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Time-Dependent ^{31}P Saturation Transfer in the Phosphoglucomutase Reaction. Characterization of the Spin System for the Cd(II) Enzyme and Evaluation of Rate Constants for the Transfer Process†

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Received April 29, 1988; Revised Manuscript Received August 16, 1988

ABSTRACT: Time-dependent ^{31}P saturation-transfer studies were conducted with the Cd^{2+}-activated form of muscle phosphoglucomutase to probe the origin of the 100-fold difference between its catalytic efficiency (in terms of k_{cat}) and that of the more efficient Mg^{2+}-activated enzyme. The present paper describes the equilibrium mixture of phosphoglucomutase and its substrate/product pair when the concentration of the Cd^{2+} enzyme approaches that of the substrate and how the nine-spin ^{31}P NMR system provided by this mixture was treated. It shows that the presence of abortive complexes is not a significant factor in the reduced activity of the Cd^{2+} enzyme since the complex of the dephosphoenzyme and glucose 1,6-bisphosphate, which accounts for a large majority of the enzyme present at equilibrium, is catalytically competent. It also shows that rate constants for saturation transfer obtained at three different ratios of enzyme to free substrate are mutually compatible. These constants, which were measured at chemical equilibrium, can be used to provide a quantitative kinetic rationale for the reduced *steady-state* activity elicited by Cd^{2+} relative to Mg^{2+} [cf. Ray, W. J., Post, C. B., & Puvathingal, J. M. (1989) *Biochemistry* (following paper in this issue)]. They also provide minimal estimates of 350 and 150 s^{-1} for the rate constants describing (PO_3^-) transfer from the Cd^{2+} phosphoenzyme to the 6-position of bound glucose 1-phosphate and to the 1-position of bound glucose 6-phosphate, respectively. These minimal estimates are compared with analogous estimates for the Mg^{2+} and Li^+ forms of the enzyme in the accompanying paper.

Phosphoglucomutase is one of a group of enzymes that conducts multiple and distinctly different bond-breaking/bond-making operations on a bound substrate before releasing its product. Thus, in the thermodynamically favorable direction (Glc-1-P → Glc-6-P),[1] the enzyme transfers its active-site (PO_3^-) group to the 6-hydroxyl group of Glc-1-P to produce bound Glc-1,6-P_2 (i.e., Glc-P_2); in a second subsequent step the enzyme accepts the 1-(PO_3^-) group from the still bound bisphosphate, thereby producing Glc-6-P and regenerating the phosphoenzyme (Ray & Peck, 1972):

$$E_P\cdot\text{Glc-1-P} \rightleftharpoons E_D\cdot\text{Glc-}P_2 \rightleftharpoons E_P\cdot\text{Glc-6-P}$$

(E_P and E_D are the phospho and dephospho forms of the enzyme, respectively.)

Efficient catalysis by muscle phosphoglucomutase requires a bound metal ion activator. Although Mg^{2+} is the most efficient activator as well as the activator that is important physiologically, a number of bivalent metal ions produce varying degrees of activation (Ray, 1969). One approach to evaluating the role of the metal ion in the bond-breaking/bond-making process is to study the origin of metal-specific differences in enzyme activation. This approach is particularly attractive for the phosphoglucomutase reaction since the equilibrium binding of the glucose monophosphates, viz., the substrate/product pair, is essentially independent of metal ion identity (Ray & Long, 1976). Hence, studies of metal-specific differences can focus on effects that the metal ion produces on the subsequent bond-breaking/bond-making process.[2]

At equilibrium in the enzymic reaction involving Mg^{2+}, the intermediate $E_D\cdot Mg\cdot$Glc-P_2 complex accounts for about half of the total enzyme, while the monophosphate complexes,

† This work was supported in part by a research grant from the U.S. Public Health Services (GM08963, to W.J.R.), by grants from the Biotechnology Resources Program of the Division of Research Resources, National Institutes of Health (RR01077, to the Purdue University Biochemical Magnetic Resources Laboratory), and by grants from the NSF Biological Instrumentation Division (8714258 and BMS8614177, to the Purdue Biological Facilities Center on Biomolecular NMR, Structure and Design).
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[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, α-D-glucose 6-phosphate, unless the β-anomer is specified; Glc-P_2 or Glc-1,6-P_2, α-D-glucose 1,6-bisphosphate; E_P and E_D, the phospho and dephospho forms of rabbit muscle phosphoglucomutase; M, a bivalent metal ion activator (of phosphoglucomutase).

[2] The insensitivity of glucose monophosphate binding to metal ion identity finds a reasonable rationale in observations which indicate that the bound bivalent metal ion interacts directly with the enzymic phosphate group (Rhyu, 1984). The metal ion thus is unlikely to act as a bridge between the enzyme and glucose monophosphate, where a metal specific effect on binding would be likely.

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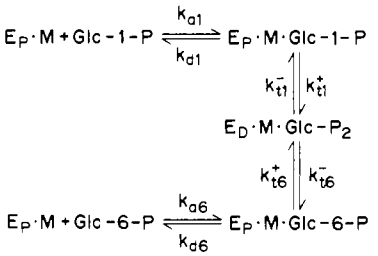
Scheme I: Phosphoglucomutase Reaction, Showing All Identified Intermediates and the Rate Constants for Their Interconversion



E_P·Mg·Glc-1-P and E_P·Mg·Glc-6-P, account for the remainder. However, with Cd^{2+} as the bound metal ion, about 90% of the equilibrium mixture is E_D·Cd·Glc-P_2. Although the predominance of the bisphosphate intermediate would make the Cd^{2+} enzyme more amenable to the study of the (PO_3^-)-transfer steps that produce this complex, the Cd^{2+} enzyme is only about 1% as active as the Mg^{2+} enzyme in initial velocity studies at saturating Glc-1-P (Ray, 1969; Ray & Long, 1976). Since Cd^{2+} is significantly larger than Mg^{2+} and exhibits a different coordination preference, its interaction with the bound bisphosphate may differ, qualitatively as well as quantitatively, from that of Mg^{2+}. Thus, catalysis by the Cd^{2+}-activated enzyme might be slower because the Gibbs energy profile of the reaction is altered, because a significant fraction of the enzyme is present as an inactive complex, or both.

We have examined Cd^{2+} activation of phosphoglucomutase by measuring (PO_3^-)-transfer rates via time-dependent ^{31}P NMR saturation transfer. Under Modus Operandi we describe the sequence of steps in the overall reaction as deduced from previous studies and the approximations used to simplify analysis of magnetization exchange in this system. Results show first that the intensities of the resonances in ^{31}P NMR spectra account for essentially all of the enzyme in the system. Next, saturation-transfer studies are described that provide assignments for ^{31}P resonances and validate the catalytic competence of the enzymic complex that predominates at chemical equilibrium. The procedure used for obtaining initial and later refined estimates of rate constants, which involved comparison of the observed time dependence of magnetization with that expected from computer simulation of the Bloch equations with variable rate constants, is outlined. The validity of these estimates is evaluated in the accompanying paper (Ray et al., 1989) by comparisons with both calculated and measured rates of ^{32}P isotope exchange and initial velocity. [A redetermination of values for the α,β-anomeric equilibrium constant of Glc-6-P and the overall equilibrium constant of the phosphoglucomutase reaction, which are required for the above analysis, also is reported (see Appendix 1).]

Modus Operandi

The reaction of muscle phosphoglucomutase is shown in Scheme I together with all intermediates that have been identified in previous studies [see Ray and Peck (1972)]. In a complex spin system such as that provided by the (PO_3^-) groups of this reaction, analysis of magnetization transfer is most easily conducted by computer simulation of the relevant Bloch equations (Forsén & Hoffmann, 1963; McConnell, 1958). Our analysis involves computing the expected time-dependent changes in spectral intensities for all resonances after selectively perturbing (saturating) one. Initial estimates for all rate constants, for T_1 relaxation times, and for equilibrium concentrations of all intermediates are required to conduct the simulation. These estimates are modified after comparison of the calculated and observed values for the magnetization of each species as a function of irradiation time. The procedure used in the computer modeling is described in Appendix 2. The remainder of this section outlines the approximations used in describing magnetization transfer via chemical exchange for the process in Scheme I.

The E_P·Cd·Glc-1-P and E_P·Cd·Glc-6-P complexes in Scheme I need not be included in a simulation model because their equilibrium concentrations are known to be small relative to other species (see Results). Thus, in a three-component equilibrium

$$X \underset{k_{-x}}{\overset{k_x}{\rightleftharpoons}} Y \underset{k_{-y}}{\overset{k_y}{\rightleftharpoons}} Z \tag{1}$$

when both $[Y_e]/[X_e]$ and $[Y_e]/[Z_e]$ are less than about 0.1, magnetization exchange from X to Z after saturating spin Z,[3] or from Z to X after saturating spin X, can be represented as

$$X \underset{k_r^{app}}{\overset{k_f^{app}}{\rightleftharpoons}} Z \tag{2}$$

and the time dependence of the exchange is approximated by single exponentials, $\exp[-(k_f^{app} + T_{1,x}^{-1})t]$ and $\exp[-(k_r^{app} + T_{1,z}^{-1})t]$. Hence, where appropriate, X ⇌ Y ⇌ Z segments of Scheme I were treated as X ⇌ Z segments (e.g., the top half of Scheme I becomes E_P·M + Glc-1-P ⇌ E_D·M·Glc-P_2).

