An Electron Spin Resonance Study of the Mn(II) and Cu(II) Complexes of the Fur Repressor Protein

Mazen Y. Hamed* and J. B. Neilands

Department of Biochemistry, University of California, Berkeley, California, U.S.

ABSTRACT

EPR spectra of Mn(II) Fur complex suggested the presence of Mn(II) in one site per Fur monomer in which Mn(II) is present in a low symmetry environment. The binding of the Mn(II) Fur complex to a DNA fragment "iron box" has a slight broadening effect on the Mn(II) signal and hence it altered the symmetry of the Mn(II) environment. We also report EPR spectra of Cu(II) Fur and Cu(II) C92S C95S mutant Fur complexes as models for Fe(II) complexes; the anisotropic g values and A values observed indicate the presence of Cu(II) in two different environments in the protein; a major axially distorted Cu(II) site bound to nitrogen and a minor distorted tetrahedral sulfur bound to the Cu(II) site. The effect of metal ion on Fur DNA binding is also discussed.

INTRODUCTION

With few exceptions, aerobic and facultative anaerobic bacteria, as well as most fungi, obtain iron from the environment or from host tissues by elaboration of ferric specific ligands called siderophores. Both the synthesis of siderophores and the transport of their ferric complexes are regulated by iron. In the case of *Escherichia coli* K-12 this regulation has been shown to be mediated by a repressor protein, Fur (ferric uptake regulation), which uses Fe(II) as corepressor. The activation of Fur to bind a specific operator sequence 5′-GATAATGATAATCATTATC (iron box) is not a sole property of Fe(II) but is shared with varying degrees of efficiency by any first-row divalent transition metal ion [1–5].

* A Fulbright visiting research associate in J. B. Neilands' laboratory.

Address reprint requests and correspondence to: Dr. M. Y. Hamed, Chemistry Department, Birzeit University, P.O. Box 14, West Bank, Via Israel.

Journal of Inorganic Biochemistry, **53**, 235–248 (1994)

0162-0134/94/$7.00

The corepressor role of the divalent transition metal ions Mn(II), Co(II), and Cu(II) as models for Fe(II) in activating the Fur to bind the DNA operator [1–6] was examined and the Fur complex with these ions was studied employing equilibrium, electronic absorption, and Fe Mössbauer spectroscopy [36]. The equilibrium and visible absorption studies revealed that Fur provides one major site for the metal ion. The Mössbauer parameters of the Fe(II) complexes of Fur and the cysteine mutants of Fur showed that Fe(II) is present in a high-spin highly distorted octahedral environment [36] and Coy et al. showed that this site is near the C-terminus [37]. The detailed description of the role of the inorganic ion requires an understanding of any possible alterations in the metal coordination sphere of the Fur complex with metal ion and also in the Fur metal ion complex with the operator.

Mn(II) and Cu(II) ions activate the Fur in vitro as surrogates for Fe(II) [2–6]. Fur dimer binds two Mn(II) ions ($K_d = 85\ \mu M$) to form a complex which then adheres to the operator [36, 37]. Also, Fur was found to regulate the genes responsible for the manganese-containing superoxide dismutase [38], which adds more importance to the study of Mn(II) Fur complex.

From the relaxation-perturbed NMR study of the Mn(II) Fur complex, Williams et al. [7, 8] suggested that Fur provides nitrogen ligands from histidine residues near the C-terminus and oxygen from carboxylate of the glutamic residues. The EPR signal of Mn(II) in protein environments has been recognized and used to study Mn(II) protein complexes by EPR in order to detect any alteration in the metal ion environment upon binding to another ligand [18]. The Cu(II) EPR signals in protein environments are well-known and their special properties, g values, hyperfine interaction, and superhyperfine interactions are known to provide a great deal of information about the metal ion environment [12, 23].

By employing an EPR study of the Mn(II) and Cu(II) complexes and Fur, this present study helps to reveal more information about the metal ion environment in the Fur complex and any possible alterations in that environment upon binding to DNA operators; also it supports our previous findings on the metal ion sites on Fur complexes with metal ions [36].

MATERIALS AND METHODS

Materials

Inorganic ions. $MnCl_2.4H_2O$ (99.99%) and $CuSO_4.5H_2O$ were purchased from Aldrich, Baker, and Alpha companies, respectively, and were used without further purification.

Fur protein. Fur protein was purified from the *E. coli* strain J R B 45 (pMON 2064) as described by Wee et al. [1]. The protein concentration was determined by both the Bradford assay and the absorbance at 275 nm (1.0 mg/ml gives an absorbance of 0.4) as reported [1]. Purity of the fractions was checked using SDS gel electrophoresis; only the fractions which gave single bands at 17 KDa were used in metal binding studies. The protein was further purified from any metal ion contaminants by dialysis against EDTA solution at constant ionic strength and the EDTA then removed by dialysis against 20 mM MOPS buffer, pH 7.2. After this process, the protein was analyzed by the atomic absorption spec-

troscopy and shown to be free from detectable levels of iron, manganese, and copper.

Native gel electrophoresis gave an indication that Fur runs as a 34 KDa dimer; this was confirmed by HPLC data obtained by K. Nakamura and C. Doyle (unpublished).

