Articles

Reaction of the Isosteric Methylenephosphonate Analog of α-D-Glucose 1-Phosphate with Phosphoglucomutase. Induced-Fit Specificity Revisited†

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ABSTRACT: The phospho form of phosphoglucomutase reacts with the isosteric methylenephosphonate analog of α-D-glucose 1-phosphate to produce the corresponding analog of α-D-glucose 1,6-bisphosphate plus the dephosphoenzyme. In a coupled reaction, $k_{cat}/K_m = 1.7 \times 10^3$ M^{-1} s^{-1}, which is about 2×10^{-5} times that for the corresponding reaction with α-D-glucose 1-phosphate. The decrease in k_{cat}/K_m is divided more or less evenly between less efficient PO_3^- transfer and decreased binding, although smaller phosphates and phosphonates bind approximately equally. There is a much smaller difference in the binding of glucose 1-methylenephosphonate 6-phosphate and glucose 1,6-bisphosphate to the dephosphoenzyme: the binding ratio is <1:35 when the glucose ring is oriented similarly. Preferred binding patterns for a number of substrates/inhibitors, studied by ^{31}P NMR and UV-difference spectroscopy, suggest that in the ground state the phosphonate group is tolerated to a much greater extent at the *catalytic* subsite than at the *phosphate-binding* subsite, where binding specificity appears to be directed toward a tetrahedral $-PO_3^{2-}$ group attached to a bridging atom that can act as a hydrogen-bond acceptor. Binding specificity at the catalytic subsite apparently is directed toward a different array, possibly $(-O\cdots PO_3\cdots O-)^{2-}$. Some of these results are considered in terms of a modified version of the "induced fit" concept of enzymic specificity, which is reexamined in view of implied thermodynamic restrictions. The internal rearrangement whereby the positions of the anionic groups of the phosphate/phosphonate are exchanged is compared with the analogous rearrangements involving glucose 1,6-bisphosphate and 1,4-butanediol bisphosphate. The supplementary material describes a three-step synthesis of 1-deoxy-α-D-glucose 1-methylenephosphonate together with a procedure for phosphorylating the phosphonate to produce an analog of α-D-glucose 1,6-bisphosphate and also describes a facile procedure for the qualitative conversion of organic phosphonates to inorganic phosphate.

Phosphoglucomutase interconverts Glc-1-P[1] and Glc-6-P via the bound intermediate, Glc-1,6-P_2. The reaction involves a succession of three chemically distinct complexes: E_P(Mg)-Glc-1-P, E_D(Mg)Glc-1,6-P_2, and E_P(Mg)Glc-6-P, where E_P and E_D are the phospho and dephospho forms of the enzyme. The presence of all three complexes in significant amounts in an equilibrium mixture (Ray & Long, 1976b) makes the normal substrates unattractive for use in X-ray diffraction studies of the crystalline enzyme. Modifying Glc-1-P so that it can act as an acceptor/donor molecule for the enzymic phosphate group only at the 6-position of the glucose molecule is one way to reduce the complexity of such a mixture. The current study describes an evaluation of one such compound: the isosteric methylenephosphonate analog of Glc-1-P, glucose 1-methylenephosphonate.

While reaction of Glc-1-MP with phosphoglucomutase is restricted to the 6-position, the mixed phosphonate/phosphate produced, Glc-1-MP-6-P, possesses a capacity for binding to the dephosphoenzyme in two different ways, like Glc-1,6-P_2. In the "6-P:distal" complex, the 6-phosphate group is bound at the phosphate-binding subsite (Ray & Post, 1990) or the site distant from the activating metal ion, and the 1-methylenephosphonate group is bound at the "proximal" or catalytic subsite, adjacent to the activating metal ion [cf. Ray et al. (1990)]. In the "1-MP:distal" complex, the positions of the phosphate and methylenephosphonate groups are reversed. In representing these binding modes, the position to the left of the -Glc- moiety designates the phosphate group bound at the proximal subsite while the group at the distal subsite is on the right, viz., E_D·P-6-Glc-1-MP and E_D·MP-1-Glc-6-P are the 1-MP:distal and 6-P:distal complexes, respectively. A mixture of these, or a complex whose structure is questionable,

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[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, glucose 6-phosphate, in solution, a mixture of α- and β-anomers, and in complexes with phosphoglucomutase the α-anomer is implied unless otherwise specified; Glc-1,6-P_2, P-6-Glc-1-P, or P-1-Glc-6-P, α-D-glucose 1,6-bisphosphate; Glc-1-MP, α-D-gluco-2,6-anhydro-1-deoxy-1-phosphonoheptulose or glucose 1-methylenephosphonate; Glc-1-MP-6-P, P-6-Glc-1-MP, or MP-1-Glc-6-P, the 6-phosphoric acid derivative of the above phosphonic acid or glucose 1-methylenephosphonate 6-phosphate; 6-dGlc-1-P, α-D-gluco-1,5-anhydro-6-deoxyglucose 1-phosphate or 6-deoxyglucose 1-phosphate; 1-dGlc-6-P, D-gluco-1,5-anhydrohexitol 6-phosphate or 1-deoxyglucose 6-phosphate; dGlc-P, either or both of the preceding deoxysugar phosphates; E_P and E_D, the phospho and dephospho forms of the enzyme; M, a bivalent metal ion; ΣE·GP(M), the equilibrium mixture of enzyme–glucose phosphate complexes obtained by treating E_P(M) with Glc-1-P or Glc-6-P; TEA, triethylammonium.

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is designated as E_D·Glc-1-MP-6-P. UV spectral differences represent one of several ways to distinguish between these complexes experimentally. Thus, Ma and Ray (1980) showed that the spectral properties of one or more of the tyrosine in the E_P·Glc-1-P complex are substantially different from those in the E_P·Glc-6-P complex and thus that the binding interactions characterizing the 1-P:distal and 6-P:distal complexes are different. Similar conclusions were drawn about the transition-state analog complexes, E_D·V-6-Glc-1-P and E_D·V-1-Glc-6-P (Ray et al., 1990). In the present study, both spectral and NMR techniques were used to examine binding patterns for Glc-1-P. For comparison, the binding patterns in the complexes of Glc-1,6-P_2 with several different metal ion forms of phosphoglucomutase also were characterized.

EXPERIMENTAL PROCEDURES

Materials. The phospho form of rabbit muscle phosphoglucomutase was prepared, demetallated, and stored in the manner described previously [Magneson et al., 1987 (miniprint section)]. Prior to use, it was diluted in the appropriate buffer. To obtain the Mg^{2+} enzyme, 2 mM Mg^{2+} plus 1 mM EDTA were used. Other metal ion forms of the enzyme were obtained by addition of an equivalent of the corresponding acetate. Preparation of [^{32}P]phosphate-labeled phosphoenzymes was conducted as described by Ray et al. (1989). Glucose-6-phosphate dehydrogenase (Sigma) was treated and stored as in the above reference. Procedures for the preparation of 1,4-butanediol *mono*phosphate and bisphosphate have been described (Ray et al., 1976a).

Instrumentation. ^{31}P NMR spectra were obtained and saturation transfer studies conducted at 80.99 MHz and a temperature of about 23 °C with Varian XL200 and XL200A spectrometers in the manner described previously (Rhyu et al., 1984; Post et al., 1989). ^{1}H NMR spectra were recorded with a Nicolet NT470 spectrometer. Ultraviolet spectral studies were conducted in the manner described by Ray et al. (1990).

Equilibrium Binding of α-Glucose 1-Methylenephosphonate to Phosphoglucomutase. Equilibrium mixtures, 50 μL, of [^{32}P]phosphoenzyme and Glc-1-MP (see the supplementary material, section 1) in the presence of Tris-HCl, Mg^{2+}, and EDTA (see the assay for α-glucose 1-methylenephosphonate) were subjected to rapid quenching in 1.0 mL of 5% trichloroacetic acid. The delivery apparatus described by Ray and Long (1976a) was employed with a more efficient stirring device (Ray & Puvathingal, 1986). After thorough centrifugation, 0.9 mL of the TCA-soluble radioactivity in 10 mL of scintillation fluid was counted.

