Role of Bivalent Cations in the Phosphoglucomutase System. IV. A Study of the Mn^{2+} Binding Site by Means of Nuclear Relaxation Measurements on Water Protons*

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ABSTRACT: The ability of Mn^{2+} to facilitate nuclear-spin relaxation of water protons, *i.e.*, the relaxivity of Mn^{2+}, is increased by about ninefold on binding to phosphoglucomutase. However, subsequent formation of a ternary complex by addition of glucose phosphate to the binary enzyme·Mn complex produces a fourfold decrease in the relaxivity of bound Mn^{2+}. The binding of other substrates as well as inhibitors produces a gradation of effects such that the relative relaxivities of the various ternary complexes are approximately parallel to the magnitude of the dissociation constants of substrates or inhibitors from these complexes, *i.e.*, the smaller the dissociation constant, the smaller the relaxivity. The temperature dependence of the longitudinal and transverse relaxation rates of water protons was measured from 0 to 50° in the presence of the binary enzyme·Mn complex or various ternary complexes involving both substrates and inhibitors. The results suggest that the number of water molecules directly bound to Mn^{2+} in the binary complex is decreased in ternary complexes involving *tightly bound* substrates or inhibitors such as glucose phosphate, xylose 1-phosphate, or glucuronic acid 1-phosphate; however, binding of those substrates and inhibitors that form *weak* ternary complexes, such as the phosphates of fructose, galactose, and mannose as well as inorganic phosphate, does not involve water displacement from Mn^{2+}. Because of the lack of water displacement by inorganic phosphate, water displacement on binding of good substrates and inhibitors probably does not involve participation of the phosphate group of these compounds. Instead, the 2- and 3-hydroxyl groups of glucose phosphate may be bound to Mn^{2+} in the enzyme·Mn·glucose phosphate complex since the binding of ribose phosphate or 2-deoxyglucose-P apparently displaces less water than does binding of glucose phosphate. Thus the metal ion in phosphoglucomutase may serve primarily to orient bound substrates at the catalytic site rather than being directly involved in bond-breaking or bond-making steps.

Phosphoglucomutase (EC 2.7.5.1) requires a bivalent metal ion for enzymatic activity. Previous studies show that a variety of metal ions will satisfy this requirement but elicit quite different activities. Thus the ratio of activities with Mg^{2+} and Zn^{2+}, the best and the poorest activators that were studied, is 140 to 1 at high and about 1000 to 1 at low pH (Ray, 1969). Ultraviolet difference spectra and solvent perturbation studies indicate that binding of Zn^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, or Cd^{2+} produces a structural alteration in the enzyme that involves several aromatic chromophores, both in the presence and in the absence of substrate. In the *absence* of substrate all metals produce an identical alteration (Peck and Ray, 1969a); in the *presence* of substrate each metal produces a characteristically different alteration that can be correlated with the activity elicited by that metal ion (Peck and Ray, 1969b). However, unequivocal evidence has not as yet been obtained that the bound metal ion participates directly in catalysis, or that the substrate and metal interact directly in the catalytically active enzyme·metal·glucose phosphate complex.

The present investigation and that described in a subsequent paper (W. J. Ray, Jr., J. S. Multani, and J. W. Long, manuscript in preparation) were undertaken to study metal–substrate interaction in the catalytically active complex by evaluating changes in the properties of bound metal ions, particularly Mn^{2+}, Ni^{2+}, and Co^{2+}, when substrates and substrate analogs were subsequently bound.

One of the properties that can be studied in the case of Mn^{2+} is its efficiency in accelerating nuclear relaxation of water protons. Such relaxation is caused by interaction of the electronic spin of the unpaired electrons of Mn^{2+} with the nuclear spins of nearby protons, and may affect protons of water molecules in direct contact with Mn^{2+} (inner sphere effect) as well as those outside the primary hydration sphere (outer sphere effect), although the latter effect is much smaller (see review by Mildvan and Cohn, 1970). Induced nuclear relaxation is propagated throughout the bulk water phase by rapid exchange between water molecules in this phase and molecules sufficiently close to Mn^{2+} to experience induced relaxation (Luz and Meiboom, 1964). Hence, processes which modulate the Mn^{2+}–proton interaction in the primary hydration sphere or affect exchange between the primary hydration sphere and bulk water may alter the efficiency of proton relaxation in bulk water (Bloembergen and Morgan, 1961; Bloembergen, 1957; Bloembergen *et al.*, 1948). Thus if binding of substrate to an enzyme·Mn complex eliminated all inner sphere water sites on the bound Mn^{2+}, thereby changing the induced relaxation from an inner to an outer

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sphere process, a substantial decrease in the efficiency, *i.e.*, rate of induced relaxation, would be expected, provided other factors remained the same. A smaller decrease in relaxation rate should be observed if, for example, four water sites were present on Mn^{2+} in an enzyme·Mn complex, but two in the enzyme·Mn^{2+}· substrate complex. The decrease in proton relaxation rate produced by binding of ADP and ATP to the pyruvate kinase·Mn^{2+} complex has been rationalized in this manner (Mildvan and Cohn, 1966).

The present report describes an investigation both of the longitudinal and tranverse proton relaxation rate of water for the binary phosphoglucomutase·Mn complex, E_P·Mn,[1] and of the effect on these processes of substrate and inhibitor binding to E_P·Mn.

Experimental Section

Materials. Procedures for the preparation of the phospho form of phosphoglucomutase (Ray and Koshland, 1962), glucose-1-P and glucose-6-P (Ray and Roscelli, 1964) have been described. A molecular weight of 62,000 (Filmer and Koshland, 1963) and an extinction coefficient of 7.7 for a 1% solution (Najjar, 1955) were used in calculations of enzyme concentration.

Ultra Pure Tris was obtained from the Mann Co. α-D-Fructose-1-P, α-D-mannose-1-P, α-D-galactose-1-P, 2-deoxy-α-D-glucose-6-P, α-D-ribose-1-P, α-D-xylose-1-P, and α-D-glucuronic acid-1-P were obtained from Sigma and used without further purification. Other reagents were of the highest purity available. The chlorides of Tris and of all metals were used throughout.

Metal-free phosphoglucomutase was prepared at pH 7.4 as described previously (Ray, 1969) except that enzyme concentrations as high as 150 mg/ml were used and EDTA was substituted for CDTA.[1] The Mn^{2+} complex of the enzyme was prepared by adding solutions of metal chloride directly to the metal-free enzyme; an excess of enzyme was maintained in most experiments.

