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Arrangement of the Phosphate- and Metal-Binding Subsites of Phosphoglucomutase. Intersubsite Relationships by Means of Inhibition Patterns†

William J. Ray, Jr.,*‡ Albert S. Mildvan,§ and James W. Long

ABSTRACT: Dissociation constants were determined for the complexes of the competitive inhibitors, inorganic phosphate and methylphosphonate, with both the phospho and dephospho forms of phosphoglucomutase. Kinetic procedures were used for measurements involving the Mg^{2+} complexes of the enzyme while water proton relaxation rates were used with the Mn^{2+} complexes. The results show that methylphosphonate can be used as a phosphate analog in studies of the phosphate-binding subsites of phosphoglucomutase. The dissociation constants for complexes of inorganic phosphate and of a series of α,ω-alkanediol bisphosphates with the Mg^{2+} forms of both the phospho- and dephosphoenzymes were determined kinetically. These and other results show that the dephosphoenzyme has a weak phosphate-binding subsite as well as a strong one; the strong subsite is also present in the phosphoenzyme and appears to be involved in the binding of the phosphate group of the normal substrates, glucose 1- and 6-phosphates. By contrast, the weak phosphate-binding subsite of the dephosphoenzyme is apparently absent in the phosphoenzyme, probably because it is occupied by the phosphate group esterified with the active-site serine residue. Thus, the weak phosphate-binding subsite appears to be the subsite at which the catalytic ($-PO_3$) transfer occurs. In such a case glucose 1,6-bisphosphate probably binds to the dephosphoenzyme in two different ways—one with the 6-phosphate at the weak subsite and the other with the 1-phosphate group at this subsite—and the interconversion of the two different complexes thus formed must be part of the catalytic cycle. However, this interconversion must occur by means of a process that does not require the complete dissociation of either complex.

If a paramagnetic metal ion, usually Mn^{2+}, is bound at the metal-activating site of an enzyme, an estimate of the distance between this site and various functional groups of a bound substrate or inhibitor sometimes can be deduced by measuring the effect of the bound metal ion on the nuclear magnetic resonance (nmr) signal of these groups (Mildvan and Cohn, 1970). Such a study involving phosphoglucomutase, Mn^{2+}, and the phosphate analog, methylphosphonate, is described in the accompanying paper (Ray and Mildvan, 1973). However, phosphoglucomutase has two phosphate-binding subsites, and the thermodynamic properties and kinetic roles of these sites must be understood in order to interpret the results of such a study. In the present paper, inhibition by inorganic phosphate and methylphosphonate is evaluated in order to show that the latter acts as a phosphate analog in the phosphoglucomutase system. Inhibition by inorganic phosphate also is contrasted with that produced by a series of α,ω-alkanediol bisphosphates, as an indication of how the binding strength varies between the phosphate-binding subsites. In addition, the observed binding patterns for inorganic phosphate are used to place restrictions on the manner in which ($-PO_3$) transfer can occur within the central complexes.

Experimental Section

Materials. The phospho form of phosphoglucomutase was isolated by means of a scaled-up and modified version of the previously described procedure (Ray and Koshland, 1962). The dephosphoenzyme was prepared from the phosphoenzyme by inducing ($-PO_3$) transfer to water by means of Xyl-1-P followed by extensive dialysis. These procedures will be described elsewhere (E. J. Peck, Jr., J. W. Long, L. Ng, J. D. Owens, and W. J. Ray, Jr., manuscripts in preparation); in the meantime, details are available on request (from W. J. R.). Enzyme activity was at least 900 units/mg for the phosphoenzyme and 850 for the dephosphoenzyme, as measured in the standard assay (National Academy of Sciences, 1972). The phosphoenzyme contained less than 7% dephosphoenzyme; the dephosphoenzyme contained less than 5% and was probably free of phosphoenzyme. The purification of the sugar phosphate substrates of phosphoglucomutase has been described (Ray and Roscelli, 1964). α-D-Xylose-1-P and α-D-2-deoxyglucose-1-P were obtained from Sigma and used without further purification. Methylphosphonate was generously supplied by Dr. Alexander Hampton, Institute for Cancer Research. ^{32}P-Labeled glucose-1-P was prepared in the manner described previously (Ray and Koshland, 1962). The prep-

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, and the Institute for Cancer Research, Philadelphia, Pennsylvania 19100. *Received March 2, 1973.* This investigation was supported by grants from the National Institutes of Health (GM-08963 and AM-13351) and the National Science Foundation (GB-29173X and GB-8579).

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aration of 1,4-butanediol-P will be described in a subsequent publication.

α,ω-Alkanediol Bisphosphates. The barium salts of the α,ω-diol bisphosphates of ethane, *n*-propane, *n*-butane, *n*-pentane, and *n*-hexane were kindly provided by Dr. Robert Barker, of the Iowa State University; those of methane, *n*-octane, and *n*-decane were prepared from the corresponding diiodo compounds *via* reaction with silver diphenyl phosphate according to the procedure used by Posternak (1957) for the preparation of α-aldose 1-phosphates, except that the higher boiling xylene was substituted for benzene, and a charcoal treatment was added after the reaction and prior to evaporating the solvent under vacuum. In addition, the residue obtained after evaporating the xylene was dissolved in cyclohexane, the solution filtered, and the filtrate evaporated under vacuum. The yields of crude α,ω-alkanediol bisdiphenyl bisphosphates at this point were in excess of 90% based on the diiodoalkanes. The phenyl blocking groups were removed by hydrogenation in ethanol at atmospheric pressure with platinum in the manner described by Posternak (1957). In each case over 90% of the theoretical uptake of hydrogen eventually was obtained; however, additional PtO_2 was always required in order to maintain a reasonable rate. In some cases a total weight of PtO_2 equal to 50% that of the starting material had been added by the end of the reaction. Even then, several hours were required to complete the process.

The reaction mixture was worked up in a manner similar to that described by Posternak although his hydrolysis step was omitted: the solution was made basic to phenolphthalein with 1 N NaOH, water was added to dissolve the precipitate, and ethanol plus benzene were removed by partial evaporation at reduced pressure. The residual aqueous solution was extracted twice with ether before the alkanediol bisphosphates were precipitated with barium and alcohol.

The barium salts of all alkanediol bisphosphates used were suspended in water and dissolved by addition of formic acid. A dilute solution of the bisphosphate was applied to a 0.9 × 20 cm column of Dowex-1 formate, 8% cross-link, and the product eluted with linear gradients of 0–2 M pyridinium formate in a total volume of 2 l. The longer the hydrocarbon chain the later the bisphosphate emerged, and the *n*-decane-1,10-diol-P_2 actually had to be eluted by subsequent application of a 2–4 M formate gradient in a total volume of 1 l. Only in the case of the methanediol-P_2 was a significant amount of inorganic phosphate found (presumably from hydrolysis during the hydrogenation), in this case about 20% of the product from the hydrogenation. The K_4 salts of the bisphosphates were isolated in the manner previously described for isolation of glucose-1,6-P_2[1] after column chromatography (Ray and Roscelli, 1964).