Assay for α-Glucose 1-Methylenephosphonate. The assay mixture, 1 mL, contained 50 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 25 μM Glc-1,6-P_2, 0.5 mM NADP, 5 μg of Glc-6-P dehydrogenase, and 0.2 mg of phosphoglucomutase. The overall optical density change at 340 nm, which was complete in about 15 min at 25 °C after addition of a 0.01-mL sample, was assessed with a Perkin-Elmer 575 spectrophotometer operated at 0–0.1 OD full scale. Steady-state kinetic studies of the reaction of the phosphonate and Glc-1,6-P_2 with phosphoglucomutase were conducted under similar conditions but with much higher phosphonate concentrations (0.7–2.9 mM), much lower concentrations of enzyme (2–5 μg/mL), and a reversed order of addition.

RESULTS

Because of the complexity of both the system and the experiments, the results are presented here in an abbreviated format. A more detailed description is available on request.

Preparation of α-D-Glucose 1-Methylenephosphonate; Reaction with Phosphoglucomutase. The three-step procedure reported in section 1 of the supplementary material involves a relatively clean chromatographic separation of the mixture of α- and β-anomers obtained from a *non*stereospecific synthesis; it is much simpler than the two previously reported *stereospecific* syntheses (BeMiller et al., 1980; Nicotra et al, 1982). The steady-state reaction of α-Glc-1-MP with phosphoglucomutase was monitored by means of the following reactions:

$$E_P + \text{Glc-1-MP} \rightarrow E_D + \text{Glc-1-MP-6-P} \quad (1)$$

$$E_D + \text{Glc-1,6-}P_2 \rightleftharpoons E_P + \text{Glc-6-P} \quad (2)$$

$$\text{Glc-6-P} + \text{NADP} \rightarrow \text{GA-6-P} + \text{NADPH} \quad (3)$$

(Equation 1 is "irreversible" under the conditions used: [Glc-1,6-P_2]$_0$ ≫ [Glc-1-MP]$_o$.) An analogous reaction becomes a multiturnover endpoint assay for α-Glc-1-MP when [E_P] ≫ [Glc-1-MP] (see Experimental Procedures). The steady-state reaction was conducted at pH 8, so that most of the Glc-1-MP was present as the dianion (pK_{a2} ≈ 7.3 at $\mu \approx 0.02$), since Glc-1-P^{2-} is the normal substrate (Rhyu et al., 1984). Under these conditions, production of NADPH is linear; the appropriate plots provide respective values for k_{cat} and K_m for the reaction in eq 1: 3.4 s^{-1} and 2 mM for Glc-1-MP^{2-}. The value of k_{cat}/K_m is smaller than that for Glc-1-P by a factor of approximately 2×10^{-5}; $^1/_{200}$ for k_{cat} and 300 for K_m. [Here, pH differences are ignored, since the value of k_{cat} for Glc-1-P is independent of pH in the neutral range (Ray, 1969).] For β-Glc-1-MP, k_{cat}/K_m is smaller by an additional factor of about 4×10^{-3}.

Equation 4 is an expansion of eq 1 analogous to the half-reaction with Glc-1-P.

$$E_P + \text{Glc-1-MP} \underset{K_{d(mono)}}{\rightleftharpoons} E_P\text{·Glc-1-MP} \overset{K_{tr}}{\rightleftharpoons} E_D\text{·Glc-1-MP-6-P} \overset{K_{d(bis)}}{\rightleftharpoons} E_D + \text{Glc-1-MP-6-P} \quad (4)$$

Because of the relative values of k_{cat} and K_m for Glc-1-P and Glc-1-MP (see above) and the large values of K_{tr} (see below) PO_3^- transfer[2] involving Glc-1-MP probably is rate-limiting, and $K_m \approx K_d$. [Slow dissociation of Glc-1-MP-6-P, which is bound more loosely than Glc-1,6-P_2 (k_d = 10 s^{-1}; Ray et al., 1989), is unlikely to limit the reaction in eq 4.] Thus, Glc-1-MP binds less tenaciously to EP than Glc-1-P (Ray & Long, 1976b) by $^1/_{800}$ (Table I).

Equilibrium mixtures of the [^{32}P]phosphoenzyme plus a large excess of Glc-1-MP were quenched rapidly with TCA [cf. Ray and Long (1976a,b)] in order to assess K_{tr}. Here, the soluble and insoluble fractions, f_s and f_i, are (E_D·Glc-1-MP-6-P* + Glc-1-MP-6-P*) and (E_P* + E_P*·Glc-1-MP), respectively, where the radioactive label is indicated by an asterisk. To simplify the results, enough E_D (1 mM) was added to the equilibrium mixture so that essentially no free Glc-1-MP-6-P was present. Hence

$$([E_P] + [E_P\text{·Glc-1-MP}])/[E_D\text{·Glc-1-MP-6-P}] = f_i/f_s \quad (5)$$

In terms of the constants in eq 4

$$[K_{d(mono)}/K_{tr}[\text{Glc-1-MP}]] + 1 = f_i/f_s \quad (6)$$

A plot of f_i/f_s versus [Glc-1-P]$^{-1}$ was linear with an apparent

[2] "PO_3^- transfer" refers to the stoichiometry of the process, not its mechanism.

Table I: Comparison of Constants for the Reaction of Phosphoglucomutase with Glucose 1-Methylenephosphonate and Glucose 1-Phosphate[a]

reactant	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$K_{d(mono)}$ (M)	K_{tr}	$K_{d(bis)}$ (M)	overall constant[b]
Glc-1-MP^{2-}	3.4[c]	1.7×10^{3} [c,d]	2×10^{-3} [c,d]	35[c]	7×10^{-7} [c]	1.2×10^{-2} [c]
Glc-1-P^{2-}	730	9×10^{7}	2.5×10^{-6} [e]	4[e]	2×10^{-8} [e]	1.2×10^{-2} [f]

[a] The process in question can be represented as

$$E_P + \text{monoP} \underset{K_{d(mono)}}{\rightleftharpoons} Ep\cdot\text{monoP} \overset{K_{tr}}{\rightleftharpoons} E_D\cdot\text{bisP} \overset{K_{d(bis)}}{\rightleftharpoons} E_D + \text{bisP}$$

where the enzyme and its complexes are in the Mg^{2+} form. The constants for Glc-1-MP refer to pH 8.0, those for Glc-1-P to pH 7.5. Both sets were obtained at 25 °C. [b] The overall constant is $K_{tr}K_{d(bis)}/K_{d(mono)}$. [c] Obtained as described under Results. [d] Calculated in terms of the dianion present. [e] Measured by Ray and Long (1976b,c) for [E_P][Glc-1-P]/[E_P·Glc-1-P]. [f] Logarithmic average of values measured by Peck et al. (1968) at pH values of 7.5 and 8.5 (see Results).

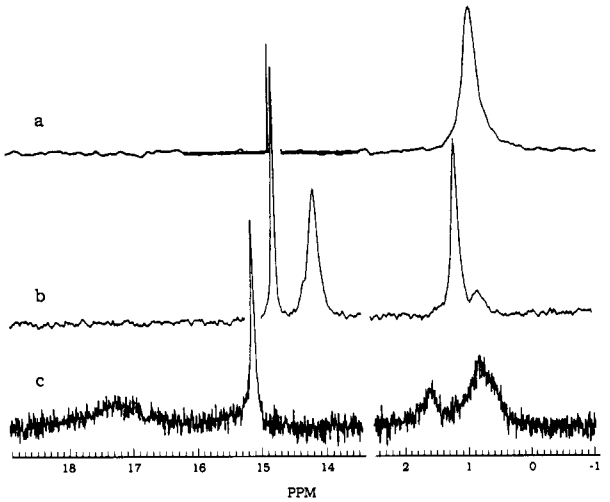


FIGURE 1: ^{31}P NMR spectra of equilibrium mixtures of phosphoglucomutase and glucose 1-methylenephosphonate. In each case, the solution initially contained 100 mg/mL of the phosphoenzyme (1.6 mM) in 20 mM Tris-HCl buffer at pH 7.4; spectra were accumulated at 22 °C. (a) Superimposed spectra of the Mg^{2+} enzyme (2 mM excess Mg^{2+} and 1 mM EDTA also present) and of Glc-1-MP, obtained separately. δ = 1.0 and 14.9 ppm, respectively. (b) Spectrum of the equilibrium mixture obtained with the Mg^{2+} form of the phosphoenzyme and a 15% excess of Glc-1-MP at pH 7.5. The resonances at frequencies of 14.7, 14.1, 1.2, and 1.0 ppm are those of Glc-1-MP, the E_D(Mg)-Glc-1-MP-6-P complex (two resonances), and E_P(Mg), respectively (see Results). (c) Spectrum analogous to spectrum b but established with the Cd^{2+} form of the phosphoenzyme (metal-free enzyme after addition of 1 equiv of $CdAc_2$) at a somewhat lower pH.[4] The resonances at frequencies of about 17.3 and 15.1 ppm are those of the methylenephosphonate of the E_D(Cd)Glc-1-MP-6-P complex and free Glc-1-MP-6-P, respectively, while those at 1.6 and 0.8 ppm are those of E_P(Cd) and the phosphate of E_D·Glc-1-MP-6-P, respectively.

ordinate intercept of 0 (essentially all ^{32}P was solubilized at high [Glc-1-MP]): $K_{d(mono)}/K_{tr}$ = 60 μM (for Glc-1-MP^{2-}) and $K_{tr} \approx 35$ (Table I).

