¹H-n.m.r. and c.d. studies of haem orientational disorder in sperm-whale myoglobin and human haemoglobin

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¹H-n.m.r. and c.d. studies on sperm-whale myoglobin show that the c.d. signal in the Soret region is inversely and linearly related to the proportion of minor isomer present. An alternative method, 'pH jump', is described for inducing orientational disorder in sperm-whale myoglobin without recourse to reconstitution.

¹H-n.m.r. studies on human haemoglobin A indicate little heterogeneity in freshly isolated haemoglobin A, but the effect is enhanced in freeze-dried Sigma haemoglobin A.

INTRODUCTION

The introduction of haem into globin, prepared by extracting non-covalently bound haem from a variety of proteins, produces a reconstituted haem protein that contains a mixture of two isomers [1–6]. The isomers, often referred to as the 'native' (major) and 'disordered' (minor) forms, may be distinguished by high-resolution proton n.m.r. and are found to be interconvertible. In sperm-whale myoglobin, at equilibrium, the native orientation is predominant (90%) with 10% of the disordered form still persisting [7]. The position of equilibrium is, however, species-dependent, with large variations, for example, between sperm-whale myoglobin and *Chironomus thummi thummi* haemoglobin [2]. N.m.r. studies have further shown that in sperm-whale myoglobin the two isomers are not the result of a dislocation of the haem but arise from a 180° rotation around the α - γ mesoaxis [8].

The kinetics of re-orientation have been investigated and are found to be pH-sensitive, with rate minima around physiological pH [7,9]. The re-orientation towards the native structure is also influenced by the nature of the ligand occupying the sixth co-ordination position. Thus, whereas sperm-whale metaquomyoglobin containing a 1:1 mixture of the two isomers interconverts to reach equilibrium (at a rate depending on pH), addition of CN⁻ ions virtually traps the two isomers in the non-equilibrium position [10].

In native human haemoglobin most of the disorder (10%) is found in the β -chains, while the α -chains appear to be only slightly disordered (2%). Upon reconstitution both subunits become significantly disordered and tend to relax towards the original equilibrium position [5,6]. The rates of re-orientation are, however, different for each subunit, with the β -chains equilibrating faster than the α -chains in the alkaline pH region whereas the opposite is true in the acid region [11].

On the basis of ligand-binding experiments we have unambiguously shown the 'disordered' isomer in sperm-whale myoglobin to be functionally equivalent to the native isomer [12]. Although contradictory to an earlier report [13], these results are in excellent agreement with recent findings [14] also showing identical ligand-binding

characteristics of the two isomers. In yellowfin-tuna myoglobin there also appears to be no functional significance of the disordered isomer [12].

Data on tetrameric haemoglobins are limited, as studies have been confined mostly to monomeric proteins lacking haem-haem interactions. However, it is these interactions that may be modified by haem rotation, leading to significant functional differences. In addition, Gersonde *et al.* [15] has shown the Bohr effect in *Chironomus thummi thummi* haemoglobin to be controlled by haem rotation. How far this is true of tetrameric haemoglobins awaits n.m.r. characterization and functional investigations of disorder in haemoglobins from a variety of sources.

In sperm-whale myoglobin a 180° rotation of the haem is not detected by absorption spectroscopy. Nevertheless, the c.d. spectrum in the Soret region can distinguish between the two isomers [16]. However, the c.d. signal in the Soret region has not yet been correlated unequivocally with the degree of haem disorder present.

Because of the potential of c.d. in monitoring haem disorder in dilute solutions, we have undertaken studies to calibrate the c.d. spectrum against the proportions of the two isomers present as determined by n.m.r. This should allow relatively easy and rapid measurement of haem disorder in sperm-whale myoglobin and may form the basis of a method for determining haem disorder in haemoglobins.

MATERIALS AND METHODS

Materials

Sperm-whale myoglobin (type II), haemin (type III) and human haemoglobin (type IV) were obtained from Sigma Chemical Co. ²H₂O (Aldrich Chemical Co.), NaN₃ (Aldrich Chemical Co.), NaCN (BDH Chemicals), K₃Fe(CN)₆ (BDH Chemicals), Na₂S₂O₄ (BDH Chemicals), CM-52 CM-cellulose (Whatman) and Sephadex G-25 (Pharmacia) were used as purchased.