After values were obtained for the apparent rate constants in the forward and reversed directions for steps analogous to those in eq 1, minimal estimates for the microscopic constants were calculated from the following inequalities by assuming steady-state conditions for Y, since under these conditions k_f^{app} and k_r^{app} are net rate constants (Cleland, 1975). Note that the expressions for k_f^{app} and k_r^{app} can be factored in two different ways:

$$k_f^{app} = \frac{k_x k_y}{k_{-x} + k_y} \leq k_x \tag{3}$$

$$\leq k_y[Y_e]/[X_e] \tag{4}$$

$$k_r^{app} = \frac{k_{-y} k_{-x}}{k_{-x} + k_y} \leq k_{-y} \tag{5}$$

$$\leq k_{-x}[Y_e]/[Z_e] \tag{6}$$

Although not immediately apparent from Scheme I, in the phosphoglucomutase reaction exchange of magnetization between the (PO_3^-) groups of substrate (Glc-1-P) and product (Glc-6-P) pools requires *two* complete catalytic cycles.[4] This feature of the reaction is shown by the sequence of steps in eq 7 and 8, where the intermediate E_P·Cd·Glc-1-P and E_P·

cycle 1: Glc-1-P* + (E_P·Cd) ⇌ E_D·Cd·P-6-Glc-1-P* ⇌ (E_P*·Cd) + Glc-6-P (7)

cycle 2: (E_P*·Cd) + Glc-1-P ⇌ E_D·Cd·P*-6-Glc-1-P ⇌ Glc-6-P* + (E_P·Cd) (8)

Cd·Glc-6-P complexes are omitted and an asterisk is used to indicate the nucleus involved in magnetization exchange.

[3] The loss of intensity at one site upon saturation of the resonance of a second site, with which the first is in chemical exchange, arises from a flow or drain of magnetization *from* the first site, rather than a transfer of spin saturation *into* the nonirradiated site (Forsén & Hoffman, 1963). However, the more common term, saturation transfer, is herein used interchangeably with magnetization exchange.

[4] The analogous transfer involving the glucose moiety would require only a single cycle, since there is no glucosyl-enzyme intermediate. Hence, one glucose moiety must be transferred from substrate to product pools in each catalytic cycle.

Table I: Matrix Showing Rate Constants for Simulation of ^{31}P Magnetization Exchange Catalyzed by Cd(II) Phosphoglucomutase[a]

	E_P·Cd	Glc-1-P	E_D·Cd·P*-1-Glc-6-P	E_D·Cd·P-1-Glc-6-P*	Glc-6-P
E_P·Cd	nd	–	k_6^{app}[Glc-6-P]	k_1^{app}[Glc-1-P]	–
Glc-1-P	–	nd	k_1^{app}[E_P·Cd]	–	–
E_D·Cd·P*-1-Glc-6-P	k_{-6}^{app}	k_{-1}^{app}	nd	–	–
E_D·Cd·P-1-Glc-6-P*	k_{-1}^{app}	–	–	nd	k_{-6}^{app}
α-Glc-6-P	–	–	–	k_6^{app}[E_P·Cd]	nd

[a] Rate constants, k_{ij} (see eq 18 in Appendix 2), for transfer from site i to site j are shown as they appear in eq 11, which also shows the representation of the exchange pathway analyzed here. Species i and j are identified in the left-hand column and the top row, respectively. Diagonal elements ($i = j$) are indicated by nd; k_{ij} values not allowed by the reaction sequence in eq 11 are indicated by –. Rate constants refer to phosphates indicated by *.

Species not observed by ^{31}P NMR because of the low concentration present are enclosed in parentheses.

The rate constants for the steps that describe the magnetization-exchange processes in eq 7 and 8 are given in matrix form in Table I. The identities of the constants in this table (see eq 11) are such that values of two rate constants, k_1^{app}[Ep·Cd] ($\equiv k_1^{obs}$) and k_6^{app}[E_P·Cd] ($\equiv k_6^{obs}$), plus values of two equilibrium constants, [Glc-1-P]/[E_D·Cd·Glc-P_2] ($= k_{-1}^{app}/k_1^{obs}$) and [α-Glc-6-P]/[E_D·Cd·Glc-P_2] ($= k_{-6}^{app}/k_6^{obs}$), together with the known concentrations of Glc-1-P and α-Glc-6-P (see Appendix 1) suffice to define the exchange process. Values for k_1^{obs} and k_6^{obs} can be measured directly in a simple exponential decay process since the loss of magnetization from the free monophosphates upon irradiation of the corresponding phosphate in E_D·Cd·Glc-P_2 is due to an isolated exchange event.

Experimental Procedures

Materials. The phospho form of phosphoglucomutase was isolated from rabbit muscle (Ray et al., 1983) and stored at 4 °C as the Mg^{2+} form in 30% ammonium sulfate that contained "crystallization buffer" (Ray, 1986). Prior to use, the enzyme was concentrated (to about 200 mg/mL) and demetalated (Rhyu et al., 1984) at 4 °C in 40 mM Bistris-HCl buffer, pH 7.2 (at room temperature), or 20 mM Tris-HCl, pH 7.4 (at room temperature). Enzyme concentrations were determined spectrophotometrically by using $E_{278} = 0.70$ and M_r 61 600 (Ray et al., 1983). Specific activities were approximately 900 units/mg (at 30 °C); less than 2% of the enzyme was in the dephospho form. The Cd^{2+} complex of the enzyme was formed immediately prior to use by adding a freshly mixed solution of $CdCl_2$ and Tris base; final pH of the mixture was 7.2. Glc-1-P and Glc-6-P were obtained from Sigma and demetalated by passing through a Chelex column equilibrated to pH 7.2 at the same ionic strength as the solutions to be demetalated; before use, deuterium oxide was passed through the same type of column.

NMR Spectroscopy. All spectra were recorded at a ^{31}P frequency of 80.99 MHz and a temperature of about 23 °C. Magnetization-exchange experiments were conducted either on a Varian XL200A spectrometer with a broad-band probe, where a selective pulse was generated by using a DANTE pulse sequence, or on a Nicolet NT200 spectrometer with a fixed-frequency probe, where a low-power radio frequency source was used to produce a selective pulse. The low-power source was operated at a 22 dB attenuation level, giving a 27-ms 90° pulse length ($\gamma H_2 \simeq 60$ Hz). Sample volumes of 1.6 and 2.6 mL were contained in spherical microcell inserts for 16- (XL200A) or 20-mm (NT200) tubes, respectively. Chemical shifts are referred to trimethyl phosphate. On this scale, the chemical shift of the internal reference included in some experiments, tetraphenylphosphonium chloride, is 19.62 ppm.

Magnetization-exchange experiments were conducted with continuous Waltz decoupling of protons at a power of 5 W. Spectra were accumulated with a 60° pulse angle, a total recovery time of 5 s or longer, and 2000–4000 scans. A spectral width of 1800 Hz was used in low-power magnetization exchange, while DANTE magnetization exchange required a spectral width of 2700–3600 Hz. Magnetization-exchange experiments included a presaturation period of varied length during which a signal was selectively irradiated, an observed pulse plus a data acquisition period, and a repetition delay. To quantify peak areas for assessing equilibrium concentrations, spectra were obtained without ^1H decoupling by using a 60° pulse angle, a 15-s total recovery time, and 5000 scans. A spectral width of 3600 Hz was used to include the internal standard tetraphenylphosphonium ion.

Selective excitation generated from a DANTE pulse train (Jasson et al., 1973; Morris & Freeman, 1978) utilized a pulse length of 1.5–2.0 μs, corresponding to a rotation angle of approximately 5°, as determined from the length of the DANTE train that produced a 90° rotation angle. A resonance was selectively irradiated with the first harmonic sideband. The transmitter frequency was set 800–1200 Hz from the irradiated resonance (well outside the frequency range of the spectrum), which required a DANTE delay of 1.25–0.8 μs. In the control spectra, the DANTE delay was decreased so that the frequency of the sideband was >150 Hz outside the spectral region. The length of the DANTE train varied according to the irradiation time period, τ. Intensities were compared in spectra with equal on- and off-resonance irradiation periods. To assess possible nonselective effects on major peaks, results obtained with the on- and off-resonance frequencies equidistant from the observed peak were compared with those obtained with the off-resonance frequency outside the spectral range. Nonselective irradiation was avoided by centering the DANTE frequency >150 or ≥110 Hz from peaks with line widths of about 20 or 2 Hz, respectively.

