Determination of the Rate and Equilibrium Constants for Oxygen and Carbon Monoxide Binding to R-state Human Hemoglobin Cross-linked between the $\alpha$ Subunits at Lysine 99*

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The kinetics of $O_2$ and CO binding to R-state human hemoglobin $A_0$ and human hemoglobin cross-linked between the $\alpha$ chains at Lys$^{99}$ residues were examined using ligand displacement and partial photolysis techniques. Oxygen equilibrium curves were measured by Imai's continuous recording method (Imai, K. (1981) Methods Enzymol. 76, 438-449). The rate of the R to T transition was determined after full laser photolysis of the carbon monoxide derivative by measuring the resultant absorbance changes at an isosbestic point for ligand binding.

Chemical cross-linking caused the R-state $O_2$ affinity of $\alpha$ subunits to decrease 6-fold compared with unmodified hemoglobin. This inhibition of $O_2$ binding was the result of both a decrease in the rate constant for ligand association and an increase in the rate constant for dissociation. The $O_2$ affinity of R-state $\beta$ subunits was reduced 2-fold because of an increase in the $O_2$ dissociation rate constant. These changes were attributed to proximal effects on the R-state hemoglobin as is the result of the covalent cross-link between $\alpha$ chain G helices. This proximal strain in cross-linked hemoglobin was also expressed as a 5-fold higher rate for the unliganded R to T allosteric transition.

The fourth $O_2$ equilibrium binding constant, $K_a$, measured by kinetic techniques, could be used to analyze equilibrium curves for either native or cross-linked hemoglobin. The resultant fitted values of the Adair constants, $a_1$, $a_2$, and $a_3$, were similar to those obtained when $K_a$ was allowed to vary, and the fits were of equal quality. When $K_a$ was fixed to the kinetically determined value, the remaining Adair constants, particularly $a_3$, became better defined.

Recent advances in protein engineering combined with an increasing clinical demand for a blood substitute have prompted the design of modified hemoglobins for use in oxygen-carrying fluids. To be considered for clinical applications the cell-free form of the protein should not dissociate into dimers, which filter through the kidneys. In addition, the $P_50$ and oxygen dissociation rate constants of the protein must be large enough to allow rapid $O_2$ unloading in respiring tissues. Generally, ideal values for these latter properties are defined by those of native, intracellular human hemoglobin in the presence of allosteric effectors.

Human hemoglobin has been site specifically cross-linked with a four-carbon fumaryl group covalently bound between $\alpha$ Lys$^{99}$ residues (aaHb), and this derivative is being examined as a potential blood substitute. aa-Cross-linked hemoglobin has been studied functionally by measurements of $O_2$ and CO binding and structurally by x-ray crystallography and resonance Raman spectroscopy (Chatterjee et al., 1986; Vandegriff et al., 1989, 1991; Larsen et al., 1990). This protein is a stable tetramer with ligand binding properties similar to those of intracellular hemoglobin (i.e. a high degree of cooperativity and reduced affinity for oxygen). The functional effects of aa-cross-linking hemoglobin appear to be manifested primarily in the later stages of ligand binding, when the protein is in the R-state. Like native hemoglobin, aaHb switches from the T to the R quaternary conformation when two to three ligands are bound (Vandegriff et al., 1989); however, precise determinations of the R- and T-state ligand binding properties of this protein have not been made.

The purpose of this study was 2-fold. First, $O_2$ and CO association and dissociation kinetics were measured to examine effects of the aa-cross-link on the ligand binding properties of R-state hemoglobin. The switchover between the T and R conformation occurs on average after 2.3 ligands have been bound to either human hemoglobin $A_0$ (Hb$A_0$) or aaHb. Consequently, kinetic measurements of the binding of the fourth ligand (i.e. Hb$O_4$: $O_2$ → Hb$O_3$) can be used to define R-state properties. The low R-state affinity of aaHb has been interpreted by a structural model in which the cross-link attenuates movement of the G helices and the joint region between the FG corner of the $\alpha$ chains and the C helix of the $\beta$ chains. This model predicts that movement of the proximal histidine toward the heme plane is still restricted after the quaternary transition and that R-state $\alpha$ helices are...
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unable to adopt a completely planar conformation (Vandegriff et al., 1989; Larsen et al., 1990). In this study, kinetic ligand binding reactions of R-state ααHb were evaluated in terms of this structural model and were consistent with its interpretation.

The second goal was to determine the equilibrium constant for the binding of the fourth O₂ molecule independently by kinetic measurements. Discrepancies between K₄ values measured by kinetic and equilibrium techniques have been reported but not resolved (Gibson and Edelstein, 1987; Philo and Lary, 1990). The techniques for measuring oxygen binding to equilibrium are usually not precise enough to allow an accurate determination of K₄ and an independent method for assigning values to this binding constant is necessary. In general, it is difficult to resolve all four binding constants from oxygen equilibrium curves using fitting procedures alone. The low levels of partially liganded intermediates which arise during oxygenation of HbAo make the intermediate binding constants difficult to evaluate (Gill et al., 1987; Vandegriff et al., 1989), and the dissociation of hemoglobin in dilute solutions causes errors in analyses which assume that only tetramers are present (Mills et al., 1976; Johnson and Ackers, 1977). Often the upper asymptote of the Hill plot cannot be defined well experimentally, and only the product K₃K₄ can be obtained with certainty (Widrow et al., 1977). Slight variations in normalizing the upper ends of HbAo equilibrium curves cause significant variation in the fitted values of K₃ and K₄ (Marden et al., 1989). Tetramer dissociation does not occur for ααHb, and partially liganded intermediates build up to a greater extent during O₂ binding for the cross-linked protein than for HbAo (Vandegriff et al., 1989). This eliminates two problems associated with the interpretation of oxygen binding data, and thus, ααHb provides a simpler system for testing the applicability of kinetically determined values of K₄ to the analysis of equilibrium curves.

MATERIALS AND METHODS

Hemoglobin Samples—HbAo and ααHb were prepared as described previously (Christensen et al., 1988; Vandegriff et al., 1989, 1991). All equilibrium and kinetic ligand binding measurements were carried out in 50 mM his-Tris, 0.1 M NaCl, and 1 mM EDTA buffer at pH 7.4, and 55 °C.