Kinetic Procedures. Standard enzymatic assays (saturating glucose-1-P, glucose-1,6-P_2, and Mg^{2+}) for both phospho and dephospho forms of phosphoglucomutase have been described (National Academy of Sciences 1972). The amount of dephosphoenzyme present in an enzyme sample was measured by means of the glucose-6-P produced on reaction with glucose-1,6-P_2, as described by Lowry and Passonneau (1969).

The binding of inhibitors to the Mg^{2+} forms of the phospho- and dephosphoenzymes at 30° and pH 7.5 (20 mM Tris-chloride) involved assays conducted, respectively, at (a) 10^{-5} M (saturating) glucose-1,6-P_2 with four subsaturating concentrations of glucose-1-P (in the range of 2–0.125K_M) and at (b) 0.125 mM (saturating) glucose-1-P and four subsaturating concentrations of glucose-1,6-P_2 (in the range of 10–0.4K_M). When methylphosphonate was the inhibitor it was present at concentrations of 6 and 2.4 mM, respectively, in the two assays; when inorganic phosphate was the inhibitor it was present at concentrations of 5 and 10 mM, respectively. Assay a involved the use of ^{32}P-labeled glucose-1-P and the procedure was analogous to that described by Ray *et al.* (1966), except that the present assay contained 2 mM Mg^{2+}–1 mM EDTA, the histidine used previously was omitted, 0.03% bovine serum albumin was substituted for the 0.02% gelatin, and the enzyme was activated (National Academy of Sciences, 1972) before use. Assay b involved the standard activation step and colorimetric procedure (National Academy of Sciences, 1972) except for the concentrations of glucose-1-P and glucose-1,6-P_2 used (see above) and the presence of the inhibitor. In both procedures, the enzyme concentration was varied so that essentially constant substrate conversions were observed under all conditions: about 15% for assay a and about 35% for assay b.

Constants in the velocity equation at subsaturating Mg^{2+} and glucose-1,6-P_2 were obtained from assays conducted at 30° and pH 8.5 (10 mM Tris) with saturating glucose-1-P, 0.25 mM. Other assay conditions and procedures were similar to those described for assay b, above, except that 32 mM histidine was used in both the activation step and the assay instead of EDTA, *e.g.*, see Ray *et al.* (1966), and an assay interval of 30 min was employed. The binding of α,ω-alkanediol bisphosphates and 1,4-butanediol-P to the dephospho form of the enzyme involved analogous assays except that constant, partially saturating concentrations of Mg^{2+}, 0.1 mM, and of glucose-1,6-P_2, 0.01 μM, were used; inhibitors usually were present in the concentration range of 2–20 μM, although concentrations of up to 100 μM were used with *n*-octane-1,8-diol-P_2 and *n*-decane-1,10-diol-P_2 and up to 3 mM with butanediol-P.[2]

The binding of α,ω-alkanediol bisphosphates to the phosphoenzyme was measured in an assay analogous to assay a, above, except that the reaction was conducted at pH 8.5 (20 mM Tris) with 0.2 mM Mg^{2+}, 32 mM histidine, and 0.1 mM glucose-1,6-P_2; inhibitor concentrations of 0.1 mM were used.

All constants obtained from substrate–velocity studies were calculated by means of standard computer techniques (Cleland, 1967).

Nmr Measurements. The longitudinal ($1/T_1$) PRR[1] of water was determined by the Carr–Purcell pulsed method (Carr and Purcell, 1954); an NMR Specialties PS60W pulsed nmr spectrometer was used. Procedures analogous to those described previously were employed (Ray and Mildvan, 1970).

[1] Abbreviations and constants used are: E_P and E_D, the phospho and dephospho forms of phosphoglucomutase; Glc-1-P, α-D-glucose 1-phosphate; hexose-P, an equilibrium mixture of hexose 1-phosphate and hexose 6-phosphate; glucose-1,6-P_2 or Glc-P_2, α-D-glucose 1,6-bisphosphate; K_A, the Michaelis constant of A; K_I, a competitive inhibition constant; K_A^{app} or K_I^{app}, apparent or measured constants; $K_{i,A}$, a kinetic constant for A; see Cleland (1963); $1/T_{1p}$, paramagnetic contribution to the longitudinal nuclear relaxation rate of water, PRR; the $-PO_3H_2$ group and all anionic groups derived from it are referred to as phospho groups and abbreviated as ($-PO_3$).

[2] Of the phosphate inhibitors used, none was hydrolyzed to an appreciable extent in 1 N sulfuric acid at 100° except methanediol-P_2. In fact, the half-time for hydrolysis of this bisphosphate in 1 mM HCl at 100° was on the order of 10 min. Because of its acid lability, appropriate blanks were subtracted from those enzyme assays which contained this inhibitor.

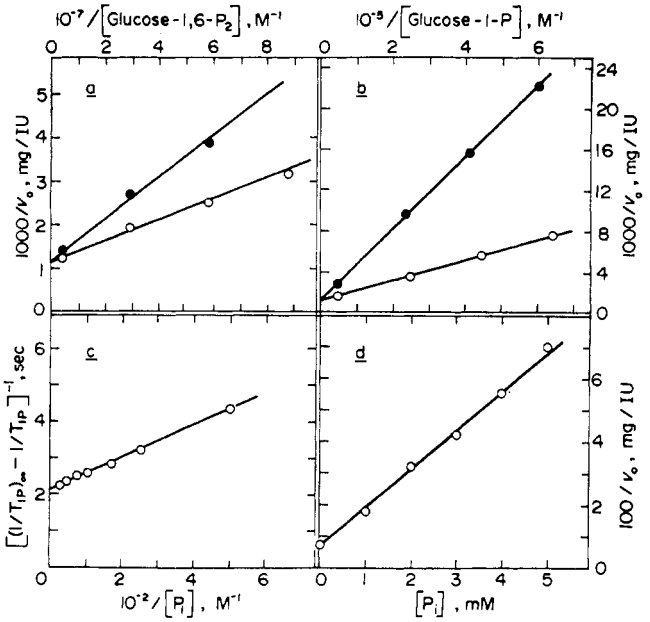


FIGURE 1: Double reciprocal plots from binding studies. All studies were conducted at pH 7.5 in the presence of 20 mM Tris-chloride; for additional details, see the Experimental Section. (a) Velocity and glucose-1,6-P_2 concentration at saturating Mn^{2+} (2 mM Mg^{2+}–1 mM EDTA) and glucose-1-P (0.125 mM). The velocity was measured by means of assay b in the presence (●) and absence (○) of 2.4 mM methylphosphonate. (b) Velocity and glucose-1-P concentration at saturating concentrations of Mg^{2+} (2 mM Mg^{2+}–1 mM EDTA) and glucose-1,6-P_2 (10 μM). The velocity was measured by means of assay a, in the presence (●) and absence (○) of 5 mM inorganic phosphate. (c) Difference in longitudinal relaxation rate of water protons in the presence of the Mn^{2+}–dephosphoenzyme complex at a given inorganic phosphate concentration, $1/T_{1p}$, and at saturating phosphate $(1/T_{1p})_\infty$. The initial solution, 0.1 ml, contained 0.1 mM Mn^{2+} and 0.12 mM dephosphoenzyme. Additions of 0.002 ml of 0.2–0.5 M inorganic phosphate, pH 7.5, were made. Relaxation rates were measured at 24° and corrected for dilution before plotting. (d) Velocity and inorganic phosphate concentration at saturating glucose-1-P (0.125 mM) and Mg^{2+} (2 mM Mg^{2+}–1 mM EDTA). Assays were conducted in a manner similar to assay b except that the glucose-1,6-P_2 concentration was maintained at 2.5×10^{-9} M (about 0.13 of its K_m value under these conditions) and inorganic phosphate concentration was varied as indicated.

