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Horse heart myoglobin reconstituted with a symmetrical heme

A circular dichroism study

Roberto Santucci [a], Franca Ascoli [b], Gerd. N. La Mar [c], Daniel W. Parish [c] and Kevin M. Smith [c]

[a] *Department of Biochemical Sciences, CNR Center of Molecular Biology, University 'La Sapienza',* [b] *Department of Experimental Medicine and Biochemical Sciences, University 'Tor Vergata', Rome, Italy and* [c] *Department of Chemistry, University of California, Davis, CA, U.S.A.*

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Proton NMR studies on myoglobins and hemoglobins reconstituted with non-natural hemes, possessing different side chains in the pyrrolic rings, have provided interesting information for the understanding of the mechanism governing heme reorientation in the globin pocket, during synthesis of the native protein in vivo or in the reconstitution process in vitro. More recently, circular dichroism (CD) studies have been reported as a qualitative, alternative tool, with respect to ^{1}H-NMR for detecting heme disorder in a reconstituted myoglobin or hemoglobin. In this paper, a CD study is reported on the reconstitution of horse heart myoglobin with protoheme XIII, a heme possessing true rotational symmetry about its α,γ-meso axis. The results obtained show that the reconstitution product with this heme, which binds to the apoprotein with high affinity, not dissimilar from that of the natural heme, is characterized by a CD spectrum with bands possessing rotational strengths much lower than in the native protein. Furthermore, the CD changes detected as a function of time, during heme reorientation, in the case of natural heme, are absent when the apoprotein is reconstituted with protoheme XIII. These data provide independent evidence for reorientation of the natural heme, which follows its insertion into the protein matrix.

1. Introduction

In recent years, high-resolution ^{1}H-NMR studies on several native and reconstituted myoglobins have revealed the existence of structural heterogeneity at the level of the heme pocket [1–5]. Isotopic labelling of individual methyl groups of natural and non-natural hemes in the reconstituted proteins have allowed precise assignments of methyl resonances giving a clear-cut demonstration that two isomeric forms of the protein exist which differ in the rotational orientation of the heme about the α,γ-meso axis. The predominant form detected by NMR is that usually found in the crystal structure of most myoglobins and hemoglobins. The reversed form was demonstrated to be present in relatively small amounts (10–20%) in many native proteins in solution, being the predominant one in only a few cases, like in *Chironomus* hemoglobin [2]. Actually, it was recently shown that this 'disordered' form occurs as the predominant one in the X-ray crystal structure at 1.9 Å resolution of *Glycera dibranchiata* hemoglobin [6], in agreement with the expectations from NMR [7,8] and CD [9] data on this protein.

Correspondence address: F. Ascoli, Department of Experimental Medicine and Biochemical Sciences, University 'Tor Vergata', via O. Raimondo, 00173 Rome, Italy.

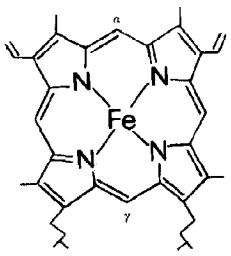

Fig. 1. Structure of symmetrical protoheme XIII.

Indeed, the two forms are interconvertible: investigation of the kinetics of sperm whale myoglobin reconstitution from apoprotein and free heme has shown that immediately after mixing, the two isomers are present in approximately equal amounts in the reconstitution mixture; the ratio of isomers then tends toward the equilibrium value typical of the native protein [10]. As mentioned above, heme heterogeneity is also detectable by CD; in fact, the optical activity induced in the heme transitions by the asymmetric protein environment is affected by the heme orientation in the globin pocket [9,11,12].

