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Observation of Cooperative Ionizations in Hemoglobin

(¹⁹F-NMR/specifically labeled proteins/molecular regulation mechanisms/hydrophobic interactions)

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ABSTRACT **¹⁹F-Nuclear magnetic resonance studies of specifically fluorinated hemoglobin derivatives have been used to determine the apparent pK_a of the histidine β146 imidazole in deoxyhemoglobin. The titration of this residue was found to be abnormally sharp, particularly in the presence of diphosphoglyceric acid. The explanation advanced for this unusual titration curve may have implications for the mechanism of cooperative ligand binding. The possible role of such ionizations is discussed in light of some chemical evidence that the cooperative binding process is governed to a greater extent by internal nonpolar forces than by electrostatic interactions of exposed groups.**

The importance of the carboxy terminal residues of the α and β chains of hemoglobin to the Bohr effect and the cooperative mechanism has been suggested by studies of enzymatically modified hemoglobin (1, 2) and hemoglobin mutants (3), and by features of the crystal structure (4). It has been proposed that electrostatic interactions involving the terminal side chains and carboxyls constrain the molecule into its deoxy form, and that the breaking of these bonds on initial ligand binding permits conformation transitions that expedite further ligand binding (4).

We have labeled human hemoglobin specifically at cysteine β93 by reaction with 1-bromo-3-trifluoroacetone. The ¹⁹F-nuclear magnetic resonance (NMR) spectrum of this fluorinated residue reflects conformational processes in the region of the $\alpha_1\beta_2$ interface where histidine β146 (the carbon terminus) is bound to aspartate β94 in deoxyhemoglobin ($Hb\text{-}deO_2$). In an earlier communication (5), the preparation and functional properties of this trifluoroacetonylated hemoglobin (Hb^{TFA}) were described and changes in the ¹⁹F-NMR spectrum as a function of binding of ligands and allosteric effectors were discussed in detail. It was reported (5) that trifluoroacetonylated oxyhemoglobin ($Hb^{TFA}O_2$) exhibits a single resonance 483 Hz upfield of trifluoroacetic acid, the chemical shift of which is invariant with pH in the range 6.0–8.3. Hb^{TFA}-deO_2 was found to exhibit an absorbtion upfield of that of $Hb^{TFA}O_2$, which had a pH-dependent chemical shift. A plot of chemical shift as a function of pH for Hb^{TFA}-deO_2 had the appearance of a sharp titration curve, suggesting that the environment of the fluorine label was being influenced directly or indirectly by an ionizable group of $pK_a = 7.4$. These earlier findings are summarized in Fig. 1.

Abbreviations: NMR, nuclear magnetic resonance; HbO_2, oxyhemoglobin; Hb-III, methemoglobin; Hb-deO_2, deoxyhemoglobin; Hb^{TFA}, trifluoroacetonylated hemoglobin.

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We now report further studies to determine the origin of the NMR shifts, and discuss implications of these findings for the mechanism of cooperativity.

MATERIALS AND METHODS

Human hemoglobin was prepared from freshly drawn citrated blood by distilled-water lysis of the saline-washed erythrocytes. Hemoglobin stock solutions were stored as the carboxy derivative at 4°, and used within 1 week of preparation. Des-His β146 hemoblogin was prepared by previously described procedures (2). The purified des-His β146 hemoglobin was fluorine-labeled by reaction with 1-trifluoro-3-bromoacetone, as described (5). Oxy, deoxy, and met derivatives of the fluorine-labeled des-His β146 hemoglobin were prepared as described (5) for Hb^{TFA}. Hb^{TFA} was carbamylated at its amino termini as described (6). Des-Tyr β145-His β146 hemoglobin was prepared from $Hb^{TFA}CO$ as described (1).

NMR spectra were taken with a Varian XL-100 spectrometer operating at 94.1 MHz, supplemented by a Fabritek model 1061 computer of average transients. Spectrum accumulation times were reduced by use of sample tubes with outer diameters of 12 mm. pH measurements were made with a Radiometer Copenhagen model 26 pH meter.

