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Kinetic characterization of the acid–alkaline transition in *Dolabella auricularia* ferric myoglobin using ^{1}H-NMR saturation transfer experiments

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The acid–alkaline transition in ferric myoglobin of the mollusc, *Dolabella auricularia*, exerts the changes in both the coordination and spin states of the heme iron. Slower transition rate, compared to the NMR time scale, in this myoglobin allowed the observation of separate signals arising from the two forms, and pH titration yielded a pK value of 7.8. ^{1}H-NMR saturation transfer experiments have been successfully used not only to provide the first signal assignments for the heme methyl proton resonances of the Met-hydroxyl form of the myoglobin, but also to determine the kinetics of the transition.

NMR; Myoglobin; Saturation transfer; Acid–alkaline transition; Hyperfine shift

1. INTRODUCTION

The acid–alkaline transition of ferric hemoproteins has been of particular interest because of the fact that it reflects characteristics of their active sites [1–7]. The rapid acid–alkaline transition rate for ferric myoglobin (metMb) of horse [4] and sperm whale [5] has been interpreted in terms of the ionization process of the distal His imidazole. On the other hand, the molecular mechanism of the relatively slow rate in the sea hare, *Aplysia limacina* [8,9], or the shark, *Galeorhinus japonicus* [10], Mbs which possess the distal Val and Gln residues, respectively, are not fully understood at present. Detailed characterization of the acid–alkaline transition for these Mbs are expected to provide chemical environments around their ligand binding sites. The thermodynamics of the acid–alkaline transition can be obtained by optical spectroscopy [1,2], ESR [3], NMR [4,5] and magnetic susceptibility measurements [1]. Only the temperature-jump method [8] and NMR line width analysis [5] have provided some kinetics of the reaction.

Dolabella auricularia is a common mollusc found on the Japanese coast and its Mb possesses the distal Val residue with an amino acid sequence similar to that of *Aplysia* Mb [11]. Here we report on the results of saturation transfer NMR experiments [12,13] of *Dolabella auricularia* metMb which not only allow quantitative determination of the acid–alkaline transition rate of this Mb, but also provide the first signal assignment of the heme methyl proton resonances of the Met-hydroxyl form of Mb (metMbOH).

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2. MATERIALS AND METHODS

Dolabella auricularia Mb was extracted from its triturative stomach and purified according to the method previously described [11]. Mb was oxidized by the addition of a 5-fold molar excess of potassium ferricyanide (Sigma). metMb was separated from the residual reagents with a Sephadex G-50 (Sigma) column equilibrated with 10 mM Bis-Tris buffer (Sigma) pH 6.8. Mb solution was concentrated to ~1 mM and then the solvent was exchanged to ^2H_2O in an Amicon ultrafiltration cell. The p^{2}H of the sample was adjusted using 0.2 M NaO^2H or ^{2}HCl and the p^{2}H was measured using a Toko model TP-10 pH meter with a Toko type CE103C electrode. The isotope effect was not considered to correct the p^{2}H value.

^{1}H-NMR spectra were recorded using a JEOL GSX-500 Fourier-transformed NMR spectrometer operating at a ^{1}H frequency of 500 MHz. A typical spectrum consisted of 3000 transients with 8k data points over a 60 kHz band width, and a 6.5 μs 90° pulse. The residual water resonance was suppressed with 50 ms presaturation decoupler pulse. Selective spin-lattice relaxation time (T_1^{sel}) was measured using the saturation-recovery method with a selective saturation pulse. Saturation transfer experiments were carried out by selectively saturating a desired peak for a variety of time and the steady-state value of the saturation transfer factor (I/I_0; I and I_0 are the signal intensities of a peak A without and with the saturation of a peak B which is connected to peak A by dynamic process, respectively) was achieved for a saturation time ≥50 ms. The spectra resulting from the saturation transfer experiments are presented in the form of difference spectrum. The saturation factor was calculated by integrating the area of peak. The signal-to-noise of the spectra was improved by apodization which introduced 50 Hz line broadening. The chemical shifts are given rela-

tive to sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H^2HO as internal reference.

