Biochemistry

Volume 16, Number 3 February 8, 1977

A Study of Conformational Changes in Two β-93 Modified Hemoglobin A's Using a Triphosphate Spin Label†

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ABSTRACT: The binding of oxygen and 1-oxyl-2,2,6,6-tetramethylpiperidine 4-triphosphate (spin-labeled triphosphate) to normal adult human hemoglobin (HbA) covalently labeled at the β-93 sulfhydryl groups with *N*-(2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (I) was studied. HbA-I was used as a model for HbA labeled at the β-93 SH groups with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II) since the binding of SLTP to HbA-II could not be measured conveniently, in the presence of the paramagnetic resonance signal of II. Both HbA-I and HbA-II can be treated as variant hemoglobins with abnormal β chains. The oxygen and SLTP binding data from HbA-I and oxygen binding data from HbA-II are consistent with a concerted transition model for cooperativity which assumes nonequivalence between α and β subunits (GCT model). The distribution of environments "seen" by conformation sensitive probes such as II and trifluoroacetone (^{19}F NMR probe) attached to the β-93 sulfhydryl groups of HbA can also be accounted for by the GCT model. It is proposed that the β-93 probes sense the dramatic change in β subunit structure resulting from the quaternary structure change (T → R) upon heme saturation as well as tertiary structure changes at the α_1-β_2 contact region resulting from ligand binding to the β-heme group. Structural changes caused by ligation of the α-hemes are not discussed.

The ability of the generalized concerted transition model to account for oxygen and organic phosphate binding data from HbA, the $\alpha^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ cyanomet hybrids of HbA, and several mutant human hemoglobins has been well documented (Ogata and McConnell, 1971, 1972a,b, 1973; Ogata et al., 1972; Coleman, 1976a). In this communication it is shown that data from oxygen binding and organic phosphate binding to two chemically modified HbA's are also consistent with the GCT model. Also it is shown that this two-state model can satisfactorily account for the distribution of environments "seen" by conformation sensitive probes covalently attached to the β-93 sulfhydryl groups of several hemoglobins.

It has been shown previously (Moffat, 1971) that bulky groups, such as certain nitroxide free radicals, when attached to β-93 cause significant structural perturbations in the fully liganded state. It is also likely that structural alterations would be present in the ligand-free state, but this has not been shown. These structural changes manifest themselves as increases (with respect to native HbA) in the oxygen affinities of the modified hemoglobins and as small decreases in their cooperative interactions (Ogawa and McConnell, 1967; Horwitz, 1969). Increases in heme ligand affinities and retention of near-normal cooperative interactions are indicative of structural perturbations which affect the β-heme groups more than the position of the T ⇌ R equilibrium. Significant cooperative interactions suggest a normal T → R transition upon saturation of the hemes. The previously studied human mutants which exhibit abnormally high ligand affinities (Hb Chesapeake (α-92, Arg → Leu), Hb Kempsey (β-99, Asp → Asn), and Hb Yakima (β-99 Asp → His)) also show negligible cooperativity (i.e., $n \cong 1$ and $T/R < 1$).

The two modified hemoglobins that were characterized in this study are designated HbA-I and HbA-II. Label I is *N*-(2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide and label II is *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide. Both labels were attached to the β-93 SH groups of HbA. Label I was chosen because it is structurally similar to label II but it is not a free radical. Thus the SLTP[1] could be used to follow the T → R transition as a function of heme saturation for HbA-I. The oxygen binding properties of HbA-I are similar to but not identical with those of HbA-II. It is

† From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. *Received May 7, 1976.* This research has been supported by the Office of Naval Research (Grant No. ONR-N00014-67-A-0112-0045) and has benefited from facilities made available to Stanford University by the Advanced Research Projects Agency through the Center for Materials Research. This work was also supported by the National Institutes of Health under Grant No. 1R01 AI 13587-01.

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[1] Abbreviations used: HbA, normal adult human hemoglobin; SLTP, spin-labeled triphosphate; GCT model, generalized concerted transition model of allosterism; DPG, 2,3-diphosphoglycerate; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; $\bar{Y}$, the fraction of the heme groups saturated with ligand; HbA-I and HbA-II, HbA covalently labeled at β-93 with labels I and II, respectively; ESR, electron spin resonance; NMR, nuclear magnetic resonance; IHP, inositol hexaphosphate.

reasonable to assume that the structural effects of both labels on HbA are qualitatively the same and that, therefore, meaningful comparisons between HbA-I and HbA-II can be made.

