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Studies on the Heterotropic Interaction of Hemoglobin

I. Mass Spectrometric Method for Determination of the pK_a of the β-146 Histidine Residue in Human Hemoglobin

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A mass spectrometric method was developed to determine pH-dependent hydrogen-deuterium exchange at the C-2 position of the imidazole ring of histidine, after converting the amino acid to the methylthiohydantoin derivative. The amount of deuterium exchange in N-acetylhistidine estimated by the present method was confirmed to be in good agreement with that determined by NMR spectrometry.

N-Acetylhistidine was deuterated at various pH's. From the amount of deuterium exchange, a pseudo-first order rate constant ($k\phi$) was calculated. A pK_a value of 7.2 for the amino acid was obtained from the relation between $k\phi$ and pH.

This method was applied to estimate the pK_a value of β-146 histidine in human hemoglobin. Human hemoglobin deuterated at various pH's was digested with carboxypeptidase A [EC 3.4.12.2] to release the β-146 histidine. The amount of deuterium exchange in the isolated histidine was determined to obtain $k\phi$. From these measurements pK_a values of 7.0 for the histidine in oxyhemoglobin and of 8.2 for that in deoxyhemoglobin were found at 36.5°, respectively.

The Bohr effect, which is the change in the oxygen affinity of hemoglobin with pH, is known to be a typical case of heterotropic interaction involving oxygen and proton binding sites in the hemoglobin molecule. While the general features of the mechanism of the effect are certainly clear, the details are still subject to uncertainty.

Perutz *et al.* (*1*) proposed that β-146 histidine, α-1 valine, and α-122 histidine residues in hemoglobin may be responsible for the alkaline Bohr effect, based on X-ray crystallographic studies. Recently, Greenfield and Williams (*2*) and Kilmartin *et al.* (*3*) measured the pK value of histidine residues in human hemoglobin by titration of the proton at the C-2 position in the imidazole ring, using proton nuclear magnetic resonance. They assigned one of the proton spectra to the β-146 histidine residue on the basis of its disappearence in des-His (β-146) hemoglobin obtained by carboxypeptidase A digestion.

On the other hand, Ohe *et al.* (*4*) have recently developed a new method for the determination of the pK_a values of individual histidine residues in pancreatic ribonuclease [EC 3. 1. 4. 22], based on pH-dependent hydrogen-tritium exchange reaction at the C-2 position of the imidazole ring of the histidine residues under mild conditions. Very recently, Markley and Kato (*5*) reported a novel NMR technique using a hydrogen-deuterium exchange method to assign NMR signals of two

Abbreviation: MTH, methylthiohydantoin.

histidine residues in soybean trypsin inhibitor after cleaving the deuterated protein with cyanogen bromide into two fragments.

In an attempt to estimate the pK_a values of individual histidines in human hemoglobin by the tritium exchange method, we succeeded in developing a mass spectrometric method using hydrogen-deuterium exchange. This paper describes the procedure and some of the results obtained.

EXPERIMENTAL

Human hemoglobin was prepared as described previously (*6*) and was concentrated to about 20 g/dl using a Sartorius collodion bag. Deuterium oxide (99.75%), deuterium chloride and deuterated acetic acid (d4) were purchased from Merck and Co. The deuterated buffers used were 0.2 M Tris-^{2}HCl and 0.2 M deuterated acetic acid-sodium acetate. Buffer materials were dissolved in deuterium oxide. Carboxypeptidase A was a product of Worthington Biochemical Corp. All reagents used for Edman degradation were of special reagent grade for sequence analysis, obtained from Wako Chemical Co.

Deuteration of N-acetylhistidine to determine its pK_a value was carried out as follows. Fifty μl of 8.8 mM N-acetylhistidine solution was added to 1.0 ml of the deuterated buffer solution at various pH's. After being sealed in a tube the mixture was incubated at 36.5° for 24 h. One ml of 2 M Tris-HCl buffer (pH 8.0) and 0.5 ml of H_2O were then added to the solution, which was further incubated at 30° for another 1 h. After desalting with Dowex 50×2 and Dowex 1×2, each sample was lyophilized. N-Acetylhistidine thus deuterated was hydrolyzed in 6 N HCl at 105° for 24 h and lyophilized.

