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# Haem disorder in modified myoglobins

## Effect of reconstitution procedures

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Apomyoglobin was reconstituted with deuterohaem derivatives under various conditions. The fraction of disordered component, which is characterized by a 180° rotation of the haem group, for the various preparations was determined by n.m.r. spectroscopy. By using the procedures described, it was shown that the fraction of disordered component is minimized if the reconstitution is carried out with high-spin ferric haem derivatives within an experimentally determined optimum pH range of 8–9.5. Use of low-spin derivatives in either the ferrous or ferric forms leads to substantial increases in the fraction of disordered form. Attempted removal of the disordered form by selective oxidation and chromatographic purification was not effective.

The widely distributed haemoproteins exhibit remarkable functional diversity despite the similarity of structure and intrinsic reactivity patterns associated with a common haem group. Elucidation of the molecular mechanisms by which the associated protein interacts with the haem group to regulate its reactivity remains the major goal of haemoprotein research.

One commonly accepted approach for the study of these protein–haem interactions involves the investigation of the structure and properties of proteins which have been reconstituted with haems bearing modified peripheral substituents (Rossi-Fanelli & Antonini, 1957, 1959; Smith & Gibson, 1959; Antonini & Brunori, 1971; Sono & Asakura, 1975; Yonetani *et al.*, 1974*a*,*b*). The validity of such studies depends on the ability to effectively reconstitute the apoprotein with modified haems so as to generate a homogeneous preparation whose structural and functional characteristics may be reliably determined while avoiding complications that may arise from minor, ill-characterized components. Thus the recent demonstration (LaMar *et al.*, 1978*a*, 1980*a*; Keller *et al.*, 1976) that some haem proteins which have been reconstituted with certain haemins may contain substantial fractions of a minor, disordered form is of great interest for structure/function studies involving haem-reconstituted proteins.

The results of X-ray-crystallographic studies of oxygen-binding haemoproteins have clearly shown that the hydrophobic edge of the haem is buried within the protein, whereas the hydrophilic propanoic acid substituents are closer to the exterior of the molecule (Perutz, 1976). This condition may be satisfied by either of two orientations of the haem group as shown in Fig. 1. Although for haemoglobin and myoglobin the native proteins exist exclusively in one orientation, LaMar and coworkers (LaMar *et al.*, 1978*a*), using n.m.r. spectroscopy, have shown that the minor, so-called 'disordered', component that arises during reconstitution of myoglobin with deuterohaemin differs from the major, native, component by the 180° rotation about the $\alpha$–$\gamma$ axis (Fig. 1). In fact, such rotational heterogeneity has also been detected in native monomeric haemoglobins (LaMar *et al.*, 1980*a*), cytochrome $b_5$ (Keller *et al.*, 1976; LaMar *et al.*, 1981*a*) and reconstituted cytochrome *c* peroxidase (Satterlee & Erman, 1981). In the case of deuterohaem-reconstituted horseradish peroxidase, only one form is obtained, which has a reversed haem orientation relative to the native protein (LaMar *et al.*, 1980*b*). Recently, Docherty & Brown (1982) have investigated the degree of haem disorder in reconstituted human haemoglobins by using a 'coupled oxidation reaction' followed by analysis of the isomer distribution of the resulting degradation product, biliverdin.

The initial study of the reconstituted deuteromyoglobin by LaMar's group (LaMar *et al.*, 1978*a*) indicated that variable amounts of disordered fraction were obtained and implied that the degree of