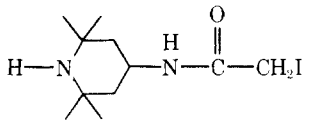
It is the intent to show from this work that sets of GCT model parameters can be selected for HbA-I and HbA-II in which the β-chain parameters are altered from their normal values in HbA and those parameters for the α chains are left unchanged. Moreover, it is shown that, within the framework of the GCT model, it is possible to account for the observed changes in the ESR spectra of HbA-II and HbA-III (defined in Materials and Methods) as a function of heme saturation (McConnell et al., 1968; Horwitz, 1969) and for the structural "states" observed by Huestis and Raftery (1972, 1973) with their ^{19}F NMR probe.

Materials and Methods

Label I, *N*-(2,2,6,6-tetramethyl-4-piperdinyl)iodoacetamide, was prepared according to the following procedure. To 16.0 g (0.078 mol) of iodoacetyl chloride (Aberhalden and Guggenheim, 1908) dissolved in 25 ml of CH_2Cl_2 was added 8.0 g (0.051 mol) of 2,2,6,6-tetramethyl-4-aminopiperidine (Aldrich) dissolved in 20 ml of CH_2Cl_2. The amine was added dropwise to the reaction flask while stirring the mixture at 0 °C. The reaction mixture turned yellow-brown and solid material began to precipitate. The reaction was terminated by washing the organic layer with 5% $NaHCO_3$. The solid material dissolved immediately and the yellow organic layer which separated was discarded. The desired product was extracted from the aqueous layer with four 150-ml portions of CH_2Cl_2 after increasing the pH of the solution to ~12 with concentrated NaOH. Evaporation of the solvent yielded 15.1 g (91%) of the crude product. This was recrystallized from hot (100 °C) toluene after filtering out the insoluble impurities. The yield of the purified product was 12.0 g and the melting point was 118–119.5 °C. Elemental analysis gave the following results. Anal. Calcd for $C_{11}H_{21}N_2OI$: C, 40.75; H, 6.53; N, 8.64; I, 39.14. Found: C, 40.93; H, 6.50; N, 8.62; I, 39.22. The 60-MHz NMR spectrum of II in $CDCl_3$ appears elsewhere (Coleman, 1974).

Spin label II, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, was prepared according to the method of McConnell and Hamilton (1968). The synthesis of the spin-labeled triphosphate, 1-oxyl-2,2,6,6-tetramethyl-4-triphosphopiperidine, is described elsewhere (Coleman, 1974; Coleman, 1976a; Ogata and McConnell, 1971). Spin label III was used by Ogawa and McConnell (Ogawa and McConnell, 1967; Ogawa et al., 1968) in their earlier studies of the ligand-induced conformational changes in horse and human hemoglobins.

To label the β-93 sulfhydryl groups with either I or II, HbA was reacted for 50 h at 0 °C with stirring in a 0.1 M PO_4^{3-} buffer at pH 7.8 (Horwitz, 1969). A large excess of the label (7.0 mg of label/100 mg of HbA) was used. Under these labeling conditions, the labels I and II react with groups other than the β-93 sulfhydryls to the extent of ca. 5% as judged by resonance spectra obtained from labeling HbA following preblocking of the sulfhydryl groups with *p*-mercuribenzoate (Ogawa and McConnell, 1967; Horwitz, 1969). Horwitz (1969) also has determined that after labeling there is ~0.5 spin label/heme group and Ogawa (1967) has shown from amino acid analysis that only two cysteines/mol of hemoglobin have been carboxymethylated during the labeling. Prior to labeling HbA was isolated chromatographically (CM-52, Whatman) from the lysate of fresh human red cells, kindly



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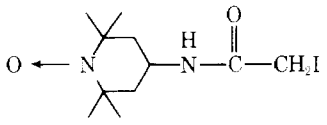

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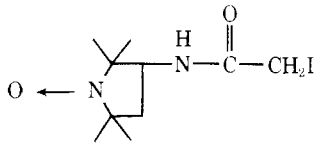

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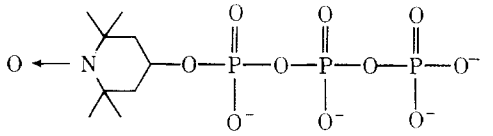

SLTP

donated by the laboratory of Dr. Christina Harbury of the Stanford Medical School. The unreacted label was removed and the solution stripped of phosphates by passing the sample through a column (2.5 × 30 cm) of Sephadex G-25 (Fine) equilibrated with 0.05 M Bistris–0.1 M Cl^- buffer, pH 7.3 at 13 °C. All experiments were performed at 13 °C in this Bistris buffer.

For the experiments with HbA-I, the hemoglobin concentration was 3.0×10^{-4} M in tetramers and the SLTP concentration was 4.6×10^{-4} M. In the case of HbA-II, the hemoglobin concentration was 2.5×10^{-4} M in tetramers and the 2,3-diphosphoglycerate concentration was 1.0×10^{-3} M. The performance of the SLTP binding experiment is described in detail elsewhere (Ogata and McConnell, 1971).

