Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 348-354

USE OF THIOCYANATE ION AS A MEDIATOR LIGAND IN CONNECTING ACIDIC AND ALKALINE FORMS OF FERRIC MYOGLOBIN *VIA* PARAMAGNETIC ^{1}H-NMR SATURATION TRANSFER

Yasuhiko Yamamoto

Department of Biomolecular Engineering, Tokyo Institute of Technology Nagatsuta, Midori-ku, Yokohama, Japan

Received September 6, 1993

SUMMARY: Paramagnetic ^{1}H-NMR saturation transfer experiments have been used successfully to connect acidic and alkaline forms of equine ferric myoglobin through met-thiocyanate form as a mediator. The saturation transfer connectivities have provided the first assignments of the heme methyl proton resonances of equine met-hydroxyl myoglobin. Without mediator ligand, the acid-alkaline transition in this myoglobin is not accessible to the present technique due to the rapid exchange process between the two forms. Analysis of the intrinsic spin-lattice relaxation time and saturation transfer factor provided the life-time of met-thiocyanate and equine met-hydroxyl myoglobins under the experimental conditions used.

The acid-alkaline transition in ferric myoglobin (metMb) reflects characteristics of interaction between Fe-bound ligand and distal amino acid residue(1-6). Acidic form of metMb possesses either hexa-coordinated heme with an Fe-bound water molecule or penta-coordinated heme(1,7-15), whereas OH^- is bound to the heme Fe in alkaline form. The heme Fe in metMb with the usual distal His (His E7) generally is hexa-coordinated(1,8,9) and Fe-bound H_2O is often absent in metMb lacking the His E7(10-15). These two acidic forms are converted to alkaline form, met-hydroxyl Mb(metMbOH$^-$), according to the scheme shown in Figure 1. The transition of hexa-coordinated metMb to metMbOH$^-$ is fast and the molecular mechanism for its rapid transition has been interpreted in terms of the influence of pH on the electronic state of the His E7 imidazole interacting with Fe-bound ligand (type I in Figure 1)(2,5,8). On the other hand, the transition of penta-coordinated metMb to metMbOH$^-$ is controlled through the diffusion of OH^- (type II) and this process is usually slower compared with the NMR time

Abbreviations: Mb, myoglobin; metMb, ferric myoglobin; metMbOH$^-$, met-hydroxyl myoglobin; metMbSCN$^-$, met-thiocyanate myoglobin; Me, methyl group.

0006-291X/93 $4.00

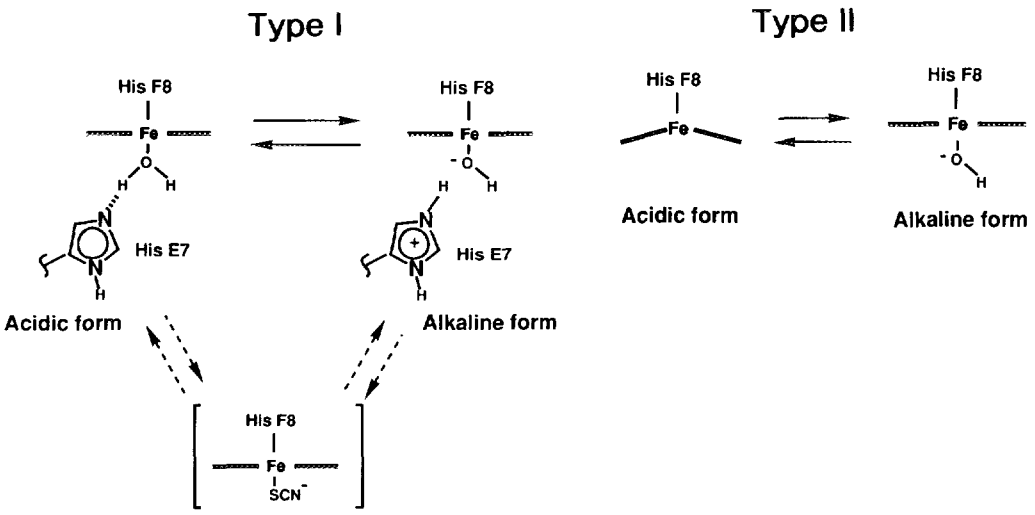

Figure 1. The acid-alkaline transition in hexa-coordinated metMb(type I) and penta-coordinated metMb(type II). Although the type I transition is fast ,compared with the NMR time scale, the use of metMbSCN$^-$ as a mediator allows to connect acidic and alkaline forms by paramagnetic ^{1}H-NMR saturation transfer experiments.

