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 (PO₃⁻) group to the 6-hydroxyl group of Glc-1-P to generate the phosphoenzyme (Ray et al., 1985). Hence, studies 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.

At equilibrium in the enzymic reaction involving Mg²⁺, the intermediate E₉-Mg·Glc-P₂ complex accounts for about half of the total enzyme, while the monophosphate complexes, E₉-P₂ and E₉-Li⁺ forms of the enzyme in the accompanying paper.

Efficient catalysis by muscle phosphoglucomutase requires a bound metal ion activator. Although Mg²⁺ 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.

At equilibrium in the enzymic reaction involving Mg²⁺, the intermediate E₉-Mg·Glc-P₂ complex accounts for about half of the total enzyme, while the monophosphate complexes,
Phosphoglucomutase Reaction, Showing All Identified Intermediates and the Rate Constants for Their Interconversion

\[
\begin{align*}
\text{Ep} \cdot \text{M} \cdot \text{Glc-1-P} & \quad \frac{k_{11}}{k_{12}} \quad \text{Ep} \cdot \text{M} \cdot \text{Glc-6-P} \\
\text{E}_p \cdot \text{M} \cdot \text{Glc-1-P} & \quad \frac{k_{11}}{k_{12}} \quad \text{E}_p \cdot \text{M} \cdot \text{Glc-6-P} \\
\text{E}_p \cdot \text{M} \cdot \text{Glc-1-P} & \quad \frac{k_{16}}{k_{16}} \quad \text{E}_p \cdot \text{M} \cdot \text{Glc-6-P}
\end{align*}
\]

Ep-Mg-Glc-1-P and Ep-Mg-Glc-6-P, account for the remainder. However, with Cd\textsuperscript{2+} as the bound metal ion, about 90\% of the equilibrium mixture is Ep-Cd-Glc-6-P. Although the predominance of the bisphosphite intermediate would make the Cd\textsuperscript{2+} enzyme more amenable to the study of the (PO\textsubscript{3}\textsuperscript{-})-transfer steps that produce this complex, the Cd\textsuperscript{2+} enzyme is only about 1\% as active as the Mg\textsuperscript{2+} enzyme in initial velocity studies at saturating Glc-1-P (Ray, 1969; Ray et al., 1989). Since Cd\textsuperscript{2+} is significantly larger than Mg\textsuperscript{2+} and exhibits a different coordination preference, its interaction with the bound bisphosphite may differ, qualitatively as well as quantitatively, from that of Mg\textsuperscript{2+}. Thus, catalysis by the Cd\textsuperscript{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\textsuperscript{2+} activation of phosphoglucomutase by measuring (PO\textsubscript{3}\textsuperscript{-})-transfer rates via time-dependent \textsuperscript{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 that the intensities of the resonances in \textsuperscript{31}P NMR spectra account for essentially all of the enzyme in the system. Next, saturation-transfer studies are described that provide assignments for \textsuperscript{31}P resonances and validate the catalytic competence of the enzyme 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 \textsuperscript{31}P isotope exchange and initial velocity. A redetermination of values for the \(\alpha,\beta\)-anomeric equilibrium constant of Glc-6-P and the overall equilibrium constant of the phosphoglucomutase reaction, which are required for the above analysis, are also 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\textsubscript{3}\textsuperscript{-}) 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 EpCd-Glc-1-P and EpCd-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

\[X \quad \frac{k_x}{k_y} Y \quad \frac{k_y}{k_x} Z\]

when both \([Y]/[X]\) and \([Y]/[Z]\) are less than about 0.1, magnetization exchange from \(X\) to \(Z\) after saturating spin \(Z\), or from \(Z\) to \(X\) after saturating spin \(X\), can be represented as

\[X \quad \frac{k_{pp}}{k_{np}} Y \quad \frac{k_{np}}{k_{pp}} Z\]

and the time dependence of the exchange is approximated by single exponentials, \(\exp\{-(k_{pp} + T_1^{-1})t\}\) and \(\exp\{-(k_{np} + T_1^{-1})t\}\). Hence, where appropriate, \(X\) \(\approx\) \(Y\) \(\approx\) \(Z\) segments of Scheme I were treated as \(X\) \(\approx\) \(Z\) segments (e.g., the top half of Scheme I becomes EpM + Glc-1-P \(\approx\) EpM-Glc-P2). 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 \(k_{pp}\) and \(k_{np}\) are net rate constants (Cleland, 1975). Note that the expressions for \(k_{pp}\) and \(k_{np}\) can be factored in two different ways:

\[k_{pp} = \frac{k_xk_y}{k_{xx} + k_{yy}} \leq k_x \leq k_y \quad (3)\]

\[k_{np} \leq k_{xx}k_{yy} - k_{xy} \leq k_{xy} \quad (4)\]

\[k_{np} = \frac{k_xk_y}{k_{xx} + k_{yy}} \leq k_x \leq k_y \quad (5)\]

\[k_{np} \leq k_{xx}k_{yy} - k_{xy} \leq k_{xy} \quad (6)\]

Although not immediately apparent from Scheme I, in the phosphoglucomutase reaction exchange of magnetization between the (PO\textsubscript{3}\textsuperscript{-}) groups of substrate (Glc-1-P) and product (Glc-6-P) pools requires two complete catalytic cycles. This feature of the reaction is shown by the sequence of steps in eq 7 and 8, where the intermediate EpCd-Glc-1-P and EpCd-Glc-6-P complexes are omitted and an asterisk is used to indicate the nucleus involved in magnetization exchange.

\[\text{Glc-1-P} + (\text{EpCd}) = \text{EpCd-Glc-1-P} \quad \text{Glc-1-P} \quad \text{EpCd-Glc-6-P} \quad \text{EpCd-Glc-6-P} + (\text{EpCd}) \quad (7)\]

\[\text{Glc-6-P} \quad \text{EpCd-Glc-1-P} \quad \text{EpCd-Glc-6-P} \quad \text{EpCd-Glc-6-P} + (\text{EpCd}) \quad (8)\]

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

---

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

\[4\quad \text{The analogous transfer involving the glucose moiety would require only a single cycle, since there is no glycosyl-enzyme intermediate. Hence, one glucose moiety must be transferred from substrate to product pools in each catalytic cycle.}\]
Table I: Matrix Showing Rate Constants for Simulation of $^{31}$P Magnetization Exchange Catalyzed by Cd(II) Phosphoglucomutase

<table>
<thead>
<tr>
<th></th>
<th>Ep-Cd</th>
<th>Glc-1-P</th>
<th>Ep-Cd-P*-1-Glc-6-P</th>
<th>Ep-Cd-P-1-Glc-6-P*</th>
<th>Glc-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ep-Cd</td>
<td>nd</td>
<td>--</td>
<td>$k_{1aPP}$[Glc-6-P]</td>
<td>$k_{1aPP}$[Glc-1-P]</td>
<td>--</td>
</tr>
<tr>
<td>Glc-1-P</td>
<td>--</td>
<td>nd</td>
<td>$k_{1aPP}$[Ep-Cd]</td>
<td>nd</td>
<td>--</td>
</tr>
<tr>
<td>Ep-Cd-P*-1-Glc-6-P</td>
<td>$k_{4aPP}$</td>
<td>$k_{1aPP}$</td>
<td>nd</td>
<td>nd</td>
<td>$k_{4aPP}$[Ep-Cd]</td>
</tr>
<tr>
<td>Ep-Cd-P-1-Glc-6-P*</td>
<td>$k_{4aPP}$</td>
<td>$k_{1aPP}$</td>
<td>nd</td>
<td>nd</td>
<td>$k_{4aPP}$[Ep-Cd]</td>
</tr>
<tr>
<td>$\alpha$-Glc-6-P</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>$k_{4aPP}$[Ep-Cd]</td>
<td>nd</td>
</tr>
</tbody>
</table>

*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 identified by nd; $k_{ij}$ values not allowed by the reaction sequence in eq 11 are indicated by --. Rate constants refer to phosphates indicated by \( \ast \).

