Binding of Triphosphate Spin Labels to Hemoglobin Kempsey

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Summary

The binding of 1-oxyl-2, 2, 6, 6-tetramethylpiperidine-4-triphosphate (I) to a mutant of human hemoglobin (Hb), Hb Kempsey (β-99 Asp→Asn), has been studied as a function of heme ligation. The (ligand-free Hb Kempsey) - (I) complex has a stoichiometry of 1.0±0.1 moles of I per mole of Hb Kempsey tetramer and a dissociation constant $1.7 \pm 0.5 \times 10^{-4}$ M at 13° in 0.05 M bis-Tris buffer, pH 7.3 and 0.1 M in Cl^-. A second spin label, N^6-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl) adenosine triphosphate, was used to probe the structure of the organic phosphate binding site in ligand-free Hb Kempsey. Neither label binds to fully liganded Hb Kempsey under these conditions. The results of these experiments are consistent with a generalized concerted transition model for cooperative ligand binding to hemoglobin.

Previous studies of the binding of triphosphate spin labels to hemoglobin (Hb) have demonstrated that cooperative oxygen binding to hemoglobin and the effect of organic phosphates on oxygen binding can be quantitatively accounted for by a generalized concerted transition model.[1,2] This model is mathematically the same as the model of Monod, Wyman and Changeux[3] except that in the generalized model, the α- and β-subunits are treated as non-equivalent.

In a study of a hemoglobin mutant, Hb Chesapeake (α-92 Arg→Leu), it was found that the observed organic phosphate and oxygen binding properties of this mutant can be accounted for in terms of simple, physically plausible

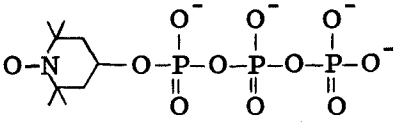
changes in the model parameters which had been determined for HbA.[2] These changes in the model parameters involve the α-subunits primarily.

Hemoglobin Kempsey is a mutant which has asparagine at position 99 of the β-subunits rather than the normal aspartic acid.[4] This mutant hemoglobin is characterized by a high oxygen affinity and low cooperativity. These properties suggested to us that by analogy with Hb Chesapeake, (a) the properties of Hb Kempsey might also be accounted for by simple changes in the model parameters obtained for HbA; and (b) the changes in the model parameters might be understood in terms of a structural perturbation involving primarily the mutated β-subunits.

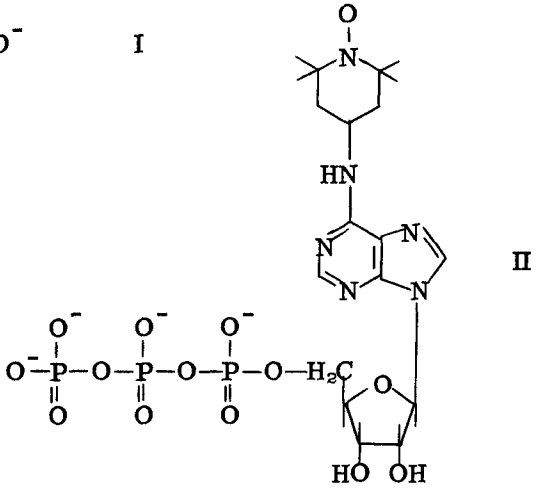
Materials and Methods

Hemoglobin Kempsey was isolated[4] from the whole blood of a patient of the Mayo Clinic in Rochester, Minnesota. The blood sample was a gift of Dr. Virgil Fairbanks. Sequence analysis[4] of the chromatographically purified mutant by Dr. Bernadine Brimhall demonstrated that the present mutant hemoglobin is identical to Hb Kempsey (β-99 Asp→Asn).

Spin labels used in this study are 1-oxyl-2, 2, 6, 6-tetramethyl-piperidine-4-triphosphate (I) and N^6-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl) adenosine triphosphate (II).[1, 2]



I



II

Binding studies were performed as described earlier[2] using a value

of 15 for η. All experiments were carried out at 13° in 0.05 M bis Tris buffer, pH 7.3 and 0.1 M in Cl^-, with hemoglobin concentrations of $\sim 3 \times 10^{-4}$ M.

