to the enzyme, the use of an acidic quenching solution may well be more effective than a basic one, since rapidly protonating the anionic group of substrate in an enzyme·substrate complex may well be the most efficient way of eliminating further catalytic activity. In fact this expectation appears to hold in the phosphoglucomutase system (Ray and Long, 1976a).

In any case, although we feel that the apparatus described here can provide adequate rates of mixing to allow the investigation of the equilibrium ratios of central complexes present in other enzymic systems, the primary problem in such studies may not be mixing, per se, but the rate of inactivation subsequent to mixing, and each case will require a separate examination.

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Thermodynamics and Mechanism of the PO_3 Transfer Process in the Phosphoglucomutase Reaction†

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ABSTRACT: The equilibria among the central complexes in the phosphoglucomutase system were evaluated by (a) using an excess of enzyme plus Mg^{2+} to prepare mixtures with glucose phosphates in which essentially no *free* glucose phosphates were present; (b) inactivating the enzyme in such mixtures by means of a procedure that prevents substantial interconversion of the central complexes; and (c) assaying the quenched mixture for glucose-1-P, glucose-1,6-P_2, and glucose-6-P. The fractional amounts of E_P·Mg·Glc-1-P, E_D·Mg·Glc-P_2, and E_P·Mg·Glc-6-P present at pH 7.5 and 24 ° C were 0.13, 0.54, and 0.33. (E_P and E_D are the phospho and dephospho forms of the enzyme, respectively.) From these fractions and the equilibrium isotope exchange constants for the three sugar phosphates, true dissociation constants can be calculated for each of the above complexes: 8.5 μM, 19 nM, and 57 μM, respectively. Relative to the rate of PO_3 transfer to water, a 3×10^{10}-fold rate increase is produced by binding glucose-1-P to the Mg^{2+}-enzyme (Ray, Jr., W. J., Long, J. W., and Owens, J. D. (1976), *Biochemistry,* the following paper in this issue.) This "substrate-induced rate effect" is equivalent to a difference of some 14 kcal in Gibbs activation energies for transfer to chemically similar hydroxyl groups, and most of this energy difference ultimately must be rationalized in terms of binding interactions involving the phosphoglucosyl moiety. Three different mechanisms for using substrate binding energy to reduce the activation energy of the subsequent catalytic step are examined as possible explanations for the substrate-induced rate effect. These mechanisms emphasize (a) enthalpic destabilization and (b) (entropic) immobilization of *reactant groups* during *formation* of the *enzyme–substrate complex,* and (c) increased binding interactions of *nonreactant groups* during the subsequent *approach to* the *transition state.* As a test for enthalpic destabilization of the enzymic phosphate group, values of $\Delta G^{\circ\prime}$ for the hydrolytic cleavage of this group in E_P and E_P·Glc-1-P are calculated from equilibria measured at pH 7.5 and 30 °C: about −1 and +1.4 kcal/mol, respectively. To test for destabilization of the acceptor hydroxyl group in the enzyme–substrate complex, $\Delta G^{\circ\prime}$ for the equilibrium, E_P·Glc-P $\rightleftharpoons$ E_D·Glc-P_2, is compared with that for the corresponding process involving the nonrigid acceptor, 1,4-butanediol monophosphate: about −0.9 and −1.9 kcal, respectively. These results are not consistent with a large enthalpic destabilization of the reactant groups in the E_P·Glc-1-P complex. To test for entropic immobilization of reactant groups, glucose 6-phosphate is considered as a bidentate ligand, and the chelate effect on the binding and subsequent enzymic transfer reaction that arises from covalently linking the sugar ring and the PO_3 group is evaluated. Reference reactions involving xylose as a PO_3 acceptor both in the presence and absence of bond (inorganic) phosphite are used. The covalently attached $CH_2OPO_3^{2-}$ group of glucose-6-P contributes about −6.2 kcal/mol to $\Delta G^{\circ\prime}$ for binding and reduces $\Delta G^{\ddagger}$ by about 7.2 kcal/mol, while $\Delta G^{\circ\prime}$ for binding of phosphite (in the presence of bound xylose) is about −5.6 kcal/mol (calculated by use of mole-fraction binding constants) and its binding reduces $\Delta G^{\ddagger}$ by about 6.2 kcal/mol. Since the overall effect (sum of $\Delta G^{\circ\prime}$ and $\Delta G^{\ddagger}$) is only about 1.6 kcal/mol more negative for the *covalently attached* $CH_2OPO_3^{2-}$ group than the *independent* HPO_3^{2-} molecule, the chelate effect for glucose-6-P is marginal, the bidentate interaction of the component parts of glucose-6-P with the enzyme does not appear to involve an unusual degree of immobilization, and the rate effect produced by immobilization of reactant groups does not approach its theoretical limit; i.e., it does not contribute overwhelmingly to the substrate-induced rate effect. To test whether binding interactions involving *nonreactant groups* markedly increase in proceeding from the enzyme–substrate complex to the transition state, an attempt was made to circumvent a possible mechanism for *preventing* maximal binding interactions between the enzyme and such groups in the enzyme–substrate complex: using the 6-hydroxymethyl group of the reactant to

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prevent optimal binding of the sugar ring plus its attached 1-phosphate group until the acceptor hydroxyl group becomes partially bonded to the enzymic phosphate group in the transition state. However, neither removal of the hydroxymethyl group of glucose-6-P, nor the contiguous enzymic phosphate group, nor both, produce an increase in binding. Hence, binding interactions involving the *nonreacting groups* probably are nearly optimal in the enzyme–substrate complex, and do not markedly increase during development of the transition state. Since none of the above concepts, taken singly, provides a rationale for a majority of the substrate-induced rate effect in the phosphoglucomutase system, a combination of effects involving more modest factors is indicated. Some of these are described in an accompanying paper in this issue.

Studies in an accompany paper show that the rate of PO_3 transfer from the Mg^{2+} form of rabbit muscle phosphoglucomutase to the 6-hydroxyl group of glucose-1-P is some 3×10^{10}-fold faster than the analogous process involving the chemically similar hydroxyl group of a water molecule (Ray et al., 1976). Most of this huge "substrate-induced rate effect", which is equivalent to a lowering of $\Delta G^{\ddagger}$ by some 14 kcal, ultimately must be accounted for in terms of binding interactions involving the phosphoglucosyl moiety. The present paper describes an examination of three different mechanisms of enzyme action each of which, by itself, appears capable of providing a rationale for rate increments in the range of that observed, and each of which uses the binding of *nonreacting groups* in a different manner: to produce enthalpic destabilization or immobilization of *reactant groups* in the enzyme–substrate complex, or to achieve a reduction in the Gibbs energy function for such groups in the transition state.

The possibility of enthalpic destabilization of reactant groups in enzyme–substrate complexes recently has received increased attention as a possible explanation of the catalytic efficiency of enzyme action (see Jencks, 1975, for a recent review). In most cases the evidence for such effects is indirect, even where the rate-determining step involves bond scission, and, in enzymic reactions where bond making is critical, it is even more difficult to determine whether or not destabilization is involved.

One way to approach this problem is to assess the change in chemical potential of a group bound to an enzyme that is produced by the subsequent binding of its reaction partner. An increased chemical potential of the first group on binding of the second would provide presumptive evidence for the generation of enthalpic destabilization during the second binding step. In group transfer reactions, the chemical potential of a group usually is assessed in terms of $\Delta G^{\circ\prime}$ for hydrolysis or a related process (under specified conditions) and it may be difficult to assess the chemical potential of a group that is noncovalently bound to an enzyme. However, a number of studies have provided values for the chemical potential of acyl groups and PO_3 groups that are covalent bound to enzymes whose reaction sequences are ping–pong (see Hinkle and Kirsch, 1971; Peck et al., 1968, and references therein). In such systems assessing $\Delta G_{hyd}^{\circ\prime}$ for the attached group in the presence of a bound acceptor should be straightforward, and in the present paper $\Delta_{hyd}G^{\circ\prime}$ for the active-site phosphate group of phosphoglucomutase is compared with the corresponding value in the presence of bound glucose-1-P.

An alternative explanation of enzymic catalysis involves an immobilization, during substrate binding, of the groups that subsequently will react. In extreme cases such a process could produce enormous rate accelerations relative to the corresponding uncatalyzed bimolecular reaction (Page and Jencks, 1971; Jencks, 1975). One approach to evaluating the importance of immobilization in a binding process is to assess the magnitude of the chelate effect associated with binding. Thus, if two different parts of a molecule are bound to an enzyme in a highly immobile manner, binding should be much more tenacious than otherwise would be predicted on the basis of the separate interactions of the component parts of the molecule (unless complicating factors are involved; see Discussion). In the present paper a chelate effect for substrate binding is evaluated by comparing the Gibbs energy changes that accompany glucose-6-P binding with those that accompany the sequential binding of xylose and inorganic phosphite.

In attempting to describe how binding interactions can produce accelerated rates in enzyme-catalyzed reactions, it seems useful in some cases to separate the overall process into events that occur during formation of the enzyme–substrate complex, as above, and events that occur during the subsequent formation of the transition state, and to also separate, at least conceptually, what happens to the *reacting* and *nonreacting* portions of the molecules in question, especially in the second of the above steps. (The reacting part of a molecule will include all nuclei whose *covalent* bonds are substantially altered in the transition state.) Since a distinction between binding and bonding of *reactant groups* becomes meaningless as the system approaches the transition state (because these terms are conceptualized in terms of ground-state phenomena), an increased binding/bonding of reactant groups to a catalyst is the sine non quo for catalysis, both in simple and complex systems. This is reflected in the transition state binding hypothesis that has been popularized among biochemists by Wolfenden (1972) and Lienhardt (1973), among others. However, the binding of *nonreacting groups* may or may not be used to facilitate catalysis, although in enzymic reactions where contact between most of the reactant molecule(s) and the enzyme is feasible, such a possibility cannot be lightly dismissed. If the enzyme–substrate complex is used as a reference point, *nonreacting groups* can produce effects either during the binding step(s) leading to this complex, as in the case of induced-fit effects (Koshland, 1963), or, if *optimal* binding interactions in the enzyme–substrate complex are *precluded*, in the subsequent attainment of the transition state. The latter possibility will be referred to as "increased binding interactions (of nonreacting groups) in the transition state."

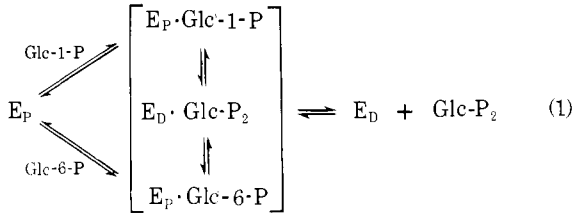
Since increased binding interactions in the transition state depend on the existence of a mechanism to *prevent* nonreacting groups from binding optimally in the enzyme–substrate complex, the possibility that such a process makes a large contribution to the substrate-induced rate effect in the phosphoglucomutase reaction was studied by attempting to eliminate features of glucose-1-P and the phospho-enzyme that might prevent optimal binding in the E_P·Glc-1-P complex, and assessing the effect of these changes on binding of the altered molecules. In this phase of the study the binding of xylose-1-P and glucose-1-P to the phospho and dephospho forms of the enzyme is compared.

In order to evaluate the results of a number of the experiments outlined above, it is necessary to know the equilibrium

constants for interconversion of the complexes, E_P·Glc-1-P, E_D·Glc-P_2, and E_P·Glc-6-P.[1] These constants can be measured if an equilibrium mixture of the above complexes can be inactivated at a sufficiently rapid rate to prevent their interconversion during the quenching step. The design and testing of a simple, inexpensive apparatus for such an operation is outlined in an accompanying paper (Ray and Long, 1976a). Some of the results described here are related to the question of the quenching efficiency attainable with this apparatus.