Magnetic Resonance Measurements. The longitudinal relaxation time, T_1, of water protons was measured by using the pulsed method of Carr and Purcell (1954) at 24.3 MHz, as described by Mildvan and Cohn (1963); the transverse relaxation time, T_2, was measured by means of the spin-echo envelope (Carr and Purcell, 1954). A NMR Specialties PS60W pulsed nuclear magnetic resonance spectrometer was used for most of these studies. The reciprocal of the measured relaxation time of the metal-free buffer or enzyme–buffer sample, $1/T_{1(0)}$, was subtracted from the reciprocal of the relaxation time measured after additions of Mn^{2+} to give the paramagnetic relaxivity, $1/T_{1P}$ or $1/T_{1P}$* (the asterisk denotes parameters related to complexes of Mn^{2+} as opposed to parameters relating to free Mn^{2+}). An analogous procedure was used for $1/T_{2P}$ and $1/T_{2P}$*. Initial volumes of 0.05 or 0.1 ml were used for all measurements; when subsequent additions were made, relaxivity values were corrected for volume changes before use in further calculations. The enhancement of the effect on proton relaxation rate when Mn^{2+} was present as a complex is defined as $\epsilon = 1/T_{1P}^*/1/T_{1P}$ (Cohn and Leigh, 1962). The effect of temperature on the proton relaxation rate of water protons was studied with a variable-temperature probe with a temperature control of ±1°.

Free Mn^{2+} was measured by determining the amplitude of its electron paramagnetic resonance spectrum (Cohn and Townsend, 1954) by using a Varian Model E-3 spectrometer at 9.5 GH_z.

Results

Since phosphoglucomutase catalyzes the transfer of the phosphate group between the 1 and 5 positions of ribose and between the 1 and 6 positions of glucose, galactose, mannose, fructose (Lowry and Passonneau, 1969), and, presumably, 2-deoxyglucose,[2] when these sugar phosphates were used to form enzyme·metal·substrate complexes they were converted into equilibrium mixtures in a much shorter time than required for making the various measurements; thus no time-dependent changes subsequent to addition of substrate were observed in any of the studies described herein. Hence, the above sugar phosphates are referred to as ribose-P, glucose-P, galactose-P, mannose-P, fructose-P, and 2-deoxyglucose-P, respectively, even though the original phosphate was, in most cases, a single isomer; complexes of these phosphates with the enzyme are designated as E_P·Mn·sugar-P.[3] At equilibrium the 5- or 6-phosphate isomers predominate over the corresponding 1-phosphates in solution; however, the distribution of bound isomers is not known.

Titration of Phosphoglucomutase with Mn^{2+} Followed by Changes in Proton Relaxation Rate. The binding of Mn^{2+} to a sample of metal-free phosphoglucomutase was followed by measuring the increase in $1/T_{1P}$* as a function of added Mn^{2+}; a control titration was conducted in the absence of the enzyme. Figure 1 indicates that enzyme-bound Mn^{2+} induces a greater proton relaxation rate than free Mn^{2+}. The sharp break in the curve at a 1:1 stoichiometry is expected because of the high concentration of enzyme, 0.102 mM, relative to the dissociation constant of the E_P·Mn complex, about 10^{-7} M, under these conditions (Ray, 1969). An enhancement, ϵ, of 9.2 may be calculated for the binary enzyme·Mn complex from the ratio of the initial slopes of these plots.

When a total of 2.35 equiv of Mn had been added, the concentration of free Mn^{2+} in both samples (with and without enzyme) was compared by means of electron paramagnetic resonance signals and the bound Mn^{2+} in the enzyme sample determined as the difference between the Mn^{2+} added and the free Mn^{2+}.[4] The bound Mn^{2+} measured in this manner was 18 ± 5% (std dev) greater than that calculated from

[1] Abbreviations used are: E_P, phosphoenzyme; E_D, dephosphoenzyme; CDTA, cyclohexyldiaminetetraacetic acid; GP or Glu-P (in equations and other abbreviated expressions), glucose phosphate.

[2] Since inversion of configuration at the 2-hydroxyl group of glucose-6-P does not eliminate the capacity for phosphate exchange with phosphoglucomutase (Lowry and Passonneau, 1969), it does not seem likely that removal of the 2-hydroxyl group would completely eliminate this potential, although 2-deoxyglucose-6-P might well be a very poor substrate; however, this has not been verified experimentally.

[3] Stoichiometry, only, is indicated by the formulation, E_P·M·sugar-P, not the position of phosphate attachment. Such complexes are actually a mixture of position isomers involving, for example, E_P·M·glucose-1-P, E_D·M·glucose-1,6-P_2, and E_P·M·glucose-6-P (Ray and Roscelli, 1966).

[4] Neither the E_P·Mn nor the E_P·Mn·glucose-P complexes give detectable signals at room temperature at either X-band or K-band frequencies, in the concentration ranges used here (G. Reed, personal communication).

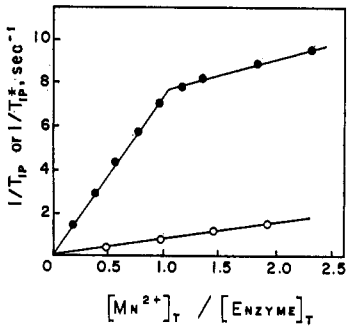


FIGURE 1: Titration of metal-free phosphoglucomutase with Mn^{2+} as followed by enhancement of proton relaxation rate. To a 0.1-ml solution of 1.02×10^{-4} M metal-free enzyme in 20 mM Tris-Cl (pH 7.4) were added 0.002-ml aliquots of 2.0 mM Mn^{2+}; the value of T_1 was measured after each addition, converted into T_{1P}^*, and corrected for volume increase (●). For comparison, 0.005-ml aliquots of Mn^{2+} were added to 0.1 ml of the same buffer solution in the absence of the enzyme (○).

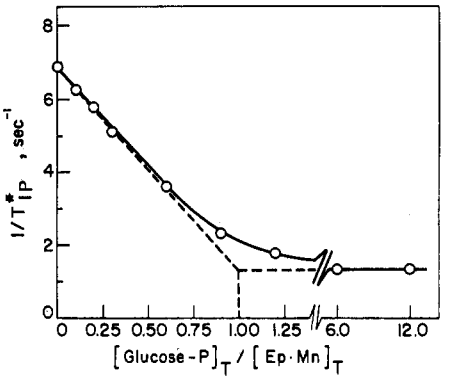


FIGURE 2: Titration of the phosphoglucomutase·Mn complex with glucose phosphate as followed by deenhancement of proton relaxation rate. To a 0.1-ml solution of 1.25×10^{-4} M enzyme containing 1×10^{-4} M Mn^{2+} in 20 mM Tris-Cl (pH 7.4) were added successive 0.002-ml aliquots of equilibrium mixtures of glucose-1-P and glucose-6-P at concentrations ranging from 0.5 to 15 mM. Values of T_{1P}^* were obtained as described in the Experimental Section. The solid line in the figure was calculated from eq 1 by using $[E_P \cdot Mn]_T = 1 \times 10^{-4}$ and $K_{d(E_P \cdot Mn \cdot GP)} = 2$ μM; corrections were made for changes in both $1/T_{1P}^*$ (before plotting the data) and in $[E_P \cdot Mn]_T$ (in calculations with eq 1) produced by volume changes during the titration.

