ESR STUDIES OF HEAT DENATURATION IN Cu MYOGLOBIN COMPLEXES

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1. Introduction

The studies of heat denaturation of proteins has involved a variety of techniques [1]. Most of them measure changes of bulk properties of proteins, they involve in a sense macroscopic measurements. On the other hand the resonance techniques, both electron spin and proton magnetic, probe localized changes in various portions of the molecule.

The use of electron spin resonance involving transition metal ions has, so far, been quite limited. We are only aware of Hollocher's measurements of the changes of ESR Fe^{3+} signal in hemoglobin during pH and heat denaturation as well as Gersonde's work in myoglobin [2–4]. The method's main limitation is the diamagnetism of the majority of proteins.

Over the period of years certain amount of information concerning electron spin resonance of copper, in copper-doped hemoglobins and myoglobin has become available [5,6]. The X-ray work of Banaszak et al. [7] has shown that copper is situated in copper-labeled myoglobin crystals in a site adjacent to histidine A10, lysine A14 and asparagine GH4 (fig.1). This implies that its nearest neighbors involve residues from two different segments of the polypeptide chain. This prompted us to investigate the behavior of Cu^{2+}, as well as of Fe^{3+}, in copper-doped myoglobin, subjected to heat treatment, with the expectation that any relative movement of the segments of the chain will manifest itself in change of the magnetic parameters of Cu^{2+}. The latter arise from Cu^{2+} spin Hamiltonian:

$$H = \mu_B \, [g_{\parallel} H_z S_z + g_{\perp} (H_x S_x + H_y S_y)] + A I_z S_z + B (I_x S_x + I_y S_y)$$

for the case of square planar (D_{4h}) symmetry of copper ion. Here μ_N is the magneton of Bohr, H, g, S and I are respectively the magnetic field, spectroscopic factor, electronic and nuclear spins. A and B are hyperfine constants.

One can however expect (fig.1) that the symmetry is less than planar and the description has to be made accordingly in terms of g_x and g_y instead of $g_{\perp}$. In addition super-hyperfine interaction of the Cu^{2+} electron with neighboring nitrogen has been detected [5]. Recent data indicate that only one nitrogen is involved [8]. This interaction is described principally by the Fermi interaction term:

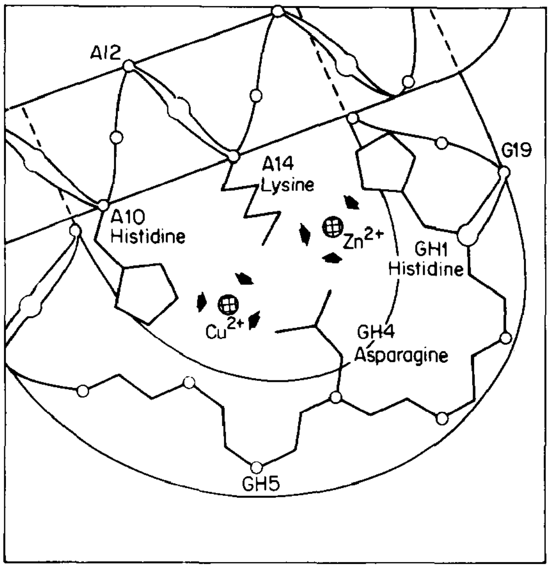

Fig.1. Position of Cu^{2+} and Zn^{2+} ions in myoglobin crystals. (From Banaszak, Watson and Kendrew, J. Mol. Biol. (1965) 12, 130).

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$$W = 4/9\, \gamma_L\, \mu_B\, \mu_N\, \alpha'^{\,2}\, |\, \psi(0)\, |^2\, S_z I_z{}^{(L)}$$

where L refers to the ligand (nitrogen), μ_N is the nuclear Bohr magneton, $\psi(0)$ is the value of the electronic wave function at the ligand nuclei and α' is the coefficient of the ligand's wave function in the molecular orbital of the ground state (B_{1g}).

2. Experimental

Myoglobin from Sigma Chemical Co. has been used (myoglobin from whale skeletal muscle, type II). Copper was added in form of $CuCl_2 \cdot 2H_2O$, and pH was adjusted with HCl or NaOH. The myoglobin concentration was 150 mg/ml and all the experiments were performed with equimolar mixtures of Cu and Mb. The solution was placed in quartz tubes and heated for 10 min, preliminary experiments showing that heating times in the range of 2 to 10 minutes gave identical results. The quartz tube was then cooled to −110°C, the temperature of most of the ESR measurements.

ESR was observed at X-band on a Varian 4502 system with a V-3400 magnet and the V-4540 variable temperature attachment.

