Arrangement of the Phosphate- and Metal-Binding Subsites of Phosphoglucomutase. Intersubsite Distance by Means of Nuclear Magnetic Resonance Measurements†

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ABSTRACT: Measurements were made of both longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates for methyl protons and phosphorus of bulk methylphosphonate in the presence of Mn^{2+} and of the Mn^{2+} complexes of the phospho and dephospho forms of phosphoglucomutase. Distances between Mn^{2+} and methyl protons of phosphorus were calculated from the paramagnetic contribution to $1/T_1$ by using the dipolar term of the Soloman–Bloembergen equation. Correlation times were determined by frequency dependences of $1/T_1$ of the methyl protons. The Mn^{2+} to methyl proton distance in the binary Mn^{2+}–methylphosphonate complex is about 4 Å while the corresponding distance in the ternary complex of Mn^{2+}, phosphoenzyme, and methylphosphonate is 10–11 Å. Hence, the phosphate-binding subsite of the phosphoenzyme is located several ångströms from the metal-binding subsite and the complex thus is an "enzyme bridge" complex. The observed interaction between Mn^{2+} and the methyl group of methylphosphonate in what appears to be a quaternary complex of Mn^{2+}, dephosphoenzyme, and two molecules of methylphosphonate is determined primarily by the methylphosphonate bound at the weak phosphate-binding subsite (presumably the site at which the enzymic transfer of the phospho group occurs)—as opposed to the methylphosphonate bound at the strong phosphate-binding subsite. Distance calculations indicate that the protons of methylphosphonate bound at the weak subsite are about 5.5 Å and the ^{31}P nucleus is about 4.9 Å from the bound Mn^{2+}. Thus, the weak phosphate-binding subsite of the dephosphoenzyme is substantially closer to the bound metal ion than is the strong phosphate-binding subsite. However, even at the weak subsite the phosphonate group probably is not bound within the coordination sphere of the metal ion, since in the binary Mn^{2+}–methylphosphonate complex, where direct coordination occurs, the ^{31}P nucleus is ~3.3 Å from Mn^{2+}. The results support an exchange mechanism of enzyme action in which two intrinsically different phosphate-binding subsites are present in the dephosphoenzyme, as described in the accompanying paper. The results also demonstrate the utility as well as some of the problems in using methylphosphonate as an analog of inorganic phosphate in binding studies involving nuclear magnetic resonance measurements.

The accompanying paper (Ray *et al.*, 1973) presents evidence for single, strong phosphate-binding subsites (K_d in the millimolar range) in both the phospho and dephospho forms of phosphoglucomutase and an additional, substantially weaker phosphate-binding subsite in the dephosphoenzyme. These results are consistent with two different mechanisms for ($-PO_3$) transfer in the phosphoglucomutase reaction. (a) In a minimal motion mechanism there would be a "single" complex of glucose 1,6-bisphosphate with the dephosphoenzyme and transfer of either of the phospho groups[1] of the bisphosphate to the active-site serine residue of the enzyme would require only minor structural alterations of this complex. (b) In an exchange mechanism there would be two substantially different $E_D \cdot$Glc-P_2 complexes;[2] in one of these transfer of the 6-phospho group of glucose-1,6-P_2 to the enzyme could occur, while in the other transfer of the 1-phospho group would be possible. To interconvert the $E_D \cdot$Glc-P_2 complexes in b, an exchange of the two phosphate groups relative to the phosphate-binding subsites of the enzyme must occur; however, the interconversion must be feasible by a process that does not involve complete dissociation of the bisphosphate. A consideration of phosphate-binding patterns indicates that in a minimal motion mechanism (a) the binding of a single phosphate molecule should produce two isomeric $E_D \cdot P_i$ complexes and (b) the "average environment" of the phosphate group in these isomeric complexes should not be substantially different from the average environment for the two phosphate groups in $E_D(P_i)_2$. By contrast, in an exchange mechanism the data indicate that binding of one equivalent of phosphate essentially should produce only one $E_D \cdot P_i$ complex, and a different average environment for the phosphate group in $E_D \cdot P_i$ and the two phosphates in $E_D(P_i)_2$ thus is expected.

In order to distinguish between these possibilities we have examined the interaction of the phosphate analog, methylphosphonate, with the phospho and dephospho forms of the enzyme. Since methylphosphonate does not bind tenaciously to the enzyme it is possible to examine the effect of exchange

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[1] The $-PO_3H_2$ group and all anionic groups derived from it are referred to as the phospho group.

[2] Abbreviations used are: PRR, longitudinal water proton relaxation rate; epr, electron paramagnetic resonance; E_P and E_D, the phospho and dephospho forms of phosphoglucomutase; glucose-1,6-P_2 or Glc-P_2, α-D-glucose 1,6-bisphosphate; ($-PO_3$), the $-PO_3H_2$ group and all anionic groups derived from it; K_d, a dissociation constant; $1/T_1$ and $1/T_2$, longitudinal and transverse relaxation rates, respectively; $1/T_{1p}$ and $1/T_{2p}$, the paramagnetic component of the above rates; p, the concentration ratio of Mn^{2+} to that of a designated entity; $1/T_{1M}$, the relaxation rate within the coordination sphere of a metal ion; τ_c, correlation time; NTA, nitrilotriacetate.

between methylphosphonate bound to the enzyme and a large excess of methylphosphonate free in solution. Proton and phosphorus nuclear magnetic resonance (nmr) spectroscopy provides a convenient way to do this if binding to the enzyme furnishes a paramagnetic environment that is absent in solution. Because the Mn^{2+} form of phosphoglucomutase is active and because Mn^{2+} is bound rather tenaciously by the enzyme (Ray, 1969) we have used bound Mn^{2+} to furnish such an environment. Bound Mn^{2+} also serves as a reference point for evaluating whether or not the phosphate-binding subsites of the dephosphoenzyme are symmetrically disposed with respect to the bound metal ion.

Experimental Section

Materials. The preparation of the phospho and dephospho forms of phosphoglucomutase is described in the accompanying paper (Ray *et al.*, 1973).

Bound metals were removed from the enzyme at pH 7.5 as described previously (Ray, 1969) except that initial protein concentrations were about 75 mg/ml. After removal of metals, some protein solutions were dialyzed for three 2-hr periods against 10 vol of 20 mM Tris buffer in D_2O in which D_2O-washed Chelex was suspended. The Tris buffer used in this process was prepared by lyophilizing Tris-HCl buffer, pH 7.5, and dissolving the residue in D_2O. Protein solutions were frozen dropwise by direct addition to liquid nitrogen and stored in liquid nitrogen until used (Yankeelov *et al.*, 1964). At this point the activity of the phosphoenzyme was at least 900 units/mg and contained less than 7% of the dephospho form. The activity of the dephosphoenzyme was at least 850 units/mg; it contained less than 3% of phosphoenzyme. Methylphosphonate was a generous gift from Dr. Alexander Hampton, Institute for Cancer Research.

Nmr measurements with methylphosphonate were conducted in solutions prepared from D_2O that had been passed through a column of Chelex resin (Bio-Rad) to remove metal ions (the Chelex was flushed with D_2O before use). A stock solution of methylphosphonate was prepared from the solid acid by suspending it in water, adjusting to pH 7.5 with KOH, and passing through a short column of Chelex. The solution was subsequently evaporated to dryness and dissolved in D_2O and the process repeated. A water solution of enzyme was used in some nmr studies involving protons; however, in such cases the volume of water was never greater than 0.02 of the final mixture. In other nmr studies solutions of enzyme in D_2O were used.

Magnetic Resonance Measurements. Longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methyl protons and the phosphorus nucleus of methylphosphonate were measured with a Varian HA-100-15 or XL-100-15 nmr spectrometer by the methods of progressive saturation and line width, as previously described (Mildvan *et al.*, 1967). The phosphorus relaxation rates were determined at 40.5 MHz with noise decoupling of the methyl protons (Nowak and Mildvan, 1972). Linear regression analyses of the appropriate data plots (Mildvan and Cohn, 1970) were used to determine the power at the onset of saturation. In addition, longitudinal relaxation rates of the methylphosphonate protons were obtained at 220 MHz with a Varian HR-220-FT nmr spectrometer, operated in the Fourier transform mode, by measuring the recovery of peak height as a function of the time interval after demagnetization of the sample using the pulse sequence: 90°, field gradient, interval, 90° (McDonald and Leigh, 1973). The temperature was maintained at 31 ± 1° during measurements with both types of instruments.

The longitudinal PRR of water at 24° was determined by pulsed methods as described in the accompanying paper (Ray *et al.*, 1973). The paramagnetic contribution to these values, $1/T_{1p}$ and $1/T_{2p}$, was determined by subtracting the appropriate diamagnetic blank.

Free Mn^{2+} was measured by determining the amplitude of its epr spectrum (Cohn and Townsend, 1954) by using a Varian Model E-3 or E-4 epr spectrometer operated at 9.15 GHz.

