Electron Paramagnetic Resonance Studies of Manganese(II) Coordination in the Phosphoglucomutase System*

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ABSTRACT: Electron paramagnetic resonance spectra are given for the Mn(II) complex of rabbit muscle phosphoglucomutase *in solution*. Spectra resemble those for powdered solids because the rotational motion of the protein complex is too slow to average the anisotropic magnetic environment of the bound ion. The scattering of fine structure transitions produced by zero-field-splitting effects is prominent in some of the spectra. Marked changes in zero-field splitting, which reflect the extent of distortion of the electronic environment of bound Mn(II) from cubic symmetry, occur when ternary complexes are subsequently formed by binding substrates and inhibitors to the enzyme. Thus, the ternary complexes of glucose 1- and 6-phosphates (equilibrium mixture), 6-deoxyglucose 1-phosphate, 1-deoxyglucose 6-phosphate, and glucuronic acid 1-phosphate with the Mn(II)·phosphoenzyme have a substantially larger zero-field splitting than the binary Mn·phosphoenzyme complex; similarly, conversion of the phosphoenzyme into the dephospho form markedly enhances splitting effects. By contrast, formation of ternary complexes with the phosphate esters of fructose, galactose, and mannose (equilibrium mixtures of 1- and 6-phosphates) has the opposite effect, and these complexes show rather low zero-field splitting; complexes with unmodified sugars and with ethylene glycol give similar spectra. Such changes reflect significant alterations in the first coordination sphere of the bound Mn(II) that in some cases would be difficult to rationalize without invoking ligand substitutions; moreover, correlations are found between the extent of zero-field splitting and the enhancement of water proton relaxivity for many of these complexes. In addition, freezing alters the electron paramagnetic resonance spectra of Mn(II)·phosphoglucomutase solutions to a marked degree although the alteration is reversed on thawing. Changes in the spectra for frozen solutions are tentatively ascribed to alterations in the protein conformation and point up possible dangers in equating structural parameters for solution and frozen samples.

The divalent cation requirement of phosphoglucomutase (EC 2.7.5.1) provides a basis for studying structural features of the enzyme and its metal ion complexes. Solvent perturbation studies show that structural changes accompany the binding of metal ions to the enzyme (Peck and Ray, 1969a); these changes appear to be identical for a number of divalent metals. Subsequent binding of the substrate (glucose 1- and 6-phosphates) causes additional changes which are moderated in a manner characteristic of the bound metal ion (Peck and Ray, 1969b). The ability of the enzyme to function with a variety of divalent transition metal ions (Ray, 1969) in place of the physiologically active ion, Mg(II) (Peck and Ray, 1971), has permitted investigation of the metal ion binding site by spectroscopic methods analogous to those used to examine structures of transition metal complexes involving small molecules (Ray and Mildvan, 1970; J. S. Multani and W. J. Ray, Jr., submitted for publication). These studies have shown that the environment of the enzyme-bound metal ion is altered upon formation of ternary complexes with substrates and substrate analogs, and the possibility of coordinate bonding between the metal and the substrate has been inferred, but not demonstrated. Electron paramagnetic resonance (epr) studies of phosphoglucomutase·Mn(II) were initiated to further study this possibility by examining the electronic environment of Mn(II) in the binary enzyme·metal complexes of phospho and dephospho forms of the enzyme and in ternary complexes with substrates or inhibitors.

Although nuclear relaxation studies of enzyme complexes containing Mn(II) have been extensive (for recent review, see Mildvan and Cohn, 1970), the virtual disappearance of epr signals for Mn(II) in macromolecular complexes in solution thus far has prevented direct use of epr spectroscopy in structural studies of such complexes. In fact, the apparent absence of epr signals for Mn(II) bound to proteins has provided a convenient analytical method for distinguishing between bound and free Mn(II) in measuring binding constants (Cohn and Townsend, 1954; Malmström *et al.*, 1958). Although rapid "homogeneous" relaxation of electron spin states for the bound ion, with concomitant and extensive line broadening might account for this absence of epr signals for protein-bound Mn(II), such an explanation is inconsistent with the sizeable enhancements of nuclear spin relaxation rates for solvent molecules which have been observed for solutions of the same Mn·protein complexes.[1] This apparent anomaly has prompted a more exhaustive search for the epr spectra of macromolecular complexes of Mn(II) and an explanation for their low intensities.

Reed and Cohn (1970) ascribe the low intensity of the solution epr spectrum for Mn(II) bound to the protein,

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[1] Longitudinal relaxation of electron spin states sufficiently rapid to effectively eliminate the epr spectrum would lead to a correlation time too short to account for the observed enhancements of the nuclear spin relaxation rate of water protons.

concanavalin A, to the solid-state character of the line shape. Thus if epr signals of Mn(II) in macromolecular complexes are not isotropically averaged by molecular tumbling, the anisotropic terms in the spin Hamiltonian will lead to a complex solid-state (*i.e.*, "powder") spectrum. The solid-state line shape causes severe experimental problems as far as observation of the Mn·protein spectrum is concerned. However, the extra effort required to obtain such spectra seems worthwhile since in principle the anisotropic magnetic interactions can be evaluated directly from the spectra, and information about the electronic environment of the bound metal ion and its response to binding of substrates and inhibitors can thus be obtained in the solution phase.

The present paper describes epr spectra for complexes formed in the phosphoglucomutase system. Some features of the interaction between the substrate and the Mn·enzyme complex are inferred from the relative magnitudes of the zero-field splitting for the various complexes. Methodology required to interpret solid-state, "powder" spectra for spin 5/2 systems in terms of an appropriate spin Hamiltonian is outlined.

Experimental Section

Materials and Assays. All sugar phosphates other than those noted below were obtained from Sigma and were used without further purification, except for glucose-1-P which was chromatographed as described previously (Ray and Roscelli, 1964). Tris base was obtained from Mann and other chemicals were analytical grade. The chlorides of Tris and manganese were used throughout. Procedures for preparing 1-deGlc-6-P, 6-deGlc-1-P, and 3-deGlc-1-P will be described in a subsequent paper. A sample of methylphosphonate was kindly provided by Dr. Alexander Hampton.