This paper deals with a CD study of the reconstitution process of horse heart myoglobin from the apoprotein and a heme possessing true rotational symmetry about the α,γ-meso axis, namely, protoheme XIII, whose structure is illustrated in fig. 1. The aim of this work is two-fold: (i) to analyze the features of the CD spectra of myoglobin reconstituted with a symmetrical heme; and (ii) to compare the CD spectra monitored during heme reorientation in the proteins reconstituted with the natural and symmetrical hemes; this was undertaken in view of the sensitivity of the CD bands to the interaction of the heme group with the neighbouring amino acids, located in the heme pocket [13]. The results obtained indicate that myoglobin containing the symmetrical heme displays absorption and CD spectra typical of most hemoglobins and myoglobins. The dichroic bands of this reconstituted protein show lower rotational strength with respect to those of the protein containing the natural heme (native or reconstituted). Finally, no changes in the CD bands are observed as a function of elapsed time after reconstitution, which further supports the phenomenon of heme reorientation in reconstituted myoglobins and hemoglobins.

2. Experimental

Myoglobin (from horse heart) and protoheme IX (hemin, type I) were used as obtained from Sigma. Protoheme XIII was synthesized as previously described [14].

Apomyoglobin was isolated using the acid-acetone method [15]. Lyophilized samples were prepared after extensive dialysis against water. Globin concentration was determined on the basis of an extinction coefficient of 12.8 $mM^{-1}\,cm^{-1}$ at 280 nm [15].

Titration of globin solution with protoheme XIII (as CO-Fe(II)-heme) was performed in 0.1 M phosphate buffer (pH 7.0) at 10 °C, under a CO atmosphere and in the presence of a few grains of dithionite. A few microliters of a concentrated buffered solution of protoheme XIII were added stepwise to the globin solution and the changes in absorbance at 422 nm were monitored 10 min after each addition.

Myoglobin was reconstituted as the ferric derivative by direct addition of the concentrated heme solution (in a small excess over the stoichiometric amount) to the buffered globin solution. The deoxygenated derivative was obtained by adding a few grains of sodium dithionite before the heme. The same procedure was followed, under a CO atmosphere, to obtain the carbomonoxy derivative.

The kinetics of reconstitution were followed by CD or spectrophotometrically, immediately after addition of a slight excess of CO-Fe(II)-heme to the apoprotein; spectra were recorded as a function of time. All reconstitution experiments were performed in 0.1 M phosphate buffer, pH 7.0 at 25 °C.

Absorbance spectra were recorded on a Cary 219 spectrophotometer. CD spectra were recorded using a Jasco J-500 N data processor. The molar ellipticity, $[\theta]$ (degree $cm^2\,dmol^{-1}$), is expressed on a heme basis.

3. Results

3.1. Titration of apomyoglobin and reconstitution with protoheme XIII

As shown in fig. 2, the titration curve of apomyoglobin with CO-protoheme XIII gives an end-point corresponding to a ratio of about 1 heme/17 000 g protein. The optical absorption spectrum of the reconstituted protein in the Soret region (see inset to fig. 2) is similar to that of the carbonmonoxy derivative of myoglobin, except for a decrease in the absorption band and a small blue-shift of the maximum (422 rather than 424 nm).

3.2. CD of reconstituted myoglobin

The dichroic spectra in the 380–600 nm range of ferric, deoxy- and carbonmonoxy derivatives of myoglobin, either native or reconstituted with symmetrical heme (recorded about 2 h after reconstitution) are shown in fig. 3. In the Soret region, the dichroic spectra of the symmetrical heme-containing myoglobin show peaks centered at about the same wavelengths of the corresponding derivatives of native myoglobin (404, 437 and 422 nm, respectively). The spectra are characterized by weak rotational strengths (between 4 and 25%, depending on the derivative, of that of the native protein). These lower ellipticities are not surprising considering the full symmetry of protoheme XIII. Carbonmonoxy and deoxy derivatives also display similar features (i.e., lower rotational strengths, small shift of the wavelength maxima) in the visible region (450–600 nm); instead, the ferric derivative containing protoheme XIII lacks CD bands in this region, suggesting (together with the very low value of the ellipticity in the Soret band, which is only approx. 4% of that of ferric myoglobin) a lower affinity of the ferric heme for the apoprotein, at least under the experimental conditions investigated.

