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Quantitative Evaluation for the Role of β146 His and β143 His Residues in the Bohr Effect of Human Hemoglobin in the Presence of 0.1 M Chloride Ion*

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Two different methods were used to determine the number of Bohr protons released upon oxygenation of human hemoglobin (Hb A) and Hb A lacking β146 His (des-His Hb A) at the pH ranging from pH 5.0 to 9.0 in the presence of 0.1 M Cl^- at 25 °C. One is the direct differential titration method, the other is based on the measurement of oxygen affinity as a function of pH. The results obtained for Hb A or des-His Hb A with two methods were completely mutually consistent. The number of Bohr protons released from des-His Hb A upon oxygenation at pH 7.5 was about 44% less than that from Hb A, while at pH 5.5 the number of Bohr protons taken up by des-His Hb A was 20% greater than that by Hb A. The differences in the number of Bohr protons between Hb A and des-His Hb A could not be simply ascribed to the lack of β146 His from Hb A.

The pK_a values, which were determined by the deuterium exchange method using ^1H NMR, were 8.0 for β146 His of deoxy-Hb A and 6.5 for that of CO Hb A, while those of β143 His were 5.2 for deoxy-Hb A and 6.0 for CO Hb A. From these pK_a values, in addition to those of α1 Val proposed for the modified CO and deoxy-Hb A with carbamylated β chains by Van Beek and De Bruin (Van Beek, G. M., and De Bruin, S. H. (1980) *Eur. J. Biochem.* 105, 353–360), it became evident that almost all (about 92%) of the alkaline Bohr protons released upon oxygenation of Hb A in the presence of 0.1 M Cl^- could be accounted for by the protons from these 2 residues, although the involvement of other histidine residues could not be denied. About half the acid Bohr protons from Hb A, which corresponds to the higher pH part (above pH 5.0) of the acid Bohr effect, could be explained by the involvement of β143 His residue. The residual acid Bohr effect in the more acidic pH region was presumably contributed by an amino acid residue with pK_a values of 4.05 and 5.95 for the deoxy- and CO Hb A, respectively, although the amino acid residue was unspecified.

In des-His Hb A, however, the acid and alkaline Bohr effects could not be satisfactorily explained if the pK_a values of β143 His and α1 Val remained unchanged in comparison with those of Hb A and moreover the involvement of other residues in the Bohr effect was not taken into account. These results indicate that removal of β-carboxyl terminal histidine may cause considerably large perturbations to the tertiary structure of the subunit and/or to the deoxy-quaternary structure of des-His Hb A, so that its acid and alkaline Bohr effects may be altered more extensively than would be expected by deletion of only 1 residue.

The oxygen affinity (p_{50}) of hemoglobin depends upon the pH when the hemoglobin shows cooperative oxygen binding. Lowering the pH from 7.0 to 6.0 reduces the oxygen affinity and raising the pH above 7.0 increases it. This latter effect of pH is called the alkaline Bohr effect, one of the important mechanisms involved in the physiological oxygen transport function of hemoglobin. On the other hand, at a pH below 6.0 the overall oxygen affinity of hemoglobin is again increased. This effect is termed the acid Bohr effect, and has not been considered to play a significant role in regulating the physiological function of hemoglobin. The total Bohr effect consists of both alkaline and acid effects. Wyman (1) proposed a hypothesis explaining the effect of protons on the change in oxygen affinity in which he assumed two independent oxygen-linked ionizable groups per heme, one responsible for the alkaline effect and the other for the acid effect. Thereafter, candidates for the ionization groups were sought by many investigators using many physicochemical means (2–13). There is evidence that the enzymatic elimination of β146 His from Hb A[1] (14), or the substitution of β146 His for Asp by point mutation (Hb Hiroshima (15)), for Arg (Hb Cochin Port Royal (16)), for Pro (Hb York (17)), and for Leu (Hb Cowtown (18)) definitely reduced the Bohr effect, especially the alkaline Bohr effect, and suggests the involvement of β146 His in the Bohr effect. According to the stereochemical mechanism proposed by Perutz (19), this His residue can form a salt-bridge with the carboxyl group of β94 Asp in the deoxy form of Hb A, but in the liganded form this salt-bridge is broken by the tertiary and/or the quaternary structure change in Hb A.

One might expect that the reduced or ruptured interaction between β146 His and β94 Asp would be brought about by the alteration of the pK_a values of β146 His on ligation of Hb A. Indeed, the direct or indirect measurement of the pK_a values of β146 His in deoxy- and CO Hb A has been carried out by two methods, ^1H NMR titration (2, 8, 10) and the deuterium exchange reaction (3–5), and such prediction for this mecha-

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[1] The abbreviations used are: Hb A, hemoglobin A; H ⇌ D exchange reaction, hydrogen ⇌ deuterium exchange reaction; D_2O, deuterium oxide; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; OEC, oxygen equilibrium curve.

nism and also the contribution of β146 His to the alkaline Bohr effect were confirmed. Using the H ⇌ D^1 exchange reaction method, Ohe and Kajita (5) extensively measured the pK_a values of all the histidine residues of α and β subunits in the deoxy- and CO form of Hb A, and found that the α20, α89, and β143 His residues can significantly contribute to the total Bohr effect in addition to the α1 Val and β146 His residues in the presence of 0.1 M Cl^-. However, the sum of the protons released from these residues on oxygenation exceeded the total Bohr protons calculated theoretically with the pK_a values for the two hypothetical ionization groups, which were previously presented by Antonini *et al.* (20).

We considered that the cause of this discrepancy might be due to the variation in experimental conditions (for example, ± Cl^-, or ± inorganic or organic phosphates such as 2,3-diphosphoglycerate or inositolhexaphosphate) employed by several investigators. As for the effect of Cl ion concentration on hemoglobin Bohr effect, the elaborate study by Rollema *et al.* (21) should be noted. They indicated that the number of Bohr protons released or taken up on oxygenation of Hb A critically depends upon the Cl^- concentration added, especially in the lower concentration range below 0.1 M. Therefore, the measurement of Bohr effect must be performed at constant Cl^- concentration throughout the experiment. If the experiment is not satisfied with such conditions, the data obtained may lead to an unavoidable, erroneous conclusion. The recent ^1H NMR titration experiment for chemical shift of histidine C2 protons as a function of pH (22, 23) had been conducted for normal and des-His Hb A in the low concentration range of Cl^- which varied from 0.005 to 0.06 M with decreasing pH.

