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Analysis of Optical Properties of Hemoglobins in Terms of the Two-state Model, Especially from Studies on Abnormal Hemoglobins with Amino Acid Substitution in the $\alpha_1\beta_2$ Contact Region*

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Circular dichroism and electronic absorption spectra of the deoxygenated and oxygenated forms of four abnormal hemoglobins with amino acid substitution in the $\alpha_1\beta_2$ contact region, Hb Kempsey (β99 Asp → Asn), Hb Yakima (β99 Asp → His), Hb Chesapeake (α92 Arg → Leu), and Hb J Capetown (α92 Arg → Glu) were measured over the wavelength region from 650 to 350 nm in the presence and absence of inositol hexaphosphate. The spectra of Hb Chesapeake and Hb J Capetown were very similar to those of Hb A in the both oxygenated and deoxygenated forms. In the oxygenated form, Hb Yakima and Hb Kempsey gave spectra identical with those of Hb A. On the other hand, the spectra of the deoxygenated Hb Yakima and Hb Kempsey were greatly different from those of normal hemoglobin with respect to the intensity of the peak and the peak position. The oxygen equilibrium curves of these hemoglobins were determined under the same conditions used for the spectral measurement to estimate the allosteric constants, *L* and *c*, of the two-state model postulated by Monod *et al.* (Monod, J., Wyman, J., and Changeux, J. P. (1965) *J. Mol. Biol.* 12, 88-118) from their oxygen affinity and Hill's *n*. We tried to verify the qualitative relation between the optical properties and the functions of these abnormal hemoglobins.

Among spectral abnormalities we chose the positive CD band in the Soret region for this analysis. We assumed that if the ellipticity of the CD band obeyed the two-state theory, then the observed (θ) might be calculated from the intrinsic θ_R and θ_T for the R and T state, and the equilibrium constant, L = T/R. From curve-fitting analysis by computer, we estimated the θ_R and θ_T as 1.1 and 1.69 × 10^5, respectively. The ellipticity of these abnormal hemoglobins in the Soret CD with and without inositol hexaphosphate was a good fit to the simulated curve of (θ) and *L*. By the same analysis, we obtained the absorption spectra for the R and T state of hemoglobin in the Soret region. The difference spectra between them was identical with the spectrum of the deoxy-T minus deoxy-R hemoglobin formed immediately after flash photolysis of HbCO and HbO_2 as demonstrated by Gibson (Gibson, Q. H. (1959) *Biochem. J.* 71, 293-303).

These results strongly suggest that the spectra of the deoxygenated hemoglobins are given by mixing of two spectra characteristic of the R and T state hemoglobins which co-exist at equilibrium with equilibrium constant, *L*. These abnormal hemoglobins with amino acid substitution at the $\alpha_1\beta_2$ contact had *c* values that differed from each other, as well as different *L* values. However, these changes in *c* could not directly correlate to the optical properties that arise mainly from heme and its environments.

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It is worth remembering the valuable works undertaken by Perutz *et al.* (1) about the influence of globin structure on the state of heme. From their evidences and comparison with the results reported by Brunori *et al.* (2), they suggested that the spectra in the Soret region of the deoxygenated hemoglobin was possibly arisen from the changes in the electronic state of heme followed by the R-T transition of quaternary structure. Their discussions on the structure of heme, however, seem to be qualitative. To analyze the quantitative relationship between the optical properties and the function of hemoglobin, we measured the oxygen equilibrium and the absorption and circular dichroic (CD) spectra of four mutant hemoglobins with $\alpha_1\beta_2$ anomaly such as Hb Kempsey, Hb Yakima, Hb Chesapeake, and Hb J Capetown. These spectra have been known to reflect sensitively the changes of electronic state and stereochemical structure of heme caused by the perturbation of heme environment due to rearrangement of subunits. Especially CD spectra have been shown to have more advantages for these purposes than electronic spectra in the Soret and visible regions (3, 4).

Of these spectrophotometric results obtained in the present work, the intensity of the Soret band (CD and absorption) for the deoxygenated forms were analyzed in terms of a simple two-state model proposed by Monod *et al.* (5). The results indicated that spectral properties of these abnormal hemoglobins in the deoxygenated forms are interpreted only by mixing of two states (T and R) having characteristic values of the Soret intensity in either CD or absorption spectra. This gives an additional proof of the existence of the R and T states in the deoxyhemoglobin that had been discovered experimentally by Gibson (6) and also proposed theoretically by Hopfield (7).

EXPERIMENTAL PROCEDURES

Materials—Pentacyclohexylammonium salt of 2,3-diphosphoglyceric acid was obtained from Boehringer, converted to the acid form by treatment with Dowex 50 resin, and used after being neutralized

with sodium hydroxide. IHP[1] and bis-Tris were purchased from Sigma.