The following two equations are important for assessing the data from the SLTP binding experiments. Equation 1 allows for the determination of the allosteric equilibrium constant ($L = T/R$) for any hemoglobin at any degree of heme saturation.

$$L = \frac{K_0 - \frac{KK_0}{K_4}}{K - K_0} \qquad (1)$$

K_0 is the dissociation constant of the SLTP–T quaternary state complex, K_4 is the dissociation constant of the SLTP–R quaternary state complex, and K is the dissociation constant of the SLTP for some equilibrium mixture of R and T states. The parameter L is the T/R ratio of the equilibrium mixture to which K applies. Experimentally, the value of K_0 is determined by measuring the dissociation constant of the SLTP–ligand-free HbA complex. It should be noted that as K becomes equal to K_0, L becomes equal to L_0, and eq 1 is unnecessary. It is assumed that L_0 for HbA ($L_0 = T/R$ in the absence of heme ligand) is 4000, which means that effectively all tetramers are in the T quaternary state. This implies that K values smaller than K_0 do not reflect a change in the T/R ratio. It is further assumed that the binding of heme ligands to the T tetramer in no way alters the SLTP dissociation constant. The value of K_4 was determined by measuring the amount of SLTP bound to HbA at full saturation of the hemes. The details of the measurement and evidence for its significance have been presented

elsewhere (Coleman, 1976a). It has been shown (Ogata and McConnell, 1971, 1972a,b; Ogata et al., 1972, Coleman, 1976a) that for all hemoglobins studied thus far the SLTP binds to ligand-free hemoglobin in an equimolar ratio.

Equation 2 is an experimental function which represents the fraction of SLTP bound to hemoglobin (under equilibrium conditions).

$$F = (l_0 - l)/l_0 \qquad (2)$$

The quantity l_0 represents the total SLTP concentration in the sample and l represents the concentration of free SLTP. Values of F can be plotted against $1 - \bar{Y}$ the fraction of ligand free hemes. The curves that result are very characteristic of the properties of the hemoglobin studied. These purely experimental functions are completely model independent and provide a good test of how well a model accounts for the effects of heme ligand binding and of organic phosphate binding on the allosteric equilibria.

Parameter Selection. A set of parameters has been chosen for HbA on the basis of several experimental results and certain key assumptions as described by Ogata and McConnell (1971) and Coleman (1976a). The only parameter of the five (K_R^α, K_R^β, K_T^α, K_T^β, and L_0) that is not measureable by direct experiment is L_0; so a value for this must be assumed. Because of the highly cooperative nature of the allosteric transition, it must be complete, i.e., there must occur a complete change from a population of low affinity tetramers to a population of high affinity tetramers. It can therefore be concluded that L_0 must be $\gg 1$. When L_0 is varied from 100 to 10 000, while holding all other parameters constant, only values around 3000–4000 yield appropriate oxygen affinities and Hill coefficients. The value of $L_0 = 4000$ gives the best overall results in the calculations. It must also be realized that L_0 corresponds to hemoglobin in the absence of organic phosphates. In the presence of 10^{-3} M free DPG, L increases about three orders of magnitude.

Model calculations using the parameter sets for HbA found in Table I are consistent with most of the available oxygen uptake and organic phosphate (SLTP and DPG) binding data (Ogata and McConnell, 1971; Coleman, 1976a; Benesch et al., 1971) and with the data of Maeda et al. (1971) on the effect of DPG on the oxygen binding curves for the mixed valency, cyanomet hybrid hemoglobins. The most recent refinements of the GCT model calculations have been submitted for publication (Coleman, 1976a).

Using the HbA parameters as a basis set, the parameter sets that apply to modified or abnormal hemoglobins are selected by changing as few parameters as possible and by changing only those most likely to reflect the structural and functional alterations in the protein induced by the modification or abnormal amino acid sequence. The SLTP binding experiments then allow the determination of shifts in the allosteric equilibrium that result from the structural perturbations.

There is no convenient analytical optimization function which would allow each parameter set to be chosen from within a predetermined error tolerance. "Best fit" refers to an inspection process whereby a given parameter is altered in order to obtain the "best" agreement between experimental and calculated oxygen uptake and SLTP (or DPG) binding data. The same parameter set must account for oxygen equilibria in *both* the absence and presence of organic phosphates. It is often difficult to select a parameter set which gives extremely accurate simultaneous agreement for these two very different conditions. Error evaluation for the model parameters and calculations is further made difficult by the lack of a detailed statistical analysis for most of the available experimental data. In addition not all measurements have been made under identical experimental conditions.

TABLE I: Model Parameters Used in Calculations.