Accordingly, we measured the Bohr effect of Hb A and des-His Hb A, by the pH dependence of oxygen affinity, p_{50}, and by direct differential titration for Bohr protons over the wide pH range from pH 5.0 to 9.0, and also pK_a values of β146 His and β143 His residues involved in the Bohr effect under conditions as same as possible. As a result, we found that in the presence of 0.1 M Cl^- β146 His and α1 Val contribute entirely to the alkaline Bohr effect and β143 His to less than 50% of total acid Bohr effect. We are now progressing the study on the mechanism of the Bohr effect of normal and des-His Hb A in the complete absence of Cl^- to avoid the effect of varying concentration of Cl^- and the involvement of Bohr groups such as β146 His and β143 His in the Bohr effect under such conditions. In a forthcoming paper, we will report the results obtained and discuss the mechanism of hemoglobin Bohr effect under this condition.

MATERIALS AND METHODS

Hemoglobins—Human hemoglobin and des-His Hb A were used in this study. Hb A was prepared from normal human blood (24), and stripped by passing through a Sephadex G-25 column equilibrated with 0.1 M NaCl. For the preparation of des-His Hb A, native α and β subunits were prepared according to the method of Bucci and Fronticelli (25). To obtain the des-His β chain, the β chain was partially digested with carboxypeptidase B (EC 3.4.12.3) at 37 °C for 4 h under a CO atmosphere in barbiturate buffer, pH 8.3 (26). The digestion process by carboxypeptidase B was monitored by high performance liquid chromatography on a TSK gel DEAE-5PW column (Toyosoda, Japan). The des-His β subunit and the des-His/Tyr β subunit were separated from the undigested β subunit by the linear gradient elution ranging from 0.01 M Na acetate, pH 6.0, to 0.1 M Na acetate, pH 6.0, on a CM-32 column. The purified preparation of des-His β subunit was confirmed to exhibit a single symmetrical peak on the same column of high performance liquid chromatography and to have lost only 1 histidine residue/Hb dimer by carboxypeptidase B digestion because only 1 Tyr residue/dimer was liberated from the preparation by carboxypeptidase A (EC 3.4.12.2) treatment. The isolated des-His β subunit was then combined with an equimolar amount of the partner subunit. The resulting des-His Hb A was purified on a CM-32 column by a stepwise increase in the pH.

Oxygen Equilibrium Curve Measurement of Hb A and Des-His Hb A under Different pH Conditions—The oxygen equilibrium curve was measured for 100 μM hemoglobin (heme basis) at 25 °C using an automatic recording apparatus (27). For the determination of the Bohr effects of these hemoglobins, 0.05 M bis-Tris acetate buffer containing 0.1 M NaCl was used for the measurements at a pH below 7.5, and 0.05 M Tris acetate buffer containing 0.1 M NaCl for those at a pH above 7.5. Bis-Tris, Tris, and acetic acid had no effect on the OEC measurement.

Differential Titration of Bohr Protons Released or Taken up by Hb A and Des-His Hb A on Oxygenation—Titration of protons released or taken up on CO binding of Hb A and des-His Hb A was performed with a handmade titration apparatus equipped with a pH stat (TOA DENPA Co.) and an optical fiber connecting with a spectrophotometer (Union Giken). The volume of the vessel was about 20 ml. The details for the titration instrument will be described elsewhere. The hemoglobin solution added to the vessel was maintained at 25 °C by a thermostated water circulator (Neslab RTE-8). Before titration, about 100 ml of hemoglobin solution (200–600 μM as heme) in 0.1 M KCl was initially deoxygenated by the continuous flow of humidified nitrogen gas and by gentle stirring in a glass bottle with three holes. The upper two holes were used for the inlet and outlet of N_2 gas, and the hole near the bottom was sealed with a rubber stopper. The deoxygenated hemoglobin solution (8–9 ml) was withdrawn from the bottle by an air-tight syringe through the rubber stopper and was transferred to the titration vessel anaerobically. The extent of deoxygenation was determined by the absobance change at 595 or 760 nm with an attached spectrophotometer. A pH electrode (TOA DENPA) was inserted horizontally near the bottom of the vessel. Immediately before the injection of CO to start the titration of Bohr protons, the deoxygenated hemoglobin solution was adjusted to the programmed pH with 0.01 N HCl or NaOH which was introduced by a computer-controlled autobiuret through a glass capillary fixed with a rubber stopper. Next, pure CO gas (about 5 ml) was injected into gas phase of the vessel. Thereafter, the volume of titrant (0.01 N HCl or NaOH) used to adjust to the set pH was recorded during CO binding. For determination of the Bohr effect of des-His Hb A, the concentration of hemoglobin was varied from 200 to 350 μM as the heme basis.

Preparation of Deuterium Oxide Solution of Hb A—Hb A were purified to remove minor hemoglobins on a CM-32 column, and then passed through a Sephadex G-25 column equilibrated with 10 mM bis-Tris acetate, pH 7.0. The eluate was adsorbed on a CM-32 column equilibrated with the same buffer. This column was washed extensively with 10 mM bis-Tris acetate, pH 7.0, in D_2O (DMM-100, Commissariat a l'Energie Atomique, France) to remove H_2O present in the column. Replacement of H_2O with D_2O in the eluted buffer was monitored by ^1H NMR. When the content of D_2O in the buffer eluted from the column exceeded 95% or more, the Hb A adsorbed by the cellulose was eluted with 50 mM Tris base in D_2O and collected. The concentration of Hb A in D_2O (95%) thus obtained was 5 mM in heme, as measured by the pyridine hemochromogen method using ε (557 nm) = 34.4 (mM^{-1} cm^{-1}). This hemoglobin solution was used for the pK_a determination of β146 His and β143 His.