Theory and Modus Operandi

The following scheme is generally accepted for the phosphoglucomutase reaction (Ray and Peck, 1972); note that in this paper E_P and E_D will represent the *Mg^{2+} complexes* of the phospho and dephospho forms of the enzyme, respectively, and that the enzyme–substrate complexes (the central complexes) also involve enzyme-bound Mg^{2+}



(1)

The following relationships hold *at equilibrium;* (CC) represents the sum of the central complexes:

$$\frac{(E_P)(\text{Glc-1-P})}{(CC)} = K_{x(\text{Glc-1-P})} \quad (2)$$

$$\frac{(E_D)(\text{Glc-}P_2)}{(CC)} = K_{x(\text{Glc-}P_2)} \quad (3)$$

$$\frac{(E_P)(\text{Glc-1-P})}{(E_P \cdot \text{Glc-1-P})} = K_{d(\text{Glc-1-P})} = K_{x(\text{Glc-1-P})}\left[\frac{(CC)}{(E_P \cdot \text{Glc-1-P})}\right] \quad (4)$$

$$\frac{(E_D)(\text{Glc-}P_2)}{(E_D \cdot \text{Glc-}P_2)} = K_{d(\text{Glc-}P_2)} = K_{x(\text{Glc-}P_2)}\left[\frac{(CC)}{(E_D \cdot \text{Glc-}P_2)}\right] \quad (5)$$

The K_x's are isotopic exchange constants measured at equilibrium in the presence of saturating concentrations of the alternative substrate (saturating bisphosphate in the case of $K_{x(\text{Glc-1-P})}$ and saturating monophosphate in the case of $K_{x(\text{Glc-}P_2)}$). It is apparent from eq 4 and 5 that the true dissociation constant, K_d, for the mono- and bisphosphates can be obtained from measured K_x values and a knowledge of the fraction of the central complexes present as E_P·Glc-1-P, E_P·Glc-6-P, and E_D·Glc-P_2, respectively.

From a knowledge of the K_x values for the mono- and bisphosphates, it is also possible to evaluate the concentration of enzyme that must be added to a solution of glucose-6-P to completely convert it to an equilibrium mixture of complexes involving *bound* glucose-1-P, glucose-6-P, and glucose-1,6-P_2, i.e., so that each sugar phosphate is present exclusively as one of the central complexes. If such an equilibrium mixture is quenched sufficiently rapidly to prevent interconversion of these complexes during the quenching step, the relative amounts of glucose-1-P, glucose-6-P, and glucose-1,6-P_2 present in quenched samples will provide a measure of the relative amounts of the central complexes in the original mixture.

Experimental Section

Materials. Phosphoglucomutase, [^{32}P]phosphoglucomutase, and [^{32}P]glucose-1-P were prepared as described previously (Long and Ray, 1973). Dilute solutions of the Mg^{2+} form of the enzyme (0.1 to 1 mg per ml) were obtained by treating the purified enzyme for 10 min at room temperature with 2 mM Mg^{2+}–1 mM EDTA in the presence of 0.1 M imidazole (recrystallized from benzene and carefully dried before use) and 20 mM Tris-Cl, pH 7.5. Further dilutions were prepared with a similar solution, which also contained 0.15 mg per ml of bovine serum albumin (crystalline) but without imidazole. Concentrated solutions of the Mg^{2+} enzyme were obtained from a "metal-free" preparation (Ray and Mildvan, 1970) by treatment with 1 mM Mg^{2+}. Uniformly labeled [^{32}P]glucose-1,6-P_2 was prepared by equilibrating [^{32}P]glucose-1-P with a 50-fold excess of the bisphosphate in the presence of phosphoglucomutase, and separating the radioactive products chromatographically (see Ray and Roscelli, 1964a). Glucose-6-P dehydrogenase (Boehringer) was dialyzed for several hours at 4 °C against 10 mM ammonium sulfate; it was subsequently frozen by dropwise addition to liquid nitrogen (cf. Yankeelov et al., 1964) and stored in liquid nitrogen prior to use. α-D-Xylose-1-P (Sigma), 100 μmol in 10 ml, was treated with glucose-6-P dehydrogenase, $NADP^+$ (Sigma), and 0.02 mol % phosphoglucomutase, Mg^{2+} form, in 20 mM Tris-Cl, pH 7.5, containing 2 mM Mg^{2+} and 1 mM EDTA for 0.5 h at room temperature. Two equal amounts of phosphoglucomutase were subsequently added at half-hour intervals. The product was chromatographically separated from traces of glucuronic acid-6-P on an anion exchange resin (see Ray and Roscelli, 1964a). A solution of 5 M xylose (Sigma) was successively treated with small amounts of decolorizing charcoal and the supernatant separated by centrifugation; the solution was passed slowly through a mixed-bed resin (Bio-Rad, AG 501-8X) and the eluent collected after thoroughly flushing the column. All other materials were of reagent grade or better.

Equilibration of Glucose 6-Phosphate with Excess Phospho-Enzyme. Solutions of enzyme and glucose-6-P were equilibrated by incubating the following materials for time intervals of several minutes to 1 h at pH 7.5 and room temperature (final concentrations are given): 20 mg per ml (0.32 mM) of phosphoglucomutase (metal-free form), 1 mM $MgCl_2$, 0.03 mM glucose 6-phosphate, and 20 mM Tris-Cl, pH 7.5. The glucose-6-P was added several minutes after other reagents were mixed. Aliquots of 50 μl were quenched by metering into 0.55 ml of KOH or $HClO_4$ solutions, 0.5 to 2 M, by means of the slow delivery–rapid quench apparatus described in an accompanying paper (Ray and Long, 1976a).

Assay of Glucose Phosphates. Enzyme samples quenched in base were allowed to stand at room temperature for a few minutes and brought to pH 1 with 5 M $HClO_4$. Samples quenched in acid were immediately brought to pH 1 with 4 N KOH. After transferring to small plastic centrifuge tubes and cooling at 4 °C for 15 min, samples were centrifuged for 5 min (Eppendorf 3200 centrifuge) at the same temperature. An aliquot of 0.01 ml of 1 M Tris was added to the supernatant, which subsequently was brought to pH 7.5 with 4 N KOH and again cooled and centrifuged as above. A 0.5-ml aliquot of the

[1] The following abbreviations are used: E_P and E_D, the Mg^{2+} complex of the phospho and dephospho forms of phosphoglucomutase, respectively; Glc-P_2, α-D-glucose 1,6-bisphosphate; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; Glc-P, an equilibrium mixture of Glc-6-P and Glc-1-P; Glc-6-S, D-glucose 6-sulfate; Glc-1-P-6-S, a mixed ester with a phosphate group attached to the 1-position and a sulfate group attached to the 6-position of α-D-glucose; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NADP^+$, nicotinamide adenine dinucleotide phosphate; $NADPH^+$, reduced $NADP^+$; P_i, inorganic phosphate.

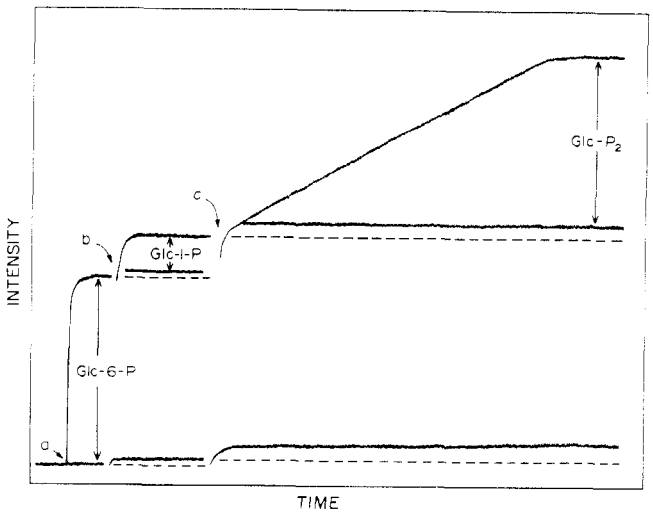


FIGURE 1: A typical recorder output obtained during analysis of a mixture of glucose-6-P, glucose-1-P, and glucose-1,6-P_2. The mixture contained a total of 0.75 nmol of sugar phosphates. The upper trace shows the fluorescence increase produced by successive additions of (a) glucose-6-P dehydrogenase, (b) Mg^{2+} and phosphoglucomutase, and (c) xylose-1-P plus more phosphoglucomutase (see Experimental Section for details). The lower trace shows the fluorescence increase produced by the same additions to an identical assay mixture, but with no glucose phosphates present. The relative amounts of the glucose phosphates are indicated by the vertical arrows.

supernatant was mixed with 1 ml of an assay solution containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 20 μM NADP and the blank fluorescence was measured on the "1 scale" of an Aminco fluorocolorimeter equipped with a blank-substract photomultiplier microphotometer. The fluorimeter was operated in conjunction with a hydrogen lamp and power supply from a Beckman DBG spectrophotometer, a Honeywell Electronik 19 recorder, which was set at 5 mV full scale, and a Sorensen ACR 500 voltage regulator; a water-jacketed cell holder also was used. An aliquot of 5 μl of a 1 mg/ml solution of dialyzed glucose-6-P dehydrogenase was added to measure the glucose-6-P present. When the fluorescence no longer increased with time, 5 μl of 0.4 M Mg^{2+} and 5 μl of a 1 μM solution (0.06 mg per ml) of phosphoglucomutase, Mg^{2+} form, were added to measure the glucose-1-P present. Finally, 5 μl of 0.1 mM phosphoglucomutase, Mg^{2+} form, and 5 μl of 0.1 M xylose-1-P in 0.1 M Tris-Cl, pH 7.5, were added to measure the glucose-1,6-P_2 present. For this assay the Mg^{2+} form of phosphoglucomutase was prepared from the metal-free form by addition of 2 mM Mg^{2+}–1 mM EDTA, but without added imidazole or serum albumin. Commercial samples of xylose-1-P were used without purification.

Isotopic Exchange Studies. $K_{x(Glc\text{-}P)}$ and $K_{x(Glc\text{-}P_2)}$ for the Mg^{2+} form of the enzyme were measured at 24 °C and pH 7.5 in a manner similar to that described in an accompanying paper for other metal forms of the enzyme (Ray and Long, 1976b). However, assay solutions contained 2 mM Mg^{2+} and 1 mM EDTA instead of the metal buffers described in that paper. In addition, $K_{x(Glc\text{-}P_2)}$ was measured at several different concentrations of both the glucose-1-P–glucose-6-P equilibrium mixture and of Mg^{2+}.

Fluorescence Assay of Acceptor Activity. One milliliter of a solution containing 4 mM magnesium chloride, 2 mM EDTA, 20 μM glucose-1,6-P_2, 0.03 mM $NADP^+$, and 40 mM Tris-Cl, pH 7.5, plus 0.01 ml of dialyzed glucose-6-P dehydrogenase, 0.2 ml of a dilute solution of phosphoglucomutase, Mg^{2+} form, and sufficient water plus PO_3 acceptor to give a total volume of 2.0 ml were combined in a fluorimeter cuvette. The increase in fluorescence with time was measured at 25 °C (see Assay of Glucose Phosphates).

The Reaction of Xylose plus Phosphite with the Phospho-Enzyme. A 0.05-ml aliquot of phosphoglucomutase, Mg^{2+} form, was added to 0.75 ml of a solution at pH 7.5 and 30 °C which contained 20 mM Tris-Cl, 2 mM magnesium chloride, 1 mM EDTA, 0.031 mM [^{32}P]glucose-1,6-P_2, 0.025 M sodium phosphite, and a variable amount of xylose. After 1 min the reaction was terminated with 0.05 ml of 1 M formic acid. The solution was diluted with 4 ml of water and transferred to a 0.6 × 1.5 cm column of Dowex 1-X8, formate form. After two washes with 0.75 ml of water, sugar monophosphates were eluted from the column with 5 ml of 0.6 M pyridine formate, pH 3. Subsequently, the bisphosphate was eluted with 5 ml of 3 M pyridine formate, pH 3. The elutes were evaporated to dryness with an air jet on a steam bath. The residue was dissolved in water and the radioactivity in an aliquot was measured with a low-background planchette counter.