Assays. The Mn·phosphoglucomutase complex present in mixtures containing both free enzyme and free Mn^{2+} was assessed by conducting an enzymatic assay with 0.01-ml aliquots of the mixture; 1 ml of a solution containing 20 mM glucose-1-P, 80 μM glucose-1,6-P_2, 10 mM EDTA, 0.8 mM NADP, and 20 mM Tris-chloride (pH 7.5) was used. Just prior to the assay, 0.01 ml of a solution that contained 1 mg/ml of glucose-6-P-dehydrogenase (Boehringer) was added. The product formed in the time interval between 1 and 11 min was measured at 240 mμ by using a Cary-14 spectrophotometer with water-jacketed cell holders. Cuvets containing the assay mixture were brought to 30° in a water bath before initiating the assay and were returned to the 30° bath for the interval between 1 and 11 min. When assays were conducted with concentrated solutions of the enzyme, *e.g.*, 0.1 mM, an initial dilution was made in a solution similar to the above assay solution but with 3 mM glucose-1-P, 5 μM glucose-1,6-P_2, and without NADP or dehydrogenase. In such cases assays were initiated 100 sec after dilution and were otherwise conducted as above.

Results

Justification of a Kinetic Assay for the Manganese(II)–Phosphoglucomutase Complex. Although Mn^{2+} dissociates rather rapidly from phosphoglucomutase at pH 7.5 in the presence of EDTA alone (half-time about 1.5 min[3]), in the presence of glucose-1-P the dissociation rate is markedly slowed, and at 20 mM glucose-1-P the slopes of product–time plots only decrease by about 30% in 10 min if low substrate conversions are employed. Moreover, under defined conditions (*e.g.*, see Experimental Section), the product formed is strictly proportional to the amount of Mn^{2+}–enzyme complex used to initiate the assay (data not shown; however, see Ray and Roscelli, 1966b; Ray, 1969). The presence of the substrate in the assay mixture does not alter the amount of Mn^{2+}–enzyme *initially* present because of the relatively high concentration of EDTA to enzyme that is used—at least 10^4:1. This concentration is sufficient to reduce enzyme activity to "zero" at metal ion equilibrium (see Ray, 1969). Moreover, when a mixture of EDTA and enzyme—10^3:1—is added to a mixture of glucose-1-P and Mn^{2+} containing 10 equiv of Mn^{2+}/equiv of enzyme, <0.1% of the activity produced by an equivalent amount of the enzyme·Mn^{2+} complex is observed. Since once the Mn^{2+}–enzyme complex is formed it is active for many minutes under these conditions (see above), the lack of activity in this experiment indicates that the presence of substrate does not significantly increase the amount of Mn^{2+} bound at the metal binding site of the enzyme under the conditions used to initiate the above assays (excess EDTA) even when free Mn^{2+} and free enzyme are initially present. In addition, in assays of mixtures containing free enzyme and free Mn^{2+}, the product formed in the above assay was strictly proportional to the amount of

[3] W. J. Ray, Jr., unpublished results.

TABLE I: Binding of Manganese(II) by Methylphosphonate.[a]

$[Mn^{2+}]_T$ (μM)	Methylphosphonate[b] (mM)	Free $[Mn^{2+}]$[c] (μM)	$K_{d,MP}^{app}$, calcd (mM)
50	7.5	35	18
50	15	24.5	14
50	30	16.4	15

[a] At pH 7.5 in the presence of 0.2 M KCl. [b] Total concentration. [c] Measured by means of the intensity of its epr spectrum.

TABLE II: Effect of Methylphosphonate on the Binding of Manganese(II) by the Phosphoenzyme.[a]

$[E_P]_T$ (mM)	$[Mn^{2+}]_T$ (mM)	Methylphosphonate (M)	Obsd Act. (Rel)[b]
0.062	0.03		690 ± 6
0.062	0.03	0.31	663 ± 8

[a] The equilibration step was conducted at pH 7.5 in the presence of 20 mM Tris chloride. [b] The enzymic assay was conducted in the presence of a large excess of EDTA (see Experimental Section) to obtain a measure of the fraction of enzyme with Mn^{2+} bound at its active site. The indicated errors are standard deviations from the mean for five assays.

enzyme solution used to initiate the assay, over a concentration range of several-fold.

In experiments where the enzyme was titrated with Mn^{2+} or equilibrated with Mn^{2+} in the presence of methylphosphonate (see the following section), Mg^{2+} contamination was always present. Although it never represented more than about 0.01 equiv relative to the total enzyme, the Mg^{2+} complex is some 20-fold more active than the Mn^{2+} complex (Ray, 1969). However, the dilution step used in connection with such experiments (excess EDTA plus substrate; see Experimental Section) plus the time inverval before the initial optical density reading was made provided sufficient time for 99% of any Mg^{2+} initially present to dissociate from the enzyme; hence, Mg^{2+} contamination presented no problem in the subsequent assay. During the same time interval about 24% of the Mn^{2+} originally present as the Mn^{2+}–enzyme complex also dissociated. However, this fraction was constant and independent of the amount of enzyme subjected to the dilution step. Hence, the response in the subsequent assay was strictly proportional to the amount of Mn^{2+}–enzyme complex present in the original aliquot.

Binding of Manganese(II) to Methylphosphonate and to the Phospho and Dephospho Forms of Phosphoglucomutase. In the concentration range of 10–30 mM, methylphosphonate substantially reduces the intensity of the epr spectrum of Mn^{2+}. Since complexes of Mn^{2+} are not expected to exhibit an appreciable epr spectrum under the conditions used (Reed and Cohn, 1970) the concentration of free Mn^{2+} can be calculated from an intensity measurement. The apparent dissociation constant for the $CH_3PO_3 \cdot Mn$ complex, obtained in this manner (see Table I), is about 15 mM at pH 7.5 and $\mu \sim 0.23$. If the dianion is the species primarily responsible for complex formation, as seems reasonable, the true K_d value would be about 7.5 mM (Ray *et al.*, 1973). This value is not much larger than the analogous value for inorganic phosphate at a similar ionic strength, about 2.5 mM, and is quite close to that of methyl phosphate, 6.5 mM (Sillen and Martell, 1964). An enhancement of the effect of Mn^{2+} on the PRR of water by methylphosphonate of 1.5 was obtained in a parallel experiment, by using the same solutions used for the epr studies.

By use of the metal–buffer technique described previously (Ray, 1969), K_d for the $E_P \cdot Mn$ complex was found to be about 3×10^{-8} M in the presence of 3–10 mM nitrilotriacetate at pH 7.5 (data not shown). A value of 2×10^{-8} M under these conditions was approximated earlier from a PRR titration curve (Ray and Mildvan, 1970). The larger value for this constant, in conjunction with the K_d^{app} for $CH_3PO_3 \cdot Mn$, indicates that under the conditions used to study the interaction of $E_P \cdot Mn$ and methylphosphonate, at the lowest Mn^{2+} concentration (see Table IV), only about 0.3% of the Mn^{2+} would be present as $CH_3PO_3 \cdot Mn$, and even less would be present as free Mn^{2+}.

In an independent study, aliquots of Mn^{2+} were equilibrated with the phosphoenzyme in the presence and absence of methylphosphonate, and the Mn^{2+} bound to the active site of the enzyme assessed kinetically as described above. The results (Table II) indicate that under the conditions used about 4.3% less Mn^{2+} was bound to the enzyme in the presence than in the absence of methylphosphonate. If a direct competition between the enzyme and methylphosphonate for Mn^{2+} is assumed, under the conditions used to study the $E_P \cdot Mn$–methylphosphonate interaction, at the lowest Mn^{2+} concentration (see Table IV), 0.6% of the total Mn^{2+} should be bound to methylphosphonate, *i.e.*, >99% of the Mn^{2+} was present in the form of complexes involving the enzyme. This value is in reasonable agreement with the percentage assessed above from the relative K_d values of $E_P \cdot Mn$ and $CH_3PO_3 \cdot Mn$, 0.3%. Since the percentage of Mn^{2+} bound to methylphosphonate, as determined by comparison of K_d values, seems less reliable than the value estimated by direct competition, the latter value (0.6%) was used as the basis for all calculations involving the phosphoenzyme.

In the case of the dephosphoenzyme, the metal–buffer technique could not be used to assess the dissociation constant for the Mn^{2+} complex—apparently because of the formation of mixed ternary complexes of Mn, NTA and the protein. Thus, the fraction of the enzyme with Mn^{2+} bound at the active site decreased markedly with Mn·NTA concentration at a constant Mn·NTA/NTA ratio—as opposed to the results with the phosphoenzyme.

On addition of 0.1 mM Mn^{2+} to 0.15 mM dephosphoenzyme, 0.01 mM free Mn^{2+} was observed by epr spectroscopy.[4] This gives a value of 7×10^{-6} M for the dissociation constant of the $E_D \cdot Mn$ complex, if a 1:1 binding stoichiometry is assumed, as is suggested by studies with the phosphoenzyme (Ray, 1969; Peck and Ray, 1969). To confirm this, an activity titration was performed in which the occupancy of the metal-activating site of the enzyme was assessed by assaying for the $E_D \cdot Mn$ complex in aliquots removed at various stages of the titration. The plot obtained, Figure 1, is curved in the region of stoichiometry, and the solid line in the figure, which represents the data quite well, is the expected titration curve if $K_{d,E_D \cdot Mn}$ is equal to 7×10^{-6} M, as as found in the epr ex-

[4] The authors are indebted to Dr. George Reed of the Johnson Foundation, University of Pennsylvania, for conducting this experiment.

