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Biofisica. — *Cyanide dissociation from ferrous myoglobins: a possible role for the distal histidine.* Nota di MAURIZIO BRUNORI (*), ANDREA BELLELLI (*) e GIOVANNI ANTONINI (**), presentata (***) dal Corrisp. M. BRUNORI.

ABSTRACT. — The reduction of cyanide saturated met myoglobin proceeds *via* a two step mechanism in which an unstable intermediate species, identified as cyanide bound ferrous myoglobin, is rapidly formed and decays slowly to the final species, unliganded ferrous myoglobin. Both reactions were investigated by time resolved optical spectroscopy in two myoglobins (from horse heart and *Aplysia limacina* buccal muscle) which differ from one another in many amino acid substitutions, but in particular because one of them (*Aplysia l.*) lacks the almost invariant distal histidine residue (E7). The different functional behaviour of the two proteins allows us to suggest a molecular mechanism for the dissociation of cyanide involving a «catalytic» role of proton transfer from the distal histidine to the bound cyanide ion.

KEY WORDS: Myoglobin; Transient spectroscopy; Ligand dissociation.

RIASSUNTO. — *Dissociazione del cianuro dalla ferro-mioglobina: possible ruolo della istidina distale.* La riduzione della ciano meta mioglobina passa attraverso un meccanismo a due stadi nel quale viene formata una specie intermedia instabile, con il cianuro legato alla mioglobina ridotta, che decade lentamente verso il prodotto finale, mioglobina ridotta. Entrambe le reazioni sono state studiate in due mioglobine (da cuore di cavallo e da muscolo buccale di *Aplysia limacina*) con tecniche di spettroscopia risolta nel tempo.

Le due mioglobine scelte per questa analisi differiscono per alcune sostituzioni di aminoacidi ed in particolare per la assenza nella mioglobina di *Aplysia l.* dell'istidina distale (E7), un residuo pressoché invariante nelle altre specie.

La cinetica di reazione nelle due proteine è diversa. L'interpretazione dei dati consente di formulare un meccanismo molecolare per la reazione di dissociazione del legante (nella forma di acido cianidrico), che assegna all'istidina distale un ruolo «catalitico» di trasferimento di protoni al cianuro legato al ferro eminico (Fe II).

INTRODUCTION.

Analysis of the molecular mechanism of biochemical reactions involving the interactions between functional proteins and small ligands has provided a wealth of information which has proved to be general and thus relevant for a variety of biological processes. The purpose of this type of investigation is to understand the basic rules controlling the dynamic aspects of binding and recognition of small molecules by proteins.

Within this framework, we have undertaken a study of the kinetics of formation and dissociation of the cyanide derivative of ferrous myoglobin as a model of molecular recognition between a heme protein and a ligand. To some extent the interest of this investigation resides in the notion (based on crystallography) that cyanide is isosteric with carbon monoxide, and that the stability of the protein-ligand complex is controlled by neighbouring amino acids on the distal and proximal sides of the heme [1, 2].

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Rapid reduction of the cyanide complex of ferric hemoglobin yields an unstable intermediate in which the ligand is bound to the ferrous heme iron [3]; this cannot be obtained as a stable species, due to the extremely small binding constant. The unstable complex dissociates irreversibly, yielding the reduced hemoprotein and free hydrocyanic acid.

In this note we report the spectroscopic properties of this intermediate and the mechanism of cyanide dissociation, which have been investigated by means of stopped-flow time resolved spectroscopy. Two myoglobins, purified respectively from horse heart and *Aplysia limacina* buccal muscle, proved to be different in the rates of reduction of the heme iron and cyanide dissociation, the myoglobin from *Aplysia l.* being slower than that from horse heart. Since the former protein lacks the distal (E7) histidine [4, 5], a model assigning a crucial role to this residue is presented as a possible description of the complex reaction time course.

Materials and methods.

Myoglobin from horse heart was purchased from Sigma Chem. Co. (USA) and used without further purification. Myoglobin from *Aplysia limacina* buccal muscle was prepared as described by Rossi Fanelli and Antonini [6]. Both proteins are stable as the ferric derivative, and the cyanide complex is easily obtained by addition of potassium cyanide in excess.

The concentration of the two proteins was determined spectrophotometrically employing a Cary 219 spectrophotometer and using the cyanide derivative of the ferric myoglobin (Mb^+CN^-).

All reagents were of Analytical grade.

A typical stopped-flow experiment was carried out as follows: a 20 μM solution of ferric myoglobin (Mb^+) in the presence of 1 mM KCN was mixed in the Gibson Durrum stopped flow apparatus with 1 to 100 mM sodium dithionite ($Na_2S_2O_4$). The absorption spectrum was recorded (over a 150 nm wavelength range) by means of a rapid scanning photodiode array spectrophotometer (Tracor Northern TN6500, USA), adapted to the 2 cm light path cell of the Gibson Durrum stopped-flow apparatus. The Tracor rapid scanning spectrophotometer is able to acquire 1024 photodiode counts in 10 ms and stores up to 64 spectra obtained at different times from start of the reaction, over the wavelength range of interest. Data were transferred to a IBM PC-AT computer for further analysis, and time courses of optical transitions recorded at different wavelengths were simultaneously fitted to a series of exponentials using the Bateman equations [7].

