Conformation and Cooperativity in Hemoglobin†

Wray H. Huestis‡ and Michael A. Raftery*,§

ABSTRACT: ^{19}F and ^{31}P nuclear magnetic resonance (NMR) spectroscopy have been used to study the ligand binding process in human hemoglobin. ^{19}F nuclear magnetic resonance studies of hemoglobin specifically trifluoroacetonylated at cysteine-β93 have permitted observation and characterization of molecular species containing two and three ligands. The behavior of these intermediate species in response to changes in pH and organic phosphate concentration is not completely consistent with any of the current theories of allostery. A model consistent with the ^{19}F and ^{31}P NMR data is proposed.

In recent years spectroscopic investigations of ligand binding to hemoglobin have yielded extensive information on molecular interactions in the cooperative oxygen binding process. Magnetic resonance has been used to study structural changes in the molecule by observation of spin-labeled allosteric effectors (Ogata and McConnell, 1972), paramagnetically shifted residues in the heme pockets (Davis et al., 1971; Ogawa and Shulman, 1972), spin-labeled hemes (Asakura, 1972), covalently attached spin-labels in the $\alpha_1\beta_2$ interface (Ogawa and McConnell, 1967; Deal et al., 1971; Ho et al., 1970), and carbon-13 resonances of mobile residues (Moon and Richards, 1972). The findings of these studies have been interpreted variously in light of X-ray crystallographic results (Perutz, 1970) as evidence in support of one of two theories of the mechanism of cooperativity—a concerted transition (Monod et al., 1965; Ogawa and Shulman, 1972) or generalized concerted transition (Ogata and McConnell, 1972) model, or a sequential (Koshland et al., 1968; Davis et al., 1971; Ogawa and McConnell, 1967; Deal et al., 1971) model. Most of the great mass of experimental findings on hemoglobin from kinetic (Roughton et al., 1955; Gibson, 1969; Riggs, 1960) as well as spectroscopic studies can be accounted for equally well by either of the above mentioned models. Much of the spectroscopic evidence presented in support of the concerted models (Ogawa and Shulman, 1972) is open to the criticism that the techniques used were not sufficiently sensitive to permit detection of forms with intermediate structure. However, the evidence that such intermediate states must exist (Davis et al., 1971; Ogawa and McConnell, 1967; Ho et al., 1970), which has been obtained mainly from studies of changes at the $\alpha_1\beta_2$ interface region, has been criticized on grounds that minute structural changes could occur within the subunits of the tetramer which do not affect the energetics of ligand binding (Ogawa and Shulman, 1972). The results of fluorine and phosphorus nuclear magnetic resonance (NMR) studies discussed in this communication (Huestis and Raftery, 1972a–d) are of sufficient sensitivity and detail to yield new evidence for energetically significant intermediate states.

Experimental Section

Materials and Methods. The studies discussed here were conducted on human hemoglobin used within 5 days of isolation from freshly drawn blood. The hemoglobin was covalently labeled with fluorine at cysteine β93 by reaction with bromotrifluoroacetone. The preparation (Huestis and Raftery, 1972a) and characterization (Lee et al., in press) of this trifluoroacetonylated hemoglobin (Hb^{TFA})[1] and the detailed procedure for ligand binding studies by ^{19}F and ^{31}P NMR (Huestis and Raftery, 1972c, 1973) have been described elsewhere. NMR studies were conducted in solutions containing 1.5–2 $\times 10^{-3}$ *M* Hb^{TFA} in a buffer 0.1 *M* in both bis-tris and NaCl. The pH values used were between 6.75 and 7.40 as specified in each case, and DPG concentrations varying from equimolar with Hb^{TFA} to a 40-fold excess were investigated, as will be discussed.

Results

Allosteric Parameters of Hb^{TFA}. Human hemoglobin contains one sulfhydryl group in each β chain which reacts readily with iodoacetate and its analogs. Modification of this residue with small, uncharged monofunctional sulfhydryl reagents generally increases oxygen affinity without serious perturbation of the Bohr effect (Benesch and Benesch, 1961) or Hill coefficient (Perutz et al., 1969). Reaction with bromotrifluoroacetone introduces a trifluoroacetonyl residue at this position. Extensive studies comparing the oxygen equilibria, Bohr effects, and DPG binding of Hb^{TFA} and Hb A (Lee et al., 1973) showed that introduction of the TFA moiety produced minimal perturbations of protein function. The oxygen affinity of Hb^{TFA} was found to be slightly higher than normal, the Hill coefficient was 2.8–2.9 compared with 3.1 for Hb A, the Bohr effect was normal, and the binding of DPG was identical with that of Hb A. Therefore it is probable that conformational processes observed in Hb^{TFA} are essentially normal.