Hydrogen ⇌ Deuterium Exchange Reaction of C2 Proton of Histidine Residues in Hb A—To the D_2O solution of Hb A were added solid bis-Tris and NaCl to make final concentrations of 50 mM and 0.1 M, respectively. This solution was placed in a air-tight chamber equipped with a TOA pH electrode and some syringe needles, and was deoxygenated by passing a nitrogen gas stream through the needle with gentle stirring of the solution. The pH of the solution containing deoxy-Hb A was adjusted to an appropriate value varying from 5.0 to 10.0 by the addition of 1 M acetic acid or 1 M NaOH in D_2O at 37 °C without correction of deuterium oxide. An aliquot of the sample (about 2 ml) was anaerobically withdrawn by a disposable syringe, and was transferred by the injection through a rubber stopper to a 6-ml test tube containing a small amount of solid sodium hydrosulfite. The air in the test tube was previously purged completely by evacuation and flushing with nitrogen. To convert deoxy-Hb A to CO form, 2 ml of pure CO gas was introduced with a air-tight syringe through the stopper. The deoxy- and CO Hb A in D_2O solution were incubated at various pH values for 100 h at 37 °C in the anaerobic chamber. During this period, neither the formation of methemoglobin nor bacterial growth was observed.

Differential Splitting of β146 His and β143 His from Deuterated Hb A—At the end of the incubation, the sample was chilled in ice and

stored at −30 °C until analysis. About 2 ml of Hb A solution (5 mM in heme) was passed through a Sephadex G-25 column equilibrated with 50 mM NH_4HCO_3, pH 8.5, and lyophilized. The dry hemoglobin powder was dissolved in the same buffer containing 2% sodium dodecyl sulfate and the solution was incubated with 25 units of carboxypeptidase A at 30 °C for 3 h. The digest was separated into two fractions on a Sephadex G-25 column. The rapidly eluted, brown fractions contained des-His/Tyr Hb A, while the slowly eluted fractions with intense absorbance at 280 nm contained 1 mol of Tyr and 1 mol of His/Hb dimer as determined by a Hitachi 835 amino acid analyzer. Both fractions were lyophilized. The former fraction was next subjected to digestion in order to obtain β143 His and the latter was dissolved in 0.5 ml of 5% trichloroacetic acid in 99.95% D_2O (Merck, Germany) for the NMR measurement. To obtain β143 His from des-His/Tyr Hb A, the hemoglobin was digested with a mixture of carboxypeptidase A and B in 50 mM NH_4HCO_3, containing 2% sodium dodecyl sulfate, pH 8.5, at 30 °C for 3 h. The incubation was terminated by addition of an equal volume of 10% trichloroacetic acid. Precipitates formed were removed by centrifugation at 3000 rpm for 10 min. The clear supernatant obtained was lyophilized. Amino acids liberated by the digestion were dissolved in 0.5 ml of D_2O (99.95%) for NMR measurement.

^1H NMR Measurement—The spectra were measured with a JEOL FX-100 NMR spectrometer using 5-mm spinning tubes at 25 MHz and 25 °C. The chemical shifts were expressed as parts/million downfield from the proton resonance of sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate. The homogated decoupling method was used to eliminate the proton resonance from HDO and to improve the resonance of NMR signals from aromatic protons. The NMR measurement conditions used were: a spectral width of 1000 Hz with a sweep time of 10 s and each spectrum was the result of 50–100 scans. The intensity ratio of the C2 to C4 proton signal was determined by computer-aided integration of two spectral areas.

RESULTS AND DISCUSSION

The Bohr Effect of Hb A and Des-His Hb A Determined by the pH Dependence of Oxygen Affinity in the Presence of 0.1 M NaCl—When bis-Tris HCl or Tris-HCl buffer is used for OEC measurement, the concentration of chloride ion can not be neglected in the lower pH region of these buffers. Therefore, as the acid component of these buffers, acetic acid was commonly used because this acid was known not to affect the OECs of Hb A and des-His Hb A. Fig. 1, *a* and *b*, show the Hill plots for OECs of Hb A and des-His Hb A at various pH values from pH 6.0 to 9.0 and 25 °C. The Hill plots measured at the pH lower than 6.0 were omitted to avoid complexity of the figure. Fig. 2 shows the variation of oxygen affinity (p_{50}) of Hb A and des-His Hb A as a function of pH ranging from pH 5.0 to 9.0. The plots above pH 6.5 were those obtained from Fig. 1 *a* and *b*. The total Bohr effect of des-His Hb A is considerably smaller than that of Hb A, but with this figure the contribution of β146 His to the Bohr effect could not be fully assessed. Consequently we attempted to calculate the number of Bohr protons released from des-His Hb A and Hb A by two independent but complementary methods as will be described below.

The linkage equation which correlates p_{50} with pH was given by Wyman (1) as follows: $\log p_{50}$ = constant + $\log (H^+ + K_1D)(H^+ + K_2D) - \log (H^+ + K_1CO)(H^+ + K_2CO)$, where K_1CO and K_2D are the ionization constants for a hypothetical oxygen-linked group responsible for the alkaline Bohr effect in CO and deoxy-Hb A, and K_2CO and K_2D are those responsible for the acid Bohr effect. As the plots in Fig. 2 span in the range from pH 5.0 to 9.0, these data must be reliable enough to simultaneously estimate the pK_a values for two assumed ionization groups involved in the acid and alkaline Bohr effects of Hb A or des-His Hb A and were fitted to the linkage equation by the curve fitting method using computer graphic display. The most consistent values for a set of pK_a were: for HbA, pK_1D = 8.0, pK_1CO = 6.85, pK_2D = 4.8, and pK_2CO = 5.5; and for des-His Hb A, pK_1D = 7.95, pK_1CO = 7.3, pK_2D = 5.35, and pK_2CO = 6.0. The total number of protons released from Hb A and des-His Hb A in the presence of 0.1 M NaCl upon oxygenation can be calculated theoretically with a set of pK_a values for individual hemoglobins at given pH values using the following equation: $dX = 4(K_1CO/$

