Molecular Dynamics of Hemoglobin Subunits as Seen by Fluorescence Spectroscopy*

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Fluorescent conjugates of \( \beta^A \) subunits and their respective heme-free derivatives have been prepared in which a 1,5-N-iodoacetyl aminoethyl-5-naphthylamine-1-sulfonate probe has been specifically placed at the \( \beta-93 \) or \( \beta-112 \) cysteine. The fluorescence anisotropy decay and static fluorescence polarization of these conjugates have been examined. Fluorescence measurements have also been made using 1-anilino-8-naphthalenesulfonate complexes, as well as the intrinsic fluorescence of the tryptophan groups. For the cases of the \( \beta-93 \) and \( \beta-112 \) conjugates there is substantial evidence for internal rotational freedom of the subunits. The internal mobility of the polypeptide is especially pronounced for the \( \beta-112 \) conjugate. In contrast, the 1-anilino-8-naphthalenesulfonate probe placed within the heme pocket shows no indication of any rotation, other than that associated with the entire \( \beta \)-subunit. Tryptophan fluorescence has been measured for the apo-\( \beta \) subunits and for the peptides \( \beta \) (1-55) from hemoglobins A and S. Perrin-Weber plots show the presence of multiple rotational modes suggesting mobility of the tryptophan groups.

The question of the possible existence of internal degrees of rotational freedom within globular proteins possessing a high degree of organized structure, reflecting the presence of molecular flexibility, has begun to draw considerable attention. There is now substantial evidence that the classical static model of protein structure derived from x-ray crystallography must in some cases be modified to account for their solution properties. Evidence for internal motions in proteins has come from a number of different experimental approaches that include tritium exchange (1, 2), magnetic resonance (3-5), electron spin resonance (6), fluorescence quenching, and fluorescence anisotropy decay (7, 8).

Internal flexibility of proteins may be relevant to the mechanism of allosteric transitions, which require that a large number of atoms shift positions in a concerted way, and to the mechanism of protein folding, which implies continuing rearrangements of the various portions of the molecule, in search of a minimum of conformational energy. Cooper (9) and Case and Karplus (10) have shown the relevance of conformational fluctuations to the stabilization of protein conformation and to ligand binding in hemoglobin.

We have earlier reported experiments using fluorescence anisotropy decay which showed the presence in hemoglobin of internal motions involving portions of the molecule smaller than a single subunit (11). In order to explore in more detail the presence of these fluctuations we have analyzed the fluorescence characteristics of several derivatives of the \( \beta \) subunits of hemoglobin A and S.

Hemoglobin \( \beta \) subunits constitute an ideal system for this kind of investigation. In fact, the presence of 2 cysteines in positions \( \beta-93 \) and \( \beta-112 \), allows the specific labeling with fluorescent probes of known positions on the surface of the molecule. After removal of the heme, ANS (12) can be introduced inside the heme pocket, providing another labeling position which this time goes deeper inside the molecule. In the heme-free derivatives the intrinsic fluorescence of the tryptophans in positions \( \beta-15 \) and \( \beta-37 \) provides additional labeling of specific sites. Finally by removing the heme and by splitting the polypeptide chain of the subunits at the methionine \( \beta-55 \) position with cyanogen bromide, derivatives are obtained with a lower amount of secondary structure than in the native \( \beta \) subunits. These derivatives can be used to assess the correlation of secondary structure and internal flexibility of the molecules.

Fluorescence anisotropy decay provides perhaps the most informative approach to detecting internal motion in proteins in solution. Interpretation of results is substantially simplified if only a single fluorescent probe is located at a known position within a protein for which considerable structural information is available. A meaningful interpretation also requires a means of distinguishing between free or hindered rotation confined to the probe and rotation which involves the polypeptide. The objective of the present study is to compare the extent of internal motion occurring in different regions of the \( \beta \) subunits of hemoglobin, subject to the above criteria. Secondary objectives are to observe the effects of heme removal and of pH and protein concentration.

**Experimental Procedures**

**Methods**

Static Polarization Measurements—Static polarization measurements have shown the relevance of conformational fluctuations to the stabilization of protein conformation and to ligand binding in hemoglobin.