The overall equilibrium constant for eq 1 (or eq 4) should be unchanged when Glc-1-P replaces Glc-1-MP, about 1.2×10^{-2} at pH 8 [estimated from the reported values of 4×10^{-2} and 4×10^{-3} at pH values of 8.5 and 7.4, respectively (Peck et al., 1968)].[3] Hence, $k_{d(bis)} \approx 7 \times 10^{-7}$ M (Table I) and is larger than the corresponding constant for Glc-1,6-P_2 by a factor of about 35. [According to this estimate, the measured value of K_{tr} for Glc-1-MP should be larger than that for Glc-1-P (Ray & Long, 1976b) by about 10-fold, as is observed.]

Figure 1a shows superimposed ^{31}P NMR spectra of the separate reactants, Glc-1-MP and E_P(Mg): $\delta(E_P)$ = 0.9 ppm independent of pH (Rhyu et al., 1984); (Glc-1-MP) $\approx$ 14.4 ppm, pH 7.4 (limiting δ at high pH, 14.75 ppm). Figure 1b shows the equilibrium mixture produced by a 15% excess of Glc-1-MP. As expected from the values for K_{mono} and K_{tr}, only two major ^{31}P resonances are observed, those of enzyme-bound Glc-1-MP-6-P. [The resonance at 0.9 ppm is from free E_P(Mg).] The resonance at 14.1 ppm is assigned to the 1-MP group on the basis of magnetization exchange studies, where bound Glc-1-MP was irradiated and free Glc-1-P observed [cf. Post et al. (1989)]. Hence, the resonance at 1.1 ppm is that of the bound 6-phosphate group.

Figure 1c shows the ^{31}P NMR spectrum of the Glc-1-MP-6-P complex when the reactant is the Cd^{2+} instead of the Mg^{2+} enzyme.[4] [The minor resonance at about 1.6 ppm is from free E_P·Cd.] The ^{31}P resonances for the 1-MP and 6-P groups, respectively, are 17.2 and 0.8 ppm (assigned as above). The unusual breadth of both is considered in a subsequent section.

^{31}P NMR Chemical Shifts of Phosphate Groups Bound at Proximal and Distal Subsites. Since neither 1-dGlc-6-P nor 6-dGlc-1-P can act as a PO_3^- acceptor or donor in the phosphoglucomutase reaction (Ray et al., 1976), their respective phosphate groups must occupy the distal subsite in their complexes with the phosphoenzyme. NMR studies from which assignments were made for the dGlc-P complexes of the Cd^{2+} enzyme are described the accompanying paper (Ray et al., 1993); related results also were obtained for the Mg^{2+} enzyme and interpreted similarly. Chemical shifts from both studies as well as previous studies (Rhyu et al., 1983) are recorded in Table II and are grouped together in the Figure 2.

Alternative Binding Modes for Dephosphoenzyme Bisphosphate Complexes. E_D(Cd) reacts slowly enough with 1,4-butanediol-P_2 [cf. Ray et al. (1976)] at pH 7 and below for the reactant complex to be studied by ^{31}P NMR. The ^{31}P NMR resonance of this complex (Figure 3a) is too broad to be rationalized in terms of exchange with the free bisphosphate, which represents less than 1% of the population under these conditions [cf. Ray et al. (1973)], or in terms of the effect of nearby hydrogens, modulated by the slow tumbling of the protein.[5] However, the line width is in accord with the averaging of different chemical shifts arising from chemical exchange between the proximal and distal subsites of the enzyme (see below). The analogous system involving 1,3-propanediol-P_2 is shown in Figure 3d.

These ^{31}P NMR spectra were simulated as in the accompanying paper by using the following two-spin system

$$P_1\sim P_4 + E_D(Cd) \underset{k_d}{\rightleftharpoons} \begin{matrix} E_D(Cd)P_1\sim P_4 \\ k_i \Big\| k_i \\ E_D(Cd)P_4\sim P_1 \end{matrix} \qquad (7)$$

The results show that the approximately Gaussian line shape

[3] The values reported are for transfer of the PO_3^- fragment of E_P to Glc-6-P and thus are smaller than those involving Glc-1-P by a factor of about $^1/_{20}$ (Post et al., 1989).

Table II: Site-Specific ^{31}P NMR Chemical Shifts of Phosphoglucomutase Complexes

type of complex[a]	complex	group present, ^{31}P chemical shift (ppm)	
		proximal site	distal site
1-P:distal(monoP)	E_P·Glc-1-P[b]	E-P, −1.6	1-P, −3.0
	E_P(Li)Glc-1-P[b]	E-P, −1.0	1-P, −3.2
	E_P(Mg)6-dGlc-1-P	E-P, +0.7	1-P, −2.6; ∼−3.1[c]
	E_P(Cd)6-dGlc-1-P	E-P, +0.9	1-P, −3.1
	$E_P(Cd)_2$6-dGlc-1-P	E-P, +2.4	1-P, −3.1
	E_P(Zn)Glc-1-P	E-P, –[d]	1-P, −3.2[d]
	E_D(Mg)V-6-Glc-1-P[e]	(6-V)	1-P, −3.2
	E_D(Li)V-6-Glc-1-P[e]	(6-V)	1-P, −3.1
	E_D(Cd)V-6-Glc-1-P[e]	(6-V)	1-P, −3.3
6-P:distal(monoP)	E_P·Glc-6-P[b]	E-P, +2.9	6-P, −0.5
	E_P(Li)Glc-6-P[b]	E-P, +2.3	6-P, −0.4
	E_P(Mg)1-dGlc-6-P	E-P, +3.2	6-P, ∼−0.4[f]
	E_P(Cd)1-dGlc-6-P	E-P, +3.8	6-P, −1.2
	E_P(Zn)Glc-6-P	E-P, +2.1[e]	6-P, −0.7[e]
	E_D(Mg)V-1-Glc-6-P[e]	(6-V)	6-P, −1.1
	E_D(Cd)V-1-Glc-6-P[e]	(6-V)	6-P, −1.1
	$E_P(Cd)_2$1-dGlc-6-P[g]	E-P, +3.8	6-P, +1.0
1-P:distal(bisP)	E_D·Glc-1,6-P_2[b]	6-P, −0.6	1-P, −3.1
	E_D(Li)Glc-1,6-P_2[b]	6-P, −1.0	1-P, −3.2
	E_D(Cd)Glc-1,6-P_2[b]	6-P, +5.2	1-P, −2.8
1-MP/6-P[h]	E_D(Mg)Glc-1-MP-6-P	(1-MP, +14.1)[h]	(6-P, +1.1)[h]
	E_D(Cd)Glc-1-MP-6-P	(1-MP, +17.2)[h]	(6-P, +0.8)[h]

[a] In complexes of the phosphoenzyme [1-P:distal(monoP) or 6-P:distal(monoP)], the enzymic phosphate must occupy the proximal subsite; hence, the other phosphate is assigned to the distal subsite. The basis for subsite binding assignments in complexes of the dephosphoenzyme [1-P:distal(bisP)] are provided under Results. [b] Rhyu et al. (1983, 1984). [c] Two complexes were observed, both with the same or nearly the same chemical shifts for the enzyme phosphate group. These may involve one versus two bound metal ions, as in the case of Cd^{2+}, or isomeric complexes. [d] Partial assignments for the Zn·CC system have been made on the basis of ^{31}P NMR saturation transfer studies (C. B. Post and W. J. Ray, Jr., unpublished work). [e] Occupancies of the proximal and distal subsites established by Ray et al. (1990). [f] Limiting chemical shift for the complex in rapid exchange with the free deoxysugar phosphate as the concentration of the free deoxysugar phosphate approaches zero. [g] Does not appear in Scheme I. [h] A proximal site/distal site assignment is not to be inferred for this complex (see Results).

