Tyrosine Ionization in Human Carbon Monoxide and Deoxyhemoglobins*

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ABSTRACT: The ionization of the tyrosine residues in human hemoglobin variants in the CO and deoxy forms was studied by spectrophotometric titration at 245 mμ. The existence of eight residues with "normal" pK (10.6), and four "abnormal" residues (by difference) with higher pK, previously demonstrated by Hermans [*Biochemistry 1*, 193 (1962)] was confirmed. Hemoglobin F which has no tyrosine in the non-α chain corresponding to the Tyr β130 of hemoglobin A had two fewer "normal" tyrosine residues than did hemoglobin A. In hemoglobin H (β_4), after the interference of cysteine was eliminated, eight "normal" and four "abnormal" tyrosine residues were observed. Two fewer "normal" residues were identified in hemoglobin A treated with carboxypeptidase A. The findings suggest that in human hemoglobin, tyrosine residues β145 and β130 are "normal" while β35 is "abnormal." Titrations on deoxyhemoglobins A and F showed two fewer "normal" residues than were present in the corresponding CO hemoglobins.

Such changes in ionization of the phenolic groups on deoxygenation were not observed in titrations of hemoglobin H or of hemoglobin A treated with carboxypeptidase A; both of these hemoglobins lack heme–heme interactions. It is suggested that two tyrosine residues undergo environmental changes with deoxygenation of hemoglobin; indirect evidence supports the idea that the residue with altered environment on reaction of hemoglobin with oxygen is β145.

Evidence concerning the configurational changes which accompany the reversible reactions of hemoglobin with oxygen has been reviewed recently (Benesch and Benesch, 1963; Rossi-Fanelli *et al.*, 1964). Crystallographic studies have revealed structural differences between human or horse deoxyhemoglobin and horse oxyhemoglobin; the most striking difference is a 7-A shift in the relative positions of the β chains (Muirhead and Perutz, 1963; Perutz *et al.*, 1964). However, the mechanisms which underlie the configurational alterations and the specific amino acid residues which are concerned remain undefined.

Hermans (1962, 1963) in studies of the ionization of the tyrosine groups in hemoglobin and myoglobin found that some phenolic groups ionized with a pK of *ca.* 10.6 ("normal" groups) and others ionized at a very high pH ("abnormal" groups). We have utilized a similar technique for studies of the participation of the tyrosine residues in the conformational changes associated with the oxygen equilibria of hemoglobin. We have also carried out spectrophotometric titrations on human hemoglobin variants with the aim of identifying the tyrosines exhibiting "normal" or "abnormal" pK values with specific residues in the known sequence of α and β chains.

Experimental Section

Isolation of Hemoglobin. Hemolysates from appropriate donors were prepared by the method of Drabkin (1949). Such hemolysates were used in some experiments without further purification, while for other experiments, hemoglobin A was isolated on IRC-50 columns by the method of Allen and co-workers (1958).

Hemoglobin F was isolated from cord blood on an Amberlite IRC-50 column with developer no. 2 (Allen *et al.*, 1958). Hemoglobin F prepared by this method was free of hemoglobin A as determined by starch gel electrophoresis. When the results of studies of purified hemoglobin F were compared with those obtained on an hemolysate which contained 95% hemoglobin F (without further purification), no significant differences were observed. Hemoglobin H, a β_4 tetramer, was prepared electrophoretically as described previously (Benesch *et al.*, 1961).

Spectrophotometric Titrations of Carboxyhemoglobins. Fresh hemolysates were exposed to CO and deionized in columns of Amberlite MB-3 or of Sephadex G-25. Hemoglobin (1 ml) of approximate concentration 3×10^{-6} M (mol wt 64,500) was mixed with 2 ml of each buffer over a pH range from 8.4 to 12.2 or 12.4. For pH 8.4–10.6, glycine–NaOH buffers, and for pH 10.4–

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11.8, ϵ-aminocaproic acid–NaOH buffers were used. For the pH range >11.8, sufficient NaOH was added to ϵ-aminocaproic acid to achieve the desired pH. Solutions were adjusted with KCl to constant ionic strength and were reexposed to CO several times during the procedure. A Beckman DU spectrophotometer was employed for one series of experiments, and a Cary Model 15 recording spectrophotometer for a second series. In each series, the optical density was measured at 245 mμ at 25°. For observations carried out on the Cary recording spectrophotometer, the hemoglobin solution at pH 8.4 was placed in the reference cell, and the difference spectrum was recorded for each buffer of higher pH. At 245 mμ, the slit width in the Beckman spectrophotometer was 0.1 and in the Cary, *ca.* 0.2. Readings were begun 30–35 sec after the mixture of hemoglobin with the buffer, and were continued at intervals of 10–20 sec. Plots of these readings were used for extrapolation to 0 time. Concentrations of hemoglobin were calculated from the optical density at 540 mμ. At pH values of 11.45 or higher, values for the optical density of hemoglobin A became time dependent, and a green color appeared in the hemoglobin solutions. At these high pH values, the 540- and 577-mμ peaks of CO hemoglobin disappeared.