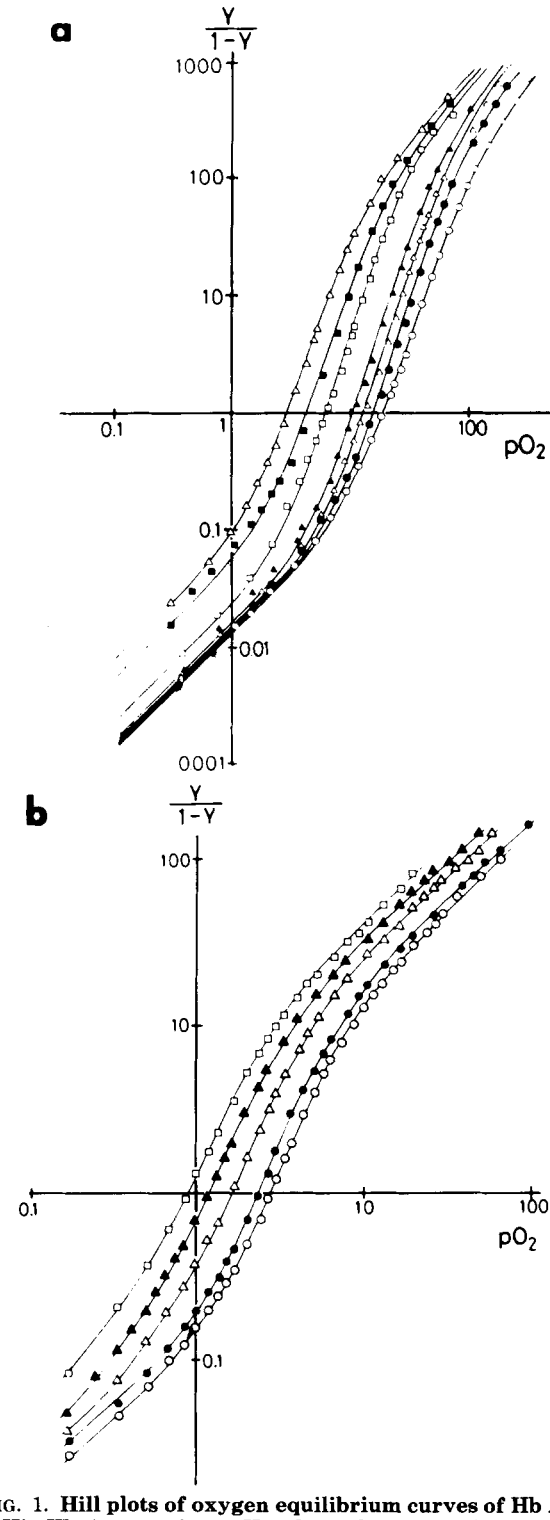

FIG. 1. **Hill plots of oxygen equilibrium curves of Hb A and des-His Hb A at various pH values.** Oxygen equilibrium curves were measured by an automatic recording apparatus at 25 °C in the presence of 0.1 M NaCl. *a*, Hill plots of oxygen binding of Hb A measured at pH 6.51, 6.77, 6.94, 7.22, 7.42, and 8.24 from *right* to *left*. *b*, Hill plots of des-His Hb A measured at pH 6.5, 7.0, 7.5, 8.1, and 8.7 from *right* to *left*. Heme concentration of hemoglobin used was 100 μM.

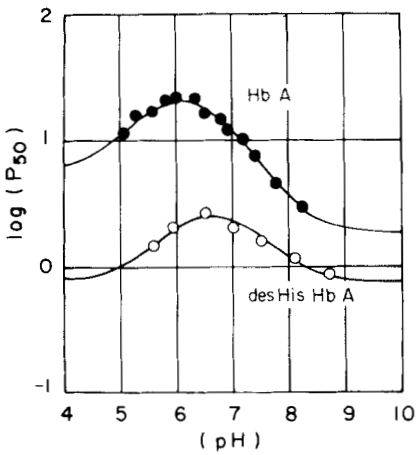

FIG. 2. **The overall oxygen affinity, p_{50}, measured in the presence of 0.1 M Cl^- as a function of pH.** ●, Hb A + 0.1 M NaCl; ○, des-His Hb A + 0.1 M NaCl. Two solid curves were calculated with the ionization constants for two hypothetical oxygen-linked groups estimated to fit the data points.

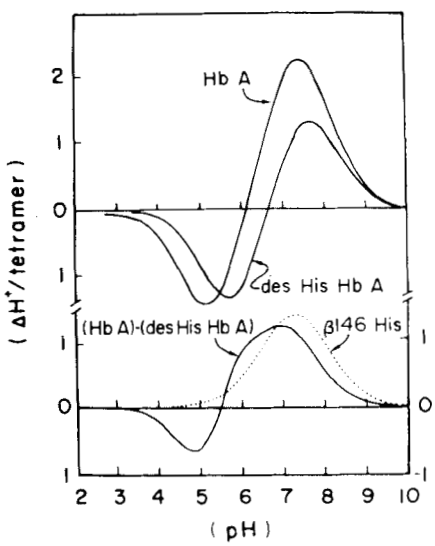

FIG. 3. **Bohr protons per hemoglobin tetramer dissociated by Hb A and des-His Hb A upon oxygenation.** The two *solid curves* in the *upper* part of this figure were calculated with the ionization constants involved in the alkaline and acid Bohr effects for Hb A and des-His Hb A, respectively. The set of ionization constants used for the calculation were as follows; $pK_1D = 8.0$, $pK_1CO = 6.85$, $pK_2D = 4.8$, $pK_2CO = 5.5$ for Hb A, and $pK_1D = 7.95$, $pK_1CO = 7.3$, $pK_2D = 5.35$, $pK_2CO = 6.0$ for des-His Hb A. The *solid curve* in the *lower* part of this figure illustrates the difference in the number of Bohr protons between Hb A and des-His Hb A as given by the curve for Hb A minus the curve for des-His Hb A. The *dotted curve* indicates the Bohr protons released from β146 His of Hb A on oxygenation, which was calculated with two pK_a values determined in this study.

$((H^+) + K_1CO) + K_2CO/((H^+) + K_2CO) - K_1D/((H^+) + K_1D) - K_2D/((H^+) + K_2D)$, where dX is the number of protons released from Hb A or des-His Hb A on oxygenation per Hb tetramer. Fig. 3 shows the Bohr effect associated proton release from Hb A and des-His Hb A on oxygenation as a function of pH ranging from pH 4.0 to 9.0. The maximum numbers calculated for protons released from Hb A and des-His Hb A are 2.28 mol/mol of tetramer and 1.43 mol/mol of tetramer at pH 7.5, respectively. The maximum proton uptakes on oxygenation of Hb A and des-His Hb A occur at pH 5.15 (1.48 mol/mol of tetramer) and pH 5.54 (1.38 mol/mol of tetramer).