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* The abbreviations used are: ANS: 1-anilino-8-naphthalenesulfonate; AEDANS: N-iodoacetyl aminoethyl-5-naphthylamine-1-sulfonate; apo-\( \beta^A \) subunits, heme-free derivative of the \( \beta \) subunits of hemoglobin A; apo-\( \beta^S \) subunits, heme-free derivative of the \( \beta \) subunits of hemoglobin S; \( \beta^A \) (1-55), peptide corresponding to the first 55 residues of the \( \beta \) subunits of hemoglobin A; \( \beta^S \) (1-55), peptide corresponding to the first 55 residues of the \( \beta \) subunits of hemoglobin S; pMB: p-mercuribenzoate.
ments were made using an Amino-Bowman spectrofluorometer which was coupled with an Amino-Dasar data acquisition system. Glan prisms purchased from Aminco were used for the polarizer and analyzer. The exciting beam was vertically polarized. A hollow cell holder was used, which permitted circulation of water from a constant temperature bath.

For each measurement, 30 determinations each were made of the intensities anisotropically and horizontally polarized components of the fluorescent radiation, plus 20 determinations of the corresponding background obtained for the solvent. These values were averaged and the background was subtracted. The polarization \( P \) was computed using the relation:

\[
P = \frac{(V - GH)/(V + GH)}{P_{\text{total}}}
\]

where \( V \) and \( H \) are the vertically and horizontally polarized intensities, respectively, and \( G \) is equal to the ratio \( V/H \) with the polarizer oriented horizontally.

In order to span a wide range of viscosity values to obtain the Perrin plot, four series of samples were measured for each experiment. The first two of these were initially in buffer and, by successive additions of sucrose, concentrations of sucrose of 9.1 and 33.3% were ultimately attained. The third series began with 50% sucrose and the sucrose level was reduced by successive additions of measured volumes of buffer until a sucrose level of 33.3% was finally attained. The fourth series was initially in 95% glycerol and was progressively diluted to 65% glycerol by buffer addition. In this way, intermediate viscosities were approached from both directions, with some overlap. In one experiment the viscosity was altered by variation of the temperature without addition of sucrose.

**Dynamic Measurements of Anisotropy**—Measurements of time-dependent anisotropy were made using an Orion 9200S fluorometer. The single photon counts corresponding to the decay of the vertically and horizontally polarized components of fluorescence were stored in a multichannel analyzer and were corrected instrumentally for background, using a solvent blank. The time/channel was usually 0.128 ns. Unpolarized exciting light was used. The lamp profile was obtained using a Ludox scattering solution. An ultraviolet filter with maximum transmission at 360 nm and half-band-pass of 20 nm intercepted the exciting beam and a 470-nm cutoff filter intercepted the fluorescent beam.

To correct for the effects of any lamp fluctuations, a calibration run was done for each experiment. A series of determinations of the ratio of the total number of vertical (\( V \)) to horizontal (\( H \)) counts in all channels were made for a time interval (20 s) which was sufficiently short to make lamp fluctuations unlikely. About 20 determinations of \( V/H \) ratio were made in each case and averaged, after rejecting values deviating from the mean by more than 5%. If \( V/H_{\text{rep}} \) and \( (V/H)_{\text{rep}} \) denote the values of the ratio for the calibration and experimental runs, respectively, then \( \alpha \), the correction factor, is equal to \( (V/H)_{\text{rep}}/(V/H)_{\text{exp}} \) and the anisotropy \( A(t) \) is given by:

\[
A(t) = \frac{\alpha (V/H)_{\text{rep}}}{2\alpha (V/H)_{\text{exp}} + 1}
\]

where \( (V/H)_{\text{rep}} \) represents the ratio of vertical to horizontal counts in the actual experiment for a particular channel, corresponding to a certain time after excitation.

The data were punched on tape and subsequently analyzed using a UNIVAC 1100/40 computer. Life-times of fluorescence were computed using the method of moments (12) and/or nonlinear least squares procedure. The best estimates of amplitudes and rotational correlation times, as indicated by a minimum value of \( \chi^2 \), were sought by computing derivatives of \( G(t) \) with respect to its parameters and derivatives of \( A(t) \) from those of \( G(t) \). \( A(t) \) parameters are then calculated and used to compute a new \( G(t) \) function in order to repeat the procedure.

