ELECTRON PARAMAGNETIC RESONANCE OF Cu^{2+}:Mb SINGLE CRYSTAL

CONFORMATIONAL CHANGES

O. R. Nascimento, S. Costa Ribeiro, *Departamento de Física, Pontifícia Universidade Católica do Rio de Janeiro, Brasil, and*
G. Bemski, *Centro de Física, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela*

ABSTRACT Copper introduced into met-myoglobin crystals occupies various sites as indicated by electron paramagnetic resonance parameters. Cu^{2+} (A) is probably liganded to histidine A10, lysine A14, and asparagine GH4 (Banaszak et al., 1965) and shows superhyperfine interaction with a single (imidazole) nitrogen. Cu^{2+} (B) and Cu^{2+} (C) correspond to other anisotropic sites described in less detail. Cu^{2+} (A) exhibits a transition to an isotropic form with a transition temperature of 40.5°C. This transition indicates a conformational change in myoglobin and could correspond to a motion of A helix away from the GH section. The transition temperature is 7°C higher than the one previously reported (Atanasov, 1971) for myoglobin in solution.

INTRODUCTION

The use of paramagnetic nitroxide spin labels has become very extensive in the last years. They have shown their usefulness by providing a substantial amount of information, particularly about static and dynamic behavior of proteins and lipids (membranes). Their use, however, is limited to cases where their introduction produces no appreciable perturbation of the biological system.

Other possible paramagnetic labels involve ions such as Cu^{2+} and Mn^{2+}. They have been used much less extensively until now (Vänngard, 1972; Chiang et al., 1975; Armbrecht et al., 1976). The present work is concerned with a study of Cu^{2+} ions bound to met-myoglobin crystals. This system has been selected for various reasons: (*a*) Myoglobin structure is well known from X-ray studies (Kendrew et al., 1961); (*b*) The exact location of Cu^{2+} ion in copper-doped myoglobin is also known from X-ray studies (Banaszak et al., 1965); (*c*) Electron paramagnetic resonance (EPR) studies of Cu-Mb complex in solution have been reported (Gurd et al., 1967). It has been therefore expected that single crystal studies of copper-doped myoglobin will provide information of structural interest. The principal result of this work has to do with observation of conformational changes in met-Mb at physiological temperatures.

METHODS

Myoglobin Crystals and Copper Doping

Myoglobin crystals were grown from lyophilized met-myoglobin (sperm whale skeletal muscle, Sigma Chemical Co., St. Louis, Mo.) by the method of Kendrew and Parrish (1956). Typical crystals, 1 × 1 × 0.5 mm, with an EPR ratio of signal to noise (for both Cu^{2+} and Fe^{3+}) at liquid nitrogen temperatures of 25, were obtained.

Crystals were doped with Cu^{2+} by immersion in a saturated equimolar solution of ammonium sulfate and copper sulfate. The efficiency of doping is greatly temperature dependent. At temperatures below 35°C and above 45°C the EPR – Cu^{2+} spectra consist of superposed isotropic (powder) and anisotropic spectra. To obtain only the anisotropic EPR spectrum, the optimum temperature is about 40°C. After 30 min at 40°C, the powder spectrum also appears.

Measurements

A Varian X-band, V-4502 spectrometer, with V-3400 magnet and V-4533 cylindrical cavity was used (Varian Associates, Palo Alto, Calif.). The signals were recorded with a XY-7005 B Hewlett-Packard recorder and the microwave frequency was measured with 5245 M and 5257 A Hewlett-Packard meters (Hewlett-Packard Co., Palo Alto, Calif.). The magnetic field was calibrated with a Cr^{3+}: MgO (Lunsford, 1967) g – marker (g = 1.9797 ± 0.0002).

The thermal treatment of the myoglobin crystals was made with a temperature-controlled heater (Model 5301 E, Artronix Inc., St. Louis, Mo.). The temperature was changed at a rate of 0.41°C/min. A Fe^{3+} EPR signal of met-myoglobin was used to orient the crystals.