The resulting histidine was converted to the methylthiohydantoin (MTH) derivative instead of to the phenylthiohydantoin derivative to increase the volatility of histidine for mass spectrometry. Coupling and subsequent cyclization were carried out according to Waterfield and Haber (*7*) with slight modifications as follows. The lyophilized sample was incubated with 0.4 ml of 0.4 M dimethylarylamine buffer (pH 9.5) and 10 μl of methyl isothiocyanate at 40° for 1 h, followed by washing with 2 ml of benzene three times. The solution was lyophilized and the residue was dissolved in 0.3 ml of 1 N HCl. After standing for 30 min at 80°, the pH of the solution was adjusted to 8.5 with 1 N NaOH. One ml of ethyl acetate was added to extract deuterated MTH-histidine. The ethyl acetate phase was subjected to mass spectrometry.

Mass spectra were obtained on a Shimadzu-LKB 9000 gas chromatograph mass spectrometer by the direct inlet method under following conditions:190° ion source temperature,[1] 3.5 eV accelerating voltage, and 70 eV ionizing voltage. The sample was ionized at 90° (10^{-7} mmHg) by raising the temperature at a rate of 20° per min. The mass spectra were scanned when the ionization of the sample reached a maximum. The amount of deuterium exchanged at the C-2 position of the histidine was calculated from the ratio of parent peaks (M^+: 210/M^++1 : 211). A Hitachi R-24B, 60 MHz, NMR spectrometer was used for calibration of the hydrogen-deuterium exchange.

The pseudo-first order rate constant ($k\phi$) for the deuterium exchange reaction was obtained as described previously (*8*). The pH of the samples was measured with a Radiometer titrator, model TTT2, at 36.5° without correction for deuterium oxide. The pK_a readings are applicable to water as well as deuterium oxide (*9*) and the Bohr effect is not affected by deuterium oxide (*10*).

RESULTS AND DISCUSSION

Mass Spectrum of Authentic MTH-histidine—The mass spectrum of MTH-histidine obtained was simple. The intensity of parent peaks was markedly higher than those of the other fragment peaks derived from the parent molecule. The ratio of M^+(210) and M^++1(211) of MTH-histidine was found to be 7.52, in good accordance with natural abundance.

Validity of the Present Method—As a preliminary experiment, the extent of deuteration in the imidazole ring of N-acetylhistidine was calibrated by the NMR technique, since it is conceivable that the deuterium exchanged for the C-2 proton may be released during the experimental procedure (*e.g.*,

[1] The spectra obtained at 290° ion source temperature showed low intensity of parent peaks, especially of the M^++1 peak, as compared with that measured at 190°.

reaction step with methyl isothiocyanate, ionization process in the mass spectrometer, *etc.*).

For this purpose, two hundred mg of N-acetylhistidine was dissolved in 50 ml of deuterium oxide (99.75%). The solution was adjusted to pH 7.0 with pyridine and incubated at 36.5°. Aliquots of the solution were taken at appropriate times and lyophilized. One hundred μg of the sample was subjected to mass spectrometry after conversion to the MTH derivative as described above. Forty mg of the sample was subjected to NMR spectroscopy as described previously (*8*).

Table I shows that the amounts of deuterium exchange at C-2 in N-acetylhistidine measured by mass spectrometry were in good agreement with those estimated by NMR within the range of experimental error. This provides strong evidence that the present method can sensitively detect the amount of deuterium exchanged without loss during the experiment.

pK_a of N-Acetyl Histidine—N-Acetylhistidine was deuterated at various pH's to determine the pK_a as a model for the histidine residue in hemoglobin, as stated above. The maximum amount of deuterium incorporated into N-acetylhistidine in 24 h was 41.3% in the alkaline region. Figure 1 shows a plot of $k\psi$ values *versus* pH for the exchange reaction. The pK_a of N-acetylhistidine was determined as a pH value corresponding to the midpoint of a sigmoidal curve according to a previous report (*8*). The pK_a and $k\psi_{max}$ values were found to be 7.2 and 2.2×10^{-2} (h^{-1}), respectively.