For selective excitation by a low-power pulse, the transmitter frequency was set to the resonant frequency. The selective and observe pulses were alternated by computer-controlled switching of the low- and high-power radio frequency transmitters during a 10-ms delay time. To collect the control spectrum, the presaturation period was set to 1 ms, a time too short to support magnetization exchange but sufficient to trigger the low-power pulse. Nonselective effects, evaluated as above, were avoided by placing the frequency of the transmitter at least 100 or 10 Hz from peaks with line widths of about 20 or 2 Hz, respectively.

Saturation-transfer rates were obtained from the fractional magnetization, the ratio of the peak intensity in the on-resonance and the control spectrum. Intensities were estimated from heights or integrated areas calculated with the standard software supplied with the spectrometer or peak heights as measured by hand. The latter procedure was more reliable for lower levels of signal to noise. A line-broadening factor of 5 Hz in the exponential apodization of the free-induction decay was generally used except where signals overlapped, i.e.,

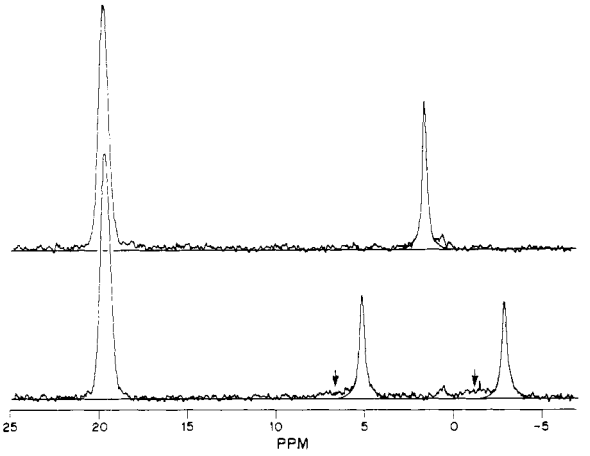


FIGURE 1: Quantitative comparisons of the ^{31}P NMR spectra of the E_P·Cd and E_D·Cd·Glc-P_2 complexes of phosphoglucomutase with that of tetraphenylphosphonium chloride. Solutions contained 40 mM Bistris-HCl, pH 7.2, 10% ^2H_2O, 1.62 mM enzyme (100 mg of protein/mL), 1.00 equiv of added Cd^{2+}, with (bottom) or without (top) 1.00 equiv of Glc-6-P, and 4.73 mM tetraphenylphosphonium chloride. Fully relaxed spectra of the enzymic complexes (5000 transients) were accumulated in the manner described under Experimental Procedures (no ^{1}H decoupling) at a rate of 4 transients/min. The areas representing the signal from the enzymic complexes were estimated from a fitted Lorentzian line shape (smooth solid lines in the figure at δ = +1.65 ppm for E_P·Cd and at δ = +5.20 and −2.75 ppm for the phosphate groups of E_D·Cd·Glc-P_2). The corresponding area for tetraphenylphosphonium chloride (δ = 19.62 ppm) was measured directly from the spectrum after smoothing the plot in the area of the base line. Relative intensity values were calculated after correcting for T_1 and recycle time (see Results). Measured relative intensities: tetraphenylphosphonium chloride, 1.00; E_P·Cd, 1.00; resonances at δ = −2.75 and +5.20, 0.76 and 0.82, respectively. The arrows in the lower spectrum indicate intensities greater than the base line that were reproducibly observed. The low-level intensity at about 0.7 ppm, which has approximately the same chemical shift as metal-free E_P, was not quantified.

for the anomers of Glc-6-P. For this case, no apodization was performed.

Transfer experiments were carried out without allowing time for full recovery of longitudinal magnetization between transients because of time limitations. This procedure effects the initial value of the magnetization (i.e., at zero irradiation time) and therefore the dynamic range of the experimental measurements but not the rate of transfer. The intensity change as a function of τ was measured from the on- and off-resonance spectra accumulated with the same effective recycle time,[5] equal to ~5 s for τ < 1 s and greater than 10 s for longer τ. Since the recycle time is $\geq T_1$ (T_1 = 5 ± 0.5 s for all resonances), the error introduced from incomplete magnetization recovery is 10% or less.[6] Although this procedure gives rise to rate constants somewhat smaller than the true rate constants, the differences are well within the acceptable range for such studies.

Results

Quantification of the ^{31}P NMR Spectral Intensities for the Cd(II) Phosphoenzyme and Its Complex with Glucose Phosphate. Figure 1 shows ^{31}P NMR spectra of E_P·Cd (top) and E_D·Cd·Glc-P_2 (bottom) obtained after a line-broadening factor of 5 Hz was applied (see Experimental Procedures). The spectra were obtained without ^{1}H decoupling to avoid a differential NOE on intensities, and with a sufficient delay between transients (15 s) for essentially complete recovery of the equilibrium magnetization for both the enzymic phosphate and the bisphosphate complex of the enzyme. [The T_1 value for E_P·Cd is 4.7 s (Rhyu et al., 1984) and for both phosphates of E_D·Cd·Glc-P_2 is 5 s (spectra and plots not shown).] Figure 1 also shows the corresponding peak for the internal standard tetraphenylphosphonium chloride.

An analysis of peak areas was conducted in the manner described in the legend of Figure 1, in most cases by using fitted Lorentzian line shapes (smooth lines in the figure). The intensity of the resonance for E_P·Cd (at 1.65 ppm) accounts for all of the expected intensity, based on the known concentrations of E_P·Cd[7] and tetraphenylphosphonium ion present,[8] and on a comparison between the areas of their resonances. (The comparison produced an average value of 100%; the precision of the area estimate was ±2%.) This comparison includes a correction for T_1 relaxation differences produced by the 15-s recycle time with the above value of T_1 for the enzymic phosphate and a value of 7.0 s for the tetraphenylphosphonium ion, measured under the conditions of the experiment.[9] In contrast, a similar analysis of the spectrum for the E_D·Cd·Glc-P_2 complex, which was produced by the addition of 1 equiv of glucose monophosphate to E_P·Cd (Rhyu et al., 1984),[10] showed that the resonances at −2.75 and +5.20 ppm each account for only 76 and 82% of a phosphate group (Figure 1, bottom) and thus, together, account for only about 80% of the two phosphate groups now present in the system. A comparable analysis of the same resonances in a spectrum obtained in the presence of excess glucose phosphates (not shown) provided intensity estimates equal to 84 and 82%, respectively, of that for E_P·Cd. In the presence of 2 equiv of Cd^{2+} (which narrows both lines somewhat and shifts both downfield by about 1 ppm; Rhyu et al., 1984), the value for the downfield resonance (now at about +6 ppm) was 82%.[11] In view of the excellent internal agreement of these values, the intensity present in the two prominent peaks at −2.75 and +5.20 ppm in the spectrum of E_D·Cd·Glc-P_2 must account for most but not all of the phosphate in the system. In fact, a weak resonance appears at 0.4 ppm (at the same chemical shift as metal-free E_P (Rhyu et al., 1983), plus intensity greater than the base line is apparent in the regions just downfield from both of the prominent spectral lines in Figure 1, bottom (see arrows in the figure). This intensity is suggestive of broad resonances and is present in the E_D·Cd·Glc-P_2 spectrum with

[5] The effective recycle time for the observed peak in the on-resonance spectrum is the time between the observed pulse and the beginning of the presaturation period for irradiation of a peak. In the off-resonance spectrum it is the time between two observe pulses, which includes the presaturation period for off-resonance irradiation.

[6] An analysis of the error produced by incomplete magnetization recovery has been made (C. B. Post, submitted for publication).

[7] An active-site titration of the dephosphoenzyme with Glc-P_2, using a procedure similar to that of Lowry and Passoneau (1969), provided a check on the value of E_{278} (Ray et al., 1983) that was used to determine the concentration of the phosphoenzyme initially present. The reported value is accurate to within ±2%.

[8] Tetraphenylphosphonium chloride is a particularly convenient internal standard because (a) its decoupled spectrum is sharp, thereby facilitating comparisons with other sharp peaks where the use of line broadening could be avoided, and (b) the width of the coupled (and unresolved) multiplet is similar to that of enzyme-bound phosphates, so that comparisons could be made between these, even when a line-broadening factor of 0.25 times the line width at half-height for the enzyme-bound phosphates was used. (Twice as large a factor could have been used.)

[9] The T_1 for tetraphenylphosphonium ion is 13.8 s in the absence of the enzyme (and at a concentration of 50 mM, instead of 5 mM) (C. B. Post, unpublished results).

[10] The E_D·Cd·Glc-P_2 complex does not dissociate to a significant extent at the concentrations employed [cf. Ray and Long (1976)].

[11] The upfield resonance for this complex overlaps that for the 1-phosphate of Glc-P_2 and was not quantified.

or without excess substrate, although the appearance of the spectrum in these regions obviously depends on the applied phase correction. Several studies (fast acquisition and variable temperature) conducted to enhance the appearance of these signals failed to produce unequivocal results. Nevertheless, when reasonable traces are passed through these regions (not shown), the area under these traces plus the area under the prominent peaks accounts for essentially all of the phosphate present (assuming T_1 for the phosphate that accounts for the minor resonances is the same as that for the phosphates that produce the prominent resonances; see above).