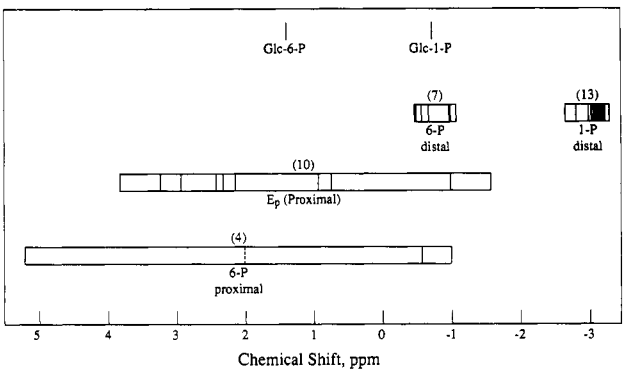


FIGURE 2: Schematic representation of the range of ^{31}P NMR chemical shifts observed for various phosphate ester groups bound at the proximal and distal substites of phosphoglucomutase. In complexes of the phosphoenzyme with monophosphate esters, by definition the enzymic phosphate group occupies the proximal subsite while the 1-phosphate and 6-phosphate esters of glucose and various derivatives, 1-P and 6-P, occupy the distal subsite. In complexes of the dephosphoenzyme, the occupancy of the proximal and distal subsites is that specified under Results and in Table II, which tabulates the chemical shifts of the phosphate esters used, together with the chemical shifts of their complexes with phosphoglucomutase (solid vertical bars in the figure). (The chemical shifts for the phosphates of $E_D(Cd)_2$Glc-P_2, which are considered anomalous, are not shown.) The ends of the various boxes represent the extremes of the observed chemical shifts, and the numbers in parentheses above the boxes indicate the number of different chemical shifts observed. In the case of 1-P:distal complexes, chemical shifts were displaced somewhat to avoid overlaps.

observed plus the excess width of the resonance relative to related complexes, about 50 Hz (Figure 3a), *and* the substantially smaller effect of the bound biphosphate on the line width of the free species (at a 1:1 ratio), about 15 Hz, can be simulated only if $k_i \gg k_d$.[6] (The spectrum with much sharper peaks in Figure 3c is that of the corresponding metal-free complex, where the chemical shift difference between proximal and distal subsites is much smaller; see Table II and Discussion.) The narrower ^{31}P NMR spectrum of the related propanediol bisphosphate complex (Figure 3d) is rationalized in terms of the shorter $(-CH_2-)_3$ bridge which reduces the chemical shift difference between the bound phosphate groups (see below) by preventing an equivalent occupation of the proximal subsite [cf. Ray et al. (1976)]. These conclusions are in accord with the relatively constant change in ^{31}P NMR chemical shift of glucose phosphate dianions and analogs produced by binding at the distal subsite, about 2 ± 0.4 ppm, irrespective of the identity of the metal ion bound adjacent to the proximal subsite, as well as the variability of the chemical shift of the phosphate group at the proximal subsite produced by changing the identity of the bound metal ion, >5 ppm (Table II and Figure 2).

The Predominant Glucose 1,6-Bisphosphate Complex. Only two prominent resonances are observed in the complexes of the bisphosphate with the Cd^{2+}, the Li^+ and the metal-free

[4] A separate study at a more carefully controlled pH verified that the chemical shift of the free Glc-1-MP was unaffected by the presence of the Cd^{2+} form of the enzyme.

[5] If the rotational correlation time for the protein is taken as 3×10^{-8} s (Rhyu et al., 1984), two protons at a ^{31}P–^1H distance of about 1.5 Å would be required to produce a 30-Hz broadening, i.e., a distance equal to the P–O distance in a normal phosphate group [cf. Sundralingham and Putkey (1970)].

[6] The ratio of k_i/k_d required to simulate the observed data depends on the difference in chemical shift of the two ^{31}P nuclei in $E_D(Cd)P\sim P$. On the basis of the average downfield shift for $-OPO_3^{2-}$ of sugar phosphates bound at the distal subsite (2 ppm, preceding section), the chemical shift difference for the $-OPO_3^{2-}$ groups of the bound bisphosphate should be about 4.5 ppm. If a more conservative estimate of 2.5 ppm is used, $k_i \approx 2000$ s and $k_d \leq 20$ s^{-1}, in which case, $k_i \geq 100k_d$. These values can be compared with those for dissociation of Glc-P_2 from the Cd^{2+} enzyme: Glc-P_2 binds ∼100-fold more tenaciously and dissociation about $^1/_{200}$ as rapidly (Ray et al., 1989).

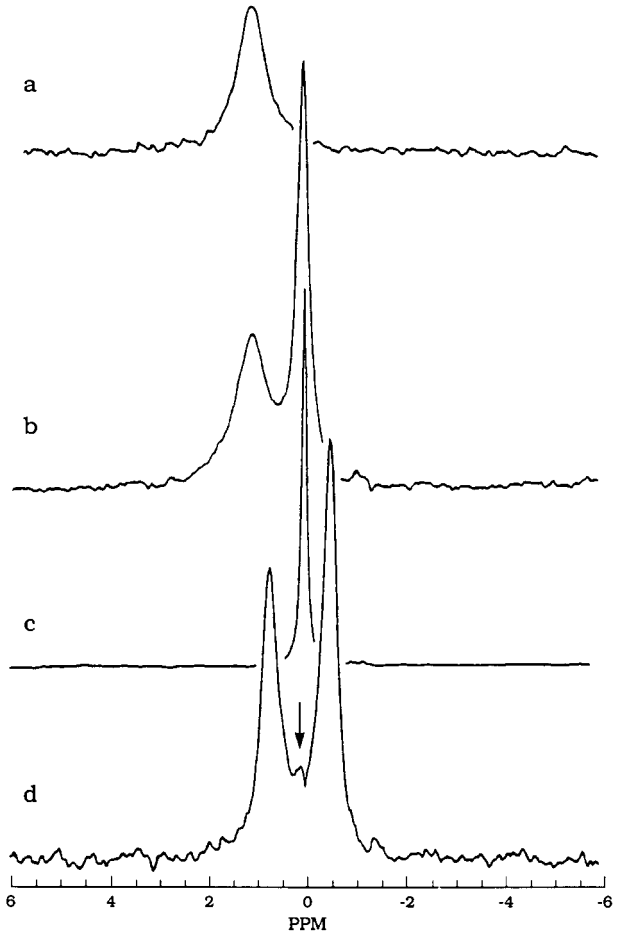


FIGURE 3: ^{31}P NMR spectra of complexes involving the dephospho form of phosphoglucomutase and the bisphosphates of butane-1,4-diol and propane-1,3-diol. Spectra were obtained as in Figure 3, except that the dephospho form of the enzyme was used. (a) Spectrum of the $E_D(Cd)$ complex of butanediol bisphosphate in the presence of a 40% excess of $E_D(Cd)$. (b) Same as spectrum a, but with a 100% excess of the bisphosphate. (c) Same as spectrum b, except that metal-free enzyme was used. (d) Same as spectrum b, but with 1,3-propanediol bisphosphate. The arrow points to the resonance from a small amount of free 1,3-propanediol phosphate that is produced during accumulation of the spectra.