The phospho form of phosphoglucomutase was isolated by means of a scaled-up and modified version of the previously described procedure (Ray and Koshland, 1962). The dephospho form of the enzyme was prepared from the phosphoenzyme by inducing phosphate transfer to water by means of Xyl-1-P (J. D. Owens, J. W. Long, and W. J. Ray, Jr., manuscript in preparation), followed by extensive dialysis. These procedures will be described elsewhere (E. J. Peck, Jr., J. W. Long, and W. J. Ray, Jr., manuscript in preparation); in the meantime details are available on request (from W. J. R.).

Bound metals were removed from the phospho and dephospho forms of the enzyme at pH 7.5 as described previously (Ray, 1969), except that initial protein concentrations were about 60 mg/ml; subsequent dialysis under reduced pressure against 20 mM Tris-chloride buffer (pH 7.5) increased protein concentrations to the range of 180–320 mg/ml; such solutions were frozen dropwise by direct addition to liquid nitrogen and stored in liquid nitrogen until used (*cf.*, Yankeelov *et al.*, 1964). At this point the activity of the phosphoenzyme was about 830 U/mg; usually it contained about 10% and always less than 20% of the dephospho form. The activity of the dephosphoenzyme was about 720 U/mg; it contained less than 5% and was probably free of phosphoenzyme.

Enzymatic assays for both phospho and dephospho forms (excess Glc-1,6-P_2) were conducted by the procedure described previously (Ray, 1967) as modified by Peck and Ray (1971). The amount of dephosphoenzyme present in a given sample was measured by means of the Glc-6-P produced on reaction with Glc-1,6-P_2, as described by Lowry and Passonneau (1969).

Epr Measurements. X-band (9.1 GHz) epr spectra were recorded with a Varian E-3 spectrometer which was equipped with a standard accessory unit for variable temperature. A constant temperature was maintained by passing precooled nitrogen through the cavity dewar; a cavity temperature of −4° was used for all spectra of enzyme solutions shown herein, and 0° for buffer solutions, but the equilibrium temperature of samples may have been a few degrees higher because of microwave absorption by the solution; spectra were also taken at room temperature (25°) but are not shown. Aqueous samples of about 40 μl were placed in capillary tubing made of high-purity quartz, 0.9-mm i.d. and 2-mm o.d. Epr spectra at K band (35 GHz) were recorded with a Varian 4503 spectrometer which was equipped with a variable-temperature accessory. Sample cells were constructed by drawing the tip of 2-mm quartz tubing to a capillary about 2 cm long and 0.3-mm i.d. Approximately 1–2 μl of solution was placed in the capillary and the end closed with a polyethylene stopper. All samples contained 50 mM Tris-chloride buffer (pH 7.5.).

At low modulation amplitudes (less than 10 G) the base-line trace of the X-band spectrometer with buffer in the sample tube was flat across the entire field scan. The use of higher modulation amplitudes (*i.e.*, 40 G) produced a sloping base line; in such cases two spectra were accumulated in a Varian C-1024 computer and two buffer traces were subtracted to correct for this slope.

Interpretation of Epr Spectra. Omitting, for simplicity, hyperfine interaction with the ^{55}Mn nucleus, in an external magnetic field, H, there are six possible spin-energy levels for Mn(II) which correspond to the six values of the electron spin quantum number, M (*i.e.*, 5/2, 3/2, ..., −5/2). Allowed epr transitions involve a change in M of ±1; hence, there are five allowed fine structure transitions. A shorthand description of the detailed spacing of the six energy levels is provided by a spin-Hamiltonian equation of the following simplified[2] form

$$\mathcal{H}_s = \mathbf{g}\beta H \cdot S + D[S_z^2 - (1/3)S(S+1)] + E[S_x^2 - S_y^2] \quad (1)$$

where D and E are, respectively, the axial and rhombic distortion parameters of the electronic quadrupole or zero-field-splitting interaction and other terms have their usual meanings (Abragam and Bleaney, 1970). The first term in eq 1 (the Zeeman interaction) has been written for an isotropic **g** tensor and is therefore invariant with changes in the orientation of the sample in the magnetic field. On the other hand, the ZFS[3] interaction (second and third terms) is anisotropic, and its effects on the energy levels will be orientation dependent. In rapidly rotating complexes (*i.e.*, $\Delta\omega\tau_r < 1$, where $\Delta\omega$ is the ZFS in angular frequency units and τ_r the rotational correlation time) the ZFS is effectively averaged to zero[4] and makes no net contribution to the spacing of energy levels. Consequently, transition frequencies for all of the fine structure components are equal and the spectrum is a single isotropic line which is a superposition of the five allowed transitions. It should be noted that motional averaging of the ZFS can

[2] A more complete Hamiltonian would contain several additional terms which are of secondary importance in describing the system.

[3] The following abbreviations are used: ZFS, zero-field splitting; E_P and E_D phospho and dephospho forms of phosphoglucomutase; sugar-P, an equilibrium mixture of sugar-1-P and sugar-6-P (when a single isomer is present, that isomer is specified). See *Biochemistry 5*, 1445, for nomenclature used in this paper.

[4] The zero-field-splitting parameters, D and E, can be expressed as a diagonal tensor operator: $D_x = E - D/3$, $D_y = -(E + D/3)$, and $D_z = 2D/3$. The time average of this operator is $(D_x + D_y + D_z)/3 = 0$.