scale(7,12,16,17). We have recently demonstrated that detailed information on the kinetics and thermodynamics of the type II transition can be obtained from the paramagnetic ^{1}H-NMR saturation transfer experiments(7,17).

We report here the results from a paramagnetic ^{1}H-NMR saturation transfer study on the type I transition in equine metMb using thiocyanate ion as a mediator ligand. Among extraneous ligands which compete with H_2O and OH^- for the binding to the heme Fe, met-thiocyanate Mb(metMbSCN$^-$) exhibits relatively sharp and well-resolved NMR signals that are desirable for the saturation transfer experiments(5). The acidic and alkaline forms in the type I transition have been successfully connected by the saturation transfer correlation through metMbSCN$^-$. The observed saturation transfer connectivities provided the first assignments of the heme methyl proton resonances of equine metMbOH$^-$.

MATERIALS AND METHODS:Equine Mb was purchased from Sigma Chemical Co. and used without further purification. 0.5 mM metMb solution was prepared in ^2H_2O. A 20-fold molar excess of potassium thiocyanate(Sigma Chemical Co.) was added to metMb to prepare metMbSCN$^-$. The p^{2}H of the sample was adjusted using 0.2M NaO^{2}H or ^{2}HCl and the p^{2}H was measured using 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-270 FT-NMR spectrometer operating at a ^{1}H frequency of 270 MHz. A typical spectrum consisted with 2000

transients with 8k data points over 50 kHz spectral width and 9.5 μs 90° pulse. The residual water resonance was suppressed with 50 ms presaturation decoupler pulse. Intrinsic spin-lattice relaxation time (T_1^{intr}) 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 50 ms. The spectra resulting from the saturation transfer experiments are presented in the form of difference spectrum. 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 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 relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H^2HO signal as internal reference.

RESULTS AND DISCUSSION:The downfield hyperfine shifted portion of the 270 MHz ^{1}H-NMR spectrum of equine metMb at p^{2}H 6.80 and 45 ℃ is illustrated in trace A of Figure 2. The assignments of the heme methyl proton resonances were made previously(15) and are indicated with the spectrum. The spectrum of metMbSCN$^-$ at p^{2}H 7.56 and 45 ℃ is illustrated in trace 2B. The heme Fe of metMbSCN$^-$ exists in high-spin state(5) and four heme methyl proton resonances, peaks B_1-B_4, are resolved in 45-65 ppm. The spectrum of metMbOH$^-$ at p^{2}H 11.31 and 45 ℃ is shown in trace 2C. Essentially low-spin character of this Mb complex at ambient temperature exhibits the heme methyl proton resonances, peaks C_1-C_4, in 25-40 ppm. The detailed spectral change from acidic form to alkaline form at 30 ℃ is shown in the left hand side of Figure 2. Similar pH-dependent spectra of equine metMb have been reported(2,5). But the present data exhibit a better resolution of the signals. With increasing pH, the signals for acidic form move toward upfield. The progressive pH-dependent shifts of the signals indicate that the acid-alkaline transition in this metMb is much faster than the NMR time scale. The pK value of 9.0 obtained from the analysis on the pH dependence of the shift (results not shown) is consistent with the previously reported value(2,5). The NMR saturation transfer technique is not applicable to system dynamically exchanging faster than the NMR time scale, such as the present case.