RESULTS AND DISCUSSION

Several chemical modifications were used to distinguish among the several ionizable groups in the vicinity of Cys β93 that might have been responsible for the pH-dependent chemical shift of Hb^{TFA}-deO_2. (*i*) Carbamylation of the amino

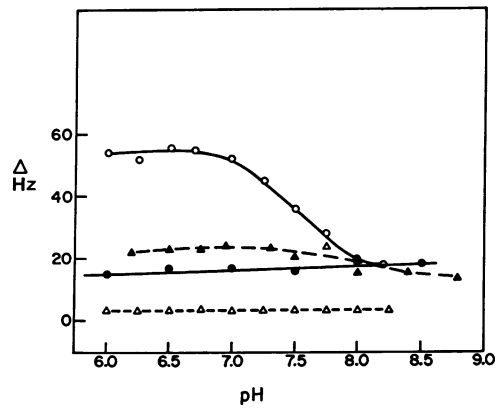

FIG. 1. Chemical shift of trifluoroacetonylated hemoglobin as a function of pH for $Hb^{TFA}O_2$, △; Hb^{TFA}-deO_2, ○; Hb^{TFA}-deO_2 des His β146, ●; Hb^{TFA}-deO_2 des [His β146-Tyr β145], ▲.

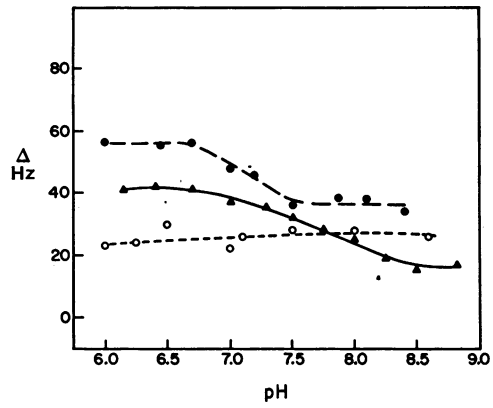

FIG. 2. Chemical shift of trifluoroacetonylated methemoglobin as a function of pH for Hb^{TFA}-III, ▲; Hb^{TFA}-III des His β146, ●; Hb^{TFA}-III des [His β146-Tyr β145], ○.

termini with isocyanate produced a small overall chemical shift change in both oxy and deoxy derivatives, but no change in the titration curve for Hb^{TFA}-deO_2. (*ii*) Binding of diphosphoglyceric acid to Hb^{TFA}-deO_2, which raises the pK_a of histidine β143 (7), did not alter the pH dependence of the NMR shift, although the titration curve appeared sharper (see Fig. 4) and an overall chemical shift change was present. (*iii*) Removal of His β146, however, resulted in disappearance of the pH-dependent chemical shift change. The chemical shift previously observed above pH 8 was present over the entire pH range (Fig. 1). The same result was obtained upon removal of His β146 and Tyr β145 by digestion with carboxypeptidase A. That the ^{19}F-NMR probe on Cys β93 should be influenced by the ionization of His β146 was not unexpected, since in deoxyhemoglobin His β146 is held about 4–5 Å away from Cys β93 by its imidazole salt bridge to Asp β94 (4). Experiments now in progress are aimed at distinguishing between charge and ring current effects in this system, by the simultaneous ^1H-^{19}F-NMR technique of Millett and Raftery (8).

Methemoglobin (Hb-III)

The extension of these experiments to other ligand forms of hemoglobin led to some surprising findings for methemoglobin (Hb^{TFA}-III). Hb^{TFA}-III exhibited a chemical shift upfield of other liganded forms (5) near that of Hb^{TFA}-deO_2, and, like Hb^{TFA}-deO_2, the chemical shift was pH-dependent (Fig. 2). The apparent pK_a of the chemical shift change was 7.8 in Hb^{TFA}-III. The origin of this ionization (or ionizations) was unknown, since methemoglobin was believed to have a conformation similar to oxyhemoglobin in the vicinity of Cys β93 (4). The NMR properties of Hb^{TFA}-III des-His β146, prepared by oxidation of $Hb^{TFA}O_2$ His β146, showed that the environment of Cys β93 was markedly altered by removal of His β146 (Fig. 2). The overall chemical shift was changed, and the pH-dependent change above pH 7.5 was eliminated. A titration curve of pK_a 7.1 remained, indicating the influence of yet another ionizable group, which was not evident in either oxy- or deoxyhemoglobin. These NMR results do not permit identification of the second group, but they do permit the conclusion that methemoglobin exhibits a conformation different from that of either oxy- or deoxyhemoglobin in this region of the $\alpha_1\beta_2$ interface. Hb^{TFA}-III does resemble Hb^{TFA}-deO_2 in the behavior of His β146, suggesting that the equilibrium conformations of their carboxyl termini are similar. However, additional processes are evident in Hb^{TFA}-III that indicate more extensive structural differences.

