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Correlation between Narrow-Banded Ultraviolet Spectra and Oxygen Equilibrium Functions in Native and Chemically Modified Human Hemoglobins†

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ABSTRACT: Correlation between narrow-banded ultraviolet spectra and oxygen equilibrium functions was studied by using human adult hemoglobin and its derivatives prepared by treating it with iodoacetamide, *N*-ethylmaleimide and carboxypeptidase A. Differential spectrum, *i.e.*, the first derivative of absorption spectrum, showed a fine structure with a maximum at 289.5 nm and two minima at 286.5 and 293.5 nm for all the hemoglobins at the position corresponding to the shoulder in the absorption spectrum. The magnitude of the fine structure in the differential spectrum for native oxyhemoglobin was twofold that for deoxy one. The magnitude of the fine structure for deoxy form and the association constant for the first oxygen molecule were increased by the chemical modifications of protein as well as by the stripping of 2,3-diphosphoglycerate, and there was a distinct correlation between the two quantities. Neither the magnitude of the fine structure for oxy form nor the association constant for the fourth oxygen molecule were affected by the chemical modifications or by 2,3-diphosphoglycerate. A fine structure also appeared around 290 nm in oxy *vs.* deoxy difference spectra of all the hemoglobins. The magnitude of the fine structure in difference spectra was closely related to the magnitude of cooperativity in oxygen binding which was measured by overall free energy of interaction among the oxygen binding sites or by Hill's coefficient. The less the cooperativity, the smaller the magnitude of the fine structure of the difference spectra whether 2,3-diphosphoglycerate was present or absent. These spectral changes cannot be explained only by heme contribution, indicating that there are contributions of aromatic chromophores. Although the aromatic groups responsible for the spectral changes cannot be explicitly identified, the present results are consistent with the idea that C3β tryptophans located at the $\alpha_1\beta_2$ contact are most probably involved. The data also suggest that the differential spectra around 290 nm are partial reflections of the conformational states the tetramer assumes in the oxy–deoxy reaction and that the oxy *vs.* deoxy difference spectra indicate the extent of the conformational changes which play key roles in the cooperative oxygen binding of hemoglobin.

In order to investigate the molecular mechanism for the allosteric effects in hemoglobin, *i.e.*, cooperative oxygen binding, Bohr effect, reciprocal binding of 2,3-diphosphoglycerate (P_2-glycerate[1]), etc., it is critically important to study the relationship between the allosteric ligand binding functions and conformational changes in the protein moiety induced by ligand binding.

Difference spectrum between oxy- and deoxyhemoglobins in the ultraviolet (uv) region from about 280 to 295 nm exhibits narrow-banded difference peaks which are superposed on the broader heme contribution and are attributable to perturbations of aromatic chromophores (Enoki and Tyuma, 1964; Briehl and Hobbs, 1970). If the perturbations are due to environmental changes around specific aromatic groups in the protein, it can be expected that the uv difference spectra reflect conformational changes in the protein and are related to the allosteric ligand binding functions of hemoglobin.

It has been found in the present study that the spectral changes in the uv region are closely related to the oxygen equilibrium functions.

Materials and Methods

Materials. Hemoglobin prepared from the blood of normal human adults (Hb A, native Hb) was freed from phosphates as described by Benesch *et al.* (1968). Three kinds of chemically modified hemoglobins were prepared from the stripped hemoglobin as follows. The reactive sulfhydryl groups of F9 (93) β-cysteines were blocked by the reaction with ten- and fivefold molar excesses of iodoacetamide and *N*-ethylmaleimide, respectively, in 0.1 M Bis-Tris buffer (pH 7.3) for 1 hr at 25° (Benesch and Bensch, 1961) and the resulting

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[1] Abbreviations used are: P_2-glycerate, 2,3-diphosphoglycerate; native Hb, Hb(AcAm), Hb(MalN), and Hb(CPase), human adult hemoglobin untreated and its derivatives prepared by treatment with iodoacetamide, *N*-ethylmaleimide, and carboxypeptidase A, respectively; *Y*, fractional saturation of hemoglobin with oxygen; *p*, oxygen pressure; P_{50}, oxygen pressure at the half-saturation; k_1 and k_4, intrinsic association constants for the first and fourth oxygen molecules, respectively; *n*, Hill's coefficient; ΔF_1, overall free energy of interaction among the oxygen binding sites; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol.

hemoglobin derivatives are designated as Hb(AcAm) and Hb(MalN), respectively. Titration experiments with *p*-mercuribenzoate showed complete block of the reactive sulfhydryl groups by *N*-ethylmaleimide and more than 90% block by iodoacetamide. The C-terminal histidine and penultimate tyrosine residues of the β chains were removed by the digestion of native Hb for 3 hr at 30° in 0.05 M Tris-HCl buffer (pH 8.0) containing carboxypeptidase A (Sigma Chemical, St. Louis, Mo.) in a weight ratio enzyme:Hb of 0.02 (Antonini *et al.*, 1961). The digestion was stopped by the addition of 1 mM hydrocinnamic acid. Amino acid analysis showed the release of 0.96 histidine and 0.89 tyrosine residues per $\alpha\beta$ dimer. This digested hemoglobin is designated as Hb(CPase). All these modified hemoglobins were freed from materials of low molecular weight such as extra sulfhydryl reagents and released amino acids by passing through a column of Sephadex G-25 equilibrated with 0.05 M Bis-Tris HCl buffer (pH 7.4). The tetrameric nature of these modified hemoglobins has been already established to be equivalent to native Hb (Antonini *et al.*, 1961; Guidotti, 1967).