Hemoglobin	Concn of Tetramers (M)	K_R^α	K_R^β	K_T^α	K_T^β	L
A	3.0×10^{-4}	0.26	0.21	20.1	43.3	4000
A (Benesch)[a]	6.0×10^{-5}	0.58	0.46	45.0	95.3	6000
A-I	2.0×10^{-4}	0.26	0.09	20.1	18.7	1000
A-II	3.0×10^{-4}	0.26	0.02	20.1	2.0	500
A[b]	2.0×10^{-3}	0.45	0.27	56.2	110.0	6000

[a] Experimental conditions: pH 7.3, 20 °C, in 0.05 M Bistris, 0.1 M Cl^-, $K_{DPG} = 1.5 \times 10^{-5}$ M. The factor of 2.2 was chosen to adjust the calculated oxygen affinities to agree with those measured at 20 °C rather than 13 °C. This single change was all that was necessary to account for the decrease in oxygen affinity which results from the increase in temperature. [b] These parameters result from adjusting the normal GCT parameters for HbA so that the calculated oxygen affinities and Hill coefficients, both in the absence and presence of DPG, correspond to the data of Huestis and Raftery measured in 0.05 M Bistris–0.1 M Cl^-, pH 7.4 at 25 °C. (Data kindly provided by W. H. Huestis.)

There has been considerable controversy about the assumption in the GCT model that K_T (i.e., K_T^α and/or K_T^β) is not significantly affected by the SLTP and DPG (inositol hexaphosphate, IHP, is not included because its effects on the heme–ligand equilibria appear to be more complex than for the other two organic phosphates). Because it is recognized that L is greatly affected by DPG and its analogues, it is very difficult to independently determine their effect on the specific heme ligand dissociation constants, K_T^α and/or K_T^β. It is certainly reasonable to assume that the binding of phosphates to the T state affects K_T^β, but the magnitude and functional importance of such an affect has not been satisfactorily demonstrated.

Mah (1973) has made a careful study of the effect on the GCT model calculations of the assumption that K_T^β is markedly altered in ligand-free tetramers that have a molecule of phosphate bound. After doing extensive comparative model calculations between the GCT model and the modified version, he was forced to conclude that the models were virtually identical. From this we concluded that, although there may be a real effect of organic phosphates on K_T^β, it is not significant enough to warrant changing and further complicating a very workable model.

The GCT model is an instructional tool which is in accord with a large amount of experimental data and remains useful because of its simplicity. Admittedly many second-order alterations can be made in any model which marginally add to its data handling capacity and often add very little which is substantive to the conceptual framework.

Results

It was found that the binding of SLTP to HbA-I as a function of heme ligation is remarkably similar to that for HbA. The dissociation constant for the SLTP–ligand-free HbA-I complex as measured by the ESR technique is $1.2 \pm 0.2 \times 10^{-5}$ M with a binding stoichiometry of 1 mol of SLTP/1 mol of ligand-free HbA-I tetramers. Since the K for HbA-I and K_0 are equal to within experimental error ($K_0 = 1.3 \pm 0.3 \times 10^{-5}$ M), it is impossible to assess differences between L and L_0 (4000) from eq 1. As long as L is large with respect to 1 (i.e.,

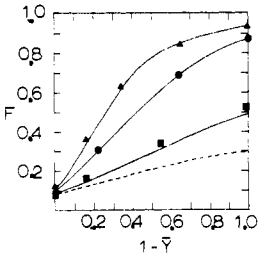

FIGURE 1: The dependence of F on $1 - \bar{Y}$ for HbA-I for three values of C_0/l_0. (▲) $C_0/l_0 = 2.6$; (●) $C_0/l_0 = 1.3$; (■) $C_0/l_0 = 0.5$. Solid curves are calculated from the GCT model. The dashed line shows the calculated dependence of F on $1 - \bar{Y}$ for HbA-II; DPG concentration is 1.0 mM. $C_0/l_0 = 0.25$.

TABLE II: Comparison of Experimental and Calculated Oxygen Binding Data.

Hemoglobin	Experimental p_{50}	Experimental n	Calculated p_{50}	Calculated n
A				
Stripped	1.8	2.8	1.8	3.0
Equimolar SLTP	3.1	2.3	3.1	2.4
A-I				
Stripped	0.82	2.7	0.80	2.7
0.47 mM SLTP	1.60	2.7	1.88	2.7
A-II				
Stripped	0.30	2.0	0.30	2.0
1.0 mM DPG	0.94	2.3	0.91	2.3

$L \geq 10$), the value measured for K will be equal to K_0, to within experimental error. For calculations with the GCT model, L was varied from 100 to 4000 in order to get the best agreement between calculations and experiment. The best value of L for HbA-I is 1000. Since HbA-I was chosen as a structural analogue to HbA-II (for which SLTP binding data could not be measured), it was presumed that L for HbA II is also large with respect to 1. The value which make the calculations agree most closely with experiment is $L = 500$.