Results

Binding of Inorganic Phosphate and Methylphosphonate to Phospho and Dephospho Forms of Phosphoglucomutase. The kinetics and mechanism of the phosphoglucomutase reaction indicate that inhibition competitive with glucose-1-P involves binding to the phosphoenzyme and inhibition competitive with glucose-1,6-P_2 involves binding to the dephosphoenzyme (Ray and Peck, 1972). Hence, the K_I values for inorganic phosphate and methylphosphonate were obtained by measuring the increase in $K^{app}_{Glc\text{-}1\text{-}P}$ or $K^{app}_{Glc\text{-}P_2}$ in the presence of these inhibitors.[3] Inhibition was strictly competitive in all cases, in that the effect of the inhibitor was completely supressed by excess substrate. Typical plots are shown in Figures 1a and 1b; the corresponding inhibition constants are given in Table I. Since these studies were conducted at a pH equal to the pK_a of methylphosphonate, 7.5 (as determined in a pH titration), and because monoanions inhibit phosphoglucomutase much less efficiently than dianions (Ray and Roscelli, 1966a), the reported inhibition constants for methylphosphonate are 0.5 of the observed values. No correction was made for the inorganic phosphate monoanion, since it only represented about 20% of the total phosphate. In these studies the concentration of Mg^{2+} was saturating with respect to the enzyme, but was substantially below the values of its effective dissociation constants for its complexes with inorganic phosphate (Sillen and Martell, 1964) and methylphosphonate[4] at pH 7.5; hence, binding of the uncomplexed inhibitor to the Mg^{2+} complex of the enzyme was evaluated.[5] For $K_{Glc\text{-}P_2}$, values obtained in these studies were quite similar to those obtained previously (Ray *et al.*, 1966); however, values for $K_{Glc\text{-}1\text{-}P}$ were 8–9 μM. The latter values are similar to the value originally reported for $K_{Glc\text{-}1\text{-}P}$ (Ray and Roscelli, 1964) but are substantially larger than the value of 5 μM (obtained by extrapolation) later reported (Ray *et al.*, 1966). We have no explanation for this difference.

Binding of inorganic phosphate and methylphosphonate to the Mn^{2+} forms of the phospho- and dephosphoenzymes was assessed by measuring the changes produced in the proton

TABLE 1: Dissociation Constants for Phosphoglucomutase–Inhibitor Complexes.

Enzyme Form	Dissociation Constant (mM)[a]			
	Mg^{2+} [b]		Mn^{2+} [b]	
	P_i [c]	$CH_3PO_3^{2-}$ [c]	P_i [c]	$CH_3PO_3^{2-}$ [c]
E_P	2[d] 1.8[d,e]	1.5[d,f]	(0.4)[g]	1.5[h]
E_D	1[e,i] 0.7[i]	1[i]	1.8[j]	

[a] Obtained at pH 7.5 and 30°. [b] Bound metal. [c] Inhibitor. [d] Determined kinetically by measuring the increase in the apparent Michaelis constant for glucose-1-P (saturating glucose-1,6-P_2) in the presence of the inhibitor. [e] Measured at pH 8.5 instead of 7.5. [f] Corrected for dianion present at pH 7.5—see Results. [g] Determined by means of titrations with glucose-P conducted in the presence and absence of the inhibitor and followed by changes in PRR (Ray and Mildvan, 1970); this value is open to question; see footnote 6. [h] Determined as in footnote *g*, except that 2-deoxyglucose-P was used as the titrant. [i] Determined kinetically by measuring the increase in the Michaelis constant for glucose-1,6-P_2 (saturating glucose-1-P) in the presence of the inhibitor. [j] Determined from a direct titration with the inhibitor as followed by changes in PRR.

[3] Glucose-1-P is itself an inhibitor competitive with glucose-1,6-P_2; $K_I \sim 0.5$ mM at pH 7.5 (Ray *et al.*, 1966). Although under the assay conditions used to study the binding of inhibitors to the dephosphoenzyme ([Glc-1-P] $\sim 0.25K_I$ in studies at pH 7.5) this effect is not serious, a minor correction in $K^{app}_{Glc\text{-}P_2}$ was made for substrate inhibition; however, no corrections were necessary in studies at pH 8.5. Minor corrections in $K^{app}_{Glc\text{-}P_2}$ also were made for Mg^{2+} inhibition competitive with glucose-1,6-P_2 (Ray and Roscelli, 1966a) in studies at both pH values.

[4] The effective dissociation constant for the Mn^{2+} complex of methylphosphonate at pH 7.5 is 15 mM (Ray and Mildvan, 1973) and Mn^{2+} is expected to bind to methylphosphonate more firmly than Mg^{2+}, as in the corresponding complexes with inorganic phosphate (Sillen and Martell, 1964).

[5] Previous studies (Ray and Roscelli, 1966a) indicate that at Mg^{2+} concentrations high enough to saturate phosphoglucomutase the Mg^{2+} complexes of the substrate are not bound by the enzyme, and we expect similar behavior relative to the Mg^{2+} complexes of inorganic phosphate and methylphosphonate.

relaxation rate (PRR) of water in titration procedures. Although neither inorganic phosphate nor methylphosphonate alters the PRR of the $E_P \cdot Mn$ complex to a sufficient extent to allow a direct titration, the dissociation constant for methylphosphonate bound to $E_P \cdot Mn$ can be assessed by titrating the $E_P \cdot Mn$ complex with 2-deoxyglucose-P in the presence and absence of methylphosphonate, in the manner previously used to evaluate inorganic phosphate binding to the same complex (Ray and Mildvan, 1970).[6] 2-Deoxyglucose-P produces a smaller enhancement change than does the glucose-P that was used previously—from 9.2 to 3.8, instead of 1.8—but is preferred over glucose-P because the dissociation constant from its ternary complex with Mn^{2+} and phosphoenzyme, about 7 μM, is larger than that of glucose-P, about 2 μM (Ray and Mildvan, 1970) and thus can be more accurately assessed under the conditions used for the titration (an $E_P \cdot Mn$ concentration of 0.1 mM was used).[7] In the presence of 26 mM methylphosphonate the apparent dissociation constant for 2-deoxyglucose-P is increased to about 70 μM; at the end of the titration the enhancement was essentially the same as that in a corresponding titration in the absence of methylphosphonate (data not shown). Such a correspondence is expected for competitive binding, and the dissociation constant of the methylphosphonate dianion from its ternary complex with Mn^{2+} and E_P, calculated on this basis, is 1.5 mM.