A previous study of a quenched equilibrium mixture produced by treating E_P·Cd with limiting amounts of glucose phosphates provided the following relative concentrations (and precision of the estimates) for the three central complexes present in this system (Ray & Long, 1976): E_P·Cd·Glc-1-P, 0.03 ± 0.005; E_D·Cd·Glc-P_2, 0.91 ± 0.01; E_P·Cd·Glc-6-P, 0.06 ± 0.005. Presumably the noise-level signals in the NMR spectrum of E_D·Cd·Glc-P_2 arise in part from the two monophosphate complexes. Whether a second bisphosphate complex, e.g., a loose encounter complex, accounts for the difference in the average of the five values (0.81) obtained by NMR and the value (0.91) obtained from the above analysis or whether 0.81 or 0.91 is the correct value for the fractional abundance of the major E_D·Cd·Glc-P_2 complex is difficult to decide. In the absence of firm evidence either way, an intermediate value, 0.85, is taken as the fractional abundance of this complex, which subsequently is referred to as the *observed* bisphosphate complex or as E_D·Cd·Glc-P_2(obs).

Assignment of Chemical Shifts to the Phosphates of Bound Glucose 1,6-Bisphosphate. Previously, chemical shifts for the phosphate groups of E_D·Cd·Glc-P_2(obs) were tentatively assigned by analogy with those of E_D·Li·Glc-P_2, where exchange with free Glc-P_2 is sufficiently rapid to conduct direct saturation-transfer studies (Rhyu et al., 1984). Such transfer studies cannot be conducted with the Cd^{2+} complex because of very slow chemical exchange with the free bisphosphate (Ray et al., 1989). In contrast, chemical exchange of the (PO_3^-) groups of E_D·Cd·Glc-P_2(obs) with excess free Glc-1-P and excess free Glc-6-P, via (PO_3^-) transfer followed by dissociation of the monophosphate complex (Scheme I), is much faster than direct chemical exchange with the free bisphosphate (as it is in the Mg^{2+} system; Ray & Roscelli, 1964) and is sufficiently rapid to permit saturation-transfer studies involving the free monophosphates. Thus, according to eq 7 and 8, when the resonance of the 1-phosphate of bound Glc-P_2 is saturated, the drain of magnetization from the phosphate of free Glc-1-P should be at least as efficient (fast) if not more efficient than when the resonance of the 6-phosphate of bound Glc-P_2 is saturated, since both transfer processes share a common step and the latter process includes two additional steps. Similarly, the drain of magnetization from the phosphate of free Glc-6-P should be at least as efficient as when the ^{31}P NMR resonance of the 6-phosphate rather than the 1-phosphate of bound Glc-P_2 is saturated. Accordingly, the saturation-transfer studies in Figure 2 (which are considered in more detail, below) show that the chemical exchange rate involving the irradiated peak at −2.75 ppm and Glc-1-P (Figure 2.B1) is faster than that between the irradiated peak at +5.2 ppm and Glc-1-P (Figure 2.B4). Similarly, the chemical exchange rate involving the irradiated peak at +5.2 ppm and Glc-6-P (Figure 2.B6) is faster than that between the irradiated peak at −2.75 ppm and Glc-6-P (Figure 2.B3). Hence, the 1-phosphate and 6-phosphate of E_D·Cd·Glc-P_2 give rise to the resonances at −2.75 and +5.20 ppm, respectively, in accord with the earlier tentative assignment. A subsequent paper will consider whether the resonances at −2.75 and +5.20 ppm arise from a single E_D·Cd·Glc-P_2 complex or two such complexes [cf. Ray et al. (1973) and Ma and Ray (1980)]. Of course, if two such complexes are involved, these must be in rapid exchange on the chemical shift time scale since the spectrum in Figure 1, bottom, has only two prominent peaks rather than four. In any case, the present treatment will consider only one such complex, in accord with Occam's "razor" (Bernal, 1954).

Is the Observed Bisphosphate Complex of the Cd^{2+} Enzyme Catalytically Competent? If the observed E_D·Cd·Glc-P_2 complex (Figure 1, bottom) is catalytically competent, when Glc-6-P irradiated, the fractional magnetization of the bound resonances must be less than or equal to that of free Glc-1-P. (In other words, in the sequence A → B → C → D, when spin D is irradiated, the approach to the new steady-state magnetization for species A, B, and C must be $\text{rate}_A \leq \text{rate}_B \leq \text{rate}_C$.) If E_D·Cd·Glc-P_2(obs) is not on the catalytic pathway, the fraction of magnetization remaining in the bound resonances (B or C, above) likely would be greater than that of Glc-1-P (when Glc-6-P is irradiated). To distinguish between these possibilities, spin-transfer studies were conducted under conditions analogous to those in the previous section (excess glucose monophosphates), except that the phosphate group of free Glc-6-P was irradiated. The results, at six different irradiation times during approach to the steady state, showed the *fraction* of the initial magnetization remaining (Figure 2.B7–B9) was substantially smaller for the 6-phosphate of E_D·Cd·Glc-P_2(obs) than for Glc-1-P and was the same, within experimental error, for Glc-1-P and the 1-phosphate of E_D·Cd·Glc-P_2(obs), as is required if E_D·Cd·Glc-P_2(obs) is an obligate intermediate in the conversion of Glc-1-P to Glc-6-P.[12] Hence, all computer simulations of magnetization exchange (see below) were conducted with sequential models.

Initial Evaluation of Kinetic Constants Required To Model ^{31}P Magnetization Transfer. Modus Operandi shows that magnetization transfer among the (PO_3^-) groups of the phosphoglucomutase system (Scheme I) can be described in terms of two apparent rate constants, which can be measured directly via simple exponential decay, and two equilibrium constants. The two rate constants are defined by the following two "half-reactions", left to right. These half-reactions include a binding step and a single (PO_3^-)-transfer step.

$$\text{Glc-1-P*} \underset{k_{-1}^{app}}{\overset{k_1^{obs}}{\rightleftharpoons}} E_D\text{·Cd·P-6-Glc-1-P*} \qquad (9)$$

$$\text{Glc-6-P*} \underset{k_{-6}^{app}}{\overset{k_6^{obs}}{\rightleftharpoons}} E_D\text{·Cd·P*-6-Glc-1-P} \qquad (10)$$

In these equations, which omit the monophosphate complexes E_P·Cd·Glc-1-P and E_P·Cd·Glc-6-P (see Modus Operandi), $k_1^{app}[E_P\text{·Cd}] = k_1^{obs}$ and $k_6^{app}[E_P\text{·Cd}] = k_6^{obs}$. The magnetization decay for Glc-1-P or Glc-6-P when the 1- or 6-phosphate, respectively, of E_D·Cd·Glc-P_2(obs) is irradiated, is shown in Figure 2.B1,B6. Under these conditions (at intermediate enzyme/substrate ratio; see Figure 2, legend), the values of the first-order rate constants, k_1^{obs} and k_6^{obs}, obtained by modeling the data with an exponential decay function

[12] A reaction sequence in which the prominent E_D·Cd·Glc-P_2 complex is an abortive complex, but in *rapid* chemical equilibrium with a different, much less abundant but competent E_D·Cd·Glc-P_2 complex that lies along a direct pathway from E_P·Cd·Glc-1-P to E_P·Cd·Glc-6-P, is in accord with the results obtained. However, this possibility seems unlikely since such a mechanism would require that the abortive complex equilibrate with the competent complex at a rate substantially faster than the rate at which the competent complex reacts or is formed.