Single Turnover Experiments. The rate of transfer of the enzymic PO_3 group to various acceptors was followed by using ^{32}P-labeled phosphoglucomutase in the manner described in an accompanying paper (Ray et al., 1976).

Results

The Metal Complexes of Phosphoglucomutase and Its Substrates. In these studies, the concentration of Mg^{2+} was maintained at sufficiently high levels to saturate the enzyme, and the presence of enzyme-bound Mg^{2+} is assumed throughout, viz., both E_P and E_D *will refer to the* Mg^{2+} *complexes of the enzyme.* However, the Mg^{2+} concentration in enzymic assays was not high enough to convert a significant fraction of a *limiting* monophosphate to its Mg^{2+} complex and, when glucose-1,6-P_2 was limiting, corrections for its inactive metal complexes (cf. Ray and Peck, 1972) were made (see below). Hence, glucose-1-P, glucose-6-P, and glucose-1,6-P_2 will refer to the metal-free forms.

The Assay of Glucose Phosphates. Figure 1 shows the results of a typical fluorimetric analysis of glucose-6-P, glucose-1-P, and glucose-1,6-P_2 in a mixture. The assay is based on the glucose-6-P dehydrogenase reaction and involves the stepwise conversion of each of the above sugar phosphates to glucuronic acid-6-P, with the coupled production of NADPH (see Experimental Section). The conversion of glucose-1,6-P_2 to glucose-6-P plus P_i (prior to reaction with the dehydrogenase) involves the following process, which is discussed in an accompanying paper (Ray et al., 1976).

$$E_P (+ \text{Xyl-1-P}) \rightarrow E_D + P_i$$

$$E_D + \text{Glc-}P_2 \rightarrow E_P + \text{Glc-6-P}$$

Analyses of the glucose phosphates present both separately and as mixtures in standard solutions showed that the fluorescence produced was linearly related to assay concentration in the range under study: 0 to 0.75 μM. Baseline changes produced by successive additions of reagents also are shown. The standard deviation for assays of a particular sugar phosphate was about 0.5% of the *total* sugar phosphates present. Because of volume changes during the workup only the *relative* amounts of glucose phosphates in quenched mixtures (see below) were calculated in most cases, and the concentration of a particular glucose phosphate in the original mixture was taken as the product of the initial concentration of glucose-6-P in the mixture and the *fraction of* of the total glucose phosphates in the quenched solution present in that form. In the few cases

TABLE I: Relative Amounts of Glucose Phosphates in Equilibrium Mixtures Quenched by Acid or Base.[a]

Glucose Phosphate	% of Total after Acid Quenching[b]				% of Total after Base Quenching[b]			
	0.5 N	1 N	2 N	Av	0.5 N	1 N	2 N	Av
Glc-1-P	15 ± 0.5	12 ± 1	13 ± 1	13 ± 1.5	7.5 ± 0	7.5 ± 0	8 ± 1	7.5 ± 0.5
Glc-P_2	52 ± 0	54 ± 3	55 ± 2	54 ± 2.5	71 ± 1	71 ± 1.5	70.5 ± 0.5	71 ± 1
Glc-6-P	33 ± 0.5	33.5 ± 3	32 ± 1	33 ± 2	21 ± 1	21.5 ± 1.5	21.5 ± 1.5	21 ± 1

[a] At pH 7.5 and 24 °C. [b] Equilibrium mixtures initially contained 0.32 mM phosphoglucomutase, 0.03 mM Glc-6-P, 1 mM Mg^{2+}, and 20 mM Tris chloride. The percent of the total glucose phosphates present in the various forms is shown after quenching with the indicated solutions and correcting for small amounts of free glucose monophosphates (see Results section). Each entry represents an average from three or four separate quenching experiments. Results and standard deviations were rounded to the nearest half percent.

where it was checked, the recovery of glucose phosphates was greater than 90% in both acid- and base-quenched samples.

The Equilibrium Distribution among the Central Complexes. Experiments described in an accompanying paper suggest that equilibrium mixtures of phosphoglucomutase and its complexes with glucose phosphates in 20 mM Tris buffer can be inactivated with 2 M base by means of a slow-delivery–rapid-quench device at a sufficient rate to prevent substantial interconversion of these complexes (Ray and Long, 1976a). However, a more stringent criterion of quenching efficiency would be obtaining the same results when an identical mixture alternatively is quenched with acid. However, Table I shows a significant difference between results obtained with acid and base quenching (columns 5 and 9), although in both cases the results were independent of the concentration of the acid or base in the quenching solution. This independence, plus the reproducibility of the results, suggests that mixing problems were not responsible for the difference obtained with the two types of quenching solutions, but that some PO_3 transfer occurred after the central complexes entered the quenching solution. As is indicated in an accompanying paper (Ray and Long, 1976a), if unfolding of the protein is a significant mechanism for inactivation by the quenching solution, quenching artifacts are likely. Apparently this is the case for the Mg^{2+} complexes of phosphoglucomutase, although no substantial differences between the results obtained with acid and base quenches were observed with metal forms of phosphoglucomutase that are somewhat less efficient, catalytically, than the Mg^{2+} form (Ray and Long, 1976b). However, even with the Mg^{2+} complex the difference in quenching conditions did not produce large differences in the results.

Since acid and base usually change equilibria in opposite directions, the average of results obtained by the two procedures might be used as an estimate of the equilibrium distribution prior to quenching—especially since the average is not greatly different from the extremes. However, for reasons outlined in an accompanying paper (Ray and Long, 1976b) we have chosen to use only the results obtained by acid quenching, although none of the conclusions reached here is significantly altered by this decision.

Even in the presence of a tenfold excess of free enzyme at a concentration of nearly 0.3 mM, equilibrium mixtures of the central complexes actually contained small amounts of free glucose monophosphates, as is indicated by the values of the equilibrium isotope exchange constants for glucose-1-P and glucose-6-P (see following section). Hence, the calculated concentrations of free glucose monophosphates were subtracted from the concentrations observed in quenched equilibrium mixtures in order to estimate the relative concentrations of the central complexes present prior to quenching, even though this correction was relatively small. In the case of the bisphosphate, such a correction was not required (see below).

Isotopic Exchange Constants at Equilibrium. Isotopic exchange constants were evaluated from double-reciprocal plots of the initial exchange rate (Boyer, 1959), at pH 7.5 and 24 °C, and the varied substrate concentration, at a saturating or near-saturating concentration of the nonvaried substrate. Such plots (not shown) were linear and resembled similar plots published previously (Ray et al., 1966). The value of the exchange constant for an equilibrium mixture of glucose-1-P and glucose-6-P ($K_{x(Glc\text{-}P)}$), 20 μM, is equivalent to the value of 1.1 μM for $K_{x(Glc\text{-}1\text{-}P)}$ observed previously under similar conditions (Ray et al., 1966), but at 30 °C ([Glc-6-P]/[Glc-1-P] = 17.3 (Atkinson et al., 1961); hence, [Glc-1-P]/[Glc-P] = 0.055). However, $K_{x(Glc\text{-}P_2)}$ was more difficult to assess since both Mg^{2+} and glucose monophosphates are inhibitors of the dephospho-enzyme, competitive with glucose-1,6-P_2; moreover, the effect observed when both are present is the product of their separate effects since Mg^{2+} inhibits by binding to glucose-1,6-P_2 and glucose monophosphates inhibit by binding to E_D (Ray and Roscelli, 1966). Hence, values of $K_{x(Glc\text{-}P_2)}^{app}$ were obtained at a constant concentration of 1 mM Mg^{2+} by using 0.5, 1.0, 1.5, and 2 mM glucose-P and extrapolating to saturating but noninhibiting glucose-P: K_I = 0.45 mM (plots not shown). Since $K_{I(Glc\text{-}1\text{-}P)}$ is reported as 0.4 mM (Ray et al., 1966) and 0.5 mM (Peck et al., 1968), both glucose-6-P and glucose-1-P bind approximately equally to E_D.[2]

Subsequently, the extrapolated value of $K_{x(Glc\text{-}P_2)}^{app}$, obtained above, was corrected for the formation of inactive complexes of glucose-1,6-P_2 and Mg^{2+}. Thus, values of $K_{x(Glc\text{-}P_2)}^{app}$ were obtained at 0.5 mM glucose-P and at Mg^{2+} concentrations of 1 to 3 mM. However, a plot of $K_{x(Glc\text{-}P_2)}^{app}$ against Mg^{2+} in this concentration range was slightly but noticeably concave (not shown). Such a curvature is expected from the results of binding studies reported previously since both Mg·Glc-P_2 and Mg_2·Glc-P_2 are inactive complexes (Ray and Roscelli, 1966). Because of this curvature, the ordinate intercept was not accurately defined. Moreover, concentrations

[2] The results of a different study also can be used to make a direct estimate of K_I for glucose-6-P (at pH 7.4 and 30 °C) from a plot of $K_{m(Glc\text{-}1\text{-}P_2)}^{app}$ for the thermodynamically unfavorable reaction, Glc-6-P → Glc-1-P, against glucose-6-P concentration (Peck et al., 1968). The value thus obtained is about 0.2 mM. However, difficulties in measuring initial velocities for the reverse reaction (Ray and Roscelli, 1964b) must have produced errors in the values of $K_{m(Glc\text{-}P_2)}^{app}$ reported in that study since (a) the data obtained in the present study show that K_I for glucose-6-P cannot possibly be as small as 0.2 mM and (b) the value of $K_{m(Glc\text{-}P_2)}$ for the reverse reaction obtained by extrapolating the data of Peck et al. (1968) to noninhibiting concentrations of glucose-6-P also is too small to correlate with some of the present data.[3]

TABLE II: Equilibrium Constants for the Complexes of Phosphoglucomutase with Acceptors and Activators.[a]

Process	Equilibrium Constant	$\Delta G^{\circ\prime}$[b] (kcal/mol)
E_P·Glc-1-P ⇌ E_P·Glc-6-P	2.5	−0.5
E_P·Glc-6-P ⇌ E_P + Glc-6-P	57 μM	+8.3
E_P·Glc-1-P ⇌ E_P + Glc-1-P	8.4 μM	+9.4
E_P·Glc-6-P ⇌ E_D·Glc-P_2	1.6	−0.3
E_P·Glc-1-P ⇌ E_D·Glc-P_2	4.2	−0.9
E_P·Xyl ⇌ E_P + Xyl	1.6 M[c]	+2.1[c]
E_P·HPO_3^{2-} ⇌ E_P + HPO_3^{2-}	2 mM[c]	+6.1[c]
E_P·HPO_3^{2-}·Xyl ⇌ E_P·HOP_3^{2-} + Xyl	3.9 M[c]	+1.6[c]
E_D·Glc-P_2 ⇌ E_D + Glc-P_2	0.019 μM	+13.1
E_D·Glc-6-P ⇌ E_D + Glc-6-P	0.5 mM	+7.0
E_P·Xyl-1-P ⇌ E_P + Xyl-1-P	28 μM	+8.7
E_D·Xyl-1-P ⇌ E_D + Xyl-1-P	0.4 mM	+7.1

[a] At pH 7.5 and 24 °C. [b] Calculated from equilibrium constants expressed as ratios of *mole fractions* (see Discussion). [c] At 30 °C instead of 24 °C.

of Mg^{2+} less than 1 mM cannot be used in such a study because maintenance of the enzyme in its Mg^{2+} complex is required and Mg^{2+} binds relatively weakly to the dephospho-enzyme (Ray and Peck, 1972). However, the value observed at 1.5 mM Mg^{2+} was about twofold larger than the value obtained by an approximate extrapolation to saturating but noninhibiting Mg^{2+}, viz., $K_{I(Mg)} \cong 1.5$ mM. This value is intermediate between the value of about 1 mM estimated from similar experiments involving initial velocity measurements, and a value of about 2 mM suggested by direct binding studies (Ray and Roscelli, 1966). Thus, the true value of $K_{x(Glc\text{-}P_2)}$ is calculated as about 1×10^{-8} M; i.e., the true value is smaller by a factor of 0.3 (i.e., 0.5×0.6) than that observed at 0.5 mM glucose-P and 1 mM Mg^{2+}, 3.2×10^{-8} M.