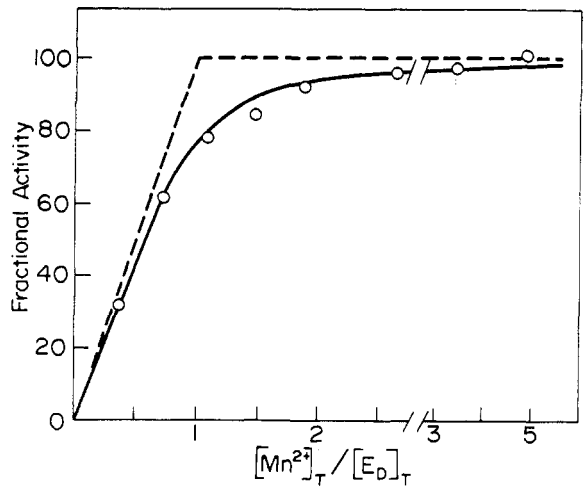


FIGURE 1: Titration of the dephosphoenzyme with Mn^{2+}. The fraction of the enzyme with Mn^{2+} bound at its active site is plotted against the Mn^{2+} to enzyme ratio. The titration was conducted at room temperature with 2 ml of 0.11×10^{-4} M dephosphoenzyme in 20 mM Tris (pH 7.5). After addition of 0.01-ml aliquots of Mn^{2+}, 0.01-ml samples were removed and assayed as described in the Experimental Section. Minor corrections for dilution were made before plotting the results. The solid line is the titration curve expected if $K_{d,E_D \cdot Mn} = 6 \times 10^{-7}$ M.

periment, and if a 1:1 binding stoichiometry is involved. As a further check on this K_d values, the fraction of enzyme with a metal at the activating site was assessed as a function of the concentrations of both enzyme and metal at a Mn^{2+} to dephosphoenzyme ratio of 0.9. Increasing the concentration of Mn^{2+} and dephosphoenzyme by 3.3-fold increased activity by 13.5%; if $K_{d,E_D \cdot Mn} = 7 \times 10^{-6}$ M, the expected increase under these conditions would be 12.5%. Hence, the above value for $K_{d,E_D \cdot Mn}$ is consistent with three independent determinations.

As a final check on $K_{d,E_D \cdot Mn}$, a titration of the dephosphoenzyme with Mn^{2+} was followed by measuring the water proton relaxation rate. From the results (not shown) a K_d of 4×10^{-6} M was estimated in the manner described previously for the phosphoenzyme (Ray and Mildvan, 1970). Since the protein concentration (0.2 mM) was too high to obtain an accurate K_d from this titration the previous value of 7×10^{-6} M was used in all calculations involving the binding of Mn^{2+} to the dephosphoenzyme. The enhancement (Mildvan and Cohn, 1970) for the $E_D \cdot Mn$ complex calculated from the initial phase of the titration was 13.8 in comparison with 9.2 for the $E_P \cdot Mn$ complex (Ray and Mildvan, 1970).

Even though $K_{d,E_D \cdot Mn}$ is in the range of 7×10^{-6} M, the competition of E_D with methylphosphonate for Mn^{2+} ($K_d \sim$ 15 mM, see above) did not appear to be sufficiently favorable to allow nmr experiments to be conducted at methylphosphonate concentrations of 0.1–0.4 M without making a correction for the presence of $CH_3PO_3 \cdot Mn$. However, methylphosphonate in this concentration range actually produced substantially smaller effects on the fraction of Mn^{2+} at the active site of the enzyme than was expected, even if K_d was taken as 4×10^{-6} M, the smaller of the above two values. Table III shows the measured fraction of Mn^{2+} bound at the active site of the dephosphoenzyme at an Mn^{2+}/enzyme ratio of 0.75, both in the absence and presence of methylphosphonate (0.1, 0.25, and 0.4 M). Also shown is the expected fraction if the K_d values for the $E_D \cdot Mn$ and $CH_3PO_3 \cdot Mn$ are 7×10^{-6} and 15 mM, respectively, as determined above. The discrepancy between the measured and expected values is

TABLE III: Effect of Methylphosphonate on the Fraction of Manganese(II) Bound at the Active Site of Dephosphoenzyme.[a]

$[Mn^{2+}]_T/[E_D]_T$[b]	Methylphosphonate (M)	Fraction at Active Site		$K^{app}_{d,E_D \cdot Mn}$[e] (μM)
		Obsd[c]	Calcd[d]	
0.75	0	0.85	0.83	7
0.75	0.1	0.83	0.54	1
0.75	0.25	0.81	0.38	0.6
0.75	0.4	0.76	0.30	0.5

[a] At pH 7.5 in the presence of 20 mM Tris-chloride. [b] The concentration of E_D was 0.11 mM. [c] Measured by assaying a sample of enzyme which had been equilibrated with Mn^{2+} under the indicated conditions. The assay was conducted in the presence of a large excess of EDTA and the results compared with a sample of enzyme treated with excess Mn^{2+} in the absence of methylphosphonate and assayed in the same manner (see Experimental Section). [d] Calculated fraction of Mn^{2+} at active site if $K_{d,E_D \cdot Mn} = 7 \times 10^{-6}$ and $K^{app}_{d,MP \cdot Mn} = 15$ mM under these conditions. [e] Value of $K^{app}_{d,E_D \cdot Mn}$ calculated from the observed fraction of Mn^{2+} bound at the active site of the enzyme, by assuming that $K^{app}_{d,MP \cdot Mn}$ is constant at 15 mM under the conditions employed.

much greater than the experimental error. Changes in K_d values with ionic strength might explain part of this discrepancy, since in neither this experiment nor in subsequent nmr experiments was the ionic strength kept constant. (All anions that have been tested are competitive inhibitors of phosphoglucomutase (Ray and Roscelli, 1966a); hence, we were reluctant to introduce extraneous anions.) However, the differences in expected and observed values seem too large to rationalize solely in this manner, although the ionic strength of the solutions used in Table III did vary from 0.02 to 0.8 (exclusive of enzyme). It seems more likely that specific binding of methylphosphonate at the active site of the enzyme decreases $K^{app}_{d,E_D \cdot Mn}$. Such a result would be suggestive of metal bridging although other possibilities can be proposed. An increased binding of Mn^{2+} by dephosphoenzyme in the presence of bound anions also might provide a rationale for the failure of metal buffer experiments involving Mn^{2+} and nitrilotriacetate, which is a dianion at the pH used (see above). In any case, a much more favorable partitioning of Mn^{2+} between the dephosphoenzyme and methylphosphonate than could be expected on the basis of the previously determined constants is indicated by the Table III data. In addition, such a rationale provides the only reasonable explanation for the small value of $1/pT_{2p}$ for the ^{31}P nucleus of bulk methylphosphonate in experiments involving Mn^{2+}, methylphosphonate, and dephosphoenzyme (see subsequent section); the observed value was only 0.14 of that which would have been obtained from the interaction of methylphosphonate and Mn^{2+} not bound to the enzyme if $K^{app}_{d,E_D \cdot Mn}$ had remained at the same value in the presence of 0.1 M methylphosphonate as in its absence.

The above observations appear to justify the use of progressively smaller values of $K^{app}_{d,E_D \cdot Mn}$ with increasing methylphosphonate concentrations in correcting the nmr results for free Mn^{2+} and Mn^{2+} bound to methylphosphonate. The actual constants used were interpolated from a plot of

$K_{d,E_D \cdot Mn}^{app}$, Table III, *vs.* methylphosphonate concentration (not shown). In all cases, both uncorrected nmr measurements and measurements corrected for free Mn^{2+} and Mn^{2+} present as its methylphosphonate complex are shown for comparison. In no case did the correction substantially alter the conclusions.