Spectrophotometric Titrations of Deoxyhemoglobin. Fresh hemolysates (<48 hr after blood had been drawn) were deionized in short columns of Amberlite MB-3 or of Sephadex G-25 without prior treatment with carbon monoxide. Buffers were the same as those used for CO hemoglobins. Prepurified N_2 was freed of traces of O_2 by the method of Meites and Meites (1948). To the N_2 purification train was attached a filtering flask (capacity 50 ml) containing 6 ml of the buffer. The purified nitrogen passed into the buffer flask and out the side arm to a tonometer with an integral quartz cuvet of 10-mm light path containing 3 ml of the hemoglobin solution. An adjustable outlet of the tonometer permitted the outflow of nitrogen. The tonometer was swirled for 10 min under N_2 at a temperature of 20–22°, the tonometer outlet was then closed, and the buffer was forced into the tonometer by the nitrogen stream. The spectrum from 700 to 500 mμ was recorded in a Cary Model 15 spectrophotometer. The proportion of methemoglobin, as calculated by the method of Benesch *et al.* (1965) from spectrophotometric measurements at 560, 576, and 540 mμ, ranged from 0 to 3.3% following deoxygenation. Deoxygenation was considered to be complete when the absorption ratio 555/540 mμ was 1.24 or higher. The absorption at 245 mμ was read in a Beckman DU spectrophotometer with thermospacers at 25°. The tonometer was opened, equilibrated with carbon monoxide, and optical density at 245 mμ was again measured. The hemoglobin concentration was obtained from final values for optical density at 540 mμ after exposure to CO (ϵ_{mM} 57.6). Except in the case of hemoglobin H, the concentrations of the hemoglobin solutions were 0.9–1.2 × 10^{-5} M prior to deoxygenation, and 3–4 × 10^{-6} M (ionic strength of 0.1) after the addition of the buffer. The concentrations of hemoglobin H solutions before deoxygenation and following dilution with buffer were 5–6 × 10^{-5} and 1.6–2 × 10^{-5} M, respectively. Deoxygenation of hemoglobin H was carried out in tonometers with integral cuvets of 2-mm light paths.

In another series of experiments the difference spectra of deoxy- *vs.* oxyhemoglobin A and deoxy- *vs.* oxycarboxypeptidase A treated hemoglobin A were determined at a pH of 10.40–10.55 and 8.40–8.50. Hemoglobin solution (2 ml) was mixed with 6 ml of the glycine buffer, to yield hemoglobin concentrations of 1.90–2.10 × 10^{-5} and ionic strength of 0.1. This solution (3 ml) was pipetted into each of two tonometers with integral cuvets of 10-mm light path. No difference spectrum was observed when spectra of these two solutions, one *vs.* the other, were recorded from 330 to 230 mμ. One of the tonometers was then deoxygenated in a nitrogen stream for 15 min as previously described. The other tonometer remained equilibrated with oxygen. According to the spectrophotometric criteria noted above, no evidence of methemoglobin was found in the recorded spectrum of either tonometer. Finally, a difference spectrum from 330 to 230 mμ was obtained, with the deoxygenated solution as reference. After reconverting the deoxyhemoglobin to oxygenated form, the base line was the same as before deoxygenation.

The pH of the solutions which had been deoxygenated was measured after exposure to CO. In these buffer solutions deoxygenation was not associated with pH changes (Bohr effect). Direct pH measurements *during* deoxygenation of a solution of hemoglobin in the same buffer system used for titration showed no change of pH.

Radiometer pH meter, Model TTT1 equipped with a G202-B or a GK2024-B (low sodium error) electrodes, standardized with standard Beckman buffers of pH 7 and 10 was used in all experiments. No correction for sodium ion error was made because of the type of electrode used. All pH readings were made at 25°. The molar extinction coefficient for hemoglobin was calculated on the basis of a molecular weight of 64,500.

Alkylation of Hemoglobin. Hemoglobins A and H were incubated with a 10 mole excess of iodoacetamide in a 0.2 M phosphate buffer, pH 7.3, for 1.5 hr at 25° (Benesch *et al.*, 1961); the solutions were then passed through Sephadex G-25 columns or were dialyzed overnight. After deionization, spectrophotometric titration of the tyrosine residues was carried out. Difference spectra at pH 10.53 (ϵ-aminocaproic acid–NaOH buffer, 0.1 ionic strength) were determined for alkylated oxyhemoglobin A *vs.* oxyhemoglobin A, and for alkylated deoxyhemoglobin A *vs.* deoxyhemoglobin A. The SH groups were titrated with PMB[1] according to the technique of Benesch and Benesch (1962a).

Carboxypeptidase Digest of Hb A. The C-terminal histidine and the penultimate tyrosine residues of the β polypeptide chains were removed by digestion with CPA by the method of Antonini and co-workers (1961).

[1] Abbreviations used: PMB, *p*-hydroxymercuribenzoate; CPA, carboxypeptidase A; NEM, *N*-ethylmaleimide.

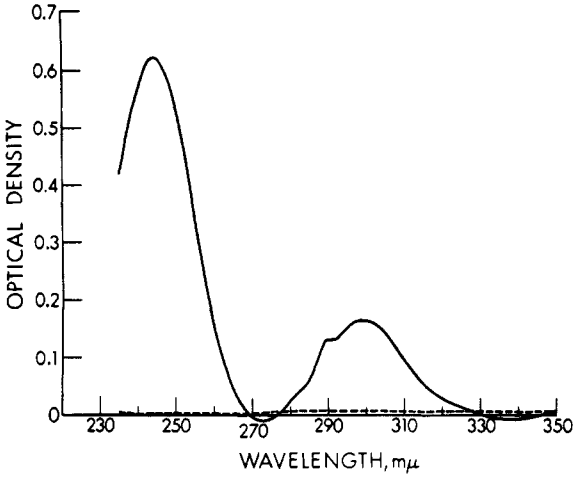

FIGURE 1: Ultraviolet difference spectrum of CO hemoglobin A, pH 11 *vs.* 8.40. Dashed line indicates base line obtained on tracing CO hemoglobin A (pH 8.40) *vs.* CO hemoglobin A (pH 8.40).

