Changes in pK_a Values of Individual Histidine Residues of Human Hemoglobin upon Reaction with Carbon Monoxide†

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ABSTRACT: We performed the deuterium-exchange reaction on human hemoglobin in its carbon monoxy and deoxy forms at various pH values and 36.5 °C. Peptides containing only one histidine residue were separated from tryptic and chymotryptic digests of the deuterated hemoglobin, except for two peptides which contained the α-87 and α-89 and the β-116 and β-117 histidine residues, respectively. The pseudo-first-order rate constant for the exchange reaction of each histidine residue was measured by using the mass spectrometric method. We obtained the following results. The pK_a values for the α-20, α-89, and β-146 histidine residues in deoxyhemoglobin decreased significantly, while that for the β-143 histidine residue increased significantly on ligation. The pseudo-first-order rate constants were virtually zero for the α-45, α-58, α-87, β-63, and β-92 histidine residues which are linked with a heme group, and also for the α-122 histidine residue which is buried at the $\alpha_1\beta_1$ contact in the hemoglobin molecule. No change was detected in the pK_a values on ligation for the other histidine residues in deoxyhemoglobin.

The change in the oxygen affinity of hemoglobin (Hb)[1] with pH (Bohr effect) has long been studied by many workers as a typical case of heterotropic interaction in an oligomeric protein. Recently, its mechanism has been studied mainly by X-ray crystallography. Perutz (1970) showed that the effect is due to the conformational change in the subunits of Hb upon ligand binding and subsequent changes in the mode of interaction among the subunits. Changes in the interaction of N-terminal groups with C-terminal groups of α and β chains and of the imidazole group of the β-146 histidine residue with the carboxyl group of β-94 aspartic acid in the β chain with conformation states were described by Perutz et al. (1968, 1969) and Perutz (1970). However, the pK_a values of the ionizing groups cannot be determined by X-ray study.

The pK_a value of the imidazole group of β-146 histidine residue was shown to change upon ligation using NMR (Greenfield & Williams, 1972; Kilmartin et al., 1973) and the deuterium-exchange method (Ohe & Kajita, 1977a,b). The pK_a value of the α-amino group of α-1 valine was also shown to change on ligation using the method of chemical modification (Kilmartin & Rossi-Bernardi, 1971; Garner et al., 1975). However, the Bohr effect cannot be quantitatively explained by the shifts in the pK_a values of these two groups, as pointed out by Kilmartin et al. (1973). Therefore, it is worthwhile to investigate the possible role of the remaining Bohr groups.

The deuterium-exchange method has been useful for determining the individual pK_a values of histidine residues in Hb, as we reported previously (Ohe & Kajita, 1977a,b). In this investigation, we determined the individual pK_a values of all deuterium-exchangeable histidine residues in HbCO and deoxy-Hb and found that the observed Bohr effect of Hb on the binding of carbon monoxide is ascribable to the α-20, α-89, and β-143 histidine residues in addition to the previously identified α-1 valine and β-146 histidine residues.

Experimental Procedures

Hb Preparation. Human Hb A was isolated from fresh venous blood by using the procedure of Benesch et al. (1968) and purified by anion-exchange chromatography (Huisman & Dozy, 1965; Dozy et al., 1968).

Buffer Solution. Deuterated acetic acid–sodium acetate, Bistris-DCl, or Tris-DCl was used at a concentration of 0.1 M. Buffer materials were dissolved in deuterium oxide (99.95%). The ionic strength of the buffer was adjusted to 0.1 with 1 M sodium chloride dissolved in deuterium oxide. All deuterium compounds (CD_3COOD, DCl, and D_2O) were purchased from Merck Co.

Deuterium-Exchange Reaction of HbCO and Deoxy-Hb. HbCO was prepared by exposing a solution of HbO_2 to an atmosphere of wet carbon monoxide for 30 min. Next, 200 μL of 4.8 mM HbCO was added to 2.1 mL of the buffer solution at various pH values and the mixture was incubated at 36.5 °C for 24 h for the deuterium-exchange reaction.

HbO_2 (5.0 mM, 200 μL) was added to 2.0 mL of the buffer solution of various pH values under N_2 gas, to which 100 μL of sodium dithionite dissolved in deuterium oxide was added. The concentration of sodium dithionite was twofold in excess of that of heme in Hb. The mixture was sealed under nitrogen and incubated at 36.5 °C for 24 h for the deuterium-exchange reaction.

The tetramer–dimer dissociation did not occur because the Hb concentration used in the present experiment was very high.

The absorption spectrum in the visible region and the pH of the incubating samples were measured with a Cary Model 14 spectrophotometer, before and after the deuterium-exchange reaction, which was carried out in the pH range of 5–9 because Hb became denatured below pH 5. Met-Hb formation during the incubation was not detected spectrophotometrically over the entire pH range used. The pH of the sample was measured with a Radiometer titrater, Model TTT2, at 36.5 °C without correction for deuterium oxide (Roberts et al., 1968). The Bohr effect is not affected by deuterium oxide (Tomita & Riggs, 1970).

Isolation of α^{HMB} and β^{HMB} Chains of Hb. Globin parts of α^{HMB} and β^{HMB} chains of deuterated Hb were separated by the method of Geraci et al. (1969) with slight modifications.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; α^{HMB} chain, the mercuribenzoate α chain; β^{HMB} chain, the mercuribenzoate β chain; *p*-HMB, *p*-(hydroxymercuri)benzoate; CM, carboxymethyl; Hb, hemoglobin; HbCO, (carbon monoxy)hemoglobin; HbO_2, oxyhemoglobin.

