^{19}F NMR Study on the Heme Electronic Structure in Oxy and Carbonmonoxy Reconstituted Myoglobins

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A ^{19}F NMR study on myoglobin reconstituted with a ring-fluorinated C_2 symmetric heme, 13,17-bis(2-carboxylatoethyl)-2,8,12,18-tetramethyl-3,7-difluoroporphyrinatoiron(II), demonstrated that the porphyrin π system is affected by the bent orientation of the Fe–O–O unit relative to the heme in oxy myoglobin and Fe $d_\pi \rightarrow$ CO π^* back-donation in carbonmonoxy myoglobin.

Characterization of the nature of O_2 and CO binding to respiratory hemoproteins is of paramount importance for understanding the molecular mechanisms responsible for the control of their physiological activities.[1] Despite detailed structural[2] and spectroscopic[3] information on as well as theoretical consideration[4] of the binding of these ligands to hemoproteins and iron–porphyrin complexes, little experimental evidence has been reported so far as to the effects of O_2 and CO binding on the heme electronic structure of hemoproteins. ^{1}H NMR is presently the most powerful spectroscopic method for elucidating the heme electronic structure of proteins. In particular, NMR studies on paramagnetic hemoproteins have provided a wealth of information on the structural and electronic properties of their active sites by virtue of the high sensitivity of paramagnetically shifted signals to a heme environment.[5] On the other hand, the heme electronic structure of diamagnetic hemoproteins has not been as fully characterized as that of paramagnetic hemoproteins by NMR.[6] One of difficulties associated with NMR characterization of the heme electronic structure of diamagnetic hemoproteins is the asymmetric nature of the heme electronic structure of native heme, the iron–protoporphyrin IX complex, which often obscures subtle changes in the heme electronic structure of diamagnetic hemoproteins. Furthermore, the sensitivity of the heme peripheral side-chain proton signals to the porphyrin π-system is relatively low due to the rather weak interaction between the pyrrole carbon p_π-orbital and the proton orbital, as manifested in the small Q values for the π spin delocalization in a paramagnetic system.[5]

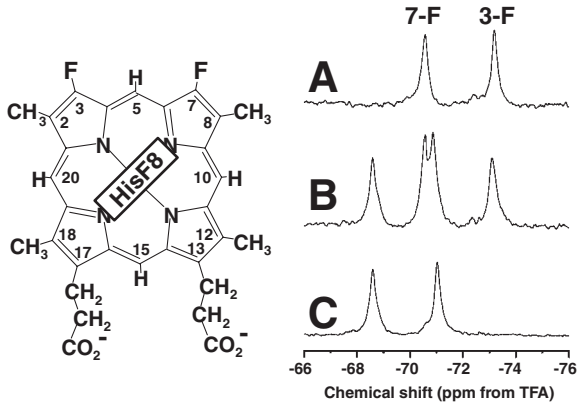

Fig. 1. The 376 MHz ^{19}F NMR spectra of Mb reconstituted with 3,7-DF, in 90% H_2O/10% ^2H_2O, pH 7.0, at 25 °C. (A) MbO_2, (B) a mixture of MbO_2 and MbCO, and (C) MbCO. The signal assignments made on the basis of observation of the nuclear Overhauser effect connectivities between ^{1}H and ^{19}F signals[7] are indicated in the spectra. Molecular structure and numbering system of 3,7-DF is indicated in the inset. The orientation of the axial His F8 imidazole ring is shown.

In order to overcome these difficulties, a ring-fluorinated C_2 symmetric heme, i.e., 13,17-bis(2-carboxylatoethyl)-2,8,12,18-tetramethyl-3,7-difluoroporphyrinatoiron(II) (3,7-DF: structure given in Fig. 1), has been synthesized,[7] and has been incorporated into the apoprotein of sperm whale myoglobin (Mb) to explore the heme electronic structure by means of ^{19}F NMR. In 3,7-DF, a change in the porphyrin π-system leads to a distortion of its C_2 symmetric electronic structure, which can be readily detected as the separation of two ^{19}F NMR signals.[7] We report herein on the results of a ^{19}F NMR study on the heme electronic structures of oxy and carbonmonoxy Mbs (MbO_2 and MbCO, respectively) reconstituted with 3,7-DF,[8] which revealed the effect of not only the orientation of the Fe–O–O unit relative to the heme, but also the Fe $d_\pi \rightarrow$ CO π^* back-donation on the heme electronic structure.

The 376 MHz ^{19}F NMR spectra of MbO_2 and MbCO reconstituted with 3,7-DF at 25 °C are shown in traces A and C of Fig. 1, respectively. Due to a protein-induced rhombic perturbation, the equivalency of the two fluorine atoms of 3,7-DF was removed and two well-resolved signals were observed in each spectrum.[7] In the spectrum of the mixture of MbO_2 and MbCO (trace B), the two sets of signals were separately observed, demonstrating that the time scale of the ligand exchange between O_2 and CO in the protein is slow compared with the NMR time scale.[9] The shift differences of more than 2.0 ppm between the corresponding signals of MbO_2 and MbCO (Table 1) were considerably larger than those observed for the heme peripheral side-chain ^{1}H signals,[6b] demonstrating the high sensitivity of the ^{19}F signals to the heme electronic structure. Furthermore, the observed ^{19}F shift difference for the present reconstituted Mbs was considerably larger than those observed for Mb reconstituted with difluorovinyl deuteroheme,[10] i.e., 0.6–1.3 ppm, demonstrating a higher sensitivity of the NMR signal for the fluoro group to the porphyrin π-system of heme than that for the fluorovinyl group.