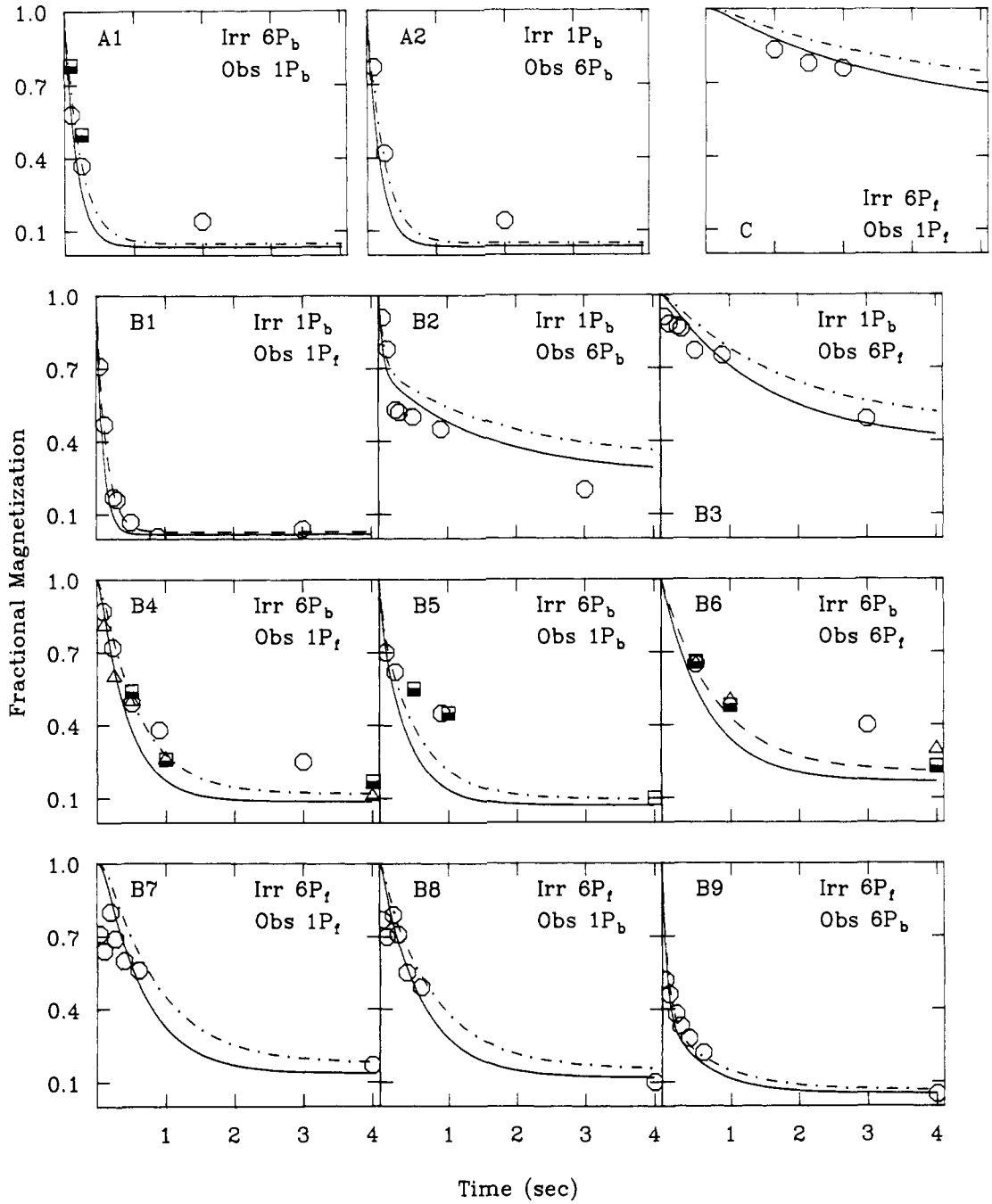


FIGURE 2: Fractional magnetization remaining in the unirradiated phosphates of the phosphoglucomutase system as a function of irradiation time. Buffer and solvent concentrations were the same as in Figure 1. The relative magnetization for each phosphate at zero time was calculated from the stoichiometry of the system (see Results) and the initial conditions by assuming that 85% of the enzyme was present as E_D·Cd·Glc-P_2 (see Results). Initial conditions were (A) (high enzyme/substrate ratio) 1.62 mM E_P·Cd plus 1.62 mM Glc-6-P, (B) (intermediate enzyme/substrate ratio) 1.62 mM E_P·Cd plus 32 mM Glc-1-P or Glc-6-P, and (C) (low enzyme/substrate ratio) 0.203 mM E_P·Cd plus 50.6 mM Glc-6-P. (Cases A–C are further differentiated in Tables II and III.) For the relative amounts the phosphates present at equilibrium, see Table III. The identity of the irradiated and observed phosphate is given for each plot. Thus, bound 1-P and bound 6-P refer to the two phosphates of E_D·Cd·Glc-P_2(obs), while free 1-P and free 6-P refer to Glc-1-P and Glc-6-P, respectively. The fractional magnetization remaining after selective irradiation either by DANTE (○ or △, for different experiments) or by low-power (▣) pulses is the ratio of peak heights in on-resonance and control spectra analogous to those in Figure 3. A simple exponential decay (- - -) was fit to the data in the plots of parts B1 and B6 to give the initial values of k_1^{obs} and k_6^{obs} listed in Table III. Simulated curves (–·–) were calculated in the manner described in Appendix 2 from the relative concentrations of the various phosphates present (see above), the measured values of T_1 (5 s, see Appendix 2), and the initial estimates of k_1^{app} and k_6^{app}. Values for k_1^{app} and k_6^{app} were adjusted, partly by trial and error, to provide the best visual fit to all of the data (—); adjusted values also are given in Table III.

(dashed curves, Figure 2) are 6.8 and 1.0 s^{-1}, respectively. Since [E_P·Cd] $\approx 1.3 \times 10^{-7}$ M (see Table III), k_1^{app} and k_6^{app} are 5.1 and 0.75×10^7 s^{-1}, respectively. From these values, estimates for the constants k_{-1}^{app} and k_{-6}^{app} for the reverse process (*cf.* eq 9 and 10) can be obtained from the concentration ratios used in the experiment (see Table III): k_{-1}^{app} = 8.0 s^{-1} and k_{-6}^{app} = 8.5 s^{-1}.

Magnetization Exchange Involving a Series of Half-Reactions. Although a simple exponential function appears adequate to represent the time dependence of magnetization transfer in the half-reactions of eq 9 and 10, a more stringent criterion for the applicability of this representation is the extent to which its use can account for transfer processes that involve a sequence of two, three, or four half-reactions, where overall

Table II: Sequences of Half-Reactions in the Cd(II) Phosphoglucomutase System Studied by Magnetization Transfer[a]

case	Glc-1-P*	⇌	E_D·P-6-Glc-1-P*	⇌	(E_P*)	⇌	E_D·P*-6-Glc-1-P	⇌	Glc-6-P*
A			obs				irrad		
A			irrad				obs		
B	obs		irrad				obs		obs
B	obs		obs				irrad		obs
B	obs		obs				obs		irrad
C	obs								irrad

[a] Each set of arrows (⇌) represents one half-reaction [binding step plus (PO_3^-)-transfer step; see Results] by which magnetization is exchanged among the phosphates in the reaction sequence indicated by the asterisks. Columns beneath phosphates so indicated show which phosphates were irradiated and which were observed in the various studies, so that the number of half-reactions (arrow sets) between irradiated and observed species is readily obtained. The various cases are further described in Table III and Figure 2.

magnetization decay no longer can be described by a simple exponential and modeling by computer simulation is required. Such transfer can be measured in a variety of ways in the present system, both in the presence and in the absence of a substantial excess of glucose monophosphates.[13] For example, although the flow of magnetization from the phosphate of free Glc-6-P to the 6-phosphate of bound Glc-P_2, considered above, involves a single half-reaction, magnetization transfer from free Glc-6-P to the 1-phosphate of bound Glc-P_2 involves three successive half-reactions; see eq 7 and 8. The various sequences of half-reactions monitored by ^{31}P magnetization exchange are shown in Table II in such a way that the number of half-reactions that constitute each sequence can be readily determined. [The number of times rate constants, k_{ij} (see Table I), are used in the simulation of a magnetization-exchange process increases with the number of half-reactions that characterize the process.]

A typical group of spectra obtained in magnetization-exchange experiments is shown in Figure 3, where the 1-phosphate of E_D·Cd·Glc-P_2(obs) was selectively saturated, here via a DANTE pulse sequence. The results from *all* such studies are shown in Figure 2, where each plot specifies which phosphate was irradiated and which was observed and where different symbols indicate different experiments. The agreement between experimental values obtained with DANTE irradiation (open symbols) and those obtained with low-power irradiation (half-filled symbols) shows that effects due to nonselective irradiation of prominent resonances (see Experimental Procedures) are negligible. [The lifetimes of the intermediates, E_P·Cd·Glc-1-P, E_P·Cd·Glc-6-P, and E_P·Cd, calculated on the basis of minimal estimates for the pertinent rate constants (see below), were too short for nonselective irradiation to significantly saturate these.]