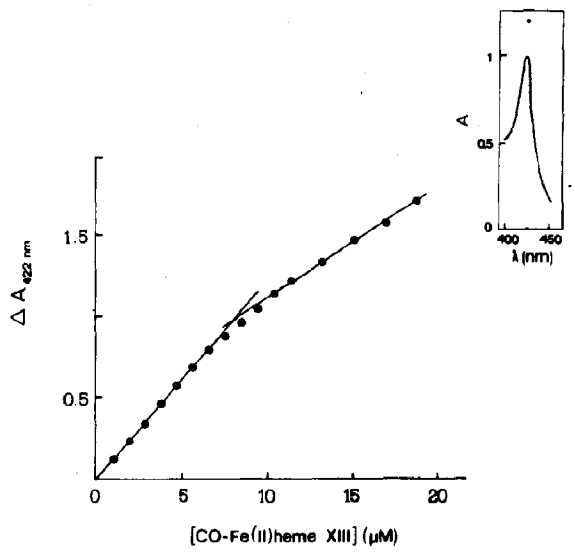

Fig. 2. Spectrophotometric titration of horse heart apomyoglobin with CO-protoheme XIII. Globin concentration: 6.5 μM. Experiments were performed in 0.1 M phosphate buffer (pH 7.0) at 10 °C. (Inset) Soret absorption spectrum of the protein reconstituted at a 1:1 molar ratio with the symmetrical heme. Asterisk indicates the expected absorbance value for native carbonmonoxymyoglobin at the same concentration (λ_{max}: 424 nm).

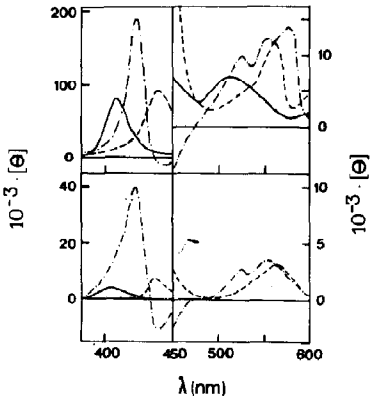

Fig. 3. CD spectra in the wavelength range 380–600 nm for the reaction of horse heart apomyoglobin with the natural (upper panel) and symmetrical heme (protoheme XIII; lower panel). Ferric (———), deoxy (- - - - -) and carbonmonoxy (–·–·–) derivatives. Experiments were performed in 0.1 M phosphate buffer (pH 7.0) at 25 °C.

3.3. Kinetics of myoglobin reconstitution as carbonmonoxy derivative

3.3.1. Optical absorption

Kinetics of carbonmonoxymyoglobin reconstitution with CO-protoheme IX and CO-proto-

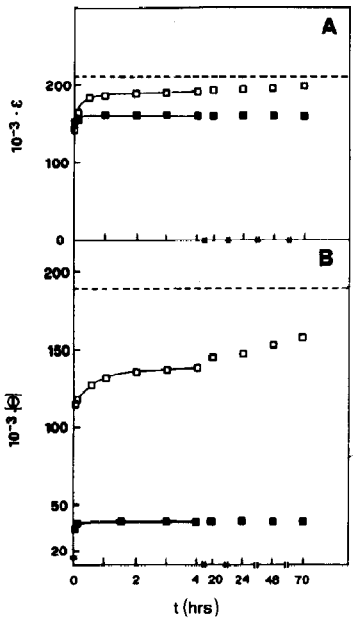

Fig. 4. Kinetics of apomyoglobin reaction with natural (□) and symmetrical heme, protoheme XIII (■), as carbonmonoxy forms. Reactions were followed spectrophotometrically (A) or by CD (B) at 424 nm (□) or 422 nm (■). Protein concentration: 6 μM, in 0.1 M phosphate buffer (pH 7.0) at 25 °C. Dashed lines refer to the molar extinction (A) and the molar ellipticity (B) values of the native carbonmonoxy derivative.

heme XIII were followed in the Soret region and the results are reported in fig. 4A. Under the conditions investigated, the reaction of apomyoglobin with CO protoheme IX appears to be a biphasic process. Within 30 s (the dead time of the measurement) the absorbance reaches about 75% of the final value and attains 85% within the first minutes. Subsequently, the reaction proceeds much more slowly and is about 98% complete within 70 h. Reconstitution with CO-protoheme XIII was followed under the same conditions; the process reaches completion in a few seconds; then the optical spectrum remained practically constant as a function of time, the final molar absorption being close to that of the native protein (about 80%, see also inset to fig. 2).