Ligand Displacement Reactions—The rates of ligand dissociation were measured by ligand displacement reactions (Olson, 1981a). For O₂ and n-butyl isocyanide dissociation, the ligands were displaced by CO. For CO dissociation, CO was displaced by NO. The reactions were measured in a Gibson-Dionex stopped-flow, rapid mixing spectrophotometer, interfaced to an OLIS model 3820 data collection system (Athens, GA). All time courses, measured at wavelengths giving the maximal absorbance change, were fitted to a two-exponential expression with equal spectral amplitudes for the two phases.

\[ \Delta A_t = 0.5 \cdot \Delta A[e^{X-Y} + e^{-X-Y}] \]  

\[ \Delta A_t = \text{the total change at time } t, \Delta A_t, \text{ is the total change in absorbance, } k_t, \text{ and } k_s, \text{ are the observed fast and slow first-order rate constants.} \]

The observed ligand replacement rates, rₓ, are equal to

\[ r_x = \frac{k_t}{[1 + k_t/X/k_y(Y)]]} \]

where X is the displaced ligand and Y is the displaced ligand dissociation. kₜ and kₛ are rate constants for the dissociation and association of ligand X, respectively, and kᵧ is the association constant for ligand Y (Olson, 1981b). The rate constants describe the last step of ligand binding, presumably representing ligand dissociation from the R conformation. To determine kₓ for O₂ dissociation, sets of rₓ versus [CO]/[O₂] were fitted to Equation 2 by iterative least squares analysis to determine kₓ and kᵧ/kₛ. For CO displacement by NO, where l = 1 and l' = 1 are the dissociation and association rate constants for the last step in CO binding, respectively, l was determined directly as rₓ, since l' \( \ll \) kₓ. For n-butyl isocyanide (nBNC) displacement by CO, kₓ was determined directly as rₓ, since kₓ < l'.

R-state Ligand Association Kinetics—O₂ association kinetics were measured using a flash photolysis apparatus equipped with a PhaseR 2100B flash lamp pumped dye laser (Durham, NH). The reactions were studied at 100 and 200% photolytic light intensities to produce complete and partial O₂ photolysis, respectively. Time courses were monitored at 436 nm and fitted to a two-exponential expression in which the spectral amplitudes of the two phases were either fixed or allowed to vary in an attempt to account for differences in the photolytic quantum yield of the α and β subunits (Morris et al., 1984). CO association kinetics were measured using a conventional barytes flash apparatus with photographic strobes (Sumpak Auto 544). The light intensity was set to 2% of full photolytic intensity to measure R-state kinetics. The time courses were monitored at 435 nm for 10 μM hemoglobin solutions equilibrated with either 0.1 or 0.2 mM CO. The time courses were fitted to Equation 1 to determine individual rates of CO binding to the α and β subunits.

Oxygen Equilibrium Curves—Oxygen equilibrium curves were measured by the continuous method of Imai (1981) and described by Vandegriff et al. (1989). The enzymatic methemoglobin reducing system of Hayashi et al. (1973) was included, and the amount of methemoglobin was < 4%. The equilibrium curves were analyzed by the Adair equation (Adair, 1925)

\[ \frac{Y(p)}{\alpha p + \beta p^2 + \gamma p^3 + \delta p^4} \]

where Y(p) is the fractional saturation of hemoglobin, p is the oxygen pressure in mm Hg, and α through δ are the overall Adair constants that are the products of the stepwise equilibrium constants (Vandegriff et al., 1989). In another version of Equation 3, δ, was constrained by the kinetic value for the fourth stepwise constant, K₄, by setting α = kₓ/K₄, where 1/4 is the statistical binding factor for the ligand.

The equilibrium curves were also fitted to the two-state Monod-Wyman-Changeux model (Monod et al., 1965) and the three-state cooperon model (Dh Cere et al., 1987; Vandegriff et al., 1988) for an estimation of the allosteric constant, L (i.e., L = [T₁]/[R]).

RESULTS

Oxygen Dissociation Rates and Subunit Assignments—Representative time courses for the displacement of O₂ from HbAo and ααHb by CO are shown in Fig. 1. All time courses were fitted to Equation 1, and there was a 2-fold difference in rates of the two phases for both hemoglobins. O₂ release from ααHb was more rapid at every ratio of [O₂]/[CO]. As shown in Fig. 2, the observed fast and slow rates in the presence and absence of para-hydroxymercurobenzoate (pMB) were fitted to the hyperbolic form of Equation 2: rₓ = kₓ([CO]/[O₂])/(kₓ/kₛ + [CO]/[O₂]).

Olson et al. (1971) assigned the fast and slow components observed in O₂ displacement reactions of hemoglobin A₀ to dissociation from the β and α subunits, respectively. Their assignments were based on two experimental results. 1) There was correspondence between the absorbance difference spectra of the fast and slow phases, respectively, with the β(O₂) minus β(CO) and α(O₂) minus α(CO) difference spectra for isolated subunits. 2) The binding of pMB to β Cys® markedly increased the O₂ dissociation rate constant for the fast component and had only a small effect on the rate constant for the slow component. These assignments have been confirmed by kinetic studies with genetically engineered human hemoglobins (Mathews et al., 1989). In the latter studies, the altered O₂ dissociation rate constants could be assigned to mutated subunits and the “normal” rate constants to native subunits. As in the earlier studies, native α and β subunits exhibited O₂ dissociation rate constants equal to ~12 s⁻¹ and ~20 s⁻¹, respectively, at 20 °C.

The two kinetic phases of O₂ dissociation from ααHb were both twice as fast as the comparable rates for HbAo. Two alternative interpretations were possible. Either the dissociation...
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Oxygen Dissociation

Fig. 1. Normalized time courses for the displacement of O₂ by CO after rapid mixing of oxy-HbA₀ (filled squares) and oxy-αα-Hb (open circles) with 1 mM CO in the stopped-flow apparatus. The reactions were carried out at 25 °C and pH 7.4 in 50 mM bis-Tris, 0.1 mM NaCl, and 1 mM EDTA buffer. Concentrations after mixing were 5 mM protein, 0.032 mM O₂, and 0.5 mM CO. The symbols show the fraction of oxyhemoglobin remaining as a function of time after mixing. Time courses were normalized to total absorbance change. Solid lines show the best fits to Equation 1. The fitted rates are 26 s⁻¹ and 10 s⁻¹ for HbA₀ and 43 s⁻¹ and 20 s⁻¹ for αα-Hb.

