Relation between Structure and Function in Hemoglobin Chesapeake*

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ABSTRACT: Hemoglobin Chesapeake ($\alpha_2^{92\,Leu}\beta_2$) is characterized by a high oxygen affinity, decreased or absent "heme–heme interaction," and a normal Bohr effect. The combination velocity constant for the reaction of CO and Hb Cheaspeake was 4.0×10^5 M^{-1} sec^{-1}, that is, twice the value of the corresponding constant in Hb A. The higher combination velocity constant seems to be the kinetic basis for the high ligand affinity in Hb Chesapeake. Two residues that undergo changes in reactivity with deoxygenation in Hb A failed to do so in Hb Chesapeake. Cysteine 93 of β-Hb does not become unreactive toward iodoacetamide nor do two tyrosine residues undergo drastic pK changes when Hb Chesapeake becomes deoxygenated. These findings, in view of the presence of a normal Bohr effect, suggest that the areas of the molecule involved in the conformational changes underlying heme–heme interaction are different and distinct from those underlying the Bohr effect. Since the amino acid substitution in Hb Chesapeake is in an area of $\alpha\beta$ contact, its unusual oxygen equilibria lend support to the idea that the interactions between α and β chains in normal Hb A may be of importance in the mechanism of the heme–heme interaction.

Studies of the oxygenation function of certain variants of human hemoglobin, notably hemoglobin H (Benesch *et al.*, 1961) and hemoglobin M_{Iwate} (Hayashi *et al.*, 1966), have resulted in significant contribution to the understanding of the structural basis underlying two important properties of hemoglobin, namely, heme–heme interaction and Bohr effect. Hemoglobin (Hb) Chesapeake which contains leucine instead of the normal arginine at α^{92} (Clegg *et al.*, 1966) was shown to exhibit significantly altered O_2 equilibria, manifested by high O_2 affinity and markedly reduced heme–heme interaction with a normal Bohr effect over a limited pH range (Charache *et al.*, 1966). In the present studies, we have confirmed the presence of this unusual constellation of functional properties in Hb Chesapeake isolated electrophoretically (rather than chromatographically) and have obtained data which suggest that Hb Chesapeake undergoes conformational changes on reaction with O_2 which differ from the oxygenation-linked changes of Hb A.

Experimental Procedure

Isolation of Hb Chesapeake. Hemolysates were prepared from samples of fresh blood according to the method of Drabkin (1946) with minor modifications. Hemoglobin Chesapeake was isolated by starch granule electrophoresis (Kunkel *et al.*, 1957) using Veronal buffer, 0.04 M at pH 8.6. Hemoglobin Chesapeake migrated more cathodically than Hb A and was eluted from the starch with 0.001 M phosphate buffer (pH 7), and the purity of the preparation was verified by starch gel electrophoresis at pH 8.6 in Tris-borate buffer. No traces of Hb A were found in any of the preparations used.

Oxygen equilibria were determined by minor modifications of the method of Allen *et al.*, (1950), and Riggs (1951) on electrophoretically isolated (Kunkel *et al.*, 1957) Hb A and Hb Chesapeake. The nitrogen used for deoxygenation was freed of O_2 by the method of Meites and Meites (1948). Deoxygenation was judged complete when the ratio OD 555:OD 540 was equal to or greater than 1.23. The tonometers were of approximately 200-ml volume with 10-mm light-path integral absorption cuvets. Hemoglobin saturations were determined spectrophotometrically in a Cary Model 14R recording spectrophotometer provided with a thermoregulated cell holder through which cold water was circulated to maintain a 10° temperature. Increments of air were added by syringe or by pipet as described by Rossi-Fanelli and Antonini (1958). Hemoglobin concentrations were about 0.05 mM (in heme), equilibrations were carried out at 10°, and buffers used were 0.1 M potassium phosphate. The pH readings were obtained using a Radiometer T111 pH meter.

Determination of SH Groups. Spectrophotometric titrations of SH groups with PMB[1] were carried

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[1] Abbreviation used: PMB, *p*-mercuribenzoate.

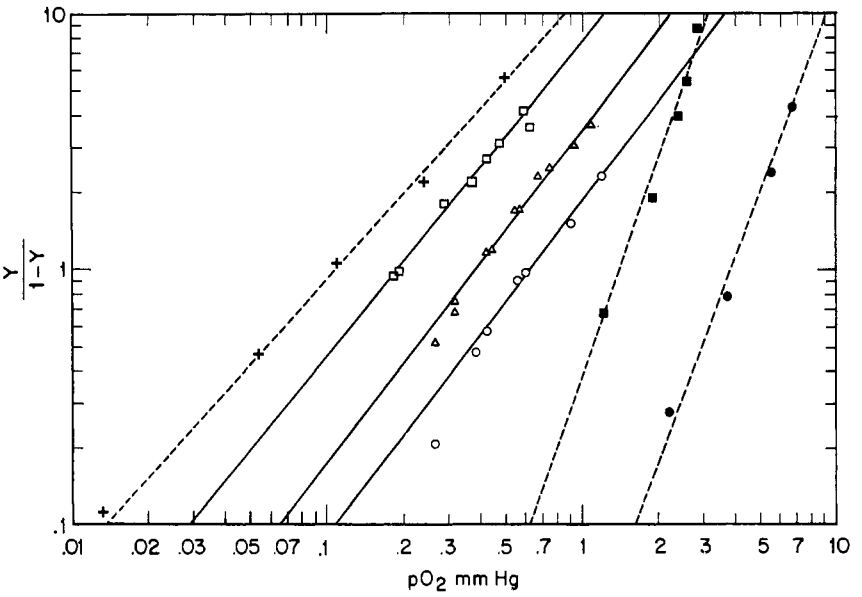

FIGURE 1: Oxygen equilibria curves of Hb Chesapeake, Hb A, and Hb α^A, plotted according to the logarithmic form of Hill's (1910) equation (see text). (--●--●--) Hb A, pH 6.70; (--■--■--) Hb A, pH 7.70; (—○—○—) Hb Chesapeake, pH 6.63; (—△—△—) Hb Chesapeake, pH 7.24; (—□—□—) Hb Chesapeake, pH 7.85; and (--+--+--) Hb α^A, pH 7.58. Temperature 10°; buffers 0.1 M phosphate.

out in oxyhemoglobin solution (about 4×10^{-5} M on heme basis) as described by Boyer (1954) and modified by Benesch and Benesch (1962a). Iodoacetamide titrations in oxy- and deoxyhemoglobin A and Chesapeake (solutions 3×10^{-3} M on heme basis) were followed by the liberation of hydrogen ions in a Radiometer pH-Stat (Benesch and Benesch, 1962a).

Tyrosine Spectrophotometric Titrations. Following electrophoresis the Hb solutions were freed from Veronal by dialysis and subsequent passage through Sephadex G-25 or MB-3 Amberlite resin. Spectrophotometric titrations of tyrosine residues at 245 mμ were measured as described previously (Nagel *et al.*, 1966). Difference spectra were recorded by placing deoxyhemoglobin Chesapeake or A in the reference cell and oxyhemoglobin Chesapeake or A in the sample cell.

Kinetic Studies. Hemoglobin A and Hb Chesapeake, isolated from the same starch block, were utilized for the study of the reaction between these hemoglobins and CO. The reaction rate was measured by the stopped-flow method (Gibson and Milnes, 1964) and by flash photolysis (Gibson, 1959). The stopped-flow experiments were performed in hemoglobin solutions dissolved in oxygen-free 0.1 M phosphate (pH 7.4), with a final concentration before mixing of 2.8×10^{-6} M (heme basis). Carbon monoxide solutions were prepared by adding 2 ml of water equilibrated with pure carbon monoxide at 1 atm of pressure and diluting with deaerated buffer. The final concentration of carbon monoxide after mixing was 1.72×10^{-5} M. Dithionite was added to both solutions which were subsequently mixed in equal volumes in the stopped-flow apparatus. The flash photolysis experiments were performed on the same solutions as the stopped-flow experiments, except that the hemoglobin solutions and the CO solution were mixed prior to the introduction into the flash apparatus. Carbon monoxide replacement experiments were performed by mixing in the stopped-flow apparatus a solution of oxyhemoglobin Chesapeake and a 5×10^{-4} M carbon monoxide solution. The reaction in this case was followed at 419 mμ.

Spectrophotometric Studies. The Soret spectra were determined in deoxyhemoglobin A and deoxyhemoglobin Chesapeake. The hemoglobin solution was placed in a tonometer with an integral cuvet of 2-mm light path and exposed to a stream of N_2 purified of traces of oxygen by the method of Meites and Meites (1948). After 10 min of gently swirling under N_2, dithionite was introduced and the flow of N_2 was continued for 3 more min. The spectrum of the solution in the sealed tonometer was traced from 450 to 400 mμ. The solution was exposed then to CO and transferred to a cuvet of 10-mm light path and the concentration was determined from the absorption at 540 mμ.

Results

Oxygen Equilibria. Figure 1 contains the data on oxygen equilibria of Hb Chesapeake and A plotted according to the logarithmic form of Hill's (1910) equation

$$\log \frac{Y}{1 - Y} = \log K + n \log p$$

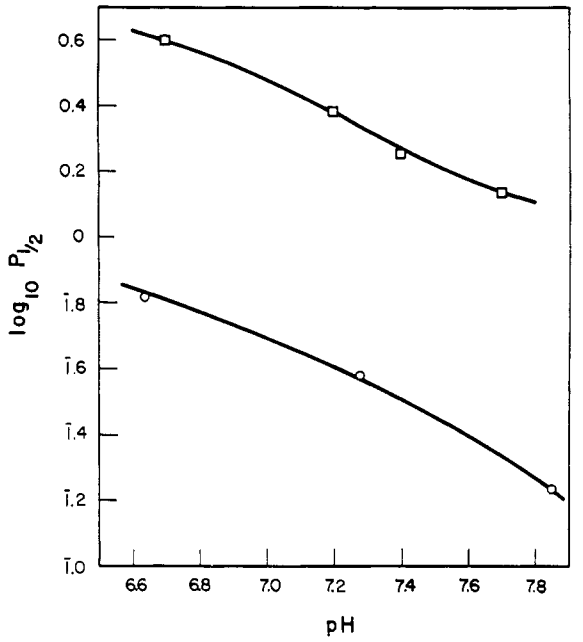

FIGURE 2: Hemoglobin A and Hb Chesapeake Bohr effect. Hb A(—□—□—)and Hb Chesapeake (—O—O—).

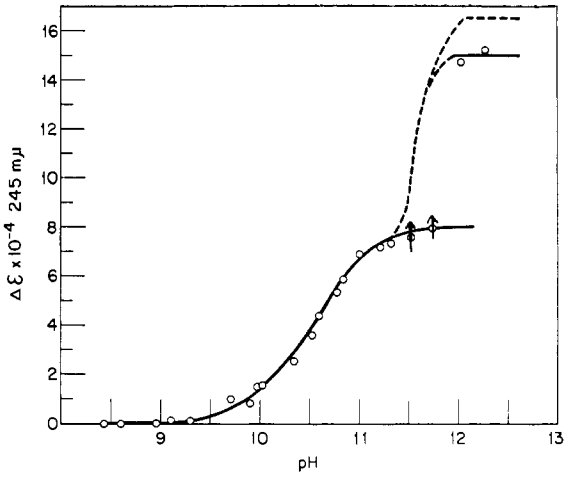

FIGURE 3: Spectrophotometric titration of Hb Chesapeake at 245 mμ. Solid line corresponds to the titration of Hb A and the open circles to the Hb Chesapeake titration. It can be observed, then, that the solid line (Hb A titration) also describes well the Hb Chesapeake titration. Between pH 11.30 and 12, dashed line is the time-dependent phase of the titration. Above pH 12, the titration is again not time dependent, and the solid horizontal line corresponds to Hb Chesapeake, dashed horizontal line to Hb A. (arrow) Time-dependent point, extrapolated to zero time. Hemoglobin concentration: 1.3×10^{-5} M (heme basis).

where Y is fractional saturation with oxygen, p is the partial pressure of oxygen with hemoglobin, and n is an exponent representing in a general way the stabilizing interactions between the oxygen binding sites. In the logarithmic plot the value of n is represented by the slope of the line. $n = 1$ indicates no heme–heme interaction; $n = 4$ indicates maximal interaction in a tetrameric molecule containing one binding site per monomer.

With hemoglobin Chesapeake solutions ranging in pH from 6.63 to 7.85, at 10°, $p_{1/2}$ values ranging from 0.660 to 0.195 mm were obtained. The designation $p_{1/2}$ corresponds to the oxygen pressure at which one-half of the hemoglobin is in oxygenated form. Hemoglobin A in solutions of pH ranging from 6.70 to 7.70 demonstrated $p_{1/2}$ values that varied with pH from 4.0 to 1.40 mm. Hemoglobin Chesapeake had a $p_{1/2}$ value about one-sixth that of Hb A. The n values of these curves were also significantly different. Hemoglobin Chesapeake curves showed an n value of approximately 1.3 and Hb A of 2.8 as a mean. We have included a comparison, the oxygen equilibria of Hb α^A chains at pH 7. It can be observed that the $p_{1/2}$ value of Hb α^A is about $^1/_{25}$ that of Hb A.

In Figure 2 we have plotted log $p_{1/2}$ *vs.* pH to compare the Bohr effect of these two hemoglobins. The Δ log $p_{1/2}/\Delta$pH values (between pH 6.6 and 7.8, where the Bohr effect is almost linear) were 0.45 in both cases. This demonstrates that in spite of significant differences in oxygen affinity, demonstrated by a low $p_{1/2}$ value and drastically decreased heme–heme interaction, the Bohr effect, in the range of pH studied, is entirely similar in Hb A and Chesapeake.

Determination of SH Groups. The titration of the SH groups with PMB demonstrated the existence of two reactive cysteines in CO Hb Chesapeake, as has been previously found in Hb A (Benesch and Benesch, 1962b) (Table I).

In the reaction of iodoacetamide with deoxyhemoglobin Chesapeake, a significant difference between this Hb and Hb A was disclosed. Deoxyhemoglobin A, as previously demonstrated by Benesch and Benesch (1962b), has no reactive groups when titrated with iodoacetamide. In contrast deoxyhemoglobin Chesapeake has 2.4 reactive SH groups (Table I).

Tyrosine Spectrophotometric Titration. The spectrophotometric titrations at 245 mμ of CO Hb Chesapeake

TABLE I: SH Group Titrations of Hb Chesapeake and A.

Hb	Method	Reactive SH Groups/ 64,500 g
Oxy Hb A	PMB	2.1
Oxy Hb Chesapeake	PMB	1.8
Deoxy Hb A	Iodoacetamide	0
Deoxy Hb Chesapeake	Iodoacetamide	2.4

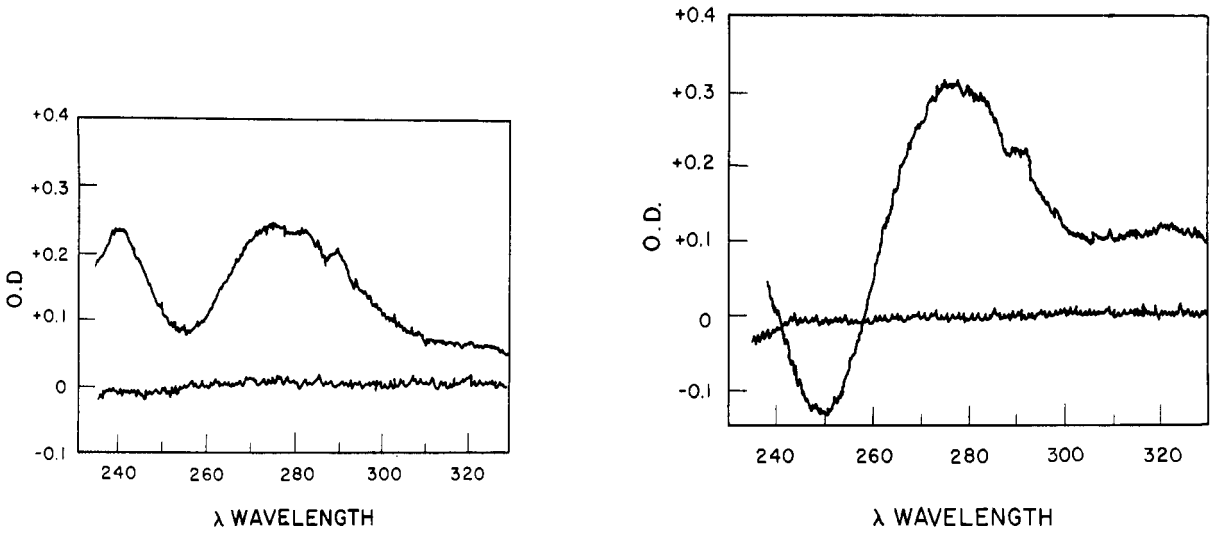

FIGURE 4: Difference spectra of oxyhemoglobin and deoxyhemoglobin A and oxyhemoglobin and deoxyhemoglobin Chesapeake. (A) (left) Difference spectra of oxyhemoglobin A, pH 10.33 (sample cell), and deoxyhemoglobin A, pH 10.35 (reference cell). Concentration: 5.68×10^{-5} M (heme basis). Base line: oxyhemoglobin A in both cells. Buffer, 0.1 ionic strength, glycine–NaOH; temperature 25°. (B) (right) Difference spectra of oxyhemoglobin Chesapeake, pH 10.30 (sample cell), and deoxyhemoglobin Chesapeake, pH 10.30 (reference cell). Concentration: 7.12×10^{-5} M (heme basis). Base line: oxyhemoglobin Chesapeake in both cells; temperature 25°.

TABLE II: Velocity Constant (l') for the Combination of Carbon Monoxide with Electrophoretically Isolated Hb A and Hb Chesapeake.

Hb	pH	Temp (°C)	Method	l' (M^{-1} sec^{-1})
Hb Chesapeake	7.4	4.2	Stopped flow	1.7×10^5
Hb Chesapeake	7.4	16.0	Stopped flow	3.6×10^5
Hb Chesapeake	7.4	19.5	Stopped flow	4.0×10^5
Hb A	7.4	19.5	Stopped flow	1.9×10^5
Hb Chesapeake	7.4	21.2	Flash photolysis	FC = 8.2×10^{6}[a]
				SC = 2.9×10^{6}[b]
Hb A	7.4	21.2	Flash photolysis	FC = 9.5×10^{6}[a]
				SC = 2.3×10^{5}[b]

[a] FC = fast component. [b] SC = slow component.

and CO Hb A are very similar (Figure 3). These curves have been interpreted to reflect the titration of eight normal tyrosines, ionizing with an apparent pK of 10.60 (Nagel *et al.*, 1966). The rest of the tyrosines present (four) have an abnormal pK, and do not ionize until denaturation occurs above pH 11.5. This coincides with the presence of time-dependent absorption changes at 245 mμ.

It has previously been shown (Nagel *et al.*, 1966) that in deoxyhemoglobin A, two normal tyrosines undergo environmental changes with deoxygenation. Deoxyhemoglobin A has six normal tyrosines instead of the eight normal tyrosines in oxy or CO Hb A. The difference spectrum between oxyhemoglobin A and deoxyhemoglobin A at pH 10–11 is particularly useful in the study of this problem. The excess of ionizable tyrosines in oxyhemoglobin A was reflected in the appearance of a tyrosine difference spectrum with a maximum at 242 mμ (Figure 4A). The other maximum of the tyrosine spectrum at 280 mμ is considerably more difficult to interpret because of contributions of other aromatic amino acids and of heme.

The difference spectrum of oxyhemoglobin Chesapeake *vs.* the deoxy form at pH 10.3 (Figure 4B) is quite different from Hb A. No tyrosine difference spectrum appears to contribute to these spectra (no maxima at 242 mμ), suggesting that no tyrosine residues undergo marked environmental changes during reaction of the Hb Chesapeake with oxygen. The negative

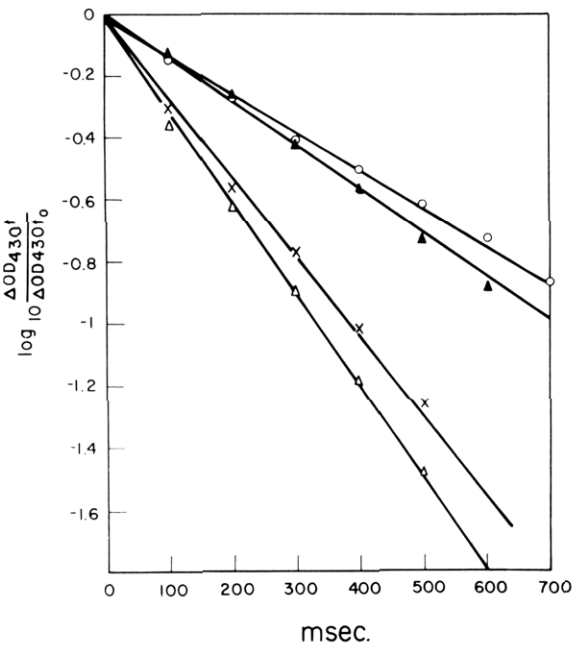

FIGURE 5: Kinetics of the combination reaction between carbon monoxide and Hb A and Hb Chesapeake, determined by the stopped-flow method. (—▲—▲—) Hb A, 19.5°; (—○—○—) Hb Chesapeake, 4.2°; (—△—△—) Hb Chesapeake, 19.5; (—×—×—) Hb Chesapeake, 16°. Abcissa: milliseconds. Ordinate: logarithm of the change in optical density at 430 mμ at time t divided by the change in optical density at 430 mμ at time t_0.

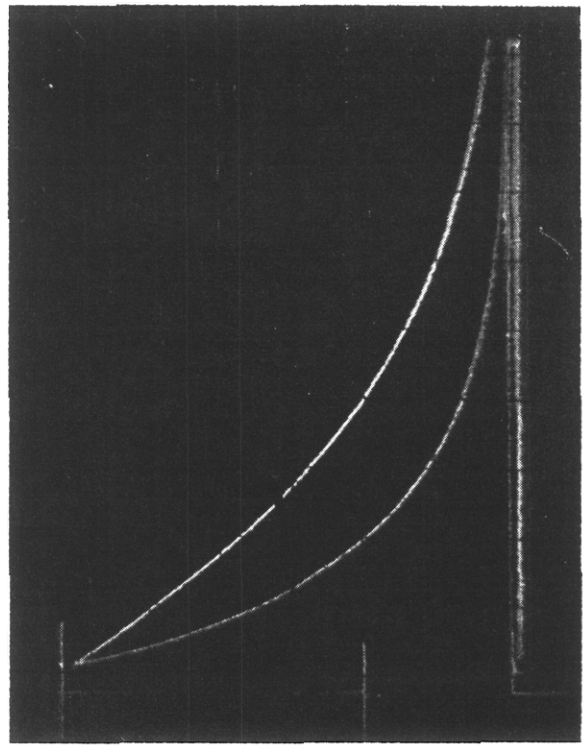

FIGURE 6: Photograph of the actual tracings in the oscilloscope screen of change of voltage produced by the reaction of carbon monoxide and Hb A and Hb Chesapeake in two successive experiments. Followed at 430 mμ and at 19.5°. Stopped-flow method. Upper curve: Hb A; lower curve: Hb Chesapeake.

difference spectra at 250 mμ have been observed also in the difference spectra of oxyhemoglobin A *vs.* deoxyhemoglobin A at pH 8.30 (Nagel *et al.*, 1966).

The difference spectrum at 245 mμ of oxyhemoglobin Chesapeake *vs.* deoxyhemoglobin Chesapeake at pH 8.3 is indistinguishable from the difference spectrum of oxyhemoglobin A *vs.* deoxyhemoglobin A at the same pH.

Kinetic Studies. The stopped-flow experiments (Figures 5 and 6, Table II) showed that the combination rate of CO with reduced Hb Chesapeake is two times that of Hb A. The flash photolysis experiments (Figure 7, Table II) demonstrated that the reaction rate of Hb Chesapeake is essentially monophasic and rapid while Hb A has a biphasic reaction with carbon monoxide. From the Arrhenius plot of the stopped-flow data an activation energy of the reaction of 10.6 kcal (which is the value usually found for Hb A) was calculated.

The rate at which CO replaces O_2 from oxyhemoglobin Chesapeake and oxyhemoglobin A is depicted in Figure 8. The reaction is followed at 419 mμ. The replacement rate appeared to be almost identical in Hb's A and Chesapeake. As in the case of Hb A, it seems that the nature of the ligand occupying the rest of the binding sites does not affect the rate in which the fourth ligand site reacts in Hb Chesapeake. If it is accepted that the curves of Hb A and Hb Chesapeake are congruent (Figure 8), the two hemoglobins must share a number of common kinetic parameters. These are: (1) the velocity of dissociation of O_2 from fully liganded Hb; and (2) the ratio of the velocity of combination of CO to the velocity of combination of O_2 for hemoglobin having one vacant site.

The curvature in the plot of Figure 8 is probably due to a distribution of the hemoglobin between states of greater and lesser aggregation and the similarity of these curves may imply that Hb A and Hb Chesapeake are alike also in the dilution-induced deaggregation.

Spectrum in the Soret Region. The spectrum of deoxyhemoglobin Chesapeake in the Soret band region (430 mμ) is indistinguishable from deoxyhemoglobin A (Figure 9). The spectra of 1:1 haptoglobin–hemoglobin complex is included in the figure for comparison.

Discussion

Examination of the presently available tentative atomic model of Hb (Perutz, 1965) suggests that the substitution in Hb Chesapeake is of particular interest. According to this model, the $FG4_\alpha$ residue is located

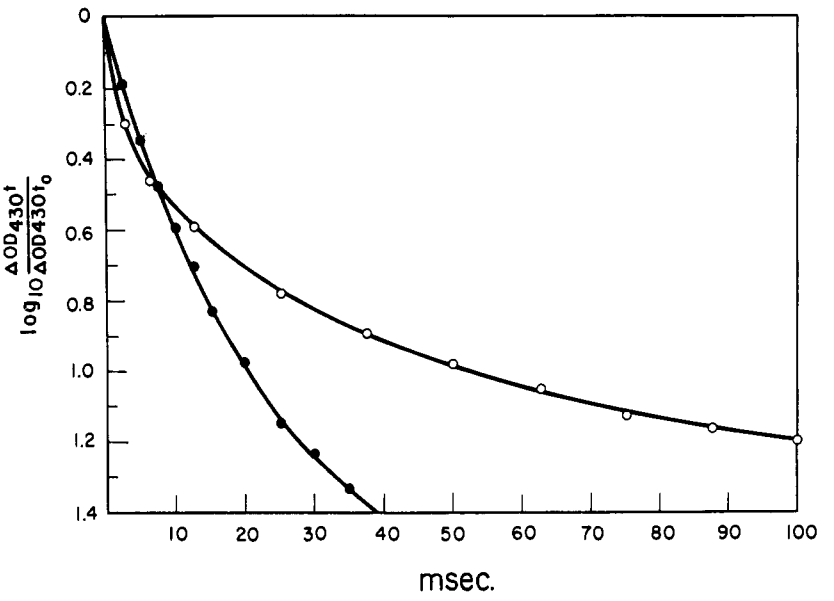

FIGURE 7: Kinetics of the combination reaction between carbon monoxide and Hb A and Hb Chesapeake. Flash photolysis method. (—o—o—) Hb A, 21.2°; (—●—●—) Hb Chesapeake, 21.2°; Flash light = 700 *J*. Abcissa and ordinate as in Figure 5.

in a largely nonpolar bridge between subunits α_1 and β_2, which intersects the line of closest approach between the hemes of these two polypeptide chains. With the data at hand it is believed that arginine $FG4_\alpha$ in horse hemoglobin and probably in human hemoglobin interacts directly with residues of the β_2 chain. The indole ring of tryptophan $C3_\beta$ is probably in contact with β and γ atoms of arginine $FG4_\alpha$. Also there is a strong possibility that the guanidine group of arginine $FG4_\alpha$ forms a hydrogen bond with an appropriate hydrogen acceptor in the β_2 chain. These considerations support the suggestion that the substitution of the polar arginine residue by the nonpolar leucine in that position probably modifies this important area of subunit contact. However, this particular area of contact is not directly involved in the reaction of dissociation to dimers by strong salt solution, a reaction which in all likelihood splits the molecule into symmetrical subunits.

Hemoglobin Chesapeake with altered oxygen equilibria and a substitution in the area of interchain con-

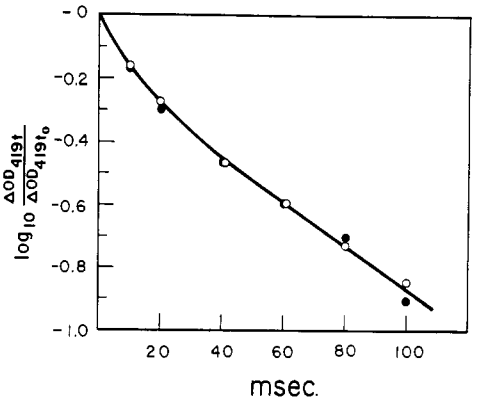

FIGURE 8: Kinetics of the replacement of O_2 bound to Hb A and Chesapeake, by carbon monoxide. Stopped-flow method. (—●—●—) Hb Chesapeake, 21°; (—o—o—) Hb A, 21°. Abcissa: logarithm of optical density change at 419 mμ at time *t* divided by the change in optical density at 419 mμ at time t_0.

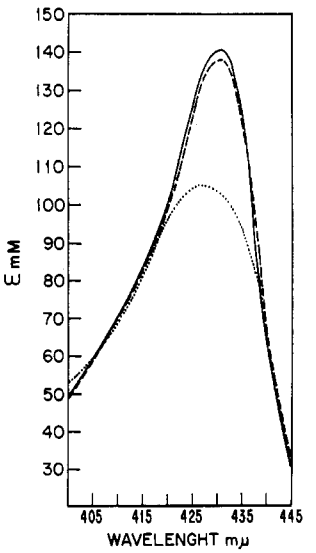

FIGURE 9: Soret spectra of deoxyhemoglobin A and deoxyhemoglobin Chesapeake. (——) deoxyhemoglobin A; (----) deoxyhemoglobin Chesapeake. (· · · ·) deoxy HbHp (1:1) included for comparison.

tact is of particular interest because of the revival of some aspects of the aggregation–deaggregation reaction as a basis of the heme–heme interaction phenomenon (Wald and Riggs, 1951; Briehl, 1963; Benesch *et al.*, 1965). Douglas *et al.* suggested in 1912 that deoxyhemoglobin is more aggregated than oxyhemoglobin and that only single Hb or Hb O_2 molecules could combine with or give off oxygen.

It was of interest then to confirm the initial oxygen equilibrium results using a different method of isolation, and to gather information on other structural–functional parameters. The oxygen equilibrium results presented here on electrophoretically isolated Hb Chesapeake are completely in accordance with previous data on the chromatographically separated component. The O_2 affinity is higher than that of Hb A but lower than that of Hb H, Hb α^A, or the hemoglobin–haptoglobin complex. There is no significant evidence, from the equilibrium curves, of heme–heme interaction and in the range studied a normal Bohr effect was observed.

The kinetic results on the combination rate of CO with Hb Chesapeake seem to demonstrate that the higher ligand combination velocity constant is the kinetic basis of the higher affinity.

Also, in two instances we have demonstrated the absence in Hb Chesapeake of a conformational change usually associated with deoxygenation in normal human hemoglobin. The $\beta^{93\,SH}$ group does not become unreactive toward iodoacetamide, nor do two tyrosines undergo environmental change when deoxygenation is completed. These two important departures from the structural behavior of normal Hb A substantiate the conclusion that a different conformational change occurs in Hb Chesapeake when it reacts with ligands.

The flash photolysis results (Figure 7) show that in Hb Chesapeake almost all the reaction is fast, of the type of the quick-reacting form postulated by Gibson (1959) to explain the initial fast rate of combination of CO with normal Hb A after flash photolysis. In Hb Chesapeake this fast reaction is followed by a second and slower phase which is nevertheless faster than the slow component in Hb A. Recent unpublished work (Q. H. Gibson and E. Antonini) suggests that at pH 7, the quick-reacting form could represent the monomeric polypeptide chains which occur in very dilute hemoglobin solutions. Then the initial fast rate may represent the Hb Chesapeake monomers, with a fast combination rate with CO comparable to that of the monomers from Hb A. The second phase would represent the reaction of the tetrameric Hb Chesapeake, which according to the stopped-flow data would be expected to be faster than Hb A.

Another important characteristic of Hb Chesapeake is the presence of a normal Bohr effect, at least between pH 6.6 and 7.85. The dissociation between the Bohr effect and heme–heme interaction has been observed previously. Antonini and coworkers have described in Hb A treated with carboxypeptidase B an n value of 2.7 with decrease in Bohr effect to about a third of the normal values (Antonini *et al.*, 1961). Also in hemoglobin reconstituted from normal globin and hemes other than protoheme, a decreased heme–heme interaction (n value of 1.7 or 1.6) and a normal Bohr effect have been observed (Antonini *et al.*, 1964). Hemoglobin Chesapeake is, however, one of the most extreme case of dissociation of Bohr effect and heme–heme interactions. These findings support the contention that the conformational changes associated with the Bohr effect are different and distinct from those associated with the heme–heme interactions, and as has been suggested (Antonini, 1965) these functional properties may involve completely different areas of the molecule.

Hemoglobin Chesapeake departs, however, from the group of Hb variants with very high affinity (Hb H, Hb α^A, HpHb, etc.) in these characteristics. (a) The oxygen affinity is not as high nor the combination rate as fast as the values observed in the very high affinity group. (b) There is no Soret band modification in the deoxy form as occurs in the very high affinity group. The 430-mμ absorption coefficient of Hb Chesapeake is indistinguishable from that of deoxyhemoglobin A. (c) The activation energy for the reaction of CO with hemoglobin Chesapeake is identical with A, unlike the very high affinity hemoglobins which have a considerably lower activation energy. (d) There is a normal Bohr effect at least between pH 6.6 and 7.85.

The evidence available at present suggests that any model designed to explain ligand binding in hemoglobin should include interchange in normal hemoglobin of two structurally distinct forms not necessarily dependent on the presence or absence of bound ligand. Based on Perutz' (1965) data we can utilize the notation R–O to depict this change. These two forms, according to available crystallographic data, differ at least in the position of the β chains which move apart by 7 A measured at the level of the hemes in the deoxyhemoglobin form compared to the oxyhemoglobin. The O structural form can perhaps be equated with a form characterized by fast reaction and high ligand affinity and abnormal Soret band spectra in the unliganded form. It is not surprising then that Hb H, Hb CPA (Antonini *et al.*, 1961), and HpHb complex (Nagel and Gibson, 1966) have all these properties in common. A likely interpretation is that these forms are fixed in the *equivalent* of a O form and can only interchange ligands without changing basic structural form.

Hemoglobin Chesapeake seems to depart from the group of hemoglobin forms with very high affinity. It is appealing to consider this hemoglobin variant as an intermediate form between R and O, and to speculate that interference with the $\alpha_1\beta_2$ interchain bond could be the structural basis of this departure from the normal conformational changes of hemoglobin secondary to its reaction with ligands. This interference could impair actual aggregation–deaggregation or hamper the conformational changes which involve areas of subunit contact.

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