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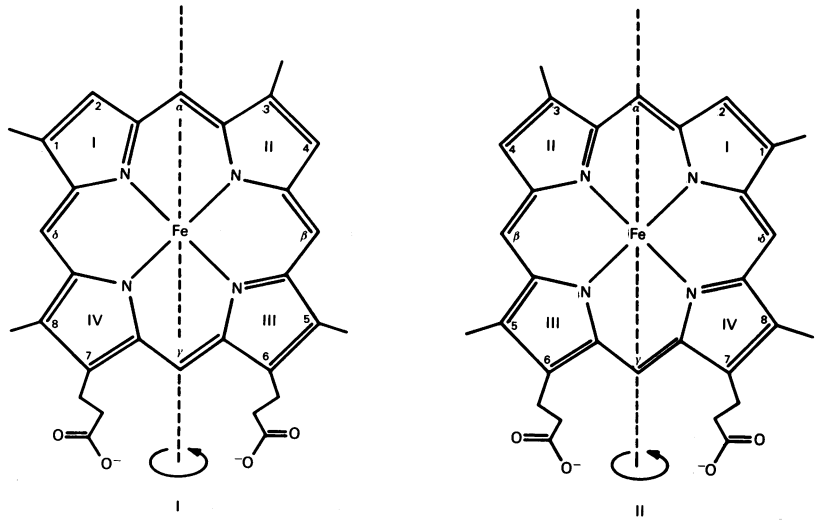


Fig. 1. *Haem orientation*

Two possible orientations of the haem group within the haem pocket are shown, which differ by a 180° rotation about the α–γ axis.

disorder may depend upon the conditions of reconstitution. Thus we have undertaken a study to determine to what extent experimental variables involved in the reconstitution procedure may effect the percentage of disordered fraction that is generated. In the present paper we wish to report the effect of solution pH and haem oxidation and spin state on the efficiency of the reconstitution procedure as determined by the percentage of disordered component that is obtained. In addition, we report the results of an attempt to remove the disordered component by selective oxidation followed by ion-exchange chromatography.

## Materials and methods

Crude deuterohaemin chloride was obtained by the resorcinol melt procedure employing a temperature of 185° for 20 minutes (Caughey *et al.*, 1966). Deuteroporphyrin IX dimethyl ester was obtained by iron removal with concurrent esterification as described by Caughey *et al.*, (1966). The porphyrin ester was purified by chromatography on deactivated (3% water) silical gel using 0.25% methanol in chloroform as eluent followed by recrystallization from chloroform/methanol. Incorporation of iron was accomplished by using anhydrous ferrous chloride in acetic acid as described by O'Keefe *et al.* (1975). Chromatography on deactivated alumina and recrystallization from chloroform/heptane yielded the oxo dimer haemin ester (Caughey *et al.*, 1966). The deuterohaemin free-acid form was obtained by the tetrahydrofuran/KOH hydrolysis procedure described by Smith (1975). The purity of the deuterohaemin sample so obtained was confirmed by t.l.c. as described by DiNello & Dolphin (1975), as well as by n.m.r. (LaMar *et al.*, 1978*b*) and the pyridine haemochromogen spectrum (Smith, 1975).

Sperm-whale myoglobin (Sigma) was used without further purification. Apomyoglobin was prepared by the butan-2-one extraction method of Teale (1959) at a pH of 2–2.5. The yields of apoprotein obtained were typically 80–90% and contained less than 0.3% metmyoglobin on the basis of electronic spectra (Tamura *et al.*, 1973).

Reconstitution of apomyoglobin with deuterohaemin was performed as described by Ogoshi *et al.* (1979), concentrations of apomyoglobin of 0.4–0.1 mM in 20 mM-phosphate buffer (at various pH values) being used. Deuterohaemin solution was prepared by dissolving 1.1 equiv. in a minimal volume of 0.1 M-NaOH, followed by dilution with distilled water to about 1 mM. The deuterohaemin solution was immediately added dropwise to a stirred solution of apomyoglobin at 4°C while maintaining the pH at a constant value by manual addition of 0.1 M HCl. On the basis of the concentration of buffer and haem solutions, we estimated that the ionic strengths of the final solutions did not vary by more than a few per cent. The mixture was allowed to stir for 4 min and was left for 2 h to ensure complete reconstitution. In all cases, extreme care was taken to ensure that the pH was maintained at the appropriate value, even in the

high-pH region where the buffering capacity of the phosphate is significantly reduced. The excess deuterohaemin was removed by gel filtration on Bio-Gel P-2 (Bio-Rad) that had been equilibrated with distilled water. The myoglobin-rich fractions were combined and immediately freeze-dried. Reconstitution was carried out at seven different values of pH (6.00, 7.00, 8.00, 9.00, 9.50, 10.0 and 11.0).