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Table I: Amino Acid Composition of Peptides from the α Chain[a]

amino acid	peptide I	II[b]	III[b]	IV	V	VI	VII	VIII[c]	IX[c]	X[c]
Lys							1.12	1.33	1.00	1.24
His	1.00	0.96	1.00	1.10	1.00	1.00	1.00	1.83	1.00	1.00
Arg										
$CySO_3H$				1.00						
Asp					3.80		1.12			
Thr				0.74	0.73	1.05				
Ser				0.69			1.92			
Glu		1.23	0.96				1.16			
Pro	1.07	1.00			1.06	1.02				
Gly			1.58				1.20			1.88
Ala		2.57	1.23		3.26	1.25	1.28	1.00	0.68	
Val			0.62	1.60	2.46	0.97	1.00			
$Met(O_2)$					0.87					
Leu		0.92		2.90	1.40		1.08			
Tyr			0.54							
Phe	2.57	0.82								

[a] Peptides were hydrolyzed in 5.7 N HCl at 105 °C in vacuo for 24 h. The results are expressed as molar ratios. [b] Peptides II and III were obtained after repeating the paper chromatography–electrophoresis step as described under Experimental Procedures. [c] Peptides VIII, IX, and X were obtained after repeating the paper chromatography step as described under Experimental Procedures.

After the deuterium-exchange reaction, the Hb solution was passed through a column of Sephadex G-25 (1.2 × 20 cm) for equilibration with a buffer containing 10 mM phosphate and 0.1 M NaCl (pH 6.1). The Hb molecule was split into α^{HMB}, the mercuribenzoate α chain, and β^{HMB}, the mercuribenzoate β chain, by adding *p*-(hydroxymercuri)benzoate (*p*-HMB) dissolved in a minimum volume of 0.1 N NaOH. The concentration of the *p*-HMB added was about sixfold in excess of that of Hb. The sample was left in a cold room at 4 °C for 12 h. Next, the solution was equilibrated with 10 mM phosphate, pH 6.5, by passage through a Sephadex G-25 column of 2.5 × 20 cm. The solution was applied to a CM-cellulose (Whatman CM-52) column of 1.5 × 2.5 cm equilibrated with 10 mM phosphate, pH 6.5. The following column chromatography was carried out at 4 °C. The deuterated β^{HMB} chain was not bound to the column and was eluted with the same buffer. The deuterated β^{HMB} chain was collected, and then the column was washed with about 10 column volumes of 10 mM phosphate, pH 7.2, in order to elute nonsplit Hb. The deuterated α^{HMB} chain was eluted from the column with 10 mM phosphate (pH 8.0). Each of the deuterated HMB chains migrated as a single band in cellulose acetate film electrophoresis at pH 8.6. The isolated chain was added to acid acetone (acetone containing 0.006 M HCl) and left for 2 h at 4 °C. The mixture was centrifuged, and the precipitated globin was dissolved in deionized water and lyophilized.

Isolation of Peptides Containing Histidine. Separation of histidine-containing peptides from deuterated globin was carried out by the technique described for tritiated bovine pancreatic ribonuclease (Ohe et al., 1974). Each deuterated α^{HMB} (20 mg) and β^{HMB} (20 mg) globin was oxidized by performic acid by the method of Hirs (1967). The oxidized globin was digested successively for 2 h with trypsin (1 mg) (Worthington Biochemical Corp.) and chymotrypsin (1 mg) (P-L Biochemicals, Inc.) in 2 mL of 1% ammonium carbonate (pH 8.0) at 36.5 °C. After removal of the salt by repeated lyophilization, the digest (16 mg) was subjected to two-dimensional paper chromatography–electrophoresis on four sheets of identical filter paper (Whatman 3MM, 58 × 68 cm). For the first dimension, descending chromatography was performed with 1-butanol–pyridine–acetic acid–water (15:10:3:12 v/v). Electrophoresis with pyridine–acetic acid–water (1:10:289 v/v, pH 3.5, 50 V/cm, 130–140 min) was employed for the second dimension. The peptide spots were detected with a 0.01% 4-phenylspiro[furan-2(3*H*),1′-phalen]-3,3′-dione (Roch fluorescamine)–acetone solution under ultraviolet illumination. One of the four filter papers was used to locate the peptides containing histidine with Pauli solution. The peptide spots containing histidine were cut out of the remaining three filter papers, and the peptides were extracted with 30% acetic acid.

Two-dimensional peptide maps of tryptic–chymotryptic digests of α and β globin after performic acid oxidation are shown in parts A and B of Figure 1. Peptides I–X in the peptide map of the α chain strongly reacted with Pauli reagent. Since peptides VIII, IX, and X were not completely separated from each other by the first chromatography (Figure 1A), they were subjected to rechromatography on the same paper in the same solvent for 2 days after the other peptides had been cut from the peptide map. Peptides II and III were also not completely separated by the first chromatography and were subjected to 4 h of reelectrophoresis conducted in the same manner as the first electrophoresis. Peptides I–VIII for the β digest were strongly positive with Pauli reagent and were clearly separated by a single paper chromatography–electrophoresis.

The peptides were hydrolyzed in 5.7 N HCl at 105 °C in vacuo for 24 h. Amino acid compositions of peptides from α and β chains are shown in Tables I and II. The location of these peptides in the primary structure of Hb A was deduced from their amino acid compositions.

α-chain peptides

$$\overset{45}{\text{Phe-Pro-His-Phe}} \quad \text{(I)}$$

$$\overset{112}{\text{Ala-Ala-His-Leu-Pro-Ala-Glu-Phe}} \quad \text{(II)}$$

$$\overset{20}{\text{Val-Gly-Ala-His-Ala-Gly-Glu-Tyr}} \quad \text{(III)}$$

$$\overset{103}{\text{Ser-His-CySO}_3\text{H-Leu-Leu-Val-Thr-Leu}} \quad \text{(IV)}$$

$$\overset{72}{\text{Thr-Asn-Ala-Val-Ala-His-Val-Asp-Asp-Met(O}_2\text{)-Pro-Asn-Ala-Leu}} \quad \text{(V)}$$

$$\overset{122}{\text{Thr-Pro-Ala-Val-His}} \quad \text{(VI)}$$

Asp-Leu-Ser-^{50}His-Gly-Ser-Ala-Gln-Val-Lys (VII)

^{87}His-Ala-^{89}His-Lys (VIII)

Ala-^{89}His-Lys (IX)

Gly-^{58}His-Gly-Lys (X)

β-chain peptides

Ser-Asp-Gly-Leu-Ala-^{77}His-Leu (I)

^{146}Tyr-His (II)