Mutant Fur protein samples were kindly supplied by C. Doyle and C. Besser.

DNA Fragment. single standard oligonucleotides, 25 merr, corresponding to the particular operator iron box of the aerobactin promoter and including three bases upstream and downstream were purchased from J. Onufer; 0.2 μmole of each single strand iron box labeled (I and II) were spin-vapped to dryness, and redissolved in 0.1 mL of 2.5 M ammonium acetate, precipitated with absolute ethanol, washed, and then dissolved in 1.9 mL TE buffer, pH 7.4. The concentration was calculated using the absorbance at 260 nm. An absorbance of 1.0 corresponds to 40 μg/ml single strand oligonucleotide.

To make double stranded DNA [1], 1.0 mL of I and II were mixed, heated to 70°C for 10 min, cooled at room temperature, and stored at −20°C. The duplex formation was complete as observed on 20% polyacrylamide gel and shown to be complete.

EPR Measurements

The Fur protein was mixed in stoichiometric amounts with the metal ion solution in 20 mM MOPS buffer, pH 7.2, unless indicated otherwise, in an eppendorf tube in a total volume of 0.2 ml. The pH was always monitored after mixing and no change was observed. Using capillary tubing and a syringe the sample was transferred to a flat cell (total volume 0.073 ml) purchased from Wilmad. For the spectra recorded at 80 K a quartz EPR tube was used; after freezing the sample it was transferred to an EPR Nitrogen dewer purchased from Wilmad.

EPR Conditions

The spectra were collected using an X-band EPR Bruker ER 200D-SRC spectrometer. The microwave frequency for the room temperature spectra was 9.82 GHz, and for the liquid nitrogen spectra was 9.39 GHz unless specified otherwise. Microwave power = 20 mW and modulation frequency = 100 KHz. Other EPR conditions are specified in the legend of each figure. Frequency was calibrated with a strong pitch sample (g = 2.0027).

The EPR spectra at 15 K were recorded on an X-band Bruker electron spin resonance ER 200 tt spectrophotometer supplied with a helium cryostat. Microwave frequency, 9.42 GHz; power, 20 mW; field modulation amplitude, 10 gauss; gain, 3.2×10^5.

RESULTS AND DISCUSSION

Mn(II) Fur Complex

Binding of Mn(II) to metal-binding sites of proteins has usually been associated with the broadening of the room temperature EPR signal of the Mn(II) [12]; several workers suggested that this loss in amplitude which accompanies protein

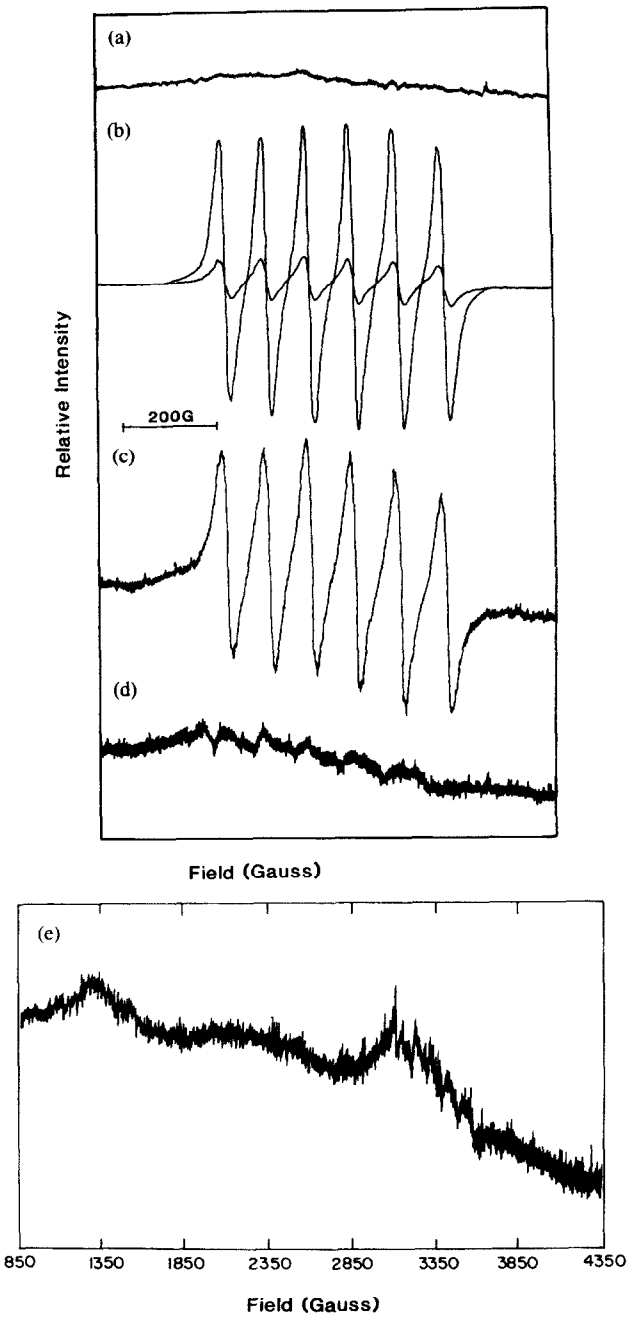


FIGURE 1. Room temperature EPR spectra of Mn(II) Fur complex: (a) base line; (b) the higher amplitude represents 220 μM Mn(II) in water; the lower amplitude spectrum represents 220 μM Mn(II) with 1.2 mM Fur (20 mM MOPS buffer, pH 7.2; Gain = 2×10^5); (c) amplification of spectrum b (gain = 4×10^5); (d) Mn(II) Fur complex at 80 K; (e) the same as spectrum d, at a wider field range (gain for d and e = 1.6×10^5). EPR conditions: microwave frequency for the room temperature (a and b) was 9.51 GHz; modulation amplitude, 6.3 gauss.

binding may result from the solid state character of the line shape conferred by the relatively long rotational correlation time of the protein [10–12].