Preparation of Hemoglobin—Human Hb A was prepared from fresh red cells by lysis with deionized water. Four abnormal hemoglobins, Hb J Capetown, Hb Chesapeake, Hb Yakima, and Hb Kempsey, were obtained from the hemolysate of patients' blood and purified. Red blood cells containing abnormal hemoglobins were stored in buffered glycerol at −80°C until use. Hemolysate from patients' blood contained approximately 30 to 40% abnormal hemoglobin and 70 to 60% Hb A. The abnormal hemoglobins were separated from Hb A by column chromatography on DEAE-cellulose (DE32, Whatman) as follows. DE32 was equilibrated with 0.01 M Tris/HCl buffer, pH 8.3, and packed into a 2-cm diameter column to a length of 60 cm for separation of Hb Yakima and Hb Kempsey and to a length of 30 cm for Hb J Capetown and Hb Chesapeake. Hemolysate, which was passed through a Sephadex G-25 column equilibrated with 0.01 M Tris/HCl buffer, pH 8.3, was applied to the column. Separation was performed by a linear gradient from 0 to 0.2 M NaCl in the same buffer solution. The purity of the abnormal hemoglobin was checked by electrophoresis on polyacrylamide slab gel in a discontinuous buffer system (8). No significant contamination of Hb A could be found in any abnormal hemoglobin preparation. All procedures were performed at 4°C.

Stripped hemoglobin was prepared by using Sephadex G-25 which was equilibrated with 0.1 M NaCl in 0.05 M bis-Tris buffer, pH 7.0, at room temperature.

Spectrophotometric Measurements—Absorption spectra were measured with a Cary model 14 recording spectrophotometer. Circular dichroic measurements were performed with a JASCO J-20 recording spectropolarimeter (Japan Spectroscopic Co., Tokyo). An aqueous solution of *d*-10-camphorsulfonic acid was used as a standard with an $\epsilon_L - \epsilon_R$ of 2.2 M^{-1} cm^{-1} at 290 nm (9). The results of CD measurements were expressed in degrees square centimeters per dmol on a heme basis. Derivative spectra were measured with a Hitachi 356 double beam spectrophotometer. Deoxygenation was carried out by repeating alternate evacuation and flushing with Q gas (helium/isobutane, 99.05:0.95) in a Thunberg-type cell with 1-cm and 0.2-cm light paths. Small quantities of sodium borohydride were added to ensure the complete deoxygenation of the sample. The addition of borohydride had no effect on absorption and CD spectra in the Soret and visible regions. Absorption and CD spectra were measured immediately after deoxygenation. Then, the samples were allowed to be oxygenated by air and the spectra were again recorded. Since Hb A and Hb J Capetown in the presence of IHP (at pH 7.0) could not be completely oxygenated by air, 100% oxygenation of hemoglobin was performed by using pure oxygen.

Oxygen Equilibrium—Oxygen equilibrium curve of each hemoglobin in the presence and absence of organic phosphate was determined by a spectrophotometric method according to Sugita and Yoneyama (10). Deoxygenation was carried out by the same method as spectrophotometric measurements. pH of the hemoglobin solution was checked by a Hitachi-Horiba pH meter after measuring oxygen equilibrium. The interaction constant, n value, was calculated from the slope of the Hill's plot at 50% saturation of oxygen. The content of Met-hemoglobin was negligible judging from good isosbestic points of titration spectra and no increase of intensity at ~630 nm during oxygen equilibrium measurements.

Data Analysis—Correlation between the intensity of the CD band and the allosteric constant (L) of hemoglobins was examined by using a FACOM 230-35 (Fijitsu, Co., Tokyo) computer according to the least square method for nonlinear function.