Bohr Effect Determined by the Differential Titration Method for Hb A and Des-His Hb A in the Presence of 0.1 M Cl^-—A typical example for differential titration was illustrated in Fig. 4. The pH profile (Fig. 5) for the number of Bohr protons released or taken up on oxygenation of Hb A which was obtained by the direct differential titration method agreed well with that obtained from pH dependence of log p_{50}. The difference in the maximum number of released Bohr protons between Hb A (Fig. 5*a*) and des-His Hb A (Fig. 5*b*) at pH 7.5 was 0.85 mol/mol of tetramer which corresponds to about 44% of that for Hb A. This percentage value closely resembled that previously proposed by Kilmartin *et al.* (2). They considered that the difference is due to lack of β146 His in Hb A and the contribution of β146 His to alkaline Bohr effect is about 40%.

The Bohr effect of des-His Hb A was reported by Kilmartin and his colleagues (14, 31). The oxygen affinity was measured as a function of pH, from 6.0 to 8.2, using 0.2 M sodium phosphate buffers, and from pH 8.4 to 10.0, using 0.05 M sodium borate buffer. Kilmartin also demonstrated the observed Bohr effect of des-His Hb A as the pH profile of the number of protons released on oxygenation (14, 32), and concluded that the pK_a values of the acid Bohr groups should be shifted to more alkaline pH values in order to consistently fit the observed data although there are few data points for des-His Hb A in the pH region below pH 6.0. When the theoretical curves for the Bohr proton release or binding as a function of pH were drawn with mutually consistent pK_a values estimated by us, the pH values were found to shift from 5.15 for Hb A to pH 5.54 for des-His Hb A and to be different from their results. This discrepancy in the pH shift may be ascribed to the fact that their assay system for Bohr effect did not contain chloride ion at the concentration of 0.1 M. Instead, phosphate buffers and borate buffers were used.

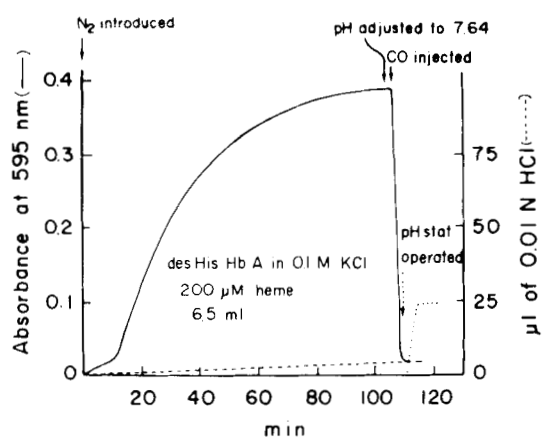

FIG. 4. **A typical trace for the measurement of Bohr protons released from des-His Hb A on CO binding.** The des-His Hb A (200 μM) in 0.1 M KCl was placed in the titration vessel and was deoxygenated by purging with N_2 gas and by mild stirring. The deoxygenation process was recorded by monitoring the absorbance change at 595 nm as a function of time up to 120 min. When the absorbance reached a plateau, pH of the hemoglobin solution was adjusted to the stated value (here pH 7.64) with 0.1 N HCl, and subsequently the pH value to be controlled by pH stat was programmed. Next, pure CO gas (about 2 ml) was injected with a gas-tight syringe to the gas phase through a rubber septum and CO binding process was monitored by the decrease in the absorbance at 595 nm, which is an isosbestic point between oxy- and CO hemoglobins. At the same time, pH stat was operated to control the pH set and the volume of 0.01 N HCl required to maintain the pH was recorded. Even after CO completely bound to the deoxy-hemoglobin, the absorbance did not come to zero. This is probably due to the increased turbidity occurring on deoxygenation.

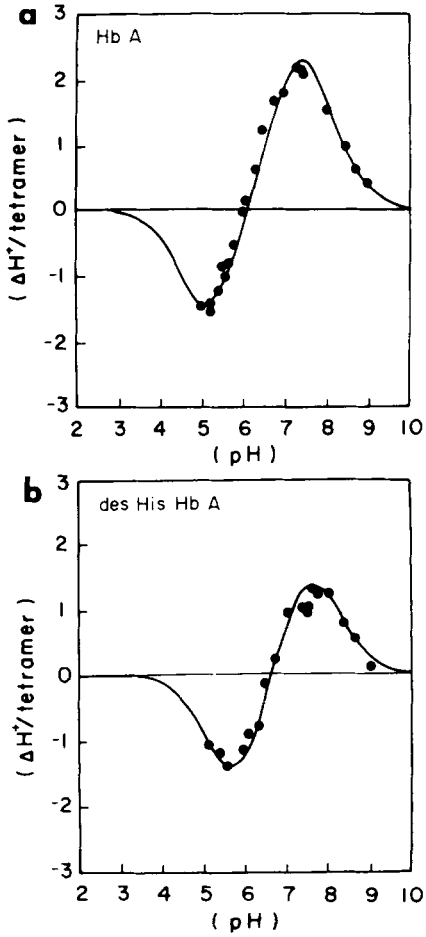

FIG. 5. **Bohr effect of des-His Hb A and Hb A in the presence of 0.1 M KCl.** The data were obtained by the pH stat method which was described under "Materials and Methods" and in the legend for Fig. 4. The number of Bohr protons released from Hb A and des-His Hb A on CO binding at a pH set was the mean values of two titration experiments and was illustrated by *closed circles*. The titration below pH 5.0 was unsuccessful because of the acid-catalyzed autoxidation of hemoglobins and denaturation. The *solid curves* indicate those calculated with four optimal pK_a values for two hypothetical ionization constants which were obtained by applying curve fitting analysis with the aid of computer graphic display to these plots. The *solid curves* for Hb A (*a*) and des-His Hb A (*b*) were identical with those described in the legend to Fig. 3.

The Bohr effect was found to depend critically on the concentration of chloride ion (21, 33). Consequently, this fact clearly indicates that the mechanism for the Bohr effect is not unique and is determined not only by alteration of protein structure, but also by the solvent conditions, namely whether the system contains chloride ion or organic phosphates (9, 26).