Coupled ionizations in deoxyhemoglobin

The situation at first appears less complicated in Hb^{TFA}-deO_2 than in Hb^{TFA}-III. It seems reasonable that the principal factor influencing the chemical shift in this case is the ionization of His β146, which thus has a pK_a of 7.4. The pK_a of this histidine is expected to lie in the range 6.7–6.8 in oxyhemoglobin (5), and a rise of about 0.7 pH units on deoxygenation would correspond well to half the total pK_a rise required to account for the alkaline Bohr effect (11). This result concurs with the findings of Kilmartin *et al.* (2) on the magnitude of the contribution of His β146 to the Bohr effect. However, this assignment is complicated by the abnormal shape of the titration curve. The chemical shift changes over a range of about one pH unit, compared with the two pH units over which 80% of a normal ionization is observed. This sharpness is accentuated when diphosphoglyceric acid is added to the deoxyhemoglobin (see Fig. 4) (5). Similar properties appear in the pK 7.1 ionization, which is observed in des-His β146 methemoglobin. Individual ionizable groups have been titrated in the active sites of lysozyme (9) and ribonuclease (10), but the curves obtained had very nearly normal shapes although the groups in question were in relatively "nonaqueous" regions. In contrast, the environment of His β146 in deoxyhemoglobin is not hydrophobic; the residue is essentially exposed to the solvent.

The steepness of these titrations can be explained by an interaction of the observed ionization with other groups in the protein that ionize in the same pH range. If a pH-dependent process occurs during the ionization of His β146, which causes its pK_a to decrease, its titration will be abnormally sharp. Consider a system of two such interacting groups, represented as HA∼∼BH, in which the pK_a of HA is controlled by the ionization state of BH and vice versa.

$$\frac{[H^+][^-A\sim\sim BH]}{[HA\sim\sim BH]} = K_{A_1} \quad (1)$$

$$\frac{[H^+][^-A\sim\sim B^-]}{[HA\sim\sim B^-]} = K_{A_2} \quad (2)$$

$$\frac{[H^+][HA\sim\sim B^-]}{[HA\sim\sim BH]} = K_{B_1} \quad (3)$$

$$\frac{[H^+][^-A\sim\sim B^-]}{[^-A\sim\sim BH]} = K_{B_2} \quad (4)$$

For this coupled ionization, the fraction **S** of A that is not ionized can be expressed as a function of $[H^+]$ and the Ks as follows:

$$S = \frac{[H^+]^2 + [H^+]K_{B_1}}{[H^+]^2 + [H^+](K_{A_1} + K_{B_1}) + K_{B_1}K_{A_2}} \quad (5)$$

Eq. (5) generates abnormally steep titration curves when $pK_{A_1} > pK_{A_2}$. Computer optimization of the slopes of these curves at S = 0.5 showed that the steepest possible titration for this model occurs in the symmetrical case, i.e., $pK_{A_1} = pK_{B_1}$ and $pK_{A_2} = pK_{B_2}$, when the $\Delta pK_a = pK_{A_1} - pK_{A_2}$ is large (≥ 4 pH units). Such a curve is shown in Fig. 3 (*curve 2*) for $\Delta pK_a = 4$ pH units, the constants being chosen to place the overall pK_a at 7.4. The slope at S = 0.5 is twice as great

as that of a normal acid titration curve. Greater slopes can be obtained by extending the model to include more mutually interacting ionizations. Systems of three and four interacting groups were analyzed as described for the double-coupled model (Fig. 3, *curves 3 and 4*). Maximum slopes at S = 0.5 approached three and four times the slope of the normal acid curve for three and four interacting groups, respectively. For any model, curves of any desired steepness less than the maximum are obtained readily by appropriate choice of constants, and no solution is unique. The data from titration of stripped Hb^{TFA}-deO_2 can be fitted by the double-coupled model ($pK_1 = pK_{A_1} = pK_{B_1} = 9.4$, $pK_2 = pK_{A_2} = pK_{B_2} = 5.4$), the triple-coupled model ($pK_1 = 8.6$, $pK_2 = 7.4$, $pK_3 = 6.2$), or the quadruple-coupled model ($pK_1 = 8.3$, $pK_2 = 7.7$, $pK_3 = 7.1$, $pK_4 = 6.5$). To obtain a curve of sufficient slope to fit the titration of Hb^{TFA}-deO_2 + diphosphoglyceric acid, at least four coupled groups are required (Fig. 4).

