Assignments of *meso* ^{1}H NMR resonances in haem proteins by selective deuteriation

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Received 1 July 1982

^{1}H NMR *Haem proteins* meso-*Proton assignment* *Deuteriation*

1. INTRODUCTION

The advent of isotopically labelled haems and their reconstitution into a variety of haem proteins has allowed the unambiguous assignments of haem ^{1}H NMR resonances in paramagnetic derivatives of these proteins [1–7]. The hyperfine shifts of haem resonances from the crowded diamagnetic region of the spectrum due to the paramagnetic iron atom has allowed easy identification and assignment upon reconstitution with selectively deuteriated haems. In the ^{1}H NMR spectra of diamagnetic porphyrins the 4 *meso*-proton resonances of the haem occur in an uncluttered region of the spectrum ($\delta \sim 10$) [8]. Here, we report the assignment of the *meso*-proton resonances of the diamagnetic ferrous oxy- and carbon monoxy-derivatives of sperm whale myoglobin (O_2Mb and COMb) and of soybean leghaemoglobin A (O_2Lb and COLb).

2. EXPERIMENTAL

Sperm whale myoglobin (Sigma) was a salt-free lyophilized powder. Soybean ferric leghaemoglobin a was a generous gift from Dr. C.A. Appleby [9]. The ^2H_2O (99.7%) was obtained from Merck, Sharp and Dohme and haemin chloride and haematoporphyrin free base, from Sigma. *p*-Toluenesulphonic acid hydrate (BDH) was recrystallised twice from ethanol and lyophilized 3 times from ^2H_2O.

2.1. *Preparation of* α, β, δ, γ meso-*tetra-deuterio-protohaemin*

Haematoporphyrin (500 mg) was dissolved in *o*-dichlorobenzene (80 ml) containing *p*-toluene [O-^{2}H]sulphonic acid (3.9 gm) and ^2H_2O (1.9 ml) and stirred in the dark under dry nitrogen at 95°C for 48 h. Dichloromethane was added and the organic phase dried over anhydrous sodium sulphate. After removal of the organic solvents by distillation at 0.6 Torr. the porphyrin was convert-

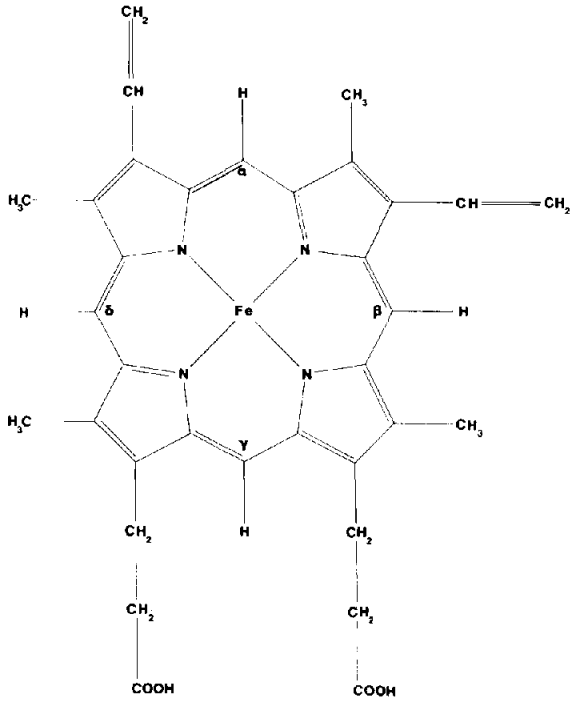

Fig.1. Chemical structure of protohaemin with *meso*-proton positions marked.

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

ed to the dimethyl ester derivative [10] and the iron inserted using ferrous sulphate/pyridine/acetic acid [11]. De-esterification was carried out [3] and the partially deuteriated haemin chloride was characterised as the bis-pyridine–ferrous protohaem complex by ^{1}H NMR [12].

2.2. *Reconstitution of haem proteins with selectively deuteriated haem*

Apomyoglobin was prepared by the 2-butanone extraction method [13,14] and reconstituted with isotopically substituted haem [15] to produce myoglobin with selectively deuteriated *meso*-protons. *Apo*-leghaemoglobin was prepared by 2-butanone extraction at −5°C and pH 3.2 (P.E. Wright and J.E. Wellington, unpublished). Reconstitution was as for myoglobin and the protein was purified on a column of Whatman DE 52-cellulose at 4°C, eluted with 13 mM sodium acetate buffer (pH 5.5) [9].

Samples for NMR were prepared as in [16,17]. Freshly prepared solutions were in 10 mM deuterated phosphate buffer. After the ^{1}H NMR spectrum was obtained on a Brüker HX-270 spectrometer, the visible spectrum was checked to ensure the absence of auto-oxidation. The pH of solutions (uncorrected for deuterium isotope effects) was measured before and after the NMR run with a Beckman 4500 pH meter and an Ingold microelectrode. Dioxane was used as an internal reference (δ 3.74) and peak areas were determined by cutting out peaks and weighing.