Leu-^{97}His-Val-Asp-Pro-Glu-Asn-Phe-Arg (III)

Val-^{2}His-Leu-Thr-Pro-Glu-Glu-Lys (IV)

Ser-Glu-Leu-^{92}His-CySO$_3$H-Asp-Lys (V)

Ala-$^{116\ 117}$His-His-Phe (VI)

Ala-^{143}His-Lys (VII)

Ala-^{63}His-Gly-Lys (VIII)

Determination of pK$_a$ Values of Histidine in Hb. Hb was deuterated at various pH values, and the peptides containing a histidine residue were separated as described above. After acid hydrolysis of the peptide, the hydrolysate was dissolved in 1% acetic acid by centrifugation to remove insoluble materials. The yield of histidine residues in each peptide was in a range of 30–50%. The clear hydrolysate (histidine content 0.5–1 μmol) was lyophilized and then subjected to Edman reaction using methyl isothiocyanate in order to measure the amount of deuterium incorporated at the C_2 position of the imidazole ring by using the mass spectrometric method described in a previous paper (Ohe & Kajita, 1977a). The effect of back-exchange of the deuterium-incorporated histidine residue was sufficiently small under the experimental conditions used for measurement of the amount of deuterium in each histidine residue (Ohe et al., 1974; Ohe & Kajita, 1977a). The pseudo-first-order rate constant (k_ψ) of the deuterium-exchange reaction of histidine was obtained by the method reported previously (Ohe & Kajita, 1977a). The hydrogen/deuterium ratios involved in the histidine residue are independent of the yield of each histidine peptide. The k_ψ vs. pH curves for all the histidine residues were fitted to the Henderson–Hasselbach equation, and the pK_a values were obtained as the midpoint of the sigmoidal k_ψ vs. pH curves. We used various buffer solutions for the titration, but the titration curve for each histidine residue was always given as a simple dissociation curve with a K_a value specific to the residue. For the curve fitting, we used a computer, Digital Equipment Corp., Model PDP, 11/40.

The pK_a readings are applicable to water as well as deuterium oxide (Roberts et al., 1969).

Results

The pseudo-first-order rate constants (k_ψ values) of the deuterium-exchange reaction for various histidine residues both in human HbCO and deoxy-Hb are plotted as a function of pH. The plots, k_ψ vs. pH, fit the theoretical sigmoidal curves well by assuming a single pK_a value for each histidine residue, except for the β-116 and β-117 histidine residues. Among the total of 19 histidine residues in the $\alpha\beta$ dimer, we found that

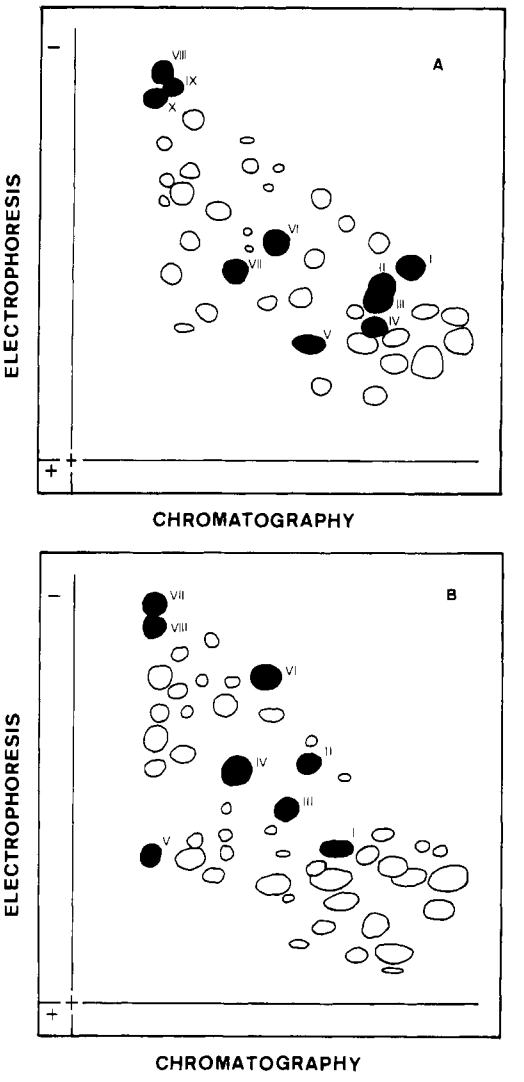

FIGURE 1: Two-dimensional chromatoelectrophoretogram of the tryptic–chymotryptic digest of (A) α-globin and (B) β-globin of Hb. First dimension: descending chromatography with 1-butanol–pyridine–acetic acid–water (15:10:3:12 v/v). Second dimension: electrophoresis with pyridine–acetic acid–water (1:10:289 v/v, pH 3.5, 50 V/cm, 130–140 min).

13 were titrable and 6 were not titrable by the deuterium exchanged. Table III summarizes the pK_a values of the histidine residues thus obtained. From the k_ψ–pH curve, the 19 histidine residues were classified qualitatively as follows.

Titrable histidine residues having different pK_a values in HbCO and deoxy-Hb: α-20, α-89 (Figure 2), β-143 (Figure 3), and β-146. Titrable histidine residues having the same pK_a value in HbCO and deoxy-Hb: α-50 (Figure 4), α-72, α-103, α-112, β-2 (Figure 5), β-77 (Figure 5), β-97 (Figure 5), and β-116+117. Nontitrable histidine residues in HbCO and deoxy-Hb: α-45, α-58, α-87 (Figure 2), α-122 (Figure 4), β-63, and β-92.

The pK_a values for the β-2 histidine residue (Figure 5) in HbCO and deoxy-Hb reasonably agreed with the values which we previously reported (Ohe & Kajita, 1977b) for the histidine residue in HbO_2 and deoxy-Hb in the absence of inositol hexaphosphate.

