The Kinetics of Assembly of Normal and Variant Human Oxyhemoglobin*

(Received for publication, July 21, 1986)

Melisenda J. McDonald‡, Susan M. Turci, Nadir T. Mrabet, Bruce P. Himelstein§, and H. Franklin Bunn¶

From the Hematology Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

The kinetics of assembly have been monitored spectrophotometrically for normal and variant human oxyhemoglobins in 0.1 M Tris, 0.1 M NaCl, 1 mM Na₂EDTA, pH 7.4, at 21.5 °C. Oxyhemoglobin versus oxy chain static difference spectra were performed and revealed subtle but significant absorption changes in both the visible and Soret regions. Kinetic experiments were performed by rapidly mixing equivalent (in heme) concentrations of $\alpha$ and $\beta^A$ chains and following the change in absorbance at 583 nm with time. A protein concentration range of 10–100 μM in heme prior to mixing, these time courses were homogeneous and followed first-order kinetics, yielding a value of 0.069 s⁻¹ for the apparent rate constant of dissociation of oxygenated $\beta^A$ chain tetramers. Under these conditions, the overall assembly of oxyhemoglobins S ($\beta^Glu→Val$) and N-Baltimore ($\beta^5Lys→Glu$) were also governed by the rates of dissociation of their respective oxygenated $\beta^A$ and $\beta^N-Baltimore$ chain tetramers with the apparent first-order rate constants of 0.044 and 0.15 s⁻¹, respectively. In the Soret region, the $\alpha\beta$ monomer combination reaction could be observed if the protein concentration (heme basis) was lowered and if protein nonequivalence ($\beta$ chain exceeded $\alpha$ chain concentration) mixing experiments were performed. A kinetic oxyhemoglobin A, oxy-α, oxy-$\beta^A$ monomer difference spectrum could be generated, and simple second-order kinetics were observed (415 nm) yielding rate constants of 2.3, 3.3, and $4.8 \times 10^5$ M⁻¹ s⁻¹ for the assembly of oxyhemoglobins S, A, and N-Baltimore, respectively. To our knowledge, this is the first kinetic study to reveal significant differences between the rate of association of $\alpha$ and $\beta$ monomers of hemoglobin A and those of two distinctly charged hemoglobin variants.

The kinetics of subunit assembly of normal adult hemoglobin (Hb A) were first investigated 2 decades ago by Antonini et al. (1). They monitored deoxyhemoglobin tetramer assembly spectrophotometrically in a stopped flow apparatus by exploiting the known spectral differences between deoxygenated subunits and intact deoxyhemoglobin. Subsequent spectrophotometric (2) and circular dichroism (3) studies have revealed three sequential processes in the reaction mechanism of assembly. A first-order dissociation of oligomeric heme-containing subunits into $\alpha$ and $\beta$ monomeric precursors must take place before two consecutive second-order processes can occur. The $\alpha$ and $\beta$ monomers combine to form $\alpha\beta$ dimers which then self-associate into intact $\alpha\beta$ hemoglobin tetramers. Experimental variables such as pH, phosphate, and protein concentration were shown to alter the kinetic profile of deoxyhemoglobin assembly and to dictate whether the rate-determining step is a first- or second-order process.

Since isolated $\alpha$- and $\beta$-heme-containing subunits possess a high affinity for ligand, it is the oxygenated form of the hemoglobin subunit which is almost certainly assembled into tetramers in vivo. The assembly of liganded Hb A has also been studied and has been found to follow the identical reaction sequence (and apparent dependence on experimental variables) seen for deoxyhemoglobin. Oxyhemoglobin assembly has been monitored by pH changes (4), time-resolved small-angle X-ray scattering techniques (5), and circular dichroism measurements (6,7), as well as visible absorbance measurements (8–10).

These visible spectrometry studies, which have been conducted in our laboratory, include the investigation of variant as well as normal oxyhemoglobin assembly. The kinetics of reconstitution of several human hemoglobins were examined to test the hypothesis that macromolecular assembly may be a post-translational determinant of hemoglobin phenotype. Our previous investigations were aimed at defining the first-order process of oxyhemoglobin assembly and were, therefore, conducted under experimental conditions which promote the stability of the oligomeric structure of liganded $\beta$ chain subunits. In this report, we extend these studies to include evaluation of the $\alpha$ and $\beta$ monomer combination process.

