difference spectrum obtained with 2-deoxy-Glc-1-P is essentially identical with that obtained with Man-1-P (—, Figure 4b) and is not shown.] A further comparison of complexes involving the ω-phosphates of these sugars with E_P·Li·Glc-6-P (Figure 4c) indicates that in each case tyrosine perturbation is less extensive than in the latter complex: valleys (↓) in the tyrosine difference region. Other less important features of these comparisons include small peaks or valleys with a half-width of ~5 nm in the 290–300-nm region that are produced by the differential perturbation of the narrow absorption bands of tryptophan residues and that usually appear superimposed on a broader valley; e.g., the arrow (↑) in Figure 4c points to a peak at ~297 nm superimposed on a broader valley (···) in

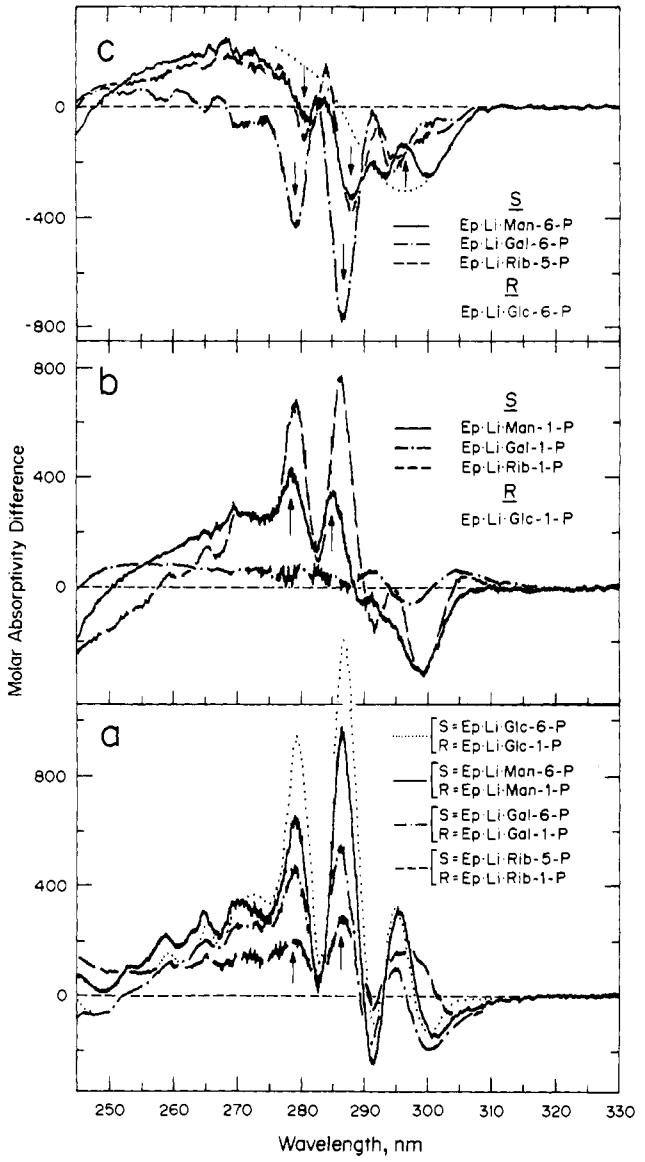


FIGURE 4: Spectral differences between complexes of phosphoglucomutase with substrates and pseudosubstrates. Procedures are described under Experimental Section, and the important components in the sample and reference cells, S and R, respectively, are shown. Concentrations of substrates and pseudosubstrates were as follows: Glc-1-P, 2 mM; Glc-6-P, 4 mM; Man-1-P, 5 mM; Man-6-P, 5 mM; Gal-1-P, 4 mM. (a) The complexes of E_P·Li with the 1- and ω-phosphates of various sugars: mannose (—); galactose (-·-); ribose (- - -); glucose (···). (b) Difference spectra with E_P·Li·Glc-1-P as the reference: E_P·Li·Man-1-P (—); E_P·Li·Gal-1-P (-·-); E_P·Li·Rib-1-P (- - -). (c) Difference spectra with E_P·Li·Glc-6-P as the reference: E_P·Li·Man-6-P (—); E_P·Li·Gal-6-P (-·-); E_P·Li·Rib-5-P (- - -).

the plot for Man-6-P (—); see also the valley in Figure 2c. Similarly, some of the tyrosine difference peaks (↑) or valleys (↓) are superimposed upon and slightly shifted by a much broader peak in the 260–290-nm region. Both of these broader features are present in other difference spectra, as was noted in a previous section.