Effect of the Phospho and Dephospho Forms of Phosphoglucomutase on the Manganese(II)–Proton Interaction Involving Bulk Methyl Phosphate; 100 MHz. The proton nmr spectrum of methylphosphonate at 100 MHz consists of a doublet, due to coupling with the ^{31}P nucleus (J = 16.0 Hz), which at pD 7.5, is centered 1.68 ppm downfield from the external standard tetramethylsilane (Figure 2a). Neither the metal-free enzyme (Figure 2b) nor the Zn^{2+}–enzyme complex (not shown) produces an appreciable effect on the line width or the power required to saturate the signal. In the presence of 2.5×10^{-4} equiv of Mn^{2+} (no enzyme) both lines undergo paramagnetic broadening (increase in $1/T_2$), Figure 2c; the radiofrequency power required for saturation also increases ($1/T_1T_2$ increases). Subsequent addition of a sufficient amount of phosphoenzyme to bind essentially all of the Mn^{2+} reduces line width somewhat and markedly decreases the saturation power (Figure 2d); however, line width and saturation power remain larger than in the absence of Mn^{2+}. On addition of a 10% excess of Zn^{2+}, relative to the enzyme, Mn^{2+} is slowly displaced from the metal-binding site of the enzyme, since Zn^{2+} binds competitively with Mn^{2+} and is bound at least 10^3-fold more strongly (Ray, 1969). After about 30 min a substantial return was observed in both line width and power at saturation in the direction of the values obtained with Mn^{2+} and methylphosphonate alone; *i.e.*, an increase is produced in both line width and saturation power (not shown).[5] Thus, on a qualitative basis, Mn^{2+} in the binary $CH_3PO_3 \cdot Mn$ complex is more effective in facilitating the relaxation of methyl protons of methylphosphonate than is Mn^{2+} in its ternary complex with methylphosphonate and phosphoenzyme.

To make a quantitative comparison of the relative Mn^{2+}–^1H interaction in the binary and ternary complexes, the effects of (a) free Mn^{2+}, (b) Mn^{2+} present in the enzyme system as $CH_3PO_3 \cdot Mn$, and (c) $E_P \cdot Mn$ complexes with unoccupied methylphosphonate binding sites must be taken into account. To do this $1/T_{1p}$ and $1/T_{2p}$ values (see Experimental Section) were multiplied by the $[CH_3PO_3^{2-}]_T/[Mn^{2+}]_T$ ratio to give $1/pT_{1p}$ and $1/_pT_{2p}$, respectively. These values were then corrected so that the final values included only the interaction in question. The constants required to make corrections for a and b, above, are discussed in the previous section; the use of a methylphosphonate concentration equal to about 30 times its K_I value (Ray *et al.*, 1973) makes the last correction unnecessary. However, to further verify this point, $1/T_{2p}$ also was measured at a methylphosphonate concentration 2.5-fold larger than that used in Figure 2; within experimental error no change in $1/pT_{2p}$ was observed. Hence, 0.1 M methylphosphonate effectively saturates the process in question.

Both measured and corrected values of $1/pT_{1p}$ and $1/pT_{2p}$ for the Mn^{2+}–^1H interaction in the presence and absence of the phosphoenzyme are given in Table IV. The ratio of $1/pT_{1p}$ values in the presence and absence of enzyme is given by ϵ_1. The ϵ_1 values indicate that the enzyme de-enhances the effect of Mn^{2+} on the longitudinal relaxation rate by a factor of about 0.025. Similar considerations apply to $1/pT_{2p}$ and ϵ_2, although the de-enhancement is not as large.

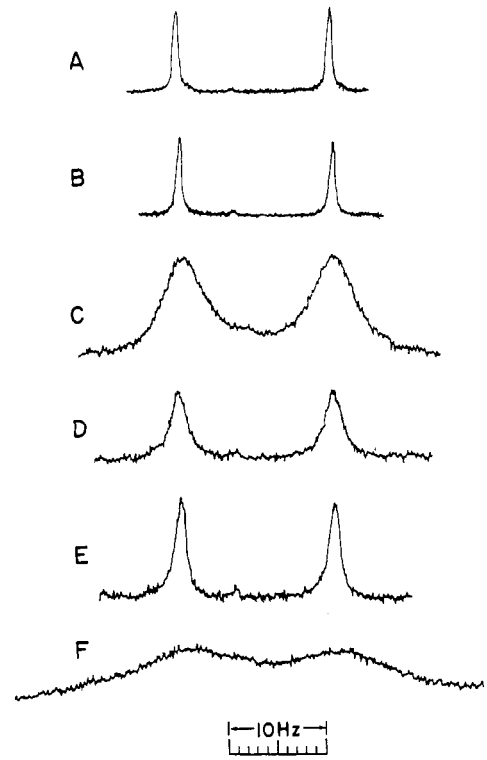


FIGURE 2: Effect of manganese(II) and its complexes with the phospho- and dephosphoenzyme on the nmr signal of the methyl protons of bulk methylphosphonate. Experiments were conducted at 24° in 94% D_2O which contained 20 mM Tris-chloride buffer (pH 7.5 in water). Samples of 0.35 ml were scanned at a rate of 0.1 Hz/sec (diamagnetic samples) or 0.4 Hz/sec (paramagnetic samples) and spectra at a radiofrequency power of about 0.2 of saturation are shown: (a) methylphosphonate, 0.1 M; (b) methylphosphonate, 0.1 M, and phosphoenzyme, 0.125 mM; (c) methylphosphonate, 0.099 M, and Mn^{2+}, 0.098 mM; (d) methylphosphonate, 0.099 M Mn^{2+}, 0.098 mM, and phosphoenzyme, 0.125 mM; (e) methylphosphonate, 0.093 M, Mn^{2+}, 0.093 mM, phosphoenzyme, 0.116 mM, and glucose-P, 28 mM; (f) methylphosphonate, 0.099 M, Mn^{2+}, 0.074 mM, and dephosphoenzyme, 0.123 mM.

In order to use $1/pT_{1p}$ to measure the Mn^{2+}–methylphosphonate distance chemical exchange must not limit its value. In the interaction involving the $E_p \cdot Mn$ complex, the approximately tenfold difference in $1/pT_{1p}$ and $1/pT_{2p}$ indicates that the former is not chemical-exchange limited, *viz.*, $1/T_{1p} \sim 1/T_{1M}$ (see Mildvan and Cohn, 1970). In the binary $CH_3PO_3 \cdot Mn$ complex, chemical exchange could limit $1/pT_{1p}$ if the lifetime of the complex were relatively long compared to T_{1M}. For a weak complex such as $CH_3PO_3 \cdot Mn$ ($K_d \sim 7.5$ mM; see above) this possibility would require unreasonably small values of the association and dissociation rate constants (*e.g.*, see Mildvan, 1970). Moreover, $1/pT_{1p}$ of the methyl protons in this complex is two orders of magnitude lower than $1/pT_{2p}$ of ^{31}P in the same complex (Table IV; *vide infra*). Hence, in the binary $CH_3PO_3 \cdot Mn$ complex, also, $1/pT_{1p}$ for the methyl protons should be equal to $1/T_{1M}$.

In the absence of chemical exchange limitations an ϵ_1 much less than 1.0 might be produced, in theory, either by an increased Mn^{2+}–methyl proton distance in the ternary complex or by a decreased value of $f(\tau_c)$, *i.e.*, $3\tau_c/(1 + \omega_I^2\tau_c^2)$. However, no combination of reasonable τ_c values for the relaxation processes in the present system could produce a decreased $f(\tau_c)$ value for the complex involving the enzyme at a proton Larmor frequency of $\omega_I = 6.28 \times 10^8$/sec (see Appendix).

[5] The final $1/pT_{2p}$ value was larger than for Mn^{2+} and methylphosphonate, alone, probably because the enzyme possesses weak ancillary metal ion binding sites. However, ancillary binding is not expected to be important at the Mn^{2+}/enzyme ratios used for other nmr experiments (see Ray and Mildvan, 1970).

TABLE IV: Effect of Manganese(II) and Its Complexes with the Phospho and Dephospho Forms of Phosphoglucomutase on the Relaxation Rates of Nuclei in Methylphosphonate.[a]

Nucleus	Frequency (MHz)	Paramagnetic Species	$1/pT_{1p}$ (sec^{-1}) Obsd	$1/pT_{1p}$ (sec^{-1}) Corrd[b]	ϵ_1	$1/pT_{2p}$ (sec^{-1}) Obsd	$1/pT_{2p}$ (sec^{-1}) Corrd[b]	ϵ_2
^{1}H	100	Mn^{2+} [c]	14,000 ± 1000	16,500	1.0	16,000 ± 1000	18,500	1.0
		$E_P \cdot Mn$[d]	500 ± 50	400	0.025	4,700 ± 100	4,600	0.25
		$E_D \cdot Mn$[e]	(7,000 ± 300)	(6,200)		(43,000 ± 10,000)	(47,000)	
		$E_D \cdot Mn$[f] (weak site)		17,000	1.4		130,000	14
^{1}H	220	Mn^{2+} [c]	5,800 ± 600	6,700	1.0			
		$E_P \cdot Mn$[g]	≤16					
		$E_D \cdot Mn$[h]		4,800	0.7			
^{31}P	40.5	Mn^{2+} [i]	6,100 ± 300	7,000	1.0	(1.0 ± 0.15) × 10^6	2.3 × 10^6	
		$E_D \cdot Mn$[j]	(6,700 ± 1000)	(6,300)		(48,000 ± 5,000)		
		$E_D \cdot Mn$[f] (weak site)		19,000	3			

[a] Data obtained at a pH or pD of 7.5 in the presence of 20 mM Tris-chloride and 0.1 M methylphosphonate at 28–31°. Except for the measurements involving ^{31}P, all studies were conducted in D_2O. The averages of two–five measurements, usually involving at least two different Mn^{2+} concentrations, are shown. The indicated errors are average deviations from the mean. [b] Corrections for free Mn^{2+} and for relaxation effects produced by free $CH_2PO_3 \cdot Mn$ in the enzymic systems were made as indicated under Results. [c] Obtained at 50–100 μM Mn^{2+}. [d] Obtained at 0.125 mM E_P and 50–100 μM Mn^{2+}. [e] Obtained at 0.125 mM E_D and 25–50 μM Mn^{2+}; however, methylphosphonate was not saturating at the concentration employed; see Results. [f] Values corrected for relaxation effects produced at the strong phosphate-binding subsite and for fractional occupancy of the weak subsite at 0.1 M methylphosphonate; see Results. [g] No detectable paramagnetic effect was obtained; the value given is equal to 2.5 times the standard error for triplicate measurements at 0.31 mM E_P and 0.25 mM Mn^{2+}. [h] Extrapolated intercept value from Figure 3. [i] Obtained at 1–2 μM Mn^{2+}. [j] Obtained at 0.15 mM E_D and 10–15 μM Mn^{2+}; however, methylphosphonate was not saturating at the concentration employed; see Results.