forms of the dephosphoenzyme (Rhyu et al., 1984). The chemical shift of one resonance is relatively independent of metal-ion binding, ±0.2 ppm; that of the other changes by more than 5 ppm (Table II and Figure 2). These two observations cannot be rationalized in terms of simple models where the fractions of the bisphosphate complex present as 1-P; distal and 6-P:distal complexes are *similar*. In such a case, slow exchange would produce four resonances, and rapid exchange would produce frequency shifts either in *both* resonances or *neither* resonance (since the results in Table II indicate that in the alternative bisphosphate complexes binding at a given site will produce a similar *change* in the chemical shift of either phosphate of P-6-Glc-1-P^{4-}). Hence, one of the isomeric $E_D(Cd)$Glc-1,6-P_2 complexes must predominate. That complex must be the 1-P:distal complex, $E_D(M)$P-6-Glc-1-P, because of the similarity between the chemical shifts of the 1-phosphate in the observed bisphosphate complex and in complexes where the 1-phosphate group must bind at the distal subsite (Table II and Figure 2) and the large effect of Cd^{2+} (and other metal ions) on the resonance of the 6-phosphate

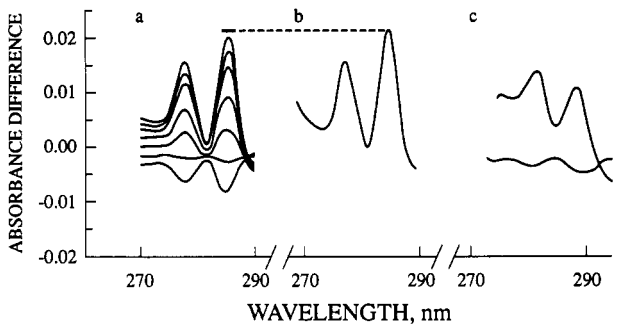


FIGURE 4: Simulated and observed binding-induced difference spectra. Spectra were obtained before and after mixing 0.800 mL each of $E_P(M)$ and glucose phosphates in a sectored cell [cf. Ray et al. (1990)]. The spectral difference was obtained by digital subtraction, after superimposing spectra in the 350–315-nm range. Except for the enzyme and the glucose phosphate, the solutions in the sectored cell were identical, 20 mM Tris-HCl buffer, pH 7.4. (a) Difference spectra simulated in the manner described under Results from the known fractional concentrations of $E_P(M)$Glc-1-P, $E_D(M)$-Glc-1,6-P_2, and $E_P(M)$Glc-6-P present when M = Zn^{2+} (20, 65, and 15%, respectively), or Cd^{2+} (3, 6, and 91%, respectively) (Ray & Long, 1976c). The difference spectra shown, from top to bottom, are those expected if the fraction of $E_D(M)$Glc-P_2 present as the 1-P:distal complex is 1.0, 0.9, 0.8, 0.6, 0.4, 0.2, and 0 for both M = Cd^{2+} and Zn^{2+} (see Results). (b) Observed spectral change produced by the binding of saturating glucose monophosphates to $E_P(Zn)$ to give $\Sigma E(Zn)GP$, viz., $[\Sigma E(Zn)GP] - [E_P(Zn) + Glc\text{-}P]$, minus the observed spectral change for the Cd^{2+} enzyme, viz., $[\Sigma E(Cd)GP] - [E_P(Cd) + Glc\text{-}P]$. (c) The spectral difference between the equilibrium mixtures obtained with $E_P(Zn)$ and $E_P(Cd)$ in the presence of saturating concentrations butane-1,4-diol mono- plus bisphosphates (bottom) and in the presence of saturating Glc-1-MP plus Glc-1-MP-6-P (top).

but not the 1-phosphate group in this complex.[7]

To confirm the above conclusion, equilibrium mixtures of the catalytically active $E_P(M)$Glc-1-P, $E_D(M)$Glc-P_2, and $E_P(M)$Glc-6-P complexes, viz., $\Sigma E(M)GP$, were examined in the tyrosine region of the UV as a function of the identity of the bound metal ion. Cd^{2+} and Zn^{2+} were used initially since the substitution of Zn^{2+} for Cd^{2+} produces the largest known shift in the fraction of $\Sigma E(M)GP$ present as the bisphosphate complex, from 0.91 to 0.15 (Ray & Long, 1976c). In all such mixtures, the spectrum of $E_D(M)$P-1-Glc-6-P is expected to be the same as that of the related $E_P(M)$Glc-6-P complex, and similarly for the alternative $E_D(M)$P-6-Glc-1-P and related $E_P(M)$Glc-1-P complexes (Ma & Ray, 1980). Hence, the spectrum of $\Sigma E(M)GP$ can be simulated if the relative concentrations of the 6-P:distal and 1-P:distal bisphosphate complexes are known. To emphasize changes produced by the above metal ion substitution, the spectral *difference* between $\Sigma E(Zn)GP$ and $\Sigma E(Cd)GP$ was simulated, instead, on the basis of spectral *differences* between $E_P(Li)$Glc-6-P and $E_P(Li)$Glc-1-P (Ma & Ray, 1990) (Figure 4a). In these simulations the fraction of 1-P:distal bisphosphate complexes was varied from 1.0 to 0. In the simulation that most closely mimics the observed spectral difference (Figure 4b), the fraction is 1.0. Differences between the spectra of $\Sigma E(Cd)$-GP and the equilibrium mixture of complexes obtained similarly with the Mn^{2+}, Ni^{2+}, Mg^{2+}, or Co^{2+} forms of the enzyme were smaller because the difference in the relative concentrations of the bisphosphate complex present is smaller (Ray & Long, 1976b). But in each case there was a good

[7] The preference of the enzyme for the $E_D(M)$P-6-Glc-1-P binding mode presumably is a reflection of the preference of $E_P(M)$ for Glc-1-P as opposed to Glc-6-P (Ray & Long, 1976a), ostensibly to balance the substantially favorable equilibrium constant, Glc-1-P ⇌ Glc-6-P, within the central complexes. [cf. Albery and Knowles (1976)].

correspondence between the observed difference spectrum and the simulated spectrum when all of the bound bisphosphate (Ray & Long, 1976) was assumed to be present as the 1-P:distal complex (spectra not shown). As a control, the spectral difference between the E_P(Zn) and E_P(Cd) complexes of butane-1,4-diol phosphate, where the 1- and 4-phosphates are identical, was recorded (Figure 4c, lower spectrum). The peaks and valleys in this spectrum do not match those expected for tyrosine difference spectra and thus are considered baseline effects.

The Predominant 1-Methylenephosphonate 6-Phosphate Complex. The spectral difference between the complexes, E_D(Cd)Glc-1-MP-6-P, and ΣE(Cd)Gp (Figure 4c, upper spectrum), is substantial because tryptophan differences are superimposed on the tyrosine differences [cf. Herskovitz and Sorensen (1968)]. [Tryptophan perturbation is substantial but essentially constant in the ΣE(M)GP complexes.] Since in a number of related complexes of the enzyme tyrosine and tryptophan spectral changes are not substantially linked,[8] the spectral differences in these two different regions induced by Glc-1-MP-6-P binding are considered separately. Thus, the intensity of the tryptophan differences produced by Glc-1-MP-6-P (290–305 nm, not shown) is only about 60% that produced on formation ΣE(M)GP and related complexes involving equilibrium mixtures of other sugar–phosphates (Ma & Ray, 1980) and actually is not much larger than that produced by the binding of inorganic phosphate. Thus, the binding of Glc-1-MP produces a less extensive conformational change, as monitored by tryptophan differences, than does Glc-1-P. In terms of tyrosine perturbation, the intensities of the 270–290-nm difference bands are in accord with a substantial population of the 6-P:distal complex, rather than a predominant presence of the 1-MP:distal complex, which predominates in the system involving Glc-P_2.

The above conclusion is in accord with various differences between ^{31}P NMR spectra of complexes involving the Mg^{2+} or Cd^{2+} forms of the dephosphoenzyme. Thus, internal exchange of bound butanediol bisphosphate broadens the ^{31}P resonance in the Cd^{2+} complex relative to the Mg^{2+} complex (Figure 2b,c) because the chemical shift of phosphorus bound at the proximal and distal subsites is substantially different. The much narrower line widths of both resonances in the complex of Glc-1-MP-6-P with the Mg^{2+} enzyme, as opposed to the Cd^{2+} enzyme (Figure 2b) can be rationalized similarly, by exchange[9] between subsites where the difference in the chemical shift of bound phosphorus is much smaller (see Table II). The paramagnetic broadening by Co^{2+}, bound adjacent to the proximal phosphate subsite (Rhyu et al., 1984), also can be interpreted in the same way. This broadening accounts for the reduced intensity of the ^{31}P NMR of the phosphoenzyme (Figure 5; middle) relative to that of the metal-free phosphoenzyme (Figure 5, top). [The failure of 0.95 equiv of added Co^{2+} to broaden the resonance of a greater fraction of the phosphoenzyme is caused by ancillary binding, which at pH 8 reduces the Co^{2+} present at the active site of the enzyme to about 0.8 equiv [cf. Ray (1972)].] Figure 5, bottom, shows the ^{31}P NMR of the 6-phosphate of Glc-1-MP-6-P bound to E_D(Co). Although the 6-phosphate resonance is extensively broadened by paramagnetic effects, the 1-MP resonance is broadened even further, to the point where it entirely merges with the baseline[10] (not shown), as expected from internal exchange when the 6-P:distal complex predominates.