The differences in tyrosine perturbation observed on binding of the various sugar phosphates might be rationalized in terms of differences in contact between tyrosine residues of the enzyme and the 2-, 3-, and 4-hydroxyl groups of bound sugar phosphates. However, the comparison of the Me_2SO perturbation difference spectrum of E_P·Li·Glc-6-P and E_P·Li·Glc-1-P (previous section) suggests that the increased spectral red shift of tyrosine caused by the binding of Glc-6-P is not the result of an increased shielding that accompanies the binding process. Hence, it seems more reasonable to suggest that the binding of the various sugar phosphates produces a gradation of conformational changes that alters the environments of internal (or only marginally exposed) tyrosines as follows: Glc-1-P ≈ Gal-1-P < Man-1-P ≈ Gal-6-P < Rib-1-P < Rib-5-P ≈ Man-6-P < Glc-6-P. (The existence of varying amounts of only two extreme conformations represented by the E_P·Li·Glc-1-P and E ·Li·Glc-6-P complexes also is possible.)

Discussion

The immediate objective of the present study was to compare the near-UV spectral properties of the reactive substrate and product complexes of phosphoglucomutase. Since in the presence of bound Mg^{2+}, the normal metal ion activator (Ray & Peck, 1972), these complexes are rapidly interconverted (k_{cat} > 1000 s^{-1}), $-PO_3$ transfer must be blocked quite efficiently if individual complexes are to be examined separately. In the present studies the transfer process is blocked by removal of bound Mg^{2+} (k_{cat} < 10^{-4} s^{-1} for the metal-free enzyme), by replacement of bound Mg^{2+} by Li^+ (k_{cat} < 10^{-5} s^{-1} for the E_P·Li complex), or by removal of the acceptor hydroxyl groups of the substrates.[5] In each case, the spectral differences between the corresponding complexes of the 1- and 6-phosphates are essentially the same (see Figure 2a). In view of the disparate ways in which $-PO_3$ transfer is blocked in these complexes, it seems reasonable to suggest that the same spectral differences would be found between the normal E_P·Mg·Glc-6-P and E_P·Mg·Glc-1-P complexes, and the subsequent analysis is based on this assumption. In these arguments, E_P will be used interchangeably with E_P·Li, since most studies were conducted with both complexes and no significant differences were observed between the two.

(1) Both Glc-1-P and Glc-6-P produce substantial spectral changes on binding to E_P; these changes are similar in overall appearance (Figure 1) but differ in detail (Figure 2a).

(2) Some of the *common features* of the above spectral changes arise from a decrease in exposure of tryptophans (probably two; see Results) to the aqueous surroundings, as is indicated by solvent perturbation difference spectroscopy. In fact, the decreased exposure of tryptophans produced by the binding of Glc-1-P (Figure 3b) is essentially the same as that produced by the binding of Glc-6-P (cf. Figure 3c).

(3) The *major* spectral *differences* produced by the binding of Glc-1-P and Glc-6-P, viz., the *major* differences between the E_P·Glc-6-P and E_P·Glc-1-P complexes, appear to arise from a differential perturbation of tyrosine residues (Figure 2a). In addition, the *size* of these differences is substantial ($\Delta\epsilon_{288}$ ≈ 1100 M^{-1} cm^{-1}). Although these differences must be produced via an alternative mechanism (see below), the magnitude of the differences is equivalent to that expected if a single tyrosine were completely exposed in E_P·Glc-1-P and completely shielded in E_P·Glc-6-P (see Results).

(4) Solvent perturbation difference spectroscopy indicates that, at most, there is a difference of only a small fraction of one residue equivalent between the exposure of tyrosines in E_P·Glc-6-P and E_P·Glc-1-P (Figure 3c). Hence, a differential shielding of tyrosines cannot be the basis for the observed spectral differences between these two complexes (see below).

(5) Since the maxima in the spectral differences between E_P·Glc-6-P and E_P·Glc-1-P are not shifted by 20% Me_2SO or

[5] Although $-PO_3$ transfer to the deoxyglucose phosphates cannot occur, a very slow transfer to water does occur in the presence of these pseudosubstrates (Ray et al., 1977).

20% sucrose, nor are the intensities of the difference peaks altered by these solutes, the tyrosines that are differentially perturbed in the above two complexes are unlikely to be external residues; viz., they probably are internal or only marginally exposed residues.