EXPERIMENTAL PROCEDURES

Preparation of Human Hemoglobins and Subunits—Erythrocytes from normal donors or from individuals with hemoglobinopathies were washed three times with 0.15 M NaCl and either used immediately or frozen in liquid nitrogen until needed. The washed red blood cells were lysed in distilled water, and the subunits of human Hb A, Hb S, and Hb N-Baltimore were prepared from these hemolysates by appropriate modifications of the method of Bucci and Fronticelli (11) as described in detail elsewhere (12). Both hemoglobins and subunits were freed of phosphate (8) prior to dialysis against the standard experimental buffer (0.1 M Tris, 0.1 M NaCl, 1 mM Na₂EDTA, pH 7.4, at 21.5 °C).

Static Difference Spectrophotometry—Static visible and Soret difference spectra of intact oxyhemoglobin versus its constituent oxy-
genated α and β subunits were generated using a Cary 2200 recording spectrophotometer in the auto-gain mode with a spectral bandwidth of 1.0 nm. Protein solutions of oxyhemoglobin and subunits were made equivalent in the 576 (or 415) nm region of the spectrum just prior to use, and an ε value of 15.37 (or 131) mM" cm" was employed to calculate protein concentration on a heme basis. Rectangular tandem-mixing cuvettes containing the appropriate oxygenated α chain, oxygenated β chain, and oxyhemoglobin A solutions were placed in a temperature-regulated compartment at 21.5 °C. The resulting spectra were recorded at the absorbance range and scan speed noted under "Results."

Spectrophotometric Kinetic Studies—Spectral changes accompanying formation of oxyhemoglobin from its oxygenated α and β subunits were monitored as a function of time in a Gibson-Durrum stopped flow device temperature-regulated at 21.5 °C. Multiple analyses were carried out on several preparations. Data collection was performed by the microcomputer-based OLIS Spectrophotometer (On-Line Instruments Systems, Jefferson, GA). Absolute static spectra were taken immediately prior to and following each kinetic experiment to confirm protein concentration (on a heme basis) and nativity of the isolated ferrous subunits. Complete recombination of the oxygenated subunits was independently verified by electrophoresis and functional criteria as previously described (8, 13).

Analysis of Spectrophotometric Data—The overall assembly of oxyhemoglobin from its constituent oxygenated α and β subunits in vitro can be described by the following reaction sequence:1

\[ α_2(O_2) \leftrightarrow 2α(O_2) \] (Ia)
\[ β_2(O_2) \leftrightarrow 2β_2(O_2) \] (IIa)
\[ α_2(O_2) + β_2(O_2) \leftrightarrow α_2β_2(O_2) \] (III)

where
\[ K_1(α) = \frac{[α_2(O_2)]^2}{[α][αO_2]} = k_4(α) \]
\[ K_1(β) = \frac{[β_2(O_2)]^2}{[β][βO_2]} = k_{10}(β) \]

and where
\[ K_2 = \frac{[β_2(O_2)]^2}{[β][βO_2]} = k_{10}(β) \] (Ib)

and
\[ K_3 = \frac{[α_2β_2(O_2)]^2}{[α][β][βO_2]} = k_{22} \] (2)

with \( K \) being the equilibrium dissociation constant and \( k' \) and \( k \) being the combination and dissociation reaction rate constants, respectively.

Under our standard experimental conditions, the values for the equilibrium dissociation constants for \( K_1(α) \), \( K_1(β) \), and \( K_2 \) have been determined and are \( 1.2 \times 10^{-6} \) M, \( 7.1 \times 10^{-7} \) M, and \( 3.0 \times 10^{-8} \) M, respectively (14, 15). The value of \( K_3 \) has recently been estimated at \( 3 \times 10^{-13} \) M (16, 17). These equilibrium values were used to determine the amount of each intermediate species of subunit assembly present at a given protein concentration.

Previous reports (8, 18) have demonstrated that no visible spectral changes are associated with either oxygenated α chain (Reaction Ia) or oxygenated αβ dimer (Reaction III) oligomerization steps. This information in conjunction with known concentrations of intermediates species has allowed us to define precisely two kinetic constants,2 \( k_1(β) \) and \( k_2 \).

At concentrations where significant oligomeric β chain exists, the rate of dissociation of these oligomers into monomers (\( k_1(β) \)) was limiting and the kinetics were first-order. An integrated rate equation of the form, \( \log(ΔA/ΔA_0) = -(1/2.303)k_1(β)t \), was used to analyze the spectral data (see "Results").