Addition of 0.5 mM KSCN to metMb solution at p^{2}H 7.32 and 45 ℃ yielded the spectrum shown in trace A of Figure 3. Signals from the both metMb and metMbSCN$^-$ ([metMbSCN$^-$]/[metMb]=3.10) are separately observed in the spectrum, indicating that the rate of the ligand exchange between H_2O and SCN$^-$ is much smaller than the difference in the resonance frequency between the two forms. The saturation transfer difference spectra resulted from selective saturation of the heme methyl proton

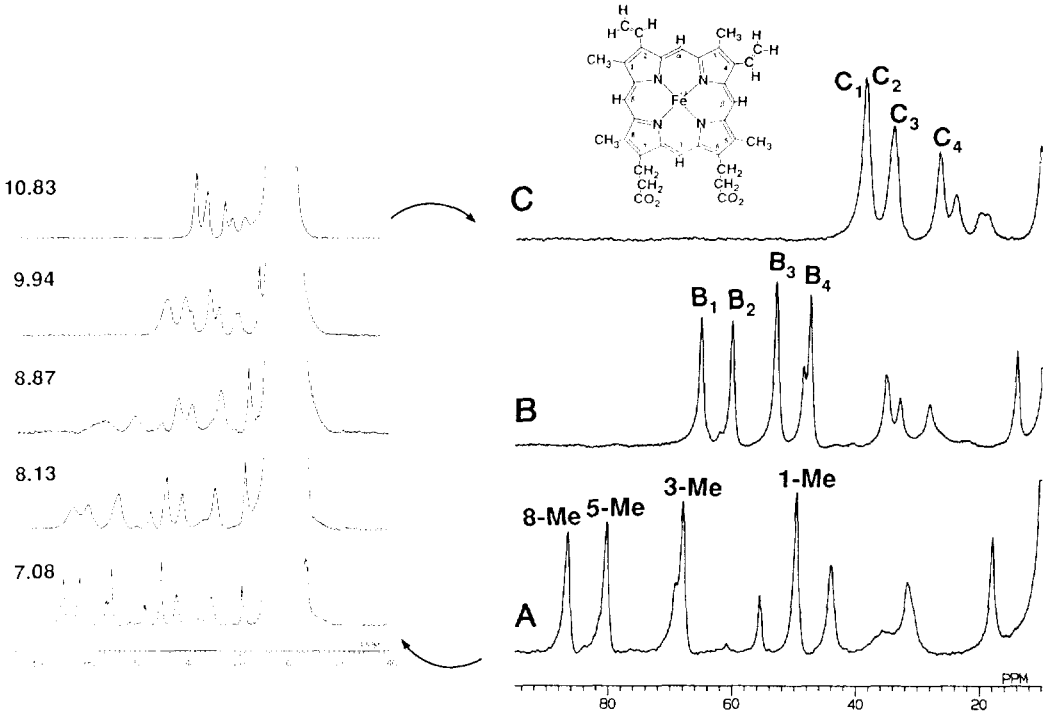

Figure 2. Downfield hyperfine shifted portion of the 270 MHz ^{1}H-NMR spectra of equine metMb in ^2H_2O, p^{2}H 6.80(A), metMbSCN$^-$ in ^2H_2O, p^{2}H 7.56(B), and metMbOH$^-$ in ^2H_2O, p^{2}H 11.31(C). The assignments of the heme methyl proton resonances of metMb(15) are indicated in the trace A. The heme methyl proton resonances of metMbSCN$^-$ and metMbOH$^-$ are labeled B_1-B_4 and C_1-C_4 in the corresponding traces. A series of spectrum illustrated on the left hand side shows the spectra of metMb at the indicated p^{2}H. Molecular structure and numbering system of heme is indicated in the inset.

