Communications

Electron Paramagnetic Resonance Spectra of Manganese(II)-Protein Complexes

MANGANESE(II)-CONCANAVALIN A*

(Received for publication, October 10, 1969)

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SUMMARY

Factors which determine the line shape in the electron paramagnetic resonance (EPR) spectra of Mn(II) bound to proteins are considered. The conclusion that a reduction in the amplitude of the Mn(II) derivative spectrum in some protein complexes may be caused by restriction of motional freedom of the bound ion rather than to line broadening caused by rapid electron spin relaxation is supported by frequency and temperature dependencies of the EPR spectra for the Mn(II)-concanavalin A complex in solution. The characteristics of the solution EPR spectra show that the environment of the bound Mn(II) is not highly distorted from cubic symmetry (zero field splitting is less than 20 gauss) and that the rotational motion of the Mn(II) is highly restricted. EPR spectra at 35.0 GHz (K band) are greatly simplified relative to spectra at 9.1 GHz (X band) thus permitting more quantitative evaluation of spectral parameters.

Previous attempts to probe the environment of the manganous ion bound to proteins by use of the electron paramagnetic resonance spectra of the complexes in solution proved unsuccessful because of an apparent absence of an observable EPR signal for the bound form of the ion. In fact, advantage was taken of this phenomenon (1-4) to determine binding constants since the amplitude of the observed derivative EPR signal could serve as a measure of the equilibrium concentration of free Mn(II) in solutions containing proteins.

Recent attempts to observe EPR spectra for Mn(II) protein complexes by use of more sensitive instrumentation have been plagued by the interfering spectrum for the equilibrium amount of free Mn(II). The observation of an EPR spectrum of the Mn(II) complex with concanavalin A in the powdered solid, and the reported high stability of this Mn(II) protein complex (5), stimulated our interest in examining the EPR spectrum of the complex in solution in order to assess the reasons for the apparent absence of EPR signals for many Mn(II) protein complexes.

For paramagnetic species such as Co(II) and Ni(II) complexes, failure to observe an EPR signal in solution can be attributed to very rapid electron spin relaxation with consequently excessively broadened lines. However, rapid electron spin relaxation need not be the underlying physical basis for the apparent absence of EPR signals for Mn(II) proteins as shown by the theoretical considerations and experiments presented in this report. In fact, the dominant effect of the macromolecule on the Mn(II) EPR signal can, in some cases, result from an "immobilization" of the ion. The spectral changes stemming from this immobilization are analogous to those observed in spectra of protein-bound organic free radicals in solution which approach the spectra observed for polycrystalline or powder samples (6).

MATERIALS AND METHODS

Concanavalin A (jackbean phytohemagglutinin) was obtained from Calbiochem as a lyophilized powder in NaCl. The powder was assayed by atomic absorption spectrophotometry and found to contain 1 g atom of manganese per 240,000 g of protein; however, the stoichiometry of manganese binding was not investigated. Solutions for EPR studies were made up from the powder and deionized water, and no additional manganese was added to the solution. The pH of solutions of the protein was 7.1 which is the isoelectric point of the protein (5), and no buffers were required to maintain the pH.

The X band (9.1 GHz) EPR spectra were recorded with a Varian E-3 spectrometer. The aqueous samples (~50 μl) were contained in quartz capillary tubing (2 mm outer diameter, 1 mm inner diameter). Sample temperature was controlled with a model E-4540 controller and E-4557 variable temperature accessory. K band (35.0 GHz) EPR spectra were recorded with a Varian model V-4503 spectrometer equipped with a model V-4561 microwave bridge accessory. Aqueous sample cells were constructed by drawing the tip of 2-mm quartz tubing to a capillary approximately 2 cm long and 0.3 mm inner diameter. The sample volume required was approximately 1 μl. The sample temperature was maintained by air flow through the cavity. The powder spectra were taken in the usual manner.