The procedure for reconstitution with various haem forms was similar to that described above (pH = 9.0). Reconstitutions with the low-spin ferric form were performed by using three different concentrations of cyanide (4, 8 and 40 equiv./equiv. of deuterohaemin) in the deuterohaemin solution. The low-spin ferrous form was prepared by addition of sodium dithionite (Virginia Chemical Co., Portsmouth, VA, U.S.A.) to the deuterohaemin solution under an atmosphere of carbon monoxide. After the mixture had been left for 2h, excess potassium ferricyanide was added in order to convert the deuterohaemin into metmyoglobin. Excess haemin and ferricyanide were removed by gel filtration on a column of Bio-Gel P-2 that had been equilibrated with distilled water. The metmyoglobin solution was immediately freeze-dried.

For the attempted removal of disordered fraction this autoxidation of reconstituted deuteromyoglobin at pH8.40 (25°C) was also carried out. The metmyoglobin was first reduced with sodium dithionite and chromatographed on a column of Bio-Gel P-2 that had been equilibrated with deoxygenated 5mM-Tris buffer, pH8.40. A visible spectrum of the eluted myoglobin solution was obtained in order to confirm complete reduction to the ferrous state (absence of a peak at 620nm). The myoglobin solution was left at room temperature in the presence of air and autoxidation of oxydeuteromyoglobin was monitored spectrally. The fraction of oxidized component was calculated by comparison of $A_{620}$ of a small sample with that of a fully oxidized sample.

After 10–15% of the oxydeuteromyoglobin had been converted into metdeuteromyoglobin, the solution was applied to a column (2.5cm × 15cm) of DEAE-cellulose (Whatman DE-52) that had been equilibrated with 5mM-Tris buffer, pH8.4, and was eluted with the same buffer (Hardman *et al.*, 1966). The metdeuteromyoglobin was eluted before the oxygenated form and was discarded. Pure oxydeuteromyoglobin, as determined by its absorption spectrum, was collected and immediately reoxidized with potassium ferricyanide. The solution was then passed through a Bio-Gel P-2 column that had been equilibrated with distilled water and was then freeze-dried.

All samples for n.m.r. spectroscopy were prepared as described by LaMar *et al.* (1978*a*). The freeze-dried myoglobin was redissolved in 0.2M-NaCl in $^2H_2O$ and the pH adjusted to 11 with $NaO^2H$. A 5-fold excess of potassium cyanide was added and the pH was adjusted to 8.5. The solution was centrifuged and the n.m.r. spectrum recorded immediately.

Proton n.m.r. spectra were recorded on a Varian XL-200 spectrometer operating at 220MHz. Between 5000 and 50000 transients were collected over a 12KHz bandwidth, in 16384 points (double-precision, 32bit) using a 4$\mu$s, 56° pulse. The $HO^2H$ peak was suppressed by homonuclear decoupling. The signal-to-noise ratio was improved by exponential sensitivity enhancement and by Gaussian apodization of the free induction decay, which introduced 5Hz line broadening. An extended filter band was used to assure less than 1% filter attenuation. The sample temperature was maintained at 37°C. All chemical shifts are given in p.p.m. and referenced to 2,2-dimethyl-2-silapentane-5-sulphonate by setting the $HO^2H$ peak at 4·63p.p.m.