^{19}F NMR Studies of Hb^{TFA}. Although cysteine-β93 apparently is not directly involved in cooperative interactions, it is in a mechanistically important region of the protein. Its immediate neighbors in the $\alpha_1\beta_2$ interface are histidine-

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‡ Postdoctoral Research Fellow of the Sloan Foundation. Present address: Chemistry Department, Stanford University, Stanford, California 94305.

§ National Institutes of Health Career Development Award Recipient.

[1] Abbreviations used are: Hb^{TFA}, trifluoroacetonylated hemoglobin A; bis-tris, bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; DPG, 2,3-diphosphoglycerate.

β92, the proximal heme ligand, and aspartate-β94, a residue which stabilizes the deoxy conformation of the β chain carboxyl terminus by means of a salt bridge to the side chain of histidine-β146. The latter interaction is directly involved in the Bohr effect (Benesch and Benesch, 1961), and indirectly linked to the cooperative mechanism since a steric requirement for its formation (Perutz, 1970) is that its neighbor residue, tyrosine-β145, must occupy a hydrophobic pocket between the F and H helices. This pocket is large enough to admit the tyrosine side chain in unliganded, but not liganded, hemoglobin. Thus the salt bridge formation at Asp-β94 indirectly reflects extensive tertiary structural changes in the subunit, which apparently are crucial to cooperative ligand binding (Huestis and Raftery, 1972b; Geraci and Sada, 1972).

The ^{19}F NMR spectrum of the TFA group at cysteine-β93 consists of a singlet (18 Hz wide at 28°) whose chemical shift is sensitive to the ligand state of the molecule (Huestis and Raftery, 1972a). Liganded forms (oxy-, carboxy-, and cyanmet-Hb^{TFA}) have similar chemical shifts around 480 Hz upfield of trifluoroacetic acid. Deoxy-Hb^{TFA} absorbs 45 Hz upfield of oxy-Hb^{TFA}. The presence of DPG (1:1) in the sample solution produces a further upfield shift of 12 Hz in the position of the deoxy-Hb^{TFA} resonance, but has no effect on the resonances of liganded forms. The resonance position of deoxy-Hb^{TFA} is pH dependent; the resonances of liganded forms are pH independent. (These findings have been described in detail elsewhere (Huestis and Raftery, 1972a,b).)

Crystallographic evidence (Perutz, 1970) has shown that the principal conformational change occurring near cysteine-β93 on ligation is the breaking of the salt bridges of histidine-β146 when tertiary structural changes force tyrosine-β145 out of its hydrophobic pocket. The changing proximity of the charged aromatic histidine side chain is a likely source for the variations in chemical shift and pH behavior of liganded and unliganded forms of Hb^{TFA}. Removal of His-β146 by carboxypeptidase B digestion (Huestis and Raftery, 1972b) of Hb^{TFA} had no effect on the NMR behavior of oxy-Hb^{TFA}, but after such modification the pH dependency of the deoxy-Hb^{TFA} chemical shift disappeared. Thus the principal structural change which can be detected by the TFA moiety is the carboxy terminus position, which is governed by tertiary structural changes in the β chain. A pH dependence (or lack thereof) in chemical shifts proves to be a useful criterion for characterizing tertiary structure in species of intermediate ligation.

^{19}F NMR Studies of the Ligand Binding Process. In the ^{19}F NMR spectrum of a partially liganded hemoglobin solution, four resonances could be detected (Figure 1A). In addition to two large resonances at positions characteristic of fully liganded and deoxy-Hb^{TFA} (designated L and D), two small resonances with intermediate chemical shifts were observed. One intermediate peak (I_2) appeared 20 Hz downfield of the deoxy peak; the other (I_3), 15 Hz upfield of the liganded peak. By means of a Lorentzian fitting program, the magnitudes of the four peaks were determined for spectra of solutions at numerous different degrees of fractional ligation. The Appendices A and B, which can be found in the supplementary material, list the data found for four different conditons of pH and DPG concentrations. The two intermediate peaks, I_2 and I_3, were found to comprise around 4 and 8% (±0.5–1%), respectively, of the total NMR integral through most of the ligation range. The small contribution of these species to the total hemoglobin