The magnetic field was measured using a home made proton flux meter. The microwave frequency was obtained on Hewlett Packard 5245 M counter.

3. Results

3.1. *Fe^{3+}*

We have studied the amplitude of the $g_\perp = 5.9$ line of the high spin Fe^{3+} in met-myoglobin, as a function of the temperature of the heat treatment, the pH and the length of time of exposure to the heat treatment. These experiments were performed for both the pure protein and for the copper–myoglobin (1:1) complex, at pH between 5 and 8.5. This pH region has not been explored by optical methods in view of precipitation of the protein during denaturation [9].

The amplitude of the Fe^{3+} line decreases upon heat treatment in a manner indicated in fig.2. The initial increase of the amplitude of the line is due to conversion of some Fe^{2+}–myoglobin, originally present in the sample to the high spin ferric state. This increase of amplitude is absent in the Cu doped sample since copper itself converts myoglobin to ferric state [6]. If we define the denaturation temperature as the temperature at which the signal is reduced to one half of its original amplitude, we see that these temperatures around 74°C for pure myoglobin and around 65°C for the copper–myoglobin samples. No low spin Fe^{3+} signals have appeared down to temperatures of −170°C.

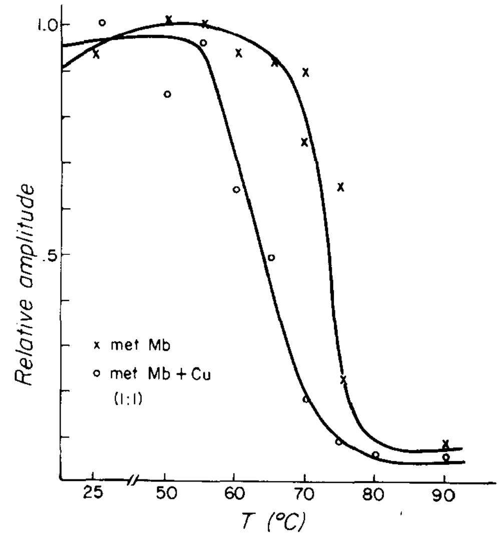

Fig.2. Changes in amplitude of the Fe^{3+} signal as a function of temperature of incubation. Metmyoglobin pH 6.4, 10 min. incubation time at each temperature. Measured at −110°C.

At pH 10 the Fe^{3+} signal is normally in the low spin state, with $g_x = 2.57$, $g_y = 2.14$, $g_z = 1.84$ [5]. After denaturation the low spin signal disappears.

3.2. *Cu^{2+}*

The magnetic parameters of Cu^{2+} in myoglobin have been obtained by Gurd et al. [5]. In frozen solution it consists of four hyperfine lines ($I_{Cu} = \frac{3}{2}$) corresponding to $g_\parallel$ and of a much larger line corresponding to $g_\perp$.

We observe changes in positions of the Cu^{2+} lines on heat treatment at physiological pH as indicated in fig.3. The changes in the amplitude of the lines are not appreciable. The super-hyperfine lines due to nitrogen, become visible as the temperature of heat treatment increases: their positions do not vary. The better resolution of these lines is due to the fact that the line width of Cu line has decreased. Computer simulations

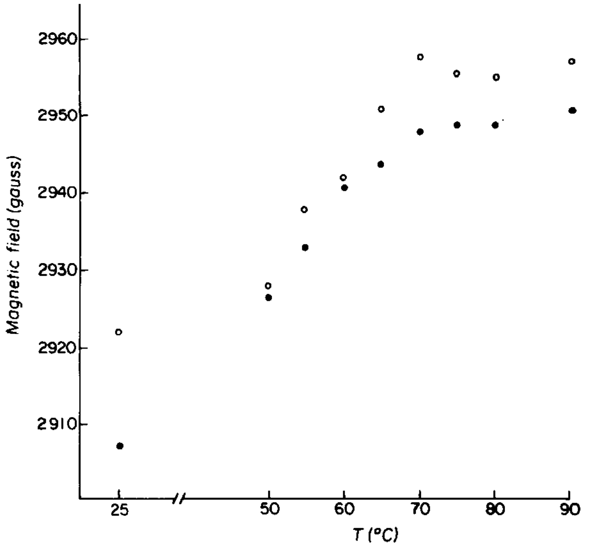

Fig.3. Changes in position of the low field Cu^{2+} lines as a function of temperature of incubation. Metmyoglobin, 10 min. incubation time at each temperature, measured at −110°C. (●—●) pH 5.4; (○—○) pH 6.4.

of the spectra show that a 5 G decrease in line width is sufficient to make the nitrogen lines visible.