RESULTS

EPR Results

Cu^{2+} in single crystal met-myoglobin gives rise to three types of anisotropic spectra. The predominant one, Cu^{2+} (A), has axial symmetry, and its magnetic parameters are given in Table I (Nascimento et al., 1975). The Cu^{2+} (A) spectrum corresponds to a single Cu ion per each of the two myoglobin molecules of the unit cell and is assumed

TABLE I

EPR PARAMETERS OF Cu^{2+} IN MYOGLOBIN IN DIFFERENT CONDITIONS

Cu^{2+}:Mb complex	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$	$A_{\perp}$
			G	G
Solution* pH 6.0	2.273	2.054	165	—
pH 10.4	2.186	2.046	190	—
Solution‡ pH 6.0	2.289	2.056	159	4
Crystal at 55°C	2.289 ±0.003	2.062 0.003	152 ± 4	10 ± 4
Dried crystal	2.270 ±0.003	2.062 ±0.003	161 ± 4	10 ± 4
Anisotropic spectrum:§ Cu^{2+} (A)	2.328 ±0.002	2.069 ±0.002	162 ± 3	20 ± 3

*Gurd et al. (1967)

‡Wanderley et al. (1975).

§Nascimento et al. (1975).

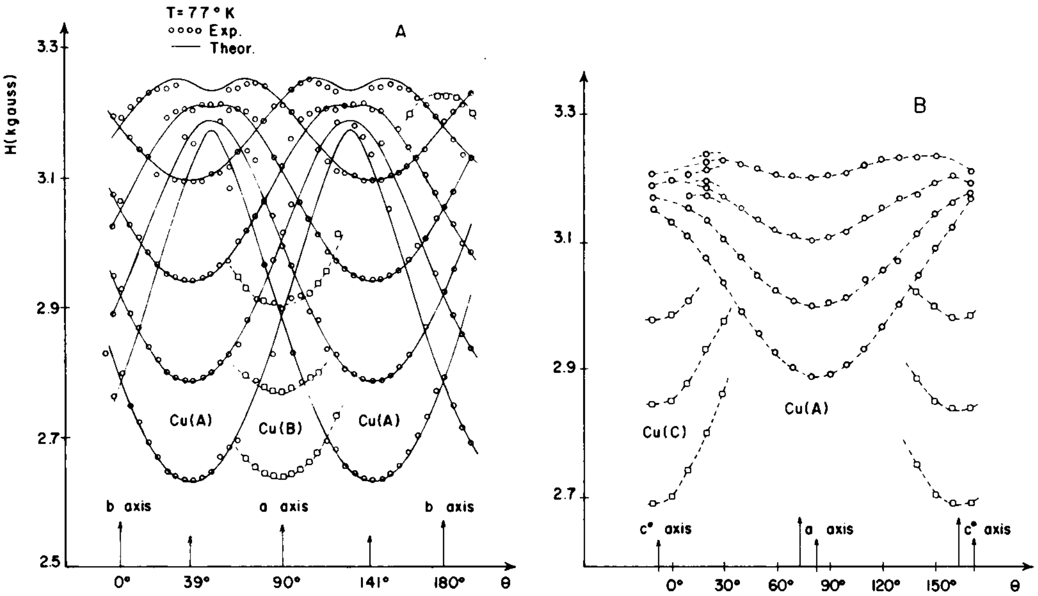

FIGURE 1 Angular variation of EPR spectra of Cu^{2+}:Mb in (A) *ab* plane and (B) *ac** plane of the single crystal. Cu^{2+} (A) was reported by Nascimento et al. (1975).

to be the same one reported by Banaszak and co-workers (1965) through X-ray determination.

The second Cu ion (Cu^{2+} (B)) was observed in the *ab* plane. The intensity of the absorption lines was at most only about 1:3 of the Cu^{2+} (A) lines. The kinetics of the production of this center by the doping process could not be properly controlled. Due to superposition with the Cu^{2+} (A) spectrum, the angular variation was only incompletely determined (see Fig. 1 A). The Cu^{2+} (B) spectrum has the following characteristics: (*a*). Only one set of four lines is observed in the *ab* plane. (*b*). The principal axes of the $\tilde{g}$ and $\tilde{A}$ tensors (assuming axial symmetry) are the same as the crystalline axes—the parallel direction coincides with *a* axis, and the perpendicular with *b* axis. (*c*). $g_{\parallel}[Cu^{2+}(B)] > g_{\parallel}[Cu^{2+}(A)]$; $A_{\parallel}[Cu^{2+}(B)] < A_{\parallel}[Cu^{2+}(A)]$; (*d*). There is no superhyperfine nitrogen spectrum associated with Cu (B).