Even though HbA-I is presumed to be a structural analogue of HbA-II, the comparison of their oxygen binding curves indicates some subtle but important differences in their properties. The $p_{50}(O_2)$ for HbA-I in the absence of organic phosphates is 0.8 mmHg and the Hill's constant is 2.7, while HbA-II under the same conditions has a $p_{50}(O_2)$ of 0.3 mmHg and $n = 2.0$. For HbA-I in the presence of 0.5 mM SLTP, the $p_{50}(O_2)$ increases to 1.60 mmHg and the cooperativity remains 2.7. For HbA-II in the presence of 1 mM DPG, the $p_{50}(O_2)$ increases to 1.0 mmHg and n increases to 2.3 (see Table II). Figures 2 and 3 compare the experimental and calculated oxygen equilibrium data for HbA-I and HbA-II, both in the absence and presence of the respective organic phosphate.

The differences between these two hemoglobins are also reflected in the GCT model parameters for each (see Table I). It can be seen that $K_R{}^\beta$ for HbA-II is fourfold lower than $K_R{}^\beta$ for HbA-I and $K_T{}^\beta$ is ninefold lower for HbA-II. These two parameters are markedly different from those of HbA. The abnormal ligand binding properties therefore can be accounted for by lowering L four- to eightfold and significantly lowering $K_R{}^\beta$ and $K_T{}^\beta$. The set of parameters of HbA-I also gives very good agreement between calculations and experiment for the function F vs. $1 - \bar{Y}$. The agreement holds for three different SLTP concentrations (see Figure 1). This experimental

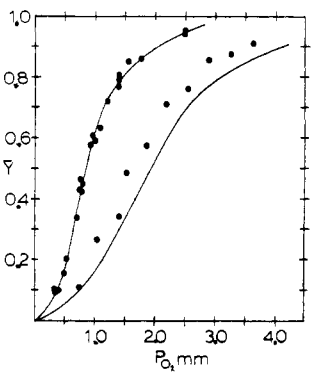

FIGURE 2: Data represent oxygen equilibrium binding curves for HbA-I in the absence of phosphates (left curve) and in the presence of 0.47 mM SLTP (right curve). The solid curves were calculated using the GCT model.

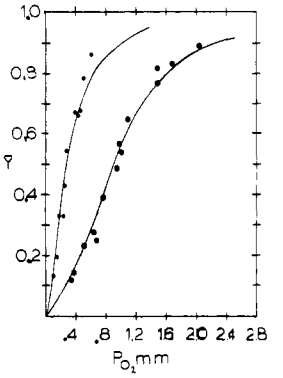

FIGURE 3: Data represent oxygen equilibrium binding curves for HbA-II in the absence of phosphates (left curve) and in the presence of 1.0 mM DPG (right curve). The solid curves were calculated using the GCT model.

function could not be measured for HbA-II but Figure 1 shows the calculated function for 1 mM DPG.

The values of K_4 for HbA-I and HbA-II were not determined experimentally, but the value assumed for *all* hemoglobins, except Hb Kansas, is the same as that measured for HbA ($2 \pm 1 \times 10^{-3}$ M, Coleman, 1976a). This assumption might not be totally accurate for HbA-I and HbA-II for two reasons: (1) the parameter selection for both modified hemoglobins indicates a functional (and probably structural) difference in the β hemes in the R state which could affect SLTP binding and (2) HbA-I bears an extra positive charge per label at pH 7.3. This might affect the SLTP binding since the label is expected to be partially exposed to the solvent in the R structure (Moffat, 1971).

It has been suggested by Perutz (1970) that the lack of isosbesty in the ESR spectra of HbA-II, as heme saturation proceeds, need not be due to the presence of quaternary states other than T or R but due to different tertiary structures within T and R. In terms of the GCT model, these structures correspond to ligand-free and liganded β chains in the T quaternary state, and ligand-free and liganded β chains in the R quaternary state.

Using the GCT model parameters for HbA-II, the fraction of each of these four structures present in solution over the course of heme saturation can be calculated. It is found that ligand-free T and liganded R predominate both in the absence and presence of DPG. In the absence of organic phosphate each of the two minor fractions comprises ~5% of the population of β chains. In the presence of 10^{-3} M DPG, liganded T_β jumps

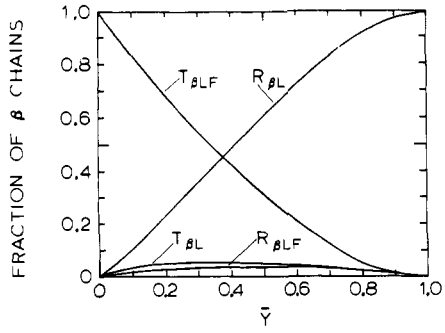

FIGURE 4: Each curve represents the fraction of β chains in the designated conformation at stages of ligation from $\bar{Y}$ = 0.0 to $\bar{Y}$ = 1.0. $T_{\beta lf}$ = ligand-free T_β chains, T_l = liganded T_β chains, $R_{\beta l}$ = liganded R_β chains, $R_{\beta lf}$ = liganded free R_β chains, l_0 = 0. Parameters used in calculations are those for HbA-II in Table I.