Hence, we conclude that the Mn^{2+}–methyl proton interaction in the ternary complex involving the phosphoenzyme involves a distance substantially greater than in the $CH_3PO_3 \cdot Mn$ complex, *i.e.*, that $CH_3PO_3^{2-}$ is not bound within the coordination sphere of Mn^{2+} in the ternary complex involving $E_P \cdot Mn$. Distance calculations described in a subsequent section support this suggestion. Hence, the complex in question is an "enzyme-bridge" complex as opposed to a "metal-bridge" complex (Mildvan, 1970).

The $E_D \cdot Mn$ complex produces a much larger effect on the relaxation of methylphosphonate protons than does $E_P \cdot Mn$.[6] The effect on line width is shown in Figure 2f at a lower Mn^{2+} to methylphosphonate ratio than in the scans involving $E_P \cdot Mn$. Even under these less favorable conditions the increased line width is substantial. Measured values of $1/pT_{1p}$ and $1/pT_{2p}$ for the interaction of the $E_D \cdot Mn$ complex with methylphosphonate (at an even lower Mn^{2+}/methylphosphonate ratio) are given in Table IV, together with the corresponding values corrected for free Mn^{2+} and $CH_3PO_3 \cdot Mn$ calculated to be present under the conditions used; also shown are the corresponding enhancement factors. All of these values appear in parentheses since subsequent experiments at 220 MHz indicated that the Mn^{2+}–^{1}H interaction was not saturated at 0.1 M methylphosphonate (see below). In fact, experiments at the higher frequency indicate that ~0.2 M methylphosphonate is required to half-saturate the Mn^{2+}–^{1}H interaction under the conditions used. Because this interaction apparently involves binding of methylphosphonate at the weak phosphate-binding subsite (see following section) and because the Mn^{2+}–methyl group interaction at the strong phosphate-binding subsite should be saturated under these conditions (see Discussion), Table IV also shows the calculated $1/pT_{1p}$ and $1/pT_{2p}$ values after correction both for the Mn^{2+}–^{1}H interaction involving the strong phosphate-binding subsite and for the partial saturation of the weak subsite. In making the former correction, we assume, as in the accompanying paper (Ray *et al.*, 1973), that the strong phosphate-binding subsite is the same or essentially the same in both the phospho- and dephosphoenzymes, and hence that the character of the Mn^{2+}–^{1}H interaction involving the strong subsite of $E_D \cdot Mn$ is the same as that found for $E_P \cdot Mn$. In making the latter correction we use the concentration dependence $1/pT_{1p}$ obtained at 220 MHz (see subsequent section). The corrected $1/pT_{1p}$ value thus obtained should be essentially free of chemical exchange limitations since it is several-fold smaller than the corresponding $1/pT_{2p}$ values of the methyl protons in the same complex, both before and after correcting as above.

After making the above corrections, the ϵ_1 value for the Mn^{2+}–^{1}H interaction at the weak subsite of the dephosphoenzyme is slightly greater than unity (see Table IV). However, the expected increase in $f(\tau_c)$ in going from $CH_3PO_3 \cdot Mn$ to a complex involving protein, Mn^{2+}, and methylphosphonate (see Appendix) is large enough to produce an ϵ_1 value much

[6] The possibility that the results observed with the phosphoenzyme can be attributed to the presence of a small amount of dephosphoenzyme that is usually present in such samples (here, ≤7%—see Experimental Section) may be discounted since (a) the phosphoenzyme binds Mn^{2+} some 200-fold more tenaciously than the dephosphoenzyme (see above) and Mn^{2+} was limiting in these studies; (b) a substantial effect of methylphosphonate concentration on the ^{1}H signal of the methyl group was observed over a concentration range where no significant concentration effect was obtained with the phosphoenzyme.

greater than unity, even if no direct coordination to Mn^{2+} takes place in the ternary complex. Hence, even when bound at the weak phosphate-binding subsite of $E_D \cdot Mn$, methylphosphonate probably is not bound within the coordination sphere of Mn^{2+}. Distance calculations from Mn^{2+} to the protons and phosphorus of methylphosphonate in the Mn^{2+}–dephosphoenzyme–methylphosphonate system (see below) support this suggestion.

Displacement of Methylphosphonate from Phosphoglucomutase–Manganese(II) Complexes by Substrate as Detected by Changes in the Relaxation Rate of its Methyl Protons. Figure 2e indicates that excess substrate almost completely eliminates the effect of the $E_P \cdot Mn$ complex on the $1/T_{2p}$ of methylphosphonate and power saturation studies suggest a similar effect on $1/pT_{1p}$. A similar change was observed on addition of glucose phosphate to the solution of $E_D \cdot Mn$ and $CH_3PO_3^{2-}$, *viz.*, line narrowing; moreover, the extent of the change was much larger since broadening produced by $Mn \cdot E_D$ is much greater than that produced with $Mn \cdot E_P$. Hence, the Mn^{2+}–^1H interactions involving methylphosphonate and the Mn^{2+} complexes of both the phospho and dephospho forms of the enzyme appear to occur at the active site of the enzyme.[7]

Effect of Phospho and Dephospho Forms of Phosphoglucomutase on the Manganese(II)–Proton Interaction Involving Bulk Methylphosphonate; 220 MHz. Proton relaxation experiments at 220 MHz were conducted with higher concentrations of the $E_P \cdot Mn$ complex than were used at 100 MHz (Table IV) but with the same methylphosphonate concentration. Even under these conditions no paramagnetic effect on T_1 of the methylphosphonate protons was detected. If 2.5 times the standard error of triplicate determinations is taken as a maximum value of $1/T_{1p}$, $1/pT_{1p} \leq 16$ sec^{-1}. We have no explanation for such a small value in comparison with the value of $1/pT_{1p}$ obtained at 100 MHz, 400 sec^{-1} (Table IV).

In contrast with the above results a substantial paramagnetic effect on T_1 of methylphosphate protons was observed at 220 MHz with the $E_D \cdot Mn$ complex. Furthermore, the effect, in terms of $1/pT_{1p}$, increased with increasing concentration of methylphosphate in the range 0.1–0.37 M. Figure 3 shows a double reciprocal plot of $1/pT_{1p}$ and methylphosphonate concentration. Both the observed results (○) and the values obtained after correcting for free Mn^{2+} and $CH_3PO_3 \cdot Mn$ (●) (see previous section) are indicated. Since the effective dissociation constant for methylphosphonate bound to the strong phosphate-binding subsite in $E_D \cdot Mn$ should be about 2 mM (Ray *et al.*, 1973), the variation of $1/pT_{1p}$ with concentration in the range of 100–380 mM probably involves the interaction of methylphosphonate with the enzyme at its weak phosphate-binding subsite, *i.e.* in a quaternary complex involving two methylphosphonates. Since the Mn^{2+}–^1H interaction at the strong phosphate subsite of $E_P \cdot Mn$ produces no detectable effects at 220 MHz, the effect observed with $E_D \cdot Mn$ must be produced primarily at the weak subsite if the assumptions stated in the section involving the 100-MHz experiments are valid. The K_d^{app} estimated from the upper plot in Figure 4 is about 0.2 M which means that K_d for the dianionic phosphonate is about 0.1 M (see Ray *et al.*, 1973). The value of $1/pT_{1p}$, extrapolated to saturating methylphosphonate in Figure 3, is shown in Table IV.

[7] It might be argued that since substrate binding by the enzyme increases Mn^{2+} binding, the observed narrowing of the nmr line can be rationalized in terms of a decrease in free Mn^{2+}. However, a comparison of columns 7 and 8 for entries 2 and 3, Table IV, indicates that this type of effect could at best produce a narrowing of only a few per cent, while, in fact, much larger changes were observed.