In the case of the $Mn \cdot E_D$ complex, binding of inorganic phosphate produced a decrease in enhancement from about 14 (Ray and Mildvan, 1973) to 7 and a direct titration thus was possible. Figure 1c shows a double reciprocal plot of the difference between the observed PRR and the PRR at saturating inorganic phosphate *vs.* phosphate concentration. The K_d thus obtained is 1.8 mM. Because essentially all of the metal ion is bound to the enzyme in the studies with Mn^{2+}, binding of the free inhibitor to the enzyme–Mn^{2+} complex is assessed.

The results of the above binding studies are compared in Table I. This comparison indicates that, at the most, only minor changes in binding interactions occur in going from inorganic phosphate to methylphosphonate in systems involving any combination of phospho- or dephosphoenzyme and Mg^{2+} or Mn^{2+}. Moreover, we also infer from these results that methylphosphonate, like inorganic phosphate, is bound at the active site of the enzyme and hence may be used as a probe of the phosphate-binding subsites of phosphoglucomutase. The failure of Mn^{2+} to produce tighter binding of phosphate and methylphosphonate than produced by Mg^{2+} suggests that the strong phosphate-binding subsite does not involve direct coordination to the metal ion (see Ray and Mildvan, 1973).

Additional kinetic studies of inhibition by inorganic phosphate competitive with glucose-1-P (binding to the phosphoenzyme) were conducted to show that the observed inhibition is produced by the binding of a single phosphate molecule. In these studies, inhibition measured at a constant, subsaturating concentration of glucose-1-P equal to $K_{Glc\text{-}1\text{-}P}/8$ (saturating Mg^{2+} and glucose-1,6-P_2) was strictly linear with inorganic phosphate from 0 to 5 mM (data not shown). If substantial amounts of an enzyme complex with two bound inorganic phosphate groups were present and if either of the bound phosphates were sufficient to prevent the normal binding of glucose-1,6-P_2 nonlinear parabolic inhibition by inorganic phosphate would have been observed under the above conditions (Cleland, 1963).

An attempt to detect the binding of a second phosphate group to the dephosphoenzyme was made by measuring inhibition competitive with glucose-1,6-P_2 (at saturating glucose-1-P), as a function of phosphate concentration. However, the observed inhibition (Figure 1d), which was strictly competitive with glucose-1,6-P_2 (not shown), was linear in the concentration range 0–5 mM phosphate at $K_{Glc\text{-}P_2}/[Glc\text{-}P_2]$ equal to about 10, and the data suggest that if a complex of $E_D \cdot Mg$ with two phosphate groups bound at its active site does exist, the dissociation constant for the second phosphate is in excess of 40 mM. Because the dephosphoenzyme should contain two phosphate-binding subsites (see Discussion), and because the dissociation constant for the first phosphate is about 1 mM (Table I), either one of the two subsites in the dephosphoenzyme is intrinsically weaker than the other by at least an order of magnitude or phosphate binding at the two subsites is markedly anticooperative.

Binding of α,ω-Alkanediol Bisphosphates to Phosphoglucomutase. The binding of alkanediol bisphosphates, $^{2-}O_3PO(-CH_2-)_nOPO_3^{2-}$, to the dephospho form of enzyme was evaluated by measuring the inhibition produced in enzyme assays conducted at saturating concentrations of glucose-1-P but at a fixed, subsaturating concentration of glucose-1,6-P_2. These inhibition studies were conducted at pH 8.5 in order to assure that the inhibitors were fully ionized; moreover, at pH 8.5 dead end substrate inhibition by glucose-1-P ceases to be a problem at the substrate concentration used (Peck *et al.*, 1968). In addition, assays were conducted at a low concentration of Mg^{2+}, 0.1 mM, in order to avoid formation of metal complexes of either the glucose-1,6-P_2 (Ray and Roscelli, 1966a) or the bisphosphate inhibitors, even though this concentration of Mg^{2+} is subsaturating in the enzymatic reaction (see below).

The intercept/slope ratio in plots of $1/v_0$ *vs.* inhibitor concentration under the above conditions gives K_I^{app}, from which the true K_I was calculated by using the parameters in the appropriate rate equation (see Ray *et al.*, 1966)

$$\frac{V_{max}}{v_0} = 1 + \frac{K_{Mg}}{[Mg^{2+}]} + \frac{K_{Glc\text{-}P_2}}{[Glc\text{-}P_2]} + \frac{K_{i,Mg}^{1,6} K_{Glc\text{-}P_2}}{[Mg^{2+}][Glc\text{-}P_2]} \quad (1)$$

Because the parameters of this equation have been evaluated only at pH 7.5, a study analogous to that described previously (Ray *et al.*, 1966) was conducted at pH 8.5. However, a 30-min assay interval was used in the present study in order to minimize the amount of phosphoenzyme present in the assay (actual enzyme concentration $< 3 \times 10^{-11}$ M) because at low glucose-1,6-P_2 the reaction of phosphoenzyme with glucose-1-P can produce glucose-1,6-P_2 (Ray and Roscelli, 1964). By using this precaution, no correction was required for glucose-1,6-P_2 produced in this manner during the assay. However, the Michaelis constant for Mg^{2+} is smaller at pH 8.5 than at 7.5 and adventituous Mg^{2+} posed a problem. Thus, at saturating

[6] As indicated by the parentheses in Table I, the accuracy of the dissociation constant for the $E_P \cdot Mn \cdot P_i$ complex is open to question because the relatively high levels of P_i that were employed in this titration (73 mM) could have altered the binding of glucose-P by means of an ionic strength effect—as well as by specific anion binding.

[7] In the titration of phosphoenzyme with 2-deoxyglucose-P, a conversion to free dephosphoenzyme and free 2-deoxyglucose-1,6-P_2 must be considered, as in the analogous titration with glucose-P (Ray and Mildvan, 1970). However, sugar diphosphates are bound to dephosphoenzyme very much more tenaciously than are sugar monophosphates to phosphoenzyme, and the complex $E_D \cdot$2-deoxyglucose-1,6-P_2 should not dissociate to a significant extent under the conditions used. Hence, only the phosphoenzyme and its complexes with methylphosphonate and with 2-deoxyglucose-P were considered in analyzing the titration of phosphoenzyme plus methylphosphonate with 2-deoxyglucose-P (see Ray and Mildvan, 1970). Under these conditions the titration assesses binding of methylphosphonate to the phosphoenzyme.