3. RESULTS AND DISCUSSION

In fig.2b partial deuteriation of the *meso*-protons is observed and virtually complete deuteriation of the vinyl CH_2 protons at 6–6.2 δ [18] which has caused the collapse of the vinyl CH resonances at δ ~8.2. Table 1 shows that the percentage of proton remaining at each *meso* position after deuteriation for 2 days is $\alpha > \beta > \delta > \gamma$ [18]. From the spectra of the *meso*-proton resonances of native and partially deuteriated COMb (fig.3) it is observed that the smallest resonance is downfield and therefore is assigned to the γ proton. The least deuteriated proton resonance (9.91 δ) corresponds to the α *meso*-proton. The differentiation between β and δ is not always unequivocal based on area measurements, particularly because of the overlapping nature of the central doublet, but fits well with the data in table 1 and also places the β-proton in an upfield position which is consistent with its position close to a phenylalanine ring (see below). Similar spectra were obtained for O_2Mb, O_2Lb and COLb and results and assignments are in table 1. In all derivatives except COLb some or all of the *meso*-proton resonances shift with change of pH [19–21]. These effects will be discussed in detail in later publications.

Studies of COMb [22], O_2Mb [23] and lupin ferric leghaemoglobin [24] reveal that the β *meso*-proton is always immediately below Phe CD1. Thus, the high-field position of the β-proton resonance

Table 1

Chemical shift data at pH 7.0 of *meso*-proton resonances and percentage residual protonation of meso-protons in diamagnetic ferrous haem proteins

meso Proton Assignment	Bis-Pyridine protohaem	Fe(II)O_2Mb		Fe(II)COMb		FE(II)O_2Lb		Fe(II)COLb	
	% proton	% proton	δ	% proton	δ	% proton	δ	% proton	δ
α	74	68	9.78	72	9.91	76	9.46	74	9.89
β	51	53	9.38	51	9.34	48	8.88	51	9.43
δ	45	–	–	43	9.85	42	9.63	48	9.85
γ	32	–	–	27	10.14	34	10.02	34	10.17
γ, δ	39	44[a]	9.89	–	–	–	–	–	–
α, δ	59	–	–	56[a]	–	–	–	60[a]	–

[a] In these cases, resolution of individual peaks was difficult, hence the sums of areas were compared

