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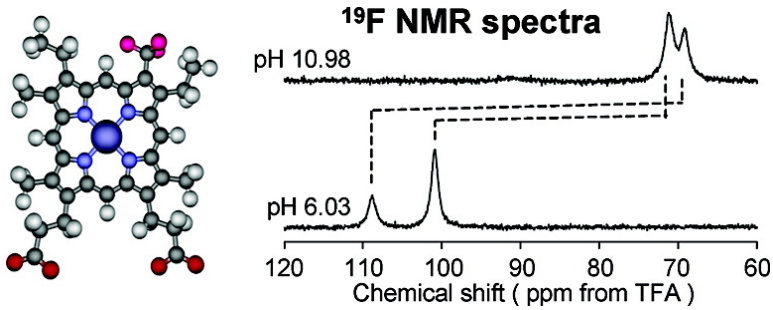
J. Am. Chem. Soc., **2005**, 127 (12), 4146-4147• DOI: 10.1021/ja043975i • Publication Date (Web): 02 March 2005



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Published on Web 03/02/2005

^{19}F NMR Characterization of the Thermodynamics and Dynamics of the Acid−Alkaline Transition in a Reconstituted Sperm Whale Metmyoglobin

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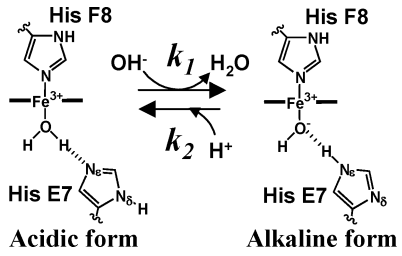
Myoglobin (Mb), an oxygen storage hemoprotein, was the first protein for which an X-ray crystal structure was determined[1] and has served for many years as a paradigm for structure−function relationships.[2] Interest in the protein remains high because a precise understanding of the molecular mechanism responsible for regulation of the functional properties of the protein is lacking. The heme active site in metmyoglobin (metMb) exhibits a characteristic pH-dependent structure change known as the acid−alkaline transition.[3] MetMb possessing highly conserved distal His E7 has H_2O and OH^- as coordinated ligands under low and high pH conditions, respectively.[3,4] As illustrated in Scheme 1, the acid−alkaline transition in metMb comprises three distinct reactions, that is, interconversion of the coordinated ligand between H_2O and OH^-, tautomerism of the His E7 imidazole, and deprotonation/protonation of His E7 $N_\delta H$, which take place simultaneously through a molecular mechanism similar to the proton relay of serine protease.[5] Furthermore, since the transition is associated with the deprotonation/protonation process, the equilibrium constant is usually represented as the "pK_a" value in analogy with the ionization equilibrium of an acid. For the scaffold of the conserved globin fold in Mbs, the pK_a value has been shown to remarkably vary with the protein, ranging from 7.2 to 10.[3,4] Thus, the acid−alkaline transition in metMb sharply reflects the structural features of the heme active site in the protein, which could be relevant to protein function.[4]

Scheme 1



We report here quantitative ^{19}F NMR characterization of the thermodynamics and dynamics of the acid−alkaline transition in sperm whale metMb reconstituted with 13,17-bis(2-carboxylatoethyl)-3,8-diethyl-2,12,18-trimethyl-7-trifluoromethylporphyrinatoiron(III)[6] (7-PF, Figure 1A). The incorporation of a CF_3 group into the heme as a peripheral side chain provided a valuable spectroscopic probe exhibiting extraordinarily high sensitivity to the heme electronic structure and a superb time scale and resolution suitable for quantitative characterization of the dynamics of the transition.[7] In addition, analysis of the functional consequences of the perturbation in the heme electronic structure, occurring on the substitution of strongly electron-withdrawing CF_3, offers a unique opportunity