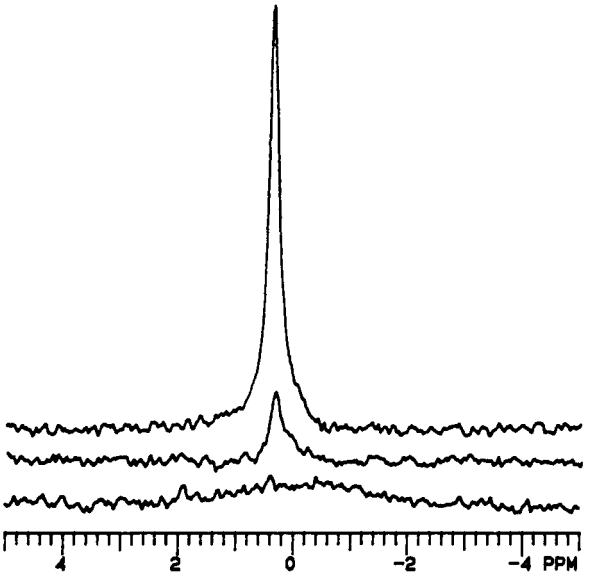


FIGURE 5: Paramagnetic effects on the ^{31}P NMR of phosphate ester groups bound at the proximal and distal subsites. NMR spectra were accmulated as in Figure 1, except that the enzyme initially was present in the metal free form.[10] The spectra are, from top to bottom, E_P, E_P plus 0.95 equiv of Co^{2+}, and the solution used for spectrum b to which has been added 1.0 equiv of Glc-1-MP.

DISCUSSION

The original goal of this research was to prepare a glucose monophosphate analog that would bind tightly to and react with the phospho form of phosphoglucomutase—one that in addition would produce a glucose bisphosphate analog that binds tightly to the dephosphoenzyme and exhibits a single or strongly preferrred binding mode. Although this goal was not realized, an evaluation of how Glc-1-MP interacts with the phosphoenzyme and how the product, Glc-1-MP-6-P, interacts with the dephosphoenzyme provides useful information about the active site of phosphoglucomutase.

Although isosteric phosphonates do not always provide tight-binding analogs of the corresponding phosphates (Engel, 1982), the dianions of methyl phosphonate and inorganic phosphite bind at the distal subsite of phosphoglucomutase as effectively as the dianions of inorganic phosphate and methylphosphate (Ray et al., 1973, 1976). In addition, the specificity of the distal subsite toward larger molecules does not appear to be particularly rigid; it can accommodate the phosphate of either Glc-1-P or Glc-6-P in such a way that their respective 6-OH and 1-OH groups can be efficiently phosphorylated by the same enzymic phosphate group (Ray & Roscelli, 1964; Ray et al., 1983). These observations suggest that the enzyme might not substantially discriminate between Glc-1-P and Glc-1-MP in terms of ground-state binding. This is not the case. The ratio of binding constants for Glc-1-MP^{2-} and Glc-1-P^{2-} is about 1:800, the k_{cat}/K_m ratio is about 10^{-5}, and the ratio

[8] Ma and Ray (1980) observe only small spectral *differences* in the 290–305-nm region relative to the 270–290-nm region, not only among the various ΣE(M)Glc-P and deoxysugar phosphate complexes but also among complexes as different as the equilibrium mixture of central complexes involving the phosphates of galactose, mannose, and ribose, although in these systems binding-induced changes in the 290–305-nm region are large on an absolute basis.

[9] The rate of this reorientation likely is some multiple of the dissociation rate for the enzyme·intermediate complex, which in the case of E_D(Mg)-Glc-1-P_2 is some 100-fold faster than for E_D(Cd)Glc-1,6-P_2 (Ray et al., 1989).

[10] Studies with free Glc-6-P (Ray et al., 1977) show that the binding of Co^{2+} to phosphate ester dianions produces broadening without a significant contact shift.

of rate constants for the PO_3^-/H^+ transfer step is about 1:200. Apparently, strong binding in a catalytically competent complex, though not in smaller molecules, requires that the bridge between the sugar and the PO_3^{2-} occupying the distal subsite accept a hydrogen bond. Knight et al. (1990) and Sem and Cleland (1990) draw the same conclusion by comparing kinetic values for 6-thioglucose-6-phosphate, 6-aminoglucose-6-phosphate, and Glc-1-P in their respective reactions with phosphoglucomutase.

The substantial population of two different binding modes observed for Glc-1-MP-6-P also was a surprise. The predominance of the 6-P:proximal complex might be expected since catalytic subsites usually are more "discriminating" than binding sites, and the binding of methyl phosphonate follows this pattern: stronger binding at the distal subsite by more than 2 orders of magnitude (Ray & Mildvan, 1973). Moreover, the preferred binding mode for Glc-1,6-P_2 is 6-P:proximal. But the 1-phosphonate of Glc-1-MP-6-P is distributed between both subsites, with the 1-MP:proximal complex predominating. Thus, if the 1-P:proximal complex of Glc-P_2 and the 1-MP:proximal complex of Glc-1-MP-6-P could be compared, where the remaining -Glc-6-P portion of the two molecules is oriented similarly, the ratio of the binding constants would be closer if not equal to 1. Hence, the bridge between the sugar and the PO_3^{2-} group bound at the distal subsite is more important for strong binding than the bridge between the sugar and the PO_3^{2-} group at the proximal subsite, in spite of suggestive evidence that general catalysis involving the hydroxyl group whose oxygen will form this bridge is a factor in the PO_3^- transfer process (Ray et al., 1976). In fact, Knight et al. (1990) and Sem and Cleland (1990) show that hydrogen bonding at the proximal bridge is not required for efficient catalysis in the reactions they studied, where the energetics of the transfer is substantially more favorable than in the normal reaction. In such cases, the primary effect of the enzyme on reactivity may well involve only the nonbridging oxygens of the phosphate group. However, it also seems probable that the proximal subsite is configured for optimal binding of a (–O···PO_3···O–) constellation instead of a tetrahedral phosphate group, as is suggested by the transition-state binding paradigm (Jencks, 1969; Wolfenden, 1972; Fersht, 1985; Page, 1987) and by the nearly 10^6-fold stronger binding of V-6-Glc-1-P and P-6-Glc-1-P (Ray & Puvathingal, 1990). If so, in the normal enzymic reaction there may well be a significant electrophilic interaction with the proximal sugar–PO_3^{2-} bridge in the transition state (partial proton transfer) but not the ground state.

In any case, the specificity of phosphoglucomutase for Glc-1-P relative to Glc-1-MP (k_{cat}/K_m ratio) is relatively large, nearly 10^5-fold. In addition, the conformational change that accompanies the binding of Glc-1-MP appears to be substantially smaller than that elicited by Glc-1-P and certainly less productive [cf. Wolfenden (1974)]. Hence, the question arises as to whether the above specificity ratio *might* be related to difference in substrate-induced conformational changes. [The binding of Glc-1-P perturbs at least two (of four) tryptophan residues (Ma & Ray, 1980), only one of which can be close to the bound substrate (Dai et al., 1992), and the binding of Glc-1-MP produces a much smaller perturbation of both (see Results).] Accordingly, in the Appendix, Koshland's induced-fit paradigm (1959) is rephrased/reformulated in a way that demonstrates how specificity can be tightly linked to conformational changes. Whether all or part of the above specificity ratio actually is related to differences between binding-driven conformational changes, or to other factors, remains to be seen.