The pK_a values for the β-146 histidine residue in HbCO and deoxy-Hb agreed well with those determined previously by using NMR (Greenfield & Williams, 1972; Kilmartin et al., 1973) and those in HbO_2 and deoxy-Hb determined by deuterium exchange (Ohe & Kajita, 1977a,b). However, the k_ψ–pH plot for this histidine residue in HbO_2 slightly deviated

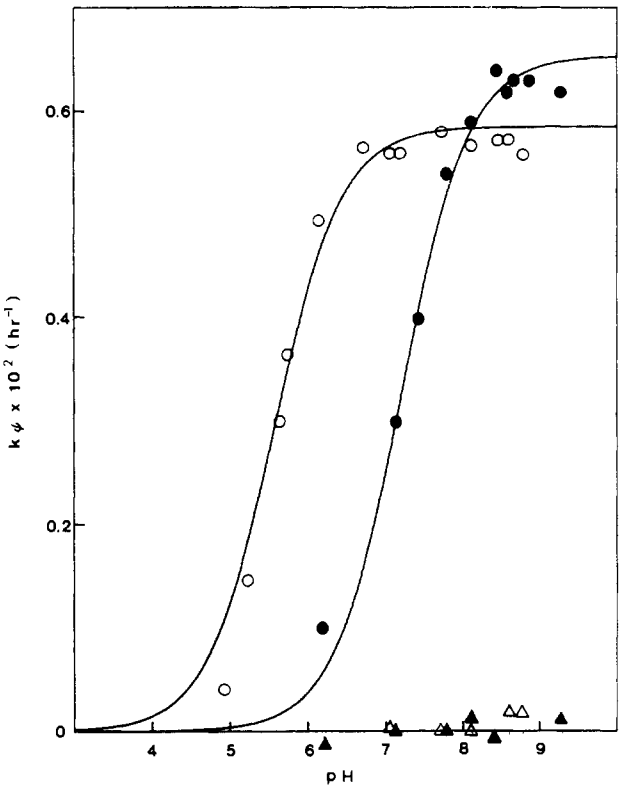

FIGURE 2: k_ψ–pH curves of the α-89 and α-87 histidine residues in Hb. ○, α-89 His in HbCO; ●, α-89 His in deoxy-Hb; △, α-87 His in HbCO; ▲, α-87 His in deoxy-Hb. The k_ψ values for α-87 histidine residue were calculated from the average of k_ψ values for the α-87 and α-89 histidine residues and that for the α-89 histidine residue.

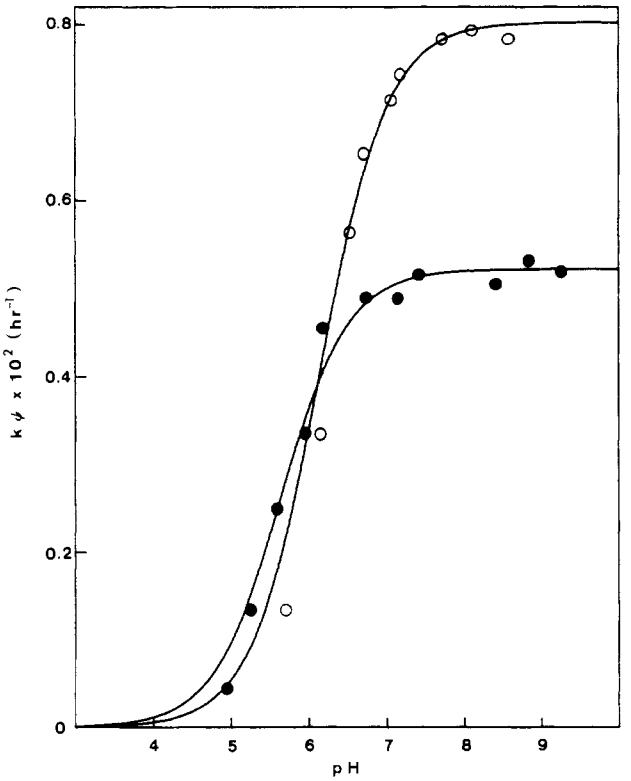

FIGURE 3: k_ψ–pH curves of the β-143 histidine residue in Hb. ○, β-143 His in HbCO; ●, β-143 His in deoxy-Hb.

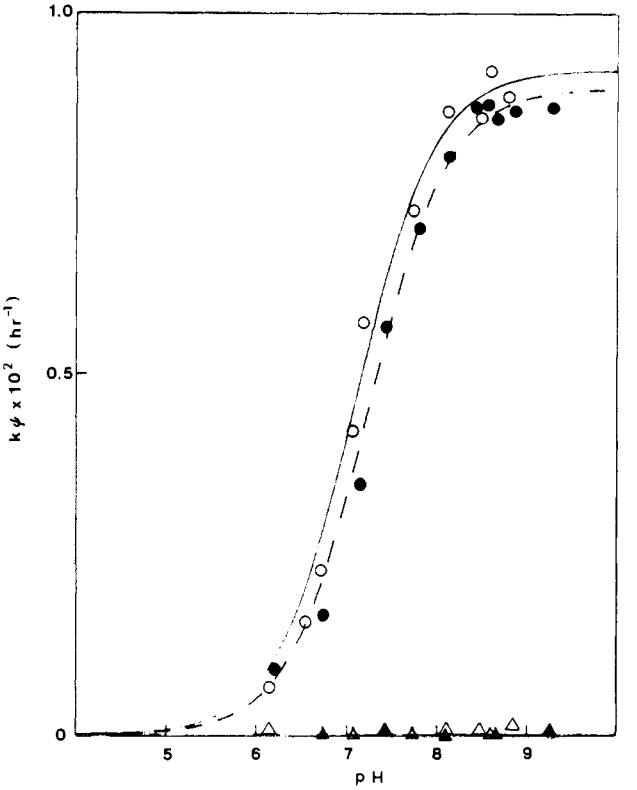

FIGURE 4: k_ψ–pH curves of the α-50 and α-122 histidine residues in Hb. ○, α-50 His in HbCO; ●, α-50 His in deoxy-Hb; △, α-122 His in HbCO; ▲, α-122 His in deoxy-Hb.