Fig. 2. Observed rates of O₂ displacement as a function of [CO]/[O₂] in the presence and absence of 2 eq of pMB/tetramer. Conditions are given in Fig. 1. [O₂] was varied, and [CO] was fixed at 1 mM before mixing. Observed rates (𝑡₀₂) are in units of reciprocal seconds. Open squares are observed rates for HbA₀. Filled squares are observed rates for HbA in the presence of pMB. Open circles are the observed rates for αα-Hb. Filled circles are the observed rates for αα-Hb in the presence of pMB. Solid and dotted lines were calculated by fitting the observed data to the hyperbolic form of Equation 2, using a least squares iterative program. A, rates for the fast phase of HbA₀. The fitted parameters are (minus pMB) 𝑘₁ = 40 s⁻¹ and 𝑘₂ = 8; (plus pMB) 𝑘₁ = 214 s⁻¹ and 𝑘₂ = 13. B, rates for the slow phase of HbA₀. The fitted parameters are (minus pMB) 𝑘₁ = 70 s⁻¹ and 𝑘₂ = 10; (plus pMB) 𝑘₁ = 213 s⁻¹ and 𝑘₂ = 14. C, rates for the slow phase of HbA. The fitted parameters are (minus pMB) 𝑘₁ = 12 s⁻¹ and 𝑘₂ = 4; (plus pMB) 𝑘₁ = 26 s⁻¹ and 𝑘₂ = 6. D, rates for the slow phase of αα-Hb. The fitted parameters are (minus pMB) 𝑘₁ = 28 s⁻¹ and 𝑘₂ = 7; (plus pMB) 𝑘₁ = 40 s⁻¹; 𝑘₂ = 7.

Oxygen dissociation rates from both the α and β subunits of ααHb were doubled, or αα-cross-linking increased the rate of O₂ release from the α chains 4-fold over the rate from HbA₀, with no change in the β subunit rate. In the latter explanation, the slow phase observed for O₂ dissociation from ααHb would represent ligand displacement in β subunits; in the former, the slow phase would still represent ligand displacement in α subunits.

To distinguish between these interpretations for ααHb, O₂ replacement was measured near an isosbestic point (574.2 nm) for the reaction in the presence and absence of 2 eq of pMB/tetramer. Fig. 3 shows that the time courses at the longer wavelength (574.6 nm) were predominantly slow, and the time courses at the shorter wavelength (574 nm) were predominantly fast. A similar pattern was observed for native HbA₀, in which the fast phase has already been assigned to β subunits. The presence of pMB increased the rates of the 574 nm time courses for both HbA₀ and ααHb. Thus, the fast and slow phases for ααHb are also a result of ligand displacement from β and α subunits, respectively. This result allows us to infer that αα-cross-linking doubles the rate of O₂ release from both subunits, accounting in part for the lower R-state O₂ affinity of the molecule as a whole (Table I).

To confirm that αα-cross-linking affects ligand release from both subunits, n-butyl isocyanide dissociation from ααHb was measured by displacement with CO. For this larger ligand, the difference between the β and α dissociation rate constants is at least 10-fold, and the assignment of the faster rate to β subunits is unambiguous (Olson and Gibson, 1971). Time courses were measured at 432 nm to provide equal amplitudes.
FIG. 3. Time courses for the displacement of O₂ by CO near an isosbestic point (574.2 nm) (represented by the dashed lines) for ligand binding in the presence and absence of pMB. Conditions are given in Fig. 1. Protein concentrations were 106 μM, and the pMB concentration was 50 μM after mixing. Double lines show time courses at 574 nm; single lines show time courses at 574.6 nm. A, HbAo; the rate of 574.6 nm time course = 4 s⁻¹; the rate of 574 nm time course = 12 s⁻¹. B, ααHb; the rate of 574.6 nm time course = 10 s⁻¹; the rate of 574 nm time course = 23 s⁻¹. C, HbAo plus pMB; the rate of 574.6 nm time course = 7 s⁻¹; the rate of 574 nm time course = 74 s⁻¹. D, ααHb plus pMB; the rate of 574.6 nm time course = 10 s⁻¹; the rate of 574 nm time course = 109 s⁻¹.

TABLE I
R-state kinetic and equilibrium constants for O₂ binding to HbAo and ααHb in 50 mM bis-Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4, 25 °C

The association (k') and dissociation (k) rate constants represent the kinetics for the last step in ligand binding to hemoglobin tetramers and are defined as R-state parameters for the α and β subunits. Association rates were measured by 20% laser photolysis of hemoglobin (100 μM in heme) in 1 atmosphere of O₂ (1.25 mM O₂). The values represent the average of four determinations for each rate constant. Dissociation rates were measured by O₂ displacement by CO. Hemoglobin (10 μM in heme) was equilibrated with O₂ at concentrations from 1.25 to 0.0625 mM and rapidly mixed with 1 mM CO. Dissociation rates (k) were determined by fitting the data in Fig. 2 to the hyperbolic form of Equation 2. Equilibrium constants for the α and β subunits were calculated from the ratio of rate constants, k'/k. Rate constants in parentheses were measured in the presence of pMB. The errors in kinetic measurements were estimated at ±20% based on the results of Mathews et al. (1989). For the equilibrium measurements of Kᵣ, the data in Fig. 7 were fitted to Equation 3. P₀ is the O₂ partial pressure at half saturation; n is the Hill coefficient.