**Quenching Experiments**—Measurements of quenching efficiency were made using a JASCO spectrophotofluorometer. Successive amounts of quencher were added to the sample, whose intensity of fluorescence was compared with a control without quencher. The relative intensities were corrected for dilution and for attenuation of the exciting beam by the inner filter effect.

**Sedimentation Equilibrium**—Sedimentation equilibrium determinations (Table 1) were made with a Beckman Model E analytical ultracentrifuge, using either interference or absorption optics following procedures elsewhere described.

**Conditions**

The buffer used in the new measurements reported here was either borate, pH 8.8-9.2, or phosphate, pH 5.4-5.7. No important difference in properties was observed for the two pH values. Temperature was normally maintained at 25 °C unless the effects of temperature were being examined. In the studies on apo-\( \beta \)-subunits and their AEDANS conjugates, the protein concentration was normally 0.1-0.2 mg/ml, whereas the effects of higher concentration were being observed. A somewhat higher concentration (0.5 mg/ml) was used in the case of the heme-containing CO-\( \beta \)-AEDANS conjugate, because of the reduced fluorescence intensity. The carbon monoxide complexes of the heme-containing subunits were used.

**Materials**

Human hemoglobins A and S, the iodoacetamide derivatives of the respective apo-\( \beta \)-subunits, and the apo-\( \beta \)-93 AEDANS conjugates were prepared by the same methods as in earlier papers (11, 15, 16). At variance with our previous report, in the case of the \( \beta \)-93 AEDANS conjugate, the protein concentration was normally 0.1-0.2 mg/ml, unless the effects of higher concentration were being observed. A somewhat higher concentration (0.5 mg/ml) was used in the case of the heme-containing CO-\( \beta \)-AEDANS conjugate, because of the reduced fluorescence intensity. The carbon monoxide complexes of the heme-containing subunits were used.

**Table 1**

| Sample          | Buffer   | Concentration interval | Mw | Cell average | At minimum concentration |
|-----------------|----------|------------------------|----|--------------|----------------------------|
| Apo-\( \beta \)A | 40 mM borate, pH 8.9 | 0.07-0.32 | 32,500 | 25,300 |
| Apo-\( \beta \)A | 40 mM phosphate, pH 5.7 | 0.06-0.44 | 25,800 | 20,000 |
| Apo-\( \beta \)A | 40 mM phosphate, pH 5.6 | 0.01-0.14 | 43,000 | 39,000 |
| Apo-\( \beta \) | 4 mM phosphate, pH 5.6 | 0.06-0.32 | 27,000 | 16,800 |
| Apo-\( \beta \)-93-AEDANS | 40 mM phosphate, pH 5.4 | 0.06-0.74 | 37,500 | 20,600 |
| Apo-\( \beta \)-93-AEDANS | 40 mM borate, pH 5.9 | 0.12-0.85 | 22,000 | 15,900 |
| Apo-\( \beta \)+ ANS | 40 mM phosphate, pH 5.6, 3.9 mM ANS | 0.06-0.28 | 27,500 | 18,500 |
sulfhydryl groups had been substituted, additional treatment with iodoacetamide ensured that all of the cysteines were either substituted with AEDANS or alkylated by iodoacetamide. Heme was removed following the procedure of Rossi-Fanelli et al. (20). Amino acid analysis indicated that only cysteines were substituted with AEDANS or alkylated by iodoacetamide. Heme was removed following the procedure of Rossi-Fanelli et al. (20). Amino acid analysis indicated that only cysteines were substituted in each case. The PA(1-55) peptide was prepared as described elsewhere (21). AEDANS was purchased from Molecular Probes, Plano, TX.

The circular dichroism of the various polypeptides was measured in the presence and absence of the fluorescent probes, following data collection and analytical procedures already described (17). In no case did the presence of the fluorescent probe appear to affect the circular dichroism spectra of the various polypeptides in the near and far ultraviolet spectral regions.

Glass-redistilled water and analytical grade reagents were used for the preparation of all solutions.