The third Cu^{2+} spectrum, Cu^{2+} (C), was observed in *ac** plane superposed on the Cu^{2+} (A) spectrum (see Fig. 1 B). The symmetries of these two centers are, however, quite different. The kinetics of its production were also difficult to control, but it appeared in general at amplitudes smaller than Cu^{2+} (A). Its characteristics are as follow:

(*a*). Only one set of four hyperfine lines is observed in the *ac** plane; (*b*). The principal axes of the $\tilde{g}$ and $\tilde{A}$ tensors in the *ac** plane are shifted by 9° with respect to the crystalline axes. The perpendicular axis is at 9° to the *a* axis and the parallel axis at 9° to the *c** axis; (*c*). $g_{\parallel}[Cu^{2+}(A)] > g_{\parallel}[Cu^{2+}(C)]$, $A_{\parallel}[Cu^{2+}(C)] < A_{\parallel}[Cu^{2+}(A)]$, and $g_{\perp}$ and $A_{\perp}$ are comparable to Cu^{2+} (A) parameters; (*d*). No superhyperfine interaction with nitrogen ligands was observed.

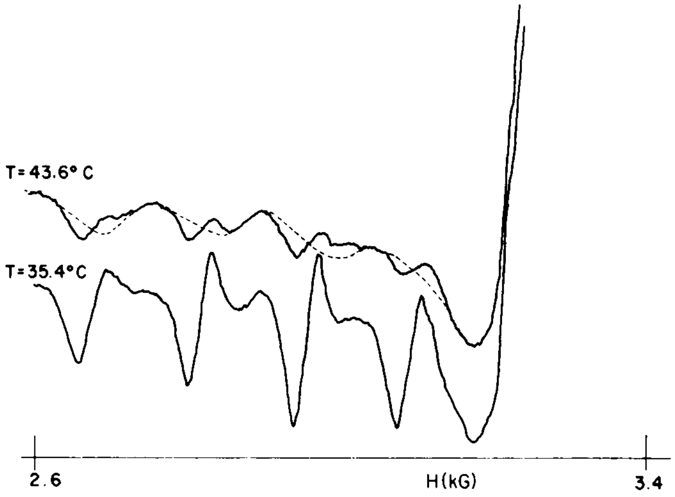

FIGURE 2 Variations of the EPR spectra of Cu^{2+}(A) as a function of the sample temperature. The dashed line corresponds to the pure powder spectra.

Thermal Variation of Cu^{2+} (A): Mb Spectrum

Once formed, the Cu^{2+} (A) ion can be preserved in the crystal at 0°C. At 25°C its anisotropic spectrum evolves toward an isotropic one in about 2 days. We have therefore studied the kinetics of this process. The temperature variation was linear, at a rate of 0.41°C/min, and the resulting variation of temperature during the time it took to register each spectrum was less than 1°. Fig. 2 shows the EPR spectra at temperatures of 35.4°C and 43.6°C. These two spectra correspond to Cu^{2+} (A) and the isotropic Cu^{2+} species, respectively. In Fig. 3 we plot the intensity of Cu^{2+} (A) signal as a function of temperature (empty circles).

Assuming that the observed transitions are quasi-static and that ΔS is independent of temperature, we obtain the following parameters: $\Delta H = 83 \pm 7$ kcal/mol, $\Delta S = 264 \pm 22$ cal/mol °K; $T_m = (40.5° \pm 0.5°)$C.

The Cu^{2+} powder spectrum parameters are included in Table I.