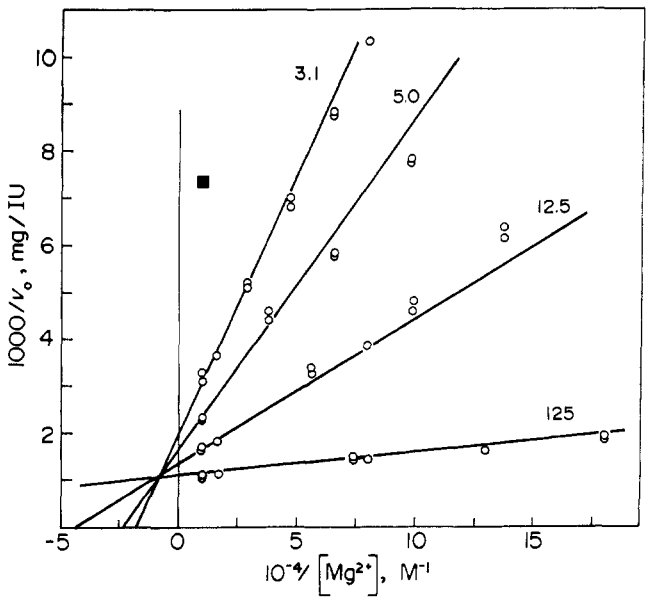


FIGURE 2: Double reciprocal plots of velocity (saturating glucose-1-P) and Mg^{2+} concentration at four subsaturating concentrations of glucose-1,6-P_2. Assays were conducted at pH 8.5 (10 mM Tris-chloride) and 30° in the presence of 0.25 mM glucose-1-P and 32 mM histidine; for other details see the Experimental Section. The Mg^{2+} concentration shown is the total concentration after correction for Mg^{2+} present in the assay solutions (1.2 μM; see Results). The lines were drawn by using the computer-generated constants given under Results. The numbers above the lines give the values of 10^8 times the glucose-1,6-P_2 concentration present in the assay. The symbol ■ indicates the expected velocity under the conditions of Mg^{2+} and glucose-1,6-P_2 concentration used in the study of alkanediol bisphosphate inhibitors (see Results).

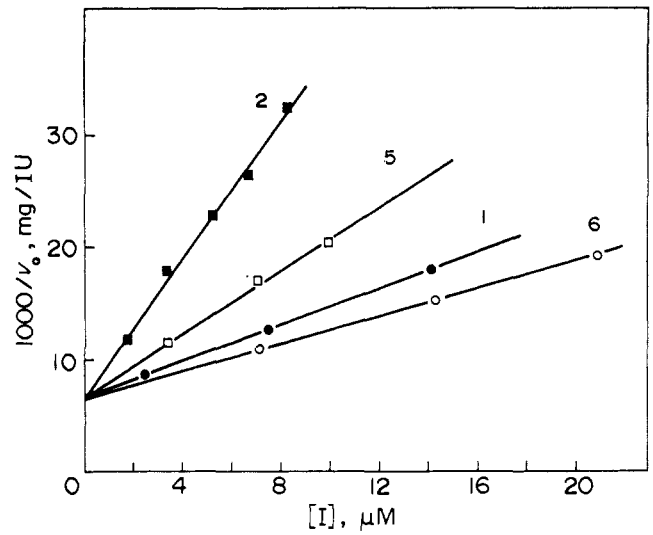


FIGURE 3: Inhibition studies with α,ω-alkanediol bisphosphates. Assays were conducted at a saturating concentration of glucose-1-P (0.25 mM) but at fixed subsaturating concentrations of glucose-1,6-P_2, 1×10^{-8} M, and Mg^{2+}, 0.1 mM, at pH 8.5 (20 mM Tris-chloride) and 30° in the presence of 32 mM histidine; for other details see the Experimental Section. The reciprocal of the velocity is plotted against inhibitor concentration and the number of the carbons in the alkanediol chain is shown above the plots.

glucose-1,6-P_2, the enzyme reaction proceeded at a rate of about 20% of V_{max} in the absence of added Mg^{2+}, although addition of a small amount of EDTA reduced this "blank" to "zero." By using a successive approximation procedure it could be shown that the observed blank corresponded to a Mg^{2+} concentration of about 1.2 μM. Hence, the true concentration of Mg^{2+} in the assay was assumed to be 1.2 μM greater than the amount actually added. This correction does not appreciably alter any of the important constants used in this study but does alter K_{Mg} by about 30% relative to the value that would have been obtained without subtracting the blank or without correcting for extraneous Mg^{2+}.

Figure 2 shows the results obtained in the above manner. The various constants together with the standard error of the estimate are given below; the lines in the figure were calculated by use of these constants: $K_{Mg} = 4.4 \pm 0.6$ μM; $K^{1,6}_{i,Mg} = 0.12 \pm 0.02$ mM; $K_{Glc\text{-}P_2} = (2.6 \pm 0.4) \times 10^{-8}$ M. The solid square in the figure shows the expected velocity under the conditions chosen for investigating alkanediol bisphosphate inhibition. The V_{max}/v_0 ratio at this point, as calculated from the above constants (and their standard errors) together with the concentrations of Mg^{2+} (0.1 mM) and glucose-1,6-P_2 (1×10^{-8} M) actually used, is 6.7 ± 1.0; the value observed in the inhibition experiments was 5.9. Errors of the size indicated have only minor effects on the calculated K_I values (see below).

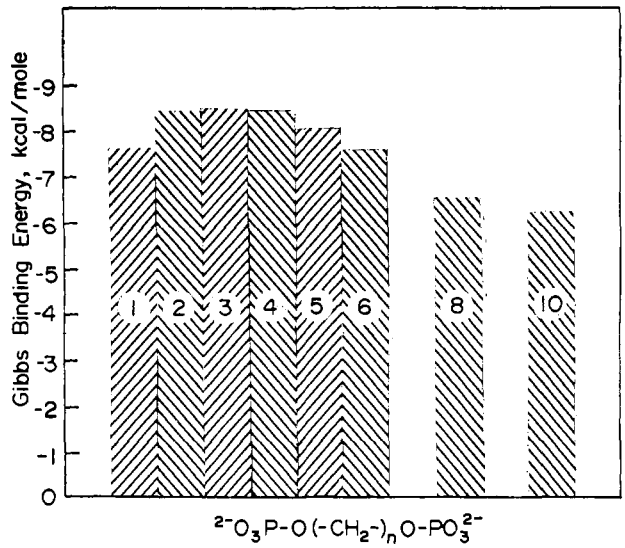
$K^{1,6}_{i,Mg}$ in eq 1 is the limiting Michaelis constant for Mg^{2+} as the concentration of glucose-1,6-P_2 approaches zero and is identical with the dissociation constant for the $E_P \cdot Mg$ complex (Ray and Roscelli, 1966b). Because in the inhibition studies, $K^{1,6}_{i,Mg}/[Mg^{2+}] = 1.2$, the free dephosphoenzyme and its Mg^{2+} complex were present in a ratio of about 1.2:1 and inhibition by the alkanediol biphosphates might involve binding to either or both of these species. In the latter case two inhibition terms of the form $(1 + [I]/K_I)$ would appear in the right-hand side of eq 1—one as a factor of the third and the other as a factor of the fourth term. The K_I values would not necessarily be the same since the factor of the third term would represent binding to the $E_D \cdot Mg$ complex while the factor of the last term would represent binding to free E_D. To distinguish these possibilities, plots of $1/v_0$ *vs.* [I] were made both at 0.1 and 0.03 mM Mg^{2+} under otherwise identical conditions (see above) for three of the inhibitors. In all three cases the slopes of the lines were unchanged, even though $K^{1,6}_{i,Mg}/[Mg^{2+}]$ was decreased from 0.8 to 0.24. Since $K^{1,6}_{i,Mg}/[Mg^{2+}]$ is a factor for the inhibition that involves binding to E_D but not for the inhibition that involves the $E_D \cdot Mg$ complex, the results indicate that binding of the alkanediol bisphosphates to $E_D \cdot Mg$ is substantially more important than binding to E_D. Accordingly inhibition was analyzed in terms of an equation that was similar to eq 1 but which contained a $(1 + [I]/K_I)$ factor associated only with the $K_{Glc\text{-}P_2}/[Glc\text{-}P_2]$ term; this equation is referred to as eq 1a.