the break point of the enzyme titration curve; this suggests an ancillary Mn^{2+} binding site or sites on the enzyme. The existence of such sites is also in accord with the observation that the slope of the upper line after the break point is 1.9-fold larger than that of the lower line (indicating a greater relaxivity after the break point than would be expected in the absence of ancillary binding). Metal binding at these ancillary sites is not required for maximal enzymatic activity (Ray, 1969) nor does such binding cause detectable ultraviolet spectral shifts (Peck and Ray, 1969a) as does binding at the primary metal binding site. An estimate of the dissociation constant for Mn^{2+} binding to an ancillary site, about 10^{-5} M, was made by using the concentration of free Mn^{2+} determined by electron paramagnetic resonance and assuming a single additional site. (In such a case the concentration of bound Mn^{2+} in excess of a 1:1 stoichiometry is equal to the concentration of the hypothetical enzyme·Mn_2^{2+} complex.)[5] Although the dissociation constant estimated in this manner is about 100-fold larger than that for the $E_P \cdot Mn$ complex (Ray, 1969), in subsequent proton relaxation rate measurements the concentration of enzyme was maintained in excess of the Mn^{2+} concentration to minimize possible complications due to ancillary Mn^{2+} binding; under the conditions used essentially all of the Mn^{2+} should be bound to the tight binding site of the enzyme when total Mn^{2+} is present in less than stoichiometric amounts.

It might be noted that the enhancement produced by Mn^{2+} binding at these sites may be estimated (Mildvan and Cohn, 1963) as about 5. Thus the observed break in the Figure 1 titration curve is the result of differences in Mn^{2+} binding at the active site as opposed to binding at ancillary sites, rather than to a large intrinsic difference in enhancement at the two sites. In accord with this conclusion, a titration curve at 1.0 instead of 0.102 mM enzyme (not shown) exhibited a much less prominant break point, although the stoichiometry at the break point was essentially the same.

Titration of the $E_p \cdot Mn$ Complex with Various Phosphates as Followed by Proton Relaxation Rate. Titration of the $E_P \cdot Mn$ complex with glucose-P produced a marked decrease or deenhancement of the Mn^{2+}-induced relaxation rate, as shown in Figure 2. The total change in $1/T_{1P}^*$, 5.52 sec^{-1}, and the limiting value of $1/T_{1P}^*$ at high glucose-P concentration 1.32 sec^{-1}, correspond to enhancements for the binary and ternary complexes of 8.9 and 1.7, respectively.

The expected course of the titration could be calculated from the initial and final values of $1/T_{1P}^*$ if the dissociation constant of glucose-P from both the binary E_P·glucose-P and ternary $E_P \cdot Mn$·glucose-P complexes[6] were known. Since the enzyme was initially in the $E_P \cdot Mn$ form and since preliminary calculations suggested that the dissociation constant of glucose-P from the $E_P \cdot Mn$·glucose-P complex was much smaller than that of the binary E·glucose-P complex (see below) binding of glucose-P by free E_P was ignored in analysis of this plot. In such a case eq 1 gives the [glucose-P]$_T$/[$E_P \cdot Mn$]$_T$ ratio required to produce a particular $1/T_{1P}^*$ value; the subscript, T, indicates the total species, both bound and free, and f, the fractional change observed in $1/T_{1P}^*$, *i.e.*, $(1/T_{1P}^* - 1.32)/5.52$, which is equal to $[E_P \cdot Mn \cdot GP]/[E_P \cdot Mn]_T$. The solid line in Figure 2, which represents the data quite well, is a plot of eq 1 using $K_{d(E_P \cdot Mn \cdot GP)} = 2$ μM. Because $[E_P \cdot Mn]_T$ was much larger than this value a rather large error in $K_{d(E_P \cdot Mn \cdot GP)}$ is expected; however, its true value is certainly between 1 and 3 μM.

$$[Glu\text{-}P]_T/[E_P \cdot Mn]_T = \frac{K_{d(EP \cdot Mn \cdot GP)}}{[E_P \cdot Mn]_T}\left(\frac{f}{1-f}\right) + f \qquad (1)$$

A second titration was conducted in the same manner except that the initial Mn^{2+} concentration was 0.67×10^{-4}

[5] An ancillary binding site or sites for Co^{2+} has also been detected; unpublished results.

[6] Dissociation constants, $K_{d(\ )}$, are defined such that the last species in the subscript identifying the K_d value is the dissociating species.

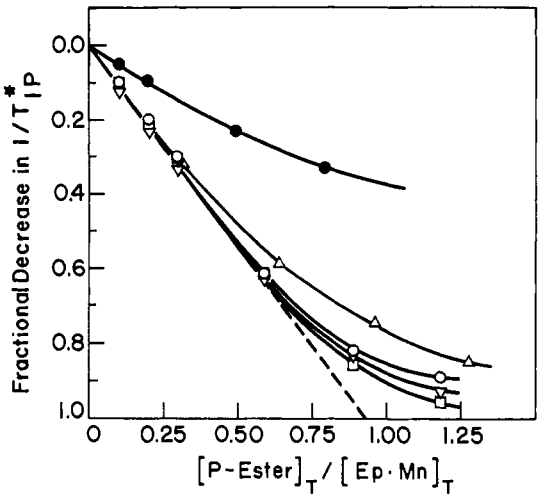


FIGURE 3: Titration of the phosphoglucomutase·Mn complex with phosphate esters as followed by deenhancement of proton relaxation rate. The titration was conducted as in Figure 2 by using glucuronic acid-1-P (□), xylose-1-P (○), 2-deoxyglucose-P (△), and ribose-P (●); a titration using glucose-P is shown for comparison (▽). The constant value of $1/T_{1P}^*$ achieved at high phosphate ester concentration (not shown) was used to calculate the fractional change in $1/T_{1P}^*$.

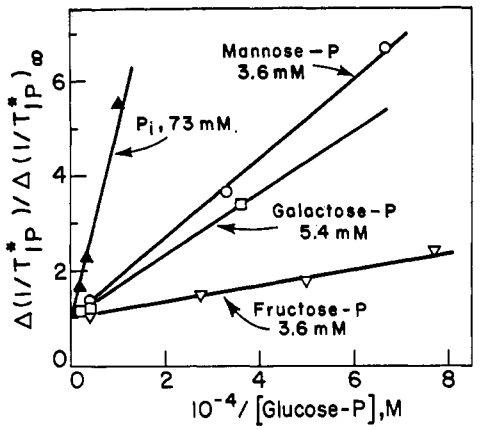


FIGURE 4: Titration of the phosphoglucomutase·Mn complex with glucose-P in the presence of inorganic phosphate and phosphate esters as followed by deenhancement of proton relaxation rate. The titration in the presence of the indicated phosphate esters was conducted as in Figure 2. The fractional change in $1/T_{1P}^*$ is plotted as a function of free glucose-P, which was estimated as described in the Results section for the ribose-P titration. The concentrations of glucose-P required for half-saturation under these conditions are 15, 67, 83, and 370 μM, respectively, in the presence of the indicated concentrations of fructose-P, galactose-P, mannose-P, and P_i.