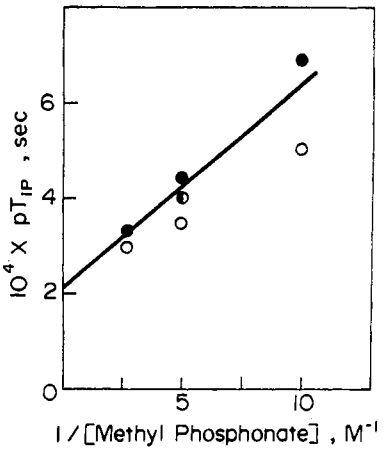


FIGURE 3: Double reciprocal plot of relaxivity of the dephosphoenzyme·Mn^{2+} complex and methylphosphonate concentration. T_1 values at 220 MHz were obtained in D_2O for the methyl protons of methylphosphonate in the presence of the dephosphoenzyme and the decrease in T_1 on addition of Mn^{2+} was measured. Conditions are listed in Table IV and the Experimental Section: (○) observed data; (●) data corrected as described under Results.

Effect of the Dephospho Form of Phosphoglucomutase on the Manganese(II)–Phosphorus Interaction Involving Bulk Methylphosphonate. At 40.5 MHz the ^{31}P nmr signal of methylphosphonate consists of a quartet due to coupling with the methyl group, $J = 16.0$ Hz, and the signal at pD 7.5 is centered 25.3 ppm downfield from the resonance of a separate sample of H_3PO_4 (64%) in D_2O (20% by volume) similarly locked on deuterons (Nowak and Mildvan, 1972). In the present study proton decoupling was employed to produce a singlet signal. Values for $1/pT_{1p}$ and $1/pT_{2p}$ for the $CH_3PO_3 \cdot Mn$ complex are recorded in Table IV, before and after correction for free Mn^{2+}. Exchange cannot limit the value of $1/pT_{1p}$ in view of the large difference between it and $1/pT_{2p}$.[8]

The corresponding $1/pT_{1p}$ value in the presence of the $E_D \cdot Mn$ complex also is given in Table IV, first after correction both for free Mn^{2+} and the contribution of $CH_3PO_3 \cdot Mn$ (values in parentheses) and finally after correcting for the fractional occupancy of the weak phosphate-binding subsite of the dephosphoenzyme at the concentration of methylphosphonate used (0.1 M)—see the previous section. Although no correction was made for a possible Mn^{2+}–^{31}P interaction involving the strong phosphate-binding subsite, such a correction is not expected to be significant, as in the analogous case involving the methyl protons of methylphosphonate (see above). Hence, the $1/pT_{1p}$ value of 19,000 is taken as a measure of the Mn^{2+}–^{31}P interaction at the weak phosphate-binding subsite. This value is only 0.15 of the $1/pT_{2p}$ value for the methyl protons of methylphosphonate at this site indicating that $1/pT_{1p}$ of ^{31}P is dominated by $1/T_{1M}$.

In the case of $1/pT_{2p}$ for phosphorus, the observed value in the presence of the $E_D \cdot Mn$ complex actually was not significantly larger than the calculated correction (see previous sections) for the $CH_3PO_3 \cdot Mn$ assumed to be present under the conditions employed. Of course, the corrected $1/pT_{2p}$ value must be at least as large as that of $1/pT_{1p}$ (Mildvan and Cohn, 1970). However, with the correlation times given in the Appendix, the dipolar contribution to the corrected $1/pT_{2p}$ value should be only about 1.3 times that for $1/pT_{1p}$, which would be

[8] The large difference in $1/pT_{1p}$ and $1/pT_{2p}$ undoubtedly reflects hyperfine interaction between Mn^{2+} and ^{31}P that is much more important than the corresponding interaction involving Mn^{2+} and ^1H in the same complex.

TABLE V: Distance Approximations for Manganese(II) to Proton or Phosphorus Nuclei in Complexes Either of Methylphosphonate and Manganese(II) or of These Entities Bound to Phosphoglucomutase.

Complex	Nucleus	$10^9\tau_c$[a] (sec)	$10^9 f(\tau_c)$[b] (sec)	$10^{-3}/T_{1M}$[c] (sec^{-1})	Distance[d] (Å)
$CH_3PO_3 \cdot Mn$	^1H	0.052	0.16	16.5[e]	3.8
				6.7[f]	4.3
	^{31}P	0.052	0.16	7.0	3.2
$CH_3PO_3 \cdot E_P \cdot Mn$	^1H	2–5	0.46–0.8	0.4	10–11
$(CH_3PO_3)_2 \cdot E_D \cdot Mn$	^1H (weak site)	2–5	0.46–0.8	17[e]	5.4–5.9
	^1H (weak site)	2–5	0.23–0.36	4.8[f]	5.1–6.3
	^{31}P (weak site)	2–5	1.6–2.0	19	4.8–4.9

[a] See Appendix. [b] The maximum and minimum $f(\tau_c)$ values for the indicated range of τ_c values. [c] Taken as equivalent to $1/pT_{1p}$ values in Table IV; see Results. [d] Where indicated, the range of distances is obtained from the maximum and minimum values of $f(\tau_c)$ used in the calculation; for protons, $d = 812\ (f(\tau_c)T_{1M})^{1/6}$; for phosphorus, $d = 601\ (f(\tau_c)T_{1M})^{1/6}$. [e] Data obtained at 100 MHz. [f] Data obtained at 220 MHz.

substantially smaller than the relaxivity contribution for the amount of $CH_3PO_3 \cdot Mn$ complex that was calculated to be present. Hence, in the present case, the observed $1/pT_{2p}$ value serves more to indicate that the calculated correction is not too large than it does to set limits on $1/pT_{2p}$ for phosphorus. In no other case did the correction for $CH_3PO_3 \cdot Mn$ approach the measured relaxivity for enzyme–Mn^{2+} complexes.

The small ϵ_1 value for the Mn^{2+}–^{31}P interaction at the weak phosphate-binding subsite of the dephosphoenzyme also is in accord with the earlier conclusion that this interaction does not involve the binding of methylphosphonate within the coordination sphere of Mn^{2+}. Distance calculations in the following section support this conclusion.

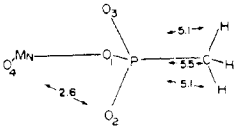
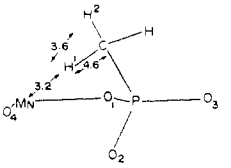
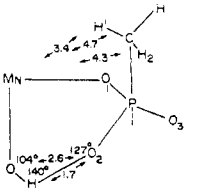
Calculation of Metal–Phosphorus and Metal–Proton Distances in Complexes of Manganese(II), Methylphosphonate, and Phosphoglucomutase. The distance between a paramagnetic metal ion such as Mn^{2+} and nuclei with which it interacts magnetically can be calculated from the paramagnetic contribution to the longitudinal relaxation rate of the nucleus while it is in the neighborhood of the metal ion, $1/T_{1M}$, and the correlation time for the interaction, τ_c (see Mildvan and Cohn, 1970). Arguments noted above and in the Appendix indicate that the measured $1/pT_{1p}$ values in Table IV are approximately equal to the corresponding $1/T_{1M}$ values, *i.e.*, the observed relaxation rates are not limited by chemical exchange. The Appendix also indicates how τ_c values were approximated from the experimental data.

Because distances are proportional to the one-sixth root of $1/T_{1M}$, an error of twofold in $1/T_{1M}$ causes a corresponding error of only 11% in the calculated distance. Average deviations of $1/pT_{1p}$ values in successive determinations, usually at different Mn^{2+} concentrations, are indicated in Table IV. In all cases the average deviation was $\leq 25\%$ of the mean; hence, random errors in $1/pT_{1p}$ values should cause distance errors of less than 4%. However, in one case, the Mn^{2+}–H^+ interaction in the $CH_3PO_3 \cdot Mn$ complex, where the same $1/pT_{1p}$ value was expected at both 100 and 220 MHz, a difference of slightly over twofold was obtained. Hence, systematic errors probably limit the accuracy of distance approximations to the region of 10–15%.

A range of τ_c values is given in Table V for each interaction involving an enzyme·Mn complex (see Appendix). In most cases a smaller range probably could have been supported; however, the indicated range suffices for the purpose of this paper. Actually it is not τ_c but $[f(\tau_c)]^{1/6}$, that appears in distance calculations. Moreover, since τ_c is in the range of ω_1, the error in $f(\tau_c)$ usually is smaller than the error in τ_c itself—see Table V. Calculated distances also are given in Table V.

Mn^{2+}–^1H and Mn^{2+}–^{31}P distances were measured with modified Dreiding models for three plausible structures for the $CH_3PO_3 \cdot Mn$ complex. Unless otherwise indicated, the following bond distances and bond angles were used: Mn–O, 2.2 Å; O–Mn–O, 90°; O–P, 1.5 Å; Mn–O–P, 120°; P–C, 1.8 Å;[9] O–P–C, 109°; C–H, 1.1 Å; P–C–H, 109°; O–H, 1.0 Å. The results are shown schematically in Chart I.