RESULTS

Absorption and CD Spectra of Four Abnormal Hemoglobins—The absorption spectra in the wavelength regions from 375 to 625 nm of the oxygenated and deoxygenated forms of four abnormal hemoglobins were measured in 0.05 M bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. The spectra of Hb Chesapeake and Hb J Capetown were almost the same as those of Hb A in both the oxygenated and deoxygenated forms. The absorption spectra of the oxygenated Hb Yakima and Hb Kempsey did not show any large differences from those of Hb A in all the spectral regions measured, indicating that the conformation surrounding the heme in the oxygenated forms of all the abnormal hemoglobins measured here were not very different from that of Hb A. On the other hand, the spectra of the deoxygenated Hb Yakima and Hb Kempsey differed markedly from those of Hb A (Fig. 1*A*). The Soret band of these two abnormal hemoglobins was broader and flatter than that of Hb A. The extinction coefficient of the maximum at 430 nm of Hb Kempsey and Hb Yakima was about 10% lower than that of Hb A. In the visible region, the absorption maximum at 555 nm found in the deoxygenated Hb A was shifted by 1 to 2 nm to longer wavelength in Hb Yakima and Hb Kempsey. The shoulder observed at ~580 nm in the deoxygenated Hb A was ambiguous in both Hb Yakima and Hb Kempsey. In order to determine the accurate positions of the absorption peaks and the shoulder, we measured the derivative spectra of the deoxygenated Hb Yakima and Hb Kempsey as well as Hb A and its subunits for comparison (Fig. 2). In the figure, one wavelength at which the curve intersects the *zero line* indicates the position of the absorption maximum or minimum and the inflection point of the derivative spectra shows the position of the shoulder. The absorption maximum of Hb Kempsey, Hb Yakima, Hb A, and its subunits was found at 557, 556, 555, and 560 nm, respectively. The shoulder of Hb Kempsey and subunits of Hb A was found at 584 nm, and that for Hb Yakima and Hb A was positioned at 581 nm. Ambiguity of the shoulder in the deoxygenated Hb Yakima and Hb Kempsey seems to come from the red shifts of absorption maximum and shoulder and also from changes of molar extinction coefficient of these two absorption bands. The derivative spectra of α and β chains were slightly different, so we superimposed the mean value of the two chains in Fig. 2.

These differences in absorption spectra of the deoxygenated Hb Yakima and Hb Kempsey were magnified in their CD

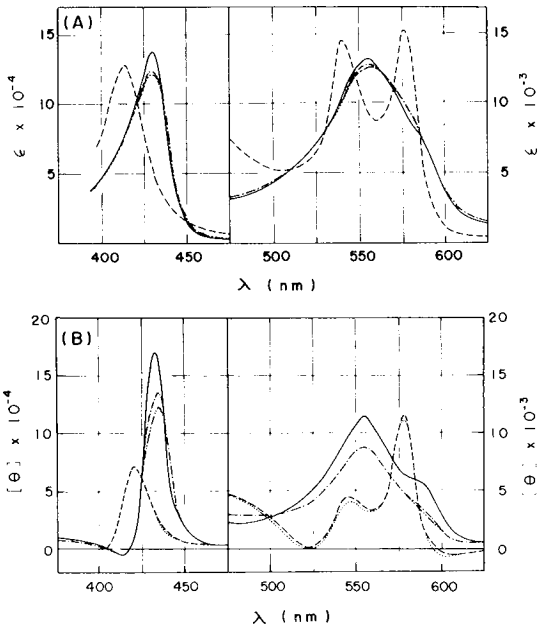

FIG. 1. Absorption and circular dichroic spectra in the Soret and visible regions of the normal and abnormal hemoglobins. *A* and *B* show the absorption and CD spectra, respectively, of the oxygenated Hb A, Hb Yakima, and Hb Kempsey (– – –), and the deoxygenated Hb A (——), Hb Yakima (-·-·-), and Hb Kempsey (----). In all cases, heme concentration was 50 μM in 0.05 M bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. A cell with a 1-cm light path was used for the region between 475 and 625 nm and a cell with a 0.2-cm light path was used for the region between 375 and 475 nm. The extinction coefficient (ϵ) (M^{-1} cm^{-1}) and the ellipticity (θ) (degrees square centimeters per dmol) are on a molar heme basis.

[1] The abbreviations used are: IHP, inositol hexaphosphate; bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

spectra. The CD spectra of the oxygenated and deoxygenated Hb Yakima and Hb Kempsey were shown in Fig. 1*B*. In this figure are also shown the CD spectra of Hb A for a comparison. The positive extremum at 433 nm which was observed in normal hemoglobin was shifted by 1.5 nm to a longer wavelength in both the deoxygenated abnormal hemoglobins. The molar ellipticity was greatly decreased, 80% in Hb Yakima and 73% in Hb Kempsey compared to that found in Hb A. In the visible region, the deoxygenated Hb A exhibited two positive extrema at 555 and 580 nm, coincident with the absorption maximum and the shoulder. On the other hand, the deoxygenated Hb Yakima and Hb Kempsey gave only one CD band at ~555 nm. In Fig. 3, we compared the CD spectra of the deoxygenated Hb Kempsey with that of isolated chains and native Hb A. The shape of the spectra of Hb Kempsey resembled that of isolated chains rather than that of Hb A. The greatly decreased ellipticity and the red shift of the Soret band that was found in the deoxygenated Hb Kempsey are also shown in the spectra of isolated chains. In the visible region, the positive band at ~580 nm that was found in the spectra of Hb A was not shown in the spectra of noncooperative isolated chains and Hb Kempsey. From these spectral similarities of Hb Kempsey to isolated chains, it is suggested that in Hb Kempsey no specific interaction exists among their subunits. In the CD spectra of Hb Chesapeake and Hb J Capetown, no significant differences from those of Hb A were observed in either the oxygenated or the deoxygenated forms. In the oxygenated forms, CD spectra of all abnormal hemoglobins used in this work are very similar to that of Hb A.