DISCUSSION

Copper can occupy various sites in the myoglobin crystal. One of those, Cu^{2+} (A), corresponds to the dominating species and corresponds probably to the one described by Banaszak et al. (1965), particularly because of the superhyperfine interaction with a single nitrogen which could correspond to the histidine A-10 imidazole nitrogen. This nitrogen is situated closer to the cupric ion than the other ones and can therefore be the only neighbor to provide a resolved superhyperfine structure, while interaction with other neighbors contributes only to line widths. The previous EPR results obtained on Cu:Mb complexes in solution do not correspond to the Cu(A) parameters, indicating that the delicate equilibrium between two states on these copper ions can

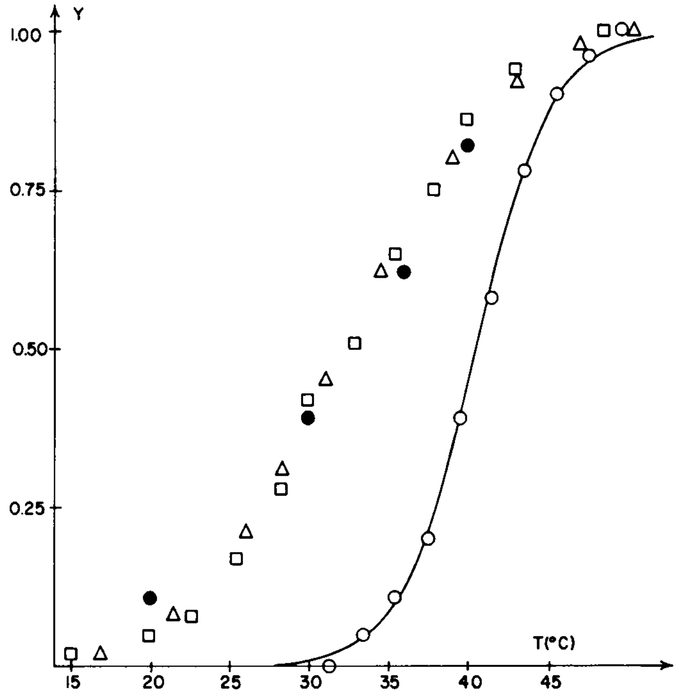

FIGURE 3 Comparison between thermal variation of the EPR signal intensity in single crystal with previous results in solution (Atanasov, 1971). ●, partial pressure of O_2 at half saturation: $Y \equiv p_{1/2O_2}$; □, rate of spin lattice relaxation of water protons: $Y \equiv$ relative T_1^{-1}; △, rotational correlation time of iminoxil spin label; ○, variation of the intensity of anisotropic EPR signal of Cu^{2+} (A). The continuous line represents the best fit: $T \equiv (1 - I/I_0)$.

easily be shifted in the direction of the isotropic one. This state probably corresponds to the one observed by Gurd et al. (1967) and by Wanderley et al. (1975). This is substantiated by the similarity between the *g* values obtained by Gurd et al. (1967) and Wanderley et al. (1975) and those of the isotropic Cu site resulting from thermal transition as observed in this investigation.

We can associate the observed transition from the low-temperature anisotropic to the high-temperature isotropic Cu spectra with a conformational change in the molecule itself. At higher temperature the A helix separates from the GH section (Fig. 4), and Cu^{2+} (A), although still bound to nitrogen of the histidine, weakens its connection to lysine A14 and to asparagine GH4 (Fig. 4). It may then become possible for the imidazole ring to rotate about the A helix, justifying the isotropic spectrum of Cu^{2+} and the modified magnetic parameters.

The two conformational isomers have been previously detected both in Mb and Hb in solution (Atanasov, 1971). In this work Atanasov, basing his conclusions on his spin label results (Fig. 3 open triangles), suggests that the conformational transition that he observes involves the CD, EF, and GH sections of myoglobin. GH is the same region where cupric ion is situated. These results are probably related to the mech-

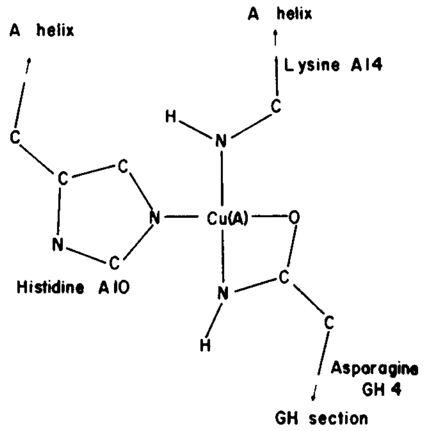

FIGURE 4 A model of chelation of the Cu^{2+} (A) ion in the myoglobin crystal.

anism of oxygenation of the molecule (Fig. 3, black circles). Our results give a transition temperature 7°C higher than the samples in solution (Fig. 3, open circles).