CHART I: Effective Metal–Proton Distances in Possible Complexes of Mn^{2+} with Methylphosphonate.[a]

Complex	Structure	d^{eff}, Å
1		5.2
2		3.55
3		3.8

[a] Standard bond angles and distances (see Results) were used to construct the various complexes, except where specifically indicated (complex 3). The following restrictions serve to further define these complexes: complex 1, Mn^{2+}–O_4 bisects $\angle O_3$–P–O_2; complex 2, Mn^{2+}–O_4 bisects $\angle$C–P–O_2 and O_4 bisects $\angle H_1$–C–H_2; complex 3, the ring is puckered so that H_3 is below O_3 and O_4 bisects $\angle H_1$–C–H_2. Pertinent distances in ångströms are indicated by arrows. Values for d^{eff} were calculated as described under Results.

[9] The P–C distance in paraffinic compounds is given as 1.87 Å (Weast, 1964). We use the value 1.8 Å because we expect a somewhat smaller distance when electronegative groups are attached to phosphorus.

In all three structures, the Mn^{2+}–^{31}P distance is the same, 3.2 Å, which is the same as the value obtained from nmr experiments (Table V). However, the methyl group can adopt a number of different positions relative to the Mn^{2+}; moreover, the Mn^{2+}–^{1}H distances usually are not the same for each hydrogen of the methyl group. In order to compare measured distances to calculated distances, the calculated value should represent an effective Mn^{2+}–^{1}H distance. Since $1/T_{1M}$ values for a relaxation process are proportional to $(1/d)^6$, where d is the distance in question, the effective Mn^{2+}–^{1}H distance for a methyl group should be given by $1/d_{eff} = (1/n\Sigma(1/d_i)^6)^{1/6}$, where $n = 3$.

Extended complexes have an effective Mn^{2+}–^{1}H distance of about 5.2 Å, as calculated with the above bond angles and distances. Complex 1 represents such a complex and is shown with the Mn–O_4 bond bisecting the O_2–P–O_3 angle to minimize unfavorable nonbonding interactions. The above distance is sufficiently greater than the range of values assessed from nmr experiments, 3.8–4.3 Å (Table V), to rule out complex 1 as an exclusive structure for $CH_3PO_3 \cdot Mn$. However, if the methyl group assumes the position occupied by O_2 or O_3 in complex 1, the effective Mn^{2+}–^{1}H distance is substantially decreased. Molecular models indicate the absence of significant unfavorable nonbonding interaction when O_4 bisects the H_1–C–H_2 angle, as in complex 2. In such a case the effective Mn^{2+}–^{1}H distance is ~3.55 Å. Although this value is sufficiently close to the observed range of values so that complex 2, alone, cannot be ruled out, it seems more reasonable to suggest that $CH_3PO_3 \cdot Mn$ actually is a mixture of complexes analogous to complexes 1 and 2. If there were equal populations of complex 1, complex 2, and a third complex analogous to complex 2, but with the methyl group below the O_4–Mn–O_1–P plane, the effective Mn^{2+}–^{1}H distance would be ~3.7 Å, which agrees well with the measured values.

A different type of complex in which one of the phosphonate oxygens is hydrogen bonded to a water molecule in the coordination sphere of Mn^{2+} to give a puckered cyclic structure also was considered. Although bond angles within the cycle are not ideal, they are not unreasonable. If the methyl group had an equal probability of being at (a) the position indicated in Chart I, complex 3, and (b) at the position occupied by O_3 in the drawing, the effective Mn^{2+}–^{1}H distance would be about 4.2 Å, which is also within the range of the measured values. A bidentate complex involving two oxygens of the phosphonate groups as ligands—especially if the two were not at equal distances from Mn^{2+}—also is not ruled out, although this possibility appears much less likely from the standpoint of bond angles and distances. In any case, in spite of the fact that the structure of $CH_3PO_3 \cdot Mn$ is not defined by the present study, it is obvious that in ternary complexes involving Mn^{2+}, methylphosphonate, and the enzyme, binding of methylphosphonate within the coordination sphere of the bound metal could produce an effective Mn^{2+}–^{1}H distance either longer or shorter than that found for the binary Mn–methylphosphonate complex by simply selecting one particular bond arrangement from among the above possibilities.

Discussion

In analyzing experiments involving the interaction of a small molecule with the metal complexes of a protein, it is necessary either to demonstrate the unimportance of an interaction between the small molecule and any metal ion not bound to the protein or to correct for such an interaction if present. In the present case, a careful analysis of the dissociation constants for complexes of Mn^{2+} with the protein and with methylphosphonate, measured in experiments conducted both separately and in mixtures of all three components, indicates that except in one experiment corrections for free $CH_3PO_3 \cdot Mn$ were only of marginal significance; however, the appropriate corrections were made anyway. Although extrapolations to other systems depend on a number of variables, from the standpoint of such corrections it appears that the phosphate analog used here, methylphosphonate, will be useful in studying the metal-binding sites of other enzymes that catalyze ($-PO_3$) transfer processes, but only when a dissociation constant for the Mn^{2+}–enzyme complex is 10 μM or less at neutral pH. However, the advantages of using methylphosphonate as a phosphate analog, where possible, are obvious; it is isoelectronic with phosphate, is approximately the same size, and can be observed much more readily by nmr techniques.

In addition to considering the above corrections, it is also necessary to demonstrate that the observed interaction occurs at the metal-binding site of the enzyme and not an ancillary site, especially in systems such as the present one where the enzyme is a "metal-activated" rather than a "metalloenzyme." The interference of bound substrate with the interactions reported here supports the viewpoint that the Mn^{2+}–methylphosphonate interactions observed do occur at the active site. In addition, the competitive nature of the inhibition produced by methylphosphonate (Ray *et al.*, 1973) indicates, at least at the strong subsite, that the observed metal–proton and metal–phosphorus interactions involve methylphosphonate bound at a position normally occupied by a phosphate group of the substrate.

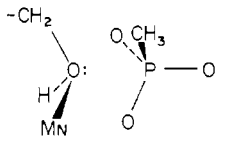
Although the present study represents an attempt to provide a quantiative estimate of the distance between bound Mn^{2+} and the phosphate-binding subsites of phosphoglucomutase, from a mechanistic standpoint one of the most important observations reported here is qualitative in nature: the magnetic interaction between methylphosphonate and $E_D \cdot Mn$ is much stronger than the analogous interaction with $E_p \cdot Mn$. This together with the observed concentration dependence of these interactions (see Results) indicates that the increased magnetic effect elicited by $E_D \cdot Mn$ occurs at a weak phosphate-binding subsite of the enzyme that is not accessible to methylphosphonate in $E_p \cdot Mn$. Since both $E_D \cdot Mn$ and $E_p \cdot Mn$ apparently have similar strong phosphate-binding subsites (see accompanying paper, Ray *et al.*, 1973), the increased magnetic effect produced by $E_D \cdot Mn$ presumably involves the weak phosphate-binding subsite that is not present in E_p, *i.e.*, the site at which the ($-PO_3$) transfer process occurs. In the accompanying paper (Ray *et al.*, 1973) this type of arrangement of subsites is used to argue that an "exchange" mechanism, with its functionally different and noninterchangeable phosphate-binding subsites, best describes the phosphoglucomutase reaction. The distance calculations below are discussed in terms of this model.

Calculated distances between Mn^{2+} and methyl protons and Mn^{2+} and the phosphorus nucleus of methylphosphonate in the binary $Mn \cdot CH_3PO_3$ complex and in complexes of these that also involve either the phospho or dephospho forms of phosphoglucomutase are shown in Table V. Chart I shows the analogous effective distances (see Results) for three conformations of the binary $CH_3PO_3 \cdot Mn$ complex, as measured from molecular models with standard bond angles and distances. Because of the differences in the Mn^{2+}–^{1}H distance in different conformations (Chart I) the Mn^{2+}–^{31}P distance is more readily interpreted. The calculated and measured values

of this distance, 3.2 Å, are 1.5 Å smaller than the corresponding distance in the complex involving methylphosphonate bound at the ($-PO_3$) transferring subsite of $E_D \cdot Mn$. Hence, methylphosphonate binding at this subsite does not appear to involve the primary coordination sphere of Mn^{2+}, although Mn^{2+} is quite close by. Indeed, the Mn^{2+}–phosphorus distance in Table IV, 4.8–4.9 Å, is too small to permit an intervening water ligand, which would require a minimum separation of about 5.5 Å. However, the latter value probably is within experimental error of the listed values.