At low protein concentrations, the αβ monomer combination reaction (\( k_2 \)) was rate-limiting, and a second-order kinetic profile was observed. These kinetic time courses were analyzed by an integrated rate equation of the form, \( 1/ΔA = (1/ΔA_0)k_2 + 1/ΔA_0t \), where \( ΔC_r = fC_r/100 \) = percent β monomer derived from \( K_1(β) \), and \( C_r \) = heme concentration after mixing (see "Results"). All data were analyzed by the method of least squares, and standard deviations have been presented.

RESULTS

Visible and Soret Static Difference Spectra—There exist small but significant spectral differences between intact oxyhemoglobin and the oxygenated derivatives of α and β chains in both the Soret and visible regions of the spectrum (19, 20). Fig. 1 displays the results of static difference spectroscopy of oxyhemoglobin A versus its constituent oxygenated subunits under experimental conditions (0.1 M Tris, 0.1 M NaCl, 1 mM Na₂EDTA, pH 7.4, at 21.5 °C) identical to those of kinetic experiments described below. At protein concentrations of 100 and 10 μM (in heme), the visible spectra for oxyhemoglobin A versus oxy-α subunits, versus oxy-β subunits, and versus oxy-α,oxy-βA subunits are displayed in Fig. 1 (A and B). The shape and magnitude of the oxyhemoglobin A versus oxy-α subunit are unaltered between 10 and 100 μM (in heme) consistent with previous findings which demonstrated an invariance in the spectrum of oxygenated α chains, as well as that of oxyhemoglobin A with protein concentration (8, 18).

In contrast, Philo et al. (18) demonstrated concentration-dependent changes in the spectrum of oxygenated β subunits, and this is readily seen in the oxyhemoglobin A versus oxy-β chain difference spectra and hence, the oxyhemoglobin A versus oxy-α,oxy-β subunit difference spectra shown in Fig. 1 (A and B) at 100 and 10 μM (in heme). A Δε value of 0.18 mm" cm" was seen at 582 and 579 nm for oxyhemoglobin A versus oxy-α,oxy-β subunit difference spectra at 100 and 10 μM (in heme), respectively.

The Soret spectra for oxyhemoglobin A versus oxy-α subunits, versus oxy-β subunits, and versus oxy-α,oxy-β subunits are displayed in Fig. 1 (C and D) for 10 and 1.0 μM (in heme). In this region of the spectrum, a 10-fold dilution again appears to alter the spectrum of the oxygenated β subunits, and the oxyhemoglobin A versus oxy-α,oxy-β subunit difference spectrum is different at 10 and 1.0 μM (in heme), respectively. A Δε value of 2.5 mm" cm" was observed at 421 and 418 nm for oxyhemoglobin A versus oxy-α,oxy-β subunit difference spectra at 10 and 1.0 μM (in heme), respectively.

The optical difference spectra presented in Fig. 1 are by no means unique. Knowles et al. (21) showed that a difference spectrum of this type could arise from any number of alterations of structure or solvent conditions which result in a shift in the position of absorption bands. This being the case, it is imperative that difference spectra be generated for the exact experimental conditions utilized in the kinetic experiments.

2 The treatment of β chain tetramer kinetics may be simplified by assuming an irreversible dissociation into monomers in the presence of α chains (\( β_4 \rightarrow 2β_2 \rightarrow 4β \)). Then by using steady state approximation with respect to the β dimer intermediate (i.e. if \( k_1 >> k_{10} \) and \( k_2 \)), the total reaction may be treated as \( β_4 \rightarrow 4β \). This value of \( k_1 \) is the first-order rate constant apparently measured in this report and will be referred to in the text as \( k_1(β) \).
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presented here.

... of oxygenated Tris, the rate-limiting step in the overall assembly of oxyhemoglobin. As demonstrated below, this is indeed the case for the studies presented here.