P_2-glycerate was obtained as the pentacyclohexylammonium salt tetrahydrate (Calbiochem, Los Angeles, Calif.) and converted into free acid by passing through a column of H-form Dowex 50 resin and its concentration was determined by titration (Benesch *et al.*, 1969). Bis-Tris was purchased from Aldrich Chemical Co., Milwaukee, Wis. Iodoacetamide and *N*-ethylmaleimide (Nakarai Chemical, Kyoto, Japan) were reagent grade and were used without further treatment.

Determination and Analysis of Oxygen Equilibrium. Oxygen equilibrium curves were determined for 6.0×10^{-5} M (on heme basis) hemoglobin in 0.05 M Bis-Tris-HCl buffer (pH 7.4) in the absence and presence of 2 mM P_2-glycerate using the automatic recording method of Imai *et al.* (1970). The curves for native Hb, Hb(AcAm), and Hb(MalN) were determined at 25°. The curves for Hb(CPase) were determined at 30° and were corrected for 25° by shifting them along the abscissa by $\Delta \log p = 0.12$ (p is oxygen pressure) toward the left hand corresponding to the heat of oxygenation of that hemoglobin (Antonini *et al.*, 1961).

The oxygen equilibrium curves were expressed by both the Hill (Wyman, 1964) and Scatchard (Edsall *et al.*, 1954) plots, *i.e.*, $\log [Y/(1 - Y)]$ *vs.* $\log p$ and $\log [Y/(1 - Y)p]$ *vs.* $4Y$ plots where Y and p are fractional saturation of hemoglobin with oxygen and partial oxygen pressure (mm), respectively. Hill's coefficient, n, an empirical measure of cooperativity in oxygen bindig, and overall oxygen affinity, $\log P_{50}$, where P_{50} is oxygen pressure at the half-saturation, were estimated from the Hill plot. Intrinsic association constants, *i.e.*, the constants corrected for statistical factors, for the first (k_1) and fourth (k_4) oxygen molecules were obtained from the extrapolated intercepts of the Scatchard plot on both the ordinates (Edsall *et al.*, 1954). Overall free energy of interaction among the oxygen binding sites, ΔF_I, which is obtained from the vertical distance between the upper and lower asymptotes of the Hill plot (Wyman, 1964), was simply estimated from the following relation: $\Delta F_I = RT \ln (k_4/k_1)$, which gives the same results as Wyman's method since the upper and lower asymptotes are expressed by $\log [Y/(1 - Y)] = \log p + \log k_4$ and $\log [Y/(1 - Y)] = \log p + \log k_1$, respectively (Tyuma *et al.*, 1971).

Ultraviolet-Differential and Difference Spectra. Differential spectra, *i.e.*, the first derivatives of absorption spectra, were determined with an on-line differential converter (Shiga *et al.*, 1971) combined with a Hitachi Model 124 recording spectrophotometer (Hitachi Co., Tokyo, Japan). This combination was employed with circuit constants of the differentiator, $C = 1$ μF and $R = 10$ MΩ, and a scanning speed of the wavelength, 1 nm/sec, which completely satisfy the conditions for an accurate derivative conversion. Deoxygenation of hemoglobin was performed in a Y-shaped tonometer (Benesch *et al.*, 1965) with a square fused silica cuvet of 10-mm light path by repeated alternative evacuations and flushings with pure nitrogen gas (99.999%), accompanied by mild shaking of the tonometer. Although the concentration of P_2-glycerate used for the differential spectrum measurements, 0.5 mM, was different from that used for the oxygen equilibrium experiments, 2 mM, comparison between the spectral and functional data will be effective, since 0.5 mM P_2-glycerate has almost saturated effect on oxygen equilibrium (Benesch *et al.*, 1968). Difference spectra of oxy- *vs.* deoxyhemoglobin in the absence and presence of P_2-glycerate were determined as previously described (Imai *et al.*, 1972) using the same spectrophotometer mentioned above. During all these spectrophotometrical measurements the bandwidth of the incident light was fixed at 1 nm over the entire range of the wavelength used. The differential and difference spectra were determined in the uv range of 240–350 nm. Reproducibility of these spectra was very good and the average readings of the spectra from two or three experiments for different preparations are given in this paper. Absorption spectra of all the samples were also determined over the range of 240–650 nm except around the Soret band before and after the determination of the differential and difference spectra. The spectra in visible range were used for the estimation of the amounts of deoxy-, oxy-, and methemoglobins by the method of Benesch *et al.* (1965). The concentration of oxygenated and reoxygenated hemoglobins was obtained from the optical density (OD) at 307 nm using the molar extinction coefficient for oxyhemoglobin (ϵ 1.58×10^4 M^{-1} cm^{-1}) (Morell *et al.*, 1964) and is expressed on heme basis in this paper. Amount of residual oxy form in the deoxygenated samples was less than 5% except for Hb(CPase) which contained no more than 10%. Amount of methemoglobin after the determination of the differential and difference spectra was less than 7% except for Hb(CPase) which contained up to 12%.