RESULTS

AEDANS Conjugates with the β4-93 and β4-112 Cysteines—In a previous paper (11) we reported measurements of decay of anisotropy for CO-HbA and its β subunits labeled with AEDANS in position β-93. In both cases the decay of anisotropy was exponential and corresponded to a single correlation time whose magnitude at 23 °C was 4.4 ns for CO-HbA and 3.4 ns for the β subunits. Due to the presence of heme, the fluorescence life-time of the label was shortened to 1-2 ns, reducing the time range of observation and probably masking the longer correlation times corresponding to the rotation of the entire molecule. In this study the investigation was extended to other conjugates of the β subunit and its heme-free derivative. The objective of this phase of the study was to compare conjugates labeled with AEDANS at the β-93 and β-112 positions and to assess the effects of heme removal.

The time decay of fluorescence anisotropy for the CO-β4-112 AEDANS-labeled subunits is shown in Fig. 1. Also, in this case the life-time of the probe was much reduced and, within the available time range of observation, the time decay of anisotropy was exponential and corresponded to a single correlation time of 2.6 ns. In the presence of 40% sucrose the decay of anisotropy was still exponential and the correlation time increased approximately 4-fold (Fig. 1).

The time decay of fluorescence anisotropy for the corresponding apo-derivative is displayed in Fig. 2. Measurement of the life-time of the probe showed the presence of a single component with a life-time of 12.5 ns, substantially enlarging the time range within which the decay of anisotropy could be observed. Still, as shown in Fig. 2, the decay of anisotropy was exponential with a single correlation time of 3.0 ns (Table II), very close to the value obtained in the presence of the heme.

![Figure 1](image1.png)  
**Fig. 1.** Time decay of fluorescence anisotropy for an AEDANS conjugate with Cys-112 of β subunits (0.5 mg/ml) in 40 mM borate buffer, pH 8.8, 25 °C. ○, no sucrose; ●, 40% sucrose.

![Figure 2](image2.png)  
**Fig. 2.** Time decay of fluorescence anisotropy for an AEDANS conjugate with Cys-112 of apo-β subunits (0.1 mg/ml) in 40 mM borate buffer, pH 8.9, 25 °C.

| Sample            | Solvent        | Concentration | τ (ns) | Aω (ns) | φω (°) | Aζ (ns) | φζ (°) |
|-------------------|----------------|---------------|--------|---------|--------|---------|--------|
| Apo-β4-93-AEDANS  | 40 mM borate, pH 8.9 | 0.1           | 13.2   | 0.06    | 0.8    | 0.06    | 9.8    |
|                   | 40 mM phosphate, pH 5.4 | 0.1           | 13.4   | 0.06    | 1.3    | 0.06    | 7.3    |
|                   | 40 mM borate, pH 9.2  | 1.0           | 13.2   | 7.3     |        |         |        |
|                   | 40 mM borate, pH 8.8  | 0.07          | 12.2   | 0.06    | 1.8    | 0.03    | 7.9    |
| Apo-β4-112-AEDANS | 40 mM borate, pH 8.9 | 0.1           | 12.5   | 3.0     |        |         |        |
|                   | 40 mM phosphate, pH 5.7 | 0.3           | 14.6   | 10.4    |        |         |        |
|                   | 40 mM phosphate, pH 5.7 | 0.09          | 14.3   | 9.1     |        |         |        |
| CO-β4-112-AEDANS  | 40 mM borate, pH 8.9 | 0.5           | 2.4°   | 2.6     |        |         |        |

*φω and φζ, the rotational correlation times, are defined by Equations S-8 and S-9, as are Aω and Aζ.

*Main component.
In both cases the correlation time was substantially less than that predicted by Equation 5-13 for an intact and rigid monomeric subunit. The implication is that even in the apo-derivative the amplitude of the corresponding rotation is sufficiently large to mask the effects of the rotation of the entire molecule.