As is indicated by eq 4 and 5, the true equilibrium constant for dissociation of a glucose phosphate from a given central complex can be calculated from the corresponding isotopic exhange constant and the relative amount of that complex present. The constants calculated in this manner for the three central complexes at pH 7.4 and 24 °C are listed in Table II.

Calculation of the Concentrations of Free Glucose Phosphates in Equilibrium Mixtures. The exchange constant, $K_{x(Glc\text{-}P)}$, in conjunction with initial concentrations of glucose-6-P and phsopho-enzyme, can be employed to calculate the concentration of free glucose phosphates (as well as the concentration of glucose phosphates bound to the enzyme) in the equilibrium mixtures used to assess the ratios of the central complexes present; see eq 2. In such calculations the total enzyme is taken as the sum of the free phospho-enzyme and the enzyme–substrate complexes, viz., a negligible amount of *free* dephospho-enzyme is assumed to be present. This follows from the value of $K_{Tr\text{-}6}^{f}$, below, even if none of the glucose-1-P were bound. The concentrations of *free* glucose-1-P and glucose-6-P calculated in this manner were 6 and 0.3% of the *total* glucose phosphates present.

The Chemical Potential of the Enzymic Phosphate Group: $(\Delta G_{Tr\text{-}6}^{\circ\prime})^f$. The Gibbs energy changes that accompany either transfer of the enzymic PO_3 group to the 6-position of *free* (as opposed to bound) glucose-1-P, $(\Delta G_{Tr\text{-}6}^{\circ\prime})^f$, eq 6, or the hydrolytic cleavage of this group from the enzyme, $\Delta G_{hyd}^{\circ\prime}(E_P)$, eq 7, can be used as a measure of the chemical potential of the enzymic phosphate group. (The corresponding equilibrium constants are $K_{Tr\text{-}6}^{f}$ and $K_{hyd}(E_P)$.)

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

$$E_P \rightleftharpoons E_D + P_i \quad (7)$$

However, for reasons noted below $(\Delta G_{Tr\text{-}6}^{\circ\prime})^f$ and $\Delta G_{hyd}^{\circ\prime}(E_P)$ will be considered separately.

As can be seen from eq 2 and 3, $K_{Tr\text{-}6}^{f}$ is equal to the ratio, $K_{x(Glc\text{-}P_2)}/K_{x(Glc\text{-}1\text{-}P)}$ (see also Peck et al., 1968). From the above values of these constants, $K_{Tr\text{-}6}^{f}$ is about 8.7×10^{-3} at 24 °C and pH 7.5.[3] This corresponds to a $(\Delta G_{Tr\text{-}6}^{\circ\prime})^f$ of about +2.8 kcal for transfer of the enzymic phosphate to the 6-position of free glucose-1-P.

By contrast, when the same transfer process involves *bound* reactants *and* products, i.e., involves two of the central complexes

$$E_P \cdot \text{Glc-1-P} \rightleftharpoons E_D \cdot \text{Glc-}P_2 \quad (8)$$

the Gibbs energy change, $(\Delta G_{Tr\text{-}6}^{\circ\prime})^b$ is about −0.9 kcal (entry 5, Table II). Thus, binding interactions in the central complexes make the PO_3-transfer process involving bound reactants and products more favorable by about 3.7 kcal/mol than the PO_3 transfer involving free reactants and free products. Note that this nearly 4 kcal/mol difference is equal to the difference in $\Delta G^{\circ\prime}$ for binding of glucose-1,6-P_2 to E_D and that for binding of glucose-6-P to E_P (see Table II) since the ratio of the equilibrium constants for eq 8 (bound reactants and products) and eq 6 (free reactants and products) is $[E_D\text{·Glc-}P_2][E_P][\text{Glc-1-P}]/[E_P\text{·Glc-1-P}][E_D][\text{Glc-}P_2]$, which in turn is equal to $K_{d(Glc\text{-}1\text{-}P)}/K_{d(Glc\text{-}P_2)}$.

Most of the difference in $K_{Tr\text{-}6}^{f}$ and $K_{Tr\text{-}6}^{b}$ values may arise from the elimination of favorable noncovalent interactions between the enzymic PO_3 group and adjacent groups on the enzyme during the transfer step in eq 6, as opposed to the retention of these interactions during the transfer step in eq 8. In addition, an increase in noncovalent interactions involving the PO_3 group that is transferred apparently occurs during or as the result of the transfer step when bound reactants and products are involved, as is indicated in the Appendix.

A value similar to $(\Delta G_{Tr\text{-}6}^{\circ\prime})^b$ can be approximated for $(\Delta G_{Tr\text{-}4}^{\circ\prime})^b$ in an analogous PO_3 transfer process, eq 9, to the

[3] The value of 9×10^{-3} for $K_{Tr\text{-}6}^{f}$ is 2.5-fold larger than that calculated from the value of $K_{Tr\text{-}1}^{f}$ for E_P + Glc-6-P ⇌ E_D + Glc-P_2, which was reported in an earlier study at 30 °C and the same pH (Peck et al., 1968). In that study two different approaches were used: (a) evaluating $K_{Tr\text{-}1}^{f}$ from an equation that involved parameters which appear in the initial velocity equations for the forward and reverse reactions and (b) determining $K_{Tr\text{-}1}^{f}$ by measuring the equilibrium constant for E_P·Mg + Glc-6-S ⇌ E_D·Mg + Glc-1-P-S and applying a correction for the smaller charge on the mixed sulfate–phosphate ester than on glucose-1,6-P_2. Apparently, difficulties in measuring initial velocities in the reverse reaction (Ray and Roscelli, 1964b) led to an underestimate of the Michaelis constant of glucose-1,6-P_2 for this process,[2] and the value of $K_{Tr\text{-}1}^{f}$ calculated from this Michaelis constant, according to a, *no longer can be considered* accurate. However, we have no explanation for the difference between the present value of $K_{Tr\text{-}6}^{f}$ (9×10^{-3}) and that calculated on the basis of the glucose 6-sulfate equilibrium, 4×10^{-3}. The temperature difference of 6 °C might account for part of this difference, but it seems unlikely to account for all of it. Although we prefer the present value, the older value differs from it by an amount equal only to 0.5 kcal/mol, in terms of the Gibbs energy function, and the difference thus is not critical.

[4] The decreased chemical potential of the enzymic phosphate in the E_P·Glc-1-P complex relative to E_P also may be caused by the problem of "solvating" the serine hydroxyl group of the enzyme, as well as the 6-hydroxyl group of bound glucose-1-P, after removal of the enzymic PO_3 group.

4-position of an acceptor which lacks a glucose ring: 1,4-butanediol-P (C_4-diol-P).

$$E_P \cdot C_4\text{-diol-P} \rightleftharpoons E_D \cdot C_4\text{-diol-}P_2 \quad (9)$$

In making this approximation we assume that $(\Delta G_{Tr\text{-}4}{}^{\circ\prime})^f$ (free reactants and products) for 1,4-butanediol-P is the same as $(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^f$ for glucose-1-P, +2.8 kcal (see above), since the chemical potential of the 6-phosphate in glucose-1,6-P_2 should be similar to that of the "4-phosphate" in 1,4-butanediol-P_2. $(\Delta G_{Tr\text{-}4}{}^{\circ\prime})^b$, eq 9, thus is equal to $(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^f$ plus the difference in the Gibbs energy change for binding 1,4-butanediol-P_2 to E_D and for binding 1,4-butanediol-P to E_P (cf. the system involving glucose-1-P, above). The former value, −8 kcal/mol at pH 7.5 and 30 °C, was obtained from inhibition measurements conducted in a manner analogous to those at pH 8.5 described previously (Ray et al., 1973): $K_I = 1.6 \times 10^{-6}$ M; the latter value, −3.3 kcal/mol, was obtained from the Michaelis constant for 1,4-butanediol-P acting as a PO_3 acceptor: 4 mM (Ray et al., 1976). Hence, $(\Delta G_{Tr\text{-}4}{}^{\circ\prime})^b$, eq 9, is about −1.9 kcal/mol, or about 1 kcal/mol more favorable than when the acceptor is bound glucose-1-P. These relationships, which are shown in the Gibbs energy diagram, Figure 2a, suggest that, although the glucose ring contributes significantly to the binding process, the binding interactions that make PO_3 transfer to the bound acceptor energetically favorable ($(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^b < 0$), in the face of the energetically unfavorable nature of the analogous PO_3 transfer involving free reactants and products ($(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^f > 0$), do not depend primarily on the glucose ring.

Binding interactions also have an effect on the Glc-1-P ⇌ Glc-6-P equilibrium. The results in Table I show that the equilibrium constant for E_P·Glc-1-P ⇌ E_P·Glc-6-P is in the range of 2.5, which is somewhat smaller than that for α-Glc-1-P ⇌ α-Glc-6-P, about 7 (Lowry and Passonneau, 1969), and substantially smaller than that for α-Glc-1-P ⇌ (α + β)-Glc-6-P, 17.3 (Atkinson et al., 1961). Thus, whether the α and β isomers of glucose-6-P are bound equally, as tentatively suggested by Gadain et al. (1974), or whether the reactive α isomer predominates, binding interactions produce some change in the α-Glc-1-P ⇌ α-Glc-6-P equilibrium. An alternative statement of this observation is that α-Glc-1-P binds somewhat more tenaciously to the phospho-enzyme than does α-Glc-6-P (see Table II). However, binding interactions alter the Glc-1-P ⇌ Glc-6-P equilibrium to a much smaller extent than $K_{Tr\text{-}6}{}^f$ (eq 6).

The Chemical Potential of the Enzymic Phosphate Group: $\Delta G_{hyd}{}^{\circ\prime}$. A value of about −1 kcal for $\Delta G_{hyd}{}^{\circ\prime}(E_P)$ at pH 7.5 can be calculated from the above value of $(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^f$, + 2.8 kcal, and $\Delta G^{\circ\prime}$ for hydrolysis of the 6-phosphate group of glucose-1,6-P_2, −3.8 kcal (cf., Ray and Peck, 1972). A somewhat similar value, −1.9 kcal at pH 8.5, was reported previously for $\Delta G_{hyd}{}^{\circ\prime}(E_P)$ (Peck et al., 1968).