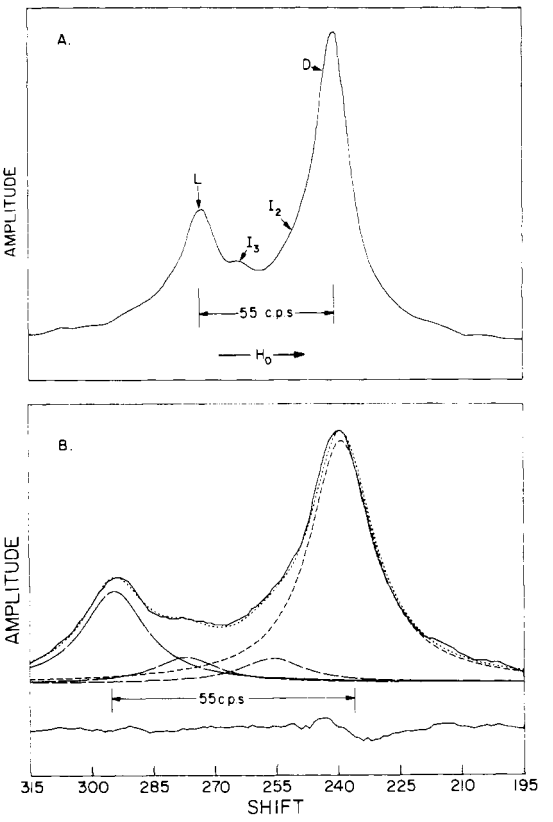

FIGURE 1: (A) ^{19}F NMR spectrum of partially carboxygenated Hb^{TFA} at $Y = 0.40$. (B) Calculated and observed NMR spectra of Hb^{TFA} when $Y = 0.40$. The solid line is the observed spectrum, the dotted line is the calculated sum of four Lorentzians, and the dashed lines are the individual Lorentzians. The bottom solid line is the difference between calculated and observed spectra. "L" is the resonance due to $Hb^{TFA}(CO)_4$, "D" is due to deoxy H^{TFA}, and I_2 and I_3 are due to species of intermediate ligation discussed in the text.

population made calculation of their magnitudes a difficult task, and reproducible, consistent quantitation was possible only when spectra with very favorable signal-to-noise ratios were computer fitted. Figure 1B shows a representative fit of a spectrum to four Lorentzians. It is worth noting that these intermediate species cannot be dismissed as mechanistically insignificant due to their low concentrations. On the contrary, in a population of molecules undergoing a positively cooperative binding process, the partially ligated species necessarily constitute a small fraction of the total concentration, but it is in these molecules that the mechanistically significant structural changes occur. Hence measurement of average characteristics of the entire population rarely yields insight into the binding process, and mechanistic information must be gleaned from small segments of the population. In this system two such intermediate species could be detected and characterized in some degree, as follows. (These analyses are based on data obtained in the presence of DPG). The NMR peaks of all four species were found to have equal line widths, and the chemical shifts of all peaks were invariant with changing ligand pressure. The chemical shift of I_2, like D, was pH dependent while L and I_3 were pH independent. The I_2 resonance shifted upfield as did that of D when DPG was added to the solution. Peak L was unaffected by the presence of DPG, while peak I_3 showed a small downfield shift under the same conditions. These findings have been described more fully elsewhere (Huestis and Raftery, 1973), and are summarized schematically in Figure 2.

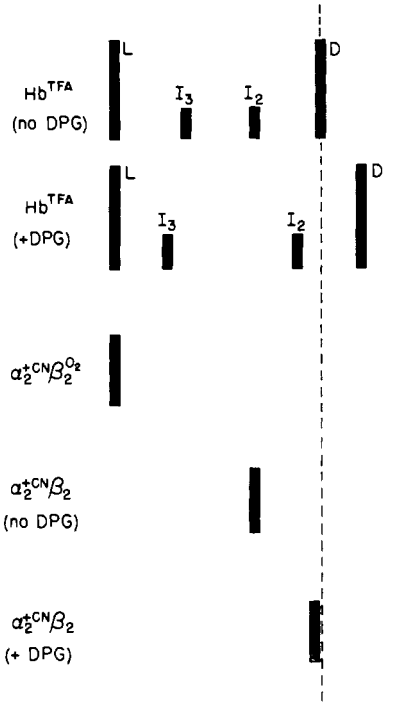

FIGURE 2: Schematic representation of the chemical shift behavior of partially liganded Hb^{TFA} and the artificial intermediate $\alpha_2^{IICN}\beta_2$, in the presence and absence of DPG.

Since the chemical shifts of the four resonances do not change with carbon monoxide pressure, exchange between species containing different numbers of ligands is slow on the NMR time scale. (In partially oxygenated samples, nonexchanging intermediate peaks could be observed only at lower temperatures.) Thus it was possible to determine the average number of carbon monoxide molecules bound to species in each NMR peak. A log–log plot of the ratio of magnitudes of any two peaks vs. ligand pressure yields a straight line the slope of which is equal to the number of moles of ligand by which species in the two peaks differ. A plot of log L/D vs. log P_{CO} gave a line of slope 4.08, so the species giving rise to peaks L and D must contain, on the average, four and zero ligands, respectively. From such plots the slope of I_2/D was found to be 1.9, L/I_2 was 2.1, I_3/D was 3.2, and L/I_3 was 0.9. Thus, peak I_2 arises primarily from species containing two bound ligands and I_3 from species with three ligands.