| Kinetics | k' (×10⁻⁶ M⁻¹ s⁻¹) | k' (×10⁻⁴ M⁻¹ s⁻¹) | k (×10⁻⁶ M⁻¹ s⁻¹) | k (×10⁻⁴ M⁻¹ s⁻¹) | Kᵣ |
|----------|----------------------|----------------------|----------------------|----------------------|----------|
| HbAo(a)  | 42 ± 8               | 47 ± 9               | 12 ± 2               | 26 ± 5               | 3.5 ± 1.0 |
| HbAo(β)  | 146 ± 29             | 122 ± 24             | 40 ± 8               | 214 ± 43             | 3.7 ± 1.1 |
| ααHb(a)  | 17 ± 3               | 17 ± 3               | 28 ± 6               | 40 ± 8               | 0.6 ± 0.2 |
| ααHb(b)  | 130 ± 26             | 88 ± 18              | 70 ± 14              | 213 ± 43             | 1.9 ± 0.6 |

Equilibria

|          | P₀ (mm Hg) | Kᵣ (equilibrium) | Kᵣ (kinetic) |
|----------|------------|------------------|--------------|
| HbAo     | 3.3        | 2.9              | 4.7 ± 0.03   | 3.6 ± 1.0           |
| ααHb     | 14.1       | 2.5              | 0.9 ± 0.01   | 0.9 ± 0.4           |

for the fast (β) and slow (α) phases. The rates of the two phases were 2.5 s⁻¹ (β) and 0.2 s⁻¹ (α) for HbAo, and 3.4 s⁻¹ and 0.4 s⁻¹ for ααHb. pMB binding increased the rates to 12.0 s⁻¹ (β) and 0.3 s⁻¹ (α) for HbAo, and 12.4 s⁻¹ and 0.4 s⁻¹ for ααHb. As in the case of O₂, the fast components for both HbAo and ααHb showed absorbance difference spectra identical to that of isolated β chains and the slow components, difference spectra identical to that of isolated α chains. These results allow unequivocal assignment of the fast and slow kinetic phases of ααHb to the β and α subunits, respectively.

Oxygen Association Rates—Partial laser photolysis time courses for oxy-HbAo and oxy-ααHb are shown in Fig. 4A. These data were fitted to a form of Equation 1 in which the relative amplitudes of the two phases were varied to determine the O₂ association rate constants (k'). Using site-directed mutants, Mathews et al. (1989) assigned the faster phase in partial photolysis experiments to O₂ rebinding to native β subunits and the slower phase to O₂ rebinding to native α subunits. Philo and Lary (1990) made the same assignments simultaneously and independently. As a further check, we added pMB to HbAo and ααHb to test whether or not modifications of β Cys₂₉ would preferentially affect the fast, β phase. As shown in Table I, the mercural had no effect on the slow rate of O₂ rebinding and decreased the fast rate by 20-30%. In the absence of pMB, the rate of O₂ association for ααHb β subunits was the same, within experimental error, as that for HbAo β subunits, but the rate of O₂ rebinding to α subunits of ααHb was ~2.5-fold lower than that to native α chains.

Rates of O₂ rebinding to HbAo after complete photolysis (Fig. 4B) agree with rates determined when only 20% of the ligand molecules were photolyzed (Fig. 4A). For native oxy-hemoglobin at high O₂ concentration (1.25 mM), ligand rebinding occurs much more rapidly than the relaxation from the R to T quaternary conformation (Sawicki and Gibson, 1977a). Thus, both types of experiments give the same result. For ααHb, however, O₂ rebinding after 100% photolysis was measurably slower toward the end of the reaction than for rebinding after partial photolysis. This decrease in rate with increasing light intensity suggests that O₂ is rebinding to a mixture of T- and R-state species and may be a result of a more rapid conformational transition to the T-state by ααHb compared with HbAo (see below).

Kinetics of Carbon Monoxide Binding—Rates of CO dissociation were measured by displacement with NO (Table III). The effect of pMB was greater on the rates of the fast phase, and by analogy to the O₂ and n-butyl isocyanide results, this phase was assigned to the β subunits. The β CO dissociation...
FIG. 4. Normalized time courses for the recombination of 1.25 mM O2 with HbA (squares) and ααHb (circles) after photolysis by a 300-ns laser flash. The reactions were carried out at 25°C and pH 7.4 in bis-Tris, 0.1 M NaCl, 1 mM EDTA buffer. Protein concentrations were 100 μM. 1-mm path length cells were used, and the time courses were monitored at 436 nm. A, the flash was filter attenuated and produced 15–20% breakdown. The symbols show the fraction of deoxyhemoglobin remaining as a function of time after the flash. Time courses were normalized to the total absorbance change. The double lines through the symbols show the best fits to Equation 1 in which the amplitudes of the two phases were allowed to vary; 56% of the total amplitudes were attributed to the faster phases. Fitted rates were 1.8 × 105 s⁻¹ and 0.5 × 105 s⁻¹ for HbA, and 1.6 × 105 s⁻¹ and 0.21 × 105 s⁻¹ for ααHb. The fast and slow phases were assigned to the β and α subunits, respectively, for both HbA, and ααHb, and those rates are given in Table I.

B, the flash was at full intensity and produced 100% photolysis. The symbols show the fraction of deoxyhemoglobin remaining as a function of time after the flash. Time courses were normalized to the total absorbance change. The solid lines are simulations of the time courses by numerical integration of Scheme 1 by the Runge-Kutta procedure, using the parameters listed in Table III. The time courses were also fit to Equation 1 with fitted amplitudes for the two phases, which gave rates of 1.8 × 105 s⁻¹ and 0.5 × 105 s⁻¹ for HbA, and 1.6 × 105 s⁻¹ and 0.16 × 105 s⁻¹ for ααHb. Those fitted curves are not shown.

rate constant for ααHb was 2-fold greater than that for HbA. The α CO dissociation rate constants were equivalent for the two proteins. CO association rate constants for the R-state hemoglobins were measured after 2% photolysis. The time courses were fitted to Equation 1, and the fast and slow phases were assigned to the β and α subunits, respectively, based on the measurements of Mathews et al. (1989) using site-directed hemoglobin mutants. The CO equilibrium constants were calculated from the ratio of the kinetic constants (Lα = l'/l). CO association rate constants for both subunits of ααHb were 2-fold lower compared with HbA (Table II), and the CO affinities of the R-state ααHb subunits were 2-4-fold lower than those for the corresponding subunits of HbA.