3. RESULTS

The pH dependence of the downfield hyperfine shifted portion of the 500 MHz ^{1}H-NMR spectrum of *Dolabella* metMb at 30°C is shown in Fig. 1. At pH 7.14, heme methyl proton signals, peaks A–D, and single proton signals, peaks a–g, are observed. The linewidth (~200 Hz) of the heme methyl proton resonance of *Dolabella* metMb is much smaller than that of well-characterized sperm whale metMb and is comparable to that of *Aplysia* metMb [9]. Peaks indicated by * and those at 25–40 ppm exhibit less than a single proton intensity and the latter disappear at acidic pH. The spectral pattern of *Dolabella* metMb at acidic pH is quite similar to that of the acidic form of *Aplysia* metMb [9]. Therefore the heme methyl proton resonances of *Dolabella* metMb may be assigned as A(8-CH_3), B(5-CH_3), C(3-CH_3), and D(1-CH_3) and then peaks labeled by * are attributed to the reversely oriented heme arising from the heme orientational disorder [14], based on the signal assignments for *Aplysia* metMb

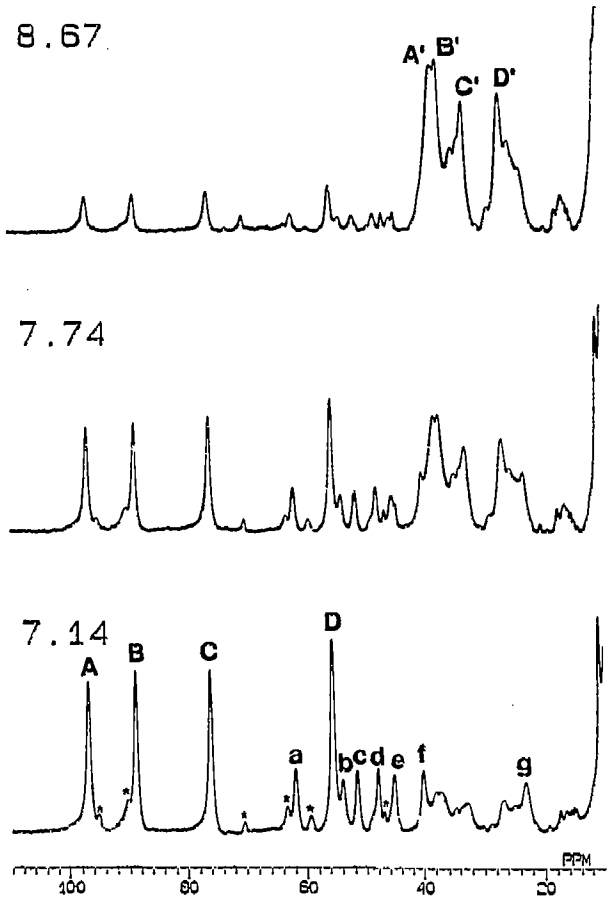

Fig. 1. Downfield hyperfine shifted portion of the 500 MHz ^{1}H-NMR spectrum of *Dolabella* metMb in ^2H_2O at 30°C and the indicated pH.