3.3.2. CD

The reconstitution reaction of apomyoglobin with the natural heme, in the CO form, monitored by CD at 424 nm as a function of time, appears to be clearly biphasic (fig. 4B). Within the first minute, an ellipticity corresponding to about 60% of that of the native protein is observable; the ellipticity then increases slowly and reaches about 80% after 24 h and 85% after 48 h. The reaction with CO-protoheme XIII was followed at 422 nm; the dichroic signal reaches its final value within few seconds and remains constant with time (up to 70 h), as intense as about 25% of that observed for the protein reconstituted with the natural heme.

4. Discussion

Reconstitution of hemoproteins with non-natural hemes possessing different substituents in the pyrrolic rings is at present of great interest, in view of the structural information that can be obtained from their ^{1}H-NMR spectra, and in particular for the understanding of the mechanisms governing the reorientation of the natural heme in the globin pocket, during formation of the native protein in vivo, or reconstitution of the holoprotein in vitro [10,16,17]. Recently, CD has been reported as a simple alternative, albeit only qualitatively, for detecting heme disorder in a given hemoprotein. The present CD study provides novel additional information, based on the study of horse heart globin reconstitution with protoheme XIII, where the substituents in the 1-vinyl and 2-methyl positions are reversed with respect to the natural heme, giving rise to a fully symmetric molecule. This heme binds to the apoprotein with high affinity in the carbonmonoxy form (even in dilute (micromolar) solutions), no different from that of the natural heme, and forms a stable holoprotein at a 1 : 1 molar ratio of the two constituents. The CD bands of the derivatives studied, all arising from the electronic transitions of porphyrin becoming optically active, are as expected, confirming specific insertion of the symmetric heme into the protein. Their ellipticity is, however, quite low in comparison to native myoglobin derivatives, and reflects the sensitivity of the CD spectrum to the symmetric conformation of the porphyrin group.

Interesting information has been obtained on the phenomenon of heme reorientation from the

changes in the Soret absorption and CD bands monitored as a function of time after reconstitution of the apomyoglobin with the two CO hemes. With the natural heme, at least two processes are evident in both CD and absorption: (a) a rapid process occurring mainly within the instrumental dead time (less than 1 min), most likely corresponding to the insertion of the heme into the protein matrix; and (b) a slow process which should reflect the change in heme orientation from that typical of the less stable form to that predominantly present in the native protein. About 3 days subsequent to reconstitution, the ellipticity amounts to approx. 98% of that of the native protein, in full agreement with the knowledge that attainment of equilibrium between the two isomers occurs on a time scale of days [18]. The apparent rate constant for the slow process is about 10^{-3} min^{-1}, in agreement with NMR data [18]. During the same period of time, very small changes in the absorbance at 422 nm are also detectable, which confirm recent results claiming that heme reorientation can be similarly studied by visible spectroscopy [19].

At variance with the above data for the natural heme, reconstitution of apomyoglobin with the symmetric heme, followed by CD (or absorbance) as a function of time, shows that the ellipticity value measured in the Soret region a few seconds after mixing remains constant throughout the time range investigated for the natural heme (from 1 min to about 3 days). Thus, all the data provide further indirect evidence for the occurrence of reorientation of the natural heme, following its insertion into the protein. In fact, a lack of changes in the CD signal after reconstitution with the symmetric heme is consistent with the idea that reorientation of such a group cannot be monitored, since its rotation around the α,γ-meso axis by 180° simply generates molecules with an identical conformation. Finally, it is of interest to point out the parallelism between the present CD data and the NMR study on sperm whale apomyoglobin reconstituted with the same symmetrical heme, where a single set of resonances was observed as a function of time [20].

In conclusion, the present results are in line with previous NMR and CD data, but provide additional, independent evidence that heme disorder tends to decrease after reconstitution, stressing again the significance of CD in the study of subtle conformational changes in hemoproteins.

Acknowledgements

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