**β Tetramer Dissociation Reaction**—We have previously shown (8-10) that under appropriate experimental conditions, the dissociation of the oxygenated non-α chain tetramer is the rate-limiting step in the overall assembly of oxyhemoglobin. Here we investigate the assembly of oxyhemoglobins S, A, and N-Baltimore under experimental conditions of 0.1 M Tris, 0.1 M NaCl, 1 mM Na₂EDTA, pH 7.4, at 21.5 °C and find that over a protein concentration range of 25–110 μM in hemoglobin prior to mixing, the assembly profile follows first-order kinetics (see Fig. 2). Semilogarithmic plots of time courses following the mixing of equivalent concentrations (on a heme basis) of oxygenated α and β^α chains are shown in Fig. 2B. These time courses are homogeneous and followed first-order kinetics, yielding a value of 0.069 (±0.0073) s⁻¹ for the apparent first-order rate constant of dissociation of oxygenated β chain tetrarmers of Hb A (k₁(β^α)). This value agrees remarkably well with that of Kawamura and Nakamura (7) who reported a value of 0.064 (±0.0072) s⁻¹ for the oxygenated β^α chain tetramer dissociation rate under identical experimental conditions using a circular dichroism stopped flow technique (see “Discussion”). In addition, the absorbance changes (Fig. 2B) accompanying the assembly of oxyhemoglobin A from its constituent oxygenated subunits correlate well with the static difference spectrophotometry experiments presented above (see Fig. 1A). Both the static and kinetic spectral measurements yield a millimolar difference extinction coefficient: value (Δε) of 0.18 mM⁻¹ cm⁻¹ at 583 nm.

The overall assembly of oxyhemoglobins S and N-Baltimore (Fig. 2, A and C) is also governed by the rates of dissociation of their respective oxygenated β^α and β^N-Baltimore chain tetrarmers. The apparent first-order rate constants k₁(β^α) and k₁(β^N-Baltimore) were 0.044 (±0.0069) and 0.15 (±0.032) s⁻¹, respectively. Thus, under conditions where the β tetramer dissociation reaction is rate-limiting, the assembly of oxyhemoglobin S precedes 1.6-fold slower than normal oxyhemoglobin A assembly and the reconstitution of oxyhemoglobin N-Baltimore is 2.2-fold more rapid than oxyhemoglobin A assembly. These ratios for β tetramer dissociation rates are similar to those reported previously (10) under experimental conditions of 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C.

In an effort to monitor a subunit assembly reaction which was subsequent to the β tetramer dissociation reaction, namely the second-order monomer combination step, we focused our attention on mixing experiments which could be conducted in the Soret region at protein concentrations ≤10 μM in heme prior to mixing. Normalized semilogarithmic plots of time courses following mixing of equivalent concentrations (10 μM in heme) of α and β^α chains at 583 nm and two distinct Soret wavelengths at 415 and 422 nm are displayed in Fig. 3A. These two Soret wavelengths were chosen because Philo et al. (18) reported that 415 nm is isosbestic and 422 nm is maximum for the β tetramer to monomer spectral change. All three plots yield k₂(β^α) values of 0.069 s⁻¹ identical to those shown in Fig. 2B. Under our experimental conditions at this protein concentration, the β tetramer reaction is still rate-limiting for the overall assembly of Hb A.

**α,β Monomer Combination Reaction**—This subunit assem-
experiment could be attempted. Under the present experimental conditions, the concentration of α chain monomer exceeds that of β chain monomer, and a deviation from simple second-order kinetics would be expected. This is indeed the case, as seen in Fig. 5A. As the time courses develop, the fitted and experimental curves diverge. Exclusive of curve 1, the error introduced by the presence of a possible slow phase (first-order β tetramer reaction) is approximately 10%. This error is very tolerable and within experimental range for the absorbance changes seen in these experiments. In the case of curve 1, a greater error, nearly 20%, is possible. (Nevertheless, a consistent difference (see Fig. 5B) is seen between assembly rates of Hb A, Hb S, and Hb N-Baltimore.) The time courses and second-order rate plots reveal a second-order rate constant (within experimental error) identical to that calculated in Fig. 4.

The overall rates of assembly of oxyhemoglobins S and N-Baltimore (Fig. 5B) are also governed by the rates of combination of oxygenated α chain monomers with oxygenated β chain monomers and N-Baltimore chain monomers, respectively. The apparent second-order rate constants $k_{\text{f}}(\text{Hb S})$ and $k_{\text{f}}(\text{Hb N-Baltimore})$ were 2.3 (±0.13) and 4.8 (±0.30) × 10^8 M\(^{-1}\) s\(^{-1}\), respectively. The reconstitution of oxyhemoglobin S proceeds 1.4-fold slower than that of normal oxyhemoglobin A, and the assembly of oxyhemoglobin N-Baltimore is 1.5-fold more rapid than oxyhemoglobin A assembly. These findings constitute the first kinetic measurements of variant human hemoglobin assembly and are discussed in detail below.