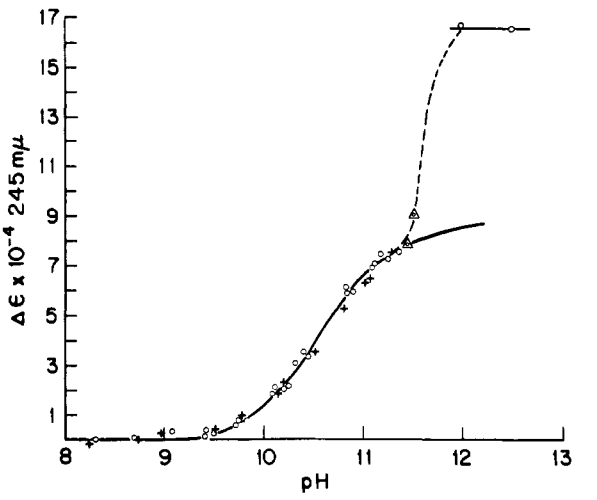

FIGURE 2: Spectrophotometric titration of CO hemoglobin A (○) and alkylated Hb A (+). The alkylated Hb A was titrated between pH 8.40 and 11.50. Points indicated by △ were time dependent, extrapolated to 0 time.

The C-terminal sequence of the β chain is

$$\text{Leu-Ala-His-Lys-Tyr-His}_{146}$$
$$\downarrow \quad \downarrow$$

The sites of CPA attack are indicated by arrows. The DFP-treated CPA and hemoglobin were incubated for 3 hr at pH 8 (Tris–HCl buffer) at 30°. After incubation the hemoglobin component (CPA Hb) and the free amino acids released by treatment with CPA were separated on Sephadex G-25. The tyrosine released from the hemoglobin was estimated spectrophotometrically utilizing a value for $\Delta\epsilon$ of 10,000 for each tyrosine. Starch gel electrophoresis was carried out by the method of Smithies (1957) or of Poulik (1957).

Materials. Iodoacetamide, lot 63B-1210, and ϵ-aminocaproic acid, lot 44B-1660, were obtained from Sigma. The sodium salts of hydroxymercuricbenzoic acid (lot B-3152), L-glycine, and L-cysteine (chromatographically pure) were obtained from Mann Research Laboratories. Carboxypeptidase A, DFP-treated, three times crystallized (COA-DFP 6131), was obtained from Worthington Biochemical Corp. All other chemicals were reagent grade.

Results

The difference spectrum of human hemoglobin (pH 11 *vs.* 8.40) with peaks at 245 and 297 mμ is shown in Figure 1. This spectrum closely resembles the difference spectra of tyrosine, cytochrome *c*, and other heme proteins (Rupley, 1964a,b; Hermans, 1962).

The equation (Hermans, 1962)

$$\text{pH} = \text{p}K + \log \frac{\Delta\epsilon}{\Delta\epsilon_{max} - \Delta\epsilon}$$

describes the spectrophotometric titrations of the tyrosyl groups. $\Delta\epsilon$ is the difference molar absorption coefficient at any given pH. $\Delta\epsilon_{max}$ is the maximum difference molar absorption of the titration curve. The adjustable parameters $\Delta\epsilon_{max}$ and pK were chosen in each case to provide the best fit with those experimental points which did not exhibit time dependency. The value for the number of "normal" tyrosine residues was obtained by dividing $\Delta\epsilon_{max}$ by $\Delta\epsilon$ 245_{max} mμ of tyrosine in human hemoglobin (12,100) (Hermans, 1962).

Hemoglobin A. The curve describing the data obtained from the titration of hemoglobin A had two phases (Figure 2). A first phase corresponded to the ionization of tyrosine residues with apparent pK in the normal range (*ca.* 10.6). In the second phase, at pH values *ca.* 11.4, absorption became time dependent. The latter part of the curve was less reliable, and extrapolation to 0 time was only approximate. The visible spectra in this high pH range showed a time-dependent disappearance of the α and β peaks of hemoglobin. Above pH 12 the color of the solution changed rapidly from that characteristic of CO hemoglobin to green, and changes became again independent of time. For CO hemoglobin A the theoretical line that best fitted the observed points was that calculated from the first phase of the curve with a pK of 10.60 and a $\Delta\epsilon_{max}$ of 92,000. Using as $\Delta\epsilon_{max}$ for each tyrosine 12,100 (Hermans, 1962), in hemoglobin A we could count eight "normal" tyrosine groups available for ionization in this pH range (Table I). Of a total of 12 tyrosine residues, by difference then, four were "abnormal." The second phase of the curve showed higher absorption than that expected for four tyrosine groups, but the changes in the heme absorption and other interfering chromophores (*e.g.*, cysteine) could account for the difference. These results were in general agreement with those obtained by Hermans (1963).