Results

Oxygen Equilibrium. Figure 1 shows the Scatchard plots of the oxygen equilibria for the native and modified hemoglobins. Table I summarizes the estimates of the oxygen equilibrium parameters, k_1, k_4, ΔF_I, n, and P_{50}, for those hemoglobins. The results for native Hb agree well with those obtained by Tyuma *et al.* (1971). The values of P_{50} and n for the modified hemoglobins in the presence of 2 mM P_2-glycerate agree essentially with those of previous investigators using inorganic phosphate buffer (Benesch and Benesch, 1961; Antonini *et al.*, 1961; Riggs, 1961; Taylor *et al.*, 1966); slight descrepancies are probably ascribable to differences in response of the hemoglobins to the phosphates.

The present results clearly indicate that k_4's for Hb(AcAm) and Hb(MalN) are insensitive to P_2-glycerate whereas k_1's are markedly reduced by the phosphate as in the case of native Hb, and that the magnitude of cooperativity measured by ΔF_I or n is increased on the addition of P_2-glycerate for Hb(AcAm) and Hb(MalN) as well as for native Hb. On the other hand the oxygen equilibrium function of Hb(CPase) is scarcely affected by P_2-glycerate, contrary to the early

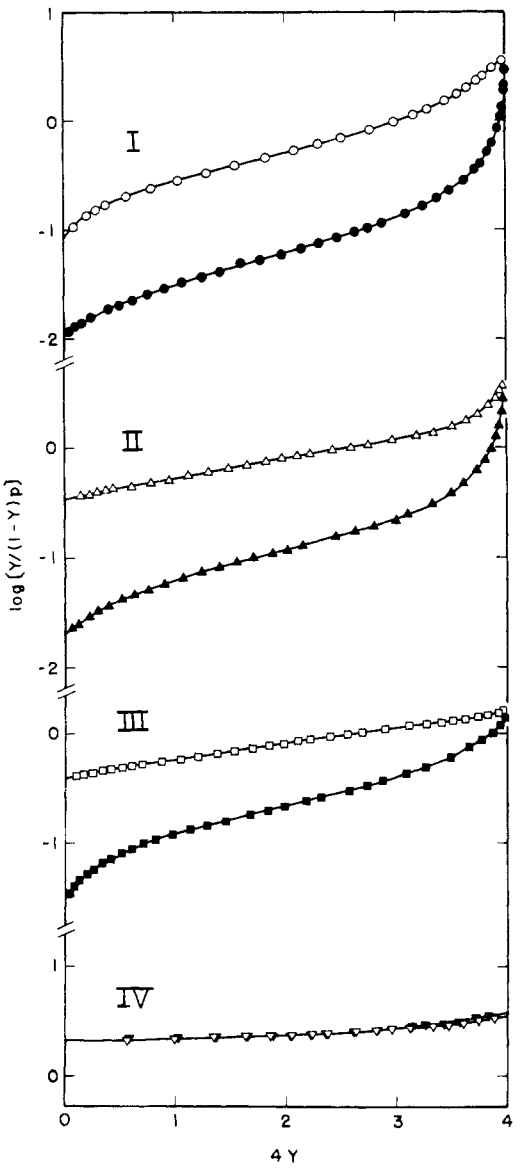

FIGURE 1: Scatchard plots of oxygenation of native Hb (I), Hb (AcAm) (II), Hb(MalN) (III), and Hb(CPase) (IV) in the presence and absence of 2 mM P_2-glycerate. Y, fractional oxygen saturation of hemoglobin; p, oxygen pressure (mm). Open and filled symbols indicate the experimental points for the absence (stripped) and presence, respectively, of 2 mM P_2-glycerate. 25°; in 0.05 M Bis-Tris buffer (pH 7.4). The plots for Hb(CPase) have been corrected for 25° (see the text).