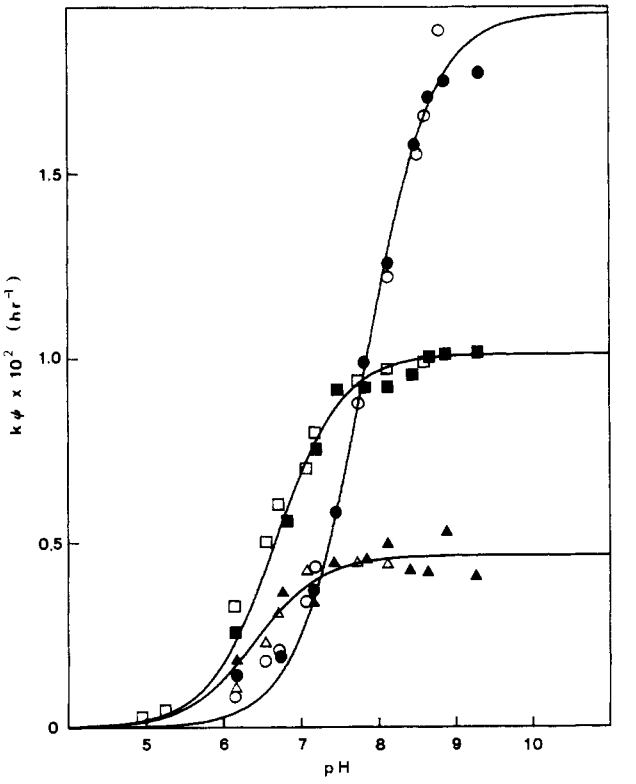

FIGURE 5: k_ψ–pH curves of the β-2, β-77, and β-97 histidine residues in Hb. ○, β-77 His in HbCO; ●, β-77 His in deoxy-Hb; □, β-2 His in HbCO; ■, β-2 His in deoxy-Hb; △, β-97 His in HbCO; ▲, β-97 His in deoxy-Hb.

from the theoretical curve, as we reported previously (Ohe & Kajita, 1977b), although the plot for the histidine residue in HbCO coincided with it (data not shown). The reason is not clear yet, but the microenvironment of the histidine residue in HbO_2 may be more unstable under changing pH conditions than that in HbCO.

As seen in Table III, the pK_a values for the α-20, α-89, and β-146 histidine residues in deoxy-Hb decreased on ligation but

Table II: Amino Acid Composition of Peptides from the β Chain[a]

amino acid	peptide I	II	III	IV	V	VI	VII	VIII
Lys				1.00	1.00		1.14	1.00
His	0.87	1.00	0.97	0.71	0.88	2.02	1.05	0.95
Arg			0.79					
$CySO_3H$					0.82			
Asp	1.06		1.79		1.18			
Thr				0.96				
Ser	1.06				1.00			
Glu			1.36	1.76	1.41			
Pro			1.00	0.98				
Gly	1.33							1.10
Ala	1.00					1.00	1.00	0.82
Val			1.00	0.76				
Leu	1.73		0.86	1.00	1.06			
Tyr		0.76						
Phe			0.79			0.96		

[a] Peptides were hydrolyzed in 5.7 N HCl at 105 °C in vacuo for 24 h. The results are expressed as molar ratios.

Table III: pK_a Values of the Histidine Residues in Hb A

chain	His residue no.	pK_a HbCO	pK_a deoxy-Hb	ΔpK_a
α	20	7.0	7.6	0.6
	45	not titrable		
	50	7.1	7.2	0.1[b]
	58	not titrable		
	72	7.1	7.3	0.2[b]
	87	not titrable		
	89	5.6	7.2	1.6
	103	7.1	7.1	0
	112	8.1	8.1	0
	122	not titrable		
β	2	6.7	6.7	0
	63	not titrable		
	77	7.8	7.8	0
	92	not titrable		
	97	6.4	6.4	0
	116[a]			
	117[a]			
	143	6.1	5.6	−0.5
	146	7.0	8.1	1.1

[a] Titrable but not determinable. [b] The difference is not significant, since the accuracy of pK_a values was ±0.1.

that for the β-143 histidine residue increased. On the other hand, the pK_a values for the α-50, α-72, α-103, α-112, β-2, β-77, and β-97 histidine residues in deoxy-Hb did not show any significant change on ligation.

Peptide VI, which was obtained from the peptide map of the tryptic–chymotryptic digest of oxidized β-globin, contained both β-116 and β-117 histidine residues. Since neither pepsin nor carboxypeptidase A could digest the peptide bond between these histidine residues in peptide VI, only the average k_ψ value of the β-116 and β-117 histidine residues could be obtained as a function of pH; it was virtually the same in HbCO and deoxy-Hb (data not shown).

The k_ψ values for the α-45, α-59, α-87, α-122, β-63, and β-92 histidine residues in HbCO and deoxy-Hb were virtually zero.

Discussion

For determination of the pK_a value of a histidine residue by the deuterium-exchange reaction, different buffer solutions were used for pH ranges of 5–6, 6–7, and 7–9. Furthermore, small amounts of the dimer might be produced during the experiments, since the concentration of Hb used was rather high. Accordingly, it is possible that the K_a value of a histidine residue is affected by the species of anions used as a buffer (Van Beck et al., 1979) and also by formation of the dimer. However, fortunately, the experimental points for titration of a histidine residue were always fitted by a simple dissociation curve with a K_a value specific to each residue, as mentioned under Results. Therefore, the effects of the dimer formation and of anions on the pK_a value are neglected in the following discussion.

Histidine Residues Involved in the Bohr Effect. The pK_a values for the α-20, α-89, and β-146 histidine residues in deoxy-Hb decreased significantly on ligation. Thus, the role of the β-146 histidine residue in the Bohr effect previously proposed by Perutz et al. (1969) was reconfirmed. Furthermore, the α-20 and α-89 histidine residues were shown to contribute to the alkaline Bohr effect. Frier & Perutz (1977) predicted that the β-143 histidine residue would be found responsible for the acid Bohr effect. Their prediction was verified by the present study which showed that the pK_a value of the β-143 histidine residue increased significantly on ligation.