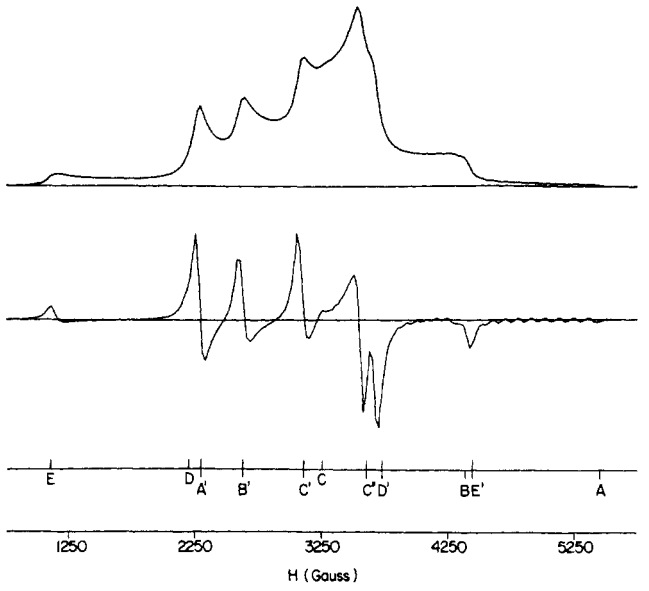

FIGURE 1: Powder spectrum (computer simulation) at X band for spin 5/2, axial symmetry, isotropic **g** tensor, no nuclear hyperfine interactions, and $D = -535$ G. Top, absorption curve; bottom, derivative curve. Unprimed letters identify the transitions for H parallel to the crystal field axis; primed letters identify transitions for H perpendicular to crystal field axis; C'' is for the $-1/2 \leftrightarrow 1/2$ transitions at $\theta = 41°48'$. Free electron resonance position, $g = 2.0023$, coincides with C. A, $5/2 \leftrightarrow 3/2$; B, $3/2 \leftrightarrow 1/2$; C, $1/2 \leftrightarrow -1/2$; D, $-3/2 \leftrightarrow -1/2$; and E, $-5/2 \leftrightarrow -3/2$. Although the spectrum is insensitive to the sign of D, transitions have been labeled assuming a negative value for D.

induce transitions (*i.e.*, relaxation) among the electron spin states. By contrast, when rotational motion is slow or the ZFS large, $\Delta\omega\tau_r > 1$, the terms, in D and E no longer vanish, and epr spectra similar to those for randomly oriented solid samples are observed. In such cases the ZFS interaction produces characteristic shifts of the energy levels of the spin system (Bleaney and Trenam, 1954; Hurd *et al.*, 1954) which are manifested in the spectrum as fine structure transitions displaced from the unperturbed or isotropic resonance position, H_0.

Magnetic field positions for the fine structure components are found from solutions to eq 1. The actual form of these solutions hinges on the relative magnitudes of the ZFS and Zeeman splitting, $\mathbf{g}\beta H$ (De Wijn and Van Balderen, 1967; Griscom and Griscom, 1967). Spectra for phosphoglucomutase-Mn(II) complexes suggest that the ZFS is less than 0.1 cm^{-1}; hence ZFS $< \mathbf{g}\beta H$. A suitable solution obtained by perturbation theory and accurate to second order in $D/g\beta H$ is given in eq 2 for the transition, $M \leftrightarrow M - 1$, in a spin 5/2 system (Perkins and Sienko, 1967; Morigaki *et al.*, 1958; Howling, 1969). Here H_r is the resonance position, H_0 is the unperturbed position, given by $h\nu/\mathbf{g}\beta$ (where ν is the spectrometer frequency), M is the electron spin quantum number for the upper state, and $f = D(3\cos^2\theta - 1) + 3E\cos 2\phi \sin^2\theta$, $b = (D - E\cos 2\phi)^2 \sin^2 2\theta + 4E^2\cos^2\theta \sin^2 2\phi$, and $c = [D\sin^2\theta + E\cos 2\phi(1 + \cos^2\theta)]^2 + 4E^2\cos^2\theta \sin^2 2\phi$. The angles ϕ and θ are the azimuthal and polar angles, respectively, of the magnetic field vector relative to the crystal field axis of the ion. The angular dependence of the $1/2 \leftrightarrow -1/2$ transition arises from the third term in eq 2 which is field dependent. The angular variations of the other transitions are dominated by the field-independent, first-order term (the second term in eq 2).

$$H_r = H_0 - [(M - {}^1/_2)f] + (1/H_0)[3.25 - 3M(M-1)b - (1.8125 - 0.75M(M-1))c] \quad (2)$$

When distortions of the complex from cubic symmetry are purely axial (*i.e.*, $D \neq 0$ and $E = 0$ in eq 1) eq 2 is greatly simplified. For randomly oriented (powder) samples the epr absorption for each fine structure component will extend from the field position where $\theta = 0°$ to the position where $\theta = 90°$ (*cf.* eq 2). These two angles correspond to extrema in the absorption curve, and therefore signals will occur at field positions corresponding to these angles in the derivative epr spectrum (Sands, 1955). For the $1/2 \leftrightarrow -1/2$ transition there is an additional angle,[5] $\theta = 41° 48'$, which corresponds to an extremum in the absorption envelope and therefore gives a derivative epr response. It should be emphasized that if one compares amplitudes of derivative epr signals for isotropic spectra with anisotropic powder spectra, the powder spectra will have smaller amplitudes (depending on the magnitude of the anisotropy) because in the powder sample only those spins whose axes lie near the angles $\theta = 0°$ or 90° contribute to the derivative signal intensity.

Figure 1 shows a computed[6] powder spectrum (absorption and derivative) for an axially distorted $S = 5/2$ system. In comparing features of this theoretical spectrum with the experimental spectra for ^{55}Mn(II) it is important to note that hyperfine coupling to the ^{55}Mn nucleus omitted in the theoretical spectrum splits each signal into six components.[7] This additional splitting produces increased overlap between fine structure components and thus adds to the complexity of the spectrum. In addition, all of the fine structure transitions need not have the same relaxation time (line width) and the relaxation time may differ for $\theta = 0°$ and 90° orientations. Moreover, additional complexities may arise from forbidden hyperfine transitions (Bleaney and Rubins, 1961) and from rhombic distortions.