(6) Although the *magnitude* of the spectral differences between the E_P·Glc-6-P and E_P·Glc-1-P complexes could be rationalized in terms of a single tyrosine whose environment is drastically different in E_P·Glc-1-P and E_P·Glc-6-P (see above) since the tyrosine or tyrosines involved appear to be either internal or marginally exposed, we favor a rationale in which several (internal) tyrosines are affected to a smaller extent, viz., a mechanism in which Glc-1-P and Glc-6-P produce conformational changes on binding which are different in detail and which, because of these differences, affect several internal tyrosines to a modest extent.

(7) A detailed analysis of the graded effects that are produced by the binding of the various sugar phosphates is premature. However, to a *first approximation* the spectral differences produced on binding of the 6-phosphates (large tryptophan and tyrosine perturbation) are less sensitive to changes in the hydroxyl groups at the 1, 2, and 3 positions[6] while differences produced on binding of the 1-phosphates (large tryptophan but minimal tyrosine perturbation) are less sensitive to changes in the hydroxyl groups at the 4 and 6 positions, as though the portion of the molecule distal to the acceptor group was the more important in producing a normal complex, at least with respect to tyrosine perturbation.

(8) In any case, the present data show that the enzyme–glucose ring interaction is different in the E_P·Glc-1-P and E_P·Glc-6-P complexes. This difference eliminates the simple form of the minimal motion mechanism (Scheme I; see below) and is in accord with conclusions drawn earlier from a more indirect approach (Ray et al., 1973; Ray & Mildvan, 1973).

(9) If the glucose ring interacts differently with the enzyme in the E_P·Glc-1-P and E_P·Glc-6-P complexes, two types of E_D·Glc-P_2 complexes probably also exist, $(E_D$·Glc-$P_2)_1$ and $(E_D$·Glc-$P_2)_6$, although these cannot be examined separately. However, since Glc-1-P binds to the enzyme substantially better than Glc-6-P (Ray & Long, 1976a), the interaction of the glucose ring with the enzyme should be energetically more favorable in the $(E_D$·Glc-$P_2)_1$ complex and this complex should predominate over the $(E_D$·Glc-$P_2)_6$ complex. In fact, a direct comparison shows that the spectrum of the E_D·Glc-P_2 complexes is quite similar to the spectrum of the E_P·Glc-1-P complex but differs from it in the manner expected if a small proportion were present as $(E_D$·Glc-$P_2)_6$.[7]

In numerous enzymic reactions a product of the first catalytic step (the initial product) serves as a substrate for a subsequent step, and phosphoglucomutase is one such reaction (Sutherland et al., 1949; Najjar & Pullman, 1954). In most reactions of this type a dissociation–association step intervenes before a second reaction takes place. An earlier study of the phosphoglucomutase reaction (Ray & Roscelli, 1964a) shows that dissociation of the intermediate E_D·Glc-P_2 complex(es) is not required for completion of the catalytic cycle and places this reaction in a second, less common category. Reactions in this category may be divided into those requiring that binding interactions between the enzyme and the initial product be substantially altered before completion of the catalytic cycle and those lacking such a requirement. The present results, which show that the initial substrate and final product complexes are structurally different, place the phosphoglucomutase reaction in the former category, although molecular models indicate that a minimal motion mechanism (Scheme I) is at least sterically feasible in this reaction.

The terms "minimal motion" and "exchange" mechanism (Scheme I) originally were formulated with only the E_D·Glc-P_2 complexes of phosphoglucomutase in mind. Although the former term seems sufficiently general in its implications, the latter represents a special case within a broader group of "internal rearrangement" mechanisms for enzymes that utilize more than one catalytic step but are able to operate without dissociation of the intermediate thus produced. The latter mechanisms can be further subdivided into (a) processes in which the original enzyme–substrate binding interactions are maintained more or less unaltered during successive catalytic steps and different groups are brought into proper position for successive reactions by changes in the enzyme itself and (b) processes in which binding interactions in the initial product complex must be altered (intramolecularly) in order to produce the second substrate complex. A distinction between these possibilities in the phosphoglucomutase reaction cannot be made at present, although we hope eventually to make such a distinction.

[6] As a first approximation, ribose 1- and 5-phosphates are considered as "hexoses" in which the 3-carbon and its 3-hydroxyl group are missing.