resonances of metMb are shown in traces 3B-3D. The saturation of the heme 8-Me signal of metMb exhibits partial saturation of peak B_1, indicating the assignment of peak B_1 to the heme 8-Me of metMbSCN$^-$. The saturation of the 5-Me signal of metMb results in partial saturation of peak B_2 in trace 3C and, although the decoupler pulse power spillage to peak B_1 with the saturation of the 3-Me signal of metMb is rather significant, the connectivity between the 3-Me signal and peak B_3 is clearly established in trace 3D. These saturation transfer connectivities provide the assignments of the 5- and 3-Me signals in metMbSCN$^-$. Since the 1-Me signal of metMb is close to peak B_4, it was not possible to separate the saturation transfer connectivity between these two peaks from the effect of the decoupler pulse power spillage. But peak B_4 is safely assigned to the 1-Me signal of metMbSCN$^-$. Thus the heme methyl proton resonances of metMbSCN$^-$ have been obtained from the known signal assignments of metMb *via* the saturation

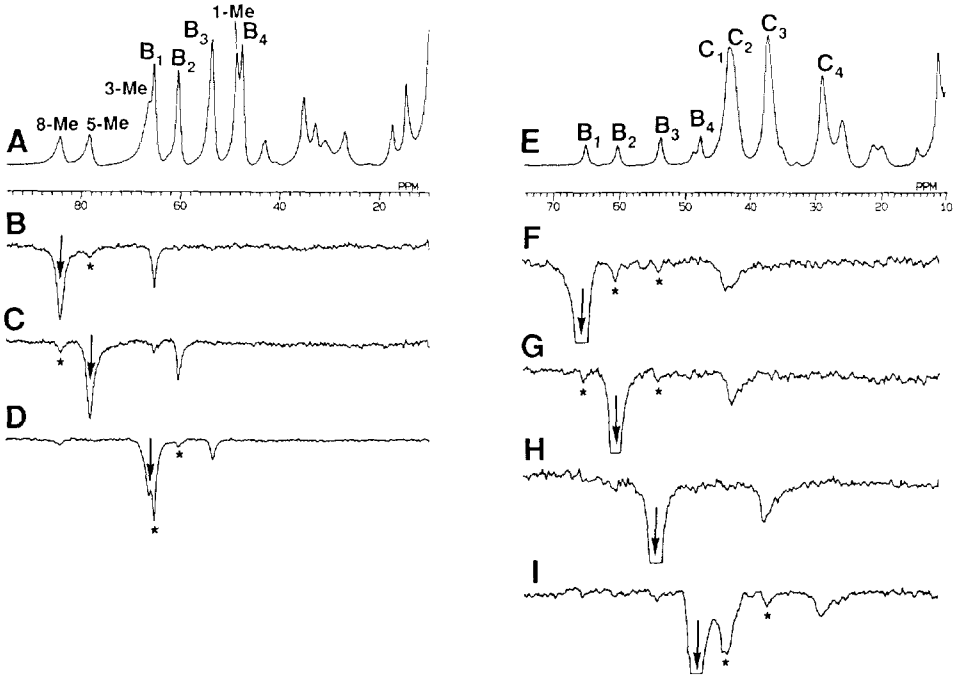

Figure 3. 270 MHz ^{1}H-NMR and saturation transfer difference spectra of equine metMb complexes at 45 ℃. (A)Downfield hyperfine shifted portion of the spectrum of the mixture of equine metMb and metMbSCN$^-$ in ^2H_2O, p^{2}H 7.32. The heme methyl proton resonances of the both forms are indicated in the trace. (B) Saturation of the heme 8-Me signal of metMb exhibiting a saturation transfer connectivity to a heme methyl proton peak B_1 of metMbSCN$^-$. (C) Saturation of the heme 5-Me signal of metMb exhibiting a saturation transfer connectivity to peak B_2 of metMbSCN$^-$. (D)Saturation of the heme 3-Me signal of metMb exhibiting a saturation transfer connectivity to peak B_3 of metMbSCN$^-$. Partial saturation of the 8-Me signal is not due to the decoupler pulse power spillage, but to the saturation transfer connectivity from peak B_1, which is partially saturated by the off-resonance effect of the irradiation pulse on the 3-Me signal. (E) Downfield hyperfine shifted portion of the spectrum of the mixture of equine metMbSCN$^-$ and metOH$^-$ in ^2H_2O, p^{2}H 10.39. (F) Saturation of peak B_1 exhibiting a saturation transfer connectivity to peak C_1. (G) Saturation of peak B_2 exhibiting a saturation transfer connectivity to peak C_2. (H) Saturation of peak B_3 exhibiting a saturation transfer connectivity to peak C_3. (I) Saturation of peak B_4 exhibiting a saturation transfer connectivity to peak C_4. Peaks due to the decoupler pulse power spillage are indicated by * .