In order to determine which subunits were liganded in the intermediate species, the artificial intermediate $\alpha_2^{IICN}\beta_2$ was prepared and the effect of α-chain ligation on the TFA chemical shift was examined. Previously discussed evidence showed that the major factor influencing the TFA chemical shift is the tertiary structure of the β chain, but small changes induced by ligation of α chains could be detected in trifluoroacetonylated $\alpha_2^{IICN}\beta_2$. The chemical shift of the oxygenated hybrid was the same as that of oxy-Hb^{TFA} (Figure 2). Deoxygenation produced a shift change of 30 Hz to higher field, placing the resonance in the same position as I_2 when DPG is absent. Addition of DPG to $\alpha_2^{IICN}\beta_2^{deO_2}$ resulted in movement of the resonance ~15 Hz to higher field. As is summarized in Figure 2, I_2 responds to DPG in the same way, exhibiting a chemical shift nearly like that of $\alpha_2^{IICN}\beta_2^{deO_2}$ under all conditions tested. Thus it is reasonable that I_2 is $\alpha_2^{CO}\beta_2^{deO_2}$, analogous to $\alpha_2^{IICN}\beta_2^{deO_2}$. It is important to note that although addition of DPG to $\alpha_2^{IICN}\beta_2^{deO_2}$ and I_2 shifts their resonances in the direction of the deoxy-Hb^{TFA} resonance, the latter resonance also shifts further to high field. Thus addition of even large excesses of DPG does *not* shift I_2 or $\alpha_2^{IICN}\beta_2^{deO_2}$ to the same position as deoxy-Hb. This suggests that I_2 and its cyanmet analog exhibit a discrete intermediate structure *which cannot be described as a simple equilibrium between a liganded (R) and an unliganded (T) structure.* If such an equilibrium were present, DPG would be expected (Ogata and McConnell, 1972; Ogawa and Shulman, 1972) to drive I_2 and D into the same structure, an effect which is not observed. Thus, the observed equilibrium must reflect a transition between intermediate structures rather than involving the deoxy structure itself.

The intermediate species I_3, containing three ligands, has undergone a substantial chemical shift change from D, yet it is not the same as L. It is a reasonable supposition that the remaining unliganded subunit is a β chain. The chemical shift of this species is sens[illegible]e to the presence of DPG but not to pH changes, sugge[illegible] that major tertiary structural rearrangements have ta[illegible] place but the quaternary structure still is sensitive to L[illegible]. An unusual feature of I_3 is its apparent symmetry. Since in early stages of ligation I_3 is larger than L (i.e., I_3 does not have one resonance hidden under L), both β chains must have the same structure near cysteine-β93 although one heme is liganded and the other is not.

Thus, the intermediate structures which can be detected are $\alpha_2^{CO}\beta_2$ and $\alpha_2^{CO}\beta^{CO}\beta$. Since the slopes of the log–log plots were found to be close to integral and since little deviation from linearity was observed at low and high values of Y, it is likely that no significant quantities of other species (e.g., $\alpha_2\beta_2^{CO}$) contribute to any of the four absorbances.

Having characterized the detectable intermediate species as to number and type of liganded chains, it is possible to infer a preferential order of ligand binding. Before and after the NMR spectrum was recorded at each ligand pressure, the fractional ligation (Y) of the solution was determined from changes in the visible spectrum at 575, 542, or 650 nm. The fractional ligation of β chains alone could be determined from the NMR spectrum as the sum of peak L ($\alpha_2^{CO}\beta_2^{CO}$) and half of peak I_3 ($\alpha_2^{CO}\beta^{CO}\beta$), divided by the total integral. This parameter (Y_β) plotted vs. Y_{total} shows a lag of approximately 10% through most of the ligation range (Figure 3b, indicating that overall ligation exceeds β-chain ligation. (The magnitude of the lag is somewhat smaller in the absence of DPG, particularly for ligands other than carbon monoxide (Huestis and Raftery, 1972d,e), but all ligands studied showed the same sort of lag in β-chain ligation.) This suggests that, on the average, equilibrium populations of partially saturated hemoglobin solutions have more α chains liganded than β chains.

This result conflicts with reports of Ho and coworkers who observed that, while oxygen apparently bound preferentially to α chains (Lindstrom and Ho, 1972), carbon monoxide appeared to bind to both chains at random (Davis et al., 1971). The source of this disagreement may be the great difficulty encountered in accurate integration of signals such as the heme pocket residues which are superimposed on undetermined numbers of "envelope" Lorentzians. A β-chain lag of 10% or less which is evident in ^{19}F NMR spectra may well be difficult to detect in spectra complicated by many overlapping lines of unknown magnitude. Moreover, it is not likely that the trifluoroacetonyl modifi-

cation of Cys-β93 could produce an artifactual lag in β-chain ligation. Hb^{TFA} has been shown to differ from Hb A only in a slight increase in overall ligand affinity (Lee et al., 1973), a predictable result since modification of Cys-β93 virtually always results in some degree of destabilization of the β chain deoxy structure. Thus if Hb^{TFA} is abnormal, it should be in the direction of a decreased β-chain lag.