The EPR spectra of the manganese(II) Fur complex, with excess Fur present, were examined at room temperature and 80 K. Both spectra show an easily detectable Mn(II) EPR signal typical of polycrystalline or powder with very broad features characterized by loss in amplitude of the $[Mn(H_2O)_6]^{+2}$ signal which has Mn(II) in cubic symmetry environment (Fig. 1). The line broadening could be attributed to an increase in the ZFS (zero field splitting) caused by axial and rhombic distortions in the manganese(II) environment, i.e., very low symmetry [12].

It is established that if the rotational correlation time is long, as for a polycrystalline (powder) or frozen solution, the ZFS in not averaged to zero and a value of one gauss or less for ZFS tends to broaden the EPR signal extensively, i.e., the separation of energy levels is not enough to give fine structures and the broad line could be a superposition of several lines resulting from allowed transitions [9, 10]. To interpret the EPR spectrum of Mn(II) Fur (the hyperfine interaction with ^{55}Mn was omitted for simplicity) there are six possible spin energy levels for Mn(II), M (i.e., $5/2, 3/2, \ldots, -5/2$). The allowed EPR transitions $\Delta M = \pm 1$, assuming an approximate cubic geometry, can be provided by the simplified spin Hamiltonian:

$$H = g\beta H.S + D[S_{x2} - 1/3S(S+1)] + E(S_{x2} - S_{y2}). \quad (1)$$

where D is a measure of the axial distortion from cubic symmetry and E is the rhombic distortion parameter and both D and E are correlated to the ZFS. The parameter $\lambda = E/D$ is often used as an expression for the degree of rhombic distortion, Bencini [12] argued that the only meaningful value for λ from the symmetry point of view is when λ takes the values between 0 (axial symmetry) and 1/3 (rhombic symmetry).

The first term of Eq. (1) describes the isotropic g tensor and does not vary with angular orientation of symmetry axis relative to the applied field. On the contrary, the ZFS terms which contain D and E are anisotropic terms and orientation dependent. This implies that if the ZFS is relatively small and at the same time the rotational correlation time of the molecule is short, the ZFS is averaged to 0 and the spectrum appears as a single isotropic line split by the nuclear spin 5/2 {Mn(II)} to six hyperfine lines as in $[Mn(H_2O)_6]^{2+}$ spectrum (Fig. 1b). But if the sample is a polycrystalline macromolecule, the ZFS is not averaged to 0 and tends to broaden the spectrum extensively (see Fig. 1b) and each EPR line can be further split by spin forbidden transitions as these transitions become more important in spectra of polycrystalline materials. Calculated plots for EPR lines for high spin Mn(II) as a function of D and E/D [12] have been used to interpret the EPR spectrum of Mn(II). The best fit for D and λ for an EPR spectrum can be estimated from the Dowsing and Gibson plots of D/h versus B/h [39] and plots by Aasa of h/D versus g/g′ [40]. The best fittings were found for E/D values equal to zero, which gave an estimated D value of 0.5 cm^{-1}. These values are indicative of axial symmetry and no or very low rhombicity which is a reasonable estimate in view of the fact that no g = 4.2 signals were seen in the Mn(II) Fur EPR spectrum (Fig. 1e) which are usually attributed to rhombic distortions [12, 39, 40].

Line broadening of the EPR signals in manganese(II) protein complexes has long been used for equilibrium studies [15–17] and proved to be effective in determining, by difference, the concentration of free and bound manganese, assuming that only free Mn(II) ions give the EPR signal [17] (see Fig. 2). This can be employed to calculate the dissociation constant of the protein metal ion complex. Titrating Fur with manganese(II) at room temperature gave evidence for a 1:1 Mn(II) complex; a dissociation constant value in the range of 100 μM could be estimated from a scatchard plot (for details see Hamed et al. [36]). Figure 2 shows that the hexaqua Mn(II) EPR signal is completely restored at one or more Mn(II) equivalents per Fur monomer [18].