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, $k_{1aPP}[Ep-Cd]$ (=$k_{1aPP}$) and $k_{4aPP}[Ep-Cd]$ (=$k_{4aPP}$), plus values of two equilibrium constants, $[Glc-1-P]/[Ep-Cd-1-Glc-P]$ (=$k_{1aPP}$) and $[\alpha-Glc-6-P]/[Ep-Cd\alpha-Glc-P]$ (=$k_{4aPP}$), together with the known concentrations of Glc-1-P and $\alpha$-Glc-6-P (see Appendix 1) suffice to define the exchange process. Values for $k_{ob}$ and $k_{oa}$ 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 Ep-Cd-Glc-P 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 $E_{275}$ = 0.70 and $M$, 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$_3$ ≈ 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 $^1$H 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 $\mu$s, 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 $\mu$s. 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, $\tau$. 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.,
The spectra were obtained without ¹H 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 T₁ value for EP-Cd is 4.7 s (Rhyu et al., 1984) and for both phosphates of EP-Cd-Glc-P₂ 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 EP-Cd (at 1.65 ppm) accounts for all of the expected intensity, based on the known concentrations of EP-Cd²⁻ and tetraphenylphosphonium ion present, 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 T₁ relaxation differences produced by the 15-s recycle time with the above value of T₁ for the enzymic phosphate and a value of 7.0 s for the tetraphenylphosphonium ion, measured under the conditions of the experiment. In contrast, a similar analysis of the spectrum for the EP-Cd-Glc-P₂ complex, which was produced by the addition of 1 equiv of glucose monophosphate to EP-Cd (Rhyu et al., 1984), 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 EP-Cd. In the presence of 2 equiv of Cd²⁺ (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%. 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 EP-Cd-Glc-P₂ 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 EP (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 EP-Cd-Glc-P₂ spectrum with a factor of 5 Hz was applied (see Experimental Procedures).
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; $E_p$-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 $E_p$-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 $E_p$-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 $E_p$-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 $E_p$-Cd-Glc-P$_2$(obs) were tentatively assigned by analogy with those of $E_p$-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$_4^-$)$^n$ groups of $E_p$-Cd-Glc-P$_2$(obs) with excess free Glc-1-P and excess free Glc-6-P, via (PO$_4^-$)$^n$ 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 31P 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 2B1) is faster than that between the irradiated peak at +5.2 ppm and Glc-1-P (Figure 2B4). Similarly, the chemical exchange rate involving the irradiated peak at +5.2 ppm and Glc-6-P (Figure 2B6) is faster than that between the irradiated peak at −2.75 ppm and Glc-6-P (Figure 2B3). Hence, the 1-phosphate and 6-phosphate of $E_p$-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 $E_p$-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 $Ca^{2+}$ Enzyme Catalytically Competent?** If the observed $E_p$-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 D is irradiated, the approach to the new steady-state magnetization for species A, B, and C must be rate$_A$ ≤ rate$_B$ ≤ rate$_C$.) If $E_p$-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 2B7–B9) was substantially smaller for the 6-phosphate of $E_p$-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 $E_p$-Cd-Glc-P$_2$(obs), as is required if $E_p$-Cd-Glc-P$_2$(obs) is an obligate intermediate in the conversion of Glc-1-P to Glc-6-P. Hence, all computer simulations of magnetization exchange (see below) were conducted with sequential models.

**Initial Evaluation of Kinetic Constants Required To Model 31P Magnetization Transfer.** Modus Operandi shows that magnetization transfer among the (PO$_4^-$)$^n$ 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$_4^-$)-transfer step.

\[
Glc-1-P^* \xrightarrow{k_{obs}} E_p$-Cd-P$_6$-Gluc-1-P^* \\
Glc-6-P^* \xrightarrow{k_{obs}} E_p$-Cd-P$_6$-Gluc-1-P
\]

In these equations, which omit the monophosphate complexes $E_p$-Cd-Glc-1-P and $E_p$-Cd-Glc-6-P (see Modus Operandi), $k_{obs}[E_p$-Cd] = $k_{obs}$ and $k_{obs}[E_p$-Cd] = $k_{obs}$. The magnetization decay for Glc-1-P or Glc-6-P when the 1- or 6-phosphate, respectively, of $E_p$-Cd-Glc-P$_2$(obs) is irradiated, is shown in Figure 2B1, B6. Under these conditions (at intermediate enzyme/substrate ratio; see Figure 2, legend), the values of the first-order rate constants, $k_{obs}$ and $k_{obs}$, obtained by modeling the data with an exponential decay function