RESULTS

The X band ($H_o = 3244$ gauss at 9.1 GHz) spectra for Mn(II)-concanavalin A in solution and in the powder, Fig. 1, show striking similarities. The powder spectrum shows partial resolution of signals which occur between the six signals in the isotropic spectrum of Mn(H₂O)₄⁺ in solution (cf. Fig. 1). Such signals are common in solid-state spectra of materials containing manganous ion and are attributed to forbidden transitions ($\Delta M = \pm 1; \Delta m \neq 0$ where $M$ and $m$ are the electron and nuclear spin quantum numbers, respectively) (7-9). Since the spectral intensity is shared between the normal and forbidden transitions, the intensities of the normal transitions may be severely depleted in the polycrystalline spectra where the forbidden transitions become important.

The broadened appearance of the X band spectrum for the protein complex in solution can be attributed either to inhomogeneous broadening caused by incomplete resolution of the
forbidden transitions, resulting from the solid-state character of the sample, or to homogeneous broadening caused by shortening of longitudinal $T_1$ or transverse $T_2$, or both, relaxation times. The temperature and frequency dependencies of the spectrum for concanavalin A provide criteria to distinguish among broadening mechanisms. If the $X$ band spectrum is primarily homogeneously broadened, then the line width should change with temperature. However, spectra taken at $0^\circ$ were virtually identical with those recorded at $28^\circ$. Because the intensities of the forbidden transitions are inversely proportional to the square of the applied magnetic field ($B_0$), they should be almost completely suppressed at the higher field, and consequently the intensities of the allowed transitions ($\Delta m = \pm 1; \Delta m = 0$) should be almost completely restored in the $K$ band spectrum ($H_0 = 12,500$ gauss at 35 GHz). The $K$ band spectrum for the protein complex in solution, Fig. 2, shows restoration of the allowed transition intensity. Consequently, the broadening in the $X$ band spectrum can be ascribed to forbidden transitions rather than fast $T_1$ relaxation. The similarity of the powder and solution $K$ band spectra, Fig. 2, further supports the contention of solid-state characteristics for EPR spectra of this Mn(II)-protein complex in solution.

The EPR spectra for solutions of concanavalin A at pH 5 show a considerable amount of the isotropic spectrum of the hexaquo complex of Mn(II) suggesting that a significant amount of Mn(II) has dissociated from the protein at this acid pH. Agrawal and Goldstein (5) have found that the protein undergoes dissociation into subunits at pH 5. The release of Mn(II) from the protein at pH 5 may be the result of dissociation of the protein as well as protonation of a ligand in the pH region between 7 and 5.

DISCUSSION

A consideration of factors which determine the EPR line shape of Mn(II) complexes includes electron spin relaxation time, spin-spin interaction, and the effects of rotational motion of the Mn(II).

An important mechanism for electron spin relaxation in paramagnetic transition metal complexes in solution is the rotational modulation of anisotropic magnetic interactions (10-12). For Mn(II) complexes the major source of magnetic anisotropy is the zero field splitting which is related to the extent of distortion of the environment of the ion from cubic symmetry (13). If the rotational motion of the complex is sufficiently rapid to satisfy the requirement $\Delta \omega^2 \tau^2 < 1$ where $\Delta \omega$ is the zero field splitting and $\tau$, the tumbling time of the complex, the anisotropy will be averaged to zero but will provide a relaxation mechanism for the electron spins (12). In macromolecular complexes, such as Mn(II) proteins of molecular weight $1 \times 10^5$, only small aniso-
tropies of the order of 1 gauss will be averaged by the slow rate of tumbling ($\tau_r \sim 10^{-7}$ to $10^{-8}$ sec), and such small anisotropies will contribute only weak relaxing fields. Thus, in slowly tumbling complexes, even relatively large distortions (zero field splittings) which might result in very short relaxation times for more rapidly tumbling complexes will not be effectively averaged and will not induce longitudinal, $T_1$, electron spin relaxation.