M instead of 1×10^{-4} M, *i.e.*, 0.53 of the above enzyme concentration. In this titration (not shown) the intersection of the initial slope and the final slope occurred at a $[Glu\text{-}P]_T/[E_P \cdot Mn]_T$ ratio of about 1.07 instead of about 1.0 as in Figure 2. This change in apparent stoichiometry suggests that binding of glucose-P by the free enzyme was becoming significant at lower $[Mn]_T/[E_P]_T$ ratios. If $K_{d(E_P \cdot GP)}$ is taken as 36 μM and $K_{d(E_P \cdot Mn \cdot GP)}$ as 2 μM, as above, the appropriate equation (which is not given but is similar to eq 1) provides an even better fit of the data than the fit obtained in Figure 2; in fact this combination of constants provides the best fit of the experimental points to the calculated line as determined by trial and error analysis of several combinations of constants. Although at present we have no independent estimate of $K_{d(E_P \cdot GP)}$ for comparison, it is clear that the Mn·enzyme complex binds the glucose phosphate about an order of magnitude more firmly than does the free enzyme.

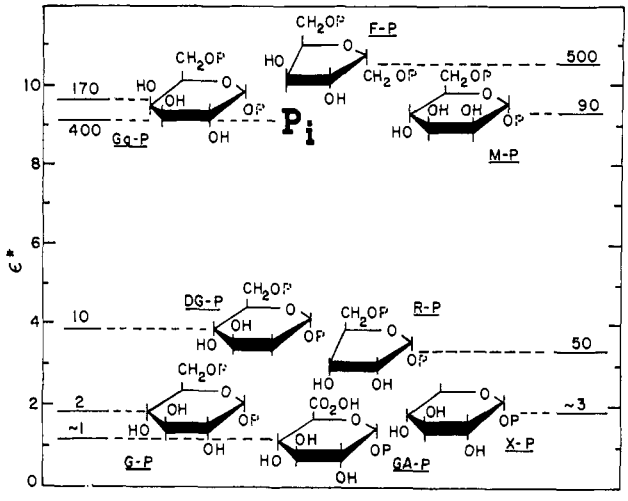
The deenhancement of proton relaxation rate produced by binding of other phosphate ester ligands was investigated by titrating the $E_P \cdot Mn$ complex in a manner analogous to that used with glucose-P. Titration curves for xylose-1-P and glucuronic acid-1-P were quite similar to that of glucose-P and are shown in Figure 3 in terms of fractional change in $1/T_{1P}^*$ as a function of the $[P\text{-ester}]_T/[E_P \cdot Mn]_T$ ratio (the enhancements for the various ternary complexes are given in Chart I). The similarity of the plots for glucose-P, xylose-1-P, and glucuronic acid-1-P suggest similar dissociation constants for the phosphate esters of these ternary complexes although xylose-1-P is evidentally bound somewhat less and glucuronic acid-1-P somewhat more tenaciously than glucose-P. Because these dissociation constants are much smaller than the concentration of $E_P \cdot Mn$ used in the titrations no attempt was made to evaluate them precisely, and their approximate values are recorded in Chart I as ~1 and ~3 μM, respectively, *i.e.*, slightly below and above the value of 2 μM estimated for glucose-P from Figure 2.

Also shown in Figure 3 are titration curves for ribose-P and 2-deoxyglucose-P; these phosphate esters are bound less tenaciously than glucose-P by factors of 5 and 25, respectively. In fact, in these titrations a reasonable measure of the amount of free phosphate ester present could be obtained as the difference between the added phosphate ester and the quantity, $f[E_P \cdot Mn]_T$, where f is the fractional change in $1/T_{1P}^*$ during the titration, *i.e.*, due to formation of the ternary complex. Plots (not shown) of $1/f$ *vs.* the reciprocal of the free phosphate ester calculated in this manner were linear and yielded values of about 10 and 50 μM, respectively, for the dissociation constants of 2-deoxyglucose-P and ribose-P from their respective ternary complexes.

Direct titrations of $E_P \cdot Mn$ with galactose-P, mannose-P, fructose-P, and P_i could not be followed accurately by proton relaxation rate techniques because the change in relaxivity produced by binding of these materials to the enzyme·Mn complex was small at best. However, a titration with glucose-P conducted in the presence of these phosphates suggested competitive binding relative to glucose-P since (a) the limiting value of ϵ at high glucose-P concentration was that characterization of the $E_P \cdot Mn \cdot$glucose-P complex, and (b) higher concentrations of glucose-P were required to affect a given fractional change in T_{1P}^* than in the absence of these phosphates.

Because of the increased apparent dissociation constant of glucose-P from its ternary complex with enzyme and Mn^{2+} in the presence of the above phosphates, a good estimate of the concentration of free glucose-P could be made in the manner described above for direct titrations with ribose-P and 2-deoxyglucose-P. Figure 4 shows double-reciprocal plots of free glucose-P concentration and fractional change in $1/T_{1P}^*$ together with the concentration of the phosphate additives. The values for the dissociation constants of the

CHART I: Enhancement of Proton Relaxation Rate in Ternary $E_P \cdot Mn \cdot$phosphate Complexes in Relationship to Both the Structure of the Phosphate Moiety and the Dissociation Constant of the Phosphate Moiety from the Ternary Complex.[a]



[a] Approximate values of the latter constants are given in terms of micromolar above the lines designating the respective ϵ values at 24°. Because the isomer distribution in those ternary complexes involving substrates is unknown, sugar phosphates that are substrates are represented with two broken-line P (phosphate) groups while sugar phosphates that are not substrates contain a single bold-face P. Abbreviations used in this chart are: F, fructose; Ga, galactose; M, mannose; DG, 2-deoxyglucose; R, ribose; G, glucose; X, xylose; GA, glucuronic acid.

phosphate additives from the ternary $E_P \cdot Mn \cdot$phosphate complexes, were estimated on the assumption that the corresponding dissociation constant for glucose-P is 2 μM (see above), and that the binding of the phosphates (both sugar phosphates and inorganic phosphate) is competitive; the constants thus determined were 0.5, 0.4, 0.17, and 0.09 mM for fructose-P, P_i, galactose-P, and mannose-P, respectively.

By way of comparison, an approximate K_d value was obtained from a direct titration of $E_P \cdot Mn$ with fructose-P, where $1/T_{1P}^*$ increased from 6.9 to 7.9 sec^{-1}. This K_d value, 1.1 mM, is in reasonable agreement with that obtained as above *via* glucose-P titrations in the presence of fructose-P, 0.5 mM. However, the K_d value for ribose-P from titration of the enzyme $\cdot$ Mn $\cdot$ ribose-P complex with glucose-P, 2×10^{-5} M, is significantly smaller than the value of 8×10^{-5} M obtained by direct titration. Since we have no unequivocal explanation for this difference, an average of the two value is subsequently used as a first approximation.