The apparent contradiction between our findings (Huestis and Raftery, 1972d) and those of Gibson and coworkers (Olson and Gibson, 1972) on the binding order of *n*-butyl isocyanide can be resolved as follows. Gibson demonstrates the presence of "fast" and "slow" binding components in partially liganded hemoglobin, and he presents several lines of evidence suggesting that the fast component (β chains) has higher affinity than the slow component (α chains). At no point does he show, however, that *in a given tetramer* unliganded β chains have higher affinity than unliganded α chains. That is, if the slow component is $(\alpha_2^{deoxy}\beta_2^{deoxy})$ and $(\alpha_2^{L}\alpha^{deoxy}\beta_2^{deoxy})$ and the fast component is $(\alpha_2^{L}\beta_2^{deoxy})$ and $(\alpha_2^{L}\beta^{L}\beta^{deoxy})$, then β chains will exhibit higher affinity than α chains but only after α chains bind. This interpretation also explains the curious finding that DPG has a larger effect on α-chain affinity than β-chain affinity, because DPG binds more strongly to the low affinity conformation in which α chains (but not β chains) bind ligands. Therefore we can agree that, while for practical purposes the β chains have higher ligand affinity, in fact the α chains bind first.

^{31}P NMR Studies of DPG Binding to Intermediate Species. ^{31}P NMR studies of DPG binding in partially liganded hemoglobin solutions were conducted to obtain information about the quaternary structures of the intermediate species. DPG, an allosteric effector found in large quantities in mammalian erythrocytes, binds strongly to deoxyhemoglobin in a well-characterized binding site between the β chains (Arnone, 1972). In liganded hemoglobin, the subunits have moved relative to one another, closing the specific binding site and expelling DPG (Perutz, 1970). Proponents (Ogata and McConnell, 1972; Ogawa and Shulman, 1972) of concerted transition models have suggested that this quaternary structural change is the "switch" from low to high affinity forms (the T and R structures of the model originally proposed by Monod et al., 1965), which produces cooperative ligand binding. Thus DPG binding has been used as the criterion indicating low affinity structure in hemoglobin, regardless of the number of ligands bound to the species in question.

The binding of DPG to species of intermediate ligation was studied by comparison of changes in the ^{31}P NMR spectrum to changes in the visible spectrum as ligand pressure was increased. DPG binding was rapid on the NMR time scale, so that the fraction of released DPG was measured as the fractional chemical shift change from the fully bound position. The fraction of free DPG was then compared with Y, with the result shown in Figure 3a. To a degree very similar to ligation of β chains, DPG release lagged behind ligand binding. Hb A and Hb^{TFA} yielded identical results in this experiment (Lee et al., 1973).

By comparing the fraction of DPG released at a given value of Y to the fractional magnitudes of the ^{19}F NMR peaks at the same Y value, the binding of DPG to the intermediates could be investigated (Huestis and Raftery, 1972c; Lee et al., 1973). This comparison showed that DPG was released more rapidly than peak L grew, but less rapidly than the sum of peaks L and I_3 grew. This indicated that

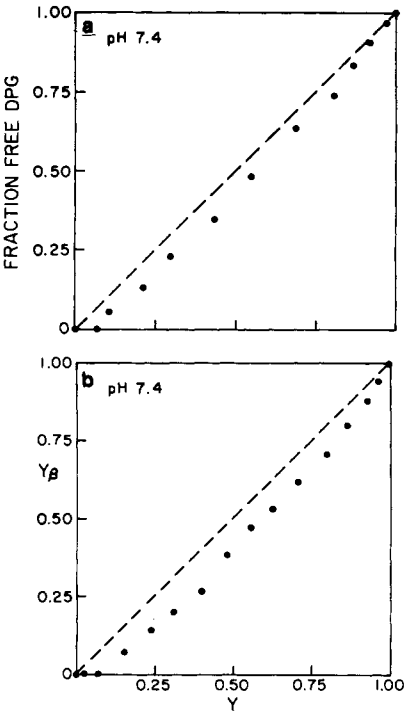

FIGURE 3: (a) The fractional release of DPG vs. total ligation (Y) HbA and Hb^{TFA} yield identical results in this experiment. (b) The increase in ligation of β chains (Y_β) vs. total ligation ($Y_{\alpha+\beta}$).