\[12\]
**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 EpCdc-Glc-P2 (see Results). Initial conditions were (A) (high enzyme/substrate ratio) 1.62 mM EpCd plus 1.62 mM Glc-6-P, (B) (intermediate enzyme/substrate ratio) 1.62 mM EpCd plus 3.2 mM Glc-1-P or Glc-6-P, and (C) (low enzyme/substrate ratio) 0.203 mM EpCd plus 50.6 mM Glc-6-P. (Cases A–C are further differentiated in Tables I and II.) 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 ED.Cd.Glc-P2(6obs), 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 (0 or A, for different experiments) or by low-power (0) 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 $k_{1app}$ and $k_{Sapp}$ 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 $k_{1app}$ and $k_{Sapp}$. Values for $k_{1app}$ and $k_{Sapp}$ 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 $[EpCdc] = 1.3 \times 10^{-7}$ M (see Table III), $k_{1app}$ and $k_{Sapp}$ are 5.1 and 0.75 $\times 10^7$ s$^{-1}$, respectively. From these values, estimates for the constants $k_{1app}$ and $k_{Sapp}$ for the reverse process (cf. eq 9 and 10) can be obtained from the concentration ratios used in the experiment (see Table III): $k_{1app} = 8.0$ s$^{-1}$ and $k_{Sapp} = 8.5$ 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...
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. For example, although the flow of magnetization from the phosphate of free Glc-6-P to the 6-phosphate of bound Glc-P₂, considered above, involves a single half-reaction, magnetization transfer from free Glc-6-P to the 1-phosphate of bound Glc-P₂ involves three successive half-reactions; see eq 7 and 8. The various sequences of half-reactions monitored by 

<table>
<thead>
<tr>
<th>Case</th>
<th>$\text{Glc-1-P}^*$</th>
<th>$\text{EpP-6-Glc-1-P}^*$</th>
<th>$(\text{Ep}^*)$</th>
<th>$\text{EpP}^*6$-Glc-1-P</th>
<th>$\text{Glc-6-P}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>obs</td>
<td></td>
<td>$\text{irrad}$</td>
<td>obs</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>obs</td>
<td>irrad</td>
<td>obs</td>
<td>obs</td>
<td>obs</td>
</tr>
<tr>
<td>C</td>
<td>obs</td>
<td>irrad</td>
<td>obs</td>
<td>obs</td>
<td>irrad</td>
</tr>
<tr>
<td>C</td>
<td>obs</td>
<td>irrad</td>
<td>obs</td>
<td>obs</td>
<td>irrad</td>
</tr>
</tbody>
</table>

*Each set of arrows (\(\rightarrow\)) represents one half-reaction (binding step plus (PO₄)⁻-transfer step; see Results) by which magnetization is exchanged among the phosphates in the reaction sequence indicated by the asterisks. Columns beneath phosphates is readily obtained. The various cases are further described in Table III and Figure 2.*

A typical group of spectra obtained in magnetization-exchange experiments is shown in Figure 3, where the 1-phosphate of Ep-Cd-Glc-P₂(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, Ep-Cd-Glc-1-P, Ep-Cd-Glc-6-P, and Ep-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 

$\text{EpP}_{\text{Cd}} + \text{Glc-1-P} \xrightarrow{k_{\text{app}}^{\text{EpP}_{\text{Cd}}}} \text{EpP}_{\text{Cd}} + \text{Glc-6-P}$

as initial estimates for these parameters. The relative concentrations of species were those listed in Table III, while values of $T_i$ for $\text{EpP}_{\text{Cd}},$ 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., $k_{\text{app}}^{\text{EpP}_{\text{Cd}}}$ and $k_{\text{app}}^{\text{EpP}_{\text{Cd}}}$ were increased from $5.1 \times 10^7$ to $7.7 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and from $0.75 \times 10^7$ to $1.0 \times 10^7 \text{M}^{-1} \text{s}^{-1}$, respectively (which provides...
### Table III: Rate Constants and Relative Concentrations Used in Computer Simulations of the Time Dependence for Magnetization Exchange

<table>
<thead>
<tr>
<th>Case</th>
<th>[enzyme]/[substrate]</th>
<th>Relative concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>free Glc-1-P</td>
<td>free α-Glc-6-P</td>
</tr>
<tr>
<td>A high</td>
<td>(0.003)³</td>
<td>(0.02)²</td>
</tr>
<tr>
<td>B intermediate</td>
<td>1.94</td>
<td>13.9</td>
</tr>
<tr>
<td>C low</td>
<td>initial value</td>
<td>final value</td>
</tr>
<tr>
<td>k_{αP}</td>
<td>5.1 x 10⁷ M⁻¹ s⁻¹</td>
<td>7.7 x 10⁷ M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>k_{βP}</td>
<td>8.0 s⁻¹</td>
<td>12 s⁻¹</td>
</tr>
<tr>
<td>k_{αP}</td>
<td>0.75 x 10⁷ M⁻¹ s⁻¹</td>
<td>1.0 x 10⁷ M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>k_{γP}</td>
<td>8.5 x 10⁶ M⁻¹ s⁻¹</td>
<td>11 s⁻¹</td>
</tr>
</tbody>
</table>