All plots of $1/v_0$ *vs.* [I] appeared to be linear; typical plots ($K_{Glc\text{-}P_2}/[Glc\text{-}P_2] = 2.6$) are shown in Figure 3. According to eq 1a the intercept/slope ratios for these plots, *viz.*, the values of K_I^{app}, are given by the following expression

$$K_I^{app} = K_I \frac{1 + K_{Mg}/[Mg^{2+}] + (K_{Glc\text{-}P_2}/[Glc\text{-}P_2])(1 + K^{1,6}_{i,Mg}/[Mg^{2+}])}{K_{Glc\text{-}P_2}/[Glc\text{-}P_2]}$$

From the values of the constants and concentrations noted above, this expression is equal to $2.5K_I$. The K_I values thus determined for the various inhibitors ranged over two orders of magnitude, from a value of 8×10^{-7} M (*n*-propane-1,3-diol-P_2) to 5×10^{-5} M (*n*-octane-1,10-diol-P_2). Chart I

CHART I: Gibbs Binding Energy for the Interaction of α,ω-Alkanediol Bisphosphates with the Mg^{2+} Form of the Dephosphoryl-enzyme.[a]



[a] Gibbs binding energies were calculated from inhibition data obtained at pH 8.5 and 30° in the manner indicated under Results. The number appearing within each area gives the number of carbons in the alkanediol.

shows the Gibbs binding energy of the various bisphosphates calculated from the measured K_I values.

Binding to the phospho form of the enzyme was investigated only for the bisphosphates of *n*-propanediol and *n*-butanediol. Only a slight inhibition was observed at a concentration of 0.1 mM (data not shown) and the calculated dissociation constant in both cases was equal to about 0.3 mM.

Binding of 1,4-butanediol monophosphate to the dephosphoenzyme was measured by the procedure described above for the bisphosphates, except that the inhibitor concentration was in the range 1–3 mM; K_I = 2 mM.

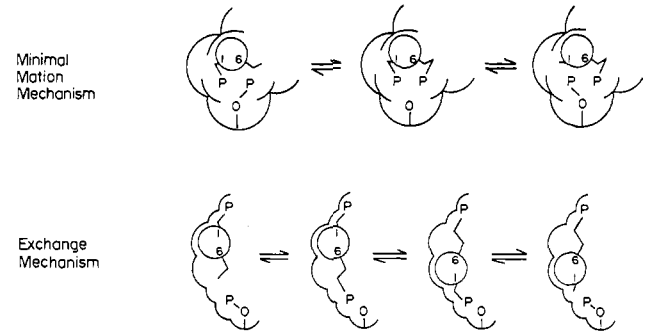
Discussion

Because there are two different phosphate groups involved in the phosphoglucomutase reaction, two successive ($-PO_3$) transfer steps must occur within the central complexes during each turnover (Ray and Peck, 1972)[8]

$$E_P\cdot\text{Glc-1-P} \rightleftharpoons E_D\cdot\text{Glc-P}_2 \; (\rightleftharpoons E_D\cdot\text{Glc-P}_2') \rightleftharpoons E_P\cdot\text{Glc-6-P}$$

Two limitations to schemes that might be proposed for this sequential transfer are provided by previous studies: (a) if only one $E_D\cdot$Glc-P_2 complex is involved, both of the phosphate groups of the bisphosphate must be positioned so that transfer of either phospho group to a single acceptor, the active-site serine residue of the enzyme, is possible with only minor structural changes; and (b) if two $E_D\cdot$Glc-P_2 complexes exist so that in one a phospho group can be transferred from the 6 position of the glucose moiety to the enzyme, while in the other phospho group transfer from the 1 position is allowed, the interconversion of these complexes must be feasible without their complete dissociation (Ray and Roscelli, 1964). Two types of mechanisms consistent with these limitations are illustrated in Scheme I. In these mechanisms,

SCHEME I: Possible Ways in Which a Sequence of Two Phosphate Transfer Steps Might Occur in the Phosphoglucomutase Reaction.



as well as in the discussion below the presence of a bound metal ion is assumed.

In a "minimal motion" or one-intermediate mechanism, corresponding to requirement a, above, the free phosphoenzyme should be a mixture of rotameric species, designated as (E_P + $_PE$), in which the active-site phosphate group interacts noncovalently with one or the other of two adjacent phosphate-binding subsites—in addition to being covalently bonded to the enzyme (see Scheme I). The distribution of the phosphate group of the enzyme between the two subsites, as well as the distribution of inorganic phosphate in the analogous ($E_D\cdot P_i$ + $P_i\cdot E_D$) complex, would depend only on their relative interaction with both subsites. In addition, when a molecule of inorganic phosphate binds to either the phosphoenzyme or the dephosphoenzyme–inorganic phosphate complex it, too, would be distributed between two alternative positions.[9] The resulting complexes will be represented as ($P_i\cdot E_P$ + $E_P\cdot P_i$) and $P_i\cdot E_D\cdot P_i$, respectively.

An "exchange" mechanism involves the interconversion of two $E_D\cdot$Glc-P_2 complexes by interchanging the positions of the two phosphate groups. In view of the variety of molecular rearrangements in which intimate ion pairs occur as intermediates, *e.g.*, in solvolysis of simple organic compounds, an interchange of the two phosphate groups in question *via* an ion-pair like process seems feasible without complete dissociation of the bisphosphate, *i.e.*, without exchange of the bound bisphosphate with free bisphosphate in solution (see b above).[10] Thus, an exchange mechanism would involve a unique subsite at which the ($-PO_3$) group of E_P interacts noncovalently with the enzyme and a separate subsite for binding of the phosphate group of glucose monophosphate (see Scheme I). Binding of inorganic phosphate to the phosphoenzyme would occur at the latter site and produce a single $E_P\cdot P_i$ complex, while binding of two inorganic phosphate

[8] A concerted transfer of both phospho groups *via* a pathway that does not involve an $E_D\cdot$Glc-P_2 intermediate is both mechanistically unattractive and without precedent.

[9] This model, *per se*, should not be taken to exclude the possibility of unequal subsite interactions such that one would observe essentially a single form of E_P and a single discrete subsite for binding of P_i to the phosphoenzyme; however, see discussion below.

[10] Molecular models indicate that an interchange of the two phosphate groups of Glc-P_2 to produce alternative $E_D\cdot$Glc-P_2 complexes, as required by the exchange mechanism, cannot be accomplished in such a way that a significant number of structural features of the glucose ring could interact with an invariant glucose binding site on the enzyme if only strain-free conformations of glucose-1,6-P_2 are considered. However, two different but partially overlapping glucose subsites might be involved, or if only one subsite is involved, it might be so constructed that it is capable of alternative interactions with the glucose ring, depending on which of the two phosphate-binding subsites is occupied by the 1-phosphate of glucose-1,6-P_2. Such alternative interactions have been postulated to explain the specificity pattern of ribonuclease (Richards *et al.*, 1969).