^1H NMR Measurement Experiment for the Measurement of $H \rightleftharpoons D$ Exchange Rate of β146 His and β143 His—The accuracy of the ^1H NMR measurement was first assessed by quantitative analysis of L-histidine in D_2O. A 0.5-ml sample of D_2O solution containing 20–320 μg of L-histidine and 1 μmol of 4-aminotriazole as an inner standard was used for the quantification of C2 and C4 protons of histidine. The standard deviation calculated with the data obtained from five measurements with the same sample was: S.D. = 20% for 20 μg of L-histidine, 9% for 89 μg, 4% for 163 μg, and 1% for 320 μg. As our NMR samples contain about 500–700 μg of L-histidine, the error must be within 4% under the conditions of NMR measurement used in this study even though 30% of C2 protons were replaced by deuterium. Fig. 6 shows the ^1H

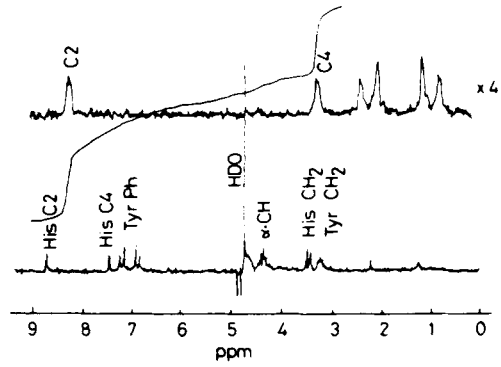

FIG. 6. **^1H NMR spectra of amino acids split off by carboxypeptidase A digestion from Hb A.** Aromatic proton region from 6.5 to 9.0 ppm was enlarged 4-fold to integrate the amounts of C2 and C4 protons.

NMR spectra of amino acids split off from Hb A by carboxypeptidase A digestion. The HDO signal that arose from exchangeable NH protons was almost eliminated by using the homogated decoupling method. For this reason, the resolution of proton resonances in the aromatic proton region was greatly improved. The imidazole C2 and C4 protons of histidine are 8.70 and 8.10 ppm downfield from the standard proton resonance. The four signals around 7.0 ppm are those from the aromatic protons of tyrosine. The amino acid analysis for the first carboxypeptidase A digest indicates that this fraction exclusively contained 1 mol of His and Tyr per Hb dimer. The acid-soluble fraction obtained by the second digestion contained 2 mol of Lys, 1 mol of His, 2 mol of Ala, 1.5 mol of Leu, 1 mol of Arg, 1 mol of Tyr, 1 mol of Ser, and 1.5 mol of Thr per dimer. Therefore, the histidine residue liberated by the first carboxypeptidase A digestion was confirmed to be β146 His, and that observed in the second digest was β143 His. The exchange ratio was determined as the ratio of the amount of C2 protons to that of C4 protons by integration curve. The exchange rate, k_H, is given by -ln(exchange ratio)/incubation time (h) (3).

Technical Problems for pK_a Determination of Histidine in Hemoglobin—The differences in temperature at which OEC measurement and $H \rightleftharpoons D$ exchange reactions were carried out should be taken into account in discussing the contribution of β146 His and β143 His to the total Bohr effect of hemoglobin. In this case, the dependence of the Bohr effect and pK_a value of histidine on solution temperature, and the effect of D_2O on pH measurement are to be pointed out. First, Antonini *et al.* (30) measured the Bohr protons released from Hb A upon oxygenation directly at various pH values and temperatures. Although direct comparison of our results with theirs is impossible, according to their data a 10 °C difference in temperature had not so large an effect on the Bohr effect of Hb A. Second, the pK_a value of L-histidine was determined by two distinct methods, one based upon pH dependence of chemical shifts of C2 protons mesured at 25 °C, and the other an $H \rightleftharpoons D$ exchange reaction of histidine performed at 37 °C. The pK_a values obtained were identical. Third, L-histidine in D_2O and H_2O was pH-titrated by using a glass electrode. The pK_a value for the histidine in H_2O solution was very similar to that for the D_2O solution. Therefore, with respect to the pH of the histidine solution, the deuterium effect was found to be neglected.

To evaluate the proportion of back-exchange of deuterated histidine residue occurring during subsequent experimental procedures, a model experiment was carried out using completely deuterated L-histidine which was prepared by the

incubation of L-histidine-free base in 99.95% D_2O at 60 °C overnight, followed by lyophilization. To 2 ml of 5 mM Hb A solution in 50 mM NH_4HCO_3, pH 8.5, containing 1% sodium dodecyl sulfate, 1 mg of the deuterated L-histidine was added and this solution incubated without carboxypeptidase A under the same conditions as those for the carboxypeptidase A digestion. The solution was applied on a Sephadex G-25 column to separate free L-histidine from Hb A. The fractions containing deuterated L-histidine were combined and lyophilized after addition of trichloroacetic acid to a final concentration of 5%. Dry powder was dissolved in 0.4 ml of D_2O for ^1H NMR measurement. As a result, back-exchange of deuterium-incorporated histidine was too small to be detected under the conditions employed in the model experiment.

Determination of pK_a Values for β146 His and β143 His in CO and Deoxy-Hb A in the Presence of 0.1 Cl^-—A pK_a value was obtained as a pH corresponding to the midpoint of the sigmoidal curve of k_ψ *versus* pH as shown in Fig. 7. In the presence of 0.1 M NaCl, the pK_a values for β146 His of deoxy- and CO Hb A were 8.0 and 6.5, respectively, and those for β143 His of deoxy- and CO Hb A were 5.2 and 6.0, respectively. Our pK_a value for β146 His of deoxy-Hb A is quite consistent with that proposed previously by Ohe and Kajita (3–5) (8.0), and Kilmartin (32) (8.1) but that for the residue of CO Hb A is 0.5 pH units lower than previous ones (7.0 and 7.1). The pK_a value for the β143 His of CO Hb A is very similar to that presented by Ohe and Kajita (5). β143 His in deoxy-Hb A had a lower pK_a value than the one (5.6) they reported. Ohe and Kajita (5) confirmed that the deuterium exchange reaction of histidine is a base-catalyzed reaction, and that the pK_a and log k_ψ, rate constant of the rate-limiting process of this reaction, for β146 His and for β143 His of deoxy- and CO Hb A follow the Brønsted equation. Fig. 8 shows that Brønsted plots for these histidine residues of CO and deoxy-Hb A are undoubtedly close to a line, indicating the complete accessibility of these residues to the solvent in both deoxy- and CO Hb A and, moreover, showing reliability of the values obtained in this study.