The preparation of sperm-whale apomyoglobin and its reconstituted product has previously been reported [12,16]. The apomyoglobin prepared by such a procedure was exhaustively dialysed against chilled water and

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freeze-dried. This material was redissolved in $^2\text{H}_2\text{O}$ to a final concentration of 1.3 mM. A 10% excess of freshly prepared haemin or haem-CO derivative [12,16] was added to the constantly stirred apoprotein solution at 4 °C. Any precipitate was removed by centrifugation. The reaction was allowed to proceed for 10 min, and a sample of the mixture was taken. The remaining protein was adjusted to pH 5.5 and incubated at 20 °C. The pH adjustment was made by addition of dilute NaO^2H (prepared by addition of a few crumbs of Na metal into $^2\text{H}_2\text{O}$) or dilute ^2HCl (prepared by dilution of conc. HCl with $^2\text{H}_2\text{O}$). Samples (3 ml) were taken from the incubated media at various time intervals and pH was adjusted to 7.8–8. The samples were kept on ice, and a 5-fold excess of NaCN (dissolved in $^2\text{H}_2\text{O}$) was added to 1 ml of each sample to quench the reorientation. These portions were then used for n.m.r. measurements.

The rest of the samples (2 ml each) were treated with a slight excess of $\text{Na}_2\text{S}_2\text{O}_4$ under an atmosphere of CO. This was passed through a Sephadex G-25 column (18 cm $\times$ 3 cm) equilibrated with 0.01 M-sodium phosphate buffer, pH 7.8. The eluted sample was treated with a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ under a CO atmosphere. For c.d. measurements dilutions were made in order that all samples had identical absorption spectra in the Soret region.

Human haemoglobin was prepared from blood (supplied by a local hospital) by a standard procedure [17]. This was dialysed against chilled water. Native Sigma haemoglobins were dissolved directly in 0.02 M-sodium phosphate buffer, pH 7.4, made in $^2\text{H}_2\text{O}$. Whenever required, the oxidation of haemoglobin to the met derivative was achieved by the addition of a few crystals of $\text{K}_3\text{Fe}(\text{CN})_6$. The excess ferricyanide was removed by gel filtration on Sephadex G-25. The interconversion between native and disordered forms was immediately quenched by adding a 10-fold excess of NaN_3 .

For n.m.r. measurements, the metazido derivative of freshly isolated haemoglobin (pre-equilibrated with 0.01 M-phosphate buffer, pH 6.3) was concentrated and buffer was exchanged on a short CM-52 CM-cellulose column (8 cm $\times$ 2 cm) equilibrated with 0.01 M-sodium phosphate buffer, pH 6.3, in $^2\text{H}_2\text{O}$. The metazide derivative was adsorbed on this column and washed with at least 1 column volume of the equilibrating buffer (in $^2\text{H}_2\text{O}$). The adsorbed protein was then eluted by 0.02 M-sodium phosphate buffer, pH 7.4, in $^2\text{H}_2\text{O}$. Excess azide was added to the sample after elution and before n.m.r. measurements.

An experiment designed to induce rotational disorder in sperm-whale myoglobin without recourse to reconstitution was carried out as follows. Sperm-whale myoglobin dissolved in 0.01 M- NaH_2PO_4 in $^2\text{H}_2\text{O}$ was adjusted to pH 2.3 by the addition of dilute ^2HCl (conc. HCl diluted with $^2\text{H}_2\text{O}$). The solution was left to stir for 10–15 min. The pH was then re-adjusted by rapid addition of 0.1 M- NaO^2H to 8.6, and the sample was held on ice. An 8-fold excess of NaCN (in $^2\text{H}_2\text{O}$) was immediately added to stop reorientation. The denatured protein was removed by centrifugation. N.m.r. and c.d. measurements were made in the usual manner.