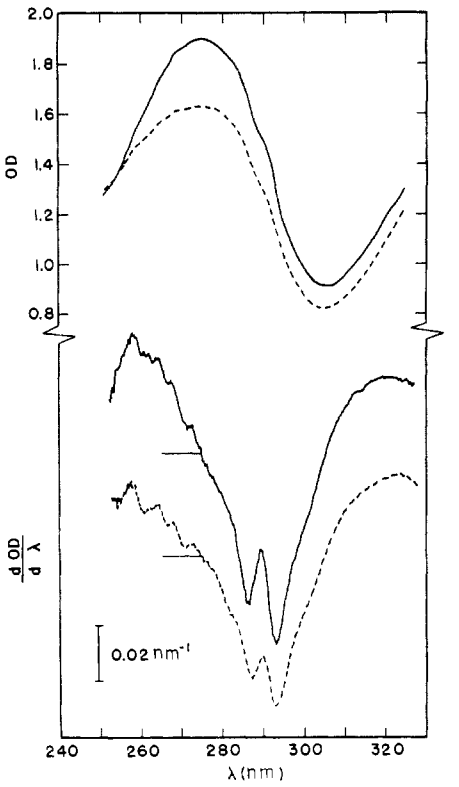

FIGURE 2: Ultraviolet absorption (upper portion) and differential (lower portion) spectra of stripped native Hb. (——) Oxy form; (- - - -) deoxy form. The horizontal lines attached to the differential spectra indicate their respective base lines. Hemoglobin concentration, 5.9×10^{-5} M; in 0.05 M Bis-Tris buffer (pH 7.4).

TABLE I: Summary of Oxygen Equilibrium Parameters.[a]

	k_1[b]	k_4[b]	ΔF_I[c]	n[d]	P_{50}[e]
Native Hb					
Stripped	0.083	4.0	2300	2.5	1.9
In 2 mM P_2-glycerate	0.011	4.0	3480	3.0	15.3
Hb(AcAm)					
Stripped	0.35	4.4	1500	1.7	1.2
In 2 mM P_2-glycerate	0.020	4.4	3190	2.7	8.5
Hb(MalN)					
Stripped	0.40	1.6	820	1.4	1.2
In 2 mM P_2-glycerate	0.032	1.6	2320	2.3	4.7
Hb(CPase)					
Stripped	2.1	3.5	300	1.2	0.44
In 2 mM P_2-glycerate	2.1	3.7	330	1.2	0.44

[a] Conditions as for Figure 1. [b] Intrinsic association constants for the first (k_1) and fourth (k_4) oxygen molecules (mm^{-1}). [c] Overall free energy of interaction among the oxygen binding sites (cal/site). [d] Hill's coefficient. [e] Oxygen pressure at the half-saturation (mm).

data of Chanutin and Curnish (1968). It is noteworthy that k_4's for Hb(AcAm) and Hb(CPase) are similar to those for native Hb whether P_2-glycerate is present or absent. The values of k_4 for Hb(MalN) are, however, somewhat smaller than those for the other hemoglobins. The cooperativities of all the modified hemoglobins are smaller than that of native Hb and decrease in order for Hb(AcAm), Hb(MalN), and Hb(CPase) both in the presence and absence of P_2-glycerate. Statistical analysis and more detailed discussion of the above data will be published elsewhere.

Ultraviolet Differential and Difference Spectra. The upper portion of Figure 2 shows the uv absorption spectra of oxy and deoxy native Hb. Both the spectra have a minute shoulder around 290 nm, the shapes of which are quite similar, making distinction between the two almost impossible. Distinguishing between them is possible, however, when we take the first derivative of the absorption spectrum, *i.e.*, differential spectrum. The differential spectra corresponding to the absorption spectra are shown in the lower portion of Figure 2. The differential spectra have fine structures which correspond to the shoulders in the absorption spectra. The fine struc-

TABLE II: Magnitude of the Fine Structures in Differential and Difference Spectra.[a]

	Magnitude of the Fine Structures		
	In Differential Spectra		
	Oxy Form	Deoxy Form	In Difference Spectra
Native Hb			
Stripped	0.0364	0.0182	0.019
In P_2-glycerate	0.0357	0.0182	0.016
Hb(AcAm)			
Stripped	0.0360	0.0209	0.014
In P_2-glycerate	0.0358	0.0193	0.014
Hb(MalN)			
Stripped	0.0346	0.0222	0.0056
In P_2-glycerate	0.0339	0.0191	0.0102
Hb(CPase)			
Stripped	0.0373	0.0299	−0.015
In P_2-glycerate	0.0360	0.0283	−0.008

[a] Readings on the spectra in Figures 3 and 4 have been corrected for the deviation of protein concentrations from 6.0×10^{-5} M and for residual oxy form in deoxygenated samples. Magnitudes of the fine structures in differential spectra are given by nm^{-1}.