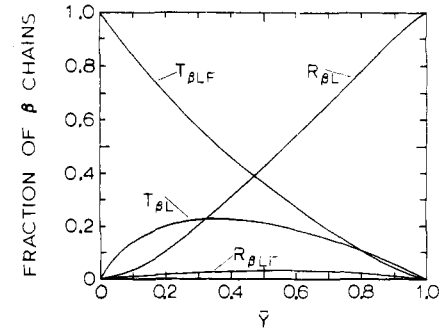

FIGURE 5: Same as Figure 4 except DPG = 1.0×10^{-3} M and Hb_0 = 2.5×10^{-4} M.

to 20% of the population and liganded R_β drops to ~1%. See Figures 4 and 5.

Discussion

Experiments with HbA-I were pursued with the hope that its oxygen and phosphate binding characteristics would be similar to those of HbA-II, so that the phosphate binding data from HbA-I could be related to HbA-II. However, the fact that I bears a positive charge at pH 7.3 must certainly affect the label's interaction with the protein. It can be seen from Table II that, indeed, the effect of I on the O_2 binding properties of HbA is less pronounced than for label II. Qualitatively, however, the results are quite similar. It can be seen from Figure 2 and Table II that there exists a difference between the experimental and calculated p_{50} values for HbA-I in the presence of SLTP. First of all it must be noted that the same parameter set is used for HbA-I in both the absence and presence of SLTP. The assumption was made that SLTP interacts with HbA-I exactly as it does with HbA. The discrepancy between the experimental and calculated O_2 uptake curves, although not terribly great, might reflect the breakdown of that assumption. The result could be explained if, because of the positive charge on the label, the affinity of SLTP for fully liganded HbA-I were somewhat greater than that for HbA.

The nearly complete release of SLTP bound to HbA-I as the hemes become fully saturated indicates a transition from a population of tetramers which bind the SLTP strongly (T) to a population of tetramers which bind this molecule weakly (R). This condition implies that $T/R \gg 1$ when $\bar{Y}$ = 0 and $T/R \ll 1$ when $\bar{Y}$ = 1.0. In addition HbA-I exhibits highly cooperative ligand binding (i.e., n = 2.7). It can be generally stated that highly cooperative hemoglobins undergo a complete allosteric transition as the fractional saturation of the hemes goes from 0 → 1.0. Since HbA-II has a Hill's coefficient of 2.0 when stripped of phosphates and 2.3 in the presence of excess DPG, it must be assumed that this hemoglobin also makes a complete transition from T → R. When the GCT model parameters for HbA-I and HbA-II are compared with those for HbA under the same conditions (see Table I), it can be seen that, even though L decreases for both variants, the values are large with respect to 1. Since the labels are bound at the α_1-β_2 interface, it is likely that their presence alters the T ⇌ R equilibrium. All abnormal hemoglobins for which GCT model parameters have been selected show T to be destabilized with respect to R in the absence of heme ligand when compared with HbA.

By further comparing the parameters it can be seen that there are large changes in K_R^β and K_T^β for both variants. For HbA-I both values are 2.5 times smaller than for HbA and for HbA-II they are factors of 10 and 20, respectively, smaller than HbA values. These parameter changes are largely responsible for the increased heme ligand affinities of HbA-I and HbA-II. It is important to note that the α-chain parameters remain unchanged.

HbA-I and HbA-II are interesting cases in the series of hemoglobin variants studied because they exhibit increases in heme-ligand affinity while at the same time they retain significant cooperative interactions. The GCT model is consistent with the oxygen binding data (both in the absence and present of organic phosphates) for these modified hemoglobins and with SLTP binding data for HbA-I. This further supports the model's applicability as a tool for understanding the fundamental allosteric effects in hemoglobin.

As reported earlier (Ogawa et al., 1968; McConnell et al., 1968; Horwitz, 1969), the ESR spectra of HbA-II do not contain isosbestic points in the low-field region as the signal changes over the course of heme saturation. Ho and co-workers (Baldassare et al., 1970) discuss the correlations between isosbesty and cooperativity in a study with HbA and several abnormal hemoglobins. The implication of the lack of isosbesty is that the probe is sensitive to more than two environments as the hemes become saturated and the conclusion of Baldassare et al. is that these environments are available to the probe only in hemoglobins with normal cooperative interactions (i.e., HbA).

Moffat (1971) has provided evidence from the analysis of the crystal structure of HbA-II that the equilibrium environment of the label in the T state is quite different from that in the R state. This is further indicated by comparing the ESR spectra of ligand-free HbA-II to fully liganded HbA-II. Moffat feels that the probe is sensitive to changes in structure in the α_1-β_2 contact region and these changes are surely a result of ligand binding and changes in quaternary structure. There is no method for determining whether large alterations in the α_1-β_2 contact region are a direct result of ligand binding to the heme groups or result from a shift in quaternary structure (from T → R).