The apo-β₁₅ subunits labeled at β₉₃ with AEDANS showed a single lifetime of the probe near 13 ns both at alkaline and at acid pH (Table II). A typical logarithmic plot of the time decay of anisotropy is shown in Fig. 3. The decay is clearly nonexponential, an initial rapid decline being followed by a more gradual decrease. At low concentrations (0.1 mg/ml) the decay of anisotropy could be fitted with two correlation times (Table II), the longer of which (8-10 ns) was in the range expected for the entire monomer unit, while the shorter (1-2 ns) must reflect internal rotation of some kind. It is of course unlikely that only two rotational modes are strictly present and the shorter time probably corresponds to some form of poorly defined average. In this range of concentration the apo-β₁₅ subunits exist primarily as monomer units, so that the viscosity was varied by the addition of sucrose at constant temperature, was approximately linear at high values of $T/\eta$, but showed pronounced downward curvature at lower values of $T/\eta$, corresponding to high solvent viscosities (Fig. 4). Extrapolation of the data obtained at high $T/\eta$ values gave a correlation time of 14 ns, whose order of magnitude is characteristic of rotation of the entire subunit (Table III), while a correlation time close to 0.9 ns was obtained from the data at low $T/\eta$ values.

**TABLE III**

| Sample                  | Solvent             | Concentration | $\tau$ | $\phi_0$ | $\phi_1$ |
|-------------------------|---------------------|---------------|--------|---------|---------|
| Apo-β₁₅                 | 40 mM phosphate, pH 5.7 | 0.2           | 3.2    | 9.7     |         |
| 25 °C                   |                     | 0.2           | 3.2    | 9.8     |         |
| 10 °C                   |                     | 0.2           | 3.2    | 7.7     |         |
| Variable temperature    | 4 mM phosphate, pH 5.6, 25 °C | 0.2           | 3.2    | <0.2    | 9.4     |
| Apo-β₁₅                 | 4 mM phosphate, pH 5.7, 25 °C | 0.2           | 3.0    | 20.0    |         |
| β₁₅ (1-55)              | 40 mM borate, pH 8.9, 25 °C | 0.1           | 3.8    | <0.2    | 10.6    |
| β₁₅ (1-55)              | 40 mM borate, pH 8.9, 25 °C | 0.2           | 3.3    | <0.2    | 3.5     |
| Apo-β₁₅-93-AEDANS       | 40 mM borate, pH 8.9, 25 °C | 0.1           | 13.0   | <0.9    | 14.1    |

**Fig. 4.** Perrin plot for an AEDANS conjugate with Cys-93 of apo-β₁₅ subunits (0.1 mg/ml) in 40 mM phosphate buffer, pH 5.4, 25 °C. ○, sucrose; ●, glycerol. Inset, enlarged version of portion of curve at high viscosities.

Fluorescence anisotropy decay measurements were made for the complex of 1,8-ANS with the apo-β₁₅ subunit, with the objective of comparing the molecular motion sensed by a probe imbedded in the heme pocket with that detected by probes covalently attached at the β₉₃ and β₁₁₂ positions. The fluorescence intensity decay of the probe was exponential, with a decay time of 14 ns (Table II). The time decay of fluorescence anisotropy was also exponential and corresponded to a single correlation time (Fig. 5) whose magnitude was in the range expected for an intact subunit (Table II).

**Tryosphum Residues of the Apo-β₁₅ and Apo-β₁₅ Subunits—**The apo-β₁₅ subunits of hemoglobins A and S contain two tryptophan groups at positions β-15 and β-37. These provide built-in fluorescent probes for monitoring localized motion of the polypeptide chain. The objective of this phase of the investigation was to monitor internal motion sensed by an intrinsic probe at these locations and to compare apo-β₁₅ and apo-β₁₅.

The fluorescence emission spectra and the polarization excitation spectra in buffer and in 50% sucrose are shown in Fig. 6 for the apo-β₁₅ subunits; the behavior of the apo-β₁₅ subunit...
Dynamics of $\beta$ Subunits

apo-$\beta^a$ and apo-$\beta^b$ tryptophans was derived from fluorescence quenching measurements, employing acrylamide as the quencher (Fig. 7). A comparison of the Stern-Volmer plots for apo-$\beta^a$ and apo-$\beta^b$ subunits with that for the model compound acetyl tryptophanamide (Fig. 7) indicated a much reduced efficiency of quenching for the former and suggests that the apo-$\beta$ tryptophans are largely shielded from the solvent. The linearity of the Stern-Volmer plots suggests further that either both residues are exposed to an approximately equivalent extent, or that the quantum yield of one residue is very low.