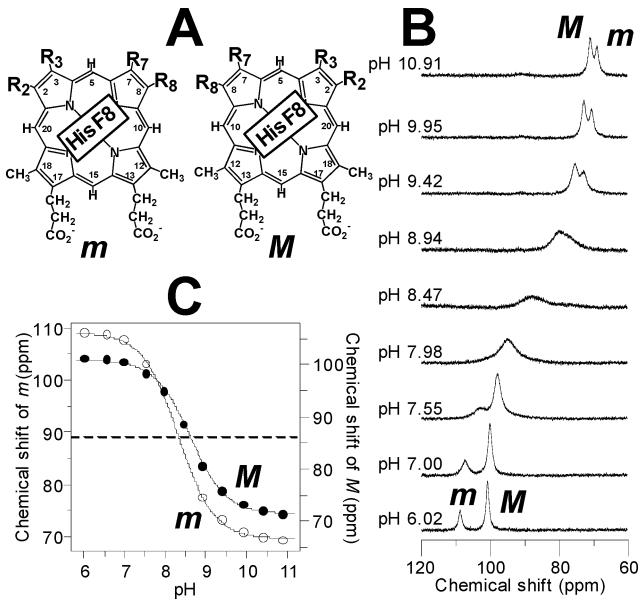


Figure 1. (A) The structures and numbering system for 7-PF ($R_2 = CH_3$, $R_3 = R_8 = C_2H_5$, $R_7 = CF_3$) and mesoheme ($R_2 = R_7 = CH_3$, $R_3 = R_8 = C_2H_5$), and two possible orientations of the heme relative to His F8 in reconstituted Mb, *m* and *M* forms. The orientation of the axial His F8 imidazole plane is shown. (B) The 470 MHz ^{19}F NMR spectra of metMb(7-PF) at 25 °C and the indicated pH values. Signals due to the *m* and *M* forms illustrated in (A) were observed. Chemical shifts of ^{19}F NMR spectra are given in parts per million downfield from trifluoroacetic acid as an external reference. (C) Plots of the shifts of the ^{19}F NMR signals of metMb(7-PF) at 25 °C as a function of pH. The pK_a values of 8.32 ± 0.03 and 8.62 ± 0.03 were obtained for the *m* and *M* forms, respectively. The vertical scales for the plots of the two signals were adjusted to highlight the difference in pK_a value between them.

to investigate the relationship between the heme electronic structure and Mb function. Comparison of the pK_a values of metMbs reconstituted with 7-PF and mesoheme (metMb(7-PF) and metMb-(Meso), respectively), which only differ in the substituent at position 7 (Figure 1A), demonstrated a significant effect of the CF_3 substitution on the pK_a value.

We first determined the pK_a value for the transition of metMb(7-PF) by ^{19}F NMR. The pH dependence of the 470 MHz ^{19}F NMR spectrum of metMb(7-PF) at 25 °C is illustrated in Figure 1B. Two signals were observed under low and high pH conditions. It has been shown that Mb(7-PF) exists as a mixture of isomers possessing two heme orientations differing by 180° rotation about the 5,10-*meso* axis[8] (Figure 1A), with a ratio of 1.0:2.2 for *m* form:*M* form at equilibrium.[7c] Therefore, the observed signals could be assigned on the basis of their intensities. With increasing pH, the signals of the *m* and *M* forms exhibited progressive upfield shifts of about 40 and 30 ppm, respectively. The acid−alkaline transition in the protein was manifested in these large pH-induced shift changes

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10.1021/ja043975i CCC: $30.25

attributable to a change in the spin state between the acidic (essentially $S = {}^5/_2$) and alkaline (mainly $S = {}^1/_2$)[9] forms. Quantitative fitting of their pH-dependent shifts to the Henderson−Hasselbach equation yielded pK_a values of 8.32 ± 0.03 and 8.62 ± 0.03 for the *m* and *M* forms of metMb(7-PF), respectively (Figure 1C). On the other hand, an optical study of metMb(7-PF) yielded a pK_a value of 8.57 ± 0.03 (see Supporting Information), which is close to the weight-averaged pK_a value of those for the *m* and *M* forms obtained with ^{19}F NMR.