Results.

The time-resolved spectroscopic analysis of the overall reaction of horse Mb is presented in fig. 1; here two series of twenty optical density spectra were accumulated in 1 s and 20 s respectively. The wavelength range presented is from 496 to 642 nm, and experimental conditions are given in table 1. The two extreme spectra represent the absorption of the initial and final species, *i.e.* Mb^+CN^- (spectrum at time = 0.01 s) and deoxy ferrous myoglobin, Mb. The intermediate species, ferrous Mb cyanide, is

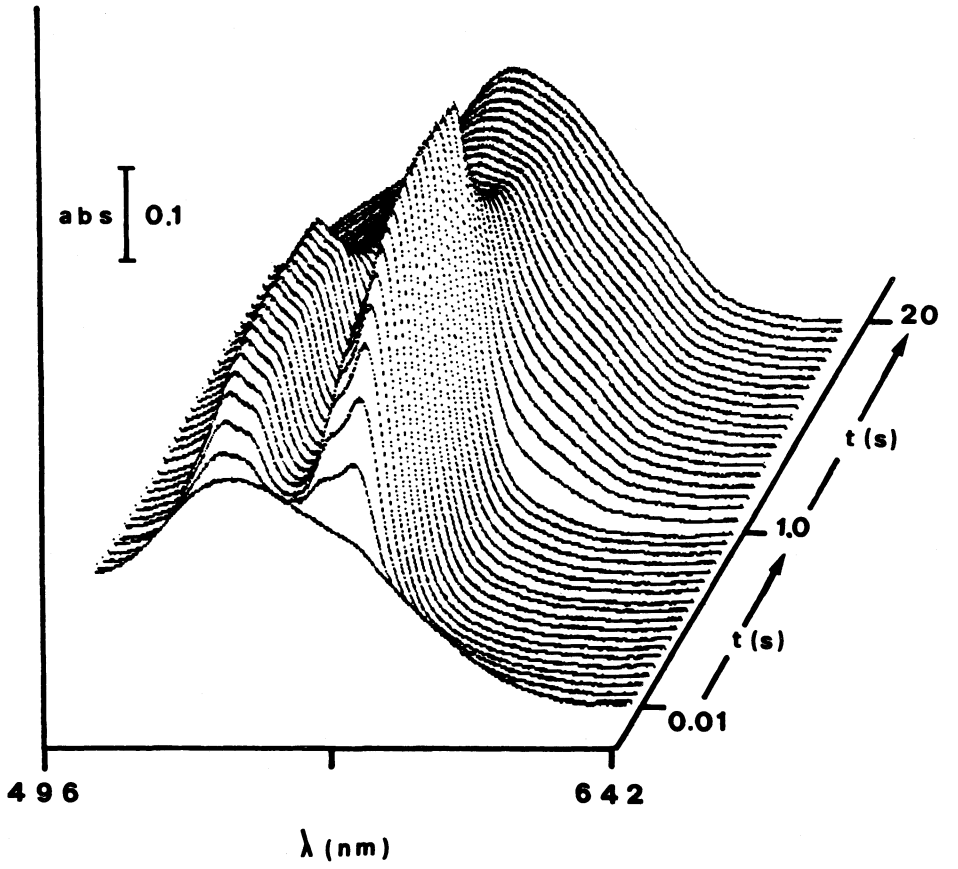

Fig. 1. – Spectral evolution during reaction of the cyanomet horse myoglobin with dithionite. Conditions as in table 1. 40 optical density spectra were collected in two series of 20 equally spaced times, the first series starting 5 ms after mixing and ending after 1 s; the second starting 1 s after mixing and ending after 20 s. Spectrum at time = 0.01 s is the absorption spectrum of Mb^+CN^-; spectrum at time = 1 s corresponds to the intermediate (ferrous cyanide Mb), and spectrum at time = 20 s corresponds to the deoxy ferrous Mb.

characterized by the absorption spectrum recorded at 1 s, since the reduction of the cyanide bound ferric heme iron by $Na_2S_2O_4$ is more than one order of magnitude faster than the decay of the intermediate. Similar data were obtained for *Aplysia l.* Mb.

The whole chemical process can be described, for both proteins, as follows:

(1) $$Mb^+CN^- \rightarrow \text{Intermediate} \rightarrow Mb + HCN$$

The first reaction in Scheme 1 is the reduction of the ferric iron driven by $Na_2S_2O_4$ in excess. This process is $Na_2S_2O_4$ concentration dependent (although of an order lower than 2; see ref. [8]), and relatively fast ($t_{1/2} < 100$ ms); its apparent rate constant for both myoglobins at pH = 7 is listed in table 1.

The second reaction in Scheme 1 conforms to a simple irreversible monomolecular decay of the intermediate, and it is usually 5 to 100 times slower than the reduction of the heme iron (see table 1).