We compared the heme electronic structures of MbO_2 and

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Table 1. ^{19}F Shifts[a] of MbO_2 and MbCO Reconstituted with 3,7-DF in 90% H_2O/10% D_2O, pH 7.0 at 25 °C

	MbO_2	MbCO	$\Delta(\delta_{O_2} - \delta_{CO})$[b]
3-F	−73.20	−70.91	−2.29
7-F	−70.60	−68.60	−2.00
$\lvert\Delta(\delta_{3\text{-F}} - \delta_{7\text{-F}})\rvert$[c]	2.60	2.31	

a) In ppm relative to trifluoroacetic acid. b) Shift difference between MbO_2 and MbCO. c) Signal separation in ppm.

MbCO reconstituted with 3,7-DF. In the absence of detailed structural information on the heme active sites in these reconstituted Mbs, the observed ^{19}F shifts could not be interpreted quantitatively in terms of the heme electronic structure. However, the electron density of a pyrrole carbon atom bearing a fluorine atom could be inferred from the observed ^{19}F shift.[11] A comparison of the ^{19}F shifts indicated that the signals for MbO_2 were upfield-shifted relative to the corresponding signals for MbCO, indicating that the electron density in the porphyrin π-system of MbCO is lower than that of MbO_2. Thus, the effect of Fe $d_\pi \rightarrow$ CO π^* back-donation[12] on the heme electronic structure of MbCO was clearly detected on ^{19}F NMR.

We next compared the in-plane asymmetry of the heme electronic structure of 3,7-DF in MbO_2 and MbCO, on the basis of the signal separation. Since C_2 symmetric 3,7-DF does not exhibit the well-documented heme orientation disorder,[13] the equivalency of the two fluorine atoms of 3,7-DF is abolished primarily by bonding interactions between the heme iron and the axial ligands, and the asymmetry in the chemical environment around the heme. X-ray crystal studies[2] demonstrated that the Fe–C–O unit in MbCO prefers a linear geometry ($\angle$Fe–C–O = 171°) to maximize the Fe $d_\pi \rightarrow$ CO π^* back-donation,[12] while the Fe–O–O unit in MbO_2 is greatly bent ($\angle$Fe–O–O = 123°), although the orientation of the His F8 imidazole with respect to the heme is essentially the same in them. It is not clear at the present if the heme coordination geometry in Mb is independent of the heme substitution. However, since the structural similarity of the heme active site in Mb possessing 3,7-DF to that of native Mb was supported by the functional and spectroscopic data,[8] the increased non-equivalency between 3- and 7-F in MbO_2, relative to that in MbCO, could be attributed to the bent Fe–O–O coordination with respect to the heme.

In conclusion, we demonstrated, from the results of a ^{19}F NMR study on Mb reconstituted with 3,7-DF, that the porphyrin π-system is affected by Fe $d_\pi \rightarrow$ CO π^* back-donation in MbCO and the bent orientation of the Fe–O–O unit relative to the heme in MbO_2. Thus, the ^{19}F NMR signals of 3,7-DF embedded in Mb are sensitive to the in-plane asymmetry of the heme electronic structure induced by coordination of axial ligands to the heme iron. The present method provides a new means for the detailed characterization of the bonding of exogenous ligands to the heme iron and the heme electronic structure in *b*-type hemoproteins in their physiologically active forms to delineate their structure–function relationships.

Experimental

NMR Sample Preparation. ApoMb was prepared from Mb (Biozyme) according to a procedure of Teale.[14] Reconstitution of apoMb with 3,7-DF[7] was carried out by the standard procedure.[15]

NMR Spectroscopy. ^{19}F NMR spectra were recorded on a Bruker AC-400P FT-NMR spectrometer operating at a ^{19}F frequency of 376 MHz. A typical spectrum consisted of 20 k transients with a 20 kHz spectral width.

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8 P_{50} values (partial pressure of O_2 when half of the total Mb is oxygenated) of 0.53 and 0.58 mmHg were obtained for Mb reconstituted with 3,7-DF and native Mb, respectively, in 100 mM phosphate at pH 7.20 and 20 °C (unpublished results). Additionally, the Val E11 γ-CH_3 ^{1}H NMR signal, which is sensitive to the orientation of the heme relative to the protein moiety in Mb, was observed at −2.73 and −2.83 ppm for MbO_2 reconstituted with 3,7-DF and native MbO_2, respectively, and at −2.29 and −2.32 ppm for MbCO reconstituted with 3,7-DF and native MbCO, respectively, at pH 7.0 and 25 °C. These functional and spectroscopic similarities among the Mbs supported that the heme active site structure was not greatly affected by the heme replacement.

9 The shift difference of 2.00 ppm between the 7-F signals of MbO_2 and MbCO (Table 1) indicated that the ligand exchange between O_2 and CO occurs at $\ll 8 \times 10^2$ s^{-1}. This rate is within the range of the values reported (R. J. Rohlfs, A. J. Mathews, T. E. Carver, J. S. Olson, B. A. Springer, K. D. Edeberg, and S. G. Sligar, *J. Biol. Chem.*, **265**, 3168 (1990)).

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