*Relative equilibrium concentrations (0.67 of the actual value) for species present at different enzyme/substrate ratios, i.e., 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). *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 $\mu$M (Ray & Long, 1976). At the intermediate enzyme/substrate ratio, $[Ep-Cd] = 1.3 x 10^{-5}$ M. *Value that did not affect simulations as long as it was low relative to total enzyme concentration. *Arbitrary value selected for computational convenience. *For definitions, see eq 11. *Values estimated from a single-exponential fit of the time dependence of the intensity loss in the resonance from free glucose monophosphates 13 (measurable concentrations). Of the other known bivalent metal ion activators (Ray, 1969), most are paramagnetic and thus are unsuitable for NMR exchange studies. *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).

The observed trends in residual magnetization are consistent with the simulated trends in the absence of (measurable concentrations of) free glucose phosphates 13 (see 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 = 16:17) (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-Cd²⁺ 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:

$$[Ep-Cd-Glc-1-P]/[Glc-1-P] = 2.9 x 10^{-2} \quad (12)$$

$$[Ep-Cd-α-Glc-6-P]/[α-Glc-6-P] = 8.8 x 10^{-3} \quad (13)$$

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

$$k_+^+ \frac{[Ep-Cd]}{k_{11}} \frac{k_{αP}^+}{k_{αP}} \frac{k_{γP}^+}{k_{γP}} \frac{Ep-Cd-Glc-P_2}{Ep-Cd-Glc-P_4}$$

$$\quad (14)$$

$$\quad (15)$$

By use of the best overall values of $k_{αP}^+$ (10.2 s⁻¹) and $k_{γP}^+$ (1.3 s⁻¹) at the intermediate enzyme/substrate ratio (see Figure 2 legend), minimum values for $k_+^+$ (transfer to bound Glc-1-P) and $k_{αP}^+$ (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 Mg²⁺ produces the most efficient transfer of the $(PO_3^-)$ moiety between donor and acceptor hydroxyl groups of bound glucose phosphates. However, the Mg²⁺ enzyme represents an intractable system for study by magnetization transfer since rapid chemical exchange between enzyme 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, Cd²⁺ produces the most attractive system for such studies, even though the Cd²⁺ enzyme is only about 1% as efficient as the Mg²⁺ enzyme in terms of $k_{cat}$. Thus, in the Cd²⁺ 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 0.1 $T_1$, 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 between the observable species can be readily measured by time-dependent $^{31}$P magnetization exchange. (Since $T_1$ relaxation counters 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 Cd²⁺ 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 $Ep-Cd-Glc-1-P$ and $Ep-Cd-Glc-6-P$, Scheme I simplifies to eq 11, where the binding of glucose monophosphates and the subsequent transfer of the enzyme 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 $^{31}$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 $A \rightarrow B \rightarrow C$ 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 $E_{0}Cd$-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 $T_I$ as well as the chemical exchange rate, $T_I$ 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 $T_I$ 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 3$T_I$; if the full, unperturbed equilibrium magnetization is to be recovered between successive transients. However, the $T_I$ 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 $T_I$, flip angle, and saturation level at long irradiation times, the error can be made relatively small: $<10\%$.

**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 $E_{0}Cd$-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. 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, $E_{0}Cd$ 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 $E_{0}Cd$-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

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14 The ultraviolet spectrum of the $E_{0}Cd$-Glc-P$_{2}$ complex (or complexes) is closely similar to that of the $E_{0}Li$-Glc-6-P complex and differs substantially from that of the $E_{0}Li$-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).

