OPTICAL ABSORPTION OF COPPER MET–MYOGLOBIN COMPLEXES

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Received 24 September 1976

1. Introduction

The optical spectrum in the visible and near ultraviolet [1] has been one of the most widely used tools in the study of proteins.

The heme moiety, the active site of the hemoprotein, absorbs strongly the visible radiation, and usually presents three optical bands, α, β and γ. These bands are attributed to electronic transitions of the porphyrin ring. There are also other bands, such as the charge transfer (C.T) ones, in the spectrum.

In low spin complexes, only α, β and γ bands are seen in the visible spectrum. The C.T. bands, on the contrary, lie in the infrared region, because of their small energy. This is not the case in the high-spin complexes, where the C.T. lines are mixed with the normal electronic transitions, producing a complicated spectrum. Furthermore, in ferric hemoproteins and their derivatives, there is generally a thermal equilibrium between these two spin forms as determined by magnetic susceptibility and EPR measurements [1].

The studies of the optical absorption bands for different myoglobin complexes (MbH_2O, MbF^-, $MbH.CO_2^-$, $MbCH_3.CO_2^-$, $MbNO_2^-$, etc.) [1] showed a correlation between their positions and intensities and the electronic and spin state of the iron ion. Nevertheless, the identification of the observed bands with the theoretically predicted ones [2] becomes difficult, due to the complexity of the spectrum.

We report here on the use of a known denaturing agent, namely Cu^{2+} ions, to induce a gradual change in the optical spectrum of Mb solutions, in order to identify the absorption bands that undergo similar changes which may be attributed to the transitions between correlated energy levels. The study of this gradual modification of the spectrum enables also the identification of an absorption band due to the Cu^{2+} ions chelated with met-myoglobin molecules. It is expected that this optical band, if observed, must have a small extinction coefficient, since the copper–Mb complex is included among the copper ion type 2, or 'non blue', as determined by the EPR parameters [3].

2. Experimental

The met-myoglobin solutions have been prepared with lyophilised powder myoglobin from Sigma Chemical Co. (myoglobin from whale skeletal muscle, type II) in 10^{-4} M concentration. Copper was added in form of $CuCl_2.2H_2O$ from E. Merck, Darmstadt. The $CuCl_2.2H_2O$ concentrations were those needed to obtain solutions with 2, 4, 6, 8, 16, 32, 48, 80, 96 copper ions per Mb molecule. The absorption measurements have been made at 20°C, after the centrifugation of the solutions during 15 min at 7000 rev/min, and after keeping the system for 2 h at 30°C, when the

Work partially supported by Brazilian Agencies CNPq, CAPES and FINEP

reaction was over [4]. An *N*-ethyl-morpholine–HCl buffer was used to fix the pH value at 6.4 ± 0.1; the ionic strength was 0.05.

The optical absorption measurements were made with a Beckman DK-2A spectrophotometer, using 1 cm and 0.1 cm quartz cells.

3. Results and discussions

3.1. *Change of the heme absorption bands in met-Mb*

The observed optical absorption spectrum between 450 nm and 1300 nm for all the Mb/Cu ratios (fig.1a and 1b) have been fitted with gaussians (in energies) using a Digital PDPll computer with intensities and widths as adjustable parameters.

The fittings have been made by minimizing (down to 2%) the root mean square deviation between the experimental and simulated spectra, and by appreciating visually the superposition of both.

In the range of 490–650 nm the gaussian positions used were those determined by a more sophisticated fitting program [5]. Outside that region, we employed the well known bands of 410 nm and 1000 nm, but the latter only appeared in the two less concentrated copper solutions and had its parameters determined by the infrared portion of the spectrum. The contribution of the Soret band (410 nm) to the description of the spectra was only important between 460 nm and 480 nm.

It was necessary to introduce two new absorption bands centered at 470 nm and 700 nm in order to fit all the spectra. The 470 nm line, not reported in literature probably due to its small amplitude compared with the neighboring strong Soret band, is present in the spectra of all solutions, including the pure met-myoglobin controls. The amplitude of the 700 nm band, not observed in the pure Mb sample, grows proportionally to the copper concentration, becoming more and more intense as the amount of copper increases (fig.1b).

The concentration dependence of the absorption bands areas, normalized to the Mb + $2Cu^{2+}$ sample spectrum, is reported in figure 2. The use of the Mb + $2Cu^{2+}$ spectrum, as reference is justifiable, for up to this concentration the copper ions do not change the MbH_2O spectrum and, furthermore, reduce to MbH_2O the fraction of MbO_2, sometimes present in the lyophilized Mb [6]. The choice of monitoring the band area instead of, as it is more common, measuring its maximum absorption amplitude or extinction coefficient, is due to the observed variation of the band width when copper concentration is varied.

The heme bands saturate when the copper concentration attains 8 ions per Mb molecule, suggesting a complete denaturation of the protein at this concentration. This has been previously observed for the 410 nm band, in EPR Fe signal and in titration measurements [7,8]. Nevertheless, the modification of the different bands in denaturation process is not the same. While the areas of the 505, 637 and 546 nm bands change by less than 20%, the area of the 410 nm