Changes in Absorption and CD Spectra by Addition of Inositol Hexaphosphate—In the previous paper (12), we reported that Hb Yakima and Hb Kempsey lacked the heme-heme interaction, whereas by the addition of IHP to hemoglobin solution both abnormal hemoglobins restored cooperativity on oxygen binding. From the results, it is suggested that the absorption and CD spectra of Hb Yakima and Hb Kempsey might change greatly in the presence of IHP on the basis of their conformational change. The absorption and CD spectra of Hb Yakima in the presence and absence of IHP are shown in Fig. 4. As the spectra of the oxygenated Hb Yakima were almost the same irrespective of the presence or absence of IHP, only the spectra of the deoxygenated form are shown in the figure. The extinction coefficient of the Soret band was increased by binding with IHP. In the visible region, the absorption peak at 556 nm was shifted to a shorter wavelength and the shoulder became apparent at ~580 nm in the spectra of Hb Yakima. The resultant spectra of Hb Yakima·IHP complex were almost the same as those of Hb A.

In the CD spectra, more marked changes were observed at the same wavelength region in which the absorption spectra changed when IHP was added to Hb Yakima solution. The extremum increased greatly and a blue shift of the peak was observed. The positive extrema at 555 and 580 nm were increased and the whole shape of the CD spectra in the deoxygenated Hb Yakima approached those of Hb A. Similar spectral changes induced by IHP were also observed in the spectra of Hb Kempsey. However, 2,3-diphosphoglyceric acid, ATP, and inorganic phosphate had no effect on the spectra of the stripped Hb Yakima and Hb Kempsey.

The effects of IHP on absorption and CD spectra of four abnormal hemoglobins and Hb A in the Soret and visible regions are summarized in Table I. Spectra of Hb Chesapeake, Hb J Capetown, and Hb A did not change significantly by the addition of IHP. The values of the extinction coefficient and the ellipticity of Hb Yakima and Hb Kempsey in the presence of IHP approached to those of normal hemoglobin.

Functional Abnormalities of Four Abnormal Hemoglobins and Effect of IHP—For the purpose of considering the correlation between optical properties of four abnormal hemoglobins and their functions, we measured oxygen equilibrium curves of these hemoglobins under the same conditions as used for the spectrophotometric measurements. Oxygen affin-

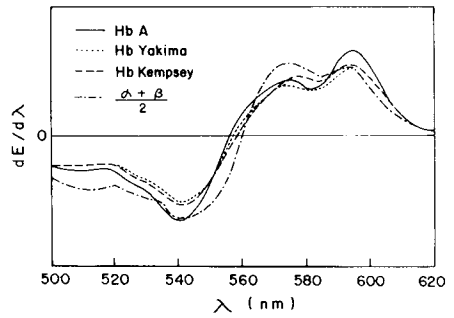

FIG. 2. Derivative spectra of the deoxygenated Hb A (——), Hb Yakima (-----), and Hb Kempsey (– – –), and the calculated means of the derivative spectra for the deoxygenated α and β chains (–·–·). Heme concentration was 100 μM. The cell used had a 1-cm light path. $d(\lambda_1-\lambda_2)$ was adjusted to 1-nm intervals. The spectra scan was carried out from longer to shorter wavelength.

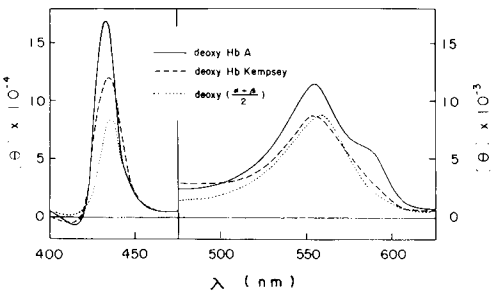

FIG. 3. Comparison between the CD spectra of the deoxygenated Hb Kempsey and the calculated means of the isolated α and β chains. The spectrum of the deoxygenated Hb A was superimposed in the figure for comparison. Conditions were the same as in Fig. 1. The CD spectra of the isolated chains were reported in the previous paper (11).