These models are functionally indistinguishable, since in each the observed ionization occurs at the central pH (7.4), the intrinsic pK_a values being unobservable. It seems likely that the same number of ionizations is involved in stripped Hb-deO_2 and in Hb-deO_2 complexed with diphosphoglyceric acid, and that diphosphoglyceric acid simply increases the magnitude of the interaction. However, present knowledge of individual ionization processes in hemoglobin is inadequate to permit application of these models to actual events in the protein. The β chain of human hemoglobin contains nine histidines, two cysteines (one in Hb^{TFA}), and one amino terminus, most of which could be expected to ionize in the pH range of interest, and many of which might be involved in a complex of interacting ionizations involving His β146. Detailed discussion of the conceivable complexities of the system is futile until more is known about the roles of individual groups in structural changes of the protein. However, such interactions must be invoked to account for the pH-dependent changes observed in Hb^{TFA}-deO_2. Since the pH-dependent chemical shift change clearly involves His β146 (either by direct influence of the ionization or indirect conformational consequence of it), cooperative ionization systems may be involved in that portion of the alkaline Bohr effect attributed to His β146 (2).

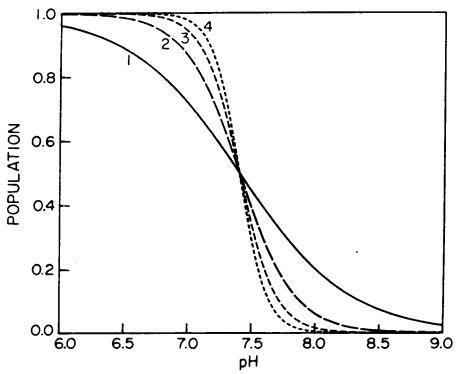

FIG. 3. Calculated ionization curves for (*1*) a single acid; (*2*) a pair of mutually interacting acids ($pK_{A1} = pK_{B1} = 9.4$, $pK_{A2} = pK_{B2} = 5.4$); (*3*) three mutually intreacting acids ($pK_1 = 9.9$, $pK_2 = 7.4$, $pK_3 = 4.9$); (*4*) four mutually interacting acids ($pK_1 = 9.9$, $pK_2 = 8.6$, $pK_3 = 6.2$, $pK_4 = 4.9$). The slope at half-titration in each case approaches the theoretical maximum for that order of interaction. All pK values were chosen to place the observed pK_a at pH 7.4.

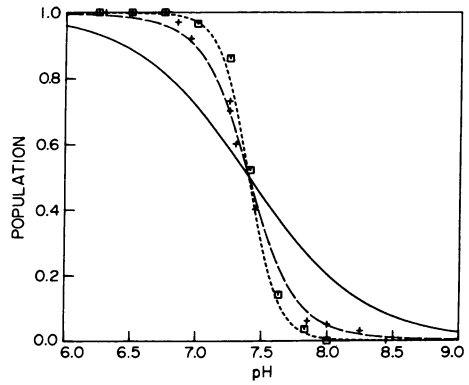

FIG. 4. Calculated ionization curves superimposed on experimental titration data for Hb^{TFA}-deO_2, +, ($pK_1 = 8.3$, $pK_2 = 7.7$, $pK_3 = 7.1$, $pK_4 = 6.5$); and for Hb^{TFA}-deO_2 + diphosphoglyceric acid, □, ($pK_1 = 9.4$, $pK_2 = 8.6$, $pK_3 = 6.2$, $pK_4 = 5.5$). *Solid line* is a single acid titration.