Evaluation of the Contribution of β146 His and β143 His to Total Bohr Effect on Hb A in the Presence of 0.1 M Cl^-—The Bohr effects expressed as the number of Bohr protons released at various pH values on oxygenation of Hb A and des-His Hb A were initially calculated with the pK_a values obtained indirectly by curve fitting analysis to their log p_{50} *versus* pH relations and were confirmed to be very accurate by the direct differential titration conducted for these hemoglobins over the wide pH range. The determination of pK_a values for β143 His and β146 His, and also α1 Val (9) were performed on carbonmonoxide-bound normal and des-His Hb A. In liganded form, the effect of species of ferrous ligands on the Bohr effect and pK_a values of these amino acids must be taken into consideration. Concerning this, Antonini *et al.* (30) showed that the Bohr effect is the same, or very nearly so, for oxygen and carbonmonoxide as with ferrous ligands. Therefore, the argument for the contribution of these amino acid residues to the Bohr effect was advanced without making any correction.

In the presence of 0.1 M NaCl, the number of protons released from β146 His and from β143 His of Hb A on oxygenation is illustrated in Fig. 9 as a function of pH together with the total number of Bohr protons from Hb A for a comparison. The theoretical curve is given by a simple addition of both curves indicating the number of protons from 2 histidine residues as a function of pH. This figure clearly shows that β143 His can account for half the acid Bohr effect

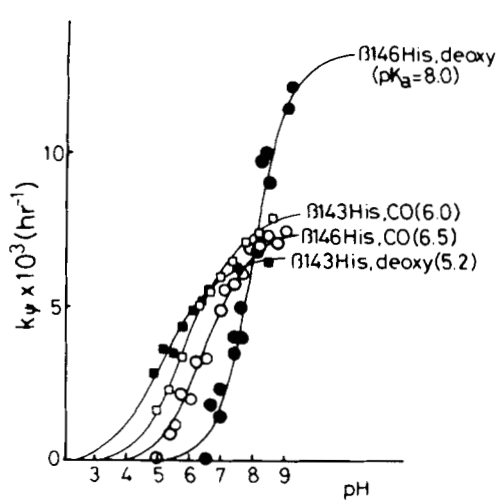

FIG. 7. **k_ψ-pH curves of β146 His and β143 His in deoxy- and CO Hb A.** ●, β146 His in deoxy-Hb A; ○, β146 His in CO Hb A; □, β143 His in CO Hb A; ■, β143 His in deoxy-Hb A.

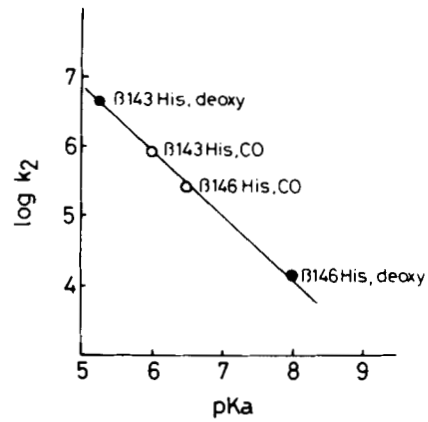

FIG. 8. **Brønsted plots of log k_2 and pK_a for β143 His and β146 His in CO and deoxy-Hb A.**

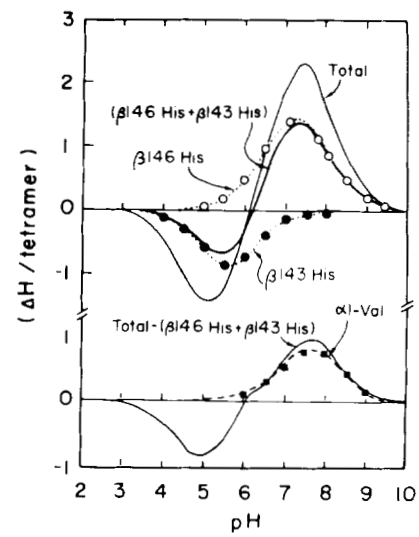

FIG. 9. **Contribution of β146 His and β143 His to the Bohr effect of Hb A.** Total Bohr protons released from Hb A on oxygenation was calculated as a function of pH with four ionization constants for two hypothetical oxygen-linked groups. The *thick solid curve* indicates the simple addition of two curves indicating the Bohr protons from β146 His (○) and β143 His (●) of Hb A on oxygenation. A *solid curve* shown in the *lower* part of this figure is the difference between total Bohr protons from Hb A and those from β146 His and β143 His. ■, Bohr protons released from α1 Val of Hb A.

at pH 5.5, and β146 His can contribute to about two-thirds of the alkaline Bohr effect at pH 7.5. The residual Bohr effect (= total − β146 His − β143 His) is also shown in the lower part of this figure. The α-amino group of N-terminal amino acid, α1 Val, of the α subunit has been pointed out as playing a role in the alkaline Bohr effect (7, 9). Van Beek and De Bruin (9) previously reported that the pK_a values for this group are 8.0 for deoxy-Hb A and 7.25 for CO Hb A in the presence of 0.1 M NaCl. These data are very similar to those obtained by Garner *et al.* (7). To assess whether this amino group can be responsible for the residual alkaline Bohr effect, the number of protons dissociated from two groups per tetramer on oxygenation was calculated with these pK_a values and plotted as a function of pH (Fig. 9). These plots considerably fit the alkaline part of the residual Bohr effect, clearly indicating that the alkaline Bohr effect for Hb A can be almost completely explained by the major involvement of two amino acids, β146 His and α1 Val for the alkaline effect, and β143 His can contribute at most to less than 50% of the acid Bohr effect.