Polarizations were measured for the apo-$\beta^a$ and apo-$\beta^b$ subunits in 4 and 40 mM phosphate buffer, pH 5.3, as a function of viscosity and Perrin plots were constructed. The excitation wavelength was 285 or 287 nm, both of which lie on a plateau in the polarization excitation spectrum. No difference in computed correlation times was observed for the two wavelengths. The average life-time of the tryptophan residues was close to 3 ns in all cases (Table III). No significant change was observed in the presence of sucrose or glycerol.

For the case of apo-$\beta^b$ subunits in 40 mM phosphate, at both 10 and 25 °C, the Perrin plots were linear down to the lowest values of $T/T_0$ examined (Fig. 8). The computed correlation times (Table III) were in the range expected for an intact monomer unit. This was also the case when viscosity was varied by alteration of temperature (Table III). Thus, no

was essentially identical. The observed value of $\lambda_{\text{max}}$ (340 nm) was characteristic of partially shielded tryptophan. The polarization excitation spectra resemble qualitatively those reported for other tryptophan-containing proteins and reflect the presence of at least three overlapping transitions of the indole fluorophore.

Further evidence for the partially shielded character of the

apo-$\beta^a$ and apo-$\beta^b$ subunits in 40 mM phosphate buffer, pH 5.7, 25 °C. Protein concentration, 0.2 mg/ml; ANS, 1.0 $\times 10^{-5}$ M.

FIG. 5. Time decay of fluorescence anisotropy for an ANS complex with apo-$\beta^a$ subunits in 40 mM phosphate buffer, pH 5.7, 25 °C. Protein concentration, 0.2 mg/ml; ANS, 1.0 $\times 10^{-5}$ M.

FIG. 6. A, fluorescence emission spectrum of apo-$\beta^a$ subunits (0.1 mg/ml); B, polarization-excitation spectrum of apo-$\beta^a$ subunits (0.1 mg/ml) in 40 mM phosphate, pH 5.7, 25 °C, in the absence and presence of 50% sucrose. ○, no sucrose; ●, sucrose present.

FIG. 7. Stern-Volmer plots for acrylamide quenching of 0.1 mg/ml of apo-$\beta^a$ (○), apo-$\beta^b$ (●), and 0.01 mM acetyl tryptophanamide (○) in 40 mM phosphate, pH 5.7, 25 °C.

FIG. 8. Perrin plot for apo-$\beta^a$ subunits (0.2 mg/ml) in 40 mM phosphate, pH 5.7, 25 °C. ○, sucrose present; ●, glycerol present.
Dynamics of β Subunits

localized motion of the tryptophans was detected under these conditions.

In the case of the apo-βS subunit, the Perrin plot in 40 mM phosphate, pH 5.6, was almost horizontal, consistent with the presence of considerable self-association (Table I). However, at the lower ionic strength of 4 mM phosphate where the aggregation was less significant, downward curvature in the Perrin plot appeared, suggesting the presence of some degree of localized mobility of the tryptophans (Fig. 9; Table III).

The downward curvature of the Perrin plots for both the apo-βA and apo-βC subunits in 4 mM phosphate gave indication of localized motions of the tryptophans with very short correlation times, whose magnitude, as estimated from the limiting slopes of the plots, was less than 100 ps.

It is of interest that the apo-βA subunit in 40 mM phosphate, pH 5.6, was almost horizontal, consistent with the presence of considerable self-association (Table I). However, at the lower ionic strength of 4 mM phosphate where the aggregation was less significant, downward curvature in the Perrin plot appeared, suggesting the presence of some degree of localized mobility of the tryptophans (Fig. 9; Table III).

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It is of interest that the apo-βA subunit in 40 mM phosphate did not show any indication of localized rotation at very low $T/\eta$ values. This may indicate either that, at this higher ionic strength, the restricted freedom of rotation of the tryptophans was lost because of involvement in a more rigid tertiary structure, or that at higher ionic strengths the tryptophans were incompletely accessible to the solvent additive and did not respond to an increment in viscosity. Support for the latter interpretation is provided by data for the quenching of tryptophan fluorescence by acrylamide (Fig. 7).