The solid state Mn(II) EPR lines can be used to detect distortion from regular geometry or alterations in the metal environment (symmetry) [9, 10, 12, 18] which are likely to take place upon binding of the protein Mn(II) complex to DNA [20–22]. Based on the finding that if the EPR signal is restored upon DNA binding, this will indicate either a release of Mn(II) to bind H_2O or a conformational change in the protein to produce greater symmetry around the metal ion. If the EPR signals suffer further broadening upon DNA binding this will indicate a tighter binding of the protein to the manganese(II) and creation of a more distorted environment, or it could indicate the presence of another

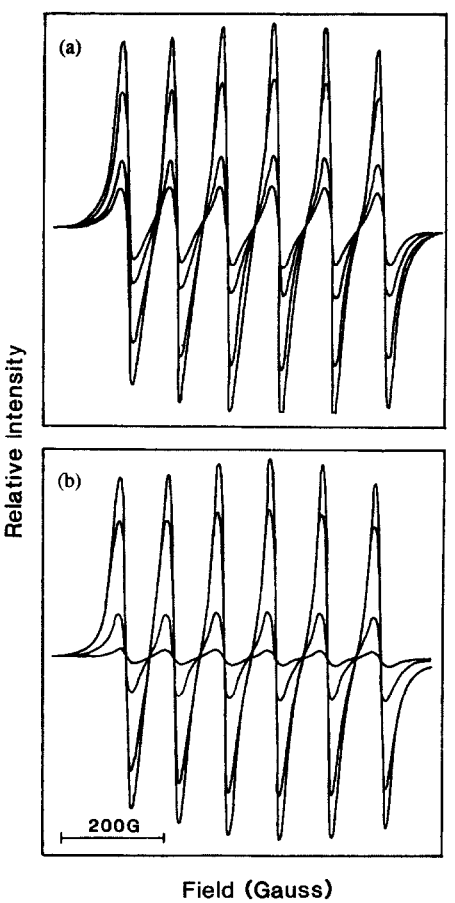


FIGURE 2. Room temperature EPR titration of 2.2 mM Fur protein with Mn(II) in 20 mM MOPS, pH 7.2; (a) Spectra of Mn(II) In H_2O. Concentrations of Mn(II) going from lower to higher spectrum amplitude were: 0.83, 1.64, 3.28, and 4.1 mM. (b) Titration of 2.2 mM Fur solution with Mn(II) in 20 mM MOPS, pH 7.2. Ratio of Mn(II) to Fur from lower to higher amplitude 0.75, 1.5, 3.2, and 4.0 equivalents Mn(II) per Fur; gain = 5×10^3. Other conditions are as for Figure 1.

macromolecule, i.e., DNA, in the immediate Mn(II) environment [9]. Upon addition of iron box DNA to the Mn(II) Fur complex, the EPR signal suffered further broadening. The results, which are shown in Figure 3, suggest a change in the distorted Mn(II) environment upon binding of the Fur manganese complex to DNA. This loss in amplitude, after correction for dilution, can be interpreted as due to possible alteration in the protein tertiary structure (conformational changes) which resulted in slight distortion in the Mn(II) environment upon binding to DNA. Most important is that Mn(II), in this case, does not seem to interact with the added DNA fragment, or a considerable change in the immediate Mn(II) environment should have been observed [9, 10, 18]. This may be interpreted as the exclusive involvement of Fur in binding to the DNA, thus excluding a direct involvement of metal ion in binding to DNA; indeed, Williams et al. [8] in a study of the Mn(II) Fur binding to DNA by NMR suggested that the positively charged amino acid residues near the N-terminus bind DNA with the exclusion of Mn(II) ion.

While manganese(II) EPR cannot distinguish octahedral from tetrahedral sites, it does tell us that Mn(II) is present in a very low symmetry site (notice the solid state character of the EPR spectrum in Figs. 1d and e).

Cu(II) Fur Complex

In vitro studies showed that Cu(II) ion activates the Fur protein to bind the DNA operator [2–4]. The EPR spectrum of Cu(II) gives information about hyperfine and superhyperfine structures which are of great importance in studying the Cu(II) ion environment in the protein, i.e., the Cu(II) geometry and distortions, nature of the ligating sites from the protein to the Cu(II), and the degree of covalency of the Cu(II)-ligand bonds [12, 18, 23].

The room temperature spectrum of Cu(II) Fur complex is shown in Figure 4. It is characterized by a broad high field signal [24, 28] with a shoulder at around 3355 gauss and poor resolution at the lower field signals, but the fine structure in the $g_{\|}$ region is clear. The general features of the spectrum resemble the low temperature spectrum in Figure 5 except that the latter is more resolved due to reduction of the spin-lattice relaxation which resulted in elimination of other

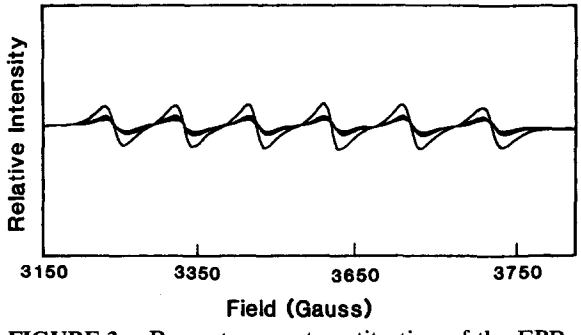


FIGURE 3. Room temperature titration of the EPR signal of Mn(II) Fur complex with DNA fragment iron box. Top spectrum is for Mn(II) in H_2O, going down in amplitude: Fur Mn(II) complex and the decrease in amplitude after adding 5 μl aliquotes of DNA solutions. ([Fur] = 3.0 mM, [Mn(II)] = 0.4 mM). EPR conditions: microwave frequency = 9.83 GHz, modulation amplitude, 4.5 gauss; gain, 5×10^3.