Chart I shows values for the enhancement of the various ternary complexes in relationship to the structure of the phosphate-containing moiety. For comparison, the average value of ϵ for the binary $E_P \cdot Mn$ complex from a number of experiments is 9.2. The approximate K_d value for dissociation of the phosphate from the ternary complex is given above the solid line that designates the enhancement (at 24°) of the ternary complex involving that phosphate. To differentiate between substrates in which the phosphate group may be at either of two positions, and inhibitors in which no phosphate isomerization can occur, the former compounds are shown with two lightly dashed "P's." Note that ternary complexes involving phosphates that are weakly bound are characterized by ϵ values close to that of the $E_P \cdot Mn$ complex and those involving phosphates that are tightly bound by low relaxivity values approaching that of $E_P \cdot Mn \cdot$glucose-P; the relaxivities of complexes involving ribose-P and 2-deoxyglucose-P are intermediate in value.

Temperature Effects on Proton Relaxation Rate. The effect of temperature on both the $1/T_{1P}^*$ and $1/T_{2P}^*$ of water protons induced by the binary $E_P \cdot Mn$ complex and by ternary complexes involving several different sugar phosphates was measured in the 0–52° temperature range. When a series of measurements was made on the same sample, measurements were made last at the higher temperatures and although some samples containing protein were slightly cloudy on cooling, representative values of T_{1P}^* at 21° were the same before and after exposure to the higher temperatures, except for the metal-free enzyme, which was used as a blank. In fact, the enzyme blank was sufficiently denatured at the higher temperatures that $T_{1(0)}$ and $T_{2(0)}$ values at temperatures above 35° had to be approximated by linear extrapolations[7] of plots of log $T_{1(0)}$ or log $T_{2(0)}$ *vs.* $1/T$ (not shown) where T ranged from 0 to 35°.[8]

The $1/T_{1P}^*$ values shown in Figure 5a as plots of log $1/T_{1P}^*$ *vs.* $1/T$ were obtained over a period of 18 months by using two different nuclear magnetic resonance spectrometers and three different enzyme preparations; both the concentrations of enzyme and of Mn^{2+} were varied as indicated in the figure legend. Figure 5b shows the corresponding $1/T_{2P}^*$ values for some of these complexes. Figure 6 shows T_{1P}^*/T_{2P}^* values as a function of $1/T$.

Since the T_{1P}^*/T_{2P}^* ratio is greater than seven-sixths at all temperatures studied (dashed line, Figure 6), the paramagnetic relaxation must involve "bound" water, *i.e.*, water whose tumbling time is much larger than 3×10^{-11} sec, the value for bulk water (Mildvan and Cohn, 1970). Such water is usually considered to be bound within the hydration sphere of Mn^{2+} in the enzyme $\cdot$ Mn complex so that its tumbling time is that characteristic of the complex, *i.e.*, in the range of 10^{-6} sec for a compact protein of the size of phosphoglucomutase (Tanford, 1961). (Water bound by the protein adjacent to the Mn^{2+} binding site might also contribute to paramagnetic relaxation although the contribution should be small since relaxation efficiency varies with the inverse sixth power of the distance from Mn^{2+} (see Discussion).)

The decrease in $1/T_{1P}^*$ with increasing temperature in the higher temperature range of the Figure 5 plots (above 20°) indicates that at least under these conditions the proton relaxation rate is not limited by the rate of exchange between bound and bulk water (Mildvan and Cohn, 1970). Thus in the upper temperature range those processes which control

[7] A linear plot of log $1/T_{1(0)}$ or log $1/T_{2(0)}$ *vs.* $1/T$ is expected from data such as those given by Luz and Meiboom (1964); linear plots for both diamagnetic relaxation parameters have also been obtained with metal-free solutions of pyruvate kinase (G. L. Cottam and A. S. Mildvan, unpublished observations).

[8] T_{1P}^* values calculated with the extrapolated $T_{1(0)}$ values were inappreciably different from those calculated with measured $T_{1(0)}$ values; however, this was not the case for T_{2P}^* values above 35°. For those complexes with high values of ϵ (*e.g.*, the fructose-P complex), T_{2P}^* at 50° calculated with the extrapolated $T_{1(0)}$ value was 6% higher than that calculated with the measured $T_{1(0)}$ value while the difference amounted to 20% for the complex with the lowest ϵ, $E_P \cdot Mn \cdot$glucose-P.

T_{1M}, the lifetime of the spin state of water protons in the primary hydration sphere of Mn^{2+}, also control the observed relaxation rate (see below).[9]

Estimation of τ_c. An estimate of the magnitude of τ_c, the correlation time for the dipolar Mn^{2+}–proton interaction can be obtained by use of the following equations, which are more extensively discussed in a review by Mildvan and Cohn (1970).

$$\left(\frac{[H_2O]}{[Mn^{2+}]}\right)\frac{1}{T_{1P}^*} = \frac{q}{T_{1M} + \tau_M} \quad (2)$$

$$\left(\frac{[H_2O]}{[Mn^{2+}]}\right)\frac{1}{T_{2P}^*} = \frac{q}{T_{2M} + \tau_M} \quad (3)$$

Here q is the number of exchangeable water molecules in the inner hydration sphere of bound Mn^{2+} and τ_M their residence time,[10] and T_{1M} and T_{2M} are the longitudinal and transverse relaxation times, respectively, for bound water. According to considerations noted above T_{1M} and T_{2M} should be much larger than τ_M in the present system, at least at temperatures above 20°; hence, under these conditions T_{1P}^*/T_{2P}^* is equal to T_{1M}/T_{2M}.

Expressions for T_{1M} and T_{2M} for complexes of Mn^{2+}, which are also discussed by Mildvan and Cohn (1970), are as follows

$$\frac{1}{T_{1M}} = \frac{3D\tau_c}{1 + \omega_I^2\tau_c^2} \quad (4)$$

$$\frac{1}{T_{2M}} = \frac{(3/2)D\tau_c}{(1 + \omega_I^2\tau_c^2)} + 2D\tau_c(1 + H\tau_e/2D\tau_c) \quad (5)$$

Here D is a collection of constants associated with the dipolar relaxation term, which has a correlation time, τ_c, and H is a collection of constants associated with the hyperfine relaxation term, which has a correlation time of τ_e, and ω_I is the proton resonance frequency.