At pH 10.6 the Cu spectrum does not change on denaturation. The super-hyperfine lines appear at exactly the same position as at pH 6.

At pH 4 the copper spectrum has parameters identical to the pH 6.4 spectrum after heat treatment to 50°C–55°C. No change in this spectrum is observed on heat treatment. The nitrogen lines are not observed.

4. Discussion

Our results concerning the decrease of the amplitude of Fe^{3+} high spin signal show marked differences with Hollocher's results in hemoglobin [3]. At −170°C the low spin spectrum is observed in hemoglobin after heat treatment, while we do not observe any low spin signal of denatured met-myoglobin. It is therefore possible that the low spin signal could be observed only at lower temperature, or that ferric iron becomes ferrous on denaturation. (Fe^{2+} signals have not been observed in hemoglobins in the S = 2 spin state).

The transition temperature for denaturation is highest for physiological pH. As obtained by ESR they are lower than those obtained in optical measurements [9]. The difference may be due to the different concentrations of myoglobin used in the two techniques, the concentrations used in the present experiments are about 3 orders of magnitude larger than in optical measurements.

Introduction of 1 copper in myoglobin is sufficient to weaken the protein's structure and decrease the transition temperature by about 10°C. This indicates that one ought to be very careful in using labels. Even though the crystalline structure was not affected [7], the copper modifies the denaturation process.

In order to interpret the Cu spectra a computer program has been prepared starting from the program of Vänngård and Aasa [11]. The best fits are obtained with the following parameters:

	$g_\parallel$	A	$g_\perp$	B
pH 6.4	2.289	159	2.056	4.0
pH 6.4 (denatured)	2.266	175	2.056	15
pH 10.6	2.193	198	2.046	12

The error in B is relatively large since the fit depends only weakly on its magnitude.

We can use Kivelson and Neiman's results to analyze our data [10]. They have correlated the spin Hamiltonian parameters with the covalency of the complex. Fig.4 shows the results. According to this

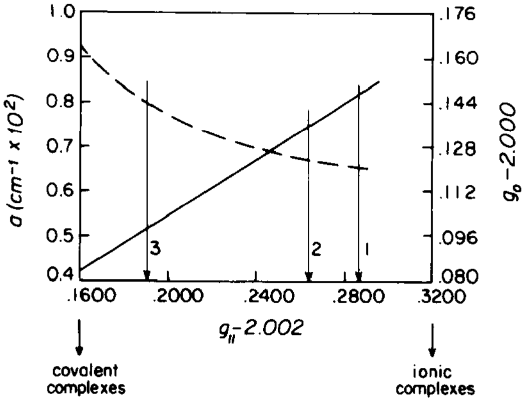

Fig.4. Experimental curves of $a = \frac{A+2B}{3}$ (– – –) and $g_0 = \frac{g_\parallel + 2g_\perp}{3}$ (—) as a function of $g_\parallel$. 1 - metmyoglobin, pH 6.4, native. 2 - metmyoglobin, pH 6.4, denatured at 80°C. 3 - metmyoglobin, pH 10.6.

interpretation the covalency of the Mb–Cu complex increases on denaturation and on increase of pH.

The tentative of simulating the interaction with nitrogen has failed because we have been considering only the isotropic interaction. Anisotropic interaction with one nitrogen has been recently observed [8].

The nitrogen lines appear at the same position in low and high pH. We cannot however fit the experimental curves keeping $g_\perp$ and B constant. We are forced to conclude that the symmetry is not D_{4h} but lower. We then have g_x and g_y instead of $g_\perp$ and A_x and A_y instead of B. If x is the Cu–N direction, then g_x and A_x remain constant, while g_y and A_y vary according to the distortion of the square planar geometry. These results are consistent with the X-ray results [7] when we consider that Cu is most strongly tied to the imidazole histidine nitrogen. This bond does not suffer any modification on heat treatment, nor at basic pH, but the weaker bonds to asparagine G H 4 and lysine A14 are affected by conformational changes of the molecule during denaturation. The direction of the changes, according to the covalency arguments [10] indicates that the region GH5 approaches the A10 histidine on denaturation. Finally, the transition temperature obtained from the analysis of Fe^{3+} and of the Cu^{2+} signals (figs.2,3) yields the same temperature between 62–65°C. This indicates that the heme region of the molecule is affected by heat treatment at the same temperature as the Cu^{2+} region. The changes of conformation at about 65°C are most probably involving the entire polypeptide chain, even if they do not lead to a random coil conformation [1].

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