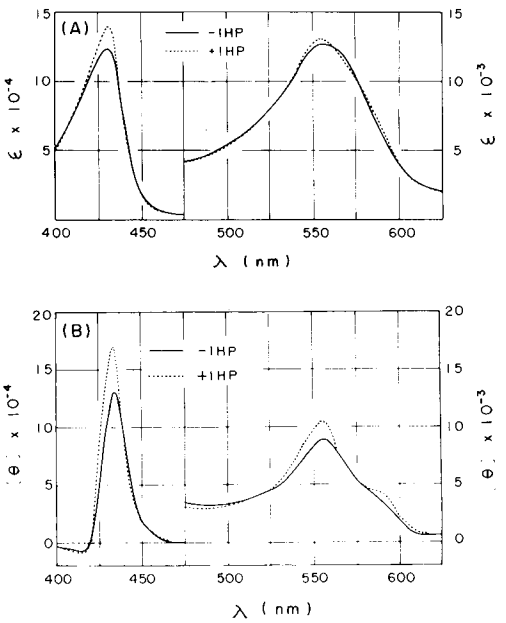

FIG. 4. Absorption (*A*) and CD (*B*) spectra of the deoxygenated Hb Yakima in the presence and absence of IHP. Heme concentration was 50 μM in 0.05 M bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. IHP concentration added was 500 μM.

ity (p_{50}) and Hill's n of four abnormal hemoglobins in the presence and absence of IHP and those of Hb A for comparison are summarized in Table II. Oxygen affinity and Hill's n of Hb J Capetown was not much different from those of Hb A. Hb Yakima and Hb Kempsey showed a very high oxygen affinity comparable to those of isolated chains. These two β-99 aspartic acid-substituted hemoglobins had no cooperative ligand binding property (n = 1.0). Hb Chesapeake had an oxygen affinity and Hill's n intermediate between Hb Yakima and Hb J Capetown.

Addition of IHP to these hemoglobins lowered the oxygen affinity of all the hemoglobins measured here and effectively increased the value of Hill's n, recovering the cooperativity of ligand binding. Functional characters of these hemoglobins presented here have no conflict with the data of Nagai *et al.* (12) (for Hb Yakima and Hb Kempsey) and of Imai (for Hb Chesapeake) (13).

In the theory of allostery of Monod *et al.* (5), the degree of cooperativity depends on L, the equilibrium between the R and T states in the absence of ligand, and on $c = K_R/K_T$, where K_R and K_T signify the microscopic dissociation constant for ligand in the R and T states.

These allosteric parameters, L and c, of four abnormal hemoglobins and normal hemoglobin in the presence and absence of IHP, calculated according to the equation cited by Edelstein (14) and Equation 1 are also shown in the third and fourth columns of Table II, respectively.

TABLE I

Summary of spectrophotometrical properties of abnormal hemoglobin and Hb A in the presence and absence of IHP

The condition of measurements were the same as described in the legends to Figs. 1 and 2. The peak positions of the absorption spectra were determined by measuring derivative spectra.

Hemoglobin	IHP	Absorption band		CD band	
		Max	$\epsilon \times 10^{-3}$	Max	$\theta \times 10^{-3}$
		nm		*nm*	
A	−	430	141	433	173
		555	13.3	555	11.1
	+	430	140	433	175
		555	13.4	555	11.5
J Capetown	−	430	140	433	171
		555	13.1	555	10.9
	+	430	140	433	170
		555	13.4	555	11.0
Chesapeake	−	430	140	433	173
		555	13.4	555	11.3
	+	430	142	433	170
		555	13.4	555	10.7
Yakima	−	430	125	435	135
		556	12.9	555	8.8
	+	430	141	433	168
		555	13.4	555	10.5
Kempsey	−	430	122	435	128
		557	12.8	555	8.7
	+	430	140	433	162
		555	13.3	555	10.9

TABLE II

Oxygen equilibrium properties and Monod-Wyman-Changeux (5) parameters of normal and abnormal hemoglobin in the absence and presence of IHP

The oxygen equilibrium curves were measured in 0.05 M bis-Tris buffer, pH 7.0, containing 0.1 M NaCl, and with or without 1 mM IHP at 25°C. The hemoglobin concentration was 50 μM (heme basis). The Monod-Wyman-Changeux parameters, L and c, were determined according to the equations as shown in the text.