For protein-bound Mn^{2+}, $H\tau_e/2D\tau_c$ in eq 5 should be substantially less than 1.0 for the following reasons. The maximum value of $H\tau_e/2D\tau_c$ for free Mn^{2+} is about 9. However, for protein-bound Mn^{2+}, values of τ_c are expected to be much larger (possibly 100-fold) and values of τ_e somewhat smaller (possibly 10-fold) than those for free Mn^{2+},[11]

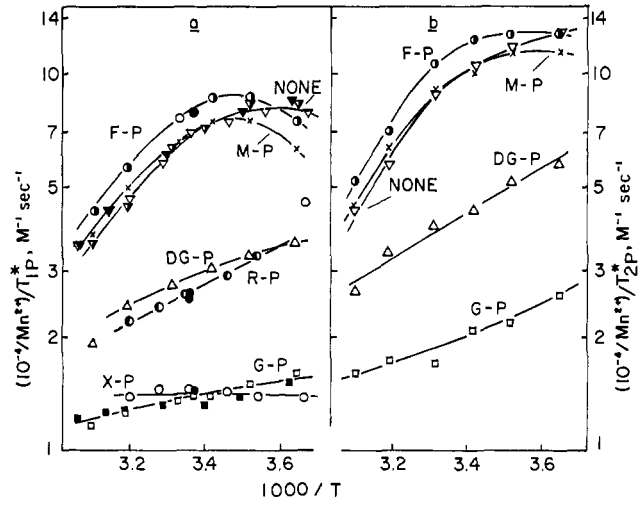


FIGURE 5: Reciprocal of the paramagnetic relaxation time *vs.* reciprocal of the absolute temperature on a semilog scale for several complexes of $E_P \cdot Mn$; (a) longitudinal relaxation; (b) transverse relaxation. Values of $(Mn^{2+})T_{1P}^*$ or $(Mn^{2+})T_{2P}^*$ where (Mn^{2+}) is the total Mn^{2+} concentration were calculated from T_1 and T_2 values (see Experimental Section) which were measured at a series of temperatures between 0 and 52° for solutions containing 0.67 to 0.9 $\times 10^{-4}$ M total Mn^{2+} in 20 mM Tris-Cl (pH 7.4) plus the additions indicated below; the concentration of phosphoglucomutase was 1.25 $\times 10^{-4}$ and 1.0 $\times 10^{-4}$ M, respectively, for measurements at 0.67 $\times 10^{-4}$ and 0.9 $\times 10^{-4}$ $[Mn^{2+}]_T$. Symbols together with the identity and concentration of additive plus the $[Mn^{2+}]_T$ concentration are given below; lines were drawn by eye.

Additive	Symbol	Concn of Additive (mM)	$[Mn^{2+}]_T$ (mM)
Fructose-P	◑	20	0.9
	○	4	0.67
None	▽		0.9
	▽		0.9
	▼		0.67
Mannose-P	×	20	0.9
Deoxyglucose-P	△	20	0.9
Ribose-P	◐	20	0.9
	●	6	0.67
Glucose-P	□	20	0.9
	■	1.5	0.67
Xylose-P	○	20	0.9
	●	4	0.67

while the value of H/D should be affected to a much smaller extent. This would produce an $H\tau_e/D\tau_c$ value of much less than 1. In such a case

$$\left[\frac{3}{2}\left(\frac{T_{1M}}{T_{2M}} - \frac{7}{6}\right)\right]^{1/2} \approx \omega_I\tau_c$$

Table I shows values of $\omega_I\tau_c$ at 25° calculated from the above expression by using the relationship $T_{1P}^*/T_{2P}^* = T_{1M}/T_{2M}$ (see above).

In view of the procedure used, values of $\omega_I\tau_c$ in Table I can be considered only as approximations. However, the

[9] The downward curvature of some of the Figure 5 plots in the lower temperature range *might* be an indication of rate-limiting water exchange below 20°; however, this is not necessarily the case (see Mildvan and Cohn, 1970).

[10] Exchange of protons, as opposed to water molecules, is not considered since significant proton exchange in methanolic solutions of Mn^{2+} does not occur over a wide temperature range (Levanon and Luz, 1968).

[11] For free Mn^{2+}, τ_c is controlled by the tumbling time of hydrated Mn^{2+}, which is about 3 $\times 10^{-11}$ sec (Bloembergen and Morgan, 1961). When bound to a macromolecule such as phosphoglucomutase the tumbling rate is decreased to the point where τ_c is controlled either by the electron-spin relaxation time, τ_s, or the lifetime of water in the hydration sphere of Mn^{2+}. If τ_c is controlled by τ_M, it should certainly be larger than 10^{-9} sec (Mildvan and Cohn, 1970) while values in the range of 10^{-9}–10^{-10} might be expected if τ_s controls τ_c. On the other hand, if for protein-bound Mn^{2+} τ_s controls τ_c it will also control τ_e. For free Mn^{2+} τ_e is about 3 $\times 10^{-8}$ sec and for bound Mn^{2+}, τ_e may be as much as an order of magnitude smaller, as suggested by electron paramagnetic resonance studies (G. Reed and W. J. Ray, Jr., unpublished observations). Hence, in the protein complexes, τ_c increases and τ_e decreases such that the resultant decrease in τ_e/τ_c can scarcely be smaller than 100-fold.

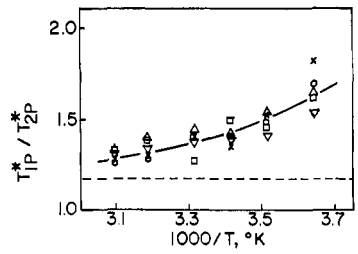


FIGURE 6: T_{1P}^*/T_{2P}^* ratios for various $E_P \cdot Mn \cdot$sugar phosphate complexes as a function of $1/T$. Data were taken from those experiments in Figure 5 in which both T_{1P}^* and T_{2P}^* were measured on the same sample. Symbols are the same as in Figure 5. The dashed line is at $T_{1P}^*/T_{2P}^* = 7/6$.

TABLE I: Approximate Values of $\omega_I\tau_c$ for Phosphoglucomutase·Mn Complexes.[a]

Sugar-P	$\omega_I\tau_c$
None	0.55
Fructose-P	0.54
Mannose-P	0.54
2-Deoxyglucose-P	0.50
Glucose-P	0.65

[a] Values were calculated as described in the Results for several temperatures and values at 24° were interpolated from plots of log $\omega_I\tau_c$ *vs.* $1/T$; ω_I is 24.3 MHz.

important point is that the $\omega_I\tau_c$ value is approximately the same for all complexes investigated, which in turn implies that T_{1M} is similar for these complexes since D is not expected to vary appreciably with environment (see equations given by Mildvan and Cohn, 1970). A relatively constant $\omega_I\tau_c$ value means that the major differences in the measured $1/T_{1P}^*$ values for the various complexes must be due to differences in q (eq 2). Thus binding of those sugar phosphates that produce substantial decreases in $1/T_{1P}^*$ at 25° (Chart I) must be accompanied by displacement of bound water from the hydration sphere of bound Mn^{2+}, whereas binding of those sugar phosphates which produce relatively small changes in $1/T_{1P}^*$ as well as the binding of P_i must not require water displacement.