The considerations above apply for complexes which are not severely distorted, i.e. the resulting zero field splitting is much smaller than the Zeeman splitting, $g\beta H$, where $g$ is the spectroscopic splitting factor, $\beta$ is the Bohr magneton, and $H$ is the magnitude of the magnetic field. For complexes in which the distortion leads to zero field splitting of the same order as $g\beta H$, the room temperature EPR spectra may be quite broad irrespective of the rate of rotation of the complex because in such highly distorted complexes the spin system and hence relaxation is strongly coupled to vibrational motions of the molecule (13).

Two additional relaxation mechanisms are important in molecules which bind more than one manganous ion at sites closer than $\sim 10$ Å or in molecules which form aggregates in which the manganous ions are brought into close proximity. In such cases, the dipolar or electron spin exchange interactions may provide relaxation mechanisms for $T_1$ or $T_2$, or both, which alter the shape of the resonance signals.

However, even in the absence of broadening resulting from $T_1$ relaxation effects or from spin-spin interaction, the EPR spectra of Mn(II)-protein complexes will not resemble the isotropic spectrum for the hexagonal manganous complex if the rotational freedom of the Mn(II) is restricted, in the limit, to the tumbling rate of the protein molecule. In fact, the incomplete averaging of anisotropic interactions resulting from slow molecular tumbling imparts solid-state characteristics to the spectral line shape which approaches that for a polycrystalline or powder sample (14).

In this respect, Mn(II) is immobilized by the protein, and if this immobilizing effect is sufficiently great, it can indeed be responsible for the apparent disappearance of the derivative EPR signal for Mn(II) upon binding to some proteins. Agrawal and Goldstein (5) suggested that at pH 7 concanavalin A is composed of 3 (or possibly 4) subunits of 68,000 molecular weight. A Stokes law calculation of the tumbling frequency gives $\sim 10^7$ sec$^{-1}$ assuming a molecular weight of $\sim 250,000$ for the protein and as pointed out earlier anisotropies greater than 1 gauss would not be averaged. The solid-state character of the EPR spectrum for Mn(II)-concanavalin A is consistent with the rotational motion of Mn(II) corresponding to that of the whole complex. The amplitude of the derivative EPR spectrum for concanavalin A at X band is much smaller than that for an equivalent amount of free Mn(II). This apparent loss of signal as pointed out above is the result of the polycrystalline line shape characteristics (15), in particular the forbidden transitions which scatter the spectral intensity (9). The apparent signal loss is less at K band but the “recovery” of signal at the higher frequency depends on the magnitude of the zero field splitting.

Conclusions regarding the nature of the manganese binding site may be inferred from the EPR data. The absence of a splitting of the five “fine structure” components (7) in the polycrystalline type spectrum signifies that the zero field splitting is less than the observed line width ($\sim 20$ gauss) of the K band spectrum. It may be concluded that the manganese binding site has almost no distortion from cubic symmetry. A lower limit on the electron spin relaxation time of the bound Mn(II) is of the same order as the Mn(II) in solution. In cases like manganese-concanavalin A, where the conditions described above hold, i.e. the bound Mn(II) resides in a highly symmetric environment, its motional freedom is greatly restricted and its spin relaxation time is long, then dramatic simplification of the EPR spectra and sharpening of the lines occurs at high frequency. In any case, a comparison of the low and high frequency spectra will distinguish between different mechanisms of broadening which are indistinguishable at X band alone and consequently will yield information concerning the macromolecular environment of the Mn(II) in the complex.

Acknowledgments—We wish to acknowledge helpful discussions with John S. Leigh, Jr., and the generosity of Dr. T. Yonetani for making the Varian 4503 EPR spectrometer available to us.

Note Added in Proof—The EPR spectrum of an aqueous solution of Mn(II)-concanavalin A at X band has recently been published (Nicolaud, C., Kali, A. J., and Yariv, J., Biophys. Acta, 194, 71 (1969)).