Mechanistic implications of ionic interactions

The observation of "cooperative" ionizations in vital residues of hemoglobin may have great significance for understanding of the detailed molecular mechanism of the cooperative transition. Simultaneously, however, determination of the effective pK_a of His β146 (or the pK_a of a major conformation change in its vicinity) raises some disturbing questions about the role of ionic forces in the cooperative process. A recent mechanistic proposal (4) states that a salt bridge between the carboxyl group of His $\beta_2$146 and Lys $\alpha_1$40 constitutes the quaternary structural constraint in the $\alpha_1\beta_2$ interface, which, along with similar electrostatic constraints at the $\alpha_1\alpha_2$ interface, produces cooperative ligand binding in the molecule. The importance of interactions at the $\alpha_1\beta_2$ interface to cooperative transition has long been recognized from the preponderance of invariant residues found there and from the diminished cooperativity found in mutants that exist. The salt bridge model designates the His $\beta_2$146–Lys $\alpha_1$40 interaction as a source of allosteric properties, and attributes lessened cooperativity in variants to weakening of this interaction. For example, the decreased cooperativity and increased oxygen affinity of hemoglobin Hiroshima (His β146 → Asp) have been attributed to loss of the His β146 imidazole–Asp β94 salt-bridge stabilization of the carboxyl salt bridge of the residue (12). If the imidazole salt bridge stabilizes the carboxyl bond and thereby contributes to the overall energy of the cooperative transition, then the titration of the imidazole, with or without associated conformation changes, should produce a decline in cooperativity as the pH is raised. This prediction is contrary to what has been observed. Within the accuracy of such experiments, the Hill coefficient of hemoglobin A has been shown to be independent of pH in the range 4.5–9 (13, 14, Lee, T. and Raftery, M. A., unpublished results), *provided* salt conditions are kept constant. This pH independence has been demonstrated by still other workers in the crucial pH range 7–8 (18). The His β146 and Arg α141 salt bridges directly involved in the proposed structural constraints are not titrated in this pH range, but the absence of any effect from removal of the stabilizing His β146 imidazole bond impels a closer examination of the role of its carboxyl bond in the mechanism.

His β146 *per se* has been shown to be expendable since cooperativity is little impaired when the histidine is removed by

carboxypeptidase B digestion ($n = 2.5$) (2). However, removal of the next residue, Tyr β145, by carboxypeptidase A abolishes cooperativity ($n = 1.0$) (2). The suggestion has been made (4) that these phenomena are due to the possibility that the Tyr β145 carboxyl group may form the salt bridge to Lys α40 in place of the formal carboxy terminus. A possibility that has not been fully discussed is that the side chain of the tyrosine may play a vital role in the transition, entering into hydrophobic interactions that are as important energetically as electrostatic ones in maintenance of the constrained deoxy structure.

The influence of the tyrosine side chains on the oxygen affinity of the β chains was discussed in a recent report (3) on the properties of mutant hemoglobins Bethesda and Rainer, in which Tyr β145 is replaced by histidine and cysteine, respectively. It was suggested that the phenolic groups contribute to tertiary constraint of the β chains, lowering oxygen affinity as long as the salt bridges of His β146 stabilize the groups in their pockets. The particular substitutions of cysteine and histidine make direct comparison of properties difficult, since the cysteine was shown to form a disulfide bond with Cys β93 and the histidine, being partially charged at physiological pH, would be expected to be less stable than tyrosine if it enters the hydrophobic F-G pocket. In both cases, the mutant residue produces structural perturbations extensive enough to disrupt the salt bridges of His β146 (15), so that the altered properties of these hemoglobins cannot be ascribed simply to loss of the tyrosine side chains. However, it is interesting to note that no other examples are known of vertebrate hemoglobins with substitutions at position β145. Mutants with residues such as glycine or alanine, which would produce no steric barrier to formation of the His β146 salt bridges, are not found. This suggests that the phenolic group is not a passive bulk that is pushed in and out of a conveniently shaped notch as salt bridges are broken and formed; rather, that the side chain itself is involved in energetically important conformational processes. As example of the sort of interactions in which a penultimate tyrosine is involved has been described (4) for Tyr $\alpha_1$140. As this tyrosine enters its pocket, it presses on the indole ring of Trp $\beta_2$37, causing it to tilt over and press on Pro $\beta_2$36. This interchain pressure pushes the β chain toward its deoxy conformation and presumably helps to keep it there.