Results

The dissociation constant of the (ligand-free Hb Kempsey)-(I) complex was found to be $1.7 \pm 0.5 \times 10^{-4}$ M, with a binding stoichiometry of 1.0 ± 0.1 moles of I per mole of Hb Kempsey tetramer. These data are comparable to those obtained for binding of I to the hybrid $\alpha_2 \beta_2^{+CN}$, where the dissociation constant is 3×10^{-4} M and the binding stoichiometry is approximately 0.8.[1] There is no apparent binding of I or II to fully liganded Hb Kempsey.

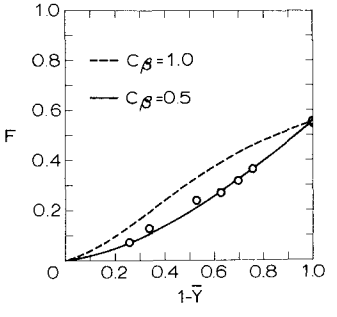


Figure 1. The dependence of the function F on $1-\bar{Y}$ determined for Hb Kempsey. The function F is defined in the text and $1-\bar{Y}$ is the fraction of ligand-free hemes. Points are experimentally determined, and the solid curve is calculated from the model using the parameters given in the text and in Table 2. $c_\beta = K_R^\beta / K_T^\beta$. For comparison, the calculated dependence for $c_\beta = 1.0$ is also given. Here $c_o = \ell_o$.

The binding of I to Hb Kempsey as a function of CO-ligation of the hemes is shown in Figure 1. The function F is defined as before,[1] and is given by the following equation.

$$F = \left(\frac{\ell_o - \ell}{c_o}\right)\left(1 + \frac{K_o}{\ell}\right) \qquad (1)$$

Here ℓ is the concentration of free (unbound) I, ℓ_o is the total concentration of I, c_o is the total concentration of Hb Kempsey and K_o is the dissociation constant of the (ligand-free HbA)-(I) complex, $K_o = 2.5 \times 10^{-5}$ M.[1,2]

In the present experiments the paramagnetic resonance spectra of I in Hb Kempsey solutions at different stages of heme ligation appeared to

exhibit isosbestic points. This could not be clearly demonstrated, however, because there was a slow reduction in the amplitude of the paramagnetic resonance signal exhibited by I in a solution of ligand-free Hb Kempsey. All measurements were carried out as quickly as possible in order to minimize the error introduced by this effect.

The Generalized Concerted Transition Model

These phosphate binding data can be accounted for by the generalized concerted transition model[1] using the parameters: L = 0.17; $K_R^\alpha = 0.36$, $K_R^\beta = 0.18$ $K_T^\alpha = 24.2$ and $K_T^\beta = 0.36$ torr oxygen. These parameters, together with the value of the dissociation constant K_o for the (ligand-free HbA)-(I) complex, account for (a) the observed dissociation constant of the (ligand-free Hb

TABLE 1

Comparison of Experimental and Calculated Oxygen Binding Data for Hemoglobin Kempsey

	p_{50} (torr)	n
Experimental	~4	low
Calculated		
$\ell_o = 0$	0.27	1.0
$\ell_o = 1.5 \times 10^{-3}$ M	0.48	1.2

Here p_{50} is the partial pressure of oxygen at $\bar{Y} = 0.50$ and n is Hill's constant. The experimental data are from ref. 4, where the conditions used were 0.05M phosphate buffer, pH 6.4 and 37°. Under these conditions, p_{50} for HbA is 23 torr oxygen. Calculated data are for 0.05M bis-Tris buffer pH 7.3, 0.1M Cl^- and 13°. Under these conditions and for $\ell_o = 1.5 \times 10^{-3}$ M, the calculated p_{50} for HbA is 5.4 torr. The parameters used in these calculations are listed in Table 2.