**(P**O**3**-) Transfer Efficiency of the Cd**(2+)

Form of Phosphoglucomutase) Minimal estimates of the rate constants for (P**O**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 \(k_4^{PP}\) and \(k_6^{PP}\) 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 (P**O**3**-) transfer involving relatively low-energy phosphates. The relative efficiencies of the Cd**(2+)

and Mg**(2+)

enzymes in both (P**O**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 \(\alpha\)-anomer of Glc-1-P (Sutherland et al., 1949), it almost certainly produces \(\alpha\)-Glc-6-P, although anomeration rapidly yields an equilibrium mixture of \(\alpha\)- and \(\beta\)-anomers. In the present spin-transfer studies, little magnetization was lost from \(\beta\)-Glc-6-P when the phosphate groups of E**2**Gcd-Glc-P**2** were irradiated for up to 4 s, during which time the magnetization for the \(\alpha\)-anomer decreased to near its limiting value (see Figure 2B3,B6). Moreover, the \(\textsuperscript{31}P\) NMR resonance of \(\beta\)-Glc-6-P is not substantially broadened by the enzyme, in contrast to that of \(\alpha\)-Glc-6-P (not shown). Hence, \(\beta\)-Glc-6-P apparently acts as an inert species with respect both to binding and, during short time intervals, to spin transfer. (Although \(\beta\)-Glc-6-P probably exhibits weak intrinsic binding to phosphoglucomutase, binding that occurs in the presence of excess \(\alpha\)-Glc-6-P likely is relatively inefficient by comparison.) Hence, in modeling the phosphoglucomutase reaction, only the binding of \(\alpha\)-Glc-6-P to the enzyme is considered. The equilibrium, \([\alpha\text{-Glc-6-P}]/[\beta\text{-Glc-6-P}]\), was measured by comparing resonance intensities of fully relaxed, proton-coupled \(\textsuperscript{31}P\) NMR spectra of the two anomers (in = 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 anomerspecific enzyme, glucose-6-phosphate dehydrogenase, and compared the rapid and slow phases of the reaction. On the basis of our value, the ratio \([\alpha\text{-Glc-6-P}]/\text{Glc-1-P} = 7.1\) when a value for the overall equilibrium constant for the phosphoglucomutase reaction \(([\alpha\text{-Glc-6-P}]/\text{Glc-1-P}]_0) = 18.7\) 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

\[
\text{A} \xrightarrow{\text{k}_a} \text{B}
\]

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, \(M_A^\infty\), are given by

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

where \(1/\tau_A = (k_A + 1/T_{1A})\) 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

\[
\frac{dM_i}{dt} = M_i^0 - M_i \sum_{j \neq q} k_{ij} M_j + \sum_{j \neq q} k_{ji} M_j
\]

The first-order chemical exchange constant, \(k_{ij}\), refers to transfer from site \(i\) to site \(j\) (cf. Table 1). 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

\[
\frac{dM}{dt} = \Omega M + K
\]

Here the elements of vector \(M\) are the time-dependent values \(M_i\); those of \(K\) are \(M_i^0/T_{1ij}\). The exchange rate matrix \(\Omega\) has elements

\[
\Omega_{ij} = -\frac{1}{T_{1ij}} - \sum_{q \neq i} k_{ij}
\]

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

\[
M = M^\infty + \exp(\Omega t) [M^0 - M^\infty]
\]

\[
M^\infty = -\Omega^{-1} K
\]

\[
M^0 = M^0_i
\]

The elements of \(M^\infty\) are the steady-state intensities at long irradiation times. To facilitate the evaluation of eq 21, the eigenvalues and eigenvectors of \(\Omega\) are used.

\[
M_i(t) = M_i^0 + \sum_j M_j^0 [M_j^0 - M_i^\infty] \exp(\lambda_{kj} t) T_{ik} T_{kj}^{-1}
\]

\(^{15}\) The use of \(n - 1\) rather than \(n\) equations assumes \(M_j = 0\) for \(i = j\). 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 $\Omega$ are $\lambda$, $T$ is the matrix of eigenvectors, and $T^{-1}$ is the inverse of $T$.

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 $\Omega$ to obtain $\lambda$ and $T$ is done with the IMSL routine EIGRF. Although a simple transform renders $\Omega$ symmetric (Grassi et al., 1986), thereby generating a well-conditioned eigenvalue problem and facilitating diagonalization, our analyses have not included symmetrization of $\Omega$. No difficulties in diagonalization have been encountered.

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The simulation procedure starts with initial estimates of the elements of $\Omega$ ($k_{ij}$ and $T_{ij}$) and the equilibrium intensities ($M^0$). 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).

**T1 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 $5.0 \pm 0.5$ s.

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

**REFERENCES**