The behavior of allosteric parameters P_{50} and n as functions of pH and ionic strength lend credence to the suggestion that subtle hydrophobic interactions such as these are no less important than electrostatic ones in the energetics of the cooperative transition. It has long been recognized that neutral salt concentration has profound influence on oxygen affinity and the Bohr effect, but not on cooperativity (13, 16). Very high concentrations (e.g., 1–4 M) of neutral salt supress the Bohr effect to a marked degree (13), and a 50-fold increase in sodium chloride concentration can produce a 10-fold increase in P_{50} for stripped hemoglobin (16). Binding of diphosphoglyceric acid, which in 1:1 molar ratio decreases oxygen affinity dramatically, is regarded as a specialized and more effective instance of the general salt effect, and indeed very high concentrations of either diphosphoglyceric acid or neutral salt can eliminate any effect from addition of the other (17). But extensive studies (13, 14, 16, and Lee, T. and Raftery, M. A., unpublished results) have failed to show dependence of the Hill coefficient on neutral salt concentration in the range $<10^{-2}$–5 M. Some implications of these findings have been discussed, particularly in reference to the role of diphosphoglyceric acid (16). The data point consistently to binding of negative salt ions in a cavity between the β chains of deoxyhemoglobin, involving six positively charged groups on the protein (His β143, Val β1, Lys β82). This binding, whether by diphosphoglyceric acid or other anions, such as chloride, stabilizes the deoxy form and thus lowers the oxygen affinity. Addition of further neutral salt to deoxyhemoglobin that is saturated with diphosphoglyceric acid lowers the oxygen affinity further, but only to a small degree. Thus it appears that most of the effective ion binding is confined to the diphosphoglyceric acid binding site. The only further effect of high-salt concentrations is the supression of the Bohr effect (13), a phenomenon that is not surprising since high-ionic strength should stabilize the protonated forms of the primary Bohr residues (His β146, Val α1) in their oxy conformation.

In view of the sensitivity of the Bohr and diphosphoglyceric acid electrostatic interactions to salt concentration, an ionic strength effect on the salt bridges of the proposed cooperative mechanism (4) would be expected. If the quaternary structural constraints afforded by the interchain salt bridges are in fact the most important energetic factors in the conformation transition, it is interesting that variation of the ionic strength of the medium has so little effect on the Hill coefficient. One might expect, in addition, that high external ion concentrations would lead to relative stabilization of the oxy conformation (similar to the effect seen for the Bohr residues), in which the eight charged groups are free in solution. This would result in increased oxygen affinity, rather than the slight decrease which is observed. On the other hand, high ionic strengths would be expected to oppose exposure of hydrophobic groups such as Tyr β145 to the medium. If indeed these hydrophobic interactions are energetically important in maintenance of the deoxy structure, some destabilization of the oxy (exposed) conformation would be expected from increased solvent ionic strength, resulting in decreased oxygen affinity.

The same considerations apply to the roles of cooperative ionizations. Unless the interacting ionizable groups are protected from the solvent or there is internal compensation for changes in their ionic character with pH and ligand binding, any allosteric property to which they contribute should be sensitive to pH and ionic strength.

CONCLUSION

A pH-dependent process having a pK_a of 7.4 has been observed in the $\alpha_1\beta_2$ interface of deoxyhemoglobin that is absent in oxyhemoglobin. Removal of His β146 abolishes the pH effect, suggesting that the observed process is a direct or indirect effect of the histidine side chain ionization. The abnormally short pH range over which this process occurs raises the possibility that other pH-dependent processes interact with it. Since it has been proposed (4) that the salt bridges of His β146 play a role in the Bohr effect and the cooperative mechanism, interactions between the His β146 ionization and other ionizations in the molecule might be significant in effecting conformation changes associated with cooperative ligand binding.

At the same time, the chemical evidence that has been discussed suggests that the cooperative transition in hemoglobin cannot be explained solely in terms of electrostatic interactions. Conformational restraints governed by several subtle

hydrophobic interactions within and between the subunits could better account for many aspects of hemoglobin's functions. Certainly the energy involved in ionic interactions such as the salt bridges must be important, but even the functions of the salt-bridged residues are complex: the role of Arg α141 as a steric spacer between the α-chains appears to be vital to normal cooperativity (17). The experimental difficulties in isolation and examination of nonpolar forces in conformational processes are great, and extensive studies of the ligand-binding process on the molecular level will be required to further our understanding of cooperativity in proteins.

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