N.m.r. measurements

The ^1H -n.m.r. spectra were obtained with a Jeol GX-400 spectrometer equipped with a 5 mm probe requiring 0.5 ml of sample or a 10 mm probe requiring 3 ml of

sample. The spectra are the sum of between 1000 and 2000 scans each. They were obtained with a 90° pulse width over a spectral width of 30000 Hz digitized with 16384 data points in the case of haemoglobin and 32768 for myoglobin. The residual HO^2H peak was decreased in intensity with a selective presaturation pulse. Spectra of myoglobin were subject to a 5 Hz broadening function or no line broadening, and spectra of haemoglobin to a 16 Hz broadening function. Chemical shifts are quoted in p.p.m. downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

C.d. measurements

C.d. measurements were made on Jasco J500 C instrument in the Soret region. A typical sample had an optical absorption of 1 unit in a 1 cm cell. The c.d. was measured at 423 nm in 0.01 M-sodium phosphate buffer, pH 7.8, at 20 °C. The parameter measured ($\Delta\text{absorption}$) was converted into the difference in molar absorption coefficients of the left (ϵ_L) and the right (ϵ_R) circularly polarized light, i.e.:

$$\Delta\epsilon = \epsilon_L - \epsilon_R$$

RESULTS AND DISCUSSION

Figs. 1(a) and 1(b) show the 400 MHz ^1H -n.m.r. spectra of the metcyano derivatives of native and freshly reconstituted sperm-whale myoglobin. Four resonances, labelled 1–4, are well resolved in the spectrum of the reconstituted form (Fig. 1b). These arise from the native and disordered forms of the protein [10]. In Fig. 1(a) resonances 1 and 4 are present at a low intensity whereas in Fig. 1(b) they are of an almost equal intensity to resonances 2 and 3. Percentage disorder was determined by measuring the relative intensities of resonances 1 and 2. Figs. 1(c)–1(g) represent a series of spectra when the reconstituted sample in Fig. 1(b) was allowed to equilibrate at pH 5.5 in the metaquo form for increasing periods of time. By comparison of the relative intensities it becomes clear that, as the two forms interconvert, resonance 2 increases and resonance 1 decreases in intensity until a spectrum similar to that of the native protein is generated. Quantitative analysis on each of these spectra gives the percentage disordered isomer present, which is plotted in Fig. 2.

C.d. measurements were also made on the same samples, first converted into the carbonmonoxy form, and values of $\Delta\epsilon$ so obtained are also plotted in Fig. 2. This Figure, relating the c.d. signal to the percentage disorder, shows a direct correlation between the intensity of the c.d. signal and the proportion of the two isomers in a given sample as determined by ^1H n.m.r. Thus with reference to our earlier work [16] it is now evident that the c.d. changes associated with non-equilibrium mixtures of the two isomers are indeed due to the presence of the disordered isomer.

The calibration in Fig. 2 is essential for easy routine measurement of disordered haem in sperm-whale myoglobin. A simple relationship relating the percentage disorder to observed c.d. in the Soret region can thus be derived from such a calibration procedure, i.e.:

$$\% \text{ disordered form} = \frac{90 - \Delta\epsilon_{423}}{0.97}$$

where $\Delta\epsilon_{423}$ is measured for the CO derivative.

Fig. 1. 400 MHz ^1H -n.m.r. spectra of metcyano derivatives of sperm-whale myoglobin at pH 7.9 in $^2\text{H}_2\text{O}$

(a) ^1H -n.m.r. spectrum of native metcyanomyoglobin; (b) ^1H -n.m.r. spectrum of reconstituted metcyanomyoglobin; (c)–(g) a series of ^1H -n.m.r. spectra of reconstituted metcyanomyoglobin after equilibration at pH 5.5 and 20°C for 75, 120, 180, 315 and 1200 min respectively. Peak assignments (in b) are in accordance with La Mar *et al.* [10]: 1 (8- CH_3) disordered, 2 (5- CH_3) native, 3 (1- CH_3) native, and 4 (3- CH_3) disordered. The protein concentration was 0.9 mM with a 10-fold excess of KCN.

Thus, although n.m.r. is the only technique for observing disorder directly, c.d. provides a speedy and simple way to monitor this disorder in sperm-whale myoglobin. More importantly, this makes possible the detection of disorder even in solutions containing a few micromolar haem.