On the other hand, the pK_a values for the α-50, α-72, α-103, α-112, β-2, β-77, and β-97 histidine residues in deoxy-Hb did not show any significant change on ligation. Furthermore, the average k_ψ value of the β-116 and β-117 histidine residues as a function of pH was virtually the same in HbCO and deoxy-Hb. These results suggest that these histidine residues have nothing to do with the Bohr effect.

Recently, the results of tritium exchange of bovine heart cytochrome *c* showed that the k_ψ value for the histidine residue coordinated with heme iron is virtually zero at various pH values (Kimura et al., 1979). In the case of Hb, the α-58, α-87, β-63, and β-92 histidine residues coordinate around heme iron and the α-45 histidine residue interacts with the propionic acid of heme. Accordingly, the deuterium-exchange reaction could not be detected for any of these histidine residues by the techniques used here. Furthermore, we could not determine the pK_a value of the α-122 histidine residue, since its k_ψ value was virtually zero. On the other hand, Nishikura (1978) obtained two kinds of peptides involving the α-122 histidine residue from the tryptic–chymotryptic digest of Hb after the tritium exchange. She reported that one peptide, A, virtually did not incorporate the tritium, while the other peptide, B, incorporated it. Our result has not given clear support to her proposal that the α-122 histidine residue is responsible for the alkaline Bohr effect.

Consequently, the pK_a values of histidine residues having k_ψ values of zero were not determined and we did not investigate whether these residues contribute to the Bohr effect.

Structural Interpretation of the pK_a Values. Minamino et al. (1977) measured the pK_a values of imidazole derivatives by means of NMR and the tritium-exchange titration method. They found that the intrinsic pK_a value of the imidazole group is about 6.5 and is lowered by a neighboring positively charged group but raised by a negatively charged group. Thus, we can estimate the effect of a neighboring charged group from orthogonal atomic coordinates for human adult deoxy-Hb at 2.5-Å resolution (Fermi, 1975) and human adult HbCO at 2.8-Å resolution (J. M. Baldwin, unpublished results) obtained from the Brookhaven Protein Data Bank.

The pK_a values of the α-20 histidine residue were 7.0 and 7.6, respectively, in HbCO and deoxy-Hb. The high pK_a value for the histidine residue in deoxy-Hb suggests an interaction between the imidazole group in the residue and a negatively charged group in the neighborhood. The distance between the δ nitrogen of the imidazole ring of the histidine residue and the carboxyl group of α-23 glutamic acid in the same chain was calculated from the X-ray data to be 7 Å in HbCO and 4 Å in deoxy-Hb, and the interaction between these groups should have raised the pK_a value of the imidazole group. However, the distance between the ϵ nitrogen of the imidazole ring of the histidine residue and the carboxyl group of the glutamic acid was also 4 Å in HbCO, which prevents a normal pK_a for the histidine residue in HbCO.

The pK_a values for the α-89 histidine residue were 5.6 and 7.2, respectively, in HbCO and deoxy-Hb. The normal pK_a value for the histidine residue in deoxy-Hb suggests that the imidazole group does not interact with other charged groups. The low pK_a value for the histidine residue in HbCO suggests a positively charged group in the proximity of the imidazole group. According to the X-ray data, the distance between the imidazole group of the α-89 histidine residue and the ϵ-amino group of α-139 lysine is 8 Å in deoxy-Hb, while that in HbCO is as close as 3 Å which would lower the pK_a values for the imidazole group.

The pK_a values for the β-143 histidine residue were 6.1 and 5.6, respectively, in HbCO and deoxy-Hb. The low pK_a value for the histidine in deoxy-Hb suggests the proximity of a positively charged group to the imidazole group. According to the X-ray data, the distance between the imidazole group of the β-143 histidine residue and the ϵ-amino group of β-144 lysine is 9 Å in HbCO and 5 Å in deoxy-Hb, which is close enough to lower the pK_a value of the imidazole group.

The pK_a values for the β-146 histidine residue were 7.0 and 8.1, respectively, in HbCO and deoxy-Hb. The electron density maps of horse (Bolton & Perutz, 1970) and human (Muirhead & Greer, 1970) deoxy-Hb clearly showed that the imidazole group of this histidine residue forms a salt bridge with the carboxyl group of β-94 aspartic acid in the same chain in deoxy-Hb, while it is free in HbO_2. Actually, the distance between the imidazole group of the histidine residue and the carboxyl group of the aspartic acid, calculated from the X-ray data, was 3 Å in deoxy-Hb. Thus, the findings of the X-ray analyses explain well the observed pK_a values for the β-146 histidine residue in HbCO and deoxy-Hb.

The α-50, α-72, α-103, β-2, and β-97 histidine residues had pK_a values which were expected for the free imidazole group. These results were consistent with the X-ray data which showed that there were no charged interactions with those histidine residues in both HbCO and deoxy-Hb.

On the other hand, the X-ray data showed that in both HbCO and deoxy-Hb the distance between the imidazole group of the α-112 histidine residue and the carboxyl group of α-27 glutamic acid is 3 Å and that the distance between the imidazole group of the β-77 histidine residue and the carboxyl group of β-73 glutamic acid is 6 Å in the same chain. The X-ray analyses may satisfactorily explain our finding that the pK_a values for both α-112 and β-77 histidine residues were higher than the normal values, i.e., 8.1 and 7.8 for the α-112 and β-77 histidine residues, respectively, and the same in both HbCO and deoxy-Hb.

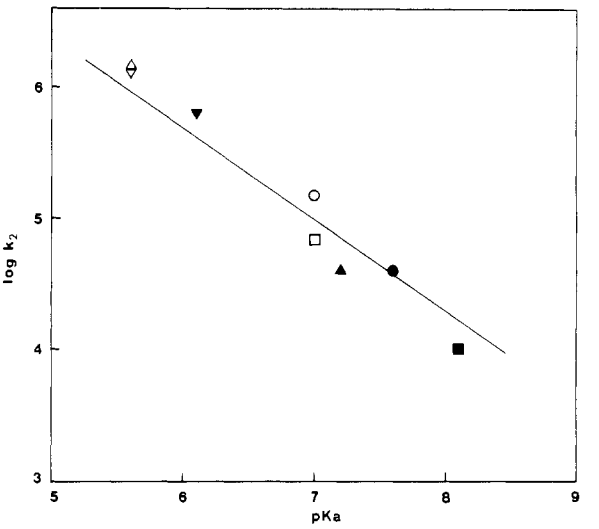

FIGURE 6: Brønsted plots for the α-20, α-89, β-143, and β-146 histidine residues in HbCO and deoxy-Hb. ○, α-20 His in HbCO; ●, α-20 His in deoxy-Hb; △, α-89 His in HbCO; ▲, α-89 in deoxy-Hb; ▽, β-143 His in HbCO; ▼, β-143 His in deoxy-Hb; □, β-146 His in HbCO; ■, β-146 His in deoxy-Hb. The line was transferred from the results of the imidazole derivatives of Minamino et al. (1977).

