Biochem. J. (1986) 237, 613–616 (Printed in Great Britain)

Characterization of haem disorder by circular dichroism

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Native and reconstituted myoglobin were prepared and their c.d. spectra recorded in the Soret region. Time-dependent changes in dichroism following reconstitution were observed and related to haem orientational disorder. Comparative c.d. studies, in agreement with n.m.r. studies, reveal that the degree and nature of this disorder are species-dependent.

INTRODUCTION

The phenomenon of haem orientational disorder is well established in respiratory carriers and may also extend to electron-transfer proteins (La Mar *et al.*, 1981; Docherty & Brown, 1982) as well as enzymes (La Mar *et al.*, 1980*a*). On the basis of high-resolution proton n.m.r. sperm-whale myoglobin, when reconstituted from its apoprotein and free haem, is shown to exist in two interconvertable forms (La Mar *et al.*, 1983, 1984; Lecomte *et al.*, 1985). These forms, although identical in their electronic absorption spectra, differ in the orientation of the haem group. In both forms the haem group resides within the hydrophobic pocket of the protein but differ in that one is rotated by 180° about the α–γ meso axis with respect to the other. This results in the haem vinyl and methyl groups being interchanged (Fig. 1).

La Mar *et al.* (1984) further showed that over a period of time following reconstitution of myoglobin the two forms interconvert until approx. 90% of haem is in the position indicated by X-ray crystallographic structure while 10% remains in the 'wrong' configuration. This equilibrium is also present in the native protein.

N.m.r. has been the only spectral technique used to date for characterizing haem rotational disorder. The present work reports a possible use of c.d. in characterizing and monitoring rotation disorder.

In its free state the haem group possesses a plane of symmetry (plane of porphyrin ring) and is therefore optically inactive. It is the protein that confers an asymmetric environmental upon the haem, giving rise to optical activity. Differences in c.d. associated with Soret region reflect differences in the immediate environment of the haem such as changes in the haem co-ordination geometry, state of ligation etc.

The reaction of globin with free haem is very fast, being complete in milliseconds when measured optically (Gibson & Antonini, 1960). The rates of change of c.d.

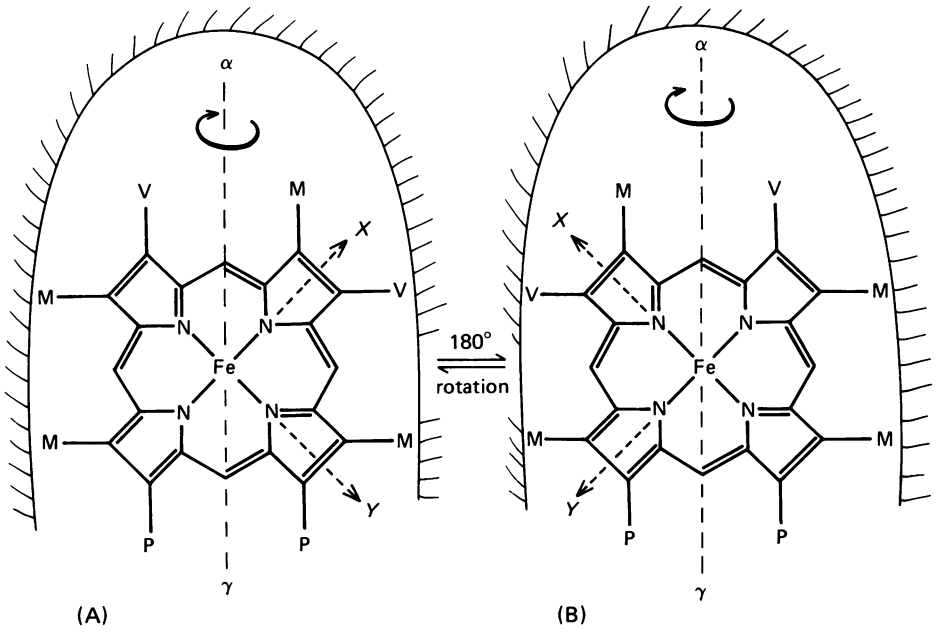

Fig. 1. Native (A) and disordered (B) forms of sperm-whale myoglobin differ by 180° rotation of haem group about the α–γ axis

The letters M, V and P represent methyl, vinyl and propionate side chains respectively.