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$^4$Although it has been assumed previously that in polycrystalline Mn(II) spectra only the $\frac{1}{2}$ to $-\frac{1}{2}$ transition is observed and that the other fine structure components are broadened beyond detectability (16), we have indeed observed fine structure splitting in polycrystalline spectra for Mn(II)-doped potassium azide where the zero field splitting is 534 gauss.
**Isoleucyl Transfer Ribonucleic Acid Synthetase Is a Single Polypeptide Chain**

(Received for publication, November 10, 1969)

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**SUMMARY**

Isoleucyl transfer RNA synthetase is not dissociable into subunits in denaturing solvents after reduction and carboxymethylation, reaction with maleic anhydride, or oxidation. Physical data is presented from electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and from equilibrium centrifugation in 6 M guanidine hydrochloride which yields a molecular weight of 114,000 ± 2,000 for the native, denatured, or alkylated enzyme. Only a single amino terminus, threonine, is detected for the oxidized protein.

Although earlier speculations suggested that all of the *E. coli* aminoacyl synthetases might have a common subunit structure, it seems more likely that recognition of a single amino acid and only a few species of tRNA does not restrict the synthetases to similar size and physical properties. Indeed, some of the enzymes purified thus far, such as the prolyl-, seryl-, and methionyl-tRNA synthetases, are composed of subunits of minimal molecular weight between 40,000 and 50,000 (3, 9-11). On the other hand, the leucyl-tRNA synthetase (12) and the valyl-tRNA synthetase (2) have molecular weights about 105,000, with no evidence of a subunit structure.

Extensive studies of the substrate and kinetic properties of the isoleucyl-tRNA synthetase from *E. coli* have been made (5, 13-16). For the native enzyme (molecular weight 112,000), there is 1 mole of isoleucyl-AMP bound (17), one binding site for tRNA<sub>ile</sub> (14), and one reactive sulfhydryl group (15). In this study we show, using the electrophoretic mobility in acrylamide gels and the sedimentation behavior under conditions which normally dissociate oligomeric proteins, that the enzyme is not dissociable into subunits and is, therefore, most probably a single polypeptide chain.

The enzyme, isoleucyl-tRNA synthetase, was that previously described (5). It exhibited a single band upon acrylamide gel electrophoresis. The molecular weight determinations of the enzyme were made by acrylamide gel electrophoresis and by equilibrium sedimentation. Acrylamide gels were run in sodium dodecyl sulfate according to the procedure of Shapiro, Vifmel.

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*This work was supported by Grant GM 13235 from the National Institutes of Health, United States Public Health Service.
† Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

**FIG. 1.** A, electrophoresis of 25 to 40 μg of isoleucyl-tRNA synthetase in sodium dodecyl sulfate-containing acrylamide gels (18) after the enzyme was subjected to various treatments. Porcine pepsin (15 μg) was added as a marker and is the faster moving band on each gel. The direction of migration was toward the bottom (anode) of the gels. a, no treatment; b, 1 hour reduction with 1% 2-mercaptoethanol; c, 3-hour reduction with 0.1 M dithiothreitol in 1% sodium dodecyl sulfate at 37°; d, reduction and carboxymethylation with iodoacetate using a modification of the procedure of Crestfield, Stein, and Moore (24). The reduction was effected in 6 M guanidine hydrochloride and 0.30 M Tris-HCl at pH 8.6 with 0.1 M 2-mercaptoethanol for 6 hours at room temperature. A 20% excess of neutralized iodoacetate over the reducing agent was added and alkylation allowed to proceed for 15 min in the dark. The solution was dialyzed at 4° in the dark. e, reaction with maleic anhydride carried out as described by Bruton and Hartley (11). B, electrophoresis in sodium dodecyl sulfate gels of isoleucyl-tRNA synthetase after oxidation at 0° with performic acid (H<sub>2</sub>O<sub>2</sub> to HCOOH, 1:9). The oxidation was terminated by 10-fold dilution of an aliquot and samples were lyophilized before dissolving in buffer containing sodium dodecyl sulfate. a, 0 min; b, 20 min; c, 40 min; d, 90 min; e, 7 hours. The direction of migration was toward the bottom (anode) of the gels.
Isoleucyl-tRNA Synthetase Is a Single Polypeptide Chain