The efficient exchange of the two phosphate groups of butanediol bisphosphate between the proximal and distal subsites of the dephosphoenzyme ($k_i \geq 2000\ s^{-1}$), without a sufficiently complete dissociation for the bound and free bisphosphate to equilibrate, plus the similar exchange involving Glc-1-MP-6-P, are manifestations of a capability associated with the Glc-P_2 intermediate that is generated in the normal enzymic reaction (Ray & Roscelli, 1964b). Although some details of this rearrangement still are unclear, the ability of the nonspecific butanediol bisphosphate to undergo what must involve rotational diffusion within an ion pair complex suggests that during the initial phases of a dissociation step the distinction between the phosphate groups that were bound at the proximal and distal subsites is lost at some point in space where there is a relatively high probability for re-formation of the bisphosphate complex. The rearrangement of these tetraanionic bisphosphates thus involves a process analogous to the internal return of ion pairs that occurs in many organic solvolyses. Presumably, the reduced efficiencies of the related reorientation involving 2,3-diphosphoglycerate in the phosphoglycerate mutase reaction (Grisolia & Cleland, 1968) and *cis*-aconitate in the aconitase reaction (Rose & O'Connell, 1967) arise from a much smaller active site cavity [cf. Dai et al. (1992)] in the case of the mutase and a much smaller difference between the properties of the intermediate and the substrate/product pair in the case of aconitase.

ACKNOWLEDGMENT

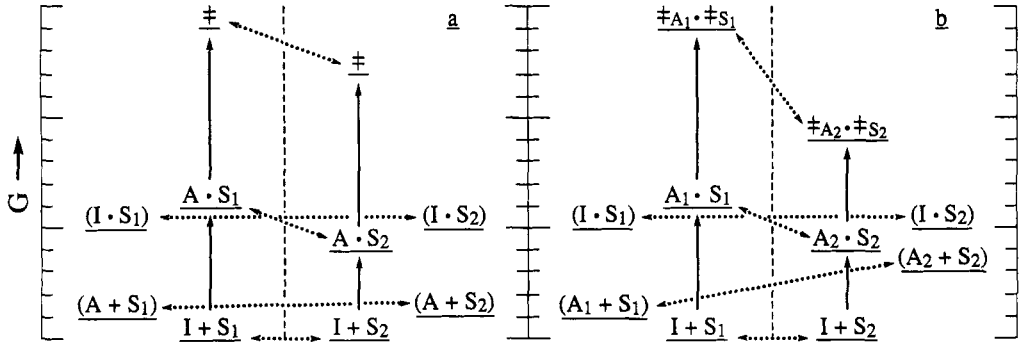
We are pleased to acknowledge numerous exchanges of views with Daniel Herschlag that substantially improved the presentation of the ideas presented in the Appendix.

APPENDIX

A Reexamination of the Induced Fit Paradigm of Enzymic Specificity. Since Koshland (1959) included phosphoglucomutase in an early list of possible examples of "induced-fit" specificity, he undoubtedly would have included the reaction of the phosphoenzyme with Glc-1-P and Glc-1-MP had the present data been available. Hence, we reconsider the concept of induced-fit specificity in light of these data, of subsequent criticism of Koshland's hypothesis (Fersht, 1974, 1985), of possible restrictions on the way induced-fit specificity can operate (Herschlag, 1988), and of the currently accepted concept that specificity is synonymous with differences in the intrinsic binding energy (Jencks, 1981) manifest in the transition state.

In order to subject Koshland's more or less embryonic paradigm to thermodynamic scrutiny, one must formalize it by defining an appropriate reaction scheme. In doing this, Fersht (1985) selected a scheme that in the final analysis is one where the induced-fit process fails to provide specificity. Herschlag (1988) considers the same scheme in detail but also discusses several interesting "exceptions" to conclusions based on that scheme which could link specificity to conformational changes. What we wish to do here is to formalize Koshland's paradigm in a different way with a slightly more complex scheme that also is more general, so that broadly based statements such as the following cannot be invalidated on an a priori basis by thermodynamic arguments: "Our data suggest that the relative reactivity of phosphoglucomutase toward Glc-1-P and Glc-1-P could arise from the inability of the latter to produce the optimal conformational change on binding". On the surface, this possibility seems reasonable since the "good" substrate binds more strongly, produces what

Scheme I: Gibbs-Energy Diagrams for the Induced-Fit Reaction Sequences in Scheme II When the Standard State for Substrates S_1 and S_2 Is Taken as Much Less Than the Respective Values of K_S and $K_S \equiv K_m$[a]



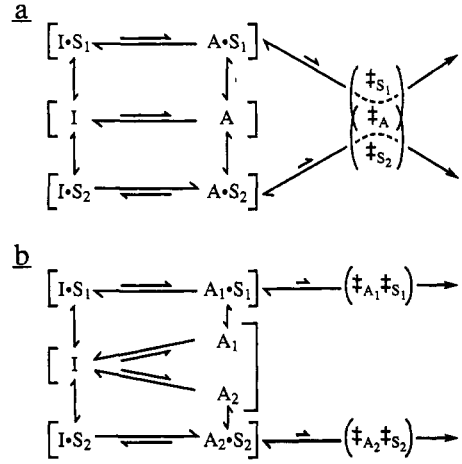
[a] The vertical dashed line indicates that the energy levels for the reactions of S_1 and S_2 have been aligned arbitrarily; the dotted arrows designate related species; species in parentheses are shown for reference; A and I designate active and inactive forms of the enzyme, respectively. (a) Diagram for induced-fit process where the active form of the enzyme is the same whether S_1 or S_2 is bound, either in the ground or transition states as in the Appendix. (b) The corresponding diagram based on the conformational heterogeneity of the enzyme where A_1 and A_2 are the conformers that bind S_1 and S_2, respectively.

appears to be a more extensive (and certainly a more productive) conformational change, and reacts more readily. This qualitative description of induced-fit specificity also seems to accord with what Koshland had in mind originally and described in the language of that day.

The similarity and difference between the way in which we formalize Koshland's paradigm and the way it was formalized earlier is considered first. As emphasized by Fersht (1985) and Herschlag (1988), it is (by definition) a difference between the intrinsic binding energy in transition states for two different substrates that ultimately provides the basis for enzymic specificity. However, the requirement in Fersht's treatment that any differences in intrinsic binding energy in the transition states for two substrates by independent of conformational differences that might develop during the two reactions is too restrictive. We thus allow differences in intrinsic binding energy in the transition state to be "inseperably" linked to substrate-specific conformational differences and base this allowance on generally accepted properties of enzymes (see below).

To clarify this point, the reaction scheme analyzed by Fersht and by Herschlag is considered first. Scheme Ia depicts two Gibbs-energy diagrams adapted from Herschlag (1988); it shows one possible relationship between the reactions of two substrates, S_1 and S_2, with an enzyme which, on binding S_2 but not S_1, is converted from an inactive form, I, to an active form A. The relationship between the reactions of S_1 and S_2 in this system is further depicted in Scheme IIa, which is adapted from Fersht (1988) and modified to include the reaction of two substrates instead of only one. It shows that, in the case of S_1, the inactive complex, $I{\cdot}S_1$, must pass through state $A{\cdot}S_1$ prior to reaction. From Scheme Ia it is obvious that in such a system those energetic differences, and presumably structural differences as well, that characterize the respective transition states also must characterize the reactive complexes $A{\cdot}S_1$ and $A{\cdot}S_2$ and that A must be the same in both $A{\cdot}S_1$ and $A{\cdot}S_2$. In other words, this reaction scheme requires that the relative binding of S_1 and S_2 in the transition state be the same as the relative binding in the $A{\cdot}S_1$ and $A{\cdot}S_2$ complexes, because A is the same in both complexes. Accordingly, in Scheme IIa we represent the respective transition states as $({}^{\ddagger}A{\cdot}{}^{\ddagger}S_1)$ and $({}^{\ddagger}A{\cdot}{}^{\ddagger}S_2)$. As pointed out by Fersht (1985) and Herschlag (1988), in such a system any specificity that the enzyme exhibits toward S_1 and S_2 must be independent of the conformational change $I \rightarrow A$ or $I{\cdot}S_2 \rightarrow A{\cdot}S_2$ since (a) the energetic toll exacted for the unfavorable conformational change by the time the transition state is reached is the same for the reaction of S_1 and S_2 and (b) any catalytic advantage obtained as the result of that toll also is the same.