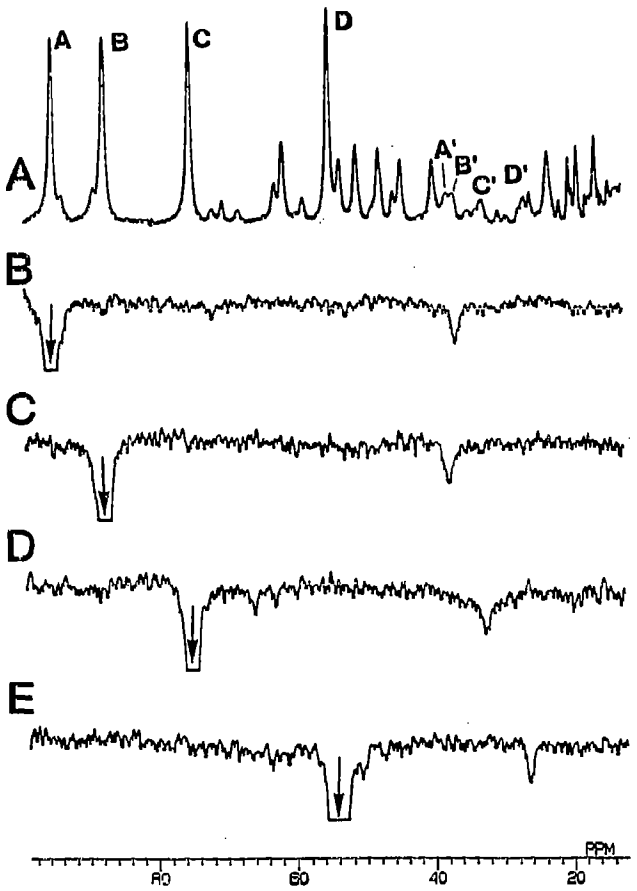

Fig. 2. Saturation-transfer difference spectra of *Dolabella* metMb in ^2H_2O at 35°C and pH 6.9. (A) Reference spectrum. (B) Saturation of the heme 8-CH_3 signal exhibits the saturation transfer factor to peak B'. (C) Saturation of the heme 5-CH_3 signal exhibits the saturation transfer factor to peak A'. (D) Saturation of the heme 3-CH_3 signal exhibits the saturation transfer factor to peak C'. (E) Saturation of the heme 1-CH_3 signal exhibits the saturation transfer factor to peak D'.

[9]. If we assume that the signal at 60 ppm arises from the heme methyl proton of the reversely oriented heme, *Dolabella* metMb exhibits ~15% reversed heme orientation. Upon raising the pH, peaks at 25–40 ppm gain intensity at the expense of peaks A–D and a–g. The spectral pattern at this chemical shift region is similar to that of metMbOH. From their signal intensities, peaks labeled A'–D' in the trace of pH 8.67 are attributed to the heme methyl proton resonances for *Dolabella* metMbOH. Analysis of the signal intensity of the heme methyl proton resonances for both acidic and alkaline forms yielded a pK value of 7.8 for the acid–alkaline transition. This pK value is similar to that reported for *Aplysia* metMb [8,9]. Furthermore, the fact that both forms of metMb exhibit separate signals indicates that the transition rate is slow compared to the NMR time scale. A slow transition rate of *Dolabella* metMb is also similar to that of *Aplysia* metMb. The line width of the heme methyl resonances for the acidic

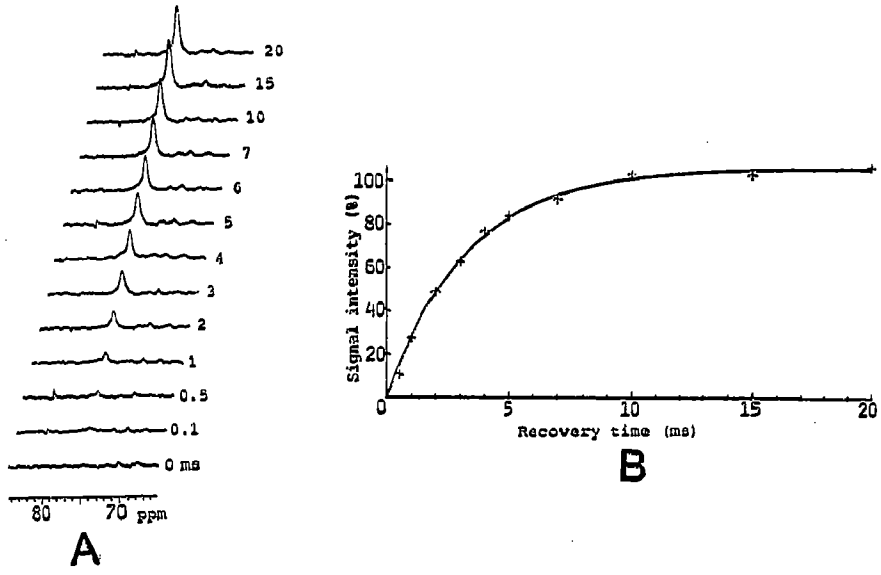

Fig. 3. (A) The recovery of the heme 3-CH_3 signal of *Dolabella* metMb in ^2H_2O at 35°C and pH 6.5, after selective saturation. (B) Plot of signal intensity vs. the recovery time. T_1^{sel} of 3.3 ± 0.3 ms was obtained.

form of *Dolabella* metMb at pH 8.67 is larger by ~35% relative to that at pH 7.14 due to exchange broadening.

The heme methyl proton resonances of *Dolabella* metMbOH were assigned with the aid of saturation transfer experiments. Saturation transfer experiments are useful to relate resonances which are connected by dynamic exchange processes with a suitable time scale [12]. The difference spectra resulting from the saturation transfer experiments at 35°C, pH 6.9, are shown in Fig. 2. The saturation of the heme 8-CH_3 signal led to partial saturation of peak B′ (trace B). The traces C–E exhibited saturation transfer connectivities between the heme 5-CH_3 signal and peak A′ (trace C), between the heme 3-CH_3 signal and peak C′ (trace D), and between the heme 1-CH_3 signal and peak D′ (trace E). These results indicate that peaks A′–D′ are assigned to heme 5-, 8-, 3- and 1-CH_3 proton resonances of *Dolabella* metMbOH, respectively.