The chemical potential of the enzymic phosphate group subsequent to substrate binding, viz., $\Delta G_{hyd}{}^{\circ\prime}$($E_P$·Glc-1-P), eq 10,

$$E_P \cdot \text{Glc-1-P} \rightleftharpoons E_D \cdot \text{Glc-1-P} + P_i \quad (10)$$

can be evaluated as the sum of the $\Delta G^{\circ\prime}$ values for the following three processes (see Table II): approximately +1.4 kcal/mol.

$$E_P \rightleftharpoons E_D + P_i \quad (11)$$

$$E_P \cdot \text{Glc-1-P} \rightleftharpoons E_P + \text{Glc-1-P} \quad (12)$$

$$E_D + \text{Glc-1-P} \rightleftharpoons E_D \cdot \text{Glc-1-P} \quad (13)$$

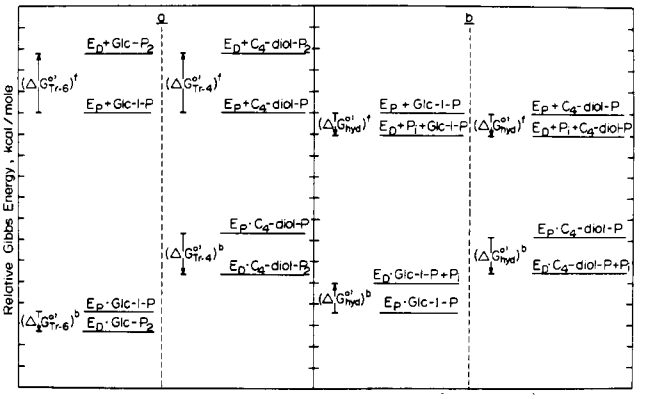


FIGURE 2: A Gibbs energy diagram showing the effect of binding glucose-1-P or 1,4-butanediol-P on (a) the energy change accompanying PO_3 transfer from phosphoglucomutase to these acceptors; and (b) the chemical potential of the enzymic phosphate group (see Results section for details). Gibbs energy changes were calculated from equilibrium constants expressed in terms of mole fractions (see Discussion).

Note, as in the previous section, that the effect of substrate binding on the chemical potential of the enzymic phosphate, $[\Delta G_{hyd}{}^{\circ\prime}(E_P\text{·Glc-1-P})] - [\Delta G_{hyd}{}^{\circ\prime}(E_P)]$, is simply the difference in $\Delta G^{\circ\prime}$ for binding of glucose-1-P to the product and reactant in the hydrolytic process E_P and E_D, respectively; this difference is about +2.4 kcal/mol.

$\Delta G^{\circ\prime}$(E_P·Glc-6-P) for hydrolysis of the enzymic phosphate in the analogous E_P·Glc-6-P complex is uncertain because of the lack of data on whether the α and β anomers of glucose-6-P are bound to the enzyme, and if both are bound, whether binding of the β anomer to E_P and E_D is the same (see above). However, these uncertainties should affect $\Delta G_{hyd}{}^{\circ\prime}$($E_P$·Glc-6-P) to only a small extent, and the value of +0.3 kcal/mol for $\Delta G_{hyd}{}^{\circ\prime}$($E_P$·Glc-6-P), obtained in a manner analogous to that used for $\Delta G_{hyd}{}^{\circ\prime}$($E_P$·Glc-1-P) (see above), is in the same range as the latter value, +1.4 kcal/mol. Thus, the chemical potential of the enzymic phosphate group changes in the presence of bound glucose phosphate in such a way that PO_3 transfer to water becomes less favorable—in fact thermodynamically unfavorable. The increased stability of the enzymic phosphate group in E_P·Glc-1-P relative to E_P apparently is caused by binding interactions involving the glucose ring since the binding of the straight-chain acceptor, 1,4-butanediol-P, does not change $\Delta G_{hyd}{}^{\circ\prime}(E_P)$ appreciably (see Figure 2b), although the energetics of the transfer process itself are similar for both bound glucose-1-P and bound 1,4-butanediol-P, viz., $(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^b \sim (\Delta G_{Tr\text{-}4}{}^{\circ\prime})^b$ (see Figure 2a). Possibly, structural changes in the enzyme that accompany glucose phosphate binding produce increased noncovalent interactions between the enzymic phosphate group and residues adjacent to it. In any case, since binding of either glucose-1-P or glucose-6-P produces changes in chemical potential that *favor* PO_3 transfer to the hydroxyl group of the bound substrate (see previous section) but *oppose* PO_3 transfer to the hydroxyl group of water, binding interactions involving glucose phosphate have *opposite* effects on $(\Delta G_{Tr\text{-}6}{}^{\circ\prime})^b$ and $\Delta G_{hyd}{}^{\circ\prime}(E_P)$.

The Binding of Substrate Analogues to the Phospho-Enzyme. The binding constants for PO_3 acceptors, and activators for PO_3 transfer to other acceptors, were taken as the reciprocals of the corresponding Michaelis constants since PO_3 transfer is slow in most of the reactions in question. (The accompanying paper describes the rates of these reactions (Ray et al., 1976).) Michaelis constants were obtained from double-reciprocal plots of transfer rate and acceptor or activator concentration. In some cases the reaction was followed directly,

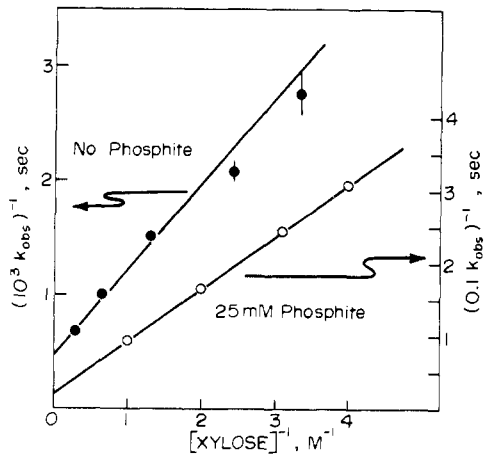


FIGURE 3: Double-reciprocal plots of the rate constant for PO_3 transfer from phosphoglucomutase to xylose in the presence and absence of inorganic phosphite. Data points, ● (xylose) and ○ (xylose + phosphite), represent the average of four and two determinations, respectively, at pH 7.5 and 30 °C. In the case of the xylose reaction, k values were obtained from plots of ln $(1 - f)$ vs. t, where f is the fraction of the PO_3 group transferred; such plots involved at least three points with f values in the range of 0.2 to 0.8. In the xylose plus phosphite reaction, product vs. time plots were linear and values of k were obtained from a single measurement. Error bars are indicated when the standard deviation exceeds the size of the data points. Assay details are described in the Experimental Section. Constants are listed in Table II.

via "single turnover" experiments, by measuring the increase with time in Cl_3CCOOH-soluble radioactivity when ^{32}P-labeled enzyme was treated with the acceptor or activator in question (Ray et al., 1976); in other cases a steady-state assay was employed. The binding constants obtained are listed in Table II; standard deviations were always less than ±20% and frequently were less than 10%. Data on product identification appear in the accompanying paper noted above. All reactions were conducted at pH 7.5; the first two reactions, below, were followed at 30 °C; the third reaction was conducted at 25 °C.

(a) The Xylose Reaction. The rate of PO_3 transfer from the phospho-enzyme to xylose to give xylose-1-P (Ray et al., 1976) was measured by means of single turnover assays. A double-reciprocal plot of rate and xylose concentration is shown in Figure 3 [(●): $k_{cat} = 2.1 \times 10^{-3}\ s^{-1}$; $K_m = 1.6$ M].

(b) The Xylose Reaction in the Presence of Bound Phosphite. The rate of PO_3 transfer in the E_P·Xyl·HPO_3 complex to give Xyl-1-P (Ray et al., 1976) was measured by means of the following reactions, which employed uniformly labeled Glc-P_2.

$$E_P + HPO_3^{2-} + Xyl \rightarrow E_D + HPO_3^{2-}\ Xyl\text{-}1\text{-}P$$

$$E_D + [^{32}P]Glc\text{-}P_2 \rightarrow [^{32}P]E_P + [^{32}P]Glc\text{-}P$$

$$[^{32}P]E_P + HPO_3^{2-} + Xyl \rightarrow E_D + HPO_3^{2-} + [^{32}P]Xyl\text{-}1\text{-}P$$

Since a saturating concentration of glucose-1,6-P_2 was employed—so that the second step was quite rapid throughout the assay—the first step was the slow step in the initial phase of the reaction. In the steady state, the analogous third step of the reaction was rate determining, and this was the step whose rate was assessed. (The labeled products of this step, glucose and xylose monophosphates, were readily separated from the unreacted bisphosphate by batch-wise chromatography on short columns of an anion-exchange resin (see Experimental Section).) The rate of formation of labeled monophosphates was proportional to enzyme concentration and was constant with time for conversions of at least 80%, provided that the glucose-P produced in the second step did not significantly compete with HPO_3^{2-} in binding to the phospho-enzyme. Concentrations of glucose-1,6-P_2 and HPO_3^{2-} of 0.03 and 25 mM, respectively, proved adequate in this respect. Double-reciprocal plots of reaction rate and xylose concentration are shown in Figure 3 (○). Under the conditions used (25 mM phosphite), the apparent values of k_{cat} and the Michaelis constant for xylose are 56 s^{-1} and 3.5 M, respectively; these become 60 s^{-1} and 3.9 M when extrapolated to saturating phosphite. The extrapolated value of k_{cat} thus is approximately 0.2 of that involving PO_3 transfer to the 1-position of glucose-6-P, under analogous conditions, about 320 s^{-1} (Ray and Peck, 1972). Similar values of k_{cat} and K_m for the xylose plus phosphite reaction also were obtained in a study that involved single turnover assays (employing $[^{32}P]E_P$); however, uncertainties in the transfer rate at high xylose concentrations were relatively large because of the rapid rate of reaction (data not shown).

(c) The Xylose 1-Phosphate Reaction. The rate of PO_3 transfer from the enzyme to water, mediated by xylose-1-P (Ray et al., 1976), was measured in an assay similar to that used by Lowry and Passonneau (1969) to study other PO_3 acceptors by (1) providing excess glucose-1,6-P_2 to re-convert the dephospho-enzyme, formed by reaction with the acceptor, to phospho-enzyme and (2) using excess $NADP^+$ and glucose-6-P dehydrogenase to oxidize the glucose-6-P thereby produced. The fluorescence of the NAPH concurrently formed was measured as a function of time (see Fluorescence Assay of Acceptor Acitivity): $k_{cat} = 3 \times 10^{-3}\ s^{-1}$; $K_m = 28\ \mu M$. Similar values were obtained in single turnover experiments conducted with purified xylose-1-P (data not shown).

Discussion

The phosphoglucomutase reaction is more complex than many other enzymic reactions in that two successive group-transfer steps are required to complete the overall process (Ray and Peck, 1972). The simplest formulation consistent with this requirement, plus the results of isotope tracer studies (Ray and Roscelli, 1964a), is as follows (note that the presence of bound Mg^{2+} is implied):[1,5]

$$E_P \cdot Glc\text{-}1\text{-}P \rightleftharpoons E_D \cdot Glc\text{-}P_2 \rightleftharpoons E_P \cdot Glc\text{-}6\text{-}P \qquad (14)$$

Although the E_D·Glc-P_2 complex usually is considered as an intermediate in the phosphoglucomutase reaction, there are several reasons for using an alternative designation in this study. Thus, in some analogue reactions, e.g., that involving the symmetrical PO_3 acceptor, $HO(CH_2)_4OPO_3^{2-}$, no comparable distinction is feasible, while in others, e.g., PO_3 transfer with water as an acceptor, a second PO_3 transfer, analogous to that in the normal reaction, does not occur. In addition, studies in an accompanying paper (Ray and Long, 1976) indicate structural similarities between the E_P·Glc-1-P and E_P·Glc-6-P complexes, but structural differences between these

[5] Recent evidence suggests the involvement of two different dephospho-enzyme–bisphosphate complexes (Ray et al., 1973). In one, the phosphate group in the 6-position of the bisphosphate is the reactive group and is correctly positioned for PO_3 transfer to the enzyme, while the 1-phosphate functions mainly in binding interactions; in the other, the positions of the two phosphates, and thus their roles, are reversed. A very rapid *internal* rearrangement interconverts these complexes; hence, a direct experimental distinction between them is difficult if not impossible. Since none of the data presented here or in the accompanying papers (Ray and Long, 1976b; Ray et al., 1976) bear on such a distinction, the results will be discussed in terms of a composite E_D·Glc-P_2 complex, as in eq 14.

complexes and E_D·Glc-P_2. Since these differences may reflect the structural differences between enzyme–substrate and enzyme–product complexes in simpler systems, in the present study, where the primary concern is with factors that affect the rate of a single PO_3-transfer step, E_P·Glc-1-P and E_P·Glc-6-P will be considered as alternative *enzyme–substrate* complexes and E·Glc-P will be used to designate their sum; correspondingly, E_D·Glc-P_2 will be considered as the *enzyme–product* complex.