One other feature of the correlation is to be noted. By extrapolation of results in Fig. 2 we can predict the c.d. of the pure native and pure disordered orientations. The

Fig. 2. Correlation of c.d. signal with percentage isomer composition

Isomer composition was determined from the ^1H -n.m.r. spectra in Fig. 1. The c.d. signal was recorded at 423 nm for the same samples as in Fig. 1 but in the carbonmonoxy form. Major and minor refer to native and disordered forms respectively.

Fig. 3. C.d. and 400 MHz ^1H -n.m.r. spectra of sperm-whale myoglobin

(a) C.d. spectra of native myoglobin before and after the 'pH jump' treatment. Myoglobin was present as the carbonmonoxy derivative in 0.01 M-sodium phosphate buffer, pH 7.4, at 20 °C. (b) ^1H -n.m.r. spectra of a similar myoglobin sample after 'pH jump'. The protein concentration was 1 mM with a 10-fold excess of KCN in $^2\text{H}_2\text{O}$ containing 0.01 M-sodium phosphate buffer, pH 8.6, at 20 °C. Peaks 1–4 are assigned as in Fig. 1.

native isomer appears to have a $\Delta\epsilon$ of approx. $90 \text{ M}^{-1} \cdot \text{cm}^{-1}$ while the disordered isomer is predicted to have a $\Delta\epsilon$ of $-7 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This almost complete cancellation of c.d. for the disordered form has also been recently predicted by other workers [14]. Thus, if the minor (disordered) isomer were ever to be isolated from a 1:1 mixture, the c.d. should allow easy characterization based on this prediction. C.d. also opens the way to monitor the kinetics of re-orientation as a function of various parameters.

Although there are no c.d. data relating to haem disorder in other haem proteins, it must be stressed that this type of calibration be carried out on wider range of proteins where disorder has been shown to exist by n.m.r.

Comparative c.d. studies, in agreement with n.m.r., seem to indicate that the degree and nature of disorder are species-dependent [2,18]. Yellowfin-tuna myoglobin exhibits a lower c.d. $\Delta\epsilon_{423}$ relative to sperm-whale myoglobin, and this may be due to higher degree (40%) of disordered component in this species [16]. Similarly in *Chironomus* haemoglobin the inverted c.d. spectra [19] may be due to 90% of the haem in an orientation resembling that of the minor component of sperm-whale myoglobin [2]. It is, however, clear that the disordered isomer in sperm-whale myoglobin has only a slightly negative $\Delta\epsilon_{423}$ value, unlike *Chironomus* haemoglobin. Structural differences, other than the haem orientation, are therefore contributing significantly to the inverted c.d. of *Chironomus* haemoglobin. Several differences indeed exist between the haem microenvironments of sperm-whale myoglobin and *Chironomus* haemoglobin [20]. The structure on the distal side of the haem in *Chironomus* haemoglobin is, for instance, modified by the displacement of histidine-E7 by isoleucine-E11 [20]. This implies that c.d. may be used to quantify haem orientation only in a given protein, and care must be

exercised when comparing the same protein from different species or indeed different haem proteins. Thus, although haem rotation can contribute significantly to the Soret c.d., the overall c.d. spectrum is the result of many specific globin-haem interactions, which vary from species to species.

The results of an experiment in which native sperm-whale myoglobin was subjected to 'pH jump' are shown in Fig. 3. From the relative intensities of methyl resonances 1 and 2 in Fig. 3(b) it is seen that rotational disorder can be induced by this method, and this is further confirmed by the diminished c.d. of a similar preparation (Fig. 3a). It appears that exposing myoglobin to pH 2.3 results in an intermediate that has low affinity for haem. This is not surprising, since haem can often be extracted from such preparations by organic solvents [21,22]. Myoglobin at this pH may thus contain relatively mobile haem, and adjustment of the pH to alkaline values results in the rapid folding of myoglobin with regained ability to bind haem. The 'pH jump' therefore mimics reconstitution and leads to a mixture of isomers. Some ($\geq 20\%$) of the myoglobin is, however, acid-denatured and is precipitated.

