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LETTER

Assignment of Proton Hyperfine NMR Resonances in Met–Cyano Monomer Hemoglobin Components from *Glycera dibranchiata* Using Deuterium-labelled Hemes

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The three major monomer hemoglobin components from *Glycera dibranchiata* have recently become objects of interest because they display the exceptional primary sequence substitution: E-7 his → leu [1–3]. The substitution has a dramatic effect on the ligand binding properties of these proteins [4–6] and is unusual in the sense that most naturally occurring E-7 point mutations in hemoglobins and myoglobins involvè replacement of the distal histidine by an amino acid which is generally polar or charged [7–10], whereas for *G. dibranchiata* monomer hemoglobins the substitution is one that replaces histidine with the less polar leucine.

Here we present initial results from our work on the three cyanide-ligated monomer methemoglobins (components II, III, IV) that complement our earlier assignments for the high-spin met-unligated forms of these same three components [11]. The primary results are partial assignments of the hyperfine shifted proton resonances, revealing that the pattern of shifts is opposite to that displayed by sperm whale metmyoglobin cyanide. Crystallography [1] has shown these two types of monomer heme proteins to have very similar three-dimensional structures and recent sequencing data confirm essentially identical heme-protein contacts for the two types [2, 3]. Based on these results, the NMR results can be taken to confirm that the heme orientation is reversed in all of the *G. dibranchiata* monomer methemoglobins compared to metmyoglobin. However, this work also reveals that the monomer hemoglobins are sensitive to apoprotein creation and reconstitution procedures. In combination with the demonstrated heme lability for these proteins [12], these results suggest that the pocket is more flexible in the monomer hemoglobins than in metmyoglobin.

Experimental

The three major monomer hemoglobin components were isolated and purified as previously described [13, 14]. The purified components were stored frozen either as lyophilized powders or 'quick-frozen' solutions in liquid nitrogen, following extensive dialysis in 0.01 M potassium phosphate buffer, pH 6.8 [11]. The details of extensive gel filtration, ion exchange, gel electrophoresis and isoelectric focusing experiments that were routinely carried out on these preparations in order to determine purity are given in refs. 11–14.

Apoprotein formation and reconstitution followed the method of Teale [15], as described in detail in ref. 11. The protohemin IX derivatives used in this study have the same nomenclature as previously described [11, 16], but are abbreviated in this work simply by indicating the deuterated substituent and its heme position (Fig. 1). In this manner, the heme in which the methyl group at substituent position 8 is perdeuterated is abbreviated in the Figures as 8-CD_3; that in which the vinyl group at position 4 is deuterated at the α-proton is designated as 4-CD=CH_2. The details of protein preparations for spectroscopy and the NMR spectroscopy itself have been previously published [11].

Results and Discussion

Figure 1 presents the downfield and upfield proton hyperfine shift regions of monomer methemoglobin–CN component III. These spectra display a larger number of resonances than expected for homogeneous sample preparations (containing but a single form) of monomeric heme proteins, indicating the presence in solution of both major and minor forms, similar to the phenomenon found for the native monomer methemoglobins [11, 12]. The downfield spectra compare favorably with those previously presented [17] and, as noted before, the major resonances form a characteristic pattern [18] for low-spin heme proteins. In this

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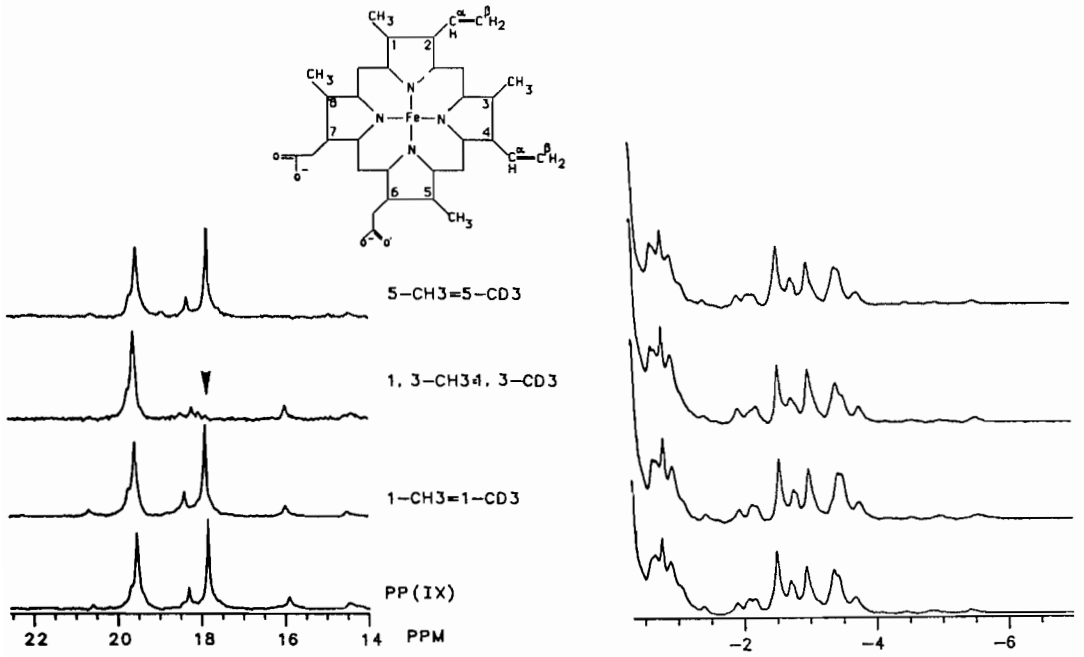

Fig. 1. (Top) Heme structure showing the numbering system used. Left: a region of the component III downfield hyperfine proton shift region showing spectral changes upon incorporation of the deuterated hemin derivatives indicated. Right: component III upfield hyperfine shift region showing spectral changes upon incorporation of the deuterated hemin derivatives indicated. Arrows indicate resonances, the intensity of which decreases as a result of reconstitution.