The phosphoglucomutase system thus is one of the few enzymic systems in which the equilibrium between enzyme–substrate and enzyme–product complexes can be measured directly (see Results); hence, it is one of the few such systems in which a thermodynamic approach can be used to evaluate the applicability of some of the current ideas about the role of binding interactions in the catalytic reaction. The phosphoglucomutase reaction is particularly attractive for such studies because of the enormous difference in the reactivity of the phosphoenzyme toward two apparently similar chemical groupings: the hydroxyl group of water and the 6-hydroxyl group of glucose-1-P. That there was a substantial difference between the reactivity of these two entities was recognized by Koshland (1958) when he initially formulated the "Induced Fit" concept of enzyme specificity. However, only recently has the actual rate of the water reaction been measured so that the magnitude of the reactivity difference could be estimated: about 3×10^{10}-fold (Ray et al., 1976). It seems obvious that differences in the interaction of the phosphoglucosyl group and the water proton with the enzyme ultimately must be responsible for this huge substrate-induced rate effect, which is equivalent to a difference in Gibbs activation energies of some 14 kcal/mol. (In fact, a comparison of the rates of PO_3 transfer from the active site analogue, phosphoserine, and from the enzyme, itself, to water, and of the rate of PO_3 transfer from the enzyme to bound glucose-1-P (Ray et al., 1976), shows that binding of the phosphoglucosyl moiety to the enzyme actually produces an increase in the rate of PO_3 transfer that is many orders of magnitude larger than the increase produced by the (imaginary) process of incorporating phosphoserine into the framework of the enzyme.) This "substrate-induced rate effect" might be the result of (a) a structural change in the enzyme (Koshland, 1963), or (b) an enthalpic destabilization of reactant groups (Jencks, 1975), or (c) an immobilization of one such group relative to the other (Page and Jencks, 1971; Jencks, 1975), or (d) a progressive increase in binding interactions involving *nonreacting groups* as the system approaches the transition state (cf. Hammes, 1964). Moreover it is difficult to decide among these possibilities on an a priori basis, and the present study was initiated in an attempt to determine the relative importance of some of them in the present system.

Ground-State Destabilization. From a thermodynamic standpoint, two of the above possibilities, ground-state destabilization and transition-state stabilization, represent variations on a central theme. Hence, it seems reasonable to briefly suggest how the former might be identified. In simple systems, ground-state destabilization usually involves only the reactants. Hence, in such systems, changes that produce rate increases accompanied by similar increases in the overall equilibrium constant are likely to be caused by ground-state destabilization, whereas rate increases caused by transition-state stabilization should not substantially alter the substrate–product ratio at equilibrium. However, in enzymic reactions, binding interactions conceivably could produce ground-state destabilization of *both* reactants and products so that the overall equilibrium constant (involving bound reactants and products) is not altered. In such a case a destabilization mechanism becomes difficult to distinguish from one involving transition-state stabilization. However, as is indicated in the introductory section, an assessment of the change in chemical potential of a group bound to an enzyme when its reaction partner subsequently is bound might be used as an indication of the importance of a destabilization mechanism in an enzymic reaction, even if reactants and products are destabilized to the same extent on binding.

Destabilization mechanisms for enzymic reactions usually are conceptualized in terms of placing a group in an environment that increases its reactivity by increasing its enthalpy (cf. Jencks, 1975). Although changes in enthalpy with changes in environment are difficult to assess, experimentally, in many such situations the concomitant entropy change would be negative, so that the change in the Gibbs energy function for the grouping in question would set an upper limit for enthalpic destabilization.

Accordingly, the Gibbs energy change accompanying hydrolysis, $\Delta G_{hyd}°'$, of the active-site phosphate group of phosphoglucomutase was assessed in the presence and absence of bound glucose monophosphates (eq 10 and 11), viz., the change in $\Delta G_{hyd}°'$ produced by substrate binding was measured as an indication of whether or not the binding process destabilizes this phosphate group. The data in Table I indicate that $\Delta G_{hyd}°'$ becomes about 2.4 kcal/mol *less favorable* when glucose-1-P is bound and about 1.3 kcal/mol less favorable when glucose-6-P is bound. These changes are *opposite* to those expected for a destabilization mechanism. However, this does not mean that *no* destabilization of the enzymic phosphate occurs on substrate binding since other concurrent changes could mask a minor destabilization effect (cf., footnote 4). What the results do mean is that other mechanisms must account for *most* of the decrease in $\Delta G^{\ddagger}$ of some 14 kcal/mol that is required to explain the 3×10^{10}-fold substrate-induced rate effect. The lack of increased binding when the 6-hydroxymethyl group of glucose-1-P is removed (to give xylose-1-P) also supports the suggestion that destabilization of reactant groups is not the source of this rate effect (see Table I).

A comparison of thermodynamic changes that accompany PO_3 transfer to the flexible acceptor, 1,4-butanediol-P, with those for the corresponding reaction with glucose-1-P (see Table I) also are in accord with the above conclusion. Although PO_3 transfer to butanediol-P is much slower than to glucose-1-P (Ray et al., 1976), the equilibrium constant for the PO_3-transfer step involving bound reactants and bound products (eq 9) actually is larger than the corresponding constant for PO_3 transfer to glucose-1-P (eq 8). Because of the nonrigid linkage between the major binding group of butanediol-P (the phosphate group) and the acceptor hydroxyl group, this increase in equilibrium constant is difficult to rationalize in terms of a mechanism in which destabilization is used to accelerate PO_3 transfer to bound glucose-1-P.

The schematics in Figure 4a,b illustrate the basis for this conclusion. In Figure 4a, the equilibrium PO_3-transfer process involving 1,4-butanediol-P is separated into two steps: (a) the step described by K_1, in which the 4-hydroxyl group of the bound acceptor shifts from one or more low energy positions to the (presumed) high enthalpy position occupied by the 6-hydroxyl group of bound glucose-1-P, and (b) a PO_3-transfer equilibrium, described by K_2, that is analogous to the corresponding equilibrium, $K_{Tr\text{-}6}{}^b$, that involves bound glucose-1-P (Figure 4b). Since the measured equilibrium for the transfer process involving bound 1,4-butanediol-P, $K_{Tr\text{-}4}{}^b$, is equal to

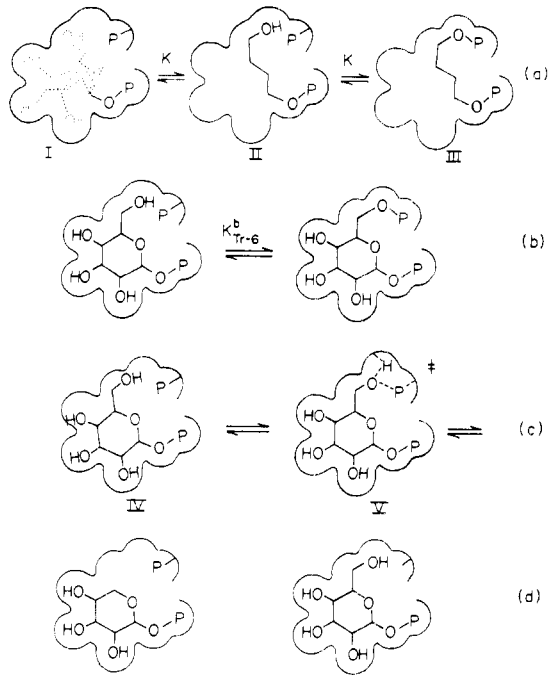


FIGURE 4: Schematic representations of possible binding interactions related to the PO_3-transfer process at the active site of phosphoglucomutase. (a) Possible sequence of events involving PO_3 transfer to 1,4-butanediol-P; I, the acceptor bound to the phospho-enzyme in such a way that its 4-hydroxyl group occupies one or more low-energy positions; II, a high-energy arrangement of the 4-hydroxyl group just prior to PO_3 transfer; III, the product of PO_3 transfer; (b) an analogous sequence involving glucose-1-P in which binding interactions produce enthalpic destabilization of the 6-hydroxyl group; (c) possible sequence for PO_3 transfer to glucose-1-P which shows how an increase in binding interactions could occur in going from the enzyme-substrate complex (IV) to the transition state (V); (d) the corresponding binding interactions for xylose-1-P and phospho-enzyme, and for glucose-1-P and dephospho-enzyme.

$K_2/[1 + (1/K_1)]$, and since K_1 must be very small if enthalpic destabilization of the acceptor hydroxyl group is quite large, $K_{Tr\text{-}4}{}^b$ should be much smaller than $K_{Tr\text{-}6}{}^b$. In fact, estimated values are $(\Delta G_{Tr\text{-}6}°)^b = -0.9$ and $(\Delta G_{Tr\text{-}4}°)^b = -1.9$ kcal/mol. Hence enthalpic destabilization of the acceptor hydroxyl group probably does not make a large contribution to the substrate-induced rate effect.

Ground-State Immobilization and the Chelate Effect. Recently, Page and Jencks (1971) and Jencks (1975) have reminded biochemists of the enormous entropic advantage that can be realized in gas-phase reactions by changing from a bimolecular to a unimolecular process—apart from enthalpic effects. They also have described model systems that give rise to large intramolecular effects in solution that cannot be enthalpic in origin and must be caused by immobilization of reactant groups in the ground state. Achieving large rate increases by means of an immobilization process in an enzymic system requires a large "intrinsic binding energy". (Intrinsic binding energy is the sum of (a) the observed binding energy, (b) the translational, rotational, and internal entropy changes that accompany the binding process, and (c) the Gibbs energy changes for all energetically unfavorable processes that are coupled with binding (Jencks and Page, 1972; Jencks, 1975), and thus would be equal to the binding energy in a hypothetical process in which b and c did not oppose binding.) Unfortunately intrinsic binding energy is difficult to evaluate experimentally, and it is correspondingly difficult to deduce either the extent to which catalysis in a given enzymic system depends on intrinsic binding energy or the extent to which immobilization of reactants groups is important.

However, as in the previous section where an evaluation of changes in the *Gibbs energy function* for reactant groups was used to place a limit on (ground state) *enthalpic effects*, so, changes in the Gibbs energy function for a reactant group during a binding process sometimes can be used as a minimal estimate for the extent of immobilization of that group in the bound reactant. Thus, evidence from changes in the Gibbs energy function that *favors* an enthalpic destabilization mechanism also could be interpreted as favoring a ground-state immobilization of reactant groups, instead. However, an unrealistically small estimate of the immobilization of a reactant group also can be obtained by such an approach. Thus, a group can be extensively immobilized *by means of* its enthalpic interactions with the enzyme so that its enthalpy is decreased (as opposed to what occurs during enthalpic destabilization) while its entropy is decreased. In such a case, the change in the Gibbs energy function for this group on binding would provide an unrealistically small measure of the concomitant immobilization. Hence, the *lack* of a large positive change in the Gibbs energy function for reactant groups during the binding process (see previous section) cannot be used to argue against possible ground-state immobilization of these groups in the phosphoglucomutase reaction.