The analysis of the saturation transfer factor, together with the selective spin-lattice relaxation time (T_1^{sel}), allows for the determination of the acid–alkaline transition rate. The spectra recorded at the elapsed time after the selective saturation of the 3-CH_3 signal (peak C) of *Dolabella* metMb at 35°C, pH 6.5, are illustrated in Fig. 3A. The plot of signal intensity against the recovery time, shown in Fig. 3B, yielded T_1^{sel} of 3.3 ± 0.3 ms and this value is comparable to those previously reported for other Mbs [10]. The absence of metMbOH at this pH ensures that this T_1^{sel} value is free from the effects of the acid–alkaline transition. The saturation of the heme 3-CH_3 signal of metMbOH (peak C′) for 50 ms yielded the saturation transfer factor (I/I_0) of 0.52 to peak C at 35°C, pH 6.9. The rate, k_{BA}, for the reaction, alkaline form → acidic form, is calculated as,

$$k_{BA} = (T_1^{sel})^{-1}(1-I/I_0)/(I/I_0).$$

Substitution of 3.3 ± 0.3 ms and 0.52 for T_1^{sel} and I/I_0 yielded a value of 280 ± 30 s^{-1} for k_{BA}. This rate is close to the value determined for *Aplysia* metMb using the temperature-jump method [8]. The rate, K_{AB}, for the reaction, acidic form → alkaline form, was calculated to be 1.3(± 0.14) × 10^7 $M^{-1} \cdot s^{-1}$ from the equilibrium population of the two forms ([metMb]/[metMbOH] = 0.15), pH and K_{BA}.

4. DISCUSSION

X-ray study [15] has revealed that *Aplysia* metMb possesses penta-coordinated heme iron in its active site. It has been shown that the heme *meso*-proton resonance can be used as a marker signal for identifying the coordination number of the heme iron in metMb [9,16]. The resemblance in the spectral features between *Aplysia* and *Dolabella* metMbs, together with the observation of the marker heme *meso*-proton signal at ~25 ppm (results not shown) and the slower acid–alkaline transition rate, strongly suggests the absence of the Fe-bound water molecule in *Dolabella* metMb. Hence, the acid–alkaline transition of *Dolabella* metMb can be written as

$$\text{metMb} + OH^- \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} \text{metMbOH}.$$

Since the pK of the above reaction for *Dolabella* metMb is close to that for *Aplysia* metMb [8], an identical amino acid residue between the two metMbs is likely to be involved in this transition.

The saturation transfer experiments enabled the determination of the kinetics of the reaction. The values of $1.3(\pm 0.14) \times 10^7$ $M^{-1} \cdot s^{-1}$ and 280 ± 30 s^{-1} for k_{AB} and k_{BA}, respectively, are roughly comparable to the dissociation and association rates for the reaction between external ligand and penta-coordinated heme in Mb [17,18]. These results indicate that the transition from metMb to metMbOH involves the insertion of OH^- to the heme active site followed by the binding of OH^- to the penta-coordinated heme iron. Hence the acid–alkaline transition of these metMbs may be considered as a model for the oxygenation process of Mb.

Saturation transfer connectivities provided the first assignment of the heme methyl proton resonances of *Dolabella* metMbOH. Although the methyl proton resonances of metMbOH exhibit comparable shifts to those for ferric low-spin complexes of which the heme methyl proton hyperfine shift pattern is primarily interpreted in terms of π-spin delocalization, the shift pattern, 1- < 3- < 8- < 5-CH_3, strongly suggests a predominant σ-spin delocalization in this complex. The small spread of the heme methyl proton hyperfine shifts (~12 ppm) indicates its small rhombic magnetic anisotropy [19].

It has been shown that the quantitative kinetic data for the acid–alkaline transition of *Dolabella* metMb can be obtained by ^{1}H-NMR saturation transfer experiments. Detailed understanding of the acid–alkaline transition in this Mb should provide important information about molecular mechanism for ligand binding process of Mb. Careful scrutiny of the acid–alkaline transition in this metMb as well as other metMbs using similar techniques is in progress in our laboratory.

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