TABLE 1. Selected hyperfine resonance assignments of cyanide-ligated *G. dibranchiata* monomer methemoglobins

Assignment	Observed chemical shift (ppm[a]) for component:		
	II	III	IV
3-CH_3	19.5	17.9	17.9
8-CH_3	20.6	19.3	18.7
5'-CH_3[b]	18.3	15.6	14.2
4-α-Vinyl	11.8	11.02	

[a]Conditions: 0.1 M potassium phosphate; 0.1 M potassium chloride; p*D* = 6.8; in D_2O at 24 °C. [b]Prime refers to the minor form with heme orientation identical to the predominant form of myoglobin.

pattern the two major resonances that lie farthest downfield, between 18–21 ppm, can reliably be attributed to two of the heme methyl resonances.

Figure 1 shows the assignment of these heme methyl resonances made by reconstituting the component III apoprotein with various deuterium-labelled protohemin IX derivatives. The resonance at 17.9 ppm disappears when the 1,3-CD_3 heme is used. The 17.9 ppm resonance is therefore assigned to the heme 3-methyl protons. The resonance at 19.4 ppm displays normal intensity throughout all of the reconstitutions. As a result of this, it must be assigned to the heme 8-methyl protons. Note also that the small resonance near 15.7 ppm disappears in the 5-CD_3 (heme) reconstituted protein. This behavior indicates its assignment to the heme 5-methyl group in the minor form; that in which the heme is in a 180° reversed orientation. Similar experiments were carried out on components II and IV (data not shown) and the heme methyl assignments are displayed in Table 1.

We have also been able to assign the heme 4-position α-vinyl resonance using protohemin IX derivatives with one or both of the vinyls deuterated. Figure 2 shows these results for component II. The 4-α-proton is assigned by virtue of its disappearance from the spectra when the heme with the perdeuterated vinyl group is used (top spectrum), or when the heme with both 2- and 4-position α-deuterons is used. Similar experiments were carried out on components III and IV and the resulting assignments are shown in Table 1. This Figure also shows the degree of sensitivity of these proteins to reconstitution procedures. This is observable as variation in the overlap of the resonances that lie between 12 and 13.5 ppm, thereby indicating this sensitivity.

These assignments indicate that, in solutions of cyanide-ligated *G. dibranchiata* methemoglobins, the major-form resonances originate from a heme orientation that is 180° reversed compared to the orientation observed in metmyoglobin–CN [18, 19]. This conclusion comes from the following considerations. (i) The reversed heme orientation for the

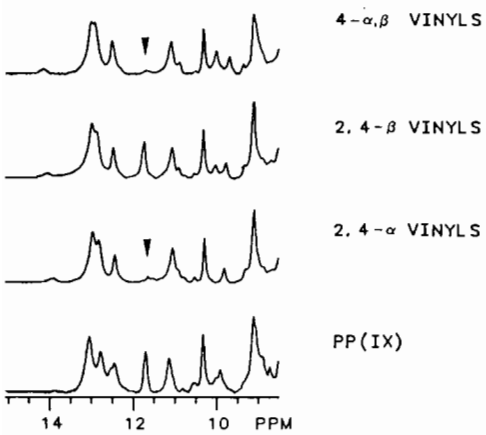

Fig. 2. Part of the component II downfield hyperfine proton shift region showing the effect of reconstituting various vinyl-deuterated protohemin IX derivatives. Arrows indicate resonance with reduced intensity.

G. dibranchiata monomer hemoglobins has been observed for two of the components in the ferrous CO-ligated form [17] and for all three components in the unligated met form [12], so that it is reasonable to expect this reversed orientation to occur in the low-spin ferric proteins as well. (ii) Heme contacts, as well as the orientation of the proximal histidine relative to the heme plane, affect the heme electronic structure and, consequently, the observed hyperfine resonance pattern [18]. Recent sequence analyses of two of the *G. dibranchiata* monomer hemoglobins show that they possess essentially identical heme-protein contacts to those in myoglobin [2, 3]. Furthermore, X-ray crystallography shows that the proximal histidine projects onto the heme plane in a similar fashion in both metmyoglobin and one of the monomer hemoglobins [1, 20, 21]. Consequently, the fact that the heme 5- and 1-methyl protons occur farthest downfield in metmyoglobin–CN, whereas the heme 8- and 3-methyl protons occur farthest downfield in the monomer methemoglobins–CN, indicates heme reversal [18].

These findings also correlate with the negative circular dichroism observed for several other derivatives of the *G. dibranchiata* monomer hemoglobin components [22], indicating that the heme reversal is detectable by both NMR and CD methods. It is an interesting coincidence that this same heme orientation (i.e. reversed relative to myoglobin) is observed for cytochrome *c* peroxidase [16], a protein that has vastly different properties from myoglobin. Moreover, recent kinetic studies of cyanide binding to the three *G. dibranchiata* monomer methemoglobins show that the observed rates were unusually slow [5, 6]. They are up to *three orders of magnitude* slower than for metmyoglobin. Given the similarities in the heme pockets for myoglobin and the monomer hemoglobins [3], it is quite possible that the heme orientation difference in these two types of proteins contributes to the unusual cyanide binding kinetics exhibited by the *G. dibranchiata* monomer methemoglobins.

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