TABLE 1. – *Kinetic rate constants for the two optical transitions observed after mixing* Mb^+CN^- *with dithionite.*

Protein	*k*1	*k*2
Horse Mb	6.5 s^{-1}	0.2 s^{-1}
Aplysia Mb	14 s^{-1}	0.033 s^{-1}

Conditions: Buffer 0.1 M phosphate pH 7, $t = 20\,°C$, $[Na_2S_2O_4] = 50$ mM; [Mb] = 10 μM; [KCN] = 1 mM, all concentrations after mixing. The rate constants were obtained by non-linear least squares fitting procedures employing the Bateman equations. Both reactions were considered irreversible first order processes, which is justified by the high dithionite concentration (100 ÷ 10 000 fold higher that the concentration of Mb).

The spectroscopic evolution of the reaction mixture was adequately described by a sequential bi-exponential system [7]. Simultaneous fit of experimental data at several wavelengths to such a model allowed us to prouve that the spectrum at $t = 1$ s in fig. 1 actually corresponds (to better than 95% accuracy) to the absorption of the cyanide complex of ferrous Mb. The spectrum of the intermediate in the visible region is characterized by two absorption bands with maxima at 570 and 536 nm for horse Mb and at 568 and 540 nm for *Aplysia l.* Mb.

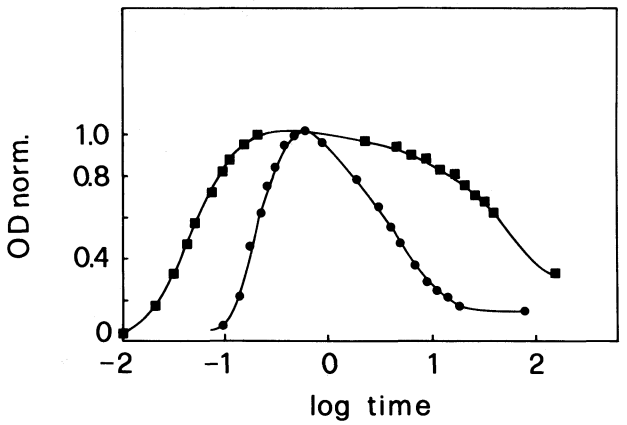

Fig. 2. – Time course of the normalized absorbance changes at 565 nm of horse heart Mb (●) and *Aplysia l.* Mb (■). Conditions as in table 1; time in s.

The time course of the normalized optical density changes at 565 nm is reported in fig. 2 to underline that in the case of *Aplysia l.* Mb the reduction of the heme iron is considerably faster and the decay of the intermediate considerably slower, as compared to horse Mb. While the relatively fast reduction of *Aplysia l.* Mb^+CN^- may have been expected (see Discussion), the slower decay of the intermediate is intringuing because of the absence of a distal histidine in *Aplysia l.* Mb.

DISCUSSION.

The reaction of Mb^+CN^- with dithionite is a bimolecular process, in which a dissociation product of $Na_2S_2O_2$ (the SO_2^- ion) is the actual reducing species [8]. This

reaction runs appreciably faster in *Aplysia l.* Mb than it does in horse Mb. Among other factors, this difference may be correlated either to the degree of heme exposure or to a different local charge at the binding site for SO_2^-. It may be noticed that the twofold difference in the reduction rate constant observed between horse and *Aplysia l.* Mb follows a general trend [9].

In discussing the decay of the ferrous cyanide derivative of the two myoglobins, one should emphasize that all hemoproteins in the ferric form bind cyanide with a very high affinity and a very small dissociation rate constant. Upon reduction of the heme iron, the affinity for cyanide (and other negatively charged ligands) becomes negligibly small ($K = 1$ M for human hemoglobin, according to ref. [3]); ligand dissociation remains relatively slow, being slower than that of oxygen for both myoglobins, and in the case of *Aplysia l.* Mb comparable to that of carbon monoxide.

The tenfold slower dissociation of cyanide from ferrous *Aplysia l.* Mb as compared to horse Mb suggests a possible role for the distal histidine in the reaction mechanism. It is known [10] that this residue forms a hydrogen bond with oxygen, and stabilizes ligand coordination to the heme iron. We suggest that dissociation of the ligand from the heme iron proceeds *via* protonation of bound CN^-. In the case of horse Mb interaction with the imidazole of His (E7) may favour rapid proton transfer from the solvent to the bound anion. This mechanism was actually proposed to account for the protonation of bound OH^- which in the case of ferric *Aplysia l.* Mb is ca. 1000 fold slower than that of sperm whale Mb [11].

In conclusion, the model presented above, based on stopped flow experiments, assumes that in horse Mb protonation of bound CN^- is accelerated by the distal imidazole at position E7 and that the bound HCN quickly dissociates from the protein, leading to the final state (ferrous Mb). In the case of *Aplysia l.* Mb, assuming the same reaction mechanism, protonation of bound CN^- would be much slower since this Mb lacks a distal His; if protonation is involved as an obligatory step, it may occurs either from the solvent or by transfer from other aminoacid side chains. The pH dependence of the decay of the unstable ferrous cyanide derivative of the two Mbs should provide a test for the proposed mechanism.

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