Computer Simulations of Saturation Transfer in the Phosphoglucomutase Reaction. Saturation transfer for all sequences of half-reactions in Figure 2 was modeled by computer simulations of eq 11 (see Appendix 2) in which the loss of magnetization from the various ^{31}P nuclei was

$$E_P\text{·Cd} + \text{Glc-1-P} \underset{k_{-1}^{app}}{\overset{k_1^{app}}{\rightleftharpoons}} E_D\text{·Cd·Glc-P}_2 \underset{k_6^{app}}{\overset{k_{-6}^{app}}{\rightleftharpoons}} E_P\text{·Cd} + \text{Glc-6-P} \quad (11)$$

calculated as a function of saturation time. The specific rates, k_{ij}, used to evaluate the elements of the relaxation matrix, Ω, are given in Table I. Values for the net rate constants, k_1^{app}, k_{-1}^{app}, k_6^{app}, and k_{-6}^{app}, obtained from single half-reactions (dashed lines, Figure 2; see the previous section) were used as initial estimates for these parameters. The relative concentrations of species were those listed in Table III, while values of T_1 for E_P·Cd, the free glucose phosphates, and the bound bisphosphate were measured directly (see Appendix 2). Dash-dot curves in Figure 2 were produced by computer simulation with the above values. After comparison of the simulated and observed losses in magnetization intensity for each species, the initial estimates for the rate constants were adjusted so that the newly simulated intensities (solid curves) were in better *overall* agreement for all transfer processes studied, as estimated visually, viz., k_1^{app} and k_6^{app} were increased from 5.1×10^7 to 7.7×10^7 M^{-1} s^{-1} and from 0.75×10^{-7} to 1.0×10^{-7} M^{-1} s^{-1}, respectively (which provides

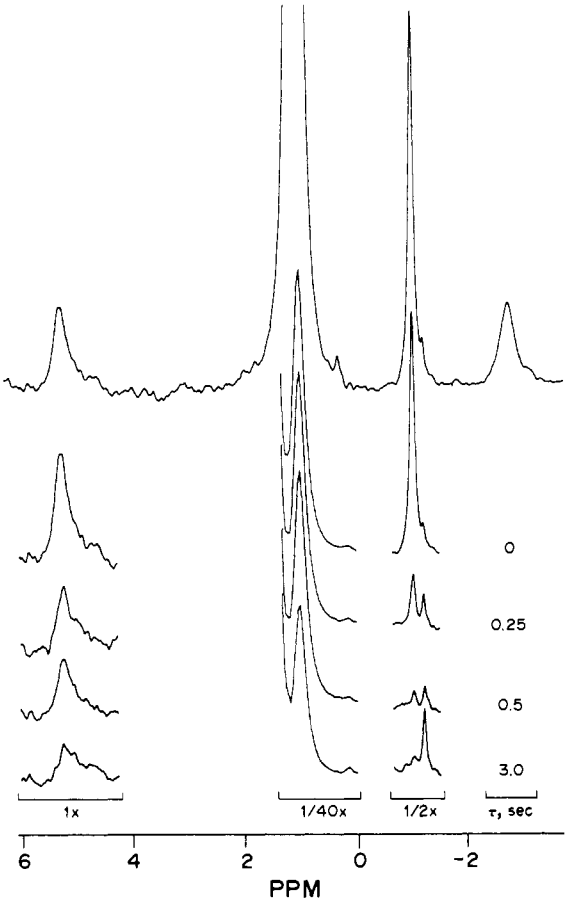


FIGURE 3: Saturation-transfer experiment with an equilibrium mixture of Glc-1-P, E_D·Cd·Glc-P_2, and Glc-6-P. The solution was the same as that used to obtain the spectrum in Figure 1 (bottom), except that 1.0 mM Glc-1-P ($\delta = -1.12$ ppm) and 18.7 mM ($\alpha + \beta$)-Glc-6-P ($\delta \approx 0.95$ ppm) also were present. Spectra obtained without irradiation (top) and after the ^{31}P resonance of the 1-phosphate of E_D·Cd·Glc-P_2(obs) was irradiated (DANTE pulse sequence) for the indicated time intervals, τ, are shown (see Experimental Procedures for details). (The small resonance at about −1.2 ppm is produced by traces of inorganic phosphate.)

[13] The mixture of E_P·Cd·Glc-1-P, E_D·Cd·Glc-P_2, and E_P·Cd·Glc-6-P complexes dissociates to E_P·Cd + Glc-1-P and Glc-6-P only to a very small extent at the concentrations used [cf. Ray and Long (1976)], but even this minor dissociation is enough to provide sufficient free glucose monophosphates to ensure that multicycle phosphate transfer is not limited by the concentration of monophosphates present.

Table III: Rate Constants and Relative Concentrations Used in Computer Simulations of the Time Dependence for Magnetization Exchange[a]

case	[enzyme]$_{tot}$/[substrate]$_f$	relative concentration: free Glc-1-P	free α-Glc-6-P	E_D·Cd·Glc-6-P	E_P·Cd[b]
A	high	(0.003)[c,d]	(0.02)[c,d]	0.85	(3.2×10^{-2})[c,d]
B	intermediate	1.0	7.2	0.85	1.3×10^{-7} [c]
C	low	1.94	13.9	0.11	0.2×10^{-6} [c]

rate constant[e]	initial value[f]	final value[g]
k_1^{app}	5.1×10^7 M^{-1} s^{-1}	7.7×10^7 M^{-1} s^{-1}
k_{-1}^{app}	8.0 s^{-1}	12 s^{-1}
k_6^{app}	0.75×10^7 M^{-1} s^{-1}	1.0×10^7 M^{-1} s^{-1}
k_{-6}^{app}	8.5 s^{-1}	11 s^{-1}

[a] Relative equilibrium concentrations (0.67 of the actual value) for species present at three different ratios of *total* enzyme to *free* substrate, viz., to free Glc-1-P plus free α-Glc-6-P, were calculated from the initial concentrations used (see Figure 2, legend) plus parameters under Results: Column 2. The same estimates (see Results) of the rate constants were used at all three enzyme/substrate ratios. Measured and estimated T_1 values required for simulations are given in Appendix 2. The curves generated for the three different enzyme/substrate ratios are shown in Figure 2.A (high), Figure 2.B (intermediate), and Figure 2.C (low). [b] Values not in parentheses were estimated from the concentrations of total substrate, total enzyme, and the equilibrium isotope exchange constant for glucose monophosphates, 2.5 μM (Ray & Long, 1976). At the intermediate enzyme/substrate ratio, [E_P·Cd] ≈ 1.3×10^{-7} M. [c] Value that did not affect simulations as long as it was low relative to total enzyme concentration. [d] Arbitrary value selected for computational convenience. [e] For definitions, see eq 11. [f] Values estimated from a single-exponential fit of the time dependence of the intensity loss in the resonance from free glucose monophosphate upon irradiation of the resonance from the corresponding phosphate group in E_D·Cd·Glc-1,6-P_2 (dashed line simulations in Figure 2). [g] Values after adjustments to achieve a good comparison of the simulated curves with the observed magnetization time dependence for *all* measured transfer processes (solid line simulations in Figure 2).

values of 12 and 11 s^{-1}, respectively, for k_{-1}^{app} and k_{-6}^{app}). The observed trends in residual magnetization are consistent with the simulated curves in studies conducted in the absence of (measurable concentrations of) free glucose phosphates[13] (Figure 2.A1,A2), in the presence of a modest excess of glucose phosphates (enzyme complex:free Glc-1-P = 1:1) (Figure 2.B1–B9), as well as in the presence of a much larger excess of glucose phosphates, where enzyme-bound phosphates were not detected (enzyme complex:free Glc-1-P = 1:16.7) (Figure 2.C). Although the initial rate constants give somewhat better agreement than the final constants in some cases, the relatively large deviations for the initial (dash-dot) curves in parts C, B2, B3, B7, and B8 of Figure 2 are reduced with the final (solid) curves. Since a reasonably good fit was obtained with a relatively small adjustment of the directly measured rate constants (see above), the model employed, i.e., Scheme I modified according to eq 11, appears to provide an adequate description of (PO_3^-) transfer in the phosphoglucomutase–Cd^{2+} system.

Minimum Values of Rate Constants for (PO_3^-) Transfer by the Cd(II) Enzyme from Saturation-Transfer Studies. By use of the relative equilibrium concentrations of the three central complexes cited in the first section under Results, the following equilibrium ratios can be evaluated from NMR studies at an intermediate enzyme/substrate ratio (Table III), where the various rate constants are defined in Scheme I:

$$\frac{[E_P\text{·Cd·Glc-1-P}]}{[\text{Glc-1-P}]} = 2.9 \times 10^{-2} \quad (12)$$

$$\frac{[E_P\text{·Cd·}\alpha\text{-Glc-6-P}]}{[\alpha\text{-Glc-6-P}]} = 8.8 \times 10^{-3} \quad (13)$$

A minimum value for k_t^+ in both of the following half-reactions (eq 14 and 15) (cf. expression involving k_y, eq 4) is given by

$$\text{Glc-1-P} \underset{k_{d1}}{\overset{k_{a1}[E_P\text{·Cd}]}{\rightleftharpoons}} (E_P\text{·Cd·Glc-1-P}) \underset{k_{t1}^-}{\overset{k_{t1}^+}{\rightleftharpoons}} E_D\text{·Cd·Glc-P}_2 \quad (14)$$

$$\text{Glc-6-P} \underset{k_{d6}}{\overset{k_{a6}[E_P\text{·Cd}]}{\rightleftharpoons}} (E_P\text{·Cd·Glc-6-P}) \underset{k_{t6}^-}{\overset{k_{t6}^+}{\rightleftharpoons}} E_D\text{·Cd·Glc-P}_2 \quad (15)$$

the ratio of the apparent rate constant, k^{app}[E_P·Cd] (i.e., k_1^{obs} and k_6^{obs}), and the equilibrium constant for the first step of the half-reaction (eq 12 or 13). By use of the best overall values of k_1^{obs} (10.2 s^{-1}) and k_6^{obs} (1.3 s^{-1}) at the intermediate enzyme/substrate ratio (see Figure 2 legend), minimum values for k_{t1}^+ (transfer to bound Glc-1-P) and k_{t6}^+ (transfer to bound Glc-6-P) under conditions used in the saturation-transfer studies can be calculated as 350 and 150 s^{-1}, respectively.