TABLE II: Binding of Monophosphates and Bisphosphates to the Dephospho Form of Phosphoglucomutase.[a]

Phosphate	K_d (M)	Bisphosphate/ Monophosphate Binding Ratio
1,4-Butanediol-P_2	9×10^{-7}	
		2.2×10^3
1,4-Butanediol-P	2×10^{-3}	
Glucose-1-P	7×10^{-4} [b]	
		8×10^3
Glucose-1,6-P_2	9×10^{-8} [c]	
		3.3×10^3
Glucose-6-P	3×10^{-4} [b]	

[a] At pH 8.5 and 30° and at Mg^{2+} concentrations sufficient to saturate the enzyme but not high enough to bind significantly to the phosphates. [b] Calculated from Figure 7 of Peck *et al.* (1968). [c] See footnote 12.

molecules to the dephosphoenzyme would involve intrinsically different subsites.

It should be pointed out that an exchange mechanism serves to minimize the complexity of the catalytic apparatus for ($-PO_3$) transfer since both 1- and 6-phosphate groups of glucose-1,6-P_2 would occupy the same position during both catalytic events. However, this mechanism also complicates the binding problem relative to the glucose ring as is indicated in Scheme I and footnote 10. By contrast, the minimal motion mechanism, while simplifying the glucose binding problem, makes the catalytic requirements more complex—especially to the extent that functional groups, such as general acids or bases, are involved in bond breaking and bond making—because the direction of nucleophilic attack on phosphorus would differ depending on whether the 1- or 6-phospho group of glucose was being transferred (see Scheme I).

The results obtained in the phosphate binding studies are helpful in further defining these two mechanisms. The *linear* inhibitions observed for inorganic phosphate under conditions where binding is to the phosphoenzyme (saturating glucose-1,6-P_2) or to the dephosphoenzyme (saturating glucose-1-P) indicate that both enzyme forms bind a *single* phosphate molecule at their active sites and the complexes thus formed have dissociation constants in the millimolar concentration range (Table I). Although the binding of a second inorganic phosphate molecule to E_D was not detected at concentrations of up to 5 mM,[11] the following arguments indicate that E_D must have a second phosphate-binding subsite. (a) A comparison of the dissociation constants for complexes of the dephosphoenzyme with monophosphates and inorganic phosphate (K_d values in the millimolar range—see Tables I and II) indicates that a phosphate-binding site in the enzyme is responsible for most of the binding interactions observed for monophosphates. A second phosphate-binding site thus is required to rationalize the observation that bisphosphates, either with or without a glucose ring, bind to E_D more strongly than the corresponding monophosphates by more than three orders of magnitude (Table II). (b) Although the monophosphate, glucose-1-P, binds to the dephosphoenzyme, presumably with its phosphate group at the strong phosphate-binding subsite, it reacts slowly if at all, and less than 2% of the enzyme is phosphorylated in 24 hr under conditions where the reaction with glucose 1,6-bisphosphate is complete within less than a second (Goodin, 1973). (c) The labeled monophosphate, [^{32}P]xylose-1-P, also binds to E_D, but reacts to produce [^{32}P]E_P very slowly if at all. However, phosphorylation of the enzyme is complete in a few minutes on addition of inorganic phosphate (10 mM) which must occupy a separate phosphate binding subsite during the reaction (Goodin, 1973). (d) Since E_P already has a strong phosphate-binding subsite (K_d in the millimolar range), removal of its phospho group should produce a second phosphate-binding subsite unless the phospho group of E_P interacts with the protein only through its covalent linkage. However, nmr spectroscopy shows that the ^{31}P nucleus of E_P is immobilized on the surface of the enzyme, *i.e.*, the phospho group is bound to the enzyme by more than just its covalent linkage and does move significantly faster than the entire molecule moves (W. J. Ray, Jr., and A. S. Mildvan, manuscript in preparation). Hence, it is reasonable to suggest that after conversion of E_P to E_D, other phosphate groups should bind to the position that was originally occupied by the phospho group of E_P.

Although all four of the above arguments are indirect, when taken together it is difficult to escape the conclusion that E_D must have two functional phosphate-binding subsites. Our inability to obtain direct evidence for a second phosphate-binding subsite by observing a complex of E_D with two equivalents of P_i (or a phenomenon attributable to such a complex) must be caused by the weak binding of the second inorganic phosphate molecule. In fact, a comparison of K_d values for complexes of E_D with 1,4-butanediol-P and the corresponding bisphosphate leads us to expect a substantially weaker interaction between $E_D \cdot P_i$ and a second P_i molecule (expected K_d, 50 mM or larger) than between E_D and P_i (K_d = 1 mM), as is indicated in the Appendix.

By contrast, there is no evidence for a second phosphate-binding subsite in E_P. Thus, the K_d values for the complexes of E_P (Mg^{2+} form) with glucose-1,6-P_2 or 1,4-butanediol-P_2 are about 5×10^{-4} (Ray and Roscelli, 1964) and 3×10^{-4} M, respectively (see Results). Since at least 1,4-butanediol-P_2 and probably glucose-1,6-P_2 as well should bind to E_P in two different ways if only one of their two phosphate groups interacts directly with the enzyme, as seems probable, these dissociation constants should be doubled before comparison with the K_d value for the $E_P \cdot P_i$ complex (see discussion in the Appendix) which is 2×10^{-3} M under the same conditions (Table I). This similarity in binding of bisphosphates and P_i to E_P is in contrast with the 1000-fold difference in their binding to E_D (see Tables I and II). In addition, there is no known phosphorylation involving E_P that occurs more rapidly in the presence of two phosphate groups (either part of the same molecule or comprising different molecules) as there is

[11] The presence of inorganic phosphate in an enzymatic reaction at concentrations in excess of about 7.5 mM presents serious technical problems in designing the assay and in following the reaction, and attempts at direct inhibition studies under such conditions have not been successful. In addition, ionic strength effects are impossible to control in such studies since phosphoglucomutase is inhibited by all common anions (Ray and Roscelli, 1966a). However, the linearity of the results obtained in the concentration range 0–5 mM indicates that the dissociation constant involving a second inorganic phosphate molecule competitively bound to E_D is not less than 40 mM.

[12] The equilibrium dissociation constant for the process ($E_P \cdot$ Glc-1-P + $E_D \cdot$ Glc-P_2 + $E_P \cdot$ Glc-6-P $\rightleftharpoons$ E_D + Glc-P_2 is given by Peck *et al.* (1968) as 4.7×10^{-6} M; since the equilibrium ratio ($E_D \cdot$ Glc-P_2)/($E_P \cdot$ Glc-1-P + $E_D \cdot$ Glc-P_2 + $E_P \cdot$ Glc-6-P) is about 0.45 (J. W. Long and W. J. Ray, Jr., manuscript in preparation), [E_D][Glc-P_2]/[$E_D \cdot$ Glc-P_2] is about 9×10^{-6} M.

in the corresponding reactions of E_D (see above). Thus, the most reasonable explanation for these observations is that the second phosphate-binding subsite in E_D is blocked by the ($-PO_3$) group in E_P.