In evaluating the above distance the following reservations must be considered. (a) Methylphosphonate, which appears to be a good phosphate analog in binding at the strong phosphate-binding subsite (Ray *et al.*, 1973), may not be as good a phosphate analog in binding to the site where ($-PO_3$) transfer occurs; perhaps inorganic phosphate would be bound differently at this site, *viz.*, within the coordination sphere of Mn^{2+}. (b) Mn^{2+} is not the natural activator of phosphoglucomutase (Peck and Ray, 1971) and is not only substantially larger than the natural activator, Mg^{2+}, but elicits a much lower activity, about 20-fold lower (Ray, 1969); perhaps in the $E_D \cdot Mg$ complex, methylphosphonate would be bound to the metal ion even though it is not in $E_D \cdot Mn$. (c) Phosphoglucomutase exhibits several characteristics of an induced-fit enzyme (Koshland, 1958; Yankeelov and Koshland, 1965); perhaps the phospho group of glucose bisphosphate that is to be transferred to the enzyme is bound within the hydration sphere of the metal ion even if inorganic phosphate and methylphosphonate are not. Because of these possibilities, the question of whether the bound metal actually participates in catalysis in a direct manner and if so, how, is not yet settled. However, an intriguing possibility consistent with the Mn^{2+}–phosphorus distance obtained here is that Mn^{2+} is coordinated with the active-site serine hydroxyl group and that methylphosphonate is bound so that the phosphorus is in a position close to that expected if it is to be the object of a nucleophilic attack by the coordinated serine hydroxyl group, see Scheme I. Although

SCHEME I



the role of the metal ion suggested by Scheme I is highly speculative at best, the possibility of "nucleophilic facilitation" by coordinated metal ions has been suggested by others on the basis of model reactions (see Busch, 1971) and is particularly attractive in a displacement on what apparently is a dianionic phosphate group.

In contrast to the Mn^{2+}–^1H distance for methylphosphonate at the weak phosphate-binding subsite, the analogous distance when methylphosphonate is bound at the strong phosphate-binding subsite indicates that this subsite is located several ångströms from the bound metal, and clearly outside the coordination sphere of the metal ion, regardless of whether its methyl group points toward or away from the metal ion. However, the orientation problem for the methyl group is sufficiently complex that we have not attempted to use the distances in Table V to produce a diagram of the active site of phosphoglucomutase.

In an earlier paper Ray and Mildvan (1970) suggested, on the basis of water proton relaxation (PRR) studies, that inorganic phosphate was not directly coordinated at the metal-binding subsite of phosphoglucomutase since, on binding, phosphate produced no substantial effect on the PRR of the $E_p \cdot Mn$ complex. This suggestion is in line with the conclusions reached here. Because of the substantial PRR de-enhancement produced by the binding of sugar phosphate substrates and inhibitors, these authors further concluded that some portion of the sugar phosphates was bound within the coordination sphere of the metal ion; if the phosphate group of such compounds is bound in the same manner as is inorganic phosphate, then only the sugar hydroxyl groups or the ring oxygen remain as likely candidates, and the 3- and 4-hydroxyl groups were suggested. However, this second suggestion clearly was based on the tacit assumption of a single $E_D \cdot Glc\text{-}P_2$ complex and the same sugar binding subsite for glucose-1-P and glucose-6-P, *i.e.*, a minimal motion mechanism (Ray *et al.*, 1973). If two different $E_D \cdot Glc\text{-}P_2$ complexes are present, as in the exchange mechanism, if these are present in comparable amounts, and if the sugar portion of bound glucose-1-P interacts differently with the enzyme than it does in the complex involving glucose-6-P, as now appears to be the case, sugar specific differences in PRR's for the ternary complex involving enzyme, Mn^{2+}, and substrate become much more difficult to interpret, since the observed enhancements represent the average effect of two structures. Moreover, Mn^{2+} cannot be involved both in the binding of sugar hydroxyl groups, as previously proposed, and in the coordination of the active-site serine, as suggested in Scheme I. Clearly additional work will be required to decide between these two possibilities.

Appendix: Calculation of Correlation Times for Manganese(II)–Methyl Proton and Manganese(II)–Phosphorus Interactions

The correlation time for the Mn^{2+}–^1H interaction in $CH_3PO_3 \cdot Mn$ is taken as the rotational correlation time for the entire complex (Mildvan *et al.*, 1967; Mildvan and Cohn, 1970). For free Mn^{2+} the analogous value is about 2.9×10^{-11} sec (Bloembergen and Morgan, 1961). Since the PRR enhancement produced by the presence of $CH_3PO_3^{2-}$ in solutions of Mn^{2+} is about 1.5 (see Results), the τ_c value in question can be calculated from the relationship, enhancement $= \tau_c^* q^* / \tau_c q$, where the parameters with and without asterisks refer, respectively, to $CH_3PO_3 \cdot Mn$ and free Mn^{2+}, and q is the number of exchangeable water molecules: 6 in the case of free Mn^{2+} and 5 for $CH_3PO_3 \cdot Mn$, if monodentate coordination is assumed. Hence, the τ_c value in question should be equal to about 5.2×10^{-11} sec. The corresponding τ_c value for $Mn \cdot HPO_4$ and $Mn \cdot FPO_3$ is 3.4×10^{-11} sec (Mildvan *et al.*, 1967).

The correlation time for the Mn^{2+}–^1H interaction in the $CH_3PO_3 \cdot E_P \cdot Mn$ complex was approximated in two ways. A maximum value was obtained from the corrected $1/pT_{1p}$ and $1/pT_{2p}$ values for the appropriate Mn^{2+}–^1H interaction in Table IV, by means of the Solomon–Bloembergen equations (see Mildvan and Cohn, 1970). In this procedure, hyperfine interaction was ignored, because methylphosphonate is not bound within the coordination sphere of Mn^{2+} in $E_P \cdot Mn$; see Results. In addition, $1/pT_{2p}$ was taken as equal to $1/T_{2M}$ because of the following considerations: if the bimolecular association rate constant for methylphosphate is as large as the minimum value of this constant for glucose-1-P, 2×10^8 M^{-1} sec^{-1} (from the ratio of V_{max}/K_m; Ray and Peck, 1972), which seems reasonable, the dissociation rate constant for $Mn \cdot E_P \cdot CH_3PO_3$ can be approximated as 3×10^5 sec^{-1} from

the K_d value for this complex (1.5 mM; Ray *et al.*, 1973). This value is much larger than $1/pT_{2p}$; hence it is unlikely that chemical exchange limits $1/pT_{2p}$. However, $1/pT_{2p}$ for the Mn^{2+}–methyl proton interaction was determined by line broadening measurements on one peak of a doublet (see Experimental Section) and such line broadening can be effected by the T_1 of the neighboring ^{31}P nucleus through a chemical exchange spin-decoupling process (Frankel, 1969; Villafranca and Mildvan, 1972). Hence, the corrected value for $1/pT_{2p}$ in Table IV must be considered as a maximum value, which means that τ_c calculated from the T_{1p}/T_{2p} ratio is also a maximum estimate, *i.e.*, $\tau_c \leq 5 \times 10^{-9}$ sec.

Alternatively, τ_c should be the same for both the Mn^{2+}–^1H interaction in question and the Mn^{2+}–water proton interaction in the *same complex*. The τ_c value for the Mn^{2+}–water proton interaction in this complex is about 3.6×10^{-9} sec.[10] This procedure appears to be less subject to experimental error and also produces a value that is consistent with the previous limiting value. However, it depends on the assumption that τ_c is essentially frequency independent in the 24.3–100 MHz range (since the Mn^{2+}–water proton interaction was assessed at the lower frequency). Hence, in order to make a reasonable allowance for errors in τ_c, its value will be taken as $2\text{–}5 \times 10^{-9}$ sec^{-1}.

A maximum value for the τ_c of the Mn^{2+}–^1H interaction involving methylphosphonate bound at the weak phosphate-binding subsite of the $E_D \cdot Mn$ complex can be approximated from the T_{1p}/T_{2p} ratio as described above for the $E_P \cdot Mn$ complex. Since methylphosphonate is bound much less tenaciously at the weak phosphate-binding subsite of $E_D \cdot Mn$ than at the strong phosphate-binding subsite (see Results), chemical exchange probably does not limit $1/pT_{2p}$ at 100 MHz (see Table IV) even though the corrected $1/pT_{2p}$ value is some 30-fold larger than for $E_P \cdot Mn$. Hence, $\tau_c \leq 5 \times 10^{-9}$ sec.

From a comparison of $1/pT_{1p}$ for the Mn^{2+}–^1H interaction at 100 and 220 MHz (Table IV), according to the Solomon–Bloembergen equations (see Mildvan and Cohn, 1970) a τ_c value of 2.2×10^{-9} is obtained. Although this appears to be the more reliable value, τ_c will be taken as $2\text{–}5 \times 10^{-9}$ in subsequent calculations for the reason noted above.

The correlation time for the Mn^{2+}–^{31}P interaction involves methylphosphonate bound at the weak phosphate binding site of $E_D \cdot Mn$; hence τ_c for this interaction is taken to be the same as τ_c for the above Mn^{2+}–^1H interaction in the *same complex*, *i.e.* $2\text{–}5 \times 10^{-9}$ sec.

All of the above τ_c values are given in Table V; also shown are the maximum and minimum values of $f(\tau_c)$ within the given range of τ_c values at the frequencies used.

[10] The τ_c value for the ternary complex of Mn^{2+}, phosphoenzyme, and inorganic phosphate was not specifically mentioned by Ray and Mildvan (1970); however, the PRR data were essentially indistinguishable for this ternary complex and the binary complex of phosphoenzyme and Mn^{2+}. In the calculation of τ_c, the hyperfine contribution to $1/T_{2M}$ was assumed to be inappreciable for the reasons stated in the above reference.

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