Tryptophan Residues of the β (1-55) Peptides of Hemoglobins A and S—Only the behavior of the tryptophan fluorescence was observed for these two peptides. The results were very similar in both cases. The fluorescence emission spectrum showed a $\lambda_{max}$ at 345 nm, indicative of some degree of shielding of the tryptophyl residues from the solvent. Perrin plots (Fig. 10), obtained in 40 mM borate at pH 8.9, showed a downward curvature at low $T/\eta$ values, suggesting the presence of internal rotation of very short correlation times (Table III). Average molecular weights of these peptides have been already reported (21). Both peptides tend to aggregate, and the peptide from hemoglobin S polymerizes more than that from hemoglobin A, especially in 40 mM borate. The large aggregation of the hemoglobin S peptide did not seem to affect the detectable rotational modes of the molecule.

DISCUSSION

Internal Rotation in Hemoglobin—The experimental results cited earlier have indicated that internal rotation of some kind is present for AEDANS conjugates labeled at the Cys-93 or Cys-112 positions of the βA and apo-βC chains. The question is whether the rotation is confined to the label itself, or whether it also reflects the motion of a portion of the polypeptide chain in proximity to the label. (The detailed theory is given in the miniprint supplement.)

Let us first ask the question whether the observed results could be accounted for by a free rotation of the label about the link joining it to a rigid protein molecule. The observed correlation time $\phi_1$ for an AEDANS-cysteine conjugate (H2O, 25 °C) is 80 ps. (This was measured for AEDANS (0.1 mM) in 0.1 M cysteine, 60% sucrose, pH 7.0, and corrected to the above conditions.) The predicted value of $\phi_1$ in Equations S-8 or S-9 would range from 0.08-0.5 ns. Already we can notice that this is 1 order of magnitude less than the values observed for the Cys-93 conjugates of βA and hemoglobin itself and for the Cys-112 conjugate of βA and apo-βA (Table II), excluding that these reflected only the free rotation of the probe.

Let us consider next the model in which the internal rotation is still confined to the probe, which undergoes hindered rotation characterized by the frequency of jumps of the probe, $w'$, as shown by Equations S-10 to S-12. Since the corresponding correlation time could have any value, depending on the magnitude of $w'$ in Equation S-11 this model could readily explain the observed values of $\phi_1$.

The dependence of $\phi_1$ upon viscosity is useful in assessing the plausibility of this model, using Equation S-14. The question arises as to whether the effective viscosity sensed by the probe is equivalent to the bulk viscosity ($\eta$) or whether it is substantially less. The latter might be the case if the effective rotating unit were partially shielded from solvent. Clearly the ratio $\phi_1^* / \phi_1$ is equal to a lower limit for $\beta$, the ratio of effective viscosities, while the upper limit is equal to $\eta / \eta'$. Thus

$$\phi_1^* / \phi_1 < \beta < \eta / \eta'$$

(4)

Values of $\phi$ and $w'$ corresponding to both limits are cited in Table IV.

The values of $\phi$ computed in this way from Equation S-14 are substantially less than the value of $\phi$ (~10 ns) predicted for the entire βA subunit for the cases of the Cys-93 conjugate of intact hemoglobin and the Cys-112 conjugate of the βA subunit (Table IV). The predicted correlation time of the entire βA subunit compared very well with experimental meas-

Fig. 9. Perrin plot for apo-βA subunits (0.1 mg/ml) in 4 mM phosphate, pH 5.7, 25 °C. ○, sucrose present; •, glycerol present.

Fig. 10. Perrin plot for βA (1-55) (0.1 mg/ml) in 40 mM borate, pH 8.9, 25 °C. ○, sucrose present; •, glycerol present.
measurements of Stryer (22) and Anderson et al. (25) on sperm whale and aplasia apo-myoglobin labeled with ANS.

Further information is obtainable from static polarization, using Equation 5-15. In this case the predicted shape of a plot of $A^{-1}$ or $1/p - \frac{1}{2}$ versus $T/\eta$ would show continuous upward curvature with increasing values of $T/\eta$. This is the opposite of what is actually observed for the Cys-93 conjugate of the apo-$\beta^a$ chain. It thus appears that the model corresponding to hindered rotation which is confined to the probe alone.