Fig. 2. The molecular weights on a logarithmic scale are plotted against the measured mobility in 10-cm sodium dodecyl sulfate-acrylamide gels (18). The proteins in order of increasing molecular weight are (1) sperm whale apomyoglobin (28), (2) porcine pepsin (29), (3) bovine plasma albumin (30), (4) isoleucyl-tRNA synthetase (experimental), and (5) E. coli β-galactosidase (21).

and Maizel (18), except that the staining procedure of Weber and Osborn (19) was used and destaining was done by diffusion. The high speed equilibrium sedimentation method of Yphantis (20) was employed with a Beckman model E ultracentrifuge, using interference optics and a six-chamber Yphantis cell. The value of $t_1$ for the synthetase was assumed to undergo no major change in 6 M guanidine hydrochloride (21-23).

Fig. 1A shows that isoleucyl-tRNA synthetase yields a single band on electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (a) and that the identical pattern is obtained after reduction with 2-mercaptoethanol (b) or dithiothreitol (c). Reduction and carboxymethylation (d) or treatment with maleic anhydride (e) also do not alter the electrophoretic behavior of the protein in these gels. The slight retardation of the protein (higher molecular weight) after treatment with maleic anhydride as seen in Band e is probably the result of the reduced capacity of the protein to bind sodium dodecyl sulfate because of its highly negative charge (25). Oxidation with performic acid and subsequent electrophoresis in the sodium dodecyl sulfate gel also yields a single band, corresponding to the denatured protein and only after an hour do traces of smaller polypeptides appear (Fig. 1B); these we attribute to chain scissions yielding fragments (26, 27).

Determination of the molecular weight of isoleucyl-tRNA synthetase under denaturing conditions with two different techniques yields essentially the same value. Fig. 2 shows a plot of the log of molecular weight versus the relative electrophoretic mobility in sodium dodecyl sulfate gel (18, 19). Based on the mobility of the isoleucyl-tRNA synthetase, relative to β-galactosidase, bovine plasma albumin, pepsin, and myoglobin, we estimate the molecular weight as 110,000 ± 10,000. Molecular weight measurements have also been performed on the carboxymethylated protein by equilibrium sedimentation in 6 M guanidine hydrochloride containing 0.1 M 2-mercaptoethanol (Fig. 3). A least squares analysis of the fringe displacement across the cell gave a value of 114,000 ± 2,000. In addition, the molecular weight of the maleyl protein was checked by equilibrium sedimentation both in 0.4 M sodium chloride-0.1 M sodium phosphate and in 6 M guanidine hydrochloride. A value of 108,000 ± 5,000 was obtained.

Isoleucyl-tRNA synthetase has a single amino terminus, threonine. Oxidized isoleucyl-tRNA synthetase was dinitrophenylated (31) in sodium bicarbonate by addition of 14C-1-fluoro-2,4-dinitrobenzene in ethanol. The dinitrophenylated protein was hydrolyzed at 110° for 12 hours in 6 N HCl. Extraction of the hydrolysate with ether was followed by chromatography on polyamide sheets using the solvent systems of Wang, Huang, and Wang (32). All of the 14C-labeled dinitrophenylated amino acid, detected by strip scanning, cochromatographed with dinitrophenylated threonine; no other dinitrophenylated amino acid was found.

We conclude from these studies that native, denatured, or alkylated isoleucyl tRNA synthetase has the same molecular weight, 114,000 ± 2,000. There is no evidence from the physical data we have collected that the enzyme is composed of subunits. This conclusion is consistent with the earlier suggestion by Baldwin and Berg (5), based on the number of tryptic peptides, that isoleucyl-tRNA synthetase could not contain identical subunits. It appears, therefore, that the single polypeptide chain of isoleucyl-tRNA synthetase contains the binding sites for amino acid, ATP, and tRNA.

Acknowledgments—We wish to thank Douglas Brutlag for his...
assistance in programming the data from the equilibrium cen-
trifugation runs. ß-Galactosidase was the kind gift of Dr.
Irving Zabin.

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