transfer connectivities. The difference in the resonance frequency between the 1-Me signal of the both forms dictates that the ligand exchange rate is ⟨300 s^{-1}. Raising the p^{2}H value of the mixture soultion of metMb and metMbSCN$^-$ to 10.39 at 45 ℃ yielded the spectrum illustrated in trace 3E. The heme methyl proton resonances, peaks C_1-C_4, of metMbOH$^-$, together with those for metMbSCN$^-$, are identified in the spectrum and the signal intensities indicate the fraction of the two forms ([metMbOH$^-$]/[metMbSCN$^-$]) is

3.30. The saturation of peaks B_1-B_4, respectively, leads to partial saturation of peaks C_1-C_4 as shown in traces 3F-3I. The observed saturation transfer connectivities directly provide unambiguous assignments of the heme methyl proton resonances of metMbOH$^-$, i.e., C_1(8-Me), C_2(5-Me), C_3(3-Me), C_4(1-Me). The heme methyl proton shift pattern in equine metMbOH$^-$ is similar to those of other metMbOH$^-$s(7). The difference in the chemical shift between the corresponding heme methyl proton resonances of metMbSCN$^-$ and metMbOH$^-$ established that the ligand exchange between SCN$^-$ and OH$^-$ is $<4500\ s^{-1}$.

The life-time (τ) of metMbSCN$^-$ and metMbOH$^-$ can be estimated from intrinsic spin-lattice relaxation time (T_1^{intr}) and saturation transfer factor (I/I_0) by the equation, $\tau^{-1}=(T_1^{intr})^{-1}(1-I/I_0)/(I/I_0)$ (18). T_1^{intr} of peak B_2 (5-Me) was measured using the saturation-recovery method as illustrated in Figure 4. The τ value of 30 ms for metMbSCN$^-$ was calculated from the obtained T_1^{intr} of 4.0 ms and I/I_0=0.88 (trace 3C). The τ value of 80 ms was similarly calculated for metMbOH$^-$ from the T_1^{intr} of the 1-Me signal of metMbOH$^-$ (1.6 ms) and I/I_0=0.98(trace 3I). These τ values are consistent with the values estimated from the diffrence in the resonance frequency between the two exchanging forms.

The present study clearly demonstrated potential utility of a mediator ligand in studying the acid-alkaline transition in metMb. If the assignments are known in one form,