Discussion

The relatively tight binding of 1 mole of Mn^{2+}/mole of phosphoglucomutase, which is accompanied by an enhancement of the proton relaxation rate of about 9-fold, makes this system attractive for studying enzyme–substrate interactions *via* nuclear magnetic resonance techniques, especially since several structurally similar substrates bind to the enzyme and produce a gradation of effects on relaxation rates. However, besides the usual difficulties encountered in unraveling the mechanistic intricacies surrounding induced nuclear relaxation in enzymatic systems, in the phosphoglucomutase system there is the added complexity of a one-substrate–one-product reaction which has three dissociable ternary complexes as intermediates (Ray and Roscelli, 1964). Thus, addition of substrate to the $E_P \cdot Mn$ system produces an equilibrium mixture of, for example, $E_P \cdot Mn \cdot$glucose-1-P, $E_D \cdot Mn \cdot$glucose-1,6-P_2, and $E_P \cdot Mn \cdot$glucose-6-P. By contrast, in a two-substrate–two-product reaction such as pyruvate kinase (Mildvan and Cohn, 1966) inactive ternary complexes such as $E \cdot Mn \cdot$ATP or $E \cdot Mn \cdot$pyruvate can be formed and studied separately—as well as abortive quaternary complexes such as $E \cdot Mn \cdot$pyruvate·ADP. However, binding of inhibitors in which no phosphate transfer is feasible, *e.g.*, P_i and xylose-1-P, produce effects which mimic the extremes of effects produced, respectively, by poorly bound substrates, such as mannose-P, and by strongly bound substrates, such as glucose-P. Hence it seems reasonable to assume that there are no significant differences in relaxivity among the isomeric ternary complexes for a particular substrate and thus that possible differences in distribution among the above isomeric complexes have no significant effect on the results. Moreover, because of the high concentrations of enzyme used, no substantial formation of *free* dephosphoenzyme is expected on addition of substrate because of the tight binding of diphosphates to the dephosphoenzyme.[12] Thus in spite of potential difficulties, the properties of the system are such that a straightforward interpretation of effects produced by the addition of various substrates can be reasonably expected.

Data relating the proton relaxation rates of various $E_P \cdot Mn \cdot$sugar phosphate complexes to the binding of the sugar phosphate moiety at room temperature are summarized in Chart I. The simplest rationale of these data is that increased binding affinity is related to decreased accessibility of bulk water to primary ligand positions of bound Mn^{2+}. Decreased accessibility might result from a decrease in number of accessible positions, a decrease in exchange rate, or both. However, since the rate of water exchange increases with increasing temperature above 20° (Figure 5) water exchange is not the process limiting the proton relaxation rate under the conditions used for measurement of the parameters recorded in Chart I.[9] Hence a decrease in the number of exchangeable water molecules in the hydration sphere of Mn^{2+} is almost certainly the basis for the major part of the decrease in relaxivity on sugar phosphate binding (see Results).

Each of the three sugar phosphates whose binding to the $E \cdot Mn$ complex is not accompanied by displacement of water from bound Mn^{2+}—fructose-P, galactose-P, and mannose-P—contains a group in at least one position of the sugar ring that is larger than the group in the corresponding position in glucose-P. Hence, formation of non-

[12] By modifying the expression given by Ray and Roscelli (1964) for the free glucose diphosphate present in a mixture prepared from phosphoenzyme and glucose monophosphate, it can be shown that the concentration of dephosphoenzyme will remain relatively insignificant, *viz.*, $E_D/E_T \ll 1$, where E_T is the total enzyme, if $(K_d/E_T)^{1/2}$ is also $\ll 1$, where K_d is the dissociation constant for $(E_D)(Glu\text{-}1,6\text{-}P_2)/(CC)$. Since $K_d \approx 10^{-6}$ M (Peck *et al.*, 1968) and the concentration of E_T used here is about 10^{-4} M, the above condition is satisfied for the glucose phosphate system. Moreover, since the Michaelis constant for fructose diphosphate given by Lowry and Passonneau (1969), about 3 μM, is probably the same as the analogous K_d value, E_D/E_T is again much less than 1 for the poorest substrate, fructose phosphate. Hence it does not appear likely that the effects observed on addition of other sugar phosphates are due to formation of free dephosphoenzyme.

productive or distorted complexes may be important in the ternary complexes involving each of these phosphates, and the lack of observable water displacement on binding probably has little significance relative to binding of the normal substrate. The possibility of forming distorted complexes is reinforced by a comparison of the dissociation constants listed in Chart I.

However, the small competitive inhibitor, P_i (Peck *et al.*, 1968), also fails to displace water on binding to the enzyme·Mn complex. If P_i binds in the same place as the corresponding group of glucose phosphate, the phosphate group of the normal substrate must not bind in the primary hydration sphere of Mn^{2+}. In such a case either a hydroxyl group or groups of glucose-P binds to Mn^{2+} or water is displaced from Mn^{2+} indirectly because of a substrate-specific structural change induced by sugar phosphate binding.

Since the ternary complex of 2-deoxyglucose-P has a significantly larger relaxivity than the corresponding complex of glucose-P it apparently displaces less water than glucose-P; hence we favor the first of the above possibilities, and further suggest that in the ternary complex the 2- and 3-hydroxyl groups of glucose-P are normally bound to Mn^{2+}. If ribose-P is considered to be like glucose-P at its 1, 2, and 5 carbons, but different at the 3 and 4 carbons the increased relaxivity of the ternary complex of ribose-P relative to that of glucose-P (Chart I) is consistent with the suggestion that in the ternary complex of glucose-P the 3-hydroxyl group is bound to Mn^{2+}. Note that the 6-hydroxyl group of glucose-P is probably not bound to Mn^{2+} since the relaxivity of xylose-P is approximately the same as that of glucose-P. We plan to test the above suggestion by studying 3- and 4-deoxyglucose phosphates.

The question arises as to whether any water remains in the primary hydration sphere of those ternary complexes in Chart I with low enhancements. The $T_{1P}*/T_{2P}*$ ratio for these complexes certainly indicates the presence of bound water (see Results); however, the bound water might reside either in the primary hydration sphere of Mn^{2+} or at a nearby binding site. Thus Koenig and Schillinger (1969) have detected water binding at discrete sites on several proteins in the absence of bound metals. Because $1/T_{1P}*$ varies as the reciprocal of the sixth power of the Mn^{2+} to proton distance, several adjacent water molecules would be required to produce an effect equal to a single water molecule in the primary hydration sphere of Mn^{2+}, and it seems more reasonable to suppose that at least one water molecule resides in the primary hydration sphere of Mn^{2+} in all complexes examined, although this cannot be unequivocally demonstrated with the present data.

In Table II the enhancement values and binding parameters for the ternary complexes of those sugar phosphates which are substrates are compared with their respective V_{max} and K_m values obtained by kinetic studies using Mg^{2+} as the activator (Lowry and Passonneau, 1969). An approximately inverse correlation exists between ϵ and V_{max} for conversion of the sugar 1-phosphates into their respective 5- or 6-phosphate isomers. A positive correlation also exists between the dissociation constants, K_d, obtained by the proton relaxation rate titrations of the phosphoglucomutase·Mn complex with an equilibrium mixture of each substrate–product pair and the Michaelis constants of the 1-phosphates of the sugars obtained with the Mg^{2+}-activated enzyme, although the absolute values of the K_M's are greater than the corresponding K_d values by as much as an order of magnitude. However, further studies will be required to determine whether water displacement on the bound divalent cation is the common denominator for all of these relationships.