Discussion

Suitability of Cd(II) Phosphoglucomutase for ^{31}P Magnetization-Transfer Studies. Activation of phosphoglucomutase by Mg^{2+} produces the most efficient transfer of the (PO_3^-) moiety between donor and acceptor hydroxyl groups of bound glucose phosphates. However, the Mg^{2+} enzyme represents an intractable system for study by magnetization transfer since rapid chemical exchange between enzymic species gives rise to a single broad peak in the ^{31}P NMR spectrum. Of the other known bivalent metal ion activators (Ray, 1969), most are paramagnetic and thus are unsuitable for NMR exchange studies. Of the remaining diamagnetic activators, Cd^{2+} produces the most attractive system for such studies, even though the Cd^{2+} enzyme is only about 1% as efficient as the Mg^{2+} enzyme in terms of k_{cat}. Thus, in the Cd^{2+} system, 80–90% of the enzyme is present as a "single complex" at equilibrium (see below)—the bisphosphate complex. In fact, there are only two phosphorus resonances from enzyme-bound glucose phosphates with sufficient intensity for quantification (see Results). Moreover, these two resonances are widely separated and do not overlap those of the free glucose monophosphates; i.e., the chemical shift dispersion is adequate for selective irradiation of each peak. In addition, the lifetimes of the observed intermediate phosphates are about $0.1T_1$, while those of the free glucose phosphates could be maintained at less than T_1 by using a high enzyme/substrate ratio. Hence, the rates of (PO_3^-) transfer among the observable species can be readily measured by time-dependent ^{31}P magnetization exchange. (Since T_1 relaxation counteracts the decline in magnetization produced by chemical exchange, the rate must be significantly faster than T_1 relaxation to be measured in such studies; see Appendix 2.) Because of these properties, it was possible to determine the rates for transfer both among the (PO_3^-) groups of the bisphosphate complex and those of the substrate and product pools in the Cd^{2+} enzyme system.

The Multisite Exchange Process and Its Simplification. As is shown in Scheme I, the phosphoglucomutase system provides at least nine different local environments for phosphate groups. An added and inherent complexity in ^{31}P magnetization-exchange studies is that two catalytic cycles are required to transfer a (PO_3^-) group between substrate and product pools (see eq 7 and 8). Because of the small relative concentrations of E_P·Cd·Glc-1-P and E_P·Cd·Glc-6-P, Scheme I simplifies to eq 11, where the binding of glucose monophosphates and the subsequent transfer of the enzymic phosphate to the bound monophosphates is telescoped into a single step. This sim-

plification is based on the assumption that during saturation transfer the magnetization of the intermediate monophosphate complex is in steady state. Although the possibility of deviations from steady-state behavior cannot be unequivocally eliminated, the absence of serious effects due to such deviations is supported by two different observations. First, the results from the saturation-transfer studies conducted at enzyme/substrate ratios that varied from about 17 to <0.1 were internally consistent (see Figure 2). Second, the rate constants for overall phosphate transfer from substrate to product pools at a lower enzyme/substrate ratio, where the steady-state approximation must hold, can be calculated from values for the apparent rate constants reported here. Comparisons of rate constants calculated in this way with constants measured in both ^{32}P isotope exchange studies at equilibrium and in initial velocity studies are made in the accompanying paper (Ray et al., 1989).

Reducing a single catalytic cycle of phosphoglucomutase to an A $\rightleftharpoons$ B $\rightleftharpoons$ C process according to eq 11 (see also Modus Operandi) means that the main features of the reaction can be defined by two equilibrium constants and two rate constants. The required equilibrium constants were determined from both NMR intensities (see Results) and previous rapid quench results obtained with an equilibrium mixture (Ray & Long, 1976). The two rate constants initially were evaluated directly by measuring the time-dependent loss of magnetization from each of the free glucose monophosphates (A or C) upon irradiation of the corresponding phosphate groups in the E_D·Cd·Glc-P_2 complex (B), since these two isolated exchange processes produce a magnetization decay that involves a single exponential that can be analyzed in a straightforward manner (see Results).

Computer Modeling of the Multisite Exchange Process. A more rigorous evaluation of rate constants than that described above was conducted by employing exchange processes that require multiple steps and double cycles of the reaction. Computer simulation of the simultaneous Bloch equations was required for analysis of magnetization decay via such processes. In such an analysis, the observed and simulated decays of magnetization for each observable species were compared and the rate constants used in subsequent simulations altered until a good fit to all of the data was achieved. Since magnetization exchange depends on T_1 as well as the chemical exchange rate, T_1 values were measured independently (by inversion recovery) for all observed NMR resonances under conditions identical with those used for saturation transfer except for the concentration of substrate (see Appendix 2). Since experimental errors in inversion recovery data were smaller than those in magnetization-transfer data, the measured values of T_1 were not altered in fitting of the simulated magnetization decay to the observed decay. A least-squares procedure was not used because the appropriate type of weighting was not obvious, in view of the varying signal to noise ratio as the intensity decayed.

Signal Averaging and Incomplete Recovery. Substantial signal averaging is required for ^{31}P spectroscopy at the low concentrations of enzyme solutions used (1.62 mM; see Results). The recycle time must be greater than $3T_1$ if the full, unperturbed equilibrium magnetization is to be recovered between successive transients. However, the T_1 values in the present system all are approximately 5 s, and using a recycle delay of 15 s to achieve nearly complete magnetization recovery would have seriously affected the attainable signal to noise ratio commensurate with the available instrument time and with the stability of the enzymic system (see below). In all saturation-transfer studies of which the authors are aware, it has been assumed, usually tacitly, that the initial magnetization at zero irradiation time (obtained from the control spectrum; see Experimental Procedures) is equal to the unperturbed equilibrium magnetization, M^0 (see Appendix 2, eq 23). We have proceeded similarly but carefully considered the error in assessing an apparent transfer rate with data obtained with incomplete recovery relative to the true rate that would be obtained with measurements utilizing complete recovery. Thus, under certain conditions of T_1, flip angle, and saturation level at long irradiation times, the error can be made relatively small: <10%.[6]

Enzyme Stability. Chemical instability of the equilibrium mixture of the enzyme and its substrate/product pair at concentrations of enzyme approaching that of the substrate did not pose a major problem in these studies, although slow generation of inorganic phosphate after addition of glucose monophosphates usually precluded the use of a given sample of enzyme for more than 48 h. Whether the hydrolytic generation of phosphate represents an inherent but very inefficient activity associated with the Cd^{2+} form of phosphoglucomutase or whether the enzyme preparation is contaminated by a very small amount of a phosphatase is not known.

Nature of the Bisphosphate Complex. Although the observed E_D·Cd·Glc-P_2 complex was shown to be catalytically competent in the overall saturation-transfer process, the present study does little to define its structure. However, spectral studies suggest that in the major fraction of the bisphosphate complex the 6-phosphate rather than the 1-phosphate is in position for phosphate transfer.[14] If a substantially unequal mixture of two complexes is indeed present, these might be in either slow or rapid exchange on the chemical shift time scale. The interpretational ambiguities produced by these uncertainties are considered in the accompanying paper (Ray et al., 1989).

Chemical Exchange Broadening. A potential problem in any saturation-transfer study is an apparent absence of one or more intermediates produced by chemical exchange broadening. Such broadening also can cause an apparent reduction in signal intensity, especially in a system where a slow conformational change separates otherwise identical reaction intermediates. For example, E_P·Cd forms two complexes with 6-deoxyglucose 1-phosphate, but only one produces a discrete NMR resonance; the other is in rapid exchange with and indistinguishable from free 6-deoxyglucose 1-phosphate (W. J. Ray, Jr., unpublished results, available on request). To rule out the importance of such effects in the present system, considerable attention was given to quantifying the ^{31}P NMR resonances attributed to E_D·Cd·Glc-P_2. The intensity of the two resonances of the bound bisphosphate were the same within experimental error, but both were about 10% less than that expected from previous rapid quench studies on equilibrium system, viz., accounted for 82 instead of 91% of the total enzyme present. Since the origin of this discrepancy is unknown, in our kinetic analysis we used a fractional abundance for the bisphosphate complex close to the average of the above two values: 0.85. Because the previously obtained values for the fractional abundance of the monophosphate complexes, 0.09, also was used, our analysis accounts for only 94% of the total enzyme present. However, this discrepancy should not

[14] The ultraviolet spectrum of the E_D·Cd·Glc-P_2 complex (or complexes) is closely similar to that of the E_P·Li·Glc-6-P complex and differs substantially from that of the E_P·Li-Glc-1-P complex (W. J. Ray, Jr., unpublished results), as might be inferred from Figure 2 of Ma and Ray (1980) and the discussion therein.

produce a serious error in the evaluation of those rate constants required to define saturation transfer in the present system (cf. eq 11).