As amino acid residues responsible for the alkaline Bohr effect of Hb A, α89 His and α20 His have also been proposed by Ohe and Kajita (5), and α122 His by Nishikura (6). The involvement of α122 His in the alkaline Bohr effect is not as great but has been plausibly discussed on the basis of evidence that human and llama hemoglobins, in which an amino acid residue of α122 was replaced by other amino acids, and mutant hemoglobins which were considered to lack the interaction with α122 His, had the decreased Bohr effect (28). Ohe and Kajita, however, failed to confirm the pK_a values of α122 His (6.1 for CO Hb A and 6.6 for deoxy-Hb A) as reported by Nishikura. She showed that the C2 proton of α122 His in peptide A was not exchangeable but was in peptide B depending upon pH and she considered that the difference may be due to the change in back-exchange rate. These controversial results cannot be explained at present. To elucidate the contribution of α122 His to the alkaline Bohr effect of Hb A in the presence of 0.1 M NaCl, more careful examination and measurement of pK_a values for this residue are needed.

Kilmartin *et al.* (29) suggested that the involvement of α89 His in the alkaline Bohr effect is highly unlikely, judging from structural and functional aspects of cat hemoglobin, which lacks the amino acid but had a normal Bohr effect. In des-Arg Hb A, however, this residue was suggested to involve the alkaline Bohr effect. On the whole, our evidence clearly indicates that the involvement of these residues in the alkaline Bohr effect is negligible under the conditions employed in this study.

As for the acid Bohr effect, β143 His is only an amino acid residue which so far has been expected to be responsible for the Bohr effect of Hb A. This idea came from extremely low pK_a values of this residue in CO (6.1) and deoxy-(5.6) Hb A, which were measured by means of mass spectra for the phenylthiohydantoin derivative of deuterated histidine by Ohe and Kajita (5) and on the decreased acid Bohr effect of Hb F, in which β143 His was replaced by Ser (27). Since the acid Bohr effect of Hb F is greatly diminished but not completely lost, the involvement of other amino acids beside β143 has been suspected. However, no additional amino acid residues have been identified to account for the residual acid Bohr effect. Our result concerning the acid Bohr effect in the presence of 0.1 M NaCl indicates that as far as our analysis is concerned, the part to which β143 His can contribute may occupy only less than 50% of the total acid Bohr effect. The residual part of the acid Bohr effect is also shown in the lower part of Fig. 9 as the differences in the number of protons taken up by Hb A on oxygenation as a function of pH. Although we cannot provide the candidate(s) for amino acid(s) responsible for this part, the involvement of an ionization group with $pK_a = 4.6$ and 5.4 for deoxy- and CO Hb A, respectively, may be tentatively proposed.

The lower pK_a value of β143 His in deoxy-Hb A than that in CO Hb A has been explained by Ohe and Kajita (95) in terms of the difference in the proximity of the positively charged ε-amino group of β144 Lys to this residue between CO and deoxy-Hb A. According to their calculation, the distance was 5 Å for deoxy-Hb A and 9 Å for CO Hb A. However, our estimation which was carried out using x-ray data of Baldwin (34) for CO Hb A and Fermi (35) for deoxy-Hb A greatly differed from theirs. The distance between the imidazole N (δ) atom of β143 His and the ε-amino N atom of β144 Lys are found to be 5.86 Å for CO Hb A and 6.47 Å for deoxy-Hb A, and those between the imidazole N (ε) atom of β143 His and the ε-amino N atom of β144 Lys are calculated 7.47 Å for CO Hb A and 8.23 Å for deoxy-Hb A. The difference in the distance are only 0.6 Å and 0.8 Å for the former and the latter, respectively. Both are considerably shorter than 4 Å which had been proposed by Ohe and Kajita (5). Therefore, the involvement of β144 Lys in a lowering of the pK_a value of β143 His may be unlikely. Instead, the ε-amino groups of $\beta_1$82 Lys and $\beta_2$82 Lys are expected to have the lowering effect on the pK_a value of β143 His. Indeed, the distance between $\beta_1$82 Lys and $\beta_1$143 His is 8.1–9.1 Å for CO Hb A and 3.6–4.9 Å for deoxy-Hb A and that between $\beta_2$82 Lys and $\beta_1$143 His is 5.9–6.2 Å for CO Hb A and 11.3–13.1 Å for deoxy-Hb A. Consequently, we assumed that the decreased pK_a value of β143 His in deoxy-Hb A may be due to the increased close proximity of the positively charged ε-amino group of β82 Lys to its residue. In des-His Hb, the pK_a values of β143 His have to shift to 4.05 for CO Hb A and to 5.95 for deoxy-Hb A to completely explain the altered acid Bohr effect if β143 His is only an acid Bohr group even in this hemoglobin. There is little shift in the pK_a value of β143 His in the CO form, but the large shift as much as 1.15 pH units to acid pH side must occur in the deoxy form. We cannot offer the molecular mechanism to explain the extraordinarily low pK_a value assumed for β143 His in the deoxy form of des-His Hb A, but it should be confirmed by the $H \rightleftharpoons D$ exchange method or the ^1H NMR titration technique for des-His Hb A in the near future. At present, we cannot account for the change in the acid Bohr effect brought about by removal of β146 His from Hb A because no crystallographic data concerning des-His Hb A so far has been presented.

Our accurate determination for the Bohr effect of normal and des-His Hb A by two independent methods at the same time and under the similar solvent condition and also for the pK_a values of β146 His in CO and deoxy-Hb A permits exact evaluation for the effect of removal of β146 His from Hb A. We first attempted to calculate the differences in overall Bohr proton release or binding between normal and des-His Hb A and presented the result in the lower part of Fig. 3 together with the Bohr protons released from β146 His on oxygenation of Hb A as a function of pH. If the change in the Bohr effect of des-His Hb A compared with that of Hb A were ascribed to the restricted effect caused by removal of β146 His alone, the overall differences in the Bohr protons from both hemoglobins should be quite equal to those from β146 His. The difference (Fig. 9), however, did not show the simple shape with a peak as assumed by the involvement of a single ionization group. This fact clearly indicates that deletion of β146 His alone greatly affects the Bohr effect of des-His Hb A through its tertiary and/or quaternary structure changes

although an idea similar to the conclusion experimentally deduced by us has been described by Saroff (36) who had attempted the theoretical analysis for Kilmartin's data concerning the Bohr effect of des-His Hb A and Hb A.

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