Hemoglobin	IHP	Hill's n	p_{50}	L	c
			mm Hg		
A	−	2.7	5.4	3.3×10^{4}	0.01
	+	2.6	54.0	3.3×10^{8}	0.004
J Capetown	−	2.2	3.0	4.1×10^{3}	0.042
	+	2.4	19.5	5.7×10^{6}	0.017
Chesapeake	−	1.2	0.72	1.0×10^{1}	0.35
	+	1.9	5.6	3.8×10^{4}	0.035
Yakima	−	1.0	0.36	1.0	1.0
	+	1.8	2.25	1.0×10^{3}	0.098
Kempsey	−	1.0	0.28	2.5×10^{-1}	1.43
	+	1.6	1.4	1.5×10^{2}	0.13

DISCUSSION

The ligand affinities and Hill's n for four abnormal hemoglobins and normal hemoglobin under various conditions (pH, ±IHP) are plotted on the same figure as shown in Fig. 5. The data for Hb Yakima, Hb Kempsey, and Hb A had already been reported by Nagai *et al.* (12) who assumed that the data could not fit one bell-shaped curve but three bell-shaped curves having different c values. The additional data concerned with Hb Chesapeake and Hb J Capetown seem to fit either one or two curves. However, that of the stripped Hb Chesapeake could not fit either curve. From these results, we suggest that hydrogen ion concentration affects oxygen affinity of various hemoglobins by changing the allosteric constant (L) but does not change the c value, whereas organic phosphate, especially IHP, changes the ligand affinity more strongly than hydrogen ion concentration and probably affects both L and c values. The changes of c indicate that the dissociation constant for oxygen binding of the T state of these abnormal and normal hemoglobins differs from each other, if we assume the dissociation constant of the R state to be constant among all hemoglobins. IHP may shift the R-T equilibrium toward the predominant T state, resulting in the rise of L, and at the same time quantitatively change the ligand affinity of the T state.

To consider the correlation between optical properties of four abnormal hemoglobins and their function, the allosteric parameters, L and c, were calculated from p_{50} and Hill's n for normal and abnormal hemoglobins in the presence and absence of IHP according to the equation of Edelstein (14) and the following equation as proposed previously by Bunn and Guidotti (15):

$$n = 1 + 3\frac{(\gamma - 1)\,(c\gamma - 1)}{(1 + \gamma)\,(c\gamma + 1)} \qquad (1)$$

where n is Hill's constant and γ is given by $p_{50}/0.4$. The c values obtained for each hemoglobin are summarized in the fourth column of Table II. Among hemoglobin species, the c values differed from each other and also from the same

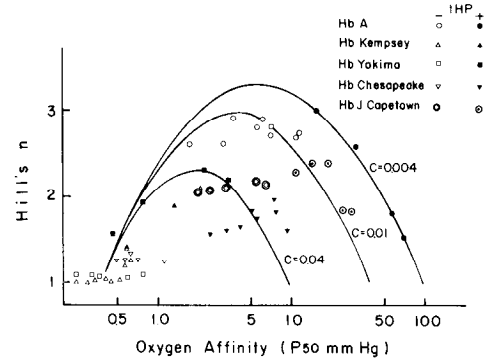

FIG. 5. Dependence of Hill's constant and allosteric parameter, c, on oxygen affinity. Plots were obtained from the data for the hemoglobins measured at various pH values in the presence and absence of IHP. pH range from 6.5 to 8.5 was usually used. Three *solid curves* indicate the theoretical ones calculated with c = 0.01, 0.04, and 0.004 according to Equation 1. *Open symbols,* absence of IHP; *closed symbols,* presence of IHP.

hemoglobin with and without IHP.

Analysis of optical properties of abnormal hemoglobins in terms of two-state model, were carried out as follows. If the intensity of the CD spectra, (θ), obeys two-state theory in which we assume the intrinsic θ_R and θ_T for the R and T state, respectively, the observed (θ) of each hemoglobin could be given by the following equation:

$$(\theta) = \frac{R}{T+R}(\theta_R) + \frac{T}{T+R}(\theta_T) \qquad (2)$$

where T/R is defined as allosteric constant L. Substituting L in Equation 2, we have Equation 3:

$$(\theta) = \frac{1}{1+L}[(\theta_R) + L(\theta_T)] \qquad (3)$$

Among the spectral abnormalities of the deoxygenated hemoglobins, we chose the positive CD band in the Soret region for this analysis. In Table I, molar ellipticity (θ) of the positive peaks in the Soret region of the deoxygenated hemoglobins was summarized. Their logarithmic values of θ were plotted against log $(1 + L)$ as shown in Fig. 6. From the observed ellipticities on normal and abnormal hemoglobins with and without IHP, the most suitable values of (θ_R) and (θ_T) which satisfy the Equation 3 were estimated by computer curve fitting analysis. As shown in Fig. 6, (θ_R) and (θ_T) were obtained as 1.1×10^5 and 1.69×10^5, respectively. These results strongly suggest that the positive CD band in the Soret region could be explained in terms of a two-state model, that is, by proportional mixing of the intrinsic (θ_R) and (θ_T) for the R and T states. In addition to the present data, those of carboxypeptidase A-digested Hb A (11), Hb Rainier ($\alpha_2\beta_2^{145Tyr \rightarrow Cys}$) (16), and Hb Hiroshima ($\alpha_2\beta_2^{146His \rightarrow Asp}$) (17) were superimposed in Fig. 6. These abnormal hemoglobins and modified hemoglobin which have altered functions also fit to this simulated curve. Addition of IHP to Hb Yakima and Hb Kempsey causes a great change in the allosteric constant, L, and the result will be a higher percentage for the T state. As Hb A and Hb J Capetown have enough values of L to show a high ellipticity in the Soret band, further addition of IHP has no effect on the CD spectra. The L of Hb Chesapeake might be a critical value for the change of the spectrum. The intensity of the hemoglobin fixed to the R state, $(\theta_R) = 1.1 \times 10^5$, is significantly larger than the mean value of α and β subunits, $(\theta) = 0.8 \times 10^5$. It is indicated that the structure of the hemoglobin

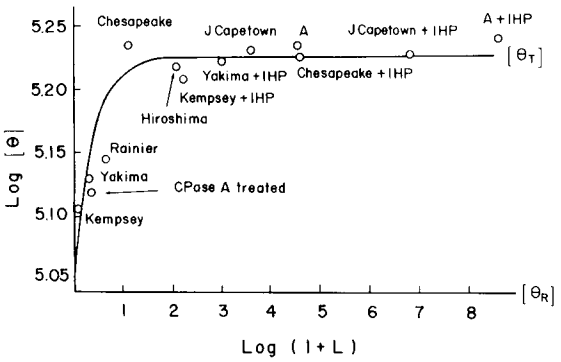

FIG. 6. Correlation between molar ellipticity at 433 nm and allosteric constant, L, of deoxygenated hemoglobins. The logarithmic values of θ of various deoxygenated hemoglobins presented in Table II are plotted against log $(1 + L)$. The values of L shown in Table I are used in this figure. The *curve* in the theoretical ones is calculated from constant θ_T and θ_R, and variable L. θ_T and θ_R indicate molar ellipticities estimated by the least squares method for the T and R state of deoxygenated hemoglobin, respectively. The data used for Hb Rainier (16), Hb Hiroshima (17), and CPase (carboxypeptidase A)-treated Hb A (11) were published elsewhere.

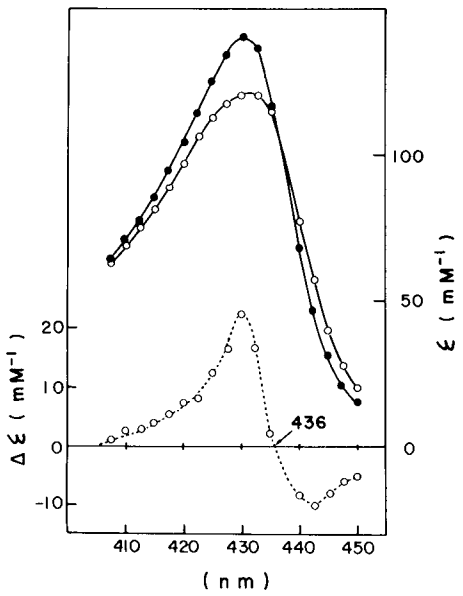

FIG. 7. Calculated absorption spectra of the R and T state of deoxygenated hemoglobin in the Soret region and difference spectrum between them. The procedure of calculation for the spectra was described in the text. ●——●, spectrum of the deoxy-T state hemoglobin; ○——○, spectrum of the deoxy-R state hemoglobin; -----, difference spectrum (T minus R spectrum). *Arrow* indicates an isosbestic point at 436 nm.

fixed to the R state could not be represented by a simple mixing of α and β chains and some interactions different from those of the T state also exist among four subunits. By expansion of these analyses to all spectral regions of the Soret band, we could get absorption and CD spectra of the specific R and T states of hemoglobin. Calculated spectra of absorption for the hemoglobin fixed to the R state and that for the T state, in addition to the difference spectrum between them, are shown in Fig. 7. Calculation of the absorption spectra for the R and T state of hemoglobin was carried out from the Soret band of Hb Kempsey, Hb Yakima, Hb A (−IHP), and Hb A (+IHP), according to a new equation, in which θ, θ_R, and θ_T, of Equation 3 were replaced by ϵ, ϵ_R, and ϵ_T, respectively, using the least squares method.