The presence of a single, strong phosphate-binding subsite in both E_P and E_D and a weaker phosphate-binding subsite in E_D, that is absent in E_P, can be readily rationalized in terms of an exchange mechanism, which implies that the phosphate-binding subsites in E_D have fixed as opposed to interchangeable functions (see above). This binding pattern also can be rationalized in terms of a minimal motion mechanism, with the following three restrictions. (a) E_P and $_PE$ (see above) should be present in approximately equal amounts because of approximately equal interactions of the phosphate group of the enzyme with both of the phosphate-binding subsites of the enzyme; otherwise conversion of (E_P + $_PE$) to E_D should produce an enzyme form that binds phosphate substantially better than (E_P + $_PE$). However, this is not the case (see above). (b) Binding of one phosphate group to the dephosphoenzyme should produce approximately equal amounts of $E_D \cdot P_i$ and $P_i \cdot E_D$ by analogy with E_P and $_PE$. (c) The weak binding of a second phosphate group to ($E_D \cdot P_i + P_i \cdot E_D$) to give $P_i \cdot E_D \cdot P_i$ must arise from anticooperative binding.[13]

The important aspect of the above restrictions for the minimal motion mechanism is that the single phosphate group in ($E_D \cdot P_i + P_i \cdot E_D$) should have an "average environment" similar to the average environment of the two phosphate groups in $P_i \cdot E_D \cdot P_i$. Alternatively, the inorganic phosphate group in ($P_i \cdot E_P + E_P \cdot P_i$) should have an average environment similar to the average environment of the two phosphate groups in $P_i \cdot E_D \cdot P_i$. The nmr studies in the accompanying paper (Ray and Mildvan, 1973) show that the latter similarity definitely does not hold. Hence, the exchange or two-intermediate mechanism with its discretely different phosphate-binding sites is more amenable to the results than is the minimal motion mechanism. Of course, changes in the structure of the active site on cleavage of the serine phosphate bond, *viz.*, during conversion (hypothetical) of $E_P \cdot P_i$ to $P_i \cdot E_D \cdot P_i$, could invalidate this argument, although any structural change that accompanies such a process must be quite small, at least in terms of detection *via* conventional techniques such as ultraviolet difference spectroscopy and circular dichroism (J. W. Long and W. J. Ray, Jr., manuscript in preparation).

Chart I shows that the binding of α,ω-alkanediol bisphosphates to the dephosphoenzyme is sensitive to the distance between the α- and ω-phosphate groups; thus the binding constants for $n = 3$ and $n = 10$ differ by nearly two orders of magnitude. The original idea in assessing the binding pattern for the bisphosphates was to deduce the distance between the phosphate-binding subsites in the dephosphoenzyme. Unfortunately, the results are difficult to interpret in such terms because of the number of factors that could be altered by a change in the chain length of the inhibitor—in addition to the actual phosphate group–binding site interaction. These include the entropy of binding caused by differences in rotational restrictions, hydrophobic binding effects, and the possible binding site flexibility of the enzyme itself. However, comparison of the binding energies for $n = 1$ and 3 (Chart I) does suggest that the two phosphate-binding subsites are not immediately adjacent to each other as would be required for a minimal motion mechanism.[14] Thus, the data in the present paper plus that in the accompanying paper (Ray and Mildvan, 1973) seem to fit more readily within the framework of the exchange mechanism.

[13] Because the system E_D + P_i contains one more hydroxyl group than does E_P, weak binding of P_i by (E_P + $_PE$) is not required simply because P_i binding by ($E_D \cdot P_i + P_i \cdot E_D$) is weak.

Appendix

Although it is not possible to obtain a precise value for the interaction of $E_D \cdot P_i$ with a second P_i group by comparison of binding data for mono- and bisphosphates, a maximum value for the extent of this interaction can be estimated by use of such data in the following manner. (In this Appendix, binding constants instead of dissociation constants are used because this usage facilitates the presentation.) In making such an estimate, the binding of the bisphosphate is considered as a two-step process. The first step involves binding of one of the phospho groups of the bisphosphate to the site at which the corresponding group of the monophosphate is bound, while the second step involves the subsequent binding of the second phospho group. The ratio of the binding constant of 1,4-butanediol-P_2 to that of 1,4-butanediol-P is about 2000:1. However, the number of phospho groups present in the case of the bisphosphate is twice that for the monophosphate at the same molar concentration. Hence, the bisphosphate is bound more strongly than the monophosphate by a factor of only 1000-fold. This factor corresponds to a *difference* in the ΔG° for the binding process of about -4.1 kcal/mol (at 30°).

However, the difference in ΔG° for binding of monophosphates and the corresponding bisphosphates is not equal to the ΔG° for binding of P_i to $E_D \cdot P_i$, even though the second step in the binding of the bisphosphate should involve analogous interactions between the enzyme and the second phospho group of the bisphosphate group or the second inorganic phosphate molecule. Thus, the binding of the second phospho group of the bisphosphate is a unimolecular process with a dimensionless equilibrium constant while the binding of the second inorganic phosphate molecule is a bimolecular process with an equilibrium constant expressed in terms of $_M{}^{-1}$. No direct comparison of calculated ΔG° values for two processes differing in this manner can be made because the comparison would depend on the (arbitrary) difinition of the unit, moles per liter. (A similar problem arises in comparison of first and second-order rate constants, *e.g.*, see Jencks, 1969.) However, if the expressions for both equilibrium constants are written in terms of mole fractions, instead of moles per liter, both equilibrium constants will be dimensionless, and a direct comparison of the difference in ΔG° values for the two processes is more satisfying, at least from the standpoint of standard states. Actually, the use of the mole-fraction convention in such a calculation, in fact, assigns a value of 55 (the molarity of water) to the "chelate effect," which operates in the second step of the binding of the bisphosphate but not in the binding of P_i to $E_D \cdot P_i$. Frequently, chelate effects are larger than 55, and very much larger values have been suggested by Page and Jencks (1971). The larger the actual value in the present system, the weaker the expected binding of P_i to $E_D \cdot P_i$, based on comparisons of the above type.

[14] Because the phosphate groups of 1,3-propanediol bisphosphate are closer together than the maximum possible separation of the phosphate groups in glucose-1,6-P_2 and because the 1,3-disphosphate is bound more tenaciously than the other bisphosphates, one might be tempted to conclude that glucose-1,6-P_2 is bound in a conformation other than the fully extended conformation. However, subsequent data do not support this conclusion (J. W. Long and W. J. Ray, Jr., manuscript in preparation).

Hence, in the mole-fraction convention, we would expect ΔG° for binding of P_i to $E_D \cdot P_i$ to be equal to or more positive than -4.1 kcal/mol, which corresponds to a *mole-fraction* binding constant of 1000 or less (20 M or less in the molar convention). This contrasts with a value of 55,000 for the binding of the first P_i molecule as expressed in the mole-fraction convention (1000 in the molar convention). Hence, the second P_i molecule is expected to bind to E_D much more weakly than the first. Experiments in the accompanying paper suggest that the binding constant for the second phosphate may well be in the range of 10 M, which is not at all unreasonable in comparison with the maximal value of 20 M estimated above.

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