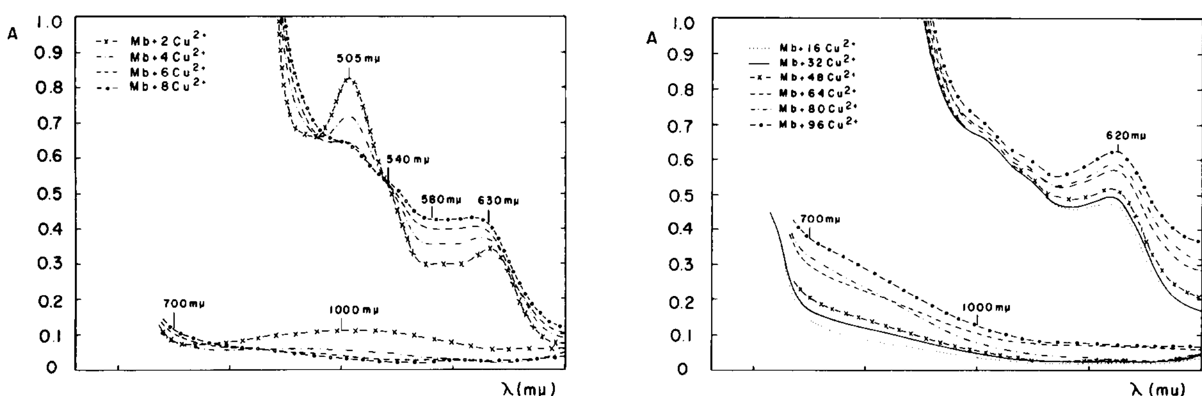

Fig.1. Optical spectra of met–myoglobin solutions doped with copper for: (a) low and (b) high concentrations of the metallic ion. The lower part of the figure corresponds to the near infrared region of the spectrum.

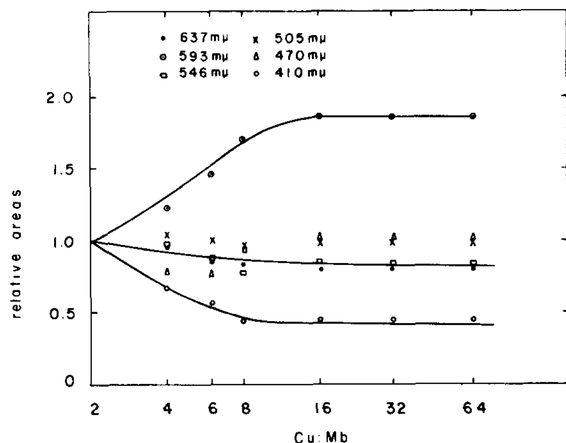

Fig.2. A semilog plot of the relative areas of the optical absorption bands against the molar ratios of Cu^{2+} to myoglobin (Mb). The areas were calculated, for each absorption band and for each metal ion concentration, dividing the value of the absorption band area by that obtained in the 1:2 copper / myoglobin solution. The experimental error is 15%.

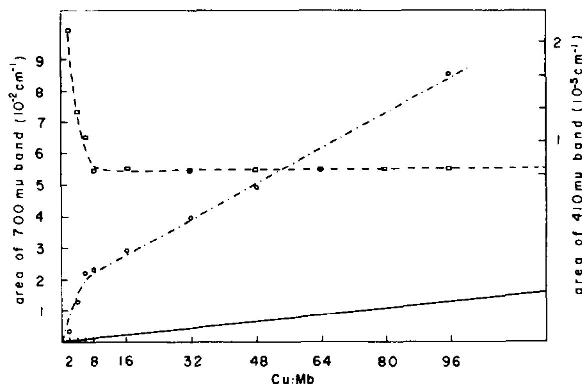

Fig.3. The variation with copper concentration of the absorption band at 700 nm (- - -) compared with that of the heme's 410 nm Soret band (—.—.—) and with the absorption of $CuCl_2$ controls.

band decreases by a factor of two, and the area of the 593 nm one grows by approximately also a factor of two.

There is no consensus, in the literature concerning the identification of the α and β bands in the Mb–H_2O optical spectrum. Williams and co-workers [1] call α and β the 593 nm and 546 nm bands, while Eaton and Hochstrasser [9] designate by α and β bands at 505 and 546 nm.

α and β bands correspond to different vibrational levels of the same electronic state (sometimes labelled Q_0 and Q_V) and, therefore, they should change in the same way when the protein is gradually denaturated. Figure 2 suggests that α and β bands correspond either to the pair of absorptions at 546 and 637 nm, or at 505 and 546 nm because of their equal rate of decrease.

3.2. *The absorption band of Cu^{2+}*

Contrasting with the heme bands, the 700 nm band does not saturate for 8 copper ions per molecule, as is shown in figure 3, where its increase is compared with that of the 410 nm band.

The intensity of the absorption at 700 nm of $CuCl_2$ controls is at least one order of magnitude smaller than of corresponding Cu–Mb solutions (fig.3). This indicates that the 700 nm band should be attributed to copper chelated to the Mb molecule.

The concentration dependence of the band areas or amplitudes (the width remaining constant) presents two distinct kinds of behaviors. For copper concentrations up to 8 per molecule the absorption amplitude varies much faster than for higher concentrations. We therefore admit the existence of two different kinds of copper binding sites. The first one related to the denaturation process monitored by the heme bands and saturated with 6 to 8 copper ions, has been already observed [7,8] and identified as the binding of copper to nitrogens of the histidine residues. The second binding site is predominant when the Mb molecule is already denaturated.

The corresponding extinction coefficients are respectively $1.2 \times 10^3\ M^{-1}\ cm^{-1}$ and $2.2 \times 10^2\ M^{-1}\ cm^{-1}$. The low values of ϵ indicate a d–d electronic transition and a highly symmetric site. This is confirmed by the existence of only one absorption band.

The detection of the copper band at the same energy for higher concentrations of copper suggests that, after the denaturation produced by the first copper ions the copper ions keep binding to other nitrogens, possibly peptide nitrogens or nitrogens belonging to lateral chains exposed by denaturation.

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