[7] The spectral differences previously observed (Ray & Long, 1976b) between a mixture of complexes that, by direct analysis, is predominately in the E_P·Glc-6-P form and one that is almost entirely in the E_D·Glc-P_2 form (cf. Figure 2c) thus appear to be caused by an altered enzyme–glucose ring interaction that *follows* the catalytic step, viz., $(E_D$·Glc-$P_2)_6 \rightarrow (E_D$·Glc-$P_2)_1$, instead of one that accompanies it, viz., E_P·Glc-6-P $\rightarrow (E_D$·Glc-$P_2)_6$, as was previously suggested (Ray & Long, 1976b).

References

Alpers, J. B., & Lam, G. K. H. (1969) *J. Biol. Chem. 244*, 200.

Ananthanarayanan, V. S., & Bigelow, C. C. (1969) *Biochemistry 8*, 3717, 3823.

Anderson, L. J., & Forster, L. S. (1972) *Biochemistry 11*, 1875.

Bigelow, C. C. (1960) *C. R. Trav. Lab. Carlsberg 31*, 305.

Britton, H. G., & Clark, J. B. (1968) *Biochem. J. 110*, 161.

Gletcher, H. G., Jr. (1963) in *Methods in Carbohydrate Chemistry II* (Whisler, R. L., & Wolfrom, M., Eds.) p 197, Academic Press, New York.

Hanna, R., & Mendicino, J. (1970) *J. Biol. Chem. 245*, 4031.

Herskovits, T. T., & Sorensen, M. (1968) *Biochemistry 7*, 2523, 2533.

Lowry, O. H., & Passonneau, J. V. (1969) *J. Biol. Chem. 244*, 910.

MacDonald, D. L. (1962) *J. Org. Chem. 27*, 1107.

Najjar, V. A. (1955) *Methods Enzymol. 1*, 294.

Najjar, V. A., & Pullman, M. E. (1954) *Science 119*, 631.

Peck, E. J., Jr., & Ray, W. J., Jr. (1969) *J. Biol. Chem. 244*, 3754.

Ray, W. J. (1978) *Biochemistry 17*, 1554.

Ray, W. J., Jr., & Roscelli, G. A. (1964a) *J. Biol. Chem. 239*, 1228.

Ray, W. J., Jr., & Roscelli, G. A. (1964b) *J. Biol. Chem. 239*, 3935.

Ray, W. J., Jr., & Mildvan, A. S. (1970) *Biochemistry 9*, 3886.

Ray, W. J., Jr., & Peck, E. J., Jr. (1972) *Enzymes, 3rd Ed. 6*, 407.

Ray, W. J., Jr., & Mildvan, A. S. (1973) *Biochemistry 12*, 37.

Ray, W. J., Jr., & Long, J. W. (1976a) *Biochemistry 15*, 3993.

Ray, W. J., Jr., & Long, J. W. (1976b) *Biochemistry 15*, 4018.

Ray, W. J., Jr., Mildvan, A. S., & Long, J. W. (1973) *Biochemistry 12*, 3724.

Ray, W. J., Jr., Long, J. W., & Owens, J. D. (1976) *Biochemistry 15*, 4006.
Ray, W. J., Jr., Mildvan, A. S., & Grutzner, J. B. (1977) *Arch. Biochem. Biophys. 184*, 453.
Ray, W. J., Jr., Szymanski, E., & Ng, L. (1978) *Biochim. Biophys. Acta 522*, 434.
Richtmeyer, N. K. (1962) in *Methods in Carbohydrate Chemistry I* (Whistler, R. L., & Wolfrom, M. L., Eds.) p 107, Academic Press, New York.
Rose, Z. B. (1968) *J. Biol. Chem. 243*, 4810.
Sandell, E. B. (1950) *Colorimetric Determination of Traces of Metals*, 2nd ed., Interscience, New York–London.
Slein, M. W. (1957) *Methods Enzymol. 3*, 154.
Sutherland, E. W., Cohn, M., Pasternak, T., & Cori, C. F. (1949) *J. Biol. Chem. 180*, 1285.
Wolfrom, M. L., & Thompson, A. (1963) in *Methods in Carbohydrate Chemistry II* (Whistler, R. L., & Wolfrom, M. L., Eds.) p 211, Academic Press, New York.