An alternative approach to evaluating the extent of reactant immobilization during binding is to measure chelate effects associated with the binding step. For example, if two closely coupled functional groups of a substrate are extensively immobilized during binding, the binding energy for the substrate should be substantially greater (more negative) than if the two groups are bound in an identical manner in a separate experiment as parts of two different molecules; i.e., a large chelate effect for substrate binding should be observed. However, chelate effects are difficult to evaluate in enzymic systems. In fact, there are very few enzymic systems where an experimental comparison has been made between the separate binding of groups A and B, and the binding of A–B. Even when such comparisons can be made, it is possible that an anomalously small chelate effect will be observed because the binding of A–B is coupled with an energy-requiring structural change in the system that does not occur when either A or B are bound separately. However, in the phosphoglucomutase system a chelate effect can be measured under conditions where this possibility does not appear to be a problem (see below).

In order to minimize the possibility that the binding of A–B produces a structural change that is not elicited when the A and B groups are not covalently joined, it seems reasonable to compare the binding of A–B with the *sequential* binding of A and B. However, A and B together cannot be bound optimally at a site designed to maximize binding of A–B (Haldane, 1930; Jencks, 1969), although the simultaneous binding of A–H and H–B within a site designed to bind ACH_2CH_2B or ACH_2OB should be feasible, and this approach was used in the present system. Thus, molecular models indicate that xylose and inorganic phosphite can interact simultaneously with a rigid binding site designed for glucose-6-P (although glucose plus phosphite or xylose plus phosphate cannot; Ray et al., 1976). Moreover, *the rate of PO_3 transfer with xylose plus phosphite is close to that for glucose-6-P* (see Results). This similarity suggests that, *if energetically unfavorable structural changes related to catalysis are produced by binding of glucose-6-P, similar changes are produced by xylose plus phosphite.* Hence, if a large chelate effect fails to appear in a comparison of the enzyme–xylose–phosphite vs. enzyme–glucose-6-P

systems, it would be difficult to blame an energetically unfavorable change in the latter system for such a failure.

Although there are a variety of ways to express the magnitude of the chelate effect for a given process, all are arbitrary in that the size of the effect depends on the standard state that is used to make comparisons. We have chosen to use a standard state in which concentrations are expressed in terms of mole fractions and to assess the chelate effect in terms of the Gibbs binding energies calculated from equilibrium constants that are formulated in this manner. This is the most common convention, although Jencks (1975) has pointed out that it is neither more fundamental nor more informative than are alternative expressions. In the mole-fraction convention, the chelate effect is (a) minimal if the sum of the $\Delta G°$ values for the sequential binding of A–H and H–B is approximately equal to that for binding ACH_2OB; (b) moderate if $\Delta G°$ for ACH_2OB is about 6 kcal more negative than for binding A–H plus H–B; and (c) is in the neighborhood of the maximum theoretical value if the above difference is in the range of −11 to −14 kcal (Page and Jencks, 1971). On this basis, the chelate effect for binding glucose-6-P to phosphoglucomutase is *minimal* since $\Delta G°$ for its binding, −8.3 kcal, is similar to the sum of the $\Delta G°$ values for binding of phosphite and the *subsequent* binding of xylose, −7.7 kcal (see Table II). (A similar value also is obtained from constants describing the *separate* binding of xylose and phosphite.) Moreover, in terms of overall binding effects (cf. Jencks and Page, 1972; Jencks, 1975), attaching the $CH_2OPO_3^{2-}$ group of glucose-6-P to the 5-position of xylose changes $\Delta G°$ for the binding step by about −6.2 kcal (Table II) and $\Delta G^{\ddagger}$ for PO_3 transfer by about −7.2 kcal (Ray et al., 1976) for an overall effect of −13.4 kcal, whereas $\Delta G°$ for binding phosphite in the presence of xylose is about −5.6 kcal[6] and $\Delta G^{\ddagger}$ for PO_3 transfer is thereby reduced by about −6.2 kcal (Ray et al., 1976) for an overall effect of −11.8 kcal. Hence, the overall effect produced by the covalently bound $CH_2OP_3^{2-}$ group of glucose-6-P is only 1.6 kcal/mol more negative than that produced by inorganic phosphite acting as a separate molecule (in the presence of saturating xylose). In fact, this small difference actually might be attributed to noncovalent binding interactions involving the missing –CH_2–O bridge in the xylose plus phosphite system. However, in any case, the size of the chelate effect in the present system clearly does not even approximate its maximal possible value (cf. Page and Jencks, 1971).

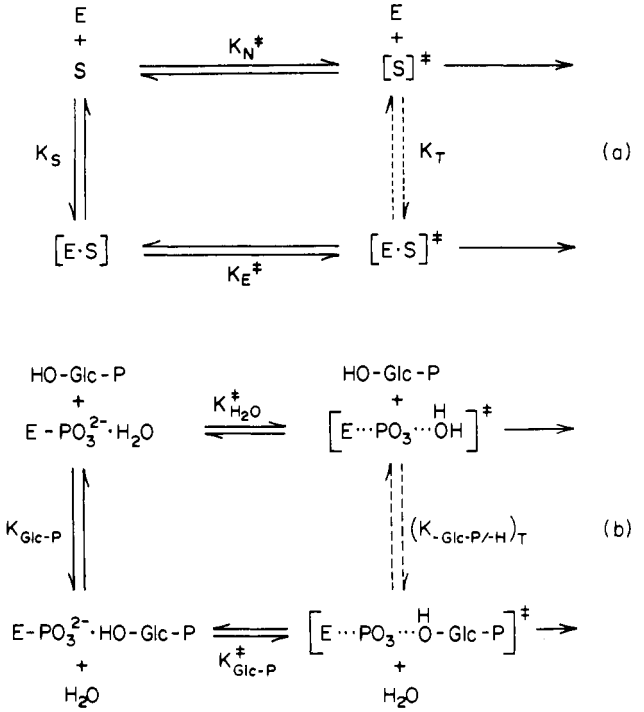
If a large chelate effect were associated with the binding of glucose-6-P, an immobilization of its component parts, relative to the enzymic binding site, would be indicated. However, the absence of a sizeable chelate effect is less informative. In fact, a variety of reasons can be offered to explain why chelate effects in simple systems are less than those calculated on the basis of the expected translational, rotational, and internal entropy changes especially for systems involving the solvent, water and it does not seem reasonable to review these here, since a unique rationale cannot be formulated to explain the small chelate effect in the present system. However, these results emphasize the difficulty of using theoretical models to assess the expected size of the chelate effect in other enzymic systems, or to infer that energetically unfavorable processes *must* accompany binding steps that fail to exhibit large chelate effects—in order to account for the "missing" chelate effect.

[6] Table II indicates that the binding energy of xylose becomes 0.5 kcal less favorable in the presence of bound phosphite; hence, the binding energy of phosphite becomes 0.5 kcal less favorable in the presence of bound xylose, if equilibrium binding is assumed.

Thus, although chelate effects substantially smaller than the maximum values can be easily rationalized, it is difficult to explain large disparities between observed and calculated chelate effects and maintain a binding model in which the bound groups are highly immobilized. Hence, because of the small chelate effect associated with glucose-6-P binding, it seems unlikely that either the glucose ring or the phosphate group of bound glucose-6-P is immobilized to a high degree, i.e., sufficiently to account for a majority of the 10^{10}-fold difference in PO_3-transfer rate involving water and glucose-6-P. In other words, if providing a "handle" to allow a high degree of immobilization of the 1-hydroxyl group were the primary function of the $CH_2OPO_3^{2-}$ group in glucose-6-P, it seems doubtful that either the binding energy or the reactivity of xylose plus phosphite would approach that of glucose-6-P, whereas both quantities actually are similar in the two systems. This does not mean that immobilization is not important in the phosphoglucomutase reaction; however, it does not appear to be overwhelmingly important.

Increased Binding Interactions in the Transition State. Although "increased binding interactions in the transition state" might at first seem to be synonymous with "transition-state binding" (Wolfenden, 1972; Lienhardt, 1973), the difference between the two can be deduced by means of the same thermodynamic box, Scheme 1a, that is used to define the transition state binding constant, K_T (Wolfenden, 1972;

SCHEME I. Thermodynamic "Boxes" for Comparing Ground-State and Transition-State Effects in (a) a General System, and in (b) the Phosphoglucomutase System.[a]



[a] Using the nomenclature of Jencks (1975), in a, $K_N^{\ddagger}$ and $K_E^{\ddagger}$ represent "equilibrium" constants for the processes leading to the transition states for the nonenzymic and enzymic reactions, respectively, K_S is the equilibrium constant for substrate binding, and K_T is the constant for a hypothetical equilibrium operation in which the nonenzymic transition state is bound/bonded to the free enzyme. In b, K_{Glc-P} and $K_{Glc-P}^{\ddagger}$ are analogous to K_S and $K_E^{\ddagger}$ in a, $K_{H_2O}^{\ddagger}$ is the equilibrium constant for the process leading to the transition state for transfer of the enzymic PO_3 group to water, and $(K_{-Glc-P/-H})_T$ is the constant for the hypothetical operation in which a proton in the transition state leading to the water reaction is replaced by the phosphoglucosyl group.

Jencks, 1975). From this scheme, the following relationship can be formulated:

$$-(\Delta G_T^\circ - \Delta G_S^\circ) = (\Delta G_N^{\ddagger} - \Delta G_E^{\ddagger}) \qquad (15)$$

The left-hand side of eq 15 can be equated with "increased transition-state binding" relative to ground-state binding, or simply "transition-state binding", while the right-hand side represents the sum of the energy contributions from all facets of the catalytic process which reduce $\Delta G_E^{\ddagger}$ relative to $\Delta G_N^{\ddagger}$, such as enthalpic destabilization, functional group catalysis, solvation effects, binding interactions of nonreacting groups, entropic effects, etc. Hence, in one sense catalysis is equivalent to (a set of particular) binding/bonding interactions and vice versa and a distinction between the two usually cannot be made.

However, the relative importance of *nonreacting groups* in chemical transformation will be different in enzymic and nonenzymic systems. If, to a first approximation, the role of *nonreacting groups* in *nonenzymatic systems* are assumed to be passive, eq 15 can be rewritten as

$$\Delta G_T^\circ - \Delta G_S^\circ = (\Delta G_E^{\ddagger} - \Delta G_N^{\ddagger})_R + (\Delta G_E^{\ddagger})_{NR} \qquad (16)$$

where the subscripts R and NR designate reacting and nonreacting parts of the substrate, viz., nuclei of the substrate whose *covalent* bonding *is* substantially altered in the transition state, as opposed to nuclei whose covalent bonding *is not* substantially altered. Since $(\Delta G_E^{\ddagger})_{NR}$ refers to the sum of the changes in the *true binding* interactions of all nonreacting groups as the enzymic system proceeds from the enzyme-substrate complex to the transition state, clearly $(\Delta G_E^{\ddagger})_{NR}$ is not the same as "transition-state binding", $(\Delta G_T^\circ - \Delta G_S^\circ)$. Moreover, while "transition-state binding" is more or less equivalent to "catalysis" and cannot be subjected to experimental verification i.e., cannot be ruled out, increased binding interactions (of nonreacting groups) in the transition state represents a bona fide catalytic mechanism that may or may not be part of the catalytic process and that at least in some cases is subject to experimental testing, i.e., can be verified or ruled out. In order to simplify discussion of the latter mechanism, "increased binding interactions in the transition-state" *will refer only to nonreacting groups;* $(\Delta G_E^{\ddagger} - \Delta G_N^{\ddagger})_R$ will be referred to as binding/bonding interaction (of reactant groups). Increased binding interactions in the transition state can facilitate an enzymic reaction only if (a) optimal binding interactions between the enzyme and a nonreacting part of the substrate molecule are *precluded* in the enzyme-substrate complex, and (b) increased binding is *coupled* with the bond making or breaking process in the reacting part of the molecule in such a way that only progress toward the transition state will optimize the binding interactions in question. Increased binding interactions in the transition state perhaps can best be visualized in terms of a continuous compensation process, of a type originally proposed by Hammes (1964), in which the energy for distorting chemical bonds in one part of the enzyme-substrate complex is partially compensated by increased binding interactions in another part.