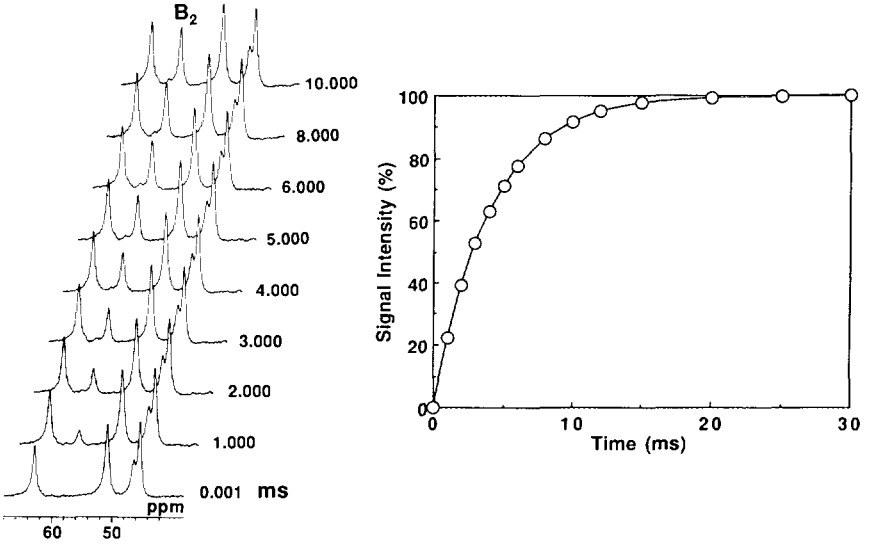

Figure 4. The recovery of the peak B_2(5-Me) of equine metMbSCN$^-$ in ^2H_2O, p^{2}H 7.56, at 45 ℃ (left) and the plot of signal intensity *vs.* the recovery time(right). T_1^{intr} of 4.0 ($\pm$0.3) ms was obtained.

the assignments in the other form can be straightforwardly and unambiguously obtained from the saturation transfer connectivities. The proposed approach should be applicable to other ferric hemoproteins, of which acid-alkaline transition is much faster compared with the NMR time scale, to connect acidic and alkaline forms and then to characterize the transition.

ACKNOWLEDGMENTS: This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Cluture (05750797), Japan. The author thanks Professors R.Chujo and Y. Inoue for their encouragement.

REFERENCES

1. Antonini, E., and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, pp.40-54, North-Holland Publishing Company, Amsterdam.
2. Iizuka, T., and Morishima,I. (1975) Biochim. Biophys. Acta 400, 143-163.
3. Sono, M., and Asakura, T. (1976) J. Biol. Chem. 251, 2664-2670.
4. McGrath, T. M., and La Mar, G. N. (1978) Biochim. Biophys. Acta 534, 99-111.
5. Morishima, I., Ogawa, S., Inubushi, T., and Iizuka, T. (1978) Adv. Biophys. 11, 217-245.
6. Gadsby, P. M. A., Peterson, J., Foote, N., Greenwood, E. C., and Thomson, A. J. (1987) Biochem. J. 246, 43-54.
7. Yamamoto, Y., Suzuki, T., and Hori, H. Accepted for publication in Biochim. Biophys. Acta.
8. Caughey, W. S. (1966) In Hemes and Hemoproteins (B. Chance, R. W. Estabrook, and T. Yonetani, Eds.) pp. 276-277, Academic Press, New York.
9. Takano, T. (1977) J. Mol. Biol. 110, 537-568.
10. Giacometti, G. M., Ascenzi, P., Brunori, M., and Bolognesi, M. (1981) J. Mol. Biol. 146, 363-374.
11. Giacometti, G. M., Ascenzi, P., Brunori, M., Rigatti, G., Giacometti, G., and Bolognesi, M. (1981) J. Mol. Biol. 151, 314-319.
12. Pande, U., La Mar, G. N., Lecomte, J. T. L., Ascoli, F., Brunori, M., Smith, K. M., Pandey, R. K., Parish, D.W., and Thanabal, V. (1986) Biochemistry 25, 5638-5646.
13. Rajarathnam, K. La Mar, G. N., Chiu, M. L., Sligar, S. G., Singh, J. P., and Smith, K. M. (1991) J. Am. Chem. Soc. 113, 7886-7892.
14. Ikeda-Saito, M., Hori, H., Andersson, L. A., Prince, R. C., Pickering, I. J., George, G. N., Sanders II, C. R., Lutz, R. S., McKelvey, E. J., and Mattera, R. (1992) J. Biol. Chem. 267, 22843-22852.
15. Yamamoto, Y., Osawa, A., Inoue, Y., Chujo,R., and Suzuki, T. (1990) Eur. J. Biochem. 192, 225-229.
16. Giacometti, G. M., Da Ros, A., Antonini, A., and Brunori, M. (1975) Biochemistry 14, 1584-1588.
17. Yamamoto, Y., Chujo, R., Inoue, Y., and Suzuki, T. (1992) FEBS Lett. 310,71-74.
18. Krishna, N. R., Huang, D. H., Glickson, J. D., Rowan III, R., and Walter, R. (1979) Biophys. J. 26, 345-366.