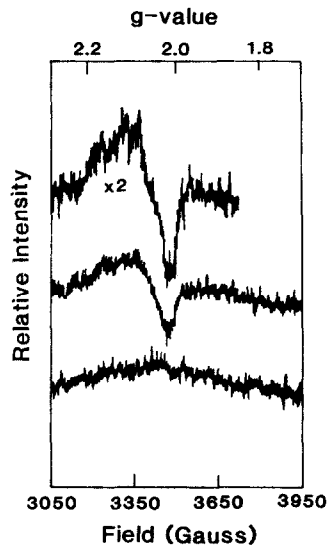


FIGURE 4. EPR spectrum of Cu(II) Fur complex at room temperature: From bottom to top: base line, Cu(II) Fur, Cu(II) Fur amplified. ([Cu(II)] = 1.5 mN, [Fur] = 1.2 mM). EPR conditions: modulation amplitude, 3.6 gauss; gain = 4 × 105.

thermally caused broadening [19, 23]. In both EPR spectra (Figs. 4 and 5), the presence of axially distorted Cu(II) is evident (D_{4h}). The need for a room temperature EPR spectrum is evident because when dealing with biological systems it is always desirable to have such data in order to make sure that no serious changes in environment took place due to glass formation or to pH changes upon freezing [23, 25, 35].

Both Table 1 and Figure 5 show the EPR parameters of the Cu(II) Fur complex as obtained from the glass sample at 80 K. Parameters for Cu(II) Fur protein are: $g_{\|} = 2.36$, $g_{\perp} = 2.04$, and $A_{\|} = 157$ gauss (other parameters are shown in Table 1). There is evidence from the EPR spectrum and the g values that the Cu(II) ions in the Fur complex are present in two different environments. The first site is typical of type 2 Cu(II) which is characterized by the A value of 158 gauss in which Cu(II) is present in a tetragonally distorted

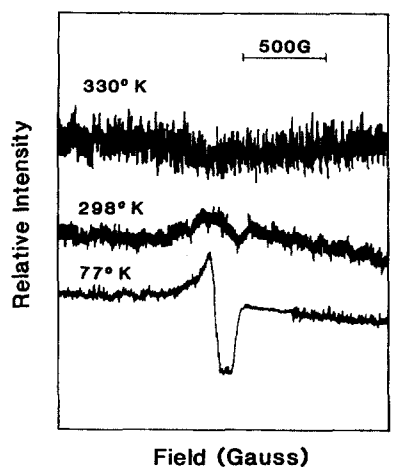


FIGURE 5. EPR spectra of Cu(II) Fur complex at various temperatures. Gain at 298 and above = 3.2×10^4; At 77, gain = 3.2×10^4. ([Cu(II)] = 1.35 mM and [Fur] = 1.2 mM). Microwave frequency at 298 and above = 9.51 GHz, modulation amplitude = 4.5 gauss.

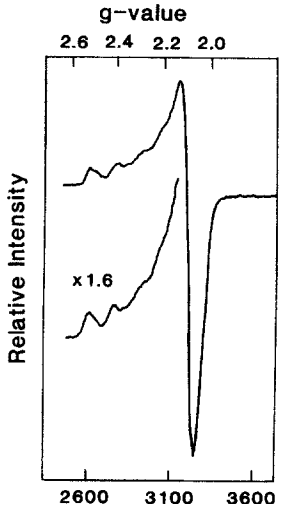
TABLE 1. EPR Parameters of the Cu(II) Fur Complex as Derived from the Spectrum Obtained at 80K Compared with the Parameters of Other Cu(II) Proteins

Complex	$g_{\|}$	$A_{\|}$ Gauss	$g_{\perp}$	$A_{\perp}$ Gauss	Refs.
Fur Cu(II)	2.36	156	2.04		
	2.30	75	2.07		
Ceruloplas min					26, 27
T 2	2.21	180	2.053		
T 1a	2.21	92	2.06	10	
T 1b	2.20	72	2.05	10	
Superoxide dismutas					
T 2	2.24	190	2.053		27
T 1	2.22	50	2.06		
Axially distorted Cu(II) bound to 3N and O	2.31	$-167v$ 10^{-4} (cm^{-1})			41