DPG must bind to D and I_2, and partially to I_3. That conclusion is consistent with the observed effects of DPG on the ^{19}F NMR peaks: I_2 and D showed ~10 Hz upfield shifts, I_3 responded differently with a 5-Hz downfield shift, and L was unchanged.

In assuming that the fraction of free DPG is simply proportional to the fractional chemical shift change, the assumption is made that the DPG binding site is the same in all species, giving rise to a single bound chemical shift. While some evidence from spin-label studies (Ogata and McConnell, 1972) supports this assumption, the apparent partial binding observed for I_3 (and the anomalous chemical shift response of the ^{19}F NMR peak) could mean that the binding site on I_3 is different. As the following discussion will show, the DPG results can be interpreted to be consistent with either a sequential or a concerted transition model depending on which interpretation of the I_3 binding is used.

Concerted Transition and Sequential Models. The ^{19}F and ^{31}P NMR results described above can be discussed in light of both currently contested models for the allosteric process. Concerted transition (Ogata and McConnell, 1972; Davis et al., 1971; Monod et al., 1965) models contend that the properties of hemoglobin are best understood as results of a conformational equilibrium between a low affinity (T) and a high affinity (R) form of the protein. In unliganded hemoglobin the position of this equilibrium is far toward the T form, but as ligand binding proceeds the equilibrium shifts in favor of the R form. (As a practical matter, a conformational "switch" generally is held to occur at a single stage of the binding process at which the R form is energetically more favorable.) Thus late binding ligands are bound with a high affinity binding constant only after early ligands bound with low affinity, producing cooperativity. An interpretation of the ^{19}F and ^{31}P NMR data in the context of this model is shown in Figure 4. Here the partial binding

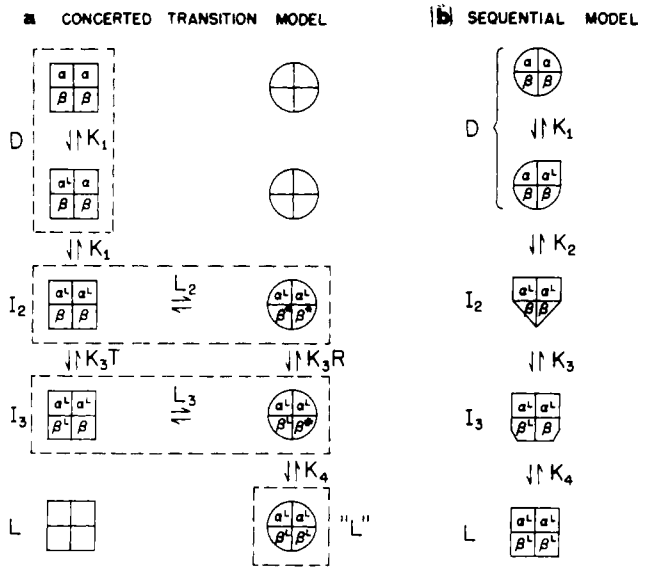

FIGURE 4: (a) Interpretation of the ^{19}F and ^{31}P NMR data by a concerted transition model. (b) Interpretation of the ^{19}F and ^{31}P NMR data by a sequential model.

of DPG to I_3 is interpreted to mean that I_3 contains both R and T structures in similar proportions with only the T structure binding DPG. According to this interpretation, the ^{31}P NMR data show that in the presence of DPG, the T to R transition occurs in the three-liganded species. Since there is evidence (Ogata and McConnell, 1972; Ogawa and Shulman, 1972) that an analog of I_2 ($\alpha_2^{IIICN}\beta_2^{deO_2}$) may undergo a transition similar to an R to T switch when DPG binds, we introduce a second pathway into the model which allows the transition to occur at the two-liganded stage in the absence of DPG. A fit of the NMR data on intermediate populations to this model would be expected to produce the following results: data obtained in the presence of DPG should exhibit small equilibrium constants for the first three binding reactions ($K_1 \simeq K_3T$) and a large equilibrium constant K_4. With DPG present, the conformational equilibrium constant L_2 (at the two-liganded stage) should be small. In the absence of DPG, L_2 should be larger, and an equilibrium constant similar to K_4 should be observed for the third ligation step. Table IA shows the equilibrium constants which were found from least-squares fits of four data sets to this model. According to expectations, values of K_{3T} were found to be only slightly larger than K_1, while K_{3R} was generally only slightly smaller than K_4. Surprisingly, however, the magnitudes of the conformational constants and hence the reaction path were found to be determined by pH as much as by DPG concentration. The data at pH 7.0 were obtained from a sample containing a 40-fold molar excess of DPG to hemoglobin, yet substantial T to R switching in I_2 is predicted from the equilibrium constant $L_2 = 1.25$. At pH 7.4 in the presence of a saturating amount of DPG, this trend is more pronounced, with a conformational equilibrium constant L_2 of 0.77. These results mean that at and above pH 7, sufficient switching from T to R states must occur in the two liganded species so that the third ligand binds primarily with the high affinity constant K_{3R}. This is not consistent with the ^{31}P NMR results, which showed that DPG is bound fully to I_2 and to about half of I_3. (Note that the conformational equilibrium constants L_3 above pH 7 predict that little DPG binding to I_3 should be observed.) Thus the structural change characterized by DPG expulsion