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that we observed are relatively low, however, taking from hours to many weeks (depending on pH) to complete, a time scale similar to that of haem orientation equilibration as monitored by n.m.r. spectroscopy. It is therefore plausible that these c.d. changes are reflections of conformational changes accompanying haem reorientation occurring after haem binding has taken place.

MATERALS AND METHODS

Sperm-whale myoglobin (type II) and haemin (type III) were purchased from Sigma Chemical Co. and used without further purification. Sephadex G-25 (fine grade) was a product of Pharmacia. Deep-frozen yellow-fin-tuna (*Thunnus albacores*) muscle tissue was obtained from Duke University Marine Laboratory (Beaufort, NC, U.S.A.). All other chemicals were analytical-reagent grade.

Apomyoglobin was prepared by extracting haem from its apoprotein with butan-2-one at pH 2.3 and 4 °C (Teale, 1959). The apoprotein was dialysed exhaustively against water followed by 0.1 M-sodium phosphate buffer, pH 7.4, at 4 °C. The final protein concentration was determined by using $\epsilon = 15.9\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ at 280 nm.

Tuna myoglobin was isolated from the muscle tissue by the method of Rice *et al.* (1979) and dialysed against 0.1 M-sodium phosphate buffer, pH 7.4.

In a typical reconstitution experiment haemin (6 mg) was dissolved in a minimum volume of 0.1 M-NaOH and then diluted to 5 ml with water. The concentration of haem was checked by the pyridine haemochromogen method (de Duve, 1948). CO–haem derivative was prepared by reduction of the ferric form with fresh $Na_2S_2O_4$ under an atmosphere of CO. Reconstitution was achieved by titration of apomyoglobin with a slight excess over the stoichiometric amount of CO–haem. The yields of the reconstituted myoglobin were typically 80–90% on the basis of electronic spectra. The reconstituted myoglobin was passed down a short Sephadex G-25 column (18 cm × 3 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4, to remove any residual free haem.

Incubations of the reconstituted material were made at pH 5.5 and pH 7.4 at 22 °C for various time intervals. Some experiments involved incubations of reconstituted myoglobin for long periods (2 months) at 22 °C. Under such conditions there exists the possibility of bacterial contamination and/or protein precipitation. However, as our samples were sealed under CO, we found no bacterial growth and the solutions remained clear. In addition, the absolute absorption spectrum remained unchanged, indicating that neither precipitation leading to changes in absorption or scattering nor changes in the chromophore had occurred.

The reconstituted myoglobin was then treated with a slight excess of dithionite under an atmosphere of CO to convert ferric myoglobin into the carbonmonoxy form.

Absorption spectra were recorded on a Perkin–Elmer type 575 spectrophotometer.

The myoglobin concentrations of solutions used for the c.d. experiments were adjusted by appropriate dilution in order that all the samples had identical absorption in the Soret region.

C.d. spectra were recorded in the Soret region on a Jasco J40CS Dichrograph coupled with a B.B.C. microcomputer and plotter. The optical path length of the cell was 1 cm.

Since changes in ligation state of haem iron affect the polarization of the porphyrin π–π^* transitions (and hence the c.d.), care was taken to ensure all samples were in carbonmonoxy form.

RESULTS AND DISCUSSION

Fig. 2(*a*) shows a typical electronic absorption spectrum of sperm-whale carbonmonoxymyoglobin for comparison with the c.d. spectra shown in Figs. 2(*b*) and 2(*c*).

These Figures show a gradual increase in differential absorption at 423 nm following reconstitution. Freshly reconstituted myoglobin [reported by La Mar *et al.* (1984) to contain a 1:1 mixture of the two isomers depicted in Fig. 1], although having an absorption spectrum closely similar to that of the native protein, exhibits a decreased dichroism in the Soret region. On incubation the absorption spectrum remains unchanged while the differential absorption at 423 nm increases and eventually approaches the same value as that of native myoglobin.

These changes must result from conformational changes that occur after haem binding, since the binding of haem to the globin is a very rapid process (milliseconds) compared with the slow c.d. changes observed. We believe these changes are due to haem reorientation within the myoglobin pocket, which is also reportedly a slow process (La Mar *et al.*, 1984).