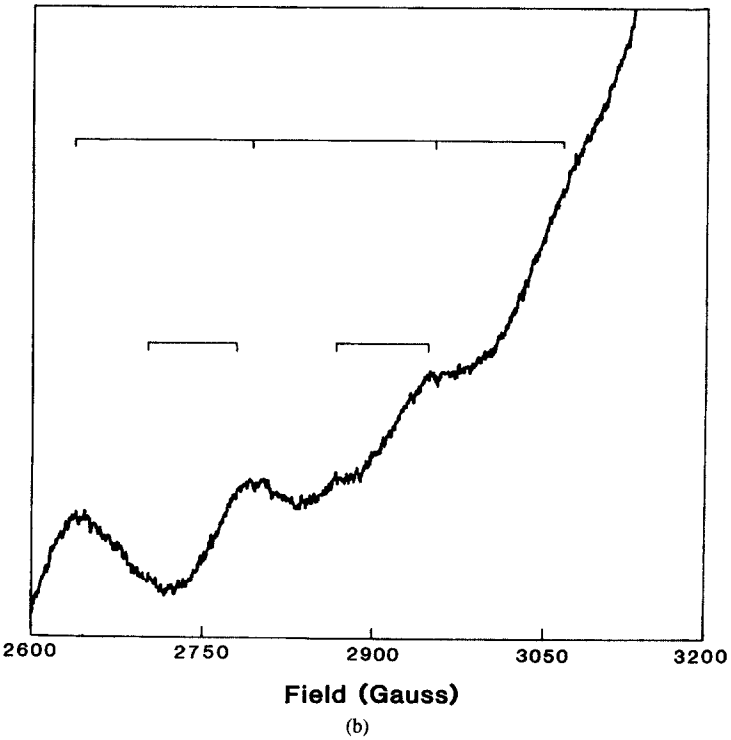
octahedral geometry, i.e., D_{4h}. Possible ligands to Cu(II) are nitrogen from histidine residues and a carboxylate oxygen [26–28] (for types of ligands see Williams et al. [7, 8] and Neilands et al. [36, 37]. The second site is minor; this site is similar to the type 1 Cu(II) which is characterized by an unusually small $A_{\|}$ value of 76 gauss (see Table 1) and Figure 6b. The ligands to the Cu(II) in this site are very well characterized in other Cu(II) proteins, as Cu(II) in a distorted tetrahedral environment bound to nitrogen and sulfur ligands [29] and the S → M(II) binding is discussed by Neilands et al. [36] and Hamed et al. [37]. Recently, this type of Cu(II) site [29] was reported to have Cu(II) in a distorted trigonal planar geometry with distant axial interaction to a methionine sulfur and a backbone carbonyl. This copper site is the one responsible for the intense blue color, a visible absorption band at 600 nm attributed to the S-Cu(II) charge transfer [27–30]. In the Cu(II) Fur complex a band in the visible region at 600 nm was observed [see Ref. 36].

The nitrogen superhyperfine structures can be seen in some of the bands; notice the fine structure in the low field bands at 2650 and 2810 gauss (Fig. 6b) these fine structures are due to the interaction of ^{14}N with the naturally abundant ^{65}Cu. From the comparison of the spectra with other reported spectra for copper(II) proteins [31, 32], it can be seen that at least two nitrogens are bound to the copper(II) in D_{4h} site; this observation has to be studied further to resolve the nitrogen superhyperfine structures using a 2–4 GHz microwave frequency [32].

In the temperature variation spectra (Fig. 5) an apparent loss in the copper(II) signal intensity was evident upon raising the temperature; this behavior is to be expected, i.e., a decrease in amplitude with temperature a typical Curie law behavior. The EPR spectrum at 15K (Fig. 7) exhibits a normal Cu(II) spectrum, the separation of the two signals at high field could be due to the anisotropic



(a)



(b)

FIGURE 6. (a) EPR spectrum of Cu(II) Fur Complex at ~ 80 K; ([Cu(II)] = 2.0 mM, [Fur] = 1.0 mM). Modulation amplitude = 4.5 gauss, gain = 1×10^4. (b) The $g_{\|}$ region of the spectrum expanded and amplified at gain = 5×10^4.

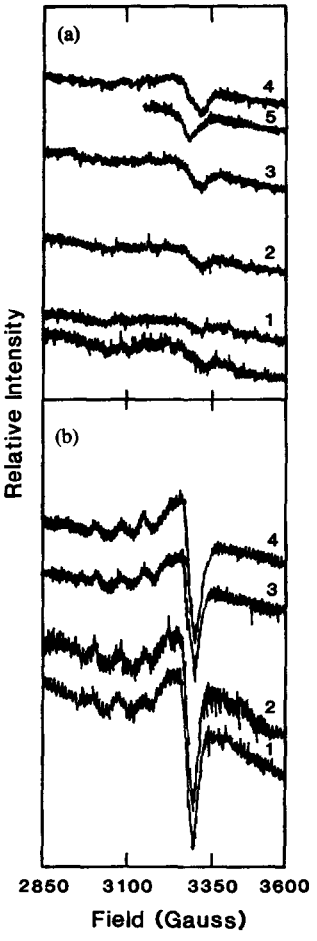


FIGURE 7. (a) Titration of Fur protein with Cu(II) at 80 K: Cu(II) concentrations starting from bottom: (1) 0.02, (2) 0.04, (3) 0.05, (4) 0.11 mM Cu(II), (5) 0.05 mM Cu(II) (for Fur was added). The bottom spectrum is spectrum 1 amplified two times ([Fur] = 0.43 mM). (b) Titration of C 92 SC 95 S double mutant Fur protein with Cu(II): Cu(II) concentrations are: (1) 0.02, (2) 0.04, (3) 0.05, and (4) 0.063 mM Cu(II) ([Fur mutant] = 0.64 mM). EPR conditions: modulation amplitude = 4.5 gauss; gain 5×10^4.

g-values or a noncoincidence of the g and hyperfine tensors. It is worth mentioning that the spectrum in Figure 7 is similar to a theoretically simulated spectrum of two interacting dipoles at a separation of 5 with g_z from one Cu(II) interacting with g_y from the other [18], although no evidence was seen for a change in the spectrum such as the appearance of new features at g = 4.