Alkylated Hemoglobin A. The blocking of β93 with iodoacetamide did not alter significantly the spectro-

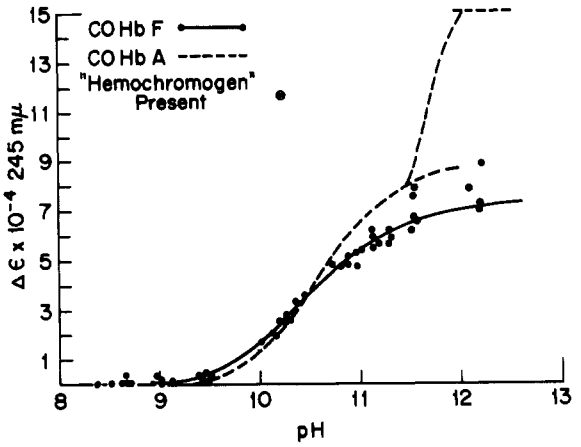

FIGURE 3: Spectrophotometric titration of CO hemoglobin F (●). For comparison the titration of CO hemoglobin A (------) is included. In the points marked ⊙, denaturation was observed, but time dependency was lacking.

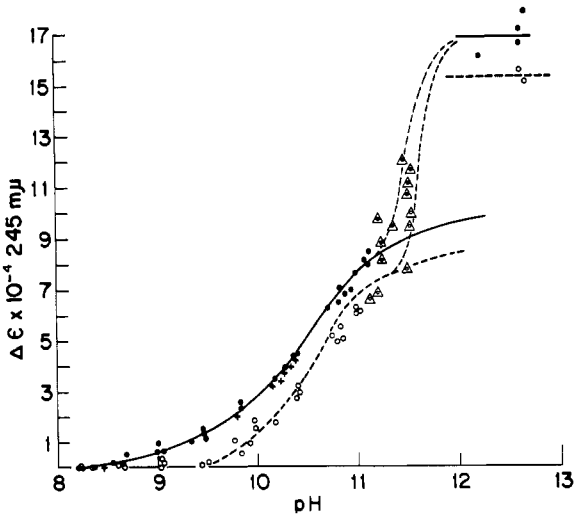

FIGURE 4: Spectrophotometric titration of CO hemoglobin H (●), deoxyhemoglobin H (+), and alkylated Hb H (○). The titration of CO hemoglobin H is included for comparison (------). Symbol △ indicates time-dependent points, extrapolated to 0 time. The dashed line indicates the titration of CO hemoglobin A.

TABLE I: Apparent pK and Maximum Δ Absorption Molar Coefficients for Carbon Monoxide and Deoxyhemoglobin A-, F- and CPA-treated A.

Hemoglobin	pK	$\Delta\epsilon \times 10^{-4}$ max	Total Residues	"Normal" Residues
CO A	10.60	9.2	12	8
Deoxy A	10.77	7.8	12	6
CO F	10.45	7.5	10	6
Deoxy F	10.65	5.3	10	4
CO CPA A	10.55	6.8	10	6
Deoxy CPA A	10.55	6.8	10	6

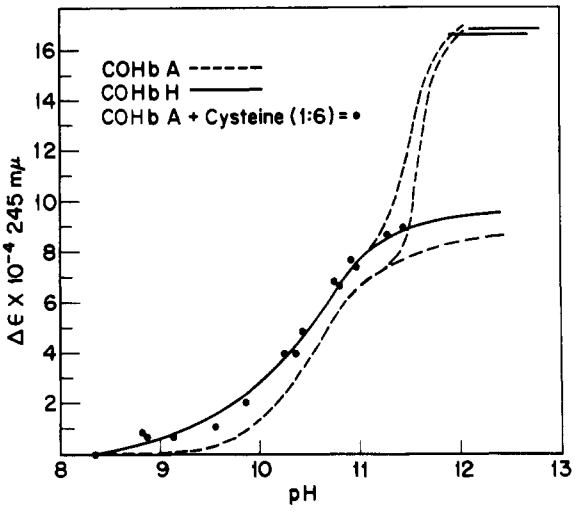

FIGURE 5: Spectrophotometric titration of CO hemoglobin A in 6 M excess of cysteine (●), between pH 8.40 and 11.50. The solid black line corresponds to the titration of CO hemoglobin H. The titration of CO hemoglobin A (------) is also included for comparison.

photometric titration of hemoglobin A (Figure 2). These titrations were not carried out >pH 11.50.

Hemoglobin F. Differences between hemoglobins F and A were observed in the first phase of the curve, *i.e.*, in the titration of the "normal" tyrosines (Figure 3). The experimental points were compatible with the presence of only six "normal" tyrosine residues in hemoglobin F (*vs.* eight in Hb A). In hemoglobin F, the apparent pK was 10.45 and $\Delta\epsilon_{max}$ 75,000 (Table I). No second phase of the curve was observed; some of the observations above pH 11.4 suggested that the formation of hemochromogen might increase the absorption at 245 mμ.

Hemoglobin H. The titration curve of hemoglobin H differed from that of CO hemoglobin A, and an unusual shape of the curve below pH 10 was noted (Figure 4). The increase in the difference molar absorbancy began at a lower pH than that observed in Hb A. Since Hb H has eight reactive SH groups (Benesch *et al.*, 1961), as contrasted with only two for Hb A, the possible interference of cysteine was considered. Hemoglobin H was alkylated with iodoacetamide by the method of Benesch and coworkers (1961). A modified hemoglobin H with only two reactive SH groups (by PMB titration) was obtained. The tyrosine titration of the alkylated hemoglobin H closely resembled that of Hb A (Figure 4). In another experiment, hemoglobin A was mixed with cysteine in a 1:6 molar ratio and the difference absorption coefficient determined immediately (to avoid the oxidation of cysteine). The results of the titration of

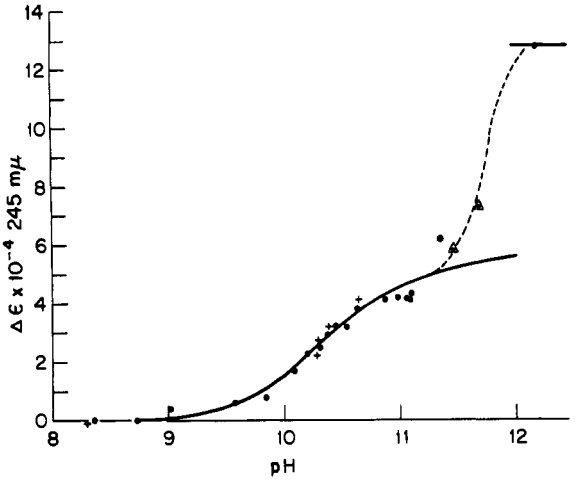

FIGURE 6: Spectrophotometric titration of CO hemoglobin A treated with carboxypeptidase A (●), and the deoxy form (+). Points indicated by △ were time-dependent points of CPA Hb A, extrapolated to 0 time.

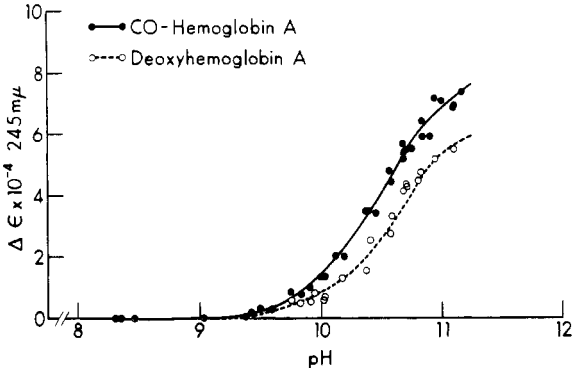

FIGURE 7: Spectrophotometric titration of CO hemoglobin A (●) and deoxyhemoglobin A (○).

Hb A in the presence of cysteine (Figure 5) resembled those obtained in unmodified Hb H. Thus, the unusual shape of the curve for Hb H was apparently related to interference by cysteine.

Carboxypeptidase Digest of Hemoglobin A. The experimental points obtained by titration of CPA Hb A fell on a two-phase curve (Figure 6). The observations were best represented by a theoretical curve calculated for a pK of 10.55 and a $\Delta\epsilon_{max}$ of 68,000 (Table I). The findings were compatible with the presence of six "normal" tyrosine groups in the first phase and four "abnormal" groups in the second phase. The results confirmed those of Hermans (1963) for CPA Hb A.

The digestion of Hb A by carboxypeptidase A resulted in the liberation of nearly 2 moles of tyrosine/mole of hemoglobin (Table II). On starch gel electrophoresis at pH 8.6, CPA Hb A exhibited mobility slightly cathodal to Hb A. The absence of significant alteration in electrophoretic mobility strongly suggests

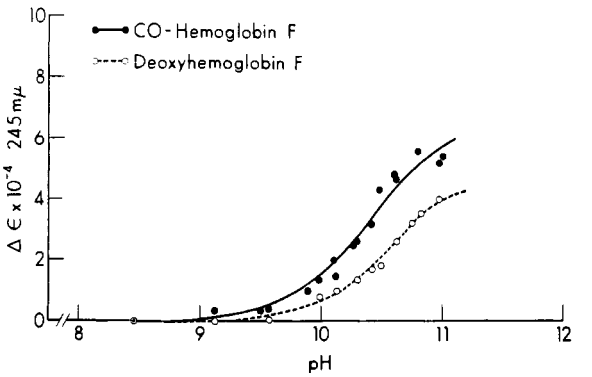

FIGURE 8: Spectrophotometric titration of CO hemoglobin F (●) and deoxyhemoglobin F (○).

TABLE II: Carboxypeptidase A Digestion of Hemoglobin A.

Date	Hb A (mg)	CPA (mg)	Tyrosine/ Mole of Hb
1-5-65	50	5	1.8
1-21-65	100	7.5	1.9
9-8-65	50	5	1.8

that the reaction with CPA ceased after the liberation of Tyr 145 since the digestion of the next amino acid (lysine) would have resulted in hemoglobin with a grossly altered net charge.

Deoxyhemoglobin A and F. The titration of deoxyhemoglobin A differed from that of CO hemoglobin A (Figure 7). The pK of the first phase was shifted to a slightly higher pH and the experimental points were consistent with a $\Delta\epsilon_{max}$ of 78,000. Thus the total normal tyrosines in deoxyhemoglobin A was six as contrasted with the eight observed in CO hemoglobin A (Table I). Above pH 11.3 the time-dependent appearance of a hemochromogen with two peaks (530 and 560 mμ) was observed. The molar absorbancy of these two peaks was compatible with the absorption constants published by Drabkin and Austin (1935) for the reduced globin hemochromogen derived from hemoglobin. Such a change was not present in CO hemoglobin A or CO hemoglobin F samples. A change in the titration curve also accompanied deoxygenation of Hb F (Figure 8): the apparent pK was shifted to a higher pH and the number of normal residues was four, two less than CO hemoglobin F. The $\Delta\epsilon_{max}$ was 53,000 (Table I).

Deoxyhemoglobin H and Deoxy-CPA Hemoglobin A. In the pH range that could be studied (up to pH 10.40), no differences between the spectra of Hb H and deoxy-Hb H were observed (Figure 4). Reduced globin hemochromogen appeared in hemoglobin H at a relatively low pH (10.4) as compared with hemoglobin A (11.3). For CPA Hb A in the deoxy form the tyrosine titration

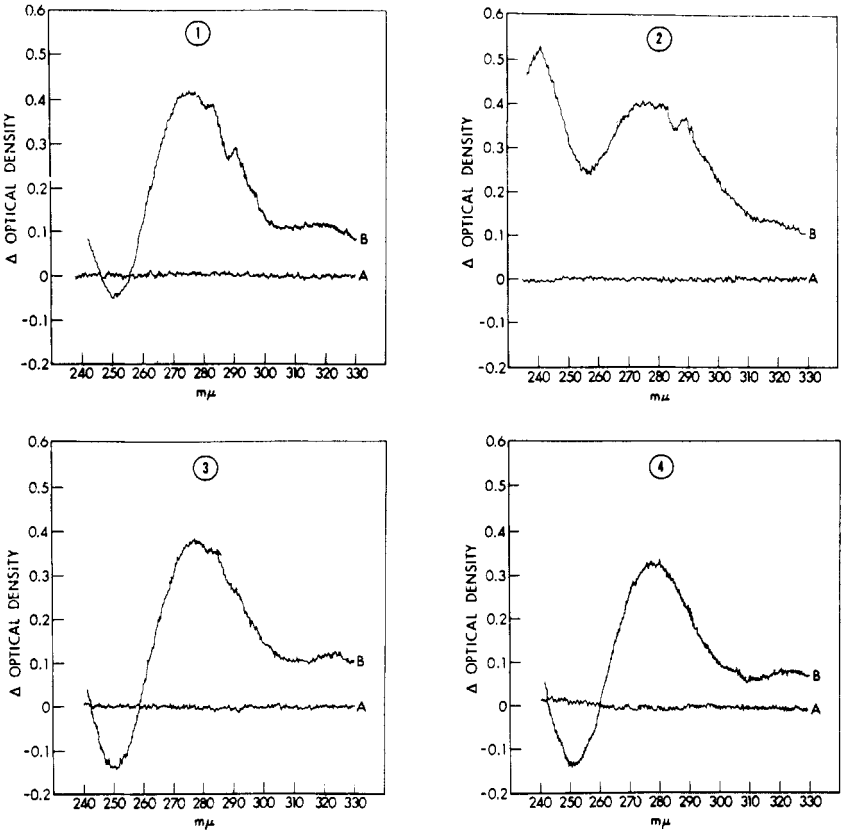

FIGURE 9: Difference spectra. (1) B represents the difference spectrum of oxyhemoglobin A (pH 8.41) *vs.* deoxyhemoglobin A (pH 8.41). A designates the base line of oxyhemoglobin A (pH 8.41) *vs.* oxyhemoglobin A (pH 8.41). Hemoglobin concentration: 2.07×10^{-5} M. (2) B represents the difference spectrum of oxyhemoglobin A (pH 10.55) *vs.* deoxyhemoglobin A (pH 10.55). A designates the base line of oxyhemoglobin A (pH 10.55) *vs.* oxyhemoglobin A (pH 10.55). Hemoglobin concentration: 1.98×10^{-5} M. (3) B represents the difference spectrum of oxy-CPA Hb A (pH 8.50) *vs.* deoxy-CPA Hb A (pH 8.50). A designates the base line of oxy-CPA Hb A (pH 8.50) *vs.* oxy-CPA Hb A (pH 8.50). Hemoglobin concentration: 2.2×10^{-5} M. (4) B represents the difference spectrum oxy-CPA Hb A (pH 10.41) *vs.* deoxy-CPA Hb A (pH 10.41). A designates the base line of oxy-CPA Hb A (pH 10.41) *vs.* oxy-CPA Hb A (pH 10.41). Hemoglobin concentration: 2.2×10^{-5} M.

curve was indistinguishable from that of the CO form (Figure 6).

Difference Spectrum of Oxyhemoglobin A vs. Deoxyhemoglobin A. The difference spectrum of oxyhemoglobin A (pH 8.41) *vs.* deoxyhemoglobin A (pH 8.41), used as a reference, demonstrated the presence of a broad peak at 275–280 mμ. At 245 mμ no significant change was observed (Figure 9).

The spectrum of oxyhemoglobin A (pH 10.55) *vs.* deoxyhemoglobin A (pH 10.55) (Figure 9) shows that in addition to the broad 275–280-mμ peak a new absorption maximum appeared at 242 mμ. The $\Delta\epsilon$ at 245 mμ is consistent with the titration of deoxyhemoglobin A (Figure 7) in which fewer tyrosines are available for ionization in the normal range when the molecule of hemoglobin becomes nonligand bound. The peak at 275–280 mμ is more difficult to interpret. A small contribution of tyrosine would be expected, but the absence of significant pH dependency in the range studied suggests that most of the absorption may be due to changes in the perturbation spectra of tryptophan and phenylalanine, secondary to conformational changes that accompany deoxygenation. A change of absorption of the heme with deoxygenation in this region might also be significant.

Difference Spectrum of Oxy-CPA Hb A vs. Deoxy-CPA Hb A. The difference spectrum of oxy-CPA Hb A (pH 8.50) *vs.* deoxy-CPA Hb A (pH 8.50) (Figure 9) resembled the corresponding difference spectrum of Hb A (Figure 9), with two exceptions: the minor peak at 288 mμ, which is probably due to tryptophan, is less evident in the spectrum of CPA Hb A than in the spectrum of Hb A, and the CPA Hb A difference spectrum has a lower absorbancy at 250 mμ.

In contrast at pH 10.55 the spectrum of oxy-CPA A *vs.* deoxy-CPA Hb A (Figure 9) is quite different from the difference spectrum of Hb A at similar pH (Figure 9) and similar to the difference spectrum of Hb A at pH

8.50. No 242 mμ peak was observed. This is consistent with the identity of the titration curves of CO-CPA Hb A and deoxy-CPA Hb A.

Difference Spectra of Oxyhemoglobin A vs. Alkylated Oxyhemoglobin A and Deoxyhemoglobin A vs. Alkylated Deoxyhemoglobin A. No significant difference spectrum was observed when oxyhemoglobin A *vs.* alkylated oxyhemoglobin A at pH 10.53 was traced. Difference spectrum of deoxyhemoglobin A *vs.* alkylated deoxyhemoglobin A at the same pH was also essentially a straight line. The concentration of the solutions used was 1.30×10^{-5} M.

Oxyhemoglobin A. The titration curves of CO and oxyhemoglobin A were indistinguishable.

Discussion

The differences in the absorption spectra between ionized and un-ionized tyrosine residues permit spectrophotometric titrations of the tyrosyl groups in proteins at either the 295- or 245-mμ peak of the difference spectrum (pH 8.4 *vs.* 12). Because of interference at 295 mμ by the absorption of heme, the 245 peak is preferred for studies of hemoglobin. At 245 mμ, cysteine residues also contribute to the observed absorption (Benesch and Benesch, 1955; Donovan, 1964), but in our studies this contribution would probably be small since cysteine has a relatively low $\Delta\epsilon$ at 245 in the pH range between 8 and 12, and hemoglobins A and F each contain only two rapidly reactive cysteine groups. Furthermore, in our studies, spectrophotometric titration curves at 245 mμ of alkylated hemoglobin A (in which no SH groups were reactive to PMB), were indistinguishable from curves obtained with unmodified hemoglobin A.

Tryptophan, another source of possible interference, exhibits changes in absorbance which are difficult to predict, as a result of perturbation of the indole chromophore by ionization of adjacent residues. Donovan *et al.* (1961) found no evidence of significant interference in titrations of model tryptophan-containing compounds at 245 mμ from pH 8.4 to 12.

Identification of the "Normal" and Abnormal Tyrosine Residues in Human Hemoglobin. The α chain of human hemoglobins A and F has three tyrosine residues: α24, α42, and α140. In the β and γ chains, residues 35 and 145 are tyrosines, but the equivalent of Tyr β130 is absent from γ chain, which therefore contains only two tyrosine residues. Comparison of the normal tyrosine residues of hemoglobin F ($\alpha_2\gamma_2$) and A ($\alpha_2\beta_2$) could, therefore, yield some information concerning the ionizability of β130, if the β and γ chains have similar conformations, particularly in the regions adjacent to the other (35 and 145) tyrosine residues. While crystallographic data are not available for hemoglobin F, similar general configurations have been found by crystallographic methods in α and β chains of horse and human hemoglobin, and in whale and horse myoglobin (Cullis *et al.*, 1962) despite differences in primary structure. The β and γ chains of human hemoglobin differ in 36 of the total of 146 residues, and the seven amino acids preceding and the seven following Tyr 35 are identical in the β and γ chains, although more variability exists in the region of Tyr 145. These observations strongly suggest that the β and γ chains have similar tertiary structure, and that comparisons of the reactivity of the tyrosine residues in the two chains are justified. Since like chains in the tetramer have equivalent conformation, observed changes would be expected to occur in two (or multiples of two) tyrosine residues.

Although in preliminary crystallographic studies Perutz and Mazzarella (1963) demonstrated that two of the four β chains of hemoglobin H closely resemble in conformation the β chains of deoxyhemoglobin A, the validity of comparing hemoglobin H, which has many unusual properties, with hemoglobin A is uncertain. The absence of anomalies in the titration curves between pH 10 and 11 in hemoglobin A, which, in this pH range, dissociates rather abruptly to dimers (Hasserodt and Vinograd, 1959), suggests that tyrosyl groups do not participate in interchain bonds between α,β dimers, and that the environments of the β chain tyrosine groups are comparable in hemoglobins A, F, and H.

At least two interpretations could be formulated from the results of studies of CPA Hb A in which two "normal" tyrosine groups were apparently lost as a result of the enzyme treatment: (1) that the β145 is "normal" in the unmodified protein and lost as a result of the CPA treatment, or (2) that β145 is "abnormal" but that a pair of other "normal" tyrosine groups of the untreated hemoglobin became "abnormal" as a result of the conformational changes in the rest of the molecule accompanying the CPA-induced loss of the β145 tyrosine and β146 histidine residues. The latter possibility appeared less likely than the first.

If our assumptions concerning comparability in conformation are correct, the following conclusions concerning the ionization of the tyrosyl groups of the β chains of hemoglobin A appear justified. Carboxyhemoglobin F has two fewer normal tyrosine groups than CO hemoglobin A, hence β130 is "normal." Because CPA-treated Hb A has two fewer "normal" tyrosine groups than unmodified Hb A, β145 also appears to be "normal." In hemoglobin H, eight tyrosines were "normal" and four were "abnormal:" since β130 and β145 appeared to be normal, the four β35 residues of Hb H and the two β35 residues of Hb A are believed to be "abnormal." The α chains must also contain two normal and one abnormal tyrosine to achieve the total of eight normal and four abnormal observed in tetrameric Hb A.

Changes in Ionization of Tyrosine Residues with Deoxygenation of Hemoglobin. In the present studies a change in the environment of the tyrosine residues with deoxygenation of hemoglobin was demonstrated. In Hb A, the number of tyrosine residues with normal p*K* was reduced from 8 to 6 with deoxygenation. Thus one tyrosine in each of two like chains became unavailable for ionization in the normal pH range. Furthermore, from a comparison of the titration of hemoglobin F and deoxyhemoglobin F in which two

tyrosine groups normal in oxyhemoglobin F became abnormal on deoxygenation suggests that in Hb A, β130 (which has no counterpart in the γ chain) is not the residue involved in the conformational changes accompanying deoxygenation.

Two of the hemoglobins studied, hemoglobin H and CPA Hb A, have been observed to have sharply decreased heme–heme interaction and to exhibit values for n in Hill's equation of *ca.* 1 (Benesch *et al.*, 1961; Antonini *et al.*, 1961). The absence of conformational changes with deoxygenation in Hb H has been suggested by the crystallographic studies of Perutz and Mazzarella (1963), and has been postulated for CPA Hb A by Zito and co-workers (1964). In the present studies, the titrations of Hb H or CPA Hb A in their deoxy forms closely resembled titrations of the CO derivatives. Therefore, the observed alterations of a pair of tyrosine groups in Hb A and F on deoxygenation appeared to be related to the conformational changes underlying heme–heme interaction, and are absent in hemoglobins which do not undergo such conformational changes.

The observed alterations in titration curves of the deoxyhemoglobins did not appear to be related to the changes in reactivity of the SH groups with deoxygenation (Benesch and Benesch, 1962b). The possibility that alterations in SH reactivity might reflect changes in ionization constants of the SH groups in deoxyhemoglobins was considered, but comparison of deoxyhemoglobin A and alkylated deoxyhemoglobin A at pH 10.53 disclosed no significant difference.

The titration curve of Hb H began to exhibit time dependency at a lower pH than did that of Hb A. Thus, Hb H was probably denatured at a less alkaline pH than was Hb A. The hemochromogen observed at *ca.* pH 11.2 in deoxyhemoglobin A was found at pH 10.4 in deoxyhemoglobin H. These findings suggested the possibility that heme–globin interaction may differ in hemoglobins A and H.

Our studies do not provide a direct method for identification of the pair of tyrosine residues which undergo environmental change during reactions with oxygen. Other indirect evidence suggests that β145 is the tyrosine residue most likely to be affected by or involved in these configurational changes. In X-ray crystallographic studies, Muirhead and Perutz (1963) demonstrated that the β chains undergo demonstrable conformational change with deoxygenation; the tyrosine residue affected by reaction with oxygen might thus be expected to be in the non-α chains. Either β (or γ) 35 or β (or γ) 145 which are tyrosines in Hb A and F might be the residues concerned. However, evidence from titrations of Hb A, F, and CPA Hb A indicated that β35 was abnormal in CO hemoglobin A and hence could not *become* abnormal on deoxygenation. Other evidence suggesting that β145 is involved in the oxygenation reaction may be summarized as follows. (1) The removal of β145 by treatment with CPA yields a Hb which lacks heme–heme interaction and low oxygen affinity (Antonini *et al.*, 1961); in CPA Hb A no change in tyrosine titration was observed on deoxygenation. (2) Carboxypeptidase A digests oxy- and deoxyhemoglobin A at different rates (Zito *et al.*, 1964), suggesting that the enzyme may recognize differences in conformation in the C-terminal end of the β chains. (3) Since, from our data, β145 is probably normal in oxyhemoglobin, this residue could become abnormal on deoxygenation. (4) A residue critically involved in the conformational changes underlying heme–heme interaction might be expected to be phylogenetically invariant, since the oxygen equilibria of mammalian hemoglobins are strikingly similar. The only tyrosine residue constant in *all* the mammalian hemoglobin for which sequence has been ascertained is Tyr 145. (5) In metmyoglobin Kendrew and co-workers (1960, 1961) showed that Tyr 145 is involved in a bond with a residue of the nonhelical FG region of the polypeptide chain; hence there appears to be no definite restriction to the formation of a bond between the β145 and the FG region of the β chain which resembles myoglobin.

On examination of the X-ray crystallographic model of horse hemoglobin (Cullis *et al.*, 1962) it appeared that, if Tyr β145 is involved in any bond, a bond to residues of the FG region would be most probable. The FG region is of particular interest in the studies of the relationship of structure and function of hemoglobin. In β93 (Cys), which is five residues removed from FG5, environmental changes with deoxygenation have been demonstrated. Benesch and co-workers (1962b) reported different reactions of cysteine β93 in oxy- and deoxyhemoglobin with NEM and iodoacetamide. The FG corner of the molecule is also one of the areas of contact with the α chains and with the side chains of the heme pyrroles. In myoglobin one of the pyrrol rings containing the vinyl side chains of the heme is in contact with the residue FG5 (Kendrew, 1962). In addition, Antonini and co-workers (1964) have observed in studies of reconstituted hemoglobins that the modification of the vinyl side chain resulted in hemoglobins with decreased heme–heme interaction.

If the participation of Tyr β145 in the conformational changes accompanying the reactions of hemoglobin with oxygen is further substantiated, an interesting possibility arises. Deoxygenation-linked modifications of the porphyrin ring might influence the FG region and structural conditions leading to the creation of a hydrogen bond between FG5 and Tyr β145 might ensue.

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