The Rate of R to T Transition in αα-Cross-linked Hemoglobin—The unliganded heme groups in human deoxyhemoglobin exhibit a substantial absorbance change when the protein switches from the R- to the T-quaternary state. This change was first characterized as the Hb* to Hb transition by Gibson (1959) and later shown to occur when isolated subunits aggregate to form deoxyhemoglobin tetramers (Brunori et al., 1988). This R to T spectral transition can also be observed at 425 nm (the isosbestic point for ligand binding) immediately after complete photolysis of carbonmonoxyhemoglobin (Sawicki and Gibson, 1976; Hofrichter et al., 1983; Marden et al., 1986). At low concentrations of CO, the rate of ligand rebinding occurs on milliseconds time scales whereas the R to T transitions occur in 100–500 μs. Time courses for the R to T transitions of completely photolyzed carbonmonoxy-HbA and ααHb were measured at 425 nm and are shown in Fig. 5 (upper panel). Estimates of the rates of the R to T transitions (kR) were determined by fitting the absorbance changes at 425 nm as described by Sawicki and Gibson (1976). Under these conditions, kR for unliganded ααHb was 5-fold higher than that for HbA. The isosbestic point for the R to T transition is 436 nm. Measurements at this wavelength showed only a small degree of CO rebindong on the time scale used to
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The association (I') and dissociation (I) rate constants represent the kinetics for the last step in ligand binding to hemoglobin tetramers and are defined as R-state parameters for the α and β subunits. CO association rates were measured by conventional flash photolysis at 2% of maximum photolytic light intensity. Hemoglobin (10 μM in heparin) was equilibrated with either 0.1 mM or 0.2 mM CO, and the time courses were monitored at 435 nm. CO dissociation rates were determined directly from the observed rates of CO displacement by NO. Hemoglobin at either of two concentrations (10 or 100 μM in heparin) was equilibrated with 0.1 mM CO and mixed rapidly with 1 atmosphere of NO. The time courses were measured at 419 nm for the lower protein concentration and 580 nm for the higher protein concentration. The rates were determined by fitting the time courses to Equation 1. The errors were estimated at ±20% based on the results of Mathews et al. (1989). The equilibrium constant, Lα, was calculated as I'/I. Rate constants in parentheses were measured in the presence of pMB.

|                        | I'         | I          | I'(+pMB) | Lα        |
|------------------------|------------|------------|----------|-----------|
| HbA0(α)                | 5 ± 1      | 0.008 ± 0.002 | 0.012 ± 0.002 | 630       |
| HbA0(β)                | 0.011 ± 0.002 | 0.043 ± 0.009 | 1,360    |
| ααHb(α)                | 3 ± 1      | 0.010 ± 0.002 | 0.014 ± 0.003 | 300       |
| ααHb(β)                | 7 ± 1      | 0.025 ± 0.005 | 0.047 ± 0.009 | 280       |

measure the conformational change (Fig. 5, middle panel). The time courses observed at 436 nm are consistent with the measured rates of I' in Table II (i.e. HbA0 showed a slightly faster rate of CO rebinding compared with ααHb).

Since the R to T transition is faster and ligand rebinding is slower for ααHb, we calculated Δε (mM⁻¹ cm⁻¹) for the R to T transition of this protein from the fitted amplitudes of the absorbance changes measured 75 μs after full photolysis of 100 μM carbonmonoxo-ααHb at wavelengths from 400 to 460 nm (Fig. 5, lower panel). These results were compared with the difference spectrum between isolated deoxy α and β chains and native deoxymemoglobin tetramers (Brunori et al., 1968). The results reported by Sawicki and Gibson (1976) for the difference spectrum between R0 (ΔA measured at 2 μs after photolysis) and T0, showed similar values of Δε compared with those in Fig. 5. The difference spectrum for ααHb is consistent with both the earlier R versus T difference spectra reported for HbA0 but does not agree in absolute value of Δε, presumably because of the contribution of ligand binding to the spectral change (i.e. the middle panel in Fig. 5 shows a small degree of ligand rebinding to ααHb 75 μs after photolysis).

Simulations of O2 Rebinding after Full Photolysis—Analysis of the 100% photolysis time course for oxy-ααHb required consideration of the rates of binding to and formation of T-state species since the R to T transition occurs at a rate comparable to ligand binding at high O2 concentration. Simulations in Fig. 4B were calculated by numerical integration of the reactions in Scheme 1, which were taken from Sawicki and Gibson (1977a). Time-dependent concentration distributions of T- and R-state hemoglobins were evaluated. These simulations take into account αβ, and αα and ββ subunits in the case of HbA0, the fraction of αα dimer present.

Initial values for k0 in Scheme 1 were set to the fitted rates for the 425 nm time courses (Fig. 5, upper panel). Initial estimates for L were obtained by allosteric modeling of equilibrium data. The value of the conformational switching factor (d), which adjusts k0 for the number of bound ligands, was taken from Sawicki and Gibson (1977a). R-state O2 rate constants for the α and β subunits were taken from Table I. Initial estimates for T-state rate constants were taken from values for unmodified hemoglobin (Sawicki and Gibson, 1977b). The fraction of dimers for HbA0 was set to 15% using 3 μM as the dimer-tetramer dissociation constant (Chu and Ackers, 1981).

The final fitted parameters in Table III allowed a good representation of the data in Fig. 4B. The R-state dissociation
rates that were slightly less for $\alpha\alpha$Hb than for $\alpha$HbA, and
simulated T-state dissociation rates that were equal for the
two hemoglobins. However, these T-state rate constants are
not well defined, especially for $\alpha$HbA, because of the small
percentage of T-state species in the reactions. As expected
from the time courses in Fig. 5 (upper panel), the distributions
of T- and R-state species as a function of time were different
for the two proteins. Fig. 6 shows that during $O_2$ rebinding
after 100% photolysis, the amount of T-state species stayed
below 8% for $\alpha$HbA but rose very quickly to over 30% for
$\alpha\alpha$Hb. Simulations over longer time scales showed that $\alpha$HbA
was completely resaturated in 100 $\mu$s, with 92% of the oxy-
hemoglobin species in the R-state and 8% in the T-state.
Levels of saturated T- and R-state species reached their final
equilibrium values (i.e. $T_4 = 0.01\%$ and $R_4 = 99.99\%$) in 2 ms.$\alpha\alpha$Hb was completely resaturated in 240 ms, with 60% of the
oxyhemoglobin species in the R-state and 40% in the T-state;
equilibrium levels of $T_4$ (0.05\%) and $R_4$ (99.95\%) were
achieved in 3 ms.