Magnetic field positions for centers of fine structure transitions are given in Figure 2 as a function of the extent of axial distortion. Aasa (1970) has provided diagrams representing solutions to eq 1 for both axial and rhombic symmetries over a wide range of ZFS. ZFS parameters for anisotropic complexes of phosphoglucomutase were estimated by comparison of the positions of transitions in both X-band and K-band spectra with the graphs in Figure 2. In this regard, predicted splitting of the $-1/2 \leftrightarrow 1/2$ transition with increas-

[5] The third term, eq 2, exhibits a maximum when $\tan^2\theta = 4/5$ or $\theta \sim 41°48'$.

[6] The simulation included 100 increments of θ between 0° and 90° at 25-G intervals. While this sampling was sufficient for smooth curves in the low-field half of the spectrum, there is apparent "noise" in the high-field portion which is indicative of insufficient angular sampling. This asymmetry arises from the second and third terms in eq 2 which are applied with opposite sign in the high-field half of the spectrum and this creates a rapid angular variation. On the other hand, the two terms have the same sign in the low-field half of the spectrum, and in this case, the angular variation is diminished. As a result derivative peaks which occur in the high-field half of the spectrum will have lower apparent intensities. This asymmetry is observed in experimental spectra.

[7] Expressions which predict the exact field positions of the ^{55}Mn hyperfine splitting are available (De Wijn and Van Balderen, 1967). These expressions (which predict a sextet splitting of each fine structure transition) come from solutions to the spin Hamiltonian in the *combined* effects of the ZFS and hyperfine tensors and are therefore valid over the region $D, A \ll \mathbf{g}\beta H$ (where A is the hyperfine coupling constant) irrespective of the magnitudes of D and A relative to each other.

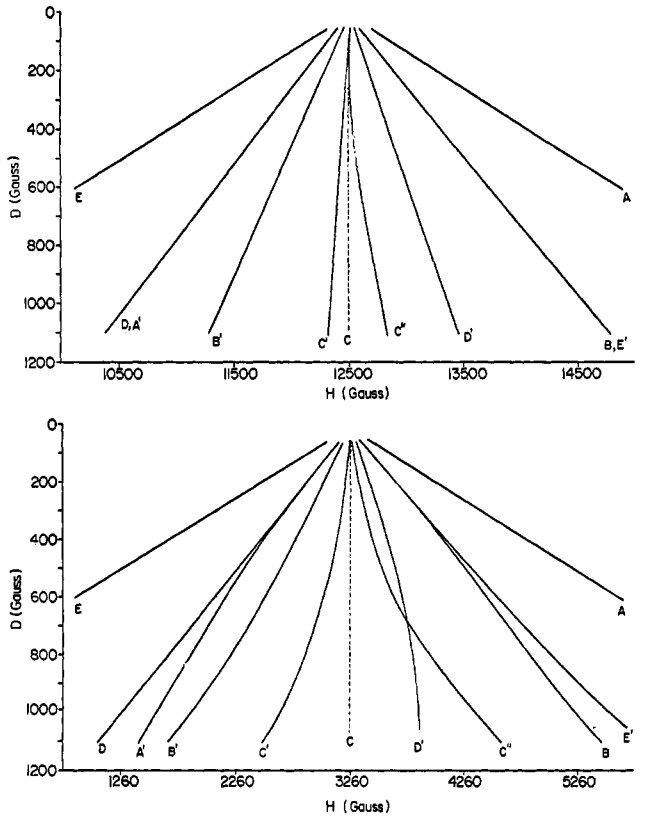

FIGURE 2: Magnetic field positions of fine structure transitions in a powder spectrum of a ^{55}Mn(II) complex with axial symmetry as a function of the zero-field splitting. Top, K band; bottom, X band. Fine structure identification is given in Figure 1. D is presumed negative (*cf.* Figure 1). Positions are obtained from eq 2. Free electron resonance position, $g = 2.0023$, coincides with C.

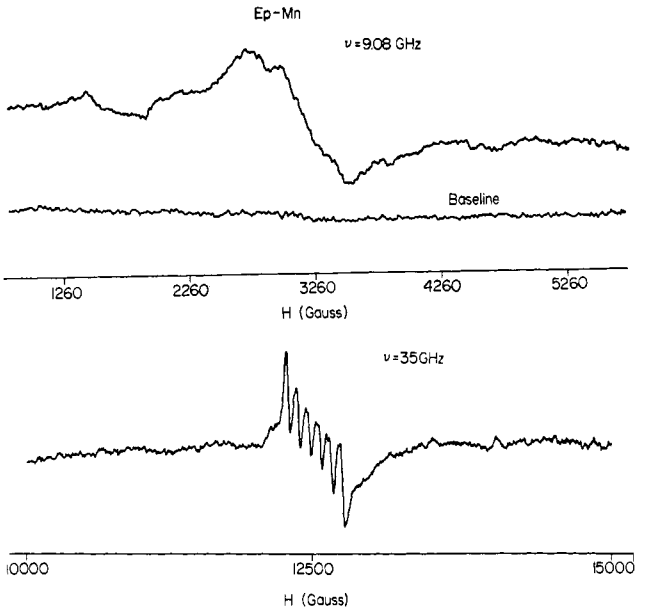

FIGURE 3: Solution epr spectra for Mn(II)·phosphoglucomutase at X-band and K-band frequencies. Solution contained 2.3 mM E_P and 1 mM $MnCl_2$.

ing D is particularly useful, especially for the K-band spectra where this transition is dominant and well resolved. Given the rather poor resolution of fine structure components in the experimental spectra, axial distortions were adequate to account for the gross features of the spectra.