EPR titration of the Fur protein and its mutant C92S C95S with Cu(II) at 77 K are shown in Figure 8; it is evident from the EPR spectra that the Cu(II) signal in the wild-type Cu(II) Fur complex is broader and less resolved than in the C92S C95S Cu(II) complex. The enhanced EPR signal in the mutant Fur complex is possible due to a different mode of binding owing to the conformational changes which was evident in the NMR spectrum of the mutant [R. J. P. Williams, private communications, see Refs. 36 and 37]; such enhanced signals can be produced upon interaction of two axially distorted Cu(II) centers in the complex. The different EPR spectra of the cystein mutant Fur with Cu(II) from that of the wild-type complex agrees with the previously reported equilibrium study in which the mutant Fur associated less numbers of Cu(II) ions with larger binding constants [36]; also, the electronic spectra were different.

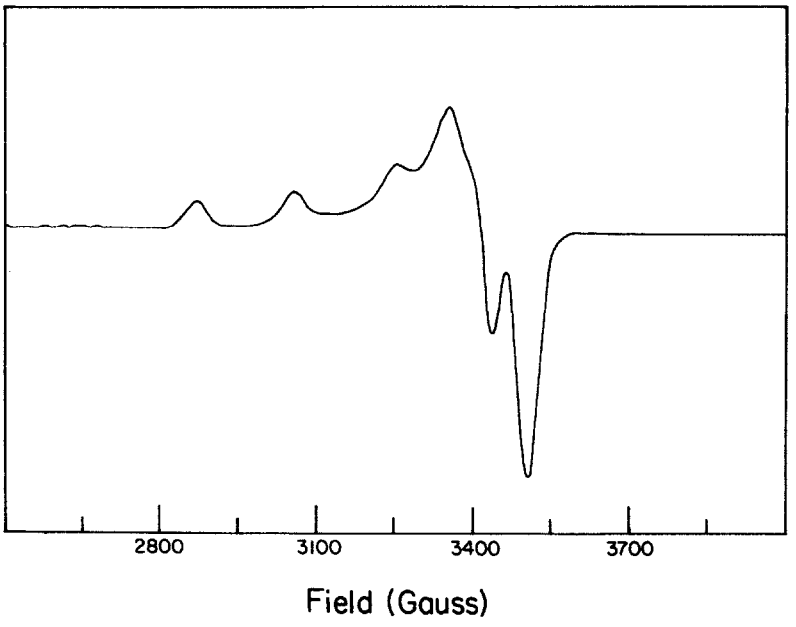


FIGURE 8. EPR spectrum of Cu(II) Fur complex in 20 mM tris buffer, pH 8.0, at 15 K. ([Cu(II)] = 0.4 mM and [Fur] = 0.3 mM).

CONCLUSION

Mn(II) is present in an axially distorted octahedral environment. Fur binds one Mn(II) ion per monomer [36, 37]. Since all metal ions bind Fur on sites in the C-terminus [37] and it is always present as a dimer, one can say Fur dimer ligates two Mn(II) ions, possibly through histidine nitrogen; NMR evidence [17, 18] showed that His 131, His 85, His 89, and His 142 or 144 are possible ligands to Mn(II), in addition to oxygens from carboxylates of Glu and Asp residues which are available in the C-terminus [7, 8].

Cu(II) is present in two different environments: a major site in which Cu(II) is present in an axially distorted environment bound to histidine nitrogen and oxygens from Asp and Glu residues and a minor site in which Cu(II) is in sulfur bound distorted tetrahedral geometry. The two Cu(II) centers exhibited an anisotropic g value with $g_{\parallel} > g_{\perp}$ indicating axial orientations. It is to be established if there are two Cu(II) centers at a distance and orientation which enables them to produce a dipole-dipole interaction, either two Cu(II) on the same molecule or on two different molecules in the dimer.

A change in the Fur Mn(II) EPR signal was observed upon binding of the complex to DNA operator; this observation rules out the possibility of Mn(II) directly participarting in binding to the DNA, unlike what was observed for other Mn(II) protein complexes when bound to a substrate [9, 10] where a more serious change took place. The above finding agrees with the previous NMR report by Williams et al. which suggested the binding of the Mn(II) Fur, complex to DNA through the positively charged residues on the N-terminus of Fur [37, 8], this part is being pursued further using the CD spectra of Fur, Mn(II) Fur, and Mn(II) Fur DNA complexes.