## Results

The proton n.m.r. spectrum of deuterometmyoglobin cyanide, which contains a significant fraction of disordered form, is shown in Fig. 2 along with the spectrum of native metmyoglobin cyanide. Most of the haem methyl resonances are hyperfine-shifted to lower field, and the chemical-shift values are comparable with the reported ones (LaMar *et al.*, 1978*a*). Three of the methyl peaks of the major component are well-resolved at 13.2, 18.1 and 24.7 p.p.m. (with reference to 2,2-dimethyl-2-silapentane-5-sulphonate) and have been assigned to 5-$CH_3$, 1-$CH_3$ and 8-$CH_3$ respectively (LaMar *et al.*, 1978*a*). For the minor component, the methyl groups at positions 3 and 8 resonate at 18.7 and 27.9p.p.m. (underlined in the Figure) respectively. The percentages of disordered component can be calculated by comparing the relative intensities of resonances at 27.9p.p.m. (minor) and 24.7p.p.m. (major). These percentages are presented in Table 1 for the various values of pH at which reconstitution was performed.

The fraction of minor component that is generated is apparently dependent upon the solution pH during the reconstitution procedure, reaching a minimum between pH8 and 10. Outside this range, under the same conditions (ionic strength, temperature etc.), significantly higher levels of disordered component are generated.

Reconstitution was also performed with haemin in the presence of various concentrations of cyanide and also in the ferrous form in the presence of carbon monoxide. In these cases the pH was maintained at 9.0. The proton n.m.r. spectrum of deuterometmyoglobin cyanide that had been reconstituted in the presence of cyanide is also shown in Fig. 2.

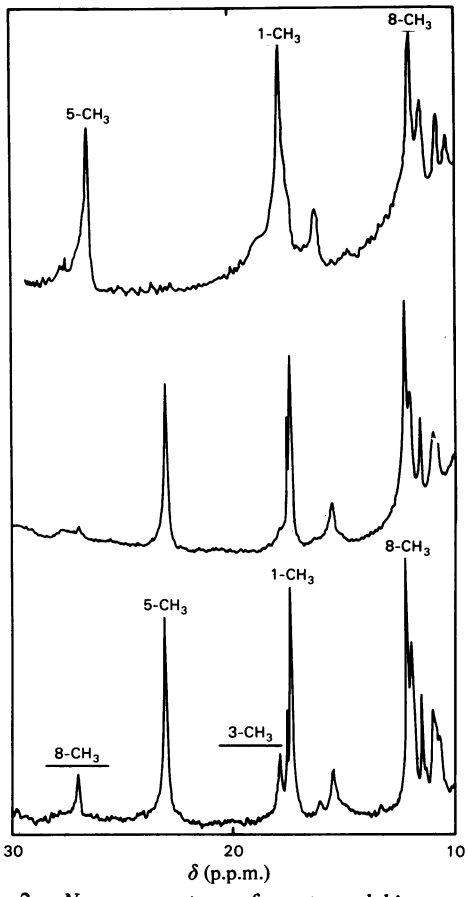


Fig. 2. *N.m.r. spectra of metmyoglobin cyanide derivatives* $^2H_2O$*/0.2*M*-NaCl, pH 8.50, at 38°C*
Top, native, protometmyoglobin cyanide; middle, deuterometmyoglobin cyanide reconstituted at pH 9; bottom, deuterometmyoglobin cyanide reconstituted in the presence of cyanide ion. Chemical shifts ($\delta$) are shown relative to 2,2-dimethyl-2-silapentane-5-sulphonate.

The n.m.r. spectra of those derivatives which had been reconstituted with low-spin forms clearly demonstrate the presence of disordered component. In both cases, the percentages of the disordered component are greater than that obtained during reconstitution in the absence of the ligands (Table 1). In addition, the percentage of minor component was dependent upon the cyanide concentration, values of 10, 13 and 17% being obtained with 5, 8 and 40 equiv. of cyanide.