Results

X-band and K-band epr spectra for millimolar solutions of the Mn(II)·phosphoenzyme complex at "−4°" (see Experimental Section) are shown in Figure 3. In all cases the concentration of (free) aquo-Mn(II) is negligible at the enzyme to Mn(II) ratio used, about 1.5 (Ray, 1969). The effective base-line trace for the X-band spectrometer (with buffer in the sample tube) is also shown. A similar trace (not shown) with apoenzyme gave no extraneous signals across the entire field scan. The solid-state character of the spectrum is apparent from the fine structure splitting as noted above. An approximate value of 250 G for the ZFS is obtained as described in the section on Interpretation of Epr Spectra. Hyperfine structure in the $1/2 \leftrightarrow -1/2$ transition (barely discernable in the X-band spectrum) is also present at room temperature (25°) in both X-band and K-band spectra (not shown) although the hyperfine components broaden markedly with increasing temperature (see below). Narrowing of the $1/2 \leftrightarrow -1/2$ transition in the K-band spectrum relative to the X-band spectrum is also observed at all temperatures.[8]

The spectrum of bound Mn(II) is remarkably sensitive to structural changes which occur upon binding of substrates or substrate analogs to the enzyme. Representative examples of such effects which reflect changes in the electronic environment of Mn(II) are illustrated in Figures 4 and 5 at X-band and Figure 6 at K-band frequencies. Table I summarizes epr spectral characteristics in terms of approximate ZFS, that were observed upon addition of a variety of such compounds. Additives which decrease, do not alter, or which increase the ZFS are grouped together in class I, II, or III, respectively.

The relatively narrow spectra obtained on binding class I additives (*e.g.*, galactose-P or glucose; see Table I and Figure 4) initially suggested that Mn(II) might be released from the enzyme in the presence of these compounds. However, the temperature dependence of the line width for the hyperfine components of this signal rules out the possibility that the signal arises from free Mn(II) or a binary Mn(II)·additive complex. Thus, in every case increasing temperature broadened spectral lines whereas increasing temperature has the opposite effect on the X-band spectra of aquo-Mn(II) and Mn(II) complexes with small molecules (Garrett and Morgan, 1966; G. H. Reed and J. S. Leigh, Jr., to be published). That the viscous, concentrated protein solutions did not alter the sign of the temperature coefficient for aquo-Mn(II) and binary Mn(II)·substrate complexes was verified by overtitrating the

[8] Such narrowing on increasing the frequency from X band to K band is produced by three processes. (1) An inverse field dependence of second-order terms reduces the field spread for the $-1/2 \leftrightarrow 1/2$ transition, since this transition does not contain a field-independent first-order term in D, *i.e.*, the term, $(M - 1/2)f$, eq 2, disappears when $M = 1/2$; in contrast, the other fine structure components, $5/2 \leftrightarrow 3/2$, *etc.*, are displaced from H_0 by the $(M - 1/2)f$ term and the effective amplitudes of these transitions, relative to that for the central transition, thus are reduced at the higher frequency. (2) Forbidden hyperfine transitions ($\Delta M = \pm 1$, $\Delta m \neq 0$) which contribute to inhomogeneous broadening of the $-1/2 \leftrightarrow 1/2$ transition also decrease with the inverse square of the field strength (Bleaney and Rubins, 1961). (3) Homogeneous relaxation of the electron spin states is frequency dependent (Reuben and Cohn, 1970; Bloembergen and Morgan, 1961; G. H. Reed and J. S. Leigh, Jr., to be published); hence, higher frequencies usually lead to longer relaxation times and thus to sharper signals.

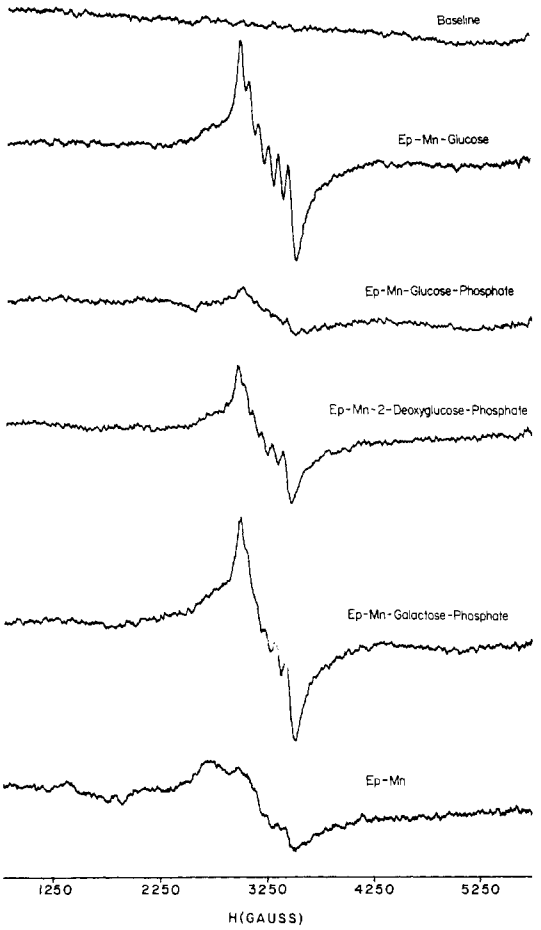

FIGURE 4: X-band epr spectra for ternary complexes of Mn(II)·phosphoglucomutase complexes. All solutions contained 2.3 mM E_P and 1.5 mM $MnCl_2$. Additive concentrations were glucose, 0.2 M; glucose phosphate, 0.04 M; 2-deoxyglucose phosphate, 0.08 M; and galactose phosphate, 0.08 M. Spectrometer settings were identical for all samples.