In conclusion, the observed behavior of the various AEDANS conjugates with Cys-93 or Cys-112 in the $\beta$ subunits cannot be explained in terms of either free or hindered rotations confined to the probe alone. Instead it appears that the observed internal rotations involve significant portions of the adjacent polypeptide chains.

**Angular Range of the Rotation of the Probe**—It is also possible to obtain an estimate of the effective angular range of an internal rotation. If the rotation of the fluorescent label is confined within a cone of semiangle $\psi$ then, if the time decay of anisotropy is given by an equation of the form of Equation S-8, $\psi$ is related to the amplitudes $A_{10}$ and $A_{30}$ by (8):

$$A_{20}/(A_{10} + A_{30}) = \cos^2 \psi (1 + \cos \psi)^2/4$$

The values of $A_{10}$ and $A_{30}$ may also be obtained from static polarization measurements. If the behavior is describable in terms of two correlation times, Equation S-3 indicates that the decay of anisotropy is given by an equation of the form of

$$A_{20}/(A_{10} + A_{30}) = \cos^2 \psi (1 + \cos \psi)^2/4$$

Equation 5 to the anisotropy decay and static polarization data described above yields the results presented in Table V. In the case of the AEDANS conjugates it is seen that the computed value of $\psi$ is considerably different for the Cys-93 and Cys-112 conjugates, being much greater for the latter. Computed values of $\psi$ are also cited for the tryptophans and indicate a substantial angular range for their rotation.

**Distribution of Internal Rotations**—In two cases, namely the ANS complex formed by the apo-$\beta^a$ subunit and the intrinsic tryptophan fluorescence of the apo-$\beta^a$ subunit in 40 mM phosphate, the time decay of fluorescence anisotropy and static polarization measurements have indicated only a single correlation time whose magnitude is in the range expected for the rotational diffusion of the entire $\beta^a$ chain. In the case of AEDANS conjugates with the $\beta$-93 sulphydryl group of the apo-$\beta^a$ subunit, dynamic anisotropy decay and static polarization indicate the presence of both a long correlation time which is probably associated with rotation of the molecule as a whole and a much shorter correlation time which must arise from internal rotation of the macromolecule. In the case of AEDANS conjugates with either the $\beta$-93 (11) or $\beta$-112 sulphydryl groups of the intact $\beta^a$ subunits, and with the $\beta$-112 cysteine of the apo-$\beta^a$ subunits, only short correlation times are detectable.

As discussed above, the short correlation times are due to internal rotations involving adjacent portions of the polypeptide chain.

A similar situation was present for the peptides $\beta$ (1-55) from hemoglobin A and S, whose Perrin plots gave evidence for the presence of a long correlation time consistent with rotational motion of the entire peptide and very short times corresponding to rotational modes of the tryptophans themselves. The interpretation of the long correlation times is in this case complicated by the association of the peptides. It may be noted that the large degree of association of the peptide from hemoglobin S does not seem to influence the longer rotational mode, supporting the idea that it reflects a motion still confined within a single molecule.

**Internal Rotation and Structure of the Various Derivatives**—As shown in Table IV the $\beta$-93 cysteine of hemoglobin is associated with a portion of the molecule whose rotational mode shows a correlation time between 4 and 6 ns. This magnitude of correlation time may be expected from a rigid sphere with a molecular weight about $\frac{1}{2}$ that of a $\beta$ subunit. In the hemoglobin subunits there are no structural domains obvious to the inspection of their tridimensional structure. Therefore, this mobility must reflect the synchronous motions of a cluster of residues belonging to different segments of the polypeptide chain.

The actual size of the rotating cluster is difficult to assess. The fit with a single exponential of the anisotropy decay may still fail to show the presence of additional short time in the very beginning of the emission of fluorescence. Also, little is known at this point with regard to the shape of the rotating unit and to the inevitable hindrance produced by the balance of the molecule. The size above mentioned of about $\frac{1}{2}$ an entire subunit must be considered an upper limit, a smaller size being more probable.

As listed in Table IV, the rotational freedom associated with cysteine $\beta$-112 shows a correlation time similar to that