The attempted removal of disordered form based on selective autoxidation was performed as described in the Materials and methods section. Separation of oxygenated and oxidized forms of deuteromyoglobin was achieved with better resolution and in higher yield on DEAE-Sephadex than with DEAE-cellulose columns. The lowest value for fraction of the disordered component obtained with this autoxidation/removal procedure was 3%.

Table 1. *Calculated percentages of disordered component from observed n.m.r. spectra*

| Sample | Disordered form (%) |
|---|---|
| Deuterohaemin, pH 6.00 | 9.4, 9.2 |
| pH 7.00 | 7.8, 7.6 |
| pH 8.00 | 4.3, 4.6 |
| pH 9.00 | 3.7, 4.1 |
| pH 9.50 | 3.9, 4.3 |
| pH 10.00 | 4.8 |
| pH 11.00 | 7.0 |
| Deuterohaemin-CN, 5 equiv. of $CN^-$ | 9.5 |
| 8 equiv. of $CN^-$ | 12.8 |
| 40 equiv. of $CN^-$ | 16.5 |
| Deuterohaem-CO | 12.0 |

## Discussion

Binding of haems and haemins to various apohaemoproteins has been shown to occur in two stages (Yonetani, 1967; Phelps & Antonini, 1969). The first stage consists of rapid and reversible non-specific binding to form an intermediate protein–haem complex. Formation of this non-specifically bound intermediate is followed by slower rearrangement resulting in 'irreversible' formation of intact reconstituted haemoprotein. The formation of the reconstituted haem protein is accompanied by a protein conformational change. Thus, in early studies of the properties of apomyoglobin, Breslow and co-workers (Breslow, 1964; Breslow *et al.*, 1965) had shown that changes in side-chain reactivity and differences in o.r.d. indicate a loss in helix content from 56% for the intact metmyoglobin to about 45% for the apoprotein. Reconstitution of the apomyoglobin with protohaemin results in restoration of the helix content to that of native metmyoglobin. There is apparently no effect of pH on the conformation of the intact metmyoglobin, although slight changes in helix content of the apoprotein are possibly brought about by variation of solution conditions (Breslow, 1964; Breslow *et al.*, 1965).

The observed pH-dependence of the fractional disorder (Table 1) could be interpreted as an indication that subtle changes in apoprotein conformation are brought about by variation in solution pH and that the conformation existing at pH 8–10 favours binding of the deuterohaemin in the native orientation. On the other hand, pH-dependent

differences in the relative rates of haem reorientation within the haem pocket in the intact deuteromethmyoglobin may also be important. In this case, the results would be interpreted as demonstrating an increased rate of conversion from 'disordered' into 'native' form at pH 8–10 relative to the rate outside of this range. Although the previously cited o.r.d. results (Breslow, 1964; Breslow *et al.*, 1965) provide no evidence for pH-dependent gross conformational differences in the intact metmyoglobin, such haem reorientation may occur.

In any case, regardless of the operative mechanisms, it is apparent that by using the procedures employed here, the percentage of disordered component obtained in the final preparation can be minimized by performing the reconstitution at pH 9.

Reconstitution with low-spin haem derivatives, either as the ferrous or ferric forms, while maintaining the optimum solution pH, results in substantial increases in the fraction of disordered form that is obtained in the final preparation. It is reasonable to assume an identical conformation of the apoprotein at this optimum solution pH, irrespective of the form of the haem derivative employed. Therefore, restricted access to the haem pocket can not be invoked to explain the observed differences in fractional disorder unless the state of aggregation of the haem derivatives differs in the three cases studied.

Ferric haems are known to dimerize in aqueous alkaline solution (Brown & Hatzikonstantinou, 1978*a*,*b*, 1979). The major stabilizing factor leading to aggregation involves $\pi$–$\pi$ interactions between pyrrole rings of partner porphyrin macrocycles (Brown & Hatzikonstantinou, 1979; White, 1978). The presence of vinyl groups in protohaemin greatly increases the stability of the dimers of this haemin (by more than 100-fold) over that of the dimers of deuterohaemin (Brown & Hatzikonstantinou, 1979). Similarly, a much decreased extent of dimerization of deuterohaemin cyanide relative to cyanide complexes of protohaemin has also been documented (LaMar *et al.*, 1981*a*,*b*).