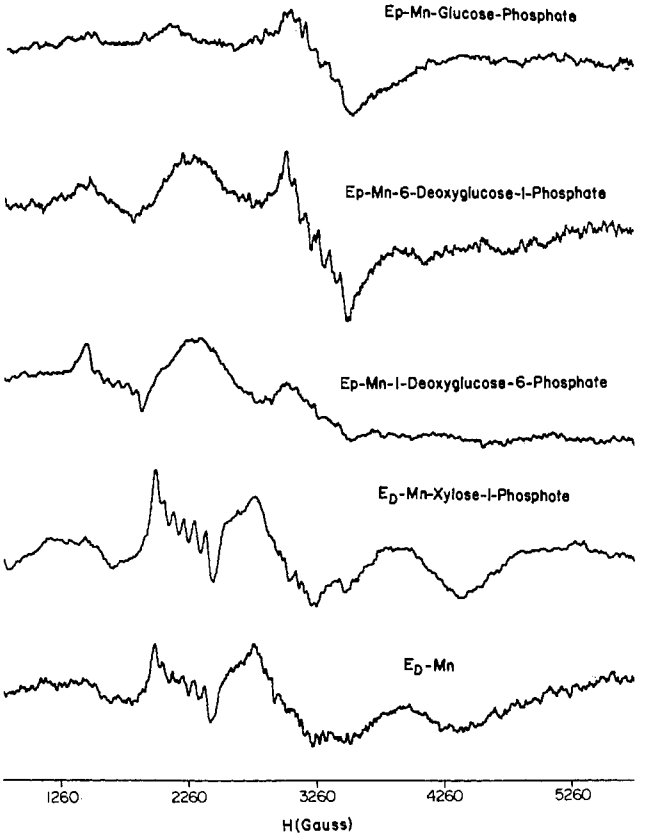

FIGURE 5: X-band epr spectra for ternary complexes of Mn(II)·phosphoglucomutase spectra were obtained from the following solutions, top to bottom; 2.0 mM E_P, 1.1 mM $MnCl_2$, 10 mM glucose phosphate, relative gain 1; 1.9 mM E_P, 0.88 mM $MnCl_2$, 15.5 mM 6-deGlc-1-P, relative gain 2; 2.3 mM E_P, 1.0 mM $MnCl_2$, 20.0 mM 1-deGlc-6-P, relative gain 1; 2.3 mM E_D (enzyme was in the phospho form prior to mixing with additive), 1.5 mM $MnCl_2$, 0.08 M Xyl-1-P (2.3 mM P_i, from hydrolysis of phosphoenzyme) relative gain 4; 1.7 mM E_D, 0.5 mM $MnCl_2$, relative gain 8. 40-G modulation amplitude and computer baseline subtraction were used for each spectrum.

enzyme or enzyme·substrate complexes with Mn(II) and examining the temperature dependence of the signals for the excess Mn(II) or Mn(II)·substrate complex.

Only those additives which are known to bind to Mn(II)·phosphoglucomutase and which still do not alter its epr spectrum are grouped in class II. In addition to P_i (Ray and Mildvan, 1970) and methylphosphonate (W. J. Ray, Jr., and A. S. Mildvan, unpublished results) the cis and trans isomers of cyclohexanediol, mannosamine, and α-methyl glucoside were without effect on the epr spectrum of Mn(II)·E_P at concentrations of 0.1 M; however, it is not known whether the latter four materials bind to the enzyme.

All class III additives produce markedly enhanced ZFS (Figures 4 and 5). Although the fine structure components of these spectra are significantly different with regard to position or apparent width, except for the complexes of GlcUA-1-P and 1-deGlc-6-P which give virtually identical spectra, the basic spectrum appears to be similar in each case. Since the class III complexes are probably rather similar structurally, the differences in these spectra demonstrate the high degree of sensitivity the epr spectrum has toward differences in the environment of the bound Mn(II).

The phosphates of glucose, 2-deoxyglucose, glucosamine, mannose, galactose, and fructose are substrates (Lowry and Passonneau, 1969; Együd and Wheland, 1963) and were present as an equilibrium mixture of the 1 and 6 isomers. Moreover, the ternary complexes formed by binding of these sugar phosphates to the Mn(II)·enzyme is an equilibrium mixture of three isomeric complexes which differ in the position of phosphate attachment, *e.g.*, E_P·Mn·Glc-1-P, E_D·Mn·Glc-P_2, and E_P·Mn·Glc-6-P (Ray and Roscelli, 1964), and in this system the relative amounts of these are unknown.[9] Although it is quite possible that the epr spectrum for each isomeric ternary complex involving a given sugar phosphate substrate is the same, this may not be the case. If not, and if one of the three forms of the complex fails to predominate over the other two, the observed spectrum will consist of a superposition of spectra for two or even three complexes, and a detailed analysis of the spectrum will not be feasible. In addition, the phosphates of mannose, galactose, and fructose are known to be poor substrates and 2-deoxyglucose phosphate may also be a poor substrate; hence, composite spectra for these sugar phosphates could be produced

[9] In the Mg(II)·phosphoglucomutase system, E_P·Mg·Glc-6-P is the prominent member of the isomeric ternary complexes; however, E_D·Mg·Glc-P_2 represents 20–30% of the total ternary complexes while the complex involving Glc-1-P represents about 5% of the total; W. J. Ray, Jr., unpublished results.

TABLE I: Zero-Field Splitting in Mn(II)·Phosphoglucomutase Complexes.[a]

Additive	Class	Concn (M)	ZFS (G)
Man-P[b]	I	0.08	$<1.5 \times 10^2$
Gal-P[b]		0.08	
Xylose		0.2	
Glucose		0.2	
Glucosamine-P[b]		0.080	
Ethylene glycol		0.113	
Fru-P[b]		0.08	
2-Deoxyglucose		0.08	
2-DeGlc-P[b]		0.08	
None	II		
Orthophosphate		0.1	$\sim 2.5 \times 10^2$
Methyl-phosphonate		0.1	
Rib-P[b]	III	0.09	$\sim 5.5 \times 10^2$
6-DeGl-1-P		0.01	$\sim 6 \times 10^2$
1-DeGlc-6-P		0.01	$\sim 6 \times 10^2$
GlcA-1-P		0.08	$\sim 6 \times 10^2$
Xyl-1-P[c]		0.08	$\sim 6.5 \times 10^2$
Glc-P[b]		0.04	$\sim 1 \times 10^3$

[a] Most solutions contain 2.3 mM E_P and 1.5 mM Mn(II). [b] Known substrate of phosphoglucomutase. [c] Causes conversion of E_P into E_D with loss of phosphate. The E_D·Mn complex shows an identical spectrum in the absence of Xyl-1-P.