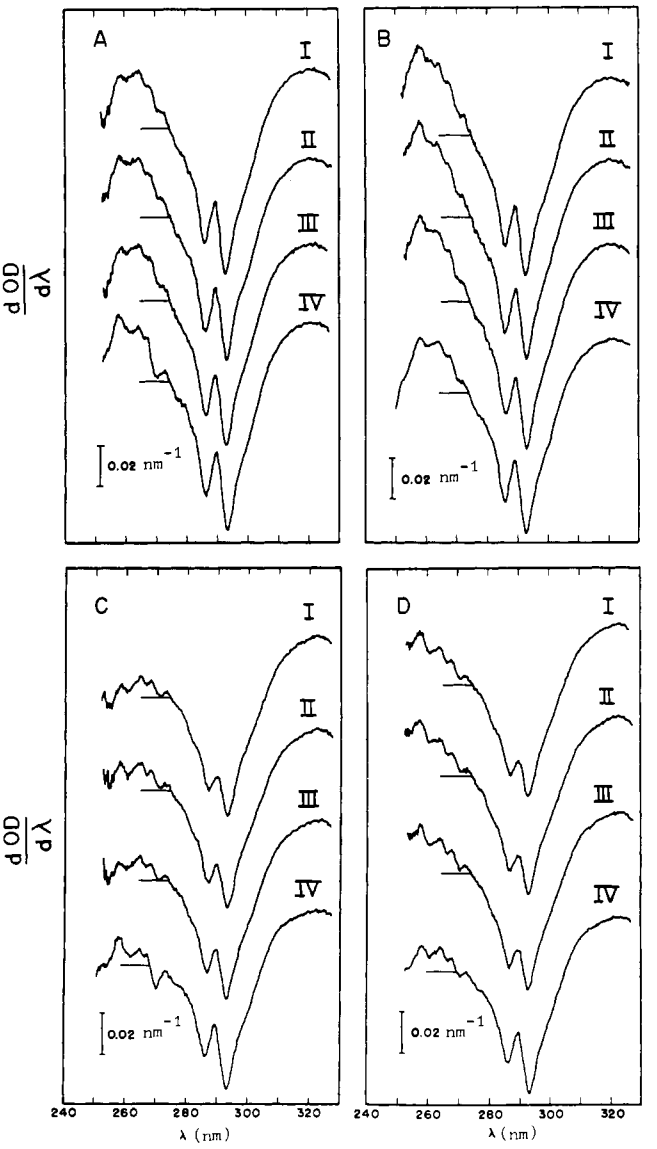

FIGURE 3: Ultraviolet differential spectra of native Hb (I), Hb (AcAm) (II), Hb(MalN) (III), and Hb(CPase) (IV) in the absence and presence of 0.5 mM P_2-glycerate. The horizontal lines attached to the spectra indicate their respective base lines. Hemoglobin concentration, $(6.0 \pm 0.3) \times 10^{-5}$ M; in 0.05 M Bis-Tris buffer (pH 7.4).

tures have a maximum at 289.5 nm and two minima at 286.5 and 293.5 nm which are identical for oxy and deoxy forms. The magnitude of the fine structures is, however, quite different: that of the oxy form is twofold of that of the deoxy form.

The uv differential spectra of the native and modified oxy- and deoxyhemoglobins were determined in the presence and absence of 0.5 mM P_2-glycerate and are compared in Figure 3. The form-dependent fine structures in the differential spectra are located in the same position but exhibit subtle differences in magnitude depending on the kind of hemoglobin and on whether P_2-glycerate is present or absent. In the range of shorter wavelength, from about 240 to 275 nm, minute but significant differences in shape, which are also form dependent, were observed but no systematic study of them was performed. In order to represent the magnitude of the fine structures of the differential spectra around 290 nm, the differences in dOD/dλ between the maximum at 289.5 nm and the minimum at 293.5 nm were read on the curves in Figure 3. Each reading was corrected for the deviation of protein concentration from 6.0×10^{-5} M and for residual oxy form in deoxygenated samples. The amount of methemoglobin was included into that of oxyhemoglobin since the differential spectrum of the former was identical with that of the latter. The corrected magnitudes of the fine structures for pure oxy and deoxyhemoglobins are listed in Table II. The magnitudes for the oxy forms are only slightly affected by either P_2-glycerate or the chemical modifications of the β chain. On the other hand the magnitudes for the deoxy form show distinct dependences on both the chain modifications and P_2-glycerate: the magnitude for deoxyhemoglobin increases and approaches that for oxyhemoglobin successively on the treatment with iodoacetamide, *N*-ethylmaleimide, and carboxypeptidase A, and P_2-glycerate decreases the magnitudes for the modified deoxyhemoglobins, making them closer to that for native deoxyhemoglobin in P_2-glycerate.

Ultraviolet difference spectra of oxy- *vs.* deoxyhemoglobin were determined in the absence and presence of 2 mM P_2-glycerate and the results are shown in Figure 4. The spectra exhibit a narrow banded peak around 290 nm, *i.e.*, around the shoulder in the absorption spectrum, superimposed on a broad band with a peak at 275 nm as previously observed by Enoki and Tyuma (1964) and Briehl and Hobbs (1970). The narrow-banded difference peak of native Hb is a notch with a maximum at 290.5 nm and a minimum at 288 nm. This becomes less distinct as the cooperativity is reduced by the chemical modifications of the hemoglobin chain, being only a shoulder for Hb(CPase) as previously observed by Nagel *et al.* (1966). A similar phenomenon is also observed in another narrow-banded spectrum with a maximum at 283 nm. In order to represent the magnitude of the fine structure appearing around 290 nm, the difference between