This is an easy and quick method for preparing non-equilibrium mixtures of the two isomers, although the yield is lower. Typical maximum yield by reconstitution is 48%, whereas that by the 'pH jump' method ranges from 40 to 43%. One advantage of the 'pH jump' method is, however, noteworthy. Samples prepared by this method do not contain excess haem, and thus can be used for functional studies with minimum purification. Purification procedures involving ion-exchange are often time-consuming and allow equilibration to proceed.

A similar 'pH jump' procedure with native human haemoglobin produces copious precipitates due to denaturation. However, preliminary experiments in which the pH of haemoglobin solutions was increased to

pH 12 for short periods showed some haem disorder. The protein is, however, very unstable under these conditions and the yield is poor.

Haem disorder has been shown to be present in native human haemoglobin [4–6,11]. Fig. 4 shows our results obtained from three different preparations of human haemoglobins. Fig. 4(a) is the ^1H -n.m.r. spectrum of the metazido derivative prepared from freshly isolated human haemoglobin. Figs. 4(b) and 4(c) are the ^1H -n.m.r. spectra from two different Sigma preparations. The methyl resonances are assigned in accordance with La Mar and co-workers [6,11]. The results show that even the native haemoglobin contains some haem disorder. A more striking observation is, however, that the Sigma haemoglobins contain significant proportions of the disordered forms. In Sigma haemoglobins the degree of disorder in each subunit varied with the batch examined. In Fig. 4(b) we see that in this particular batch of freeze-dried haemoglobin A the haem disorder is present predominantly in the β -chains. This is indicated by the appearance of resonance 3 and an increase in the intensity of resonance 7. These resonances arise (respectively) from the 8-CH_3 and the 4-CH (vinyl) protons of the disordered haem orientation in the β -chain [6,11].

In contrast, the results in Fig. 4(c) indicate that the haem disorder in this batch of haemoglobin A is mainly confined to the α -chains. This conclusion is supported by the increase in intensity of resonance 1, which (seen as a shoulder) arises from the 8-CH_3 of the disordered haem in the α -chains [6,11].

By comparison of the spectra in Fig. 4 it is clear that the resonances observed in Fig. 4(c) do not lie directly below those observed in the other two spectra. This is due to Fig. 4(c) being recorded at 25°C whereas the other spectra were obtained at 35°C . The assignment of resonance $8'$ in Fig. 4(c) is ambiguous, but it can be attributed to 5-CH_3^α or 5-CH_3^β of the disordered isomer [6,11]. However, since this resonance remained unresolved in Fig. 4(b) which is significantly disordered in the β -chain, it is likely to originate from the 5-CH_3^α group.

Whether the observed differences in disorder are the result of different preparation conditions, freeze-drying or individual variations is not clear. The last may be unlikely, since our results on freshly isolated human haemoglobin are in agreement with those reported by La Mar and co-workers [6,11]. Whatever the reason, the Sigma haemoglobins seem to contain various degrees of disordered haem, which in these cases was largely confined to either the α - or the β -subunits. Since preparation of the reconstituted haemoglobin in which all the sites are fully saturated with haem is difficult

Fig. 4. 400 MHz ^1H -n.m.r. spectra of metazido derivatives of native human haemoglobins in 0.02 M-sodium phosphate buffer, pH 7.3, at 35°C (a and b) or 25°C (c) in $^2\text{H}_2\text{O}$ in the presence of 10-fold excess of NaN_3 .

(a) Freshly prepared haemoglobin A; (b) and (c) two different batches of Sigma (type IV) haemoglobin A preparations. Peak assignments are in accordance with Yamamoto & La Mar [11]: 1 (8-CH_3^α) disordered, 2 (5-CH_3^α) native, 3 (8-CH_3^β) disordered, 4 (5-CH_3^β) native, 5 (1-CH_2^α) native, 6 (1-CH_2^β) native, 7 (4-CH^α) disordered, 8 (8-CH_3^β) native, and 9 (8-CH_3^α) native.

(unlike reconstituted myoglobin), the Sigma haemoglobins may prove useful to the study of haem rotational disorder and its functional consequences in human haemoglobin.