Table I: Carbon Monoxide Binding Constants (in mm^{-1}) Obtained by Fitting ^{19}F NMR Data to (A) the Modified Concerted Transition Model and (B) the Sequential Model.

A. Modified Concerted Transition Model	K_1	K_{3T}	K_{3R}	K_4	L_2[a]	L_3[a]
pH 6.75 + DPG	3.1	10	152	150	16.7	1.1
pH 7.0 + DPG	6.8	8	96	99	1.25	0.1
pH 7.4 + DPG	5.3	8	100	124	0.77	0.06
pH 6.75 *no* DPG	6.3	3	54	66	1.25	0.07

B. Sequential Model	K_1	K_2	K_3	K_4
pH 6.75 + DPG	6	2	29	76
pH 7.0 + DPG	10	9	34	132
pH 7.4 + DPG	10	7	38	148
pH 6.75 *no* DPG	15	5	30	63

[a] $L = [T]/[R]$.

Table II

	L_0[a]	L_1	L_2	L_3	L_4	c
pH 6.75 + DPG	3846	250	16.7	1.1	0.07	0.066
pH 7.0 + DPG	181	15	1.25	0.1	0.009	0.083
pH 7.4 + DPG	120	9.6	0.77	0.06	0.005	0.080
pH 6.75 no DPG	400	22	1.25	0.07	0.004	0.056

[a] $L = [T]/[R]$, calculated from fitted values of L_2 and c for all stages of ligand binding.

occurs at a ligation stage *later* than the onset of high affinity binding, in contradiction to a concerted transition model which assumes that conformational changes other than those accompanying DPG expulsion do not affect the energetics of binding (Ogawa and Shulman, 1972).[2] A second objection which might be raised to the modified concerted transition model summarized in Figure 4 is the incorrect implication that the "T" forms of I_2 and I_3 have the same structure as the deoxy species. As was mentioned earlier, the chemical shift behavior of I_2 and I_3 is indicative of structural differences between these species and ligand-free (or fully liganded) molecules. There is no evidence that the deoxyhemoglobin structure contributes to the supposed equilibria in I_2 and I_3 (or in $\alpha_2^{IIICN}\beta_2^{deO_2}$); indeed, their chemical shifts in the presence of DPG indicate the contrary.

Thus, analyses of these data according to a concerted transition model meet with two problems. Species of intermediate ligation can be observed which differ from either liganded or unliganded molecules in a manner which cannot be explained by equilibria between T and R structures governed by DPG binding. Analysis of the populations of these intermediate species, moreover, leads to a discrepancy in the position of T to R "switch" predicted from ^{31}P NMR data and from conformational equilibrium constants obtained by data fitting. However, a concerted transition

[2] Having determined the allosteric equilibrium constants L_2 and L_3 which fit the ^{19}F NMR populations, values of L at other stages of ligation can be calculated from the fundamental relations $L_1 = L_0c$, $L_2 = L_1c$, ... $L_4 = L_3c$ implicit in concerted transition models (Monod et al., 1965; Ogata and McConnell, 1972), where c is the ratio of ligand binding constants K_T/K_R. The results in Table II show that except below pH 7, L_0 values are not consistent with the predictions of concerted transition models. Above pH 7, L_0 is at least an order of magnitude too small to produce the observed degree of cooperativity (Ogata and McConnell, 1972; Monod et al., 1965).

model which placed greater emphasis on the role of pH in stabilizing the R and T forms might well be more successful in accounting for these results.

The data from ^{19}F NMR studies are, in addition, reasonably consistent with a sequential allosteric model (Koshland et al., 1968) such as is shown in Figure 4b. The sequential model, in one simple extreme, attributes cooperativity to gradual structural changes in the molecule induced by the binding of successive ligands. In the case of positive cooperativity, binding of each ligand alters the structure of the other subunits so that the next ligand binds more readily. To convert the concerted transition model to a sequential model (Figure 4) for the purposes of these data, we need only treat I_2 and I_3 as discrete conformational states and allow the binding constants to vary freely to fit the data. (No attempt is made to separate the actual ligand binding constants from any accompanying conformational equilibrium constants.) In this mechanism the partial binding of DPG to I_3 simply means that I_3 has a structure which binds DPG less strongly. The results of data fitting are shown in Table IB. K_1 and K_2 were found to be nearly equal, indicating that the two α chains bound ligands with low affinity and little or no cooperativity. K_4 was large and strongly pH dependent, and K_3 was intermediate in magnitude.