Kempsey)-(I) complex, 1.7×10^{-4}M;* (b) the high oxygen affinity and low cooperativity in oxygen binding of Hb Kempsey (Table 1); and (c) and binding of I to Hb Kempsey as a function of CO saturation of the heme groups. The experimental and calculated dependence of F on $1-\bar{Y}$ are compared in Figure 1. In this Figure, F is equal to $\bar{T}$, the fraction of tetramers in the T state. The parameters used here for Hb Kempsey are compared in Table 2 with those used to fit the properties of HbA, Hb Chesapeake and the two hybrids $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$.

TABLE 2

Model Parameters Used to Fit Experimental Data

Hemoglobin	L	K_R^α	γ	c_α	c_β
A	3000	0.36	2	0.0149	0.0055
$\alpha_2{}^{+CN}\beta_2$	3000	0.36	2	0.0149	0.0055
$\alpha_2\beta_2{}^{+CN}$	3000	0.36	2	0.0149	0.0055
Chesapeake	0.53	0.36	2	1.0	0.0055
Kempsey	0.17	0.36	2	0.0149	0.5

In all calculations, $c_o = 3 \times 10^{-4}$. $\gamma = K_R^\alpha / K_R^\beta$, $c_\alpha = K_R^\alpha / K_T^\alpha$ and $c_\beta = K_R^\beta / K_T^\beta$. For $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$, when the ferrous subunits are ligand-free, $[T]/[R]$ is equal to Lc_α^2 and Lc_β^2, respectively. L refers to stripped (phosphate-free) hemoglobin in 0.1 M Cl^- and increases when phosphate is added (see ref. 1).

* As with the earlier analysis of the properties of Hb Chesapeake given in reference 2, we have assumed that the organic phosphate-binding or "T" state (which we identify with the quaternary structure that Perutz refers to as the "deoxy" structure[5]) is identical for both HbA and Hb Kempsey. Therefore, the decreased affinity of I for Hb Kempsey relative to HbA is attributed to a change in the equilibrium between structures which bind I (T structures) and those which do not (R structures).

Discussion

A previous study[2] demonstrated that the oxygen and organic phosphate binding properties of Hb Chesapeake can be accounted for simply by assuming that the amino acid substitution (α-92 Arg→Leu) "locks" the Hb Chesapeake α-subunits in high oxygen affinity structures resembling the structures of normal liganded α-subunits. Further, it was suggested that there is a class of abnormal hemoglobins whose properties can be understood in terms of a locking of the α- or β-subunits in their high or low affinity structures.[2]

The present results can similarly be accounted for by assuming that the substitution (β-99 Asp→Asn) locks the β-subunits of Hb Kempsey in structures resembling those of normal liganded β-subunits. Evidence for this comes from the parameters of the generalized concerted transition model which best account for the present results and the similarity between the affinities for I exhibited by ligand-free Hb Kempsey and the hybrid $\alpha_2\beta_2^{+CN}$. Thus, ligand-free Hb Chesapeake has an affinity for I similar to that of $\alpha_2^{+CN}\beta_2$ and ligand-free Hb Kempsey has an affinity for I similar to that of $\alpha_2\beta_2^{+CN}$. Unlike Hb Chesapeake, where the parameters of the generalized concerted transition model give $K_R^\alpha/K_T^\alpha = 1.0$, with Hb Kempsey, $K_R^\beta/K_T^\beta = 0.5$. These values suggest that with Hb Chesapeake, the α-subunits are completely locked in their high affinity structures, independent of the quaternary structure of the tetramer and of the presence of α-heme ligands. With Hb Kempse the fact that $K_R^\beta/K_T^\beta < 1$ suggests that the structures of the β-subunits are not completely locked in one form but are weakly dependent on the quaternary structure of the tetramer and on the presence of ligands on the β-hemes. This dependence is greatly reduced relative to HbA, however, where $K_R^\beta/K_T^\beta = 0.0055$.[2]