Can the c.d. in the Soret region be used to probe the nature and degree of disorder in human haemoglobin? The interpretation of c.d. data for haemoglobin is expected to be complicated owing to the presence of disordered haem in both the α - and the β -subunits, which may contribute differently to the c.d. spectrum. Thus knowing the total differential absorption will not be informative enough to reveal the location of disorder. Whether the disorder in α - and β -chains can be resolved separately by c.d. remains to be investigated. Current work is directed towards this end, and our preliminary n.m.r. and c.d. measurements on metazido human haemoglobin seem to indicate that the positive and the negative c.d. bands, observed for this derivative, are perturbed differently depending on whether the disorder is in the α - or the β -chains. This may form the basis of distinguishing haem disorder, between α - and β -chains, by c.d.

This research was supported by Science and Engineering Research Council Grants (GR/D/33784 and GR/D/80339), the former for purchasing the n.m.r. spectrometer.

REFERENCES

1. La Mar, G. N., Budd, D. L., Viscio, D. B., Smith, K. M. & Langry, K. C. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5755–5759
2. La Mar, G. N., Smith, K. M., Gersonde, K., Sick, H. & Overkamp, M. (1980) *J. Biol. Chem.* **255**, 66–70
3. La Mar, G. N., Roff, J. S., Smith, K. M. & Langry, K. C. (1980) *J. Am. Chem. Soc.* **102**, 4833–4835
4. Docherty, J. C. & Brown, S. B. (1982) *Biochem. J.* **207**, 583–587
5. Jue, T. & La Mar, G. N. (1984) *Biochem. Biophys. Res. Commun.* **119**, 640–645
6. La Mar, G. N., Yamamoto, Y., Jue, T., Smith, K. M. & Pandey, R. K. (1985) *Biochemistry* **24**, 3826–3831
7. La Mar, G. N., Toi, H. & Krishnamoorthi, R. (1984) *J. Am. Chem. Soc.* **106**, 6395–6401
8. Lecomte, J. T. J., Johnson, R. D. & La Mar, G. N. (1985) *Biochim. Biophys. Acta* **829**, 268–274
9. Ahmad, M. B. & Kincaid, J. R. (1983) *Biochem. J.* **215**, 117–122
10. La Mar, G. N., Davis, N. L., Parish, D. W. & Smith, K. M. (1983) *J. Mol. Biol.* **168**, 887–896
11. Yamamoto, Y. & La Mar, G. N. (1986) *Biochemistry* **25**, 5288–5297
12. Aojula, H. S., Wilson, M. T. & Morrison, I. E. G. (1987) *Biochem. J.* **243**, 205–210
13. Livingston, D. J., Davis, N. L., La Mar, G. N. & Brown, W. D. (1984) *J. Am. Chem. Soc.* **106**, 3025–3026
14. Light, W. R., Rohlfs, R. J., Palmer, G. & Olson, J. S. (1987) *J. Biol. Chem.* **262**, 46–52
15. Gersonde, K., Sick, H., Overkamp, M., Smith, K. M. & Parish, D. W. (1986) *Eur. J. Biochem.* **157**, 393–404
16. Aojula, H. S., Wilson, M. T. & Drake, A. (1986) *Biochem. J.* **237**, 613–616
17. Antonini, E. & Brunori, M. (1971) *Haemoglobin and Myoglobin in their Reactions with Ligands*, pp. 2–5, North-Holland, Amsterdam and London
18. Levy, M. J., La Mar, G. N., Jue, T., Smith, K. M., Pandey, R. K., Smith, W. S., Livingston, D. J. & Brown, W. D. (1985) *J. Biol. Chem.* **260**, 13694–13698
19. Formanek, H. & Engel, J. (1968) *Biochim. Biophys. Acta* **160**, 151–158
20. Osmulski, P. A. & Leyko, W. (1986) *Comp. Biochem. Physiol. B* **85**, 701–722
21. Teale, F. W. J. (1959) *Biochim. Biophys. Acta* **35**, 543
22. Ascoli, F., Rossi-Fanelli, M. R. & Antonini, E. (1981) *Methods Enzymol.* **76**, 72–87

Received 27 July 1987/28 September 1987; accepted 17 November 1987