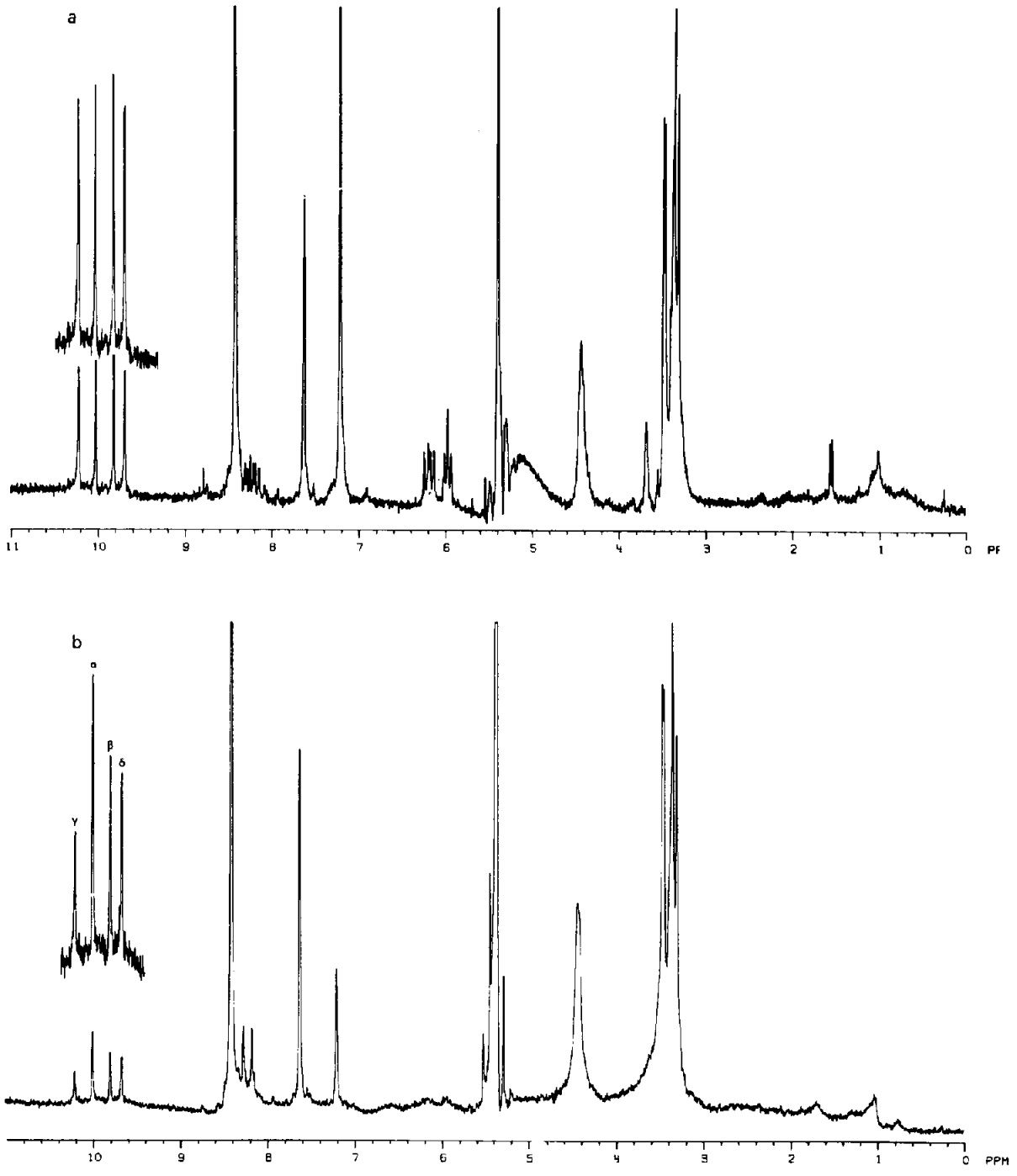

Fig.2. ^1H NMR spectra at 270 MHz of bis-pyridine protohaem in ^2H_2O at 20°C of: (a) non-deuteriated sample; (b) deuteriated sample with assignments of the *meso*-proton resonances shown [18]. Strong resonances at δ 5.4 and δ 7–9 are due to ^2H_2O and pyridine, respectively.

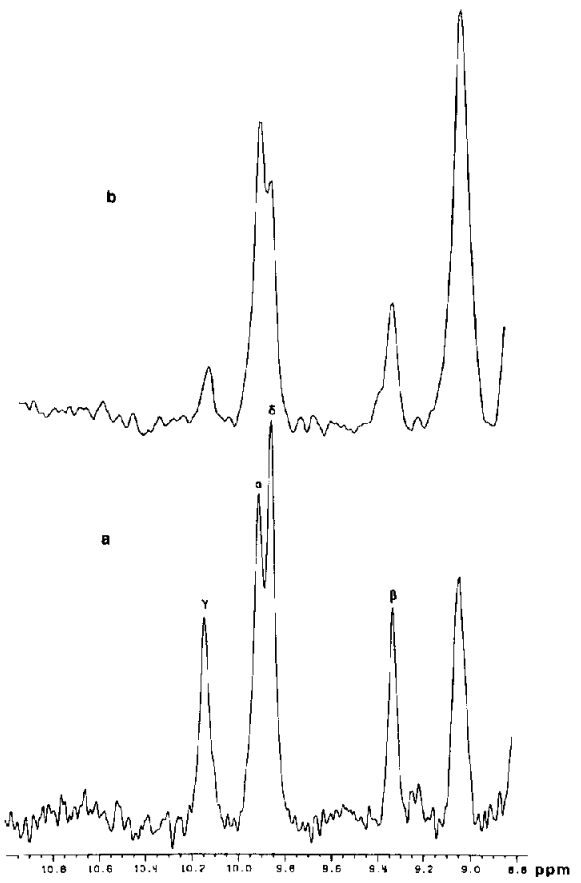

Fig.3. ^1H NMR spectra at 270 MHz of 5 mM solutions in ^2H_2O at pH meter reading 6.62 and 40°C of the *meso*-proton resonances of: (a) native COMb (4000 scans); (b) COMb reconstituted with partially deuteriated haem (8000 scans). The assignment of peaks is by partial deuteriation. The two spectra are not plotted on the same vertical scale and the resonance at δ 9.05 possibly belongs to a buried NH.

in all cases in table 1 is due to the diamagnetic shielding by nearby Phe CD1.

In O_2Lb all *meso*-proton resonances lie upfield of their position in COLb whereas in myoglobin there are only small differences between corresponding protons for the 2 derivatives. The large −0.6 ppm upfield shift of the β-proton resonance in O_2Lb as compared with COLb may be due to movement of the ring of Phe CD1 with respect to the position of the β *meso*-proton, which may not be possible in the case of myoglobin because of its smaller, more constricted haem cavity [19].

These assignments will be used to elucidate the structural changes in the region of the haem that result from change of pH in MbO_2 (acid Bohr effect) and from binding of different ligands.

ACKNOWLEDGEMENTS

We thank Dr C.A. Appleby for the gift of leghaemoglobin and helpful discussions, Dr P.E. Wright and Mrs J.E. Wellington for use of unpublished experimental procedures, the staff of the National NMR Centre for obtaining the spectra and the Australian Research Grants Committee for financial support.

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