While the results of these studies of Hb Chesapeake and Hb Kempsey are most easily interpreted by assuming that the α- or β-subunits of these mutants are locked in high oxygen affinity structures resembling those of liganded α- and β-subunits, this interpretation may well be incorrect or at least oversimplified. As Perutz has emphasized to us (private communicatio the X-ray crystal structures of liganded and ligand-free Hb Chesapeake show no evidence of a locking of the α-subunits in structures similar to those of liganded α-subunits. Indeed, these studies find no significant structural

differences between normal ligand-free human hemoglobin and ligand-free Hb Chesapeake.[6] Further, the resonance spectra of label II bound to ligand-free Hb Kempsey and to $\alpha_2\beta_2^{+CN}$ are similar but not identical, implying non-identical structures. Also, the ligation dependence of the resonance spectrum of Hb Kempsey covalently spin-labeled at cysteine β-93 (C. Ho, private communication) is significantly different from that of β-93 spin-labeled $\alpha_2\beta_2^{+CN}$.[7]

In fitting the present results to the generalized concerted transition model, we have assumed that the structure of the phosphate binding site (in the T state) is identical for both Hb Kempsey and HbA. Therefore, a spin label which is sensitive to the quaternary structure of a hemoglobin molecule should exhibit the same paramagnetic resonance spectrum when it is bound to ligand-free Hb Kempsey as it does when bound to ligand-free HbA. The spectrum exhibited by label II when bound to hemoglobin is more informative than that exhibited by label I.[2] We have used II to probe the structure of the organic phosphate binding site in ligand-free Hb Kempsey. The observed results are shown in Figure 2. The spectrum due to II bound to Hb Kempsey does differ from that due to II bound to HbA. Though these spectral differences are significant, we do not feel that they are due to structural differences large enough to invalidate the present analysis. It is known that the relatively small[5,8] structural differences among hemoglobin derivatives having different heme ligands can cause large differences in the paramagnetic resonance spectra exhibited by spin labels covalently attached to these derivatives.[9] Also, while the shapes of these spectra are different, their intensities relative to that of II free in solution are quite similar. Therefore, any error introduced by using the same value of η (see ref. 1) for HbA and Hb Kempsey is likely to be small.

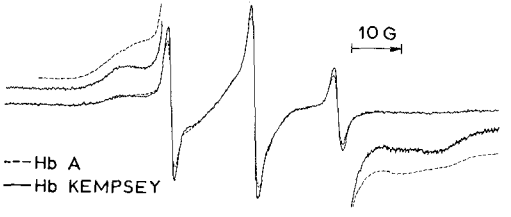


Figure 2. Paramagnetic resonance spectra of label II in solutions of ligand-free Hb Kempsey (——) and ligand-free HbA (---). Portions of the spectra at higher gain arise from II bound to hemoglobin. For HbA $c_0/\ell_0 = 5$.

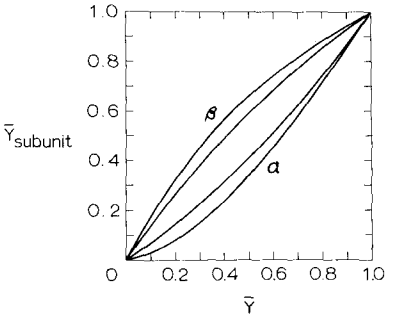


Figure 3. Calculated dependence of the fraction of liganded α-subunits ($\bar{Y}_\alpha$) and β-subunits ($\bar{Y}_\beta$) as a function of overall heme ligation ($\bar{Y}$) for Hb Kempsey. The curves are calculated using the parameters given in the text and in Table 2. The inner pair of curves are for $\ell_o = 0$ and the outer pair are for $\ell_o = 1.5 \times 10^{-3}$M.

The parameters used to fit the present results to Hb Kempsey can be used to predict the relative saturation levels of the α- and β-subunits in Hb Kempsey as a function of total heme ligation. This is shown in Figure 3. In contrast with Hb Chesapeake, where the α-subunits bind ligand preferentially, in Hb Kempsey the β-subunits are predicted to bind ligand preferentially. Unfortunately, no experimental data are available at present to test this latter p

Acknowledgments

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