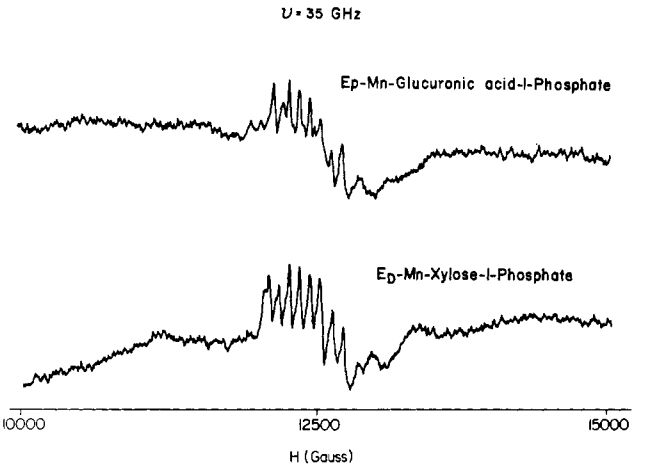

FIGURE 6: K-band epr spectra for ternary complexes of Mn(II)·phosphoglucomutase. Solutions contained 2.3 mM E_P and 1.5 mM $MnCl_2$. Additive concentrations were 0.08 M. (Note Xyl-1-P produces E_D.) Corresponding X-band spectrum for glucuronic acid phosphate is identical with that for 1-deGlc-6-P (see Figure 5).

by the presence of both productive and nonproductive complexes; however, ambiguities related to multiple binding modes do not seem likely for those class I complexes which elicit only narrow bands in the central region of the spectrum.

Multiple complexes of the former type (those involving position isomers of the phosphate group) are definitely absent for ternary complexes in which a phosphate transfer is not possible, *viz.*, the class III complexes of glucuronic acid 1-phosphate, 1-deoxyglucose 6-phosphate, and 6-deoxyglucose 1-phosphate; although multiple binding modes of the latter type are not excluded they seem less probable because of the close correspondence of these sugar phosphates to the normal substrate. Xyl-1-P is not a substrate for phosphoglucomutase; however, Xyl-1-P induces a rapid transfer of the phosphate group of E_P to water to produce the dephosphoenzyme (J. D. Owens, J. M. Long, and W. J. Ray, Jr., unpublished results). In accord with these observations, the spectrum obtained after addition of Xyl-1-P to E_P·Mn is identical with that for purified E_D·Mn in the absence of Xyl-1-P (*cf.* Figure 5).

The concentrations of all sugar phosphates in Table I (except Glcn-P) are known to be saturating, as is the concentration of inorganic phosphate and methylphosphonate (Ray and Mildvan, 1970; W. J. Ray, Jr., and A. S. Mildvan, unpublished data). Of the other additives, all of the class I materials seem to have produced the maximum effect at the concentration used.

Discussion

In contrast to many epr studies of paramagnetic complexes of transition metal ions and biopolymers, the experiments described here were performed in the solution phase, and a brief comparison of the relative advantages of solution phase *vs.* solid-phase studies seems in order.[10] Because of dielectric losses from aqueous solutions (Poole, 1967), sample volumes are restricted in solution studies, and this restriction limits the attainable signal-to-noise ratio at a given concentration. On the other hand, dielectric losses are diminished in frozen solutions, and substantially larger sample volumes are thus feasible. Also, improved resolution of fine structure components may be possible in the frozen state. However, in solution phase, the spectra of ternary or quaternary complexes can be examined under conditions where chemical equilibria also can be determined. Moreover, the spectrum of Mn(II), at least in its complex with phosphoglucomutase, appears to be rather sensitive to what may be conformational changes in the protein (see below) and conformation may well be influenced by the freezing process. In fact, frozen solutions of E_P·Mn and E_P·Mn·Glc-P produce spectra markedly different from those obtained in the solution phase, although the enzyme is stable toward freezing and thawing. Also, in contrast with the obvious differences in solution, spectra for rapidly frozen solutions[11] of E_P·Mn and E_P·Mn·Glc-P (not shown) are quite similar and both appear to consist of a mixture of very sharp and quite broad components; the sharp component resembles the spectrum for aquo-Mn(II) in nonaggregated, frozen solutions (Ross, 1965), although no significant amount of free Mn(II) was present when freezing was initiated and the rate at which Mn(II) dissociates from the enzyme (30°) is very much slower than the rate of freezing (Ray, 1969). However, since aqueous solutions typically undergo solvent–solute segregation during freezing, proteins can be exposed to unexpectedly large salt or pH gradients during the freezing process (Ross, 1965; Taborsky, 1970). A reversible alteration

[10] The following discussion applies specifically to Mn(II) since electron spin relaxation times for complexes with this ion are relatively long even in the vicinity of 273–300°K. Electron spin relaxation times for other transition metal ions may be too short to permit investigation in this temperature range.

[11] Rapid freezing was obtained by quickly immersing an epr sample tube, which contained the enzyme solution and which had been cooled to 0°, into partially frozen solution of isopentane.

in the protein structure induced in this manner is the most reasonable rationale for the present results, and served as a note of caution that the possibility of structural changes on freezing should not be ignored—even when the freezing is relatively rapid.

In principle, detailed molecular properties of the Mn(II)·ligand environment can be obtained from the zero-field-splitting (ZFS) parameters. However, the extent of ZFS for Mn(II) in a given ligand environment is difficult to predict theroetically (see, for example, Sharma *et al.*, 1966), and at present only a limited number of solid-state spectra for Mn(II) complexes with potential protein-like ligands are available for comparison. Most of these involve EDTA and its common analogs, but the spectra are sufficiently diverse to show that both the types of ligands and their relative positions in the coordination sphere of Mn(II) are important in determining the extent of ZFS, as is expected from crystal field theory. Although these spectra actually show qualitative similarities with those for some of the phosphoglucomutase complexes (G. H. Reed and J. S. Leigh, Jr., to be published), further studies of model complexes will be necessary before possible alternatives for Mn(II) coordination in $E_P \cdot Mn$ can be evaluated. However, one can use changes in ZFS of the Mn(II) enzyme on binding of substrates or inhibitors to indicate that a change has occurred in the ligand sphere of bound Mn.