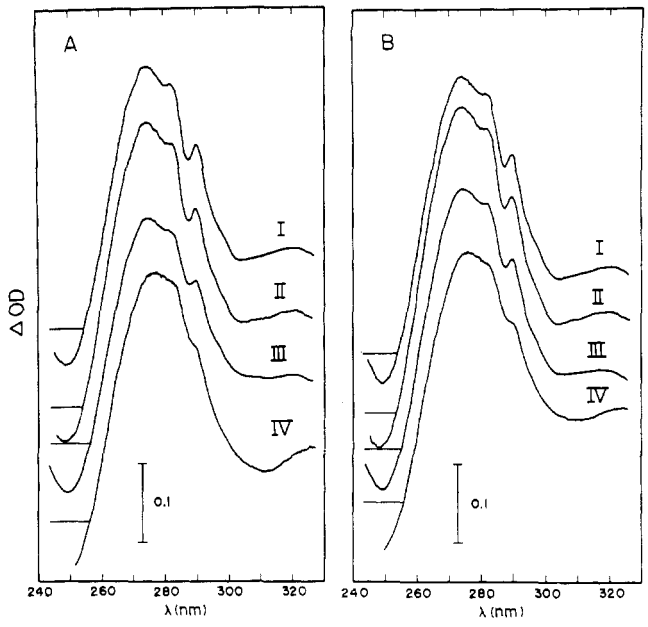

FIGURE 4: Ultraviolet difference spectra of oxy- *vs.* deoxyhemoglobin in the absence (A) and presence (B) of 2 mM P_2-glycerate. (I) Native Hb, (II) Hb(AcAm), (III) Hb(MalN), and (IV) Hb(CPase). The horizontal lines attached to the spectra indicate their respective base lines. Hemoglobin concentration, $(6.0 \pm 0.3) \times 10^{-5}$ M; in 0.05 M Bis-Tris buffer (pH 7.4). The difference between the protein concentration of the sample and that of its reference is less than 2%.

ΔOD at 290.5 nm and that at 288 nm was read on the curves in Figure 4. Each reading was corrected for deviation of protein concentration from 6.0×10^{-5} M. The corrected values of the magnitude of the fine structures of difference spectra are listed in Table II. The magnitude of the fine structures for the stripped hemoglobins is reduced in the following order: native Hb, Hb(AcAm), Hb(MalN), and Hb(CPase) as mentioned above. P_2-glycerate slightly decreases the magnitude for native Hb but that for Hb(AcAm) remains unchanged and those for Hb(MalN) and Hb(CPase) are increased.

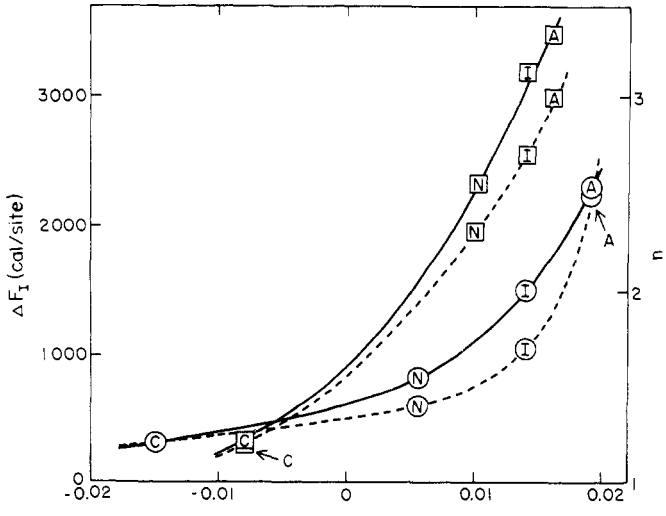

FIGURE 5: Dependence of the cooperativity in oxygen binding, ΔF_1 or n, upon the magnitude of the fine structures in oxy *vs.* deoxy difference spectra. Circles and squares stand for the stripped and P_2-glycerate-added hemoglobins, respectively. The letters in these symbols, A, I, N, and C, indicate native Hb, Hb(AcAm), Hb(MalN), and Hb(CPase), respectively. Solid lines are ΔF_1 *vs.* magnitude of the fine structures plots and broken lines n *vs.* magnitude of the fine structures plots.

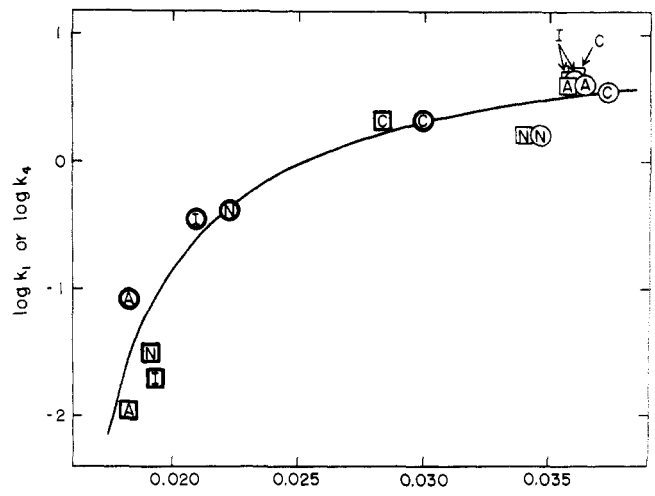

FIGURE 6: Dependence of log k_1 and log k_4 upon the magnitude of the fine structures in differential spectra. Bold circles and squares stand for the plot of log k_1 against the magnitude of the fine structures for deoxyhemoglobin and fine circles and squares stand for that of log k_4 against the magnitude of the fine structures for oxyhemoglobin. Other symbols as for Figure 5.