A simple sequential model such as shown in Figure 4 encounters one principal difficulty in describing the NMR data, and that is the apparent symmetry exhibited by the β chain NMR spectra throughout ligand binding. Binding of one ligand to an α chain did not produce detectable changes in the β chains (or appreciably increase the affinity of the second α chain). However, ligation of both α chains produced structural changes which could be detected in the β chains. Binding of the third ligand to a β chain produced a species in which the β-chain structure near the ^{19}F probe was the same in the unliganded and liganded chain. The symmetry of these intermediates suggests that the structure of each subunit is governed by stabilizing interactions from the other three, so that no single change in structure or ligation can alter structure in the remainder of the molecule unless it is reinforced by changes from other subunits. The symmetry thus produced is not inconsistent with a sequential binding mechanism; it requires a high order of complexity in the envisioned interactions.

Despite the problems encountered by both of these models, the populations of observed species could be fitted adequately using the parameters of either (Raftery and Huestis, 1973). Although successful data fitting often is cited in defense of these models, it should be recognized that data analysis of this kind had not been useful in distinguishing between them. The utility of the ^{19}F and ^{31}P NMR data lies rather in the behavior of the intermediate species, from which information about their conformational interactions can be obtained. The ligand binding process shown in Figure 5 summarizes these findings. (It should be noted that the conclusions which follow arise from analysis only of those species which can be detected by structural features at the ^{19}F NMR probe site, and whose existence is required to obtain good fits of the spectra. These analyses do not exclude the possibility that small populations of other intermediate species could exist, due to ligand binding by other pathways.) Deoxy-Hb^{TFA}, with a quaternary structure amenable to DPG binding and a pH-dependent tertiary structure, binds a first ligand preferentially to an α chain. This event produces no detectable changes in the DPG binding site or the $\alpha_1\beta_2$ interface near cysteine-β93. The

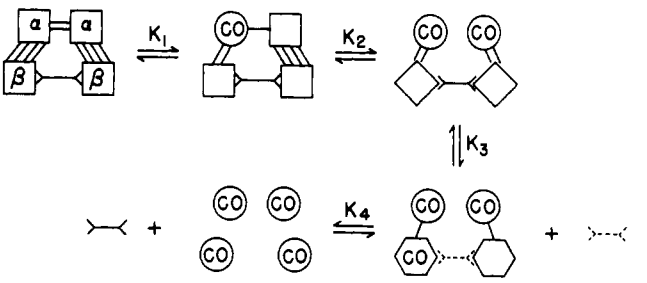

FIGURE 5: A schematic representation of structural changes observed as ligand binding progresses. ><= DPG.

second ligand also binds primarily to an α chain, and the combined effect from both α chains is sufficient to induce structural changes in the β chains detectable at Cys-β93. Despite the fact that this effect must be exerted through changes in α-β (quaternary) interactions, DPG remains bound. The exact nature of the structural change is not apparent, since the new species still has a pH dependent chemical shift. Particularly above pH 7, this change results in a higher affinity for the third ligand (see Table I). The third ligand binding is accompanied by more profound changes in tertiary and quaternary structure. The three-liganded species I_3 is not pH dependent, indicating major alterations in tertiary structure, and quaternary structural changes are apparent from the decrease in DPG binding. These changes confer still higher ligand affinity on the remaining chain, which binds the final ligand and changes further to the fully liganded form (including a further change in a β chain already liganded in I_3).

The ligation process which emerges from the NMR studies is not entirely consistent with either a concerted transition or a simple sequential allosteric model, but it contains some features of each. The significant mechanistic findings are: (1) in unliganded hemoglobin, α chains exhibit higher ligand affinity than β chains, so that on the average the binding sequence is $\alpha,\alpha,\beta,\beta$; (2) the quaternary structural change (or changes) associated with DPG release occurs after binding of the third ligand and before binding of the fourth; (3) prior to the quaternary changes which expel DPG, other quaternary interactions permit the ligation state of the α chains to influence the structure of the $\alpha_1\beta_2$ interface region and the ligand affinity of the β chains; (4) the observed structural symmetry of the β chains suggests that structures of differing ligand affinity are stabilized by intricate interactions dependent on the state of the entire tetramer. Thus any "cooperative mechanism" based on the energetics of changes in a small region of the molecule or in a few interacting groups runs a risk of oversimplification.

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Supplementary Material Available

Appendices A and B will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N. W., Washington, D. C. 20036. Remit check or money order for

$4.00 for photocopy or $2.50 for microfiche, referring to code number BIO-75-1886.

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