Further evidence that the dichroic changes are due to reorientation of haem to its native form is provided by comparing Figs. 2(*b*) and 2(*c*). Reconstituted myoglobin incubated at pH 5.5, a procedure reported to lead to rapid equilibration of the two orientational isomers (Ahmad & Kincaid, 1983; La Mar *et al.*, 1984), also rapidly leads to enhanced c.d. at 423 nm. The equilibration time at pH 5.5 was in the order of hours, whereas that at pH 7.4 was in the order of days to many weeks. These equilibration times are in agreement with the equilibration times measured by n.m.r. spectroscopy (Krishnamoorthi & La Mar, 1983; La Mar *et al.*, 1984).

In addition, n.m.r. investigations have revealed that the degree and nature of disorientation of haem are species-dependent. As shown in Fig. 2(*c*), native yellow-fin-tuna myoglobin, known to possess a 3:2 mixture of orientation isomers (Levy *et al.*, 1985), has a relatively low differential absorption at 423 nm, resembling the freshly reconstituted sperm-whale myoglobin, which is (almost) a 1:1 mixture.

Furthermore, *Chironomus thummi thummi* monomeric haemoglobin, which has its haem group inverted relative to sperm-whale myoglobin (La Mar *et al.*, 1980*b*), has a c.d. spectrum that is also inverted, showing negative Cotton effect in the Soret region (Formaneck & Engel, 1968). These dichroism differences observed among preparations of the same protein from different species may be due to the differences in relative haem orientation and to the degree of haem disorder present. However, when making such comparisons it must be noted that the actual c.d. will also reflect other haem environmental differences between species as well as haem orientational differences.

It is not surprising that the two orientational forms have significantly different c.d. spectra. The reorientation