One feature that is common to many of the X-band spectra of Mn(II)·phosphoglucomutase is a signal (often with resolved ^{55}Mn hyperfine structure) which occurs near half-field value (*i.e.*, about 1600 G) and which is not a part of an axial line shape. Forbidden fine structure transitions (*i.e.*, $\Delta M = \pm 2$) which increase in intensity with $(D/g\beta H)^2$ can occur at this field value (De Wijn and Van Balderen, 1967) and probably account for this signal.

Most of the complexes of $E_P \cdot Mn(II)$ studied here can be divided into three classes based on (a) their dissociation constant, (b) their enhancement of the relaxation rate of water protons (Ray and Mildvan, 1970; W. J. Ray, Jr., and A. S. Mildvan, unpublished results), and (c) the size of their ZFS relative to the binary $E_P \cdot Mn(II)$ complex. Class I (Table I) includes those complexes involving ethylene glycol, unmodified sugars, and weakly bound sugar phosphates and is characterized by relatively isotropic epr spectra with decreased ZFS (relative to $E_P \cdot Mn(II)$) but little or no change in enhancement. Class II (Table I) includes complexes which elicit no change in ZFS and little if any enhancement change, while class III includes complexes of tightly bound sugar phosphates that are similar to the normal substrate and that have highly anisotropic epr spectra with markedly increased ZFS and markedly decreased enhancements of proton relaxation rate. Two sugar phosphates, Rib-P and 2-deGlc-P are in an intermediate class by the criteria of binding and enhancement; however, 2-deGlc-P gives an epr spectrum that resembles other class I spectra, although it may be a superposition of signals from a mixture of complexes with high and low ZFS components. On the other hand, the spectrum for Rib-P (not shown) resembles that of 6-deGlc-1-P (*cf.* Figure 5). Note that if strong deenhancement in this system is caused by replacement of water in the coordination sphere of bound Mn(II), as suggested by Ray and Mildvan (1970), the formation of all class III complexes would involve water replacement. Moreover, a substitution within the coordination sphere of Mn(II) could provide a rationale for the large increase in ZFS produced by binding of the class III additives. The marked decrease in ZFS produced by class I complexes might also involve ligand substitutions or rearrangements within the Mn(II) coordination sphere. The absence of a significant change in enhancement for this class was initially construed as evidence for no change in hydration number (Ray and Mildvan, 1970). However, because of the decreases in apparent epr line widths which accompany binding of class I additives one cannot rule out the possibility that decreases in hydration number are fortuitously compensated by increases in correlation time.

Although generalizations may be somewhat premature, a decrease in ZFS (*i.e.*, an increased symmetry of the electronic environment of Mn(II)) is produced by substrate analogs lacking a phosphate ester group and may well be mediated by vicinal hydroxyl groups. However, neither the cis nor trans isomers of 1,2-cyclohexanediol produce an epr change nor does α-methyl glucoside or mannoseamine, although it is not clear whether these compounds are actually bound to the enzyme at the concentrations used (0.1 M). Those sugar phosphates that produce a similar decrease in ZFS probably do so primarily because of their sugar ring, since the binding of inorganic phosphate, alone, does not produce such an effect, nor does binding of inorganic phosphate plus the free sugars alter the spectrum significantly.

By contrast, compounds which produce a highly anisotropic spectrum with a markedly increased ZFS relative to that for the binary $E_P \cdot Mn(II)$ complex contain both a phosphate group and a glucose ring (except Rib-P) that is unmodified at the 2, 3, and 4 positions. Changes at any one of these positions can convert the class III into a class I response. Moreover, binding of the phosphate group, alone, cannot explain the class III response, since no changes are brought about by inorganic phosphate. At present it is not clear whether the class I change, which is presumably produced by hydroxyl groups, is also produced by class III additives together with additional changes, or whether class I and III additives produce changes which are entirely different on a molecular level.

It should be emphasized that *none* of our observations require direct coordination of Mn(II) to either the phosphate group of the phosphoenzyme, or to hydroxyl groups of the substrate. In fact the observation of opposite changes in ZFS for binding of class I and III additives tends to suggest that the conformational response of the protein to the moiety bound rather than direct metal–substrate bonding may be the primary source of the epr spectral changes.

The present observation of fine-structure splitting in the solution spectrum for $E_P \cdot Mn$ and its ternary complexes confirms the explanation of low apparent amplitudes for derivative epr spectra of Mn(II)–protein complexes given by Reed and Cohn (1970). Thus the powder line shape rather than homogeneous electron spin relaxation is the dominant mechanism for the apparent loss of signal, and larger ZFS thus will lead to lower derivative amplitudes.

Although the high electronic and nuclear spin multiplicities of Mn(II) produce complex epr patterns, such spectra are rich in detailed molecular information partly because of these complexities. Unlike the isoelectronic complexes of high-spin Fe(III), the symmetry distortions for Mn(II) complexes are typically small (*i.e.*, <0.1 cm^{-1}) and are therefore, readily measureable by epr. The sensitivity of these distortion parameters to structural changes in the phosphoglucomutase complexes is particularly encouraging. In this sense, Mn(II) functions as a natural spin label at the active center of phosphoglucomutase. Whereas spectra for nitroxide free-radical labels offer information relating primarily to the motional

freedom of the label group, spectra for Mn(II) can, *in principle*, provide insight into (1) interactions of the bound metal ion with solvent, (2) the geometry and symmetry of the coordination sphere of the metal ion, and (3) the types of ligands to the ion. Although the phenomenological aspects of the present data have been emphasized in this report, one can reasonably look forward to advances in the theoretical and experimental aspects of Mn(II) epr which will allow a more detailed interpretation of such results.

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