Correlation between the Narrow-Banded Ultraviolet Spectra and the Oxygen Equilibrium Parameters. There is a distinct correlation between the magnitude of the fine structures in difference spectra and the magnitude of cooperativity in oxygen binding as demonstrated in Figure 5. The magnitude of the fine structures becomes larger as the cooperativity increases. The correlation in the presence of P_2-glycerate is, however, somewhat different from that in its absence.

It is reasonable to consider that the fine structure in differential spectra are partial reflections of environmental states for aromatic groups in oxy- and deoxyhemoglobins. Therefore the magnitudes of the fine structures were compared with k_1 and k_4 (or with log k_1 and log k_4) which represent "initial" and "final" oxygen affinities for hemoglobin, *i.e.*, the affinities for Hb and $Hb(O_2)_3$, respectively. As observed in Figure 6, the magnitude of the fine structures in differential spectra is closely related to the microsocopic oxygen affinities, log k_1 and log k_4. The increase in the initial oxygen affinity, caused by the chemical modifications of hemoglobin chains or by the stripping of P_2-glycerate is accompanied by an increase in the magnitude of the fine structures. The increase in the microscopic oxygen affinity caused by the cooperative effect in oxygen binding, *i.e.*, its increase from k_1 to k_4, is also accompanied by the increase in the magnitude of the fine structures. The magnitude of the fine structures for oxyhemoglobin and also the final oxygen affinity are affected so much by neither the chain modifications nor the addition of P_2-glycerate.

Discussion

The differential spectrum technique employed in the present study clearly demonstrated the subtle changes in shape of the narrow-banded uv absorption spectra around 290 nm. The fact that the differential spectrum is influenced by the chemical modifications of hemoglobin chains and by the presence of P_2-glycerate implies that the spectral changes are

attributable not only to the heme contribution but also to the conformational states around aromatic chromophores.

It is generally accepted that deoxyhemoglobin is in a constrained state, where the reactivity of hemes toward oxygen is lowered, and successive oxygenation of the hemes removes the constraints among the subunits, making the other deoxyhemes more reactive. Detailed explanations for the constraints were given by Perutz (1970). The distinct correlation between the magnitude of the fine structures in differential spectra and the microscopic oxygen affinities suggests that the magnitude of the fine structures is a partial reflection of the degree of the constraints in hemoglobin molecule, although we cannot specify the nature and location of the constraints at present. Large and small magnitudes of the fine structures will correspond to less and more constrained forms, respectively. The decrease in the magnitude on the addition of P_2-glycerate to deoxyhemoglobin may imply that the phosphate makes the deoxy form more constrained.

The above consideration leads us to expect that the magnitudes of the fine structures in oxy *vs.* deoxy difference spectra around 290 nm represent an extent of the oxygenation-induced conformation changes around aromatic chromophores. If this is valid, the dependence of the cooperativity, measured by ΔF_I or n, upon the magnitude of the fine structures depicted in Figure 5 suggests that the extent of the conformational changes is maximum in native Hb and decreases in the modified hemoglobins. The interval between the plots for P_2-glycerate-added and stripped hemoglobins along the ordinate in Figure 5 represents an extra ΔF_I (Tyuma *et al.*, 1971) probably appended by the formation of additional salt bridges between the P_2-glycerate molecule and the β subunits (Perutz, 1970). The increases in ΔF_I due to the phosphate in Hb(MalN), Hb(AcAm), and native Hb are accompanied by an increase, no change, and a decrease, respectively, in the magnitude of the fine structures. A phenomenon similar to that observed for Hb(MalN) was already found in a genetically modified hemoglobin, Hiroshima (HC3β, histidine $\rightarrow$ aspartate), which resembles Hb(MalN) in oxygen equilibrium functions (Imai *et al.*, 1972). The present study confirms and extends the previous observation.

The present result with respect to the correlation of the cooperativity with the magnitude of the fine structures in difference spectra suggests that the perturbations of aromatic chromophores are related to changes of quaternary conformation although they may be also related to intrasubunit conformational changes. Judging from the location of the narrow banded difference peak the aromatic groups will involve tryptophans and/or tyrosines and not phenylalanines. Tryptophans are most probable since the shoulder in absorption spectrum around 290 nm is characteristic of them. Briehl and Hobbs (1970) tentatively identified the chromophores responsible for the narrow-banded difference spectra with C3β tryptophans. Their conclusion was based on the fact that the difference spectra resemble those of tryptophan and indole; that human adult hemoglobin has three tryptophan residues per $\alpha\beta$ dimer, two of which at A12α and A12β are buried in their own subunits, whereas the third at C3β is located at $\alpha_1\beta_2$ contact; and that the narrow banded difference spectra are diminished in the isolated chains (Briehl and Ranny, 1970; Ueda *et al.*, 1970). Bolton and Perutz (1970) demonstrated from their analysis of X-ray data that the C3β tryptophan residues have contacts with five residues of the partner α chains in oxyhemoglobin which decrease to four in deoxyhemoglobin, and thus they undergo an environmental change during oxygenation. Because the conformational change at the $\alpha_1\beta_2$ contact is considered to be essential to the cooperative oxygen binding (Perutz *et al.*, 1968; Perutz and Lehmann, 1968), the present results showing a relationship between cooperativity and the magnitude of the fine structures in difference spectra are consistent with the conclusion of Briehl and Hobbs that the C3β tryptophans are responsible for the narrow-banded difference spectra.