**Equilibrium Binding**—Oxygen equilibrium curves for
$\alpha$HbA and $\alpha\alpha$Hb are shown in Fig. 7. $\alpha\alpha$Hb has a higher
$P_o$ and slightly reduced cooperativity (Table I). The upper
ends of the curves were normalized by extrapolating to the
final saturation at infinite $P_o$ from graphical analysis of
percent saturation ($Y$) versus $1/P_o$. Equilibrium binding
constants were evaluated by fitting the data to the Adair
equation (Equation 3).

Fitted values of $\alpha_1$ and $\alpha_2$ for $\alpha$HbA were sensitive to
normalization, and $K_c$ was difficult to determine precisely solely
from equilibrium binding data. Variation in the upper end of
the $HbA$ curve by as little as $-0.3%$ changed the fitted value
of $\alpha_1$ from $9 \times 10^{-4}$ to $1 \times 10^{-12}$ mm Hg$^{-1}$. Precise values of $a_3$
are required to calculate $K_c$, and with this variation in $a_3$, the
fitted value of $K_c$ changed from 7.65 to $10^{-12}$ mm Hg$^{-1}$. Thus,
it is clear that $K_c$ and $K_4$ are highly correlated for native
$HbA$. Analysis of the $\alpha\alpha$Hb equilibrium curve was less sen-
sitive to normalization. The same variation in the upper end
of the curve changed $a_1$ from $9 \times 10^{-3}$ to $4 \times 10^{-3}$ mm Hg$^{-1}$,
producing only a 2-fold variation in $K_c$ from 1.55 to 3.2 mm
Hg$^{-1}$. $K_c$ was assigned by using normalization factors that
gave the minimal residual sum of squares. The fitted values
of $K_c$ were 7.65 mm Hg$^{-1}$ and 1.55 mm Hg$^{-1}$ (4.7 $\times 10^{-1}$
and $0.9 \times 10^{5}$ M$^{-1}$) for $HbA$ and $\alpha\alpha$Hb, respectively, and
the residuals are shown in Fig. 8.

**Kinetic Determination of $K_c$**—$K_c$ can also be computed from
the ratio of association and dissociation rate constants ob-
tained in the partial photolysis and ligand displacement
experiments. In these kinetic experiments, differences between
the $\alpha$ and $\beta$ subunits were resolved, and the rate parameters
were assigned to ligand binding to the R-state quaternary
conformation. Equilibrium constants for $O_2$ binding to the
last unliganded $\alpha$ or $\beta$ subunit were computed from the ratios
$K_{\alpha\alpha} = k_{\alpha\alpha}/k_{\alpha\alpha}$, and $K_{\beta\beta} = k_{\beta\beta}/k_{\beta\beta}$. If the subunits are not
equivalent, the intrinsic value of $K_c$ for equilibrium binding
is defined as $K_c = 2K_{\alpha\beta}K_{\beta\alpha}/(K_{\alpha\alpha} + K_{\beta\beta})$. The
technically determined values of $K_c$ for $HbA$ and $\alpha\alpha$Hb were
(3.6 $\pm 1.0$) $\times 10^{4}$ M$^{-1}$ (5.9 mm Hg$^{-1}$) and (0.9 $\pm 0.4$) $\times 10^{4}$ M$^{-1}$ (1.5 mm
Hg$^{-1}$), respectively, and are similar to those obtained from
fitting the $O_2$ binding curves.

The equilibrium data in Fig. 7 were refitted to the Adair
equation (Equation 3).

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**Table III**

Final values in Scheme 1 for simulated time courses after 100%
photolysis of oxyhemoglobin in 50 mM bis Tris, 0.1 M NaCl, 1 mM
EDTA, pH 7.4, 25 °C

Initial estimates for $k_0$, the rate of transition in deoxyhemoglobin
conformation from the R- to the T-state, were determined from the
time courses at 425 nm (Fig. 5, upper panel). $L$ is the allosteric
constant that gives the ratio of deoxyhemoglobin in the T- and R-
states (i.e. $[T]/[R]$). $D$ is the conformational switching factor that
adjusts the rate of conformational change with the number of ligands
bound (Sawicki and Gibson, 1977a). Initial estimates for R-state $O_2$
association rate constants for the $\alpha$ ($k_{\alpha\alpha}$) and $\beta$ ($k_{\beta\beta}$) subunits were taken from the ratios of the partial photolysis time courses for oxy-
hemoglobin (Fig. 4A and Table I). R-state $O_2$ dissociation rate con-
stants for the $\alpha$ ($k_{\alpha\alpha}$) and $\beta$ ($k_{\beta\beta}$) subunits were taken from the fitted
dissociation rates from the $O_2$ displacement reactions (Table I). Initial
estimates of the T-state $O_2$ association ($k_{\alpha\alpha}$ and $k_{\beta\beta}$) and dissociation
($k_{\alpha\alpha}$ and $k_{\beta\beta}$) rate constants were taken from the literature (Sawicki
and Gibson, 1977b). $F$ is the fraction of dimers in solution.