The range of temperature in which this transition is observed is smaller in the crystalline state with the consequent increase of the change in enthalpy (83 *vs.* 50 kcal/mol). These results point to the possibility of a slight alteration in the structure of the molecule in the crystalline form as compared to the solution, with the crystal preserving the low-temperature conformation to higher temperatures. Such slight differences have been already suggested in the spin label investigation of McConnell et al. (1969) and in infrared measurements (Brumberger and Cheng, 1974). This slight difference does not invalidate the extrapolation of the X-ray results obtained in single crystals to the molecules in solution.

Finally we see that drying of the crystal affects Cu^{2+} parameters quite strongly (Table I). This implies a global conformational change in the molecule that goes beyond the changes of the spin state of the Fe^{3+} as indicated by Mossbauer (Trautwein et al., 1970) and EPR experiments (Yonetani and Schleyer, 1967).

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REFERENCES

ARMBRECHT, H. J., T. E. GUNTER, J. S. PUSKIN, and A. R. TEREPKA. 1976. An electron paramagnetic resonance study of Mn^{2+} uptake by the chick chorioallantoic membrane. *Biochim. Biophys. Acta.* **426**:557

ATANASOV, B. P. 1971. Possible structural and functional differences of the two conformers of myoglobin-like molecules with respect to the haem-haem interaction. *Nature (Lond.).* **233**:560.

BANASZAK, L. J., H. C. WATSON, and J. C. KENDREW. 1965. The binding of cupric and zinc ions to crystalline sperm whale myoglobin. *J. Mol. Biol.* **12**:130.

BRUMBERGER, H., and B. CHENG. 1974. Conformational changes of polypeptides on crystallization. *Biopolymers.* **13**:2653

CHIANG, S. C., R. F. SPRECHER, F. K. SCHWEIGHARDT, and N. C. LI. 1975. Interaction of copper (II) and manganese (II) with hemoglobin. *J. Inorg. Nucl. Chem.* **37**:2381

GURD, R. N., K. E. FALK, B. G. MALMSTRÖM, and T. VÄNNGARD. 1967. A magnetic resonance study of sperm whale ferrimyoglobin and its complex with 1 cupric ion. *J. Biol. Chem.* **242**:5724

KENDREW, J. C., and R. G. PARRISH. 1956. The crystal of myoglobin. III. Sperm-whale myoglobin. *Proc. R. Soc. Lond. A Math. Phys. Sci.* **238**:305.

KENDREW, J. C., H. C. WATSON, B. E. STRONDBERG, R. E. DICKERSON, D. C. PHYLLIPS, and V. C. SHOSW. 1961. A partial determination by X-ray methods, and its correlation with chemical data. *Nature (Lond.).* **190**:666

LUNSFORD, J. H. 1967. EPR Study of NO absorbed on magnesium oxide. *J. Chem. Phys.* **46**:4347

MCCONNELL, H. M., W. DEAL, and R. T. OGATA. 1969. Spin-labelled hemoglobin derivatives in solution, polycrystalline suspensions and single crystals. *Biochemistry.* **8**:2580

NASCIMENTO, O. R., S. COSTA RIBEIRO, and G. BEMSKI. 1975. Electron spin resonance of copper-labelled myoglobin crystals. *FEBS (Fed. Eur. Bio. Chem. Soc.) Lett.* **53**:190

TRAUTWEIN, A., H. EICHER, and A. MAYER. 1970. Electronic structure quadrupole splitting chemical shift and susceptibility of ferrous iron in anhydrohemoglobin, anhydromyoglobin and bispyridinehemin. *J. Chem. Phys.* **52**:2473

VÄNNGARD, T. 1972. Biological Applications of Electron Spin Resonance. Interscience, John Wiley & Sons, Inc., New York.

YONETANI, T., and H. SCHLEYER. 1967. Electromagnetic properties of hemoproteins. *J. Biol. Chem.* **242**: 3919, 3926.

WANDERLEY, S. R., S. COSTA RIBEIRO, and G. BEMSKI. 1975. ESR studies of heat denaturation in Cu-myoglobin complexes. FEBS *(Fed. Eur. Biochem. Soc.).* Lett. **53**:53