**DISCUSSION**

Of prime importance is the origin of the spectral change accompanying hemoglobin assembly. The difference spectrum for oxyhemoglobin A and its constituent chains is distinct from that reported for deoxyhemoglobin A and its constituent subunits. The reconstitution of unliganded hemoglobin yields color changes that are 2-fold greater in the visible and at least 5-fold larger in the Soret region of the spectrum than the corresponding changes accompanying liganded hemoglobin formation (20). This larger color change is what prompted earlier studies of assembly of deoxyhemoglobin subunits by Antonini et al. (1). McGovern et al. (2), upon reinvestigation of the assembly process, found that the majority of absorbance change following mixing of unliganded subunits was associated with the αβ dimer formation rather than tetramer aggregation step.

In direct contrast to that seen for the assembly of deoxyhemoglobin, no absorbance change is associated with the αβ dimer aggregation step of liganded hemoglobin formation. Both this laboratory and that of Philo et al. (18) reported an invariance in the spectrum of liganded Hb A with concentration. This means that the formation of liganded hemoglobin involves two and only two possible color-producing reactions, one of which is first-order (β tetramer dissociation) and the other of which is second-order (α and β monomer combination).

Our results indicate that the overall color change in both kinetic instances is attributed to a change in the oxygenated β\(^a\) chain tetramer with concentration.

A concentration-dependent spectral change for oxygenated β\(^a\) chains in the standard buffer system employed here has been previously demonstrated by Philo et al. (18). These workers reported a red-shifted spectrum for the β\(^a\) tetramer to β\(^a\) monomer reaction. We have found a 3-nm red shift for the oxyhemoglobin A versus oxygenated β\(^a\) spectrum and a corresponding 3-nm blue shift for the oxyhemoglobin A versus oxygenated αβ chain spectrum upon protein dilution. Furthermore, the spectra depicted in Fig. 1 (A and B) are quali-

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**Fig. 3. Rate plots of oxyhemoglobin A assembly.** A, normalized semilogarithmic plots of time courses followed at 583 nm (O), 415 nm (■), and 422 nm (X) for [α] = [β\(^a\)] = 10 \(\mu\)M in heme before mixing (ΔAA\(_t\)) (1 cm) values were 0.0012, 0.0120, and 0.0147, respectively. A 1-mm slit was used, and a 3-nm dispersion was expected. A $k_1(\beta\(^a\))$ value of 0.069 s\(^{-1}\) was obtained. B, second-order reciprocal absorbance plots of time courses followed at 415 nm [α] = 5 and [β\(^a\)] = 10 \(\mu\)M in heme before mixing (ΔAA\(_t\)) (1 cm) value of 0.0120 was obtained. A value of $k_1(\beta\(^a\))$ = 3.3 × 10^8 M\(^{-1}\) s\(^{-1}\) was seen. B displays two independent kinetic experiments performed on separate days. Finally, a rapid phase corresponding to monomer combination is not seen in the time courses depicted in A. Presumably, this is due to the loss of this phase in the "dead time" of the stopped flow experiment.

**Fig. 4. Time courses and kinetic difference spectrum for oxy-α and oxy-β monomer combination.** Protein concentration is identical to that of Fig. 3B. A, time courses at five distinct Soret wavelengths. Each curve and a value is fitted as described under "Experimental Procedures." All time courses yield $k_1$ values of 3.3 × 10^8 M\(^{-1}\) s\(^{-1}\). B, a Soret kinetic difference spectrum derived from 21 independent time courses.