Resonance Raman Spectroscopy of Arsanilazocarboxypeptidase A: Conformational Equilibria in Solution and Crystal Phases†

R. K. Scheule, H. E. Van Wart,‡ B. L. Vallee,* and H. A. Scheraga*

ABSTRACT: Resonance Raman spectroscopy has been employed to explore the environment of the azoTyr-248 residue of arsanilazotyrosine-248 carboxypeptidase A in solution and in crystal phases. In solution near pH 6, azoTyr is protonated and studies of model compounds indicate that azoTyr-248 is in an aqueous environment. At pH 8.5, the spectrum of the arsanilazoenzyme correlates well with those of model azophenols forming complexes with zinc, in accord with earlier data demonstrating an intramolecular complex between azoTyr-248 and the active-site zinc atom of the enzyme. Zinc is bound to the phenolic oxygen and an azo nitrogen atom of azoTyr, with the azo group in the planar trans conformation. At pH 11, this intramolecular complex is dissociated and azoTyr-248 then exists as the ionized azophenolate species. Resonance Raman studies of *o*-hydroxyazobenzene models for azoTyr show that in solution the azophenol forms of these molecules coexist in two different conformations which differ with respect to the presence or absence of an intramolecular hydrogen bond between the phenolic proton and a nitrogen atom of the azo group. Each of these conformations exhibits characteristic ν^{NN} and $\nu^{\phi N}$ bands, with intensities proportional to their concentrations. The relative amounts of these two forms which are in equilibrium depend on the capacity of the local environment to act as hydrogen-bond acceptors. Hence, the relative intensities of both the pair of ν^{NN} and the pair of $\nu^{\phi N}$ bands in the resonance Raman spectrum of azocarboxypeptidase provide specific information about the state of hydrogen bonding of the phenolic proton of azoTyr-248, which has been thought by some to be transferred to the substrate during catalysis. In solution, azoTyr-248 is predominantly hydrogen-bonded *intra*molecularly and exists in an "aqueous-like" environment. However, crystallization apparently induces a conformational change that enables the phenolic proton of Tyr-248 to form an *inter*molecular hydrogen bond to a group of the protein. This interaction may be related to the marked reduction of the enzyme's activity brought about by crystallization.

Arsanilazocarboxypeptidase A,[1] the derivative of carboxypeptidase A, obtained by selective chemical modification of its active-site Tyr-248 residue with diazotized arsanilic acid, has been studied intensively in our laboratories. In particular, absorption and circular dichroism spectra have characterized both its local conformation as a function of its physical state (Johansen & Vallee, 1971, 1973, 1975) and its interaction with inhibitors (Johansen et al., 1976; Alter & Vallee, 1978). Recently, we have further elucidated chemical details pertinent to these molecular events by means of resonance Raman (rR)[2] spectroscopy (Scheule et al., 1977, 1979).

The rR spectra of the azoenzyme and the apoazoenzyme in solution (Scheule et al., 1977) identify the same pH-dependent species detected earlier by absorption spectrometry. In addition, the rR spectra provide structural details of the intramolecular coordination complex between azoTyr-248 and the active-site zinc atom and have similar potential for the conformation of the rest of the enzyme. However, for this purpose more extensive band assignments are required. This has now been achieved through the study of the rR spectra of several azophenols and their isotopically substituted derivatives which serve as models of azotyrosine (Scheule et al., 1979). The resultant data are employed here to identify the vibrational bands in the rR spectra of azocarboxypeptidase, and these assignments are used to interpret the spectral changes in the enzyme induced by various environmental perturbations, including crystallization and changes in pH. Crystallization is known to affect the spectral properties of

† From The Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853 (R.K.S. and H.A.S.), and the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115 (H.E.V.W. and B.L.V.). *Received October 1, 1979.* This work was supported by Research Grants GM-14312 and GM-15003 from the National Institutes of Health, U.S. Public Health Service, and from the National Science Foundation (PCM 75-08691).

‡ Present address: Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306.

[1] In order to simplify the presentation, "arsanilazotyrosine-248 carboxypeptidase", "arsanilazocarboxypeptidase", "azocarboxypeptidase", and "azoenzyme" are all terms used interchangeably with monoarsanilazotyrosine-248 zinc carboxypeptidase A; "apoazoenzyme" and "apoenzyme" are used interchangeably with apoarsanilazotyrosine-248 carboxypeptidase; "arsanilazotyrosine-248", "azotyrosine-248", "azoTyr-248", "azotyrosine", and "azoprobe" are all terms used interchangeably with monoarsanilazotyrosine-248.

[2] Abbreviations used: rR, resonance Raman; DAC, monoarsanilazo-*p*-cresol; DAT, monoarsanilazo-*N*-acetyltyrosine; DMS, dimethyl suberimidate.

0006-2960/80/0419-0759$01.00/0