Accessibility of Histidine Residues to the Solvent. According to the mechanism of the hydrogen exchange (Matsuo et al., 1972; Ohe et al., 1974), the k_2 value, which is the second-order rate constant of the rate-determining step leading to an ylide intermediate, is determined by the accessibility of the histidine residue to water. On the other hand, it is well-known that in the base-catalyzed reaction the relationship between pK_a and log k_2 is given by the Brønsted equation. Minamino et al. (1977) showed that the Brønsted equation can be applied to simple imidazole derivatives which seem freely accessible to solvent. They also found from results of the tritium-exchange reaction of bovine pancreatic ribonuclease that the reactivity of a histidine residue located at the surface of a protein molecule is close to the line on the Brønsted plot of the imidazole derivatives, while the reactivity for a histidine residue buried in the molecule lies significantly below the line.

As shown in Figure 6, we found that log k_2 vs. pK_a plots for the α-20, α-89, β-143, and β-146 histidine residues involved in the Bohr effect satisfactorily agreed with the plot previously reported for free imidazole derivatives, thus indicating the complete accessibility of these histidine residues to the solvent in both HbCO and deoxy-Hb. The k_2 values for histidine residues having the same pK_a values in HbCO and deoxy-Hb were not determined.

The fact that the k_2 value for the α-122 histidine residue is virtually zero might be because it is most deeply buried in the $\alpha_1\beta_1$ contact in the Hb molecule. In the case of the bovine pancreatic ribonuclease molecule, we previously found that the histidine residue most deeply buried in the molecule has a very small k_2 value (Ohe et al., 1974). Thus, we concluded that in the case of histidine residues involved in the Bohr effect, proton exchange easily occurs between the Hb molecule and solvent and that the heme-linked and α-122 histidine residues probably are not involved in the Bohr effect, since their protons

cannot be easily exchanged. Thus, our results strongly suggested that α-122 histidine residue is not involved in the Bohr effect, although Perutz (1970) previously proposed the involvement of the histidine residue in the Bohr effect.

pK_a Values Obtained by Other Methods. Recently, Ho & Russu (1978) assigned the proton signals of specific histidine residues in human adult Hb by using abnormal Hb by the NMR method. Their results showed that the pK_a values for β-2 histidine residue were 6.51 in HbCO and 6.38 in deoxy-Hb, respectively. They also found that the pK_a values for β-143 histidine residue were 6.25 in deoxy-Hb and not observable in HbCO. These pK_a values measured by them did not agree with those obtained by us. The reason for the discrepancies is not clear. Furthermore, they found that the pK_a values for β-146 histidine residue were 7.90 in HbCO and 8.0 in deoxy-Hb, respectively. The pK_a value for β-146 histidine residue in HbCO obtained by us is in good agreement with that obtained by means of NMR by Kilmartin et al. (1973) and did not agree with that obtained by Ho & Russu (1978).

Very recently, Matthew et al. (1979) applied the modified Tanford–Kirkwood theory in a detailed study of electrostatic effect in Hb. They divided theoretically titrable and masked histidine residues from the atomic coordinate. They suggested that α-45 histidine residue is titrable, but we actually found that the histidine residue was not titrable. They suggested that α-103, β-97, and β-116 histidine residues are masked, but we actually found that these histidine residues were titrable. The pK_a values of many histidine residues theoretically obtained by Matthew et al. (1979) did not agree with those obtained by us. Especially, they suggested that the pK_a value for α-20 histidine residue in deoxy-Hb is 6.75, but we found it as 7.6. They also suggested that the pK_a value for α-89 histidine residue in HbO_2 is 7.20, but we found that the pK_a value in HbCO was 5.6. The pK_a value for β-143 histidine residue in HbO_2 suggested by them (4.00) is much different from the value in HbCO obtained by us (6.1). Their pK_a value for β-146 histidine residue (8.21) is also different from our observed value in HbCO (7.0).

Interpretation of the Bohr Effect. Perutz et al. (1969) proposed a stereochemical mechanism to account for the contribution of the β-146 histidine residue to the Bohr effect. According to them, the histidine residue is exposed to the solvent in HbO_2 and is bridged to the carboxyl group of β-94 aspartic acid in deoxy-Hb. It has a normal pK_a value in HbO_2 and a high value in deoxy-Hb. These predictions by Perutz and co-workers were verified by directly measuring the pK_a value of the β-146 histidine residue, as mentioned above. In addition, we found that the pK_a value of the β-143 histidine residue also changes on ligation. In deoxy-Hb, the imidazole group of the β-143 histidine residue had a low pK_a value, indicating that it interacts with a positively charged group, most likely with the ϵ-amino group of β-144 lysine. In HbCO, it had a slightly low pK_a value, indicating that the imidazole group interacts only slightly with the ϵ-amino group of β-144 lysine (Figure 7).