In the phosphoglucomutase system the nonreacting group is the phosphoglucosyl moiety of glucose-1-P. In fact, in the final analysis, this group must provide sufficient binding interactions to account for the 14-kcal difference in the Gibbs activation energy for PO_3 transfer to water and to the hydroxyl group of bound glucose-1-P, as can be verified by reference to the thermodynamic "box" in Scheme 1b. The processes along the left-hand side and bottom of this box are analogous to the corresponding processes in Scheme 1a; however, the top of the box represents the "equilibrium" process leading to the transition state for the enzymic PO_3 transfer with water as an acceptor, while the right-hand side represents a hypothetical process in which a proton in the transition state leading to the water reaction is replaced by the phosphoglucosyl moiety, in an operation analogous to that involved in binding/bonding a transition state to an enzyme, except that in the present operation no new interactions with covalent character are produced. Since the bonds that are made and broken are similar for both the upper and lower processes, since the sum of the Gibbs energy changes in a cyclic process is zero, and since the difference in Gibbs activation energies for PO_3 transfer from the enzyme to water and to bound glucose-P is some 14 kcal/mol, the latter energy difference must be associated with *binding* interactions of *nonreacting groups* that appear either in the substrate binding step (left-hand step) or in the approach to the transition state (lower step), or both. To the extent that these binding interactions develop *during* approach to the transition state from the enzyme-substrate complex, the substrate-induced rate effect would arise from "increased binding interactions in the transition state".

In order to evaluate the possibility of increased binding interactions in the transition state in the phosphoglucomutase reaction, an attempt was made to uncouple the substrate-binding step from the structural restraints that must prevent optimal binding interactions in the enzyme-substrate complex if this mechanism is to operate. The most logical such restraint involves the 6-hydroxymethyl group of glucose-1-P. The possibility that unfavorable steric interactions between this group and the enzymic PO_3 group in the enzyme-substrate complex would prevent optimal binding of the phosphoxylosyl portion of glucose-1-P, but allow these interactions to be optimized in the transition state is illustrated, schematically, in Figure 4c. In such a case, removal of the 6-hydroxymethyl group or the enzymic PO_3 group (Figure 4d), or both, should allow binding interactions between the rest of the enzyme and the sugar phosphate to be optimized in the respective binding steps, and thus lead to a greatly enhanced binding relative to glucose-1-P and the phospho-enzyme. However, none of the above operations produced increased binding, and in fact, all produced small decreases (see Table II).

The alternative, that conformational changes in the enzyme, itself, that are controlled by the character of the bonding at the enzymic PO_3 group, might serve as the required restraint to binding in the enzyme-substrate complex, that is subsequently relaxed in the transition state, seems remote—especially since the complete removal of this group produces no increase in ground-state binding effect (see above). "Increased binding interactions in the transition state" thus does not appear to provide a rationale for the substrate-induced rate effect in the phosphoglucomutase system. Note, however, that this is not equivalent to the statement that (the all-inclusive) transition-state binding does not provide a rationale for this effect; in fact the latter statement is incorrect.

Summary and Conclusions

Many of the classical reaction mechanisms in physical organic chemistry that serve as the basis for our understanding of enzymic mechanisms—rudimentary as that understanding is at present—were worked out by systematically eliminating all but one of the reasonable mechanistic alternatives for a particular reaction. Such an approach has not been used frequently in studies of enzymic reactions, partly because a consideration of so many different alternatives would be required, and partly because experiments to exclude a given possibility

are difficult to design. In the present study a process of elimination was attempted partly because the basic problem was to provide a rationale for the *rate difference* for two closely related reactions, i.e., for the substrate-induced rate effect. By contrast, a comprehensive rationale of catalysis in the phosphoglucomutase system must also include an explanation for the difference in the rate at which the simple, active site analogue, phosphoserine, transfers its PO_3 group to water and the rate of PO_3 transfer from the phospho-enzyme to water, and will be considerably more difficult to formulate than the rationale sought in this study. An additional reason for attempting to use a process of elimination was that the difference in rates of the reactions under consideration was sufficiently large that factors which produced variations in rate of up to several fold were small enough, by comparison, to be ignored, at least to a first approximation.

Even though the substrate-induced rate effect in the phosphoglucomutase system is unusually large, there are three different mechanisms, each of which, *by itself,* could provide a rationale for most of this effect and all of which ultimately depend on binding interactions between the enzyme and the substrate. Two of these can be considered as ground-state effects: enthalpic destabilization and immobilization of reactant groups in the enzyme–substrate complex; the third involves a transition state effect—increased binding interactions of *nonreacting groups* in the transition state. The primary objective of this paper was to determine whether any one of these possibilities is either overwhelmingly important or accounts for a majority of the substrate-induced rate effect—and in all three cases the conclusion is negative, although the overall effect probably involves contributions from all three factors.

Thus, the failure to observe the type of binding pattern that would be expected if an increase in binding interactions occurred in the transition state must be interpreted in light of the probability that the energy change accompanying the replacement of one hydrogen of a water molecule by the phosphoglucosyl group will not be the same in the ground state and the transition state, since the other H–O bond probably is weakened (by stretching) in the transition state (Ray et al., 1976). Because this possibility cannot be examined by means of the approach used here and because of the many possible compensating effects that can occur during binding processes in enzymic systems, it would be unrealistic to conclude from the present results that transition-state effects are not responsible for part of the substrate-induced rate effect, although it does seem probable that ground-state effects are more important. In such a case, the present system provides a marked contrast with that of lysozyme, where it appears that binding interactions at subsites D and E are either entirely absent or nearly so in the ground-state enzyme–substrate complex, and develop only as the system approaches the transition state (Holler et al., 1975).

The failure to detect any evidence of ground-state enthalpic destabilization of reactant groups also should not be interpreted as ruling out the possibility that such a factor contributes to the substrate-induced rate effect. In addition, the possibility that substrate-binding produces an enthalpic destabilization of catalytic groups in the enzyme was not tested.

Of the three factors that might produce very large rate effects in enzymic processes, immobilization of reactant groups in the ground state is the most difficult to verify. Even the procedure used here is not likely to be generally applicable since the size of the expected chelate effect for a given substrate-binding step is correspondingly difficult to evaluate (Jencks, 1975). Only in extreme cases, where either a relatively modest degree or a very high degree of reactant-group immobilization is involved, is the evaluation of chelate effects likely to offer some insight into the extent of reactant-group immobilization in the enzyme–substrate complex.

Although the lack of a large chelate effect in the substrate-binding step indicates that immobilization of the bound glucose ring and thus of the attacking hydroxyl group in the enzyme–substrate complex does not account for a majority of the substrate-induced rate effect in the present system, it should be pointed that one of the reactant groups, the enzymic phosphate, is relatively immobile with respect to the catalyst (the rest of the enzyme) *prior* to substrate binding. (The lack of independent motion of the enzymic PO_3 group is indicated by the large T_1/T_2 ratio of about 200 for the relaxation processes involving the ^{31}P nucleus of the phospho-enzyme: W. J. Ray, Jr., A. S. Mildvan, J. B. Grutzner, manuscript in preparation.) Presumably this immobilization, relative to the rest of the enzyme, is produced by the same interactions that account for the increased thermodynamic stability of the enzymic phosphate group relative to that of serine phosphate (see Results).

Although it may not be surprising that no single mechanism for utilizing substrate binding energy to produce rate effects adequately accounts for the substrate-induced rate effect in the present system, the present demonstration makes the alternative approach to rationalizing the substrate-induced rate effect in the accompanying paper (Ray et al., 1976) seem much more palatable viz., the lack of a single large factor requires that the overall effect be rationalized in terms of several more modest factors.

Acknowledgment

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Appendix

The changes in the Gibbs energy function calculated from the equilibrium constants for the following processes allow an assessment of the effect of noncovalent interactions on the chemical potential of the enzymic phosphate group of phosphoglucomutase (in the presence of bound Mg^{2+}).

	$\Delta G^{\circ\prime}$ (kcal/mol)	
Ser-P + Glc-1-P $\rightleftharpoons$ Ser + Glc-P_2	+0.5	(A1)
E_P + Glc-1-P $\rightleftharpoons$ E_D + Glc-P_2	+2.8	(A2)
E_P · Glc-1-P $\rightleftharpoons$ E_D · Glc-P_2	−0.9	(A3)
(Ser-P · Glc-1-P $\rightleftharpoons$ Ser · Glc-P_2)	(~0)	(A4)

$\Delta G^{\circ\prime}$ for process A1 can be evaluated from data summarized by Ray and Peck (1972) and is positive primarily because of unfavorable electrostatic effects. Since the same *covalent* bonds are involved, the more unfavorable value of $\Delta G^{\circ\prime}$ for process A2 demonstrates the existence of noncovalent interactions in E_P that oppose PO_3 transfer, i.e., reduce the chemical potential of the enzymic phosphate. Presumable these involve the PO_3 group and adjacent side chains of the enzyme (see Discussion). The less unfavorable value of $\Delta G^{\circ\prime}$ for process A3 shows that noncovalent interactions in E_P-Glc-1-P must increase in importance during or as the result of PO_3 transfer involving bound reactants and products. The extent to which

noncovalent interactions increase during this process may be approximated as about −1 kcal/mol, by comparison with the *hypothetical* transfer in process A4, where electrostatic effects have been eliminated by appropriately positioning the PO_3^{2-} group *prior* to transfer and moving it an inappreciable distance during transfer (as is presumably the case in process A3). A somewhat larger value of about 3 kcal/mol can be calculated for the increase in noncovalent interactions during the analogous process involving E_P·Glc-6-P and E_D·Glc-P_2. (The same changes in noncovalent interactions are obtained by comparing values of $\Delta G°'$ for hydrolysis of Ser-P, E_P, E_P·Glc-1-P, and (Ser-P·Glc-1-P): see parameters in the Results and the review by Ray and Peck (1972).)

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An Analysis of the Substrate-Induced Rate Effect in the Phosphoglucomutase System†

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ABSTRACT: The rate constant for the catalytic transfer of the active-site PO_3 group from rabbit muscle phosphoglucomutase to the hydroxyl group of a water molecule is about 3×10^{-8} s^{-1} under optimal reaction conditions, but in the absence of the normal substrate, viz., at pH 7.5 and 30 °C, in the presence of saturating Mg^{2+}; the corresponding constant for transfer to the 6-hydroxyl group of glucose 1-phosphate under analogous conditions, about 1000 s^{-1}, is larger than this by some 3×10^{10}-fold. Since no single factor appears to be capable of providing a rationale for a majority of this "substrate-induced rate effect" (Ray, Jr., W. J., and Long, J. W. (1976), *Biochemistry,* the preceding paper in this issue), the change in the PO_3-transfer rate produced by binding various parts of the phosphoglucosyl moiety to the enzyme, both separately and concurrently, was investigated. The rate of PO_3 transfer to water is increased by up to 1000-fold by binding entities that provide the active site with a second PO_3 group, e.g., ethyl phosphate or inorganic phosphite. Using an alcoholic acceptor further increases transfer efficiency (in the presence of bound phosphite): increase with methanol, about 2000-fold on a molar basis. The reactivities of ten other primary aliphatic alcohols vary by nearly 600-fold as the acidity of the PO_3 acceptor is varied over a 4000-fold range. Although no straightforward relationship is observed between the efficiency of an alcohol as an acceptor and its acidity—presumably because of complications due to steric effects, for example—an increased

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