Assuming identical protein conformations in all three experiments as well as essentially monomeric haem derivatives, and thus negligible influence of steric factors, it seems likely that the observed variation in fractional disorder arises from differences in the relative rates of conversion of the disordered orientation into the native orientation. Evidently, the rate of transformation to the native orientation is substantially slower for the metcyanide- or carbonmonoxy-deuteromyoglobins than for the metaquo form. Some support for this interpretation is provided by the observation that, after 1 day, the metcyanide derivative of reconstituted deuteromyoglobin exhibits the same percentage of disordered component as is observed within 2 h after the incorporation reaction.

It is worth mentioning at this point that the results obtained here are consistent with the studies of reconstituted haemoglobin recently reported by Docherty & Brown (1982). These workers found a substantial increase in the percentage of disordered form which arises if reconstitution is carried out with the carbon monoxide derivatives of the protohaem. Although the method of analysis was an indirect method based on the 'coupled oxidation' of haem in intact protein, these workers were careful to demonstrate the validity of their procedure for determining percentage disorder.

In an attempt to remove the persistent minor fraction of disordered form, we have evaluated a procedure based on the premise that the disordered component may be destabilized with respect to autoxidation. However, the results presented above indicate that these procedures are not effective. Inasmuch as the structural parameters of the haem pocket may not be significantly altered by the 180° rotation of the deuterohaem moiety, the stabilization of the bound dioxygen molecule (Yonetani *et al.*, 1974*b*; Caughey *et al.*, 1975; Ikeda-Saito *et al.*, 1977; Tucker *et al.*, 1978) may not be affected. It is also possible that, either in the high-spin ferric derivative that results from autoxidation or in the oxidized preparation generated by addition of ferricyanide to the separated 'native' form, the haem may subsequently undergo reorientation to produce a small percentage of disordered component. The work by LeMar *et al.* (1978*a*), which demonstrated some degree of interconvertibility between the native and disordered components upon reduction and reoxidation with ferricyanide, provides some support for this interpretation. Similarly, the work by Docherty & Brown (1982) also supports this interpretation. In their study of disorder in haemoglobin they were able to demonstrate that, in native metaquo forms, which are known to undergo haem exchange (Bunn & Jandl, 1966, 1968), an equilibrium exists between the two forms depicted in Fig. 1 and that the ratio of the two components becomes fixed upon conversion into oxy- or deoxyhaemoglobin. Thus elimination of the disordered component by selective oxidation and chromatographic purification, by the procedure described here, may be ineffective because of interconversion of a fraction (~3%) of the purified native form to disordered form.

Our purpose in undertaking the present studies was to determine the effect of experimental procedures on the fraction of disordered form that may arise during reconstitution of apomyoglobin with modified haems. Although several factors may contribute to the control of this process, certain conditions have been shown to minimize this disorder. Thus it is beneficial to reconstitute the apoprotein in the absence of strong-field ligands at a

solution pH of 8–9.5. This avoids formation of low-spin derivatives which either generate greater fractions of disordered form or substantially decrease the rate of conversion of this form into the native form. By using this procedure it is possible to decrease the percentage of disordered form to very low levels (~3%). This is true even in the case of deuterohaemin, which, as a result of the significantly decreased steric interactions associated with the replacement of vinyl groups by sterically non-demanding hydrogen atoms, would be expected to exhibit the greatest degree of fractional disorder of all the commonly utilized modified haems. Finally, consistent with the interconvertibility demonstrated by LaMar *et al.* (1978*a*), we have not been able to devise an efficient procedure for selective oxidation and chromatographic removal of the disordered component.

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