| $HbA$ | $\alpha\alpha$Hb |
|-------|----------------|
| $k_0$ (s$^{-1}$) | 13,000, 75,000 |
| $L$ | 125,000, 34,000 |
| $d$ | 10, 10 |
| $k_{\alpha\alpha}$ (M$^{-1}$ s$^{-1}$) | 42, 17 |
| $k_{\beta\beta}$ (M$^{-1}$ s$^{-1}$) | 140, 140 |
| $k_{\alpha\alpha}$ (s$^{-1}$) | 12, 28 |
| $k_{\beta\beta}$ (s$^{-1}$) | 40, 70 |
| $k_{\alpha\alpha}$ (M$^{-1}$ s$^{-1}$) | 2, 1 |
| $k_{\beta\beta}$ (M$^{-1}$ s$^{-1}$) | 40, 30 |
| $k_{\alpha\alpha}$ (s$^{-1}$) | 100, 100 |
| $k_{\beta\beta}$ (s$^{-1}$) | 3,000, 3,000 |
| $K_{\alpha\alpha}$ (M) | 100, 100 |
| $F$ (%) | 15, 0 |
| $O_2$ (M) | 1,250, 1,250 |

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FIG. 6. Simulated time courses for the formation and disappearance of individual species during oxygen rebinding to $A$, $\text{HbA}_0$, and $B$, $\alpha\alpha\text{Hb}$ after 100% laser photolysis. The concentrations of R- and T-state species were calculated by numerical integration of Scheme 1 with the parameters in Table III and are plotted as percent of total hemoglobin. These populations correspond to the simulated time courses in Fig. 4B. Solid lines represent the population of deoxy R-state species as a function of time; double lines represent the oxy R-state species; dotted lines are deoxy T-state species; and dashed lines are oxy T-state species.

Discussion

Structural Effects of α Lys$^9$ Cross-linking—Resonance Raman and absorption spectroscopy of transient photoproducts suggest that the tertiary conformation of ααHb R-state subunits are more "T-state like" (Larsen et al., 1990). These data suggest that the cross-link partially inhibits in-plane movement of the α iron atom even in the R quaternary conformation and also exerts proximal constraints on the β heme which are not as readily interpreted. These structural changes are manifested as 2-6-fold decreases in $K_{\text{R}}$ and $K_{\text{T}}$ when comparing ααHb with HbA0 (Tables I and II).

An alternative interpretation is that the cross-link increases the value of $L$, the allosteric conformational equilibrium constant, inhibiting the quaternary transition until three or four ligands are bound. This would cause a decrease in the apparent equilibrium constant for the binding of the fourth ligand molecule. However, this explanation is ruled out by our previous equilibrium binding studies, which have shown that $L$ for the cross-linked hemoglobin is smaller than that for HbA0 and that the switch from T- to R-states occurs after 2.3 ligands...
Ligand Binding to R-state αα-Cross-linked Hemoglobin

Oxygen Equilibrium Curves

**FIG. 7.** Oxygen equilibrium curves of HbAo and ααHb. Conditions were pH 7.4 and 25 °C in 50 mM bis-Tris, 0.1 mM NaCl, and 1 mM EDTA buffer in the presence of the methemoglobin reductase system of Hayashi *et al.* (1973). Protein concentrations were 100 μM. Squares represent HbAo, and circles represent ααHb. The inset shows a Hill plot of the data, where the abscissas are log \( P_{O_2} \) and the ordinates are \( \log(Y/1-Y) \). Solid lines are fitted curves to the data by the Adair equation (Equation 3) in which \( a_4 \) was unconstrained. The fitted values of the Adair constants for HbAo are \( a_1 = 0.135 \pm 0.003 \) mm Hg, \( a_2 = 9.04 \times 10^{-3} \pm 7.0 \times 10^{-4} \) mm Hg\(^2\), \( a_3 = 8.95 \times 10^{-4} \pm 7.7 \times 10^{-5} \) mm Hg\(^3\), \( a_4 = 8.58 \times 10^{-4} \pm 7.7 \times 10^{-5} \) mm Hg\(^4\); for ααHb \( a_1 = 0.0795 \pm 0.0011 \) mm Hg, \( a_2 = 2.70 \times 10^{-3} \pm 9.4 \times 10^{-5} \) mm Hg\(^2\), \( a_3 = 9.03 \times 10^{-5} \pm 4.4 \times 10^{-6} \) mm Hg\(^3\), \( a_4 = 3.49 \times 10^{-4} \pm 1.2 \times 10^{-5} \) mm Hg\(^4\).

have been bound (Vandegriff *et al.*, 1989). The ααHb \( O_2 \) binding data were poorly fitted when \( L \) was set to values equal to or greater than \( L \) for HbAo. \( L \) was better determined for both ααHb and HbAo when \( K_R \) was constrained by the kinetic measurement of \( K_r \). Thus, it is clear that the cross-link lowers \( O_2 \) affinity by affecting the intrinsic ligand binding properties of the R-state subunits rather than by increasing the allosteric constant.

**Kinetic Differences between the Subunits in αα-Cross-linked Hemoglobin**—The association rate constants for \( O_2 \) and CO binding to the α subunits in ααHb are 2–3-fold smaller than those for α subunits in HbAo (Tables I and II). The altered configuration of the proximal heme pocket in ααHb places the R-state iron atom further out of the plane of the heme ring (Larsen *et al.*, 1990), which could account for the decreased reactivity with ligands. Since there is little difference in the position of the E helix in the R- and T-states of native α chains (Baldwin and Chothia, 1979; Shaanan, 1983), the decrease in \( k_{so} \) is not likely to be caused by distal steric interactions. The \( O_2 \) dissociation rate constant from R-state α subunits is increased 2-fold by the cross-link, presumably because of the added proximal strain that inhibits association although distal hydrogen bonding effects cannot be ruled out. The net result of these rate changes is a 6-fold lowering of \( K_R \), for \( O_2 \) binding.

In the case of R-state β subunits, cross-linking causes no changes in \( k_{bd} \) for \( O_2 \) binding, a 2-fold increase in \( k_{bd} \), and as a result, only a 50% decrease in \( O_2 \) affinity. In contrast, the rate of CO rebinding to β subunits in ααHb is decreased compared with HbAo, and the rate of CO dissociation increases 2-fold. Thus, CO affinity decreases roughly 4-fold, and discrimination against CO in favor of \( O_2 \) binding is increased in β subunits by the chemical cross-link.