We next analyzed the effect of the CF_3 substitution on the pK_a value on the basis of comparison between metMb(7-PF) and metMb(Meso). The optical study yielded a pK_a value of 9.43 ± 0.03 for metMb(Meso) (see Supporting Information), and therefore, the substitution of CF_3 for the heme peripheral CH_3 side chain at position 7 lowered the pK_a value by about 1 pH unit. Ferriheme in metMb carries a net positive charge and, hence, needs to be stabilized through neutralization on electrostatic interaction with nearby polar groups in the hydrophobic heme pocket of the protein. As far as the stability of the ferriheme in the protein is concerned, the alkaline form is more stable than the acidic one because of neutralization of the cationic character of the ferriheme by the coordinated OH^-. The substitution of strongly electron-withdrawing CF_3 is expected to partly enhance the positive charge at the heme iron of ferriheme, which in turn further destabilizes the acidic form. Consequently, the equilibrium of the transition is shifted toward the alkaline form by the CF_3 substitution, leading to a decrease in the pK_a value, as observed by comparison of metMb(7-PF) and metMb(Meso). These results constitute persuasive evidence for a previous proposal that the reactivity of the heme iron is regulated through the electronic nature of heme peripheral side chains.[4c]

We found a distinct difference in the pK_a value between the *m* and *M* forms of metMb(7-PF) (Figure 1C). The characterization of the dynamics of the transition in the *M* form indicated that the k_2 value is diffusion-controlled (see below), and therefore, the k_2 values of the *m* and *M* forms should be similar. Consequently, the difference in pK_a value between the two forms can be attributed to the effect of the heme orientation, with respect to the protein, on the k_1 value, and, hence, the stability of the acidic form. In the acidic form, the hydrogen bond between the coordinated ligand and His E7 contributes not only to stabilization of the H_2O coordination to the heme iron but also to neutralization of the cationic character of the ferriheme by the partial negative charge of the oxygen atom, induced by polarization of the O−H bond due to the hydrogen bond. Therefore, the stronger hydrogen-bonding interaction could result in a higher pK_a value because of the greater stability of the acidic form.[10] The difference in the strength of the hydrogen-bonding interaction between the *m* and *M* forms could arise from displacement of the heme iron, relative to the protein, possibly exerted by differential heme−protein contacts in the two forms.[11]

Finally, we determined, from the results of ^{19}F NMR line shape analysis, the dynamics of the transition in the *M* form at pH 8.62 and 25 °C.[12] As shown in the series of spectra in Figure 1C, the signals were narrowest in the extreme pH regions and considerably broad at the pK_a value.[13] Excess line broadening could arise from exchange of the CF_3 group between the acidic and alkaline forms of the protein. Using the familiar equation for the fast exchange limit,[13] the reaction rate of $(1.1 \pm 0.2) \times 10^5$ s^{-1} was obtained for the k_2 value of the *M* form of metMb(7-PF) (see Supporting Information). Additionally, using the obtained k_2 value, combined with the pK_a value of 8.62 ± 0.03, the value of $(2.6 \pm 0.5) \times 10^{10}$ $M^{-1}\cdot s^{-1}$ was determined for the k_1 value. Both the k_1 and k_2 values were within the ranges estimated previously for native sperm whale metMb and metMbs reconstituted with various chemically modified hemes.[4b,c] The obtained k_2 value for metMb(7-PF) was comparable to the exchange rate of histidyl imidazole NH protons fully exposed to the solvent,[14] indicating that the deprotonation/protonation of His E7 $N_\delta H$ associated with the transition is essentially a diffusion-controlled process. In fact, according to the X-ray crystal structure,[15] the His E7 $N_\delta H$ proton is completely exposed to the solvent, although the His E7 residue is mostly buried in the protein matrix. Furthermore, analysis of the temperature dependence of the k_2 value using Arrhenius plots yielded a value of 20.9 ± 3 kJ mol^{-1} for the activation energy of the transition. The relatively small activation energy of the transition is consistent with the absence of a large conformation change in the protein upon the transition.

In summary, we have shown that ^{19}F NMR in combination with fluorinated heme can be used for quantitative characterization of the dynamic processes occurring at the heme active site in Mb. The present method is a new means of studying in detail structural properties relevant to functional regulation of various *b*-type hemoproteins to delineate their structure−function relationships.

Acknowledgment. This work was partly supported by a Grant-in-Aid (No. 15550143) from MEXT, and by the University of Tsukuba, Research Project.

Supporting Information Available: Figures SI 1−9, Table SI1, and calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA043975I