Scheme II: Reaction Pathways for Comparing the Effect of Binding-Driven Conformational Changes on Enzymic Specificity[a]



[a] Symbols in panel a, which is taken from Fersht (1985) and expanded to include a second substrate, are as follows: A, catalytically active conformer of the enzyme; I, inactive conformer; S_1 and S_2, two different substrates, of which the binding of S_2 produces the catalytically active form of the enzyme; ‡A, enzymic component of the transition state which is thermodynamically equivalent whether S_1 or S_2 are bound; $^{\ddagger}S_1$ and $^{\ddagger}S_2$, a component of the transition state provided by substrates S_1 and S_2, respectively. States with the same composition are enclosed in brackets. Relative values of critical equilibrium constants are indicated by the relative length of appropriate arrows. In panel b, two different conformers of the enzyme, A_1, and A_2, are active, and the two substrates utilize different transition states, $^{\ddagger}A_1{}^{\ddagger}S_1$, and $^{\ddagger}A_2{}^{\ddagger}S_2$.

But we see no reason to restrict the induced-fit paradigm in the above manner, particularly since the conformational heterogeneity that now is known to characterize enzymes, together with the ability of ligand binding to alter the conformational profiles that describe that heterogeneity, provide a way to circumvent these restrictions. Thus, the conformational effects produced by the binding of two different substrates need not be the same, either in the reactive enzyme–substrate complex or in the transition state. Hence, we adopt the formalization of Koshland's paradigm shown in Scheme IIb, where the conformational heterogeneity of the enzyme is abbreviated as $(I \rightleftharpoons A_1 \rightleftharpoons A_2)$, with both A_1 and A_2 as minor components of the system in the absence of substrates.

Similarly, we represent the two enzyme–substrate complexes as ($I{\cdot}S_1 \rightleftharpoons A_1{\cdot}S_1$) and ($I{\cdot}S_2 \rightleftharpoons A_2{\cdot}S_2$); the reactive substrate complexes are $A_1{\cdot}S_1$ and $A_2{\cdot}S_2$, and the respective transition states are ($^{\ddagger}A_1{\cdot}^{\ddagger}S_1$) and ($^{\ddagger}A_2{\cdot}^{\ddagger}S_2$).

Scheme Ib is one of several energy level diagrams that can be constructed for the reaction sequences of Scheme IIb. Although other features of Scheme Ib are considered below, the difference between the active forms of the enzyme in the reaction involving S_1 as opposed to S_2, viz., differences between the enzyme in $A_1{\cdot}S_1$ and $A_2{\cdot}S_2$, means that the energetics of the conformational change associated with ($I \rightleftharpoons A_1 \rightleftharpoons A_2$) + $S_1 \rightarrow {}^{\ddagger}A_1{\cdot}^{\ddagger}S_1$ is different from that associated with ($I \rightleftharpoons A_1 \rightleftharpoons A_2$) + $S_2 \rightarrow$ ($^{\ddagger}A_2{\cdot}^{\ddagger}S_2$), so that the specificity of the system will depend on conformational differences between the reactive forms of the enzyme, A_1 and A_2. Although such differences may not be large (however, see below), they do preclude a general "factoring" of the conformational change from the two reactions, as well as the equivalent generalization (Fersht, 1985) that thermodynamic arguments can be used to invalidate induced-fit specificity schemes.

The question immediately arises, are we quibbling? Are energetic differences between A_1 and A_2 really large enough to provide a rationale for the sometimes very large specificity differences that are observed for substrates that structurally are quite similar? Although energetic differences between A_1 and A_2 may not be large, there is another aspect of the specificity problem that could make such differences critical. Thus, enzymic specificity patterns that are observed today are the result of evolutionary development, and energetic difference between A_1 and A_2 conceivably could provide a beginning for the development of a much higher degree of specificity than can be rationalized in terms of those differences per se, as well as the development of specificity that is inseparably intertwined with binding-driven conformational changes.

Actually, the developmental aspects of specificity can be viewed in two different ways. The view emphasized by Fersht (1985) and Herschlag (1988) (rephrased) is that by comparing observed specificity with that which *might be obtained* in an idealized system, it becomes obvious that an omniscient designer would never choose a binding-driven conformational change in an enzyme solely to maximize specificity. Thus, other factors being equal, a higher degree of specificity can be realized by maximizing differences in intrinsic binding energy in the transition state rather than "wasting" those differences at the level of substrate binding (Fersht, 1985).

We agree that the above view is certainly correct when one assumes that the active form of the enzyme is unique, as in Schemes Ia and IIa [and when no distinction is made between binding sites and catalytic sites, see Menger (1992), which is not the point under consideration here]. But there is an alternative way to view the design problem since the operations of nature generally are unlike those of an omniscient designer. In fact, evolution proceeds by random or near random changes with overall efficiency as a directional arrow, and it is easy to differentiate between the following possible scenarios for rationalizing the development of induced-fit specificity. In the first scenario, specificity unrelated to binding-driven conformational changes in an enzyme is a *fait accompli*. At that point, no increase in specificity can be obtained by an additional evolutionary event whose sole effect is to change the stable form of the free enzyme from an active to an inactive form so that a binding-driven conformational change is required for reactivation [cf. Fersht (1985), Herschlag (1988), and Scheme IIa]. But the same negative conclusion does not apply to the reverse scenario. Thus, in a primitive enzymic system with little or no specificity and marginal overall efficiency, an evolutionary event that provides an enzyme with the capacity to utilize more intrinsic binding energy in the transition state for the reaction of S_2, but changes its capacity to react with S_1 to a smaller extent, could be an event which requires that binding of S_2 drive a conformational change which is not the same as that produced by, or does not occur on binding of, S_1. Subsequent evolutionary events might then improve the efficiency of the reaction of S_2, and at the same time the specificity relative to S_1, thereby "inseparably" intertwining overall efficiency, specificity, and the induced-fit process. Hence, in Scheme IIb, the energy requirement for $A_2{\cdot}S_2 \rightarrow {}^{\ddagger}A_2{\cdot}^{\ddagger}S_2$ is shown as less than that for $A_1{\cdot}S_1 \rightarrow {}^{\ddagger}A_1{\cdot}^{\ddagger}S_1$.

We offer no cartoons to suggest how a smaller energy difference between $A_1{\cdot}S_1$ and $A_2{\cdot}S_2$ might be parlayed into a much larger difference between $^{\ddagger}A_1{\cdot}^{\ddagger}S_1$ and $^{\ddagger}A_2{\cdot}^{\ddagger}S_2$, although such are available on request. However, Herschlag's suggestion (Herschlag, 1988) that a substrate-surrounding process of the type described by Wolfenden (1972, 1987) can serve as a vehicle for producing specificity in an enzymic reaction provides one starting point for constructing such cartoons. Although Schemes Ib and IIb do not depend on substrate-surrounding constructs, all thermodynamically sound cartoons that accord with these two schemes must couple the conformational change with the attainment of additional intrinsic binding energy in the transition state, as is pointed out by Herschlag (1988). (Herschlag also notes a possible relationship between evolutionary changes and an induced-fit process that could apply to multidomain proteins.)

Our suggestion about the reaction specificity of phosphoglucomutase thus neither violates thermodynamic principles nor requires an unreasonable evolutionary scenario. But as noted under Discussion, whether or not it is true remains to be seen. On the other hand, the above considerations suggest that Koshland's induced-fit paradigm is alive and well.

SUPPLEMENTARY MATERIAL AVAILABLE

Section 1 describing a three-step synthesis/isolation of 1-deoxy-α-D-glucose 1-methylenephosphonate, beginning with 2,3,4,6-tetrabenzylglucose and tetraethylmethylenebisphosphonate, that can be used to prepare gram amounts of the phosphonate/phosphate, and also describing the phosphorylation of this 1-methylenephosphonate at the 6-position of glucose by α-D-glucose-1,6-bisphosphate in the presence of phosphoglucomutase, NADPH, and Glc-6-P dehydrogenase, together with a procedure for isolating the phosphonate/phosphate, and section 2 describing a simple and rapid "wet-ash" procedure for the quantitative conversion of organic phosphonates to inorganic phosphate in such a way that the product is readily measured via its heteropolymolybdate complex (8 pages). Ordering information is given on any current masthead page.

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