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Fig. 5. Time courses and rate plots of assembly of normal and variant oxyhemoglobins. Each curve is fitted as described under "Experimental Procedures." A, time courses and second-order plots for oxyhemoglobin A assembly at five distinct protein concentrations (no greater than 10% deviation was seen between repetitive determinations of chain concentrations): curve 1, [α] = 5 and [β] = 10 μM in heme before mixing (C_T = 1.8 μM); curve 2, [α] = 4 and [β] = 8 μM in heme before mixing (C_T = 1.6 μM); curve 3, [α] = 3 and [β] = 7 μM in heme before mixing (C_T = 1.4 μM); curve 4, [α] = 2 and [β] = 5.5 μM in heme before mixing (C_T = 1 μM); curve 5, [α] = 1 and [β] = 1.0 μM in heme before mixing (C_T = 0.5 μM). B, time courses and second-order plots of oxyhemoglobin S (△) and oxyhemoglobin N-Baltimore (■) assembly. Protein concentrations correspond to those in A. A solid line in the reciprocal plots represents the oxyhemoglobin A control. Values of C_T were calculated (as described under "Experimental Procedures") from a previously reported equilibrium value (10) for β^3 and estimated for β^2, β^4, and β^5 chains by monitoring pH changes accompanying tetramer formation. They observed a first-order process at high heme concentrations (>170 μM) which they attributed to a rate-limiting dissociation of liganded β^3 chain tetramers (see Ref. 8) and a second-order process at lower concentrations which yielded rates similar to those of deoxy subunits (5 × 10^6 M^-1 s^-1). This novel method of monitoring recombination kinetics required the absence of buffers and was carried out in 0.1 M KCl at 25°C. Further studies of the effect of pH, salts, and ionic strength on the assembly of oxyhemoglobin may explain this less than 2-fold difference between our value of k; and that of Rollema et al. (4).

Incki et al. (5) studied the reconstitution of liganded hemoglobin from isolated human carbon monox-α and β^4 chains by time-recorded small-angle x-ray scattering techniques. They found that at pH 7.4 in 0.1 M potassium phosphate buffers and a protein concentration of 2 mM (in heme), the overall kinetic profile of assembly followed first-order kinetics. They attributed this process to the dissociation of the β^4 tetramer to monomer. This agrees with previous findings (6, 8, 9) which demonstrate that at high protein concentrations the β^4 tetramer dissociation reaction can be rate-limiting in the assembly of hemoglobin.

Kawamura and Nakamura (6, 7) have investigated the kinetics of assembly of oxyhemoglobin A from isolated α and β^4 chains by the use of a circular dichroism stopped flow apparatus. They demonstrated, following mixing of equivalent
concentrations (heme basis) of α and βA chains, that the time
courses of the circular dichroism change in the Soret region
exhibited both a rapid and slow phase. In 0.1 mol potassium
phosphate buffer, pH 7.5, at 25 °C (6), this slow phase was a
first-order reaction with a rate constant of 0.0026 s⁻¹ and was
attributed to the dissociation of the βA chain tetramers. The
rapid phase assigned to the combination of oxy-α and β
monomers to dimers yielded a second-order rate constant of
7.5 × 10⁴ M⁻¹ s⁻¹. More recent studies by these workers (7) in
the buffer system employed here have shown an even greater
rate of assembly (1.9 × 10⁴ M⁻¹ s⁻¹).

Our spectrophotometric studies here on nonequivalent mix-
tions of oxygenned α and βA chains indicate a k₂ value for
oxyhemoglobin A nearly 10-fold less than that reported in
this identical buffer system by Kawamura and Nakamura (7).
This is especially noteworthy since our value of k₂(Hb A)
was identical to that found by these workers (see “Results” and
Fig. 2B). The two spectroscopic techniques may be probing
different stages of assembly. Circular dichroism captures any
alteration in secondary structure. Such structural perturba-
tions could occur in a region of the protein remote from the
heme pocket and could take place prior to the heme pocket,
itself, undergoing a conformational change. It would be this
later event at the heme environment which our spectropho-
tometric technique would detect. This could account for the
differences in k₂ values obtained by these two distinct tech-
niques. Nevertheless, it would be of special interest if the
assembly of several variant hemoglobins could be monitored
by circular dichroism.

A recent report from our laboratory (23) has shown that
electrostatic attraction may govern oxyhemoglobin assembly.
Although direct measurements of rate constants are not pos-
sible with chain competition experiments, nonetheless, rela-
tive rates of monomer association can be estimated. The ratio
of experimental values of k₂(Hb X)/k₂(Hb A) was 2.6 and
0.41 for Hb N-Baltimore and Hb S, respectively. These ratios
are in qualitative agreement with the direct kinetic measure-
ments reported here, which yielded ratios of 1.5 and 0.66,
respectively. Quantitative differences are most likely attri-
buted to different buffer, pH, and temperature conditions.
Although the technique we employed here has an inherent
error due to the small color changes seen upon oxyhemoglobin
assembly (and large deviations are presented for any given
value of k₂), paired kinetic experiments between normal and
variant β subunits nevertheless showed a consistent, repro-
ducible difference in monomer assembly rates.

Acknowledgment—We thank Adrianna Morris for preparation of
this manuscript.

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