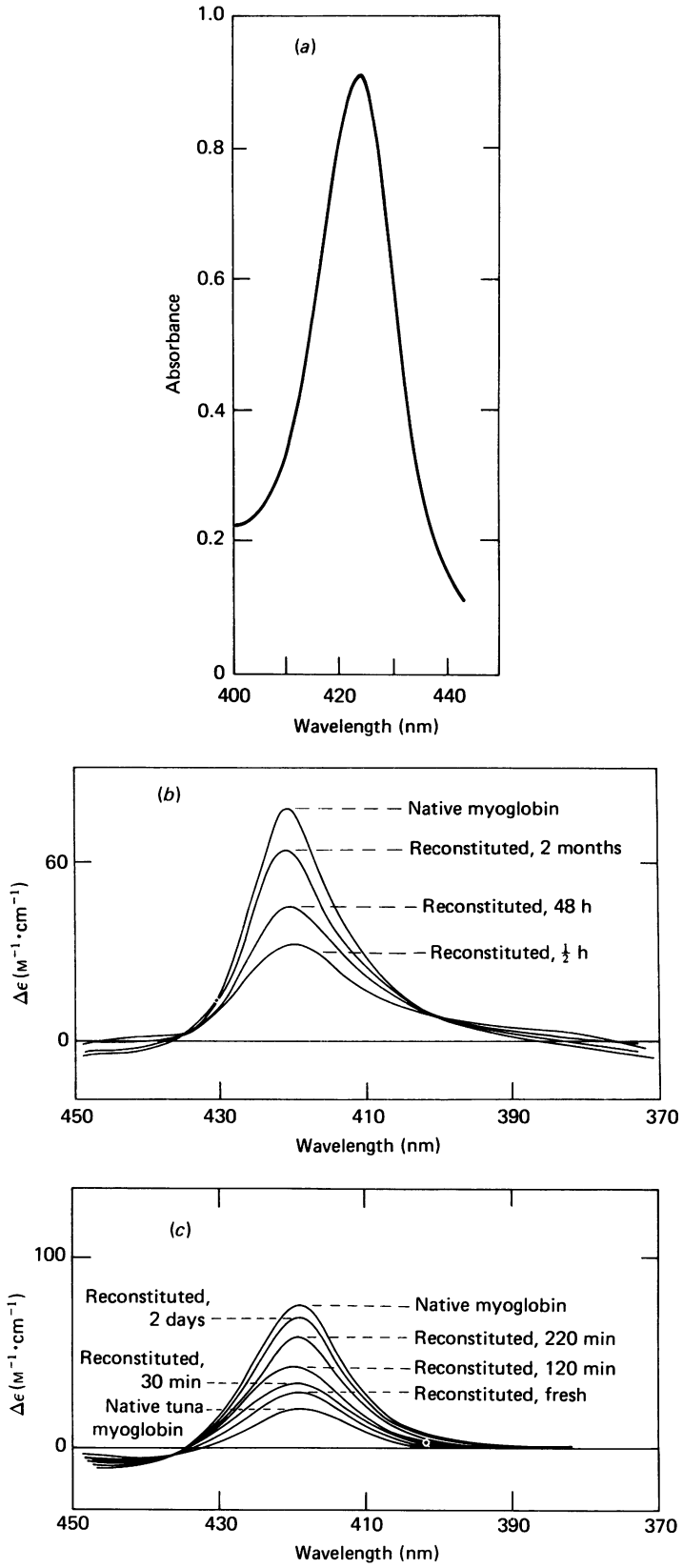

Fig. 2. Absorption and c.d. spectra of carbonmonoxymyoglobins measured in 0.1 M-sodium phosphate buffer, pH 7.4, at 20 °C

All samples had identical absorption spectra. (*a*) Typical absorption spectrum of sperm-whale myoglobin (4.9 μM) used for c.d. measurements. (*b*) C.d. spectra of native and reconstituted sperm-whale myoglobin at pH 7.4. The time elapsed from reconstitution is indicated. (*c*) C.d. spectra of native and reconstituted sperm-whale myoglobin compared to native yellow-fin-tuna myoglobin. The reconstituted protein was incubated at pH 5.5 and 20 °C for the indicated times and then diluted to pH 7.4 for spectra.

of haem about the α–γ meso axis effectively generates a mirror-image change in the porphyrin localized electric-transition dipole-moment directions. This will not change the absolute transition moment directions in plane and along the rotation (α–γ) or orthogonal axes, but it will change the direction of other transitions. The degenerate Soret bands polarized along X or Y (Fig. 1) are of this latter class, and from a dipole–dipole coupling point of view (Myer, 1978; Hsu & Woody, 1971) this will be sufficient to account for a decreased c.d. in a mixture of 'rotamers' if not a change in sign with complete rotamer inversion. Rotation by 180° about the α–γ meso axis exchanges the methyl groups at positions 1 and 3 for the vinyl groups at positions 2 and 4, and this will modulate haem methyl and vinyl peripheral contacts. Such alterations in haem–protein contacts may also perturb dipole–dipole coupling between haem transitions and π–π^* transitions of nearby aromatic residues, which are largely responsible for Soret optical activity. Although many factors may contribute to rotational strength, haem rotational disorder must clearly be considered as one of these. Certainly this may provide the simplest and indeed the only explanation why the c.d. spectrum of myoglobin changes with time following reconstitution without implying any major conformational change in protein structure. Haem orientational disorder also occurs in human haemoglobin.

Docherty & Brown (1982) have measured haem disorder in reconstituted haemoglobin A by the 'coupled oxidation' approach in which the haem is degraded to various possible biliverdin isomers (α, β, γ and δ). By analysis of the proportions of the isomers they concluded that reconstituted haemoglobin contained both orientational isomers but with only 20% of the disordered form. This disorder in haemoglobin should be detected by c.d., and, in fact, Konishi & Suzuki (1985) have reported c.d. stopped-flow studies on human haemoglobin reconstituted from haem–caffeine and found a slow increase in c.d. in the Soret region following haem binding. We suspect this slow change to be due to reorientation of the haem within the protein pocket.

If the c.d. changes we observed are indeed due to reorientation of haem, this would provide us with an alternative technique to n.m.r. for measuring haem disorder. The advantages of using c.d. over n.m.r. are obvious, because of its simplicity, speed and, most importantly for proteins, use of dilute (few micromolar) solutions.

Studies on the functional consequences of haem disorder have indicated that the disordered form has a higher affinity for O_2 than has the form predominant in the native protein (Livingston *et al.*, 1984). We have undertaken investigations of the ligand-binding kinetics of native and reconstituted myoglobins by fast reaction techiques. Our preliminary results indicate no differences between the O_2 'off' rates of native and reconstituted sperm-whale myoglobin. Tuna myoglobin also shows monophasic behaviour.

We thank Peter Udrarhelyi of Birkbech College, London, for c.d. measurements.

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Received 10 March 1986/2 May 1986; accepted 12 May 1986