Mutant hemoglobins which lack cooperativity (e.g., Hb Chesapeake, Hb Kempsey, and Hb Yakima) possess abnormal α_1-β_2 contacts (Morimoto et al., 1971; Perutz and Lehmann, 1968). For this reason it is very difficult to predict what kinds of environments would be available to probe II over the course of heme saturation. It also appears that, in addition to the specific structure in the α_1-β_2 contact region, the structure of the probe is very important in determining which environments are accessible. Probe III, for instance, gives spectra which show isosbesty for HbA. Because so little is known about the structure in solution of the α_1-β_2 contact region and about how probes attached to β-93 interact with this region, unequivocal

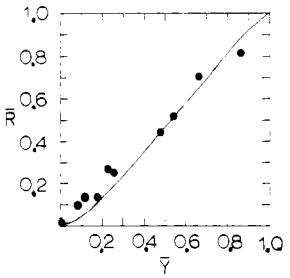

FIGURE 6: The data points are taken from Ogawa (1967) and represent the fractional spectral change with heme saturation ($\bar{Y}$) for HbA-III. The solid curve represents the calculation of the fraction of tetramers in the R conformation ($\bar{R}$) plotted as a function of $\bar{Y}$.

interpretations of the spectral information from these probes are impossible.

McConnell et al. (1968) and Horwitz (1969) have shown that tetraaquoferric HbA labeled with II exhibits a large spectral change and isosbestic points over the course of heme saturation with CN^- (pH 7.5 in 0.1 M PO_4^{3-}). Coleman (1976b) has demonstrated that at pH 6.5 in the presence of SLTP aquoferric HbA binds CN^- cooperatively ($n \simeq 1.5$). Perutz (1972) and Perutz et al. (1974) also provide evidence for a T → R equilibrium in aquoferric HbA at pH 6.5 in the presence of organic phosphates. The occurrence of a T → R transition as aquoferric HbA becomes saturated with CN^- provides a reasonable explanation for the spectral change with label II.

A plausible interpretation of the spin-label spectra which is consistent with a two-state model (GCT model) can be made. It is possible to imagine four distinct tertiary structures that the β subunits could have, as mentioned in the Results section. For HbA-II the GCT model calculations (using the parameters in Table I) do show three of the four tertiary structures make up significant fractions of the β-chain population, both in the absence and presence of DPG (see Figures 4 and 5). If the tertiary structure in the region of the label is different for ligand-free β-subunits than for liganded ones, in either the T or R states, then spectral isosbesty would not result. If there is no significant change in the tertiary structure of the β subunit in the region of the label in either quaternary state, isosbesty would be observed.

In light of the GCT model, the following hypothesis can be made. If a probe attached to β-93 gives rise to a series of spectra that do not contain isosbestic points, then it is sensitive to the large structural changes associated with the T → R transition *and* sensitive to tertiary structural changes which arise from ligand binding to the β-heme group and which perturb the α_1-β_2 contacts. If spectra from such a probe give rise to isosbestic points, then the label is sensitive only to the quaternary structural chnge (T → R). It is important to acknowledge the possible influence that ligation of the α-heme groups might have on the signal of β-93 labels. At present this will be considered (a priori) as a second-order effect and the discussion will not be further complicated by the inclusion of this point.

In his doctoral thesis Ogawa (1967) presents data from HbA labeled with III in which he plots fractional spectral change against $\bar{Y}$. To within experimental error these data agree well with calculations of $\bar{R}$, the fraction of tetramers with the R conformation, plotted against $\bar{Y}$, using the GCT model and the parameters chosen for HbA-II. See Figure 6. The deviation between the data and the calculated line could be due, in part, to either the presence of small amounts of met HbA II or to incomplete removal of oxygen from this high affinity hemoglobin. It is assumed that the parameters for HbA-II and HbA-III are identical. The notion that label III is able to measure the position of the T ⇌ R equilibrium is further corroborated by Ogawa and McConnell (1968). They show that in the presence of 0.05 M PO_4^{3-} the $\alpha_2^{+CN}\beta_2$(III) spectrum shows a greater change upon saturation of the β hemes than does the $\alpha_2\beta_2^{+CN}$(III) spectrum upon saturation of the α hemes. This is completely consistent with the measurements of Ogata and McConnell (1971) with the SLTP which established the basis of the GCT model, i.e., saturation of the α hemes shifts the T ⇌ R equilibrium to a lesser degree than saturation of the β hemes.