These values were in accord with those determined by the tritium exchange method (*8*). The results appear to confirm that exchanged deuterium was not released from histidine throughout the experimental procedure. Deuteration of N-acetylhistidine was also performed in the presence of 1.1 mM sodium dithionite. No significant change of the pK_a value was observed in the presence and absence of sodium dithionite, suggesting that the deuterium exchange reaction was not affected by sodium dithionite, which will be used in later experiments to deoxygenate hemoglobin.

pK_a of β-146 Histidine in Hemoglobin—Since it was expected from the above results that the present method might be applicable to estimate the pK_a of histidine in hemoglobin, the following experiment was carried out. Fifty μl of 19.1 g/dl oxyhemoglobin was added to 1.0 ml of deuterated buffer at various pH's. The mixture was incubated at 36.5° for 24 h. Then, 1.0 ml of 2 M Tris-HCl buffer (pH 8.0) and 0.5 ml of 0.96 mg/ml of carboxypeptidase A were added and the mixture was digested at 30° for 1 h, following the method of Antonini *et al.* (*11*). After digestion, the solution was deproteinized by trichloroacetic acid treatment. The results of amino acid analysis of the desalted supernatant showed that histidine and tyrosine was present in an equimolar ratio, whereas other amino acids were present only in trace amounts, in accordance with the results of Antonini *et al.* (*11*). The yield of β-146 histidine was about 46%. The tyrosine and deuterated histidine thus isolated were converted to MTH derivatives as described above. After the MTH-tyrosine had been extracted with

TABLE I. Comparison of deuterium contents in N-acetylhistidine as measured by the present method and by the NMR technique.

Incubation time (h)	Amount of deuterium in N-acetylhistidine	
	Present method[a]	NMR technique[b]
22	16.2±1.2	16.9±2.1
28	20.6±1.3	21.0±1.8
50	33.8±1.1	33.5±1.5

[a] Average of 20 measurements per sample. [b] Average of 10 measurements per sample.

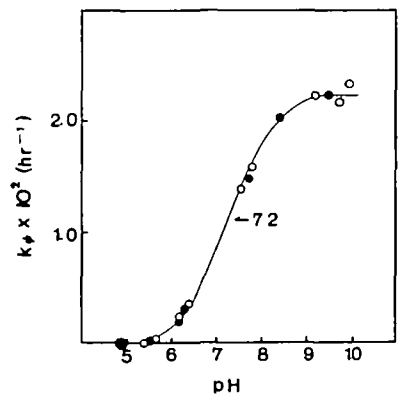

Fig. 1. Plot of the pseudo-first order rate constants ($k\psi$) for the deuterium exchange of N-acetylhistidine as a function of pH at 36.5°. ○, in the absence of $Na_2S_2O_4$; ●, in the presence of 1.1 mM $Na_2S_2O_4$. The concentration of N-acetylhistidine was 0.42 mM. μ=0.2.

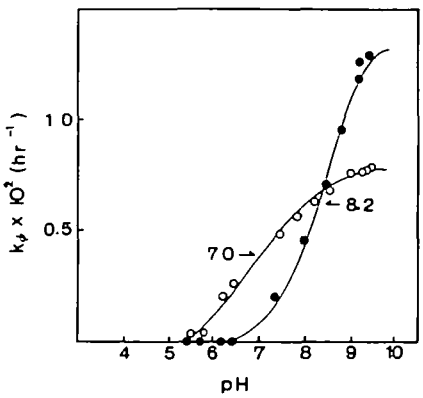

Fig. 2. Effect of oxygenation on the pK_a value of β-146 histidine in human hemoglobin at 36.5°. ○, oxyhemoglobin; ●, deoxyhemoglobin. The concentration of hemoglobin was 9 1 mg/ml. μ=0.2.