**R to T Transition in αα-Cross-linked Hemoglobin**—The slower rate of \( O_2 \) rebinding after 100% photolysis of ααHb is the result of both a lower rate of \( O_2 \) binding to R-state α chains and a faster transition from the R to the T quaternary conformation. Simulations of time courses in Fig. 4B by numerical integration of Scheme 1 revealed higher levels of T-state intermediates during \( O_2 \) rebinding to ααHb (Fig. 6). Rates of \( O_2 \) association with R-state ααHb were underestimated by ~20% when transition to the T-state was unac-
counted for. Only small amounts of T-state intermediates were formed during the O₂ rebinding reaction after 100% photolysis of HbAo.

The rates of the R- to T-state transition were also measured directly at 425 nm after complete photolysis of the CO complexes of HbAo and ααHb. The observed values of $k_0$ were 1.3 $\times$ 10⁹ and 7.3 $\times$ 10⁹ s⁻¹ for HbAo and ααHb, respectively. The rate for HbAo is similar to that reported by Sawicki and Gibson (1976, 1977a), and that for ααHb is nearly identical to $k_0$ obtained by analyzing O₂ rebinding as shown in Figs. 4B and 6. Thus, it is clear that the α Lys⁹⁸ cross-link significantly enhances the rate of switching from the R- to the T-state as might be expected if it stabilizes the subunits in intermediate tertiary conformations.

The Advantage of Determining $K_4$ Kinetically—Finally, the kinetic data for O₂ binding and dissociation were used to compute the Adair equilibrium constants for the last step in ligand binding to HbAo and ααHb. The resultant values of $K_4$ were very similar to those obtained by curve fitting of O₂ equilibrium binding data. The kinetically determined value for $K_4$ was used to reduce the number of fitted Adair constants from four to three, and the equilibrium curves were reanalyzed. Fixing $K_4$ had no effect on the size or distribution of the residuals of the fit; however, the confidence limits of $K_3$ were markedly improved. These data show unambiguously that $K_4$ can be determined independently from kinetic measurements and then used to analyze equilibrium data. This procedure prevents $\alpha_3$ from becoming zero or negative when the equilibrium curve is analyzed, avoiding the necessity of obtaining parameter values that are chemically unacceptable.

REFERENCES

Adair, G. S. (1925) J. Biol. Chem. 63, 529-545
Baldwin, J., and Chothia, C. (1979) J. Mol. Biol. 129, 175-220
Brunori, M., Antonini, E., Wyman, J., and Anderson, S. R. (1968) J. Mol. Biol. 34, 357-359
Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, P. H., Arnone, A., and Walder, J. A. (1986) J. Biol. Chem. 261, 9929-9937
Christensen, S. M., Medina, F., Winslow, R. M., Snell, S. M., Zegna, A., and Marini, M. A. (1988) J. Biochem. Biophys. Methods 17, 143-154
Chu, A. H., and Ackers, G. K. (1981) *J. Biol. Chem.* **256**, 1199–1205
Di Cera, E., Robert, C. H., and Gill, S. J. (1987) *Biochemistry* **26**, 4003–4008
Gibson, Q. H. (1959) *Biochem. J.* **71**, 283–303
Gibson, Q. H., and Edelstein, S. J. (1987) *J. Biol. Chem.* **262**, 516–519
Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., and Robert, C. H. (1987) *Biochemistry* **26**, 3995–4002
Hayashi, A., Suzuki, T., and Shin, M. (1973) *Biochim. Biophys. Acta* **310**, 309–316
Hofrichter, J., Sommer, J. H., Henry, E. R., and Eaton, W. A. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 2235–2239
Imai, K. (1981) *Methods Enzymol.* **76**, 438–449
Johnson, M. L., and Ackers, G. K. (1977) *Biophys. Chem.* **7**, 77–80
Larsen, R. W., Chavez, M. D., Ondrias, M. R., Courtney, S. H., Friedman, J. M., Lin, M. J., and Hirsch, R. E. (1990) *J. Biol. Chem.* **265**, 4449–4454
Marden, M. C., Hazard, E. S., and Gibson, Q. H. (1986) *Biochemistry* **25**, 7591–7596
Marden, M. C., Kister, J., Poyart, C., and Edelstein, S. J. (1989) *J. Mol. Biol.* **208**, 341–345
Mathews, A. J., Rohlfis, R. J., Olson, J. S., Tame, J., Renaud, J.-P., and Nagai, K. (1989) *J. Biol. Chem.* **264**, 16573–16583
Mills, F. C., Johnson, M. L., and Ackers, G. K. (1976) *Biochemistry* **15**, 5350–5362
Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
Morris, R. J., Gibson, Q. H., Ikeda-Saito, M., and Yonetani, T. (1984) *J. Biol. Chem.* **259**, 6701–6703
Olson, J. S. (1981a) *Methods Enzymol.* **76**, 631–651
Olson, J. S. (1981b) *Methods Enzymol.* **76**, 652–667
Olson, J. S., and Gibson, Q. H. (1971) *J. Biol. Chem.* **246**, 5241–5253
Olson, J. S., Andersen, M. E., and Gibson, Q. H. (1971) *J. Biol. Chem.* **246**, 5919–5923
Philo, J. S., and Lary, J. W. (1990) *J. Biol. Chem.* **265**, 139–143
Sawicki, C. A., and Gibson, Q. H. (1978) *J. Biol. Chem.* **251**, 1533–1542
Sawicki, C. A., and Gibson, Q. H. (1977a) *J. Biol. Chem.* **252**, 5783–5788
Sawicki, C. A., and Gibson, Q. H. (1977b) *J. Biol. Chem.* **252**, 7538–7547
Shaanan, B. (1983) *J. Mol. Biol.* **171**, 31–59
Vandegriff, K. D., Medina, F., Marini, M. A., and Winslow, R. M. (1989) *J. Biol. Chem.* **264**, 17824–17833
Vandegriff, K. D., Benazzi, L., Ripamonti, M., Perrella, M., Le Tellier, Y. C., Zegna, A., and Winslow, R. M. (1991) *J. Biol. Chem.* **266**, 2697–2700
Winslow, R. M., Swenberg, M.-L., Berger, R. L., Shrager, R. I., Luzzana, M., Samaja, M., and Rossi-Bernardi, L. (1977) *J. Biol. Chem.* **252**, 2331–2337