REFERENCES

1. S. Wee, J. B. Neilands, M. L. Bittner, B. C. Hemming, B. L. Haymore, and R. Seetharam, *Biol. Metals* **1**, 62 (1988).
2. V. de Lorenzo, S. Wee, M. Herrero, and J. B. Neilands, *J. Bacteriol.* **169(6)**, 2624 (1987).
3. A. Bagg and J. B. Neilands, *Biochemistry* **26**, 5471 (1987).
4. A. Bagg and J. B. Neilands, *Microbiol. Rev.* **51(4)**, 509 (1987).
5. V. De Lorenzo, M. Herrero, F. Giovannini, and J. B. Neilands, *J. Mol. Biol.* **203**, 875 (1988).
6. A. Bendereif and J. B. Neilands, *J. Bacteriol.* **162(3)**, 1039 (1985).
7. T. Saito and R. J. P. Williams, *Eur. J. Biochem.* **197(1)**, 29 (1991).
8. T. Saito and R. J. P. Williams, *Eur. J. Biochem.* **197(1)**, 39 (1991).
9. P. H. Hafner, Goodsaid-Zalduondo, and J. E. Coleman, *J. Biol. Chem.* **249(20)**, 6693 (1974).
10. G. H. Reed and W. J. Ray, Jr., *Biochemistry* **10(17)**, 3190 (1971).
11. M. Cohn and G. H. Reed, *J. Biol. Chem.* **245(3)**, 662 (1970).
12. A. Bencini and G. Dante, in *Transition Metal Chemistry: A Series of Advances*, A. Gordon, B. Melson, and N. Figgis, Eds., Marcel Dekker, Inc., NY and Basil, 1982, Vol. 8, pp. 1–178.
13. J. C. de Paula, W. F. Beck, and G. W. Brudvig, *J. Am. Chem. Soc.* **108**, 4002 (1986).
14. J. S. Leigh, Jr., *J. Chem. Phys.* **52(52)**, 2608 (1970).
15. A. S. Mildvan and M. Cohn, *J. Biol. Chem.* **240(1)**, 238 (1965).
16. M. Cohn and J. Townsend, *Nature* **173**, 1090 (1954).
17. D. M. Tener and K. H. Mayro, *Eur. J. Biochem.* **189**, 559 (1990).
18. G. Plamer, in *Advances in Inorganic Biochemistry*, D. W. Darnell and R. G. Wilkins, Eds., Elsevier/N. Holland, NY, Amsterdam, Oxford, 1980, Vol. II, pp. 153–182.
19. A. Hudson and G. R. Luckhurst, *Chem. Rev.* **69**, 191 (1969).
20. J. Eisenger, R. G. Shulman, and B. M. Szymaski, *J. Chem. Phys.* **36(7)**, 1721 (1962).
21. A. Danchin and M. Guerron, *Eur. J. Chem.* **16**, 532 (1970).
22. A. A. Schreier and P. R. Schimmel, *J. Mol. Biol.* **86**, 601 (1974).
23. J. F. Boas, in *Copper proteins and copper enzymes*, R. Lonti, Ed., CRC Press Inc., 1984, Vol. I, pp. 5–53.
24. C. E. Brown, D. W. Vidrine, R. L. Julian, and W. Froncisz, *J. Chem. Soc., Dalton*, 2371 (1982).
25. D. L. Williams-Smith, R. C. Bray, M. J. Barber, A. D. Tsopanaskis, and S. P. Vincent, *Biochem. J.* **167**, 593 (1977).
26. P. O. Gunnerson, U. Nylen, and G. Petterson, *Eur. J. Biochem.* **34**, 74 (1973).
27. L. Ryden, in *Copper proteins and copper enzymes*, R. Lonti, Ed., CRC Press, Inc., 1984, Vol. III, pp. 37–91.
28. E. I. Solomon, in *Metal Clusters in Proteins, ACS Symposium Ser. 372*: A symp. sponsored by Inorg. Div. of ACS at 194th meeting of ACS, New Orleans, LA, Q. Lourence, Jr., Ed., ACS Washington, DC, 1987, pp. 2–23.
29. W. H. Armstrong, *ibid*, pp. 2–27.
30. H. B. Gray and B. G. Malmstrom, *Inorg. Chem.* **68(8)**, 2316 (1964).
31. A. K. Wiersemsa and J. J. Windle, *J. Phys. Chem.* **68(8)**, 2316 (1964).
32. G. Rakhit, W. E. Antholine, W. Froncisz, J. S. Hyde, J. R. Pilbrow, G. R. Sinclair, and S. Bibundhendra, *J. Inorg. Biochem.* **25**, 217 (1985).
33. A. Zuberbuhler and H. S. Mason, in *Magnetic Resonance in Biological Systems*: Proc. 2nd Int. Conf. Stockholm, A. Ehrenberg, B. G. Malmstrom, and T. Vanngard, Eds., Pergamon Press, Oxford, London, NY, 1967, Vol. 9, pp. 187–196.

34. T. Vanngard, *ibid*, pp. 213–219.
35. R. Basosi, W. E. Antholine, W. Froncisz, and J. S. Hyde, *J. Chem. Phys.* **81(11)**, 4849 (1984).
36. M. Y. Hamed and J. B. Neilands, *J. Inorg. Biochem.*, in press (1993).
37. M. Coy and J. B. Neilands, *Biochemistry* **30**, 8201 (1991).
38. E. C. Neiderhoffer, C. M. Maranjo, K. L. Bradley, and J. A. Fee, *J. Bacteriol.* **172(4)**, 1930 (1990).
39. R. D. Dowsing and J. F. Gibson, *J. Chem. Phys.* **50**, 294 (1969).
40. R. Aasa, *J. Chem. Phys.* **52**, 3919 (1970).
41. R. Calvo, M. C. Passeggi, R. A. Isaacson, M. Y. Okamura, and G. Feher, *Biophys. J.* **58**, 149 (1990).

Accepted March 26, 1993