Huestis and Raftery (1972, 1973) have reported that a ^{19}F NMR probe attached to the β-93 sulfhydryl groups of HbA is sensitive to four different environments over the course of heme ligation. This assessment is made on the basis of fitting Lorentzian curves to the experimental spectra at several values for $\bar{Y}$. It is clear that of the four environments accessible to the probe, two predominate and two are only slightly populated. This is in excellent agreement with the calculations of the GCT model using the parameters for HbA (see footnote *b*, Table I) in Table I. The two large peaks from the NMR spectra, D and L, correspond to ligand-free T_β and liganded R_β, respectively. The two minor peaks I_2 and I_3 correspond to the populations of liganded T_β and ligand-free R_β, respectively. The fractional distributions of β-chain conformations as a function of overall heme saturation in the absence of phosphate and in the presence of a twofold excess of DPG are very similar to the curves shown in Figures 4 and 5 except that liganded T_β is at most 5% of the β chains in the presence of DPG.

From the preceding discussion it is possible to conclude that the environment of labels attached to β-93 is greatly altered by the change in quaternary structure associated with ligand binding. The absence of isosbestic points indicates that subtle changes in environment at the α_1-β_2 interface induced by β-heme ligand binding are sensed by some probes (such as II and the trifluoroacetone of Huestis and Raftery). Specific structural features of some probes, i.e., III, limit their accessibility to certain sites in the protein structure and thereby make their spectra less complex. It can be argued that III is sensitive to a single environment in the T quaternary structure and a single environment in the R quaternary structure, thus giving rise to spectra containing isosbestic points. This feature should also make III a good probe for measuring the fractional change from T structures to R structures over the course of heme saturation. In summary, the data from these probes are *consistent with* a generalized concerted transition model. This by no means constitutes a proof of the model but demonstrates that the GCT model accounts for the experimental findings obtained with the ^{19}F and spin labels discussed here.

Acknowledgments

I thank Dr. William Fitch for his advice in the preparation of label I and Mr. Jim Crapuchettes for his instruction in the operation of the PDP-8 computer which was used for the calculations. I also thank Dr. R. L. Baldwin and Dr. Wray Huestis for their helpful suggestions in the preparation of this manuscript.

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The Structure of Chromatin: Interaction of Ethidium Bromide with Native and Denatured Chromatin†

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ABSTRACT: The binding of ethidium bromide, as monitored by fluorescence enhancement, to chromatin prepared by nuclease digestion has been compared with the binding of the dye to sheared chromatin. The nuclease preparation (native chromatin) is characterized by a high affinity region of the Scatchard plot (r = 0–0.025, $K_1 = 1 \times 10^6$ M^{-1}), a transition (r = 0.025–0.05), and a low affinity region (r = 0.05–0.12, $K_2 = 3 \times 10^5$ M^{-1}). The final amount of ethidium bromide bound per base is 0.12 as compared with 0.20 for free DNA. Sheared chromatin has the two regions of high and low affinity ($K_1 = 2 \times 10^6$ M^{-1}, $K_2 = 5 \times 10^5$ M^{-1}) as originally shown by Angerer and Moudrianakis (1972), but the transition is much reduced or absent. Binding of the dye to native chromatin is independent of salt at concentrations ranging from 0.2 mM EDTA to 10 mM Tris-Cl, 10 mM NaCl, 0.2 mM EDTA, while sheared chromatin and DNA both bind ethidium bromide electrostatically as well as by intercalation at the low salt concentration, leading to extensive energy transfer. Thus the phosphate groups in native chromatin are unavailable to external cations even at very low salt. Polarization of fluorescence of ethidium bromide intercalated into native chromatin at low r is very high, indicating a highly rigid structure. As r approaches 0.02, there is a very rapid depolarization; at r = 0.03, the polarization is no greater than that of the dye intercalated into DNA. Depolarization is not due to energy transfer. The Scatchard plot derived for the bulk preparation of native chromatin is very similar to the one derived for the monomer ν body. These results indicate that the DNA in native chromatin is in a very rigid form, with its phosphate anions neutralized by structural components, not by free salt. Ethidium bromide intercalation appears partially to disrupt this structure, perhaps by unwinding, leading to slight changes in its properties.

Chromatin in the nucleus appears to be a highly organized structure. The basic unit of this structure is a core of histones (H2A, H2B, H3, and H4) associated with 200 base pairs of DNA (Kornberg and Thomas, 1974; Noll, 1974; Van Holde et al., 1974; Burgoyne et al., 1974; Oudet et al., 1975; Senior et al., 1975).

In such a structure, the DNA is under constraints which may be similar to the topological constraints existing in circular DNA. It has been shown (Oudet et al., 1975; Griffith, 1975; Germond et al., 1975) that the association of the DNA with the histone core, during chromatin reconstitution, alters the supercoiling of a circular DNA. So it is conceivable that, in the

† From the Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510. *Received June 14, 1976.* This work was supported by United States Public Health Service Grants GM 19481 and GM 21012.

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