TABLE II: Comparison of Enhancement and Binding Parameters with Kinetic Constants for Various Sugar Phosphates.

ϵ^a	Glucose-P < Ribose-P < Mannose-P < Galactose-P < Fructose-P
$V_{max}{}^b$	Glucose-P > Mannose-P > Ribose-P > (Galactose-P) > Fructose-P
$K_d{}^a$	Glucose-P < Ribose-P < Mannose-P < Galactose-P < Fructose-P
$K_m{}^b$	Glucose-P < (Galactose-P) < Mannose-P < Ribose-P < Fructose-P

[a] From Chart I. [b] For the conversion of the 1- into the 5- or 6-phosphate isomer; from Lowry and Passonneau (1969). For sugar phosphates in parentheses only lower limits for K_m and V_{max} values were given; hence these inequalities are only approximate.

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References

Bloembergen, N. (1957), *J. Chem. Phys. 27*, 572.
Bloembergen, N., and Morgan, L. O. (1961), *J. Chem. Phys. 34*, 842.
Bloembergen, N., Purcell, E. M., and Pound, R. V. (1948), *Phys. Rev. 73*, 679.
Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 3*, 630.
Cohn, M., and Leigh, J. S. (1962), *Nature 193*, 1037.
Cohn, M., and Townsend, J. (1954), *Nature 173*, 1090.
Filmer, D. L., and Koshland, D. E., Jr. (1963), *Biochim. Biophys. Acta 77*, 334.
Koenig, S. H., and Schillinger, W. E. (1969), *J. Biol. Chem. 244*, 3283, 6520.
Levanon, H., and Luz, Z. (1968), *J. Chem. Phys. 49*, 2031.
Lowry, O. H., and Passonneau, J. V. (1969), *J. Biol. Chem. 244*, 910.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
Mildvan, A. S., and Cohn, M. (1963), *Biochemistry 2*, 910.
Mildvan, A. S., and Cohn, M. (1966), *J. Biol. Chem. 241*, 1178.
Mildvan, A. S., and Cohn, M. (1970), *Advan. Enzymol.* (in press).
Najjar, V. A. (1955), *Methods Enzymol. 1*, 294.
Peck, E. J., Kirkpatrick, D. S., and Ray, W. J., Jr. (1968), *Biochemistry 7*, 152.
Peck, E. J., Jr., and Ray, W. J., Jr. (1969a), *J. Biol. Chem. 244*, 3748.
Peck, E. J., Jr., and Ray, W. J., Jr. (1969b), *J. Biol. Chem.. 244*, 3754.
Ray, W. J., Jr. (1969), *J. Biol. Chem. 244*, 3740.

Ray, W. J., Jr., and Koshland, D. E., Jr. (1962), *J. Biol. Chem. 237*, 2493.
Ray, W. J., Jr., and Roscelli, G. A. (1964), *J. Biol. Chem. 239*, 1228.
Ray, W. J., Jr., and Roscelli, G. (1966), *J. Biol. Chem. 241*, 2596.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 444.

Influence of pH on the Kinetics of Complex Formation between Aromatic Sulfonamides and Human Carbonic Anhydrase*

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ABSTRACT: The kinetics of complex formation between aromatic sulfonamides and human carbonic anhydrases B and C have been examined over the pH range, 5.0–10.8 by stopped-flow kinetic measurements of fluorescence quenching. The apparent bimolecular association rate constant is highly pH dependent whereas the dissociation rate shows no variance with pH. The kinetic constants and their pH dependence are similar for the Zn and Co(II) forms of the enzyme. Correlation between the visible d–d absorption spectra of the Co(II) enzyme and the association kinetics suggest that the pH dependence of the association process is governed by a proton-dependent equilibrium between different coordination forms of the enzyme. Combination appears to occur between the neutral sulfonamide species and the coordination form of carbonic anhydrase which predominates at alkaline pH. The pH-insensitive dissociation rate is also consistent with spectroscopic evidence for a fixed coordination state of the sulfonamide complex. Carboxymethylation of a single histidine residue in human carbonic anhydrase B markedly affects the sulfonamide binding kinetics. The pH-independent dissociation rate is increased 45-fold while the pH dependence of association is shifted to higher pH with little change in the intrinsic rate constant.

Co(II) enzyme spectral changes upon carboxymethylation also show an alteration in the pH dependence of the equilibrium between coordination forms of the enzyme which is in good accord with the association rate–pH profile. A mechanism for sulfonamide complex formation involving its combination as a neutral species and subsequent transfer of the sulfonamido proton to a nucleophilic group on the enzyme is proposed.

Aromatic and heterocyclic sulfonamides form a group of specific inhibitors of the metalloenzyme, carbonic anhydrase, possessing a wide range of affinity constants (Maren, 1967; Taylor *et al.*, 1970). The crystal structure of a human carbonic anhydrase C–acetoxymercurisulfanilamide complex shows that the sulfonamido group resides in close vicinity to the coordination sphere of the metal (Fridborg *et al.*, 1967). Replacement of the single Zn atom in the enzyme by Co generate a visible d–d spectrum characteristic of the coordinated ligands and their geometric arrangement around the metal (Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1968). Changes in the Co(II) spectrum accompanying sulfonamide binding further support the concept that the sulfonamide may function as a metalloligand. Fluorescence enhancement observed with the sulfonamide, dansylamide, suggests that the essential aromatic ring of this inhibitor is bound in a relatively hydrophobic environment in the protein (Chen and Kernohan, 1967). Thus stabilization of the complex may be conferred through both ligand–metal bonding and hydrophobic interaction involving the unsaturated ring of the sulfonamide. Indeed, much of the specificity inherent in this reaction may be a consequence of the spatial arrangement of these interacting sites and the sequence of component steps involved in complex formation.

Studies employing rapid reaction techniques to examine the kinetics of ligand–metalloprotein complexes have largely been confined to small inorganic ligands and hemoproteins where advantage can be taken of the large spectral change accompanying ligand binding (Ellis and Dunford, 1968; Ver Ploeg and Alberty, 1968; Duffey *et al.*, 1966; Goldsack *et al.*, 1966). Studies of the pH–rate dependence have proved helpful in characterizing the ionizing groups affecting the reactivity of the protein. The sulfonamide–carbonic anhydrase system introduces the possibility of additional involvement of a hydrophobic interaction at a site distinct from the ligand–metal coordination sphere. A kinetic investigation of complex formation should help delineate the relative free-energy contributions arising from the various interactions stabilizing the complex.

Using stopped-flow instrumentation coupled with a fluorescence detection system, we have previously examined

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