The present results demonstrated that the α-20 and α-89 histidine residues also contribute to the Bohr effect. We suggest that in the α chain the interaction of the imidazole group of the α-89 histidine residue and a positively charged group, which is most likely the ϵ-amino group of α-139 lysine, takes place in HbCO, since it had a low pK_a value. However, the residue seems to be free in deoxy-Hb, since it had a normal pK_a value. We also suggest that the imidazole group of the α-20 histidine residue interacts with a negatively charged

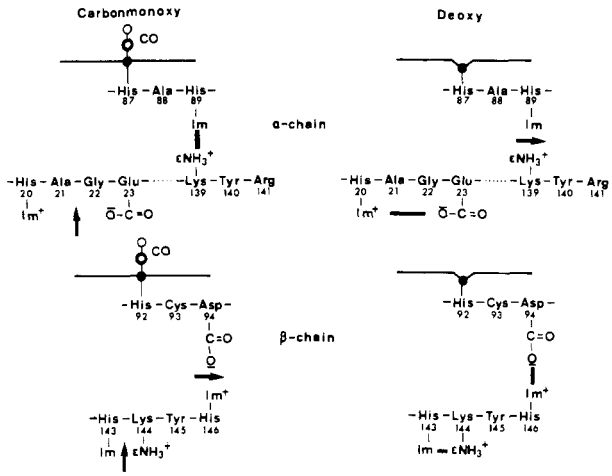

FIGURE 7: Schematic figure of the α chain and the β chain in HbCO and deoxy-Hb.

group, most likely with the carboxyl group of α-23 glutamic acid, in deoxy-Hb because it had high pK_a value. But the residue seems to be free in HbCO, since it had a normal pK_a value (Figure 7).

Perutz et al. (1974a–c) assumed that the low oxygen affinity of deoxy-Hb is due to the constraints which oppose the movement of the iron atom and the proximal histidine residue that is needed for combination with oxygen. Perutz (1970) also proposed that the carboxyl groups and the α-amino groups in the α and β chains are involved in the allosteric mechanism of Hb. Therefore, it is noteworthy that the interacting state between the β-146 histidine and β-94 aspartic acid residues is similar to that between the α-89 histidine and α-139 lysine residues on the tertiary structure in the Hb molecule (i.e., β-146 His, HC 3; β-94 Asp, FG 1; α-139 Lys, HC 1; α-89 His, FG 1) and that the α-89 histidine and β-94 aspartic acid residues are located very near the proximal histidine residue (α-87, β-92). It is also interesting to note that the α-139 lysine residue is located near the C-terminal region of the α chain which is located near the N-terminal region of another α chain, while the β-146 histidine residue in the β chain is located near the N-terminal region of another β chain.

Interpretation of the Acid and Alkaline Bohr Effects. German & Wyman (1937) showed that at pH values below 6 HbO_2 binds more protons than deoxy-Hb does, while at pH values above 6 deoxy-Hb binds more protons. These phenomena are called the acid and alkaline Bohr effects, respectively. Wyman (1939) originally showed that the Bohr effect in mammalian Hb can be interpreted by assuming two independent oxygen-linked ionizable groups per heme, one responsible for the alkaline and the other for the acid Bohr effect.

We estimated the contribution of pK_a changes in the α-20, α-89, β-143, and β-146 histidine residues at 36.5 °C to the Bohr effect by using the method of Kilmartin et al. (1973). The contribution to the Bohr effect of α-amino groups of α and β chains measured at 26 °C by Garner et al. (1975) was also calculated, and the results are shown in Figure 8. The total change in the charge of these Bohr groups (A) shows a bell-shaped curve with a maximal number of released protons of 1.5/$\alpha\beta$ dimer at pH 7.3 on ligation. On the other hand, the measured Bohr effect can be accounted for theoretically by the contribution of two alkaline Bohr groups changing these pK_a values from 7.63 to 6.23 and two acid Bohr groups changing them from 5.49 to 6.29 per $\alpha\beta$ dimer at 30 °C on oxygenation in human Hb, as reported by Anonini et al. (1965). As seen in Figure 8, the total Bohr effect (A) cal-

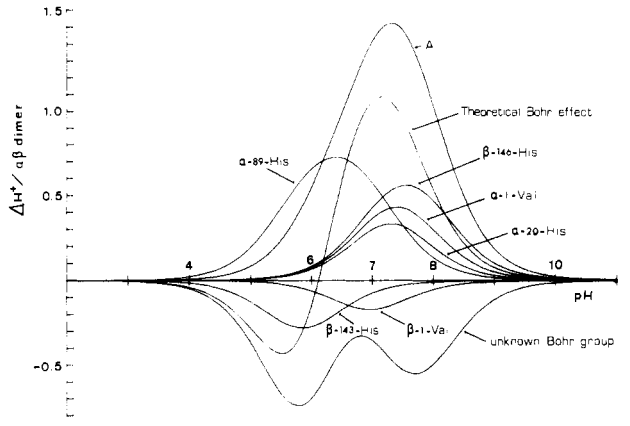

FIGURE 8: Number of protons per $\alpha\beta$ dimer released from Hb on oxygenation. (A) was obtained from the pK_a changes of the α-20, α-89, β-143, and β-146 histidine residues from the present results and the α-amino groups of the α and β chains from Garner et al. (1975). The curve for the theoretical Bohr effect is that calculated by Antonini et al. (1965).

culated from the changes in pK_a values of four histidine residues and two α-amino groups ("total" Bohr effect) does not fit the Bohr effect calculated by Antonini et al. (1965) ("theoretical" Bohr effect). We obtained two peaks, one having a maximal number of uptaken protons of 0.7 at pH 5.8 and the other having a maximum of 0.5 at pH 7.7 per $\alpha\beta$ dimer on oxygenation, when the total Bohr effect (A) was subtracted from the theoretical Bohr effect. The difference between pK_a values for the Bohr groups due to temperature was small, although we did not correct for temperature. The two peaks obtained may involve the carboxyl groups and ϵ-amino groups, since they alter the pK_a values for the imidazole groups of the histidine residues and the α-amino groups involved in the Bohr effect. However, a possibility remains that the disagreement between the total Bohr effect obtained by us and the theoretical Bohr effect is due to the situation that in our experiments we used various kinds of buffer solutions of ion strength at 0.1, which influenced the total Bohr effect.

Finally, it should be noted that the amount of Bohr protons at the physiological pH of 7.4 in the blood is mainly determined by change in the pK_a values of α-89 and β-146 histidine residues. This fact may suggest that these two histidine residues are particularly essential for the heterotropic interaction in Hb in the blood.

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