ethyl acetate from the water phase, the pH of the water phase was adjusted to 8.5 and the deuterated MTH-histidine was extracted with ethyl acetate. The amount of deuterium exchanged in the β-146 histidine residue was measured mass spectrometrically. Deuteration of deoxyhemoglobin was performed in the same manner except that the reaction was carried out under nitrogen in a sealed test tube after the addition of sodium dithionite in two-fold excess with respect to heme concentration. The maximum amounts of deuterium incorporated into β-146 histidine in oxy- and deoxy-hemoglobin within 24 h in the alkaline region were 15.9% and 26.6%, respectively.

Figure 2 shows the relation of $k\phi$ and pH in the deuteration of β-146 histidine in oxy- and deoxy-hemoglobin. Plots obtained for deoxyhemoglobin coincided well with the theoretical curve (*4*), whereas those for oxyhemoglobin showed a slight deviation from the theoretical curve. Similar results have been reported in the case of pancreatic ribonuclease (*12*). The interpretation of this deviation will be discussed in a subsequent paper. The pK_a of β-146 histidine in oxyhemoglobin was found to be 7.0, while that in deoxyhemoglobin was found to be 8.2, at 36.5°. These pK_a values agree well with those reported by Kilmartin *et al.* (*3*) (7.1 in carbomonoxy-hemoglobin and 8.0 in deoxyhemoglobin at 30°). A decrease of 1.2 in the pK_a value on oxygenation confirms the proposed role of β-146 histidine in the alkaline Bohr effect. According to Perutz *et al.* (*1*) β-146 histidine could account for maximum of half of the alkaline Bohr effect by forming a salt bridge between its imidazole group and the carboxyl group of β-94 aspartic acid in deoxyhemoglobin, which would raise the pK_a of the imidazole group. The remaining part of the Bohr effect may be attributed to α-122 histidine in addition to the α-amino group of α-1 valine.

The pK_a value of α-122 histidine remains to be determined to confirm the role of the residue in the alkaline Bohr effect. It may also be worthwhile to investigate the possible role of other histidine residues, which are assumed to be irrelevant to the Bohr effect. The results of experiments along this line will be published elsewhere.

REFERENCES

1. Perutz, M.F., Muirhead, H., Mazzarella, L., Crowther, R.A., Greer, J., & Kilmartin, J.V. (1969) *Nature* **222**, 1240–1243
2. Greenfield, N.J. & Williams, M.N. (1972) *Biochim. Biophys. Acta* **257**, 187–197
3. Kilmartin, J.V., Breen, J.J., Roberts, G.C.K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.* **70**, 1246–1249
4. Ohe, M., Matsuo, H., Sakiyama, F., & Narita, K. (1974) *J. Biochem.* **75**, 1197–1200
5. Markley, J.L. & Kato, I. (1975) *Biochemistry* **14**, 3234–3237
6. Suzuki, M., Kajita, A., & Hanaoka, E. (1954) *J. Biochem.* **41**, 401–418
7. Waterfield, M. & Haber, E. (1970) *Biochemistry* **9**, 832–839
8. Matsuo, H., Ohe, M , Sakiyama, F., & Narita, K. (1972) *J. Biochem.* **72**, 1057–1060
9. Roberts, G.C.K., Meadows, D.H., & Jardezky, O. (1968) *J. Am. Chem. Soc.* **90**, 1042–1055
10. Tomita, S. & Riggs, A. (1970) *J. Biol. Chem.* **245**, 3104–3109
11. Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., & Caputo, A. (1961) *J. Biol. Chem.* **236**, 60–63
12. Kangawa, K., Matsuo, H , & Narita, K (1976) *Seibutsu-butsuri* (in Japanese) **16**, 145–153