Anderson *et al.* (1971) showed that above pH 10 dissociation of hemoglobin tetramer to dimer is accompanied by spectral changes in the uv region and suggested that the latter were due to environmental changes around C7α tyrosine and C3β tryptophan which are located at the cleavage surface, *i.e.*, the $\alpha_1\beta_2$ contact, and become exposed to solvent upon dissociation. The effect of dissociation on spectral changes has no bearing on the results of this study which are concerned with the dependence of spectral changes on the kind of hemoglobin studied. Under the pH conditions used here the dissociation properties of native Hb, Hb(AcAm), and Hb(CPase) are the same (Guidotti, 1967; Antonini *et al.*, 1961) and Hb(MalN) is less dissociated than native Hb (Guidotti, 1967).

Although at present we cannot explicitly specify the relation between the spectral changes observed in this study and specific conformational changes in the protein, it seems reasonable to suggest that the differential spectra around 290 nm are partial reflections of the conformational states the tetramer assumes in the oxy–deoxy reaction and that the oxy *vs.* deoxy difference spectra indicate the extent of the conformational changes which play key roles in the cooperative oxygen binding of hemoglobin.

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Effects of Anions and Ligands on the Tertiary Structure around Ligand Binding Site in Human Adult Hemoglobin†

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ABSTRACT: We have studied the tertiary structure of the heme pockets of human adult carbon monoxide hemoglobin and oxyhemoglobin by investigating the ring-current shifted proton resonances in the 250-MHz nmr spectra. We have found that the conformation of the E11 valine residues in α and β chains relative to the heme plane is quite dependent on the nature of the anions and the pD of the solution as well as on the nature of the ligands. The E11 valines are located on the distal histidine side of the heme plane and are believed to play a vital role in the cooperative oxygenation of hemoglobin. We have attempted to correlate the structural transitions manifested by the differences in the ring-current shifts with the known functional effects produced by the different buffer systems. We have suggested a possible relationship between the conformation of the E11 valine methyl groups and the ligand affinity and have proposed a structural mechanism for the effects of anion binding on tertiary structure of the heme pockets in hemoglobin.

The nuclear magnetic resonance (nmr)[1] spectrum of human carbon monoxide hemoglobin A (HbCO A) is known to contain ring-current shifted proton resonances that arise from local magnetic fields produced by the delocalized π electrons in the heme groups (Ho *et al.*, 1970; Shulman *et al.*, 1970; Wüthrich *et al.*, 1972; Lindstrom *et al.*, 1972). In a previous report we have shown that the resonances at 6.58 and 5.86 ppm upfield from HDO can be assigned to the γ_1 and γ_2 methyl groups of the β67 (E11) valines, respectively (Lindstrom *et al.*, 1972). The other resonances have been assigned according to whether they are due to α or β chain residues. There is reasonably good evidence that the resonance at 6.48 ppm upfield from HDO is the γ_1 methyl of the α63 (E11) valine (Lindstrom *et al.*, 1972). The E11 valine residues are located on the distal histidine side of the heme plane, situated next to the ligand binding site, and are believed to play a vital role in the stereochemical mechanism for the cooperative oxygenation of hemoglobin (Perutz, 1970). Since the magnitude of the ring-current shifts is extremely sensitive to the geometrical relationship between the affected protons and the heme plane, these resonances are very sensitive to changes in the tertiary structure of the heme pockets. Our assignments of the E11 valine methyls allow us to probe the tertiary structure of the heme pockets with unprecedented sensitivity.

We have determined that the tertiary structure of the heme pockets is quite dependent on the nature of the supporting electrolyte and also on the nature of the ligand. The type of buffer, concentrations of added ions, and the pH of the solution all affect the tertiary structure of the heme pocket in a specific manner. We have attempted to relate these tertiary structural changes to the known functional effects of the perturbing conditions. In some cases, such as variations in pH and the use of phosphates, the tertiary structural changes may be correlated with changes in ligand affinities in hemoglobin.

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[1] Abbreviations used are: nmr, nuclear magnetic resonance; Hb A, human adult hemoglobin; HbCO, carbon monoxide hemoglobin; HbO_2, oxyhemoglobin; ppm, parts per million; DGP, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Tris, tris(hydroxymethyl)-aminomethane; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; mes, 2-(*N*-morpholino)ethanesulfonic acid.