(PO_3^-)-Transfer Efficiency of the Cd^{2+} Form of Phosphoglucomutase. Minimal estimates of the rate constants for (PO_3^-) transfer in the thermodynamically favorable direction, viz., from the phosphoenzyme to bound Glc-1-P or bound Glc-6-P (eq 14 and 15), were obtained from the measured values of k_1^{app} and k_6^{app} plus the relevant equilibrium constants (see Results). These values, 350 and 150 s^{-1}, show that in spite of its rather poor efficiency as a catalyst, the Cd^{2+} enzyme is quite respectable in its ability to facilitate (PO_3^-) transfer involving relatively low-energy phosphates. The relative efficiencies of the Cd^{2+} and Mg^{2+} enzymes in both (PO_3^-) transfer and catalysis are compared in the accompanying paper (Ray et al., 1989).

ACKNOWLEDGMENTS

We are grateful to Drs. C. R. Jones and R. E. Santini for their help with regard to instrumentation and to Dr. J. M. Puvathingal for preparation of the enzyme.

APPENDIX 1

The Equilibrium Constant for the Phosphoglucomutase Reaction and the Anomeric Equilibrium for Glucose 6-Phosphate. Since the muscle phosphoglucomutase reaction is specific for the α-anomer of Glc-1-P (Sutherland et al., 1949), it almost certainly produces α-Glc-6-P, although anomerization rapidly yields an equilibrium mixture of α- and β-anomers. In the present spin-transfer studies, little magnetization was lost from β-Glc-6-P when the phosphate groups of E_D·Cd·Glc-P_2 were irradiated for up to 4 s, during which time the magnetization for the α-anomer decreased to near its limiting value (see Figure 2.B3,B6). Moreover, the ^{31}P NMR resonance of β-Glc-6-P is not substantially broadened by the enzyme, in contrast to that of α-Glc-6-P (not shown). Hence, β-Glc-6-P apparently acts as an inert species with respect both to binding and, during short time intervals, to spin transfer. (Although β-Glc-6-P probably exhibits weak intrinsic binding to phosphoglucomutase, binding that occurs in the presence of excess α-Glc-6-P likely is relatively inefficient by comparison.) Hence, in modeling the phosphoglucomutase reaction, only the binding of α-Glc-6-P to the enzyme is considered. The equilibrium, [α-Glc-6-P]/[β-Glc-6-P], was measured by comparing resonance intensities of fully relaxed, proton-coupled ^{31}P NMR spectra of the two anomers (δ = 0.92 and 0.98 ppm, respectively). The value obtained, 0.62, is somewhat different from the value of 0.7 reported by Lowry and Passoneau (1968), who treated the anomeric mixture of Glc-6-P with the anomer-specific enzyme, glucose-6-phosphate dehydrogenase, and compared the rapid and slow phases of the reaction. On the basis of our value, the ratio α-Glc-6-P/Glc-1-P is equal to 7.1 when a value for the overall equilibrium constant for the phosphoglucomutase reaction ([α-Glc-6-P] + [β-Glc-6-P])/[Glc-1-P]) of 18.7 is used. The latter value was obtained from integrated areas of a fully relaxed spectrum of the three sugar phosphates in the presence of a catalytic amount of phosphoglucomutase. The value previously reported is 17 ± 2 (Atkinson et al., 1961).

APPENDIX 2

Multisite Exchange. For a nucleus in chemical exchange between multiple sites, the exchange between any two sites is described by the process

$$A \underset{k_B}{\overset{k_A}{\rightleftharpoons}} B$$

Selective irradiation at the resonance of site B leads to a decrease in the component of A magnetization aligned with the static field—the longitudinal or z component—due to a net flow of magnetization from A to B. In the special case of only two sites, the time dependence of the z component of A magnetization, M_A, and the steady-state intensity (i.e., after prolonged saturation of B) in site A, M_A^∞, are given by

$$M_A(t) = M_A^0\left[\frac{\tau_A}{T_{1,A}} + k_A\tau_A \exp\left(\frac{-t}{\tau_A}\right)\right] \quad (16)$$

$$M_A^\infty = M_A^0\left(\frac{1}{1 + k_A T_{1,A}}\right) \quad (17)$$

where $1/\tau_A$ equals ($k_A + 1/T_{1,A}$) and the intensity of A at zero irradiation time is M_A^0, the unperturbed equilibrium magnetization.

For the general case of exchange between n sites, the time dependence of the spectral intensities when site q is saturated is described by a set of ($n - 1$) simultaneous equations[15] of the form

$$\frac{dM_i}{dt} = \frac{M_i^0 - M_i}{T_{1,i}} - \sum_{j\neq i,q} k_{ij}M_i + \sum_{j\neq i,q} k_{ji}M_j \quad (18)$$

The first-order chemical exchange constant, k_{ij}, refers to transfer from site i to site j (cf. Table I). One method of simulating an exchange system involves numerical integration of eq 18. A time step equal to 0.1–0.01 of the smallest value of $1/(M_i^0 k_{ij})$ is required for convergence with the method. The large number of computations necessary in numerical integration is avoided by using a matrix solution to eq 18, which in matrix form becomes

$$\frac{d\mathbf{M}}{dt} = \Omega\mathbf{M} + \mathbf{K} \quad (19)$$

Here the elements of vector $\mathbf{M}$ are the time-dependent values M_i; those of $\mathbf{K}$ are $M_i^0/T_{1,i}$. The exchange rate matrix Ω has elements

$$\Omega_{ii} = -\frac{1}{T_{1,i}} - \sum_{j\neq i,q} k_{ij} \quad (20)$$

$$\Omega_{ij} = k_{ji}$$

The solution to eq 19 takes a form similar to eq 16.

$$\mathbf{M} = \mathbf{M}^\infty + \exp(\Omega t)[\mathbf{M}' - \mathbf{M}^\infty] \quad (21)$$

$$\mathbf{M}^\infty = -\Omega^{-1}\mathbf{K} \quad (22)$$

$$\mathbf{M}' = M_i^0 \quad (23)$$

The elements of $\mathbf{M}^\infty$ are the steady-state intensities at long irradiation times. To facilitate the evaluation of eq 21, the eigenvalues and eigenvectors of Ω are used.

$$M_i(t) = M_i^\infty + \sum_j J_{ij}[M_j^0 - M_j^\infty] \quad (24)$$

$$J_{ij} = \sum_k \exp(\lambda_k t) T_{ik} T_{kj}^{-1} \quad (25)$$

[15] The use of $n - 1$ rather than n equations assumes $M_q = 0$ for $t = 0$. Instantaneous saturation of the resonance of site q is not consistent with the properties of nuclear relaxation. Nonetheless, the error introduced by this assumption is negligible except when both the irradiated spin is at a high relative concentration and its relaxation rates plus the power of the selective pulse is such that the Torrey oscillation frequency is comparable to the exchange rate.

The eigenvalues of Ω are λ_i, $\mathbf{T}$ is the matrix of eigenvectors, and $\mathbf{T}^{-1}$ is the inverse of $\mathbf{T}$.

To perform the multisite exchange simulation, a FORTRAN program, which utilizes the International Mathematics Statistical Library (IMSL), was written and is available upon request (C.B.P.). Diagonalization of Ω to obtain λ and $\mathbf{T}$ is done with the IMSL routine EIGRF. Although a simple transform renders Ω symmetric (Grassi et al., 1986), thereby generating a well-conditioned eigenvalue problem and facilitating diagonalization, our analyses have not included symmetrization of Ω. No difficulties in diagonalization have been encountered.

The simulation procedure starts with initial estimates of the elements of Ω (k_{ij} and T_1) and the equilibrium intensities (M_i^0). The magnetization of spin i at any time t is then obtained by eq 24, and the time-dependent decay of the magnetization can be computed. Better estimates of the parameters indicated by a comparison of the simulated decay with experimental results are used in an iterative fashion. Alternatively, a nonlinear least-squares analysis has been applied by others (Muhandiran et al., 1987).

T_1 Relaxation Measurements. Values for spin–lattice relaxation times are needed in the analysis of chemical exchange rates from magnetization-transfer experiments. Although T_1 values could be treated in the computer simulation as variables, similar to k_{ij} values, they were independently measured in nonselective inversion recovery experiments. This procedure aided the analysis by decreasing the number of parameters. Because sample conditions affect the spin–lattice relaxation time, conditions close to those used for magnetization-transfer experiments were employed when T_1 was measured. In particular, the effect of viscosity on T_1 can be large (see Results). When T_1 values are measured, the possible influence of chemical exchange was negated by eliminating excess free ligand for enzyme-bound sites or by using a sufficiently large ratio of ligand to enzymic binding sites for small molecules. The T_1 values for all resonances in Figure 3 are 5.0 ± 0.5 s.

Registry No. Glc-1-P, 59-56-3; Glc-6-P, 15209-11-7; Cd, 7440-43-9; phosphoglucomutase, 9001-81-4.

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