Evidence for the two forms of deoxyhemoglobin described here was first discovered by Gibson (6) when he observed that flash photolysis of carbonmonoxy hemoglobin resulted in a slow and a fast reacting phase (Hb and Hb*) which could be distinguished by the strength of their absorption at 430 nm, and more recently was shown by laser photolysis of oxyhemoglobin A (18, 19). For the characteristic spectra of the quaternary R structure of deoxygenated hemoglobin, Perutz and his collaborators (20) showed the spectra of *N*-ethylsuccinimide-des-Arg hemoglobin, des-Arg-Tyr hemoglobin, and Hb Kempsey. They compared the difference spectrum of *N*-ethylsuccinimide-des-Arg hemoglobin in the Soret band with the kinetic difference spectrum between deoxyhemoglobin A and the sum of the free deoxy α and β subunits discovered by Brunori *et al.* (2). They concluded that the two difference spectra were identical. However, the difference spectra obtained by flash photolysis of oxyhemoglobin, reported by Sawicki and Gibson (19), are not the same as those shown by Perutz *et al.* (1) and Brunori *et al.* (2), especially in the intensity of the peaks. The former spectra have a peak at 431 nm of the extinction coefficient of 28 $mM^{-1} \cdot cm^{-1}$ and a negative trough at 422 nm of 12 $mM^{-1} \cdot cm^{-1}$, and the latter spectra have a peak at 430 nm of the extinction coefficient of 17 mM^{-1} and a negative trough at 422 nm of 7 $mM^{-1} \cdot cm^{-1}$. In the difference spectra between quaternary R and T structures in

the deoxygenated form calculated in the present paper, the extinction coefficient of a peak at 430 nm is 25 $mM^{-1} \cdot cm^{-1}$ and that of a negative trough at 425.5 nm is 10 $mM^{-1} \cdot cm^{-1}$, and an isosbestic point seems to exist at 436 nm. Judging from the spectral properties, the difference spectrum calculated by us in this paper is rather similar to that formed by 97% photolysis of oxyhemoglobin (19). The low intensity of the difference spectra shown by Perutz *et al.* (20) might be due to co-existence of the T conformation in the deoxy-*N*-ethylsuccinimide-des-Arg hemoglobin. Generally, Hb Yakima and Hb Kempsey were considered to remain in the quaternary of the R type even when fully deoxygenated. However, according to the present analysis of the Soret band in terms of the two states, the spectrum of Hb Yakima comes from the 50% R structure and the 50% T structure (L = T/R = 1.0 for Hb Yakima).

Perutz *et al.* (20) pointed out that in the absorption bands of the deoxyhemoglobin, a blue shift of all absorption bands will occur through the R and T structure transition. In the absorption spectra of Hb Yakima and Hb Kempsey, the peak in the visible band of the deoxygenated form exhibited a red shift by 1 or 2 nm. However, we failed to observe the red shift of the Soret absorption band of Hb Yakima and Hb Kempsey. It must be due to experimental difficulties, since the shift of the Soret band of these hemoglobins may be too small to detect. In the CD spectra of Hb Yakima and Hb Kempsey, red shifts of all extrema in the Soret and visible regions were observed. It is suggested that the extent of the wavelength shifts of the spectra depends on the length of the bond distance from the porphyrin to the iron (20). The porphyrin-iron distance of Hb Yakima and Hb Kempsey might be shorter than that of normal hemoglobin. It might be improbable, however, that the geometry of the heme of deoxyhemoglobins is assumed to be heterogenous among abnormal hemoglobins, if our considerations are adopted on intensity changes of CD and absorption spectra in solution as discussed above. Rather, wavelength shifts and intensity changes seen in some deoxyhemoglobins should be considered uniformly by alteration of the equilibrium constant, L, existing between the hemoglobin species, with spectra characteristic of the deoxy-R and -T state.

In the absence of IHP, it seems likely that the oxygen affinities of the T state of various hemoglobins, K_T, are different from each other, because all the hemoglobins examined by us have different c values as shown in Table I, whereas K_R, the oxygen affinity of the R state, is thought to be similar among all the stripped hemoglobins, and this assumption has been indeed considered by several investigators (13, 21). IHP also greatly reduced c values compared with those of the corresponding stripped hemoglobins, indicating that K_T of the hemoglobin was greatly decreased by IHP binding. What are the structural properties governing the oxygen affinity for the deoxyhemoglobin which was affected by amino acid substitutions due to point mutations, or by binding of allosteric effectors?

Although the complete answer for this problem could not be provided at this time, we could at least claim that the electronic and stereostructure of the heme in the deoxygenated hemoglobins play, only in part, the role in regulation of the oxygen affinity. Other structural factors determining the c value of hemoglobin will be described in detail in a forthcoming paper.

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Analysis of optical properties of hemoglobins in terms of the two-state model, especially from studies on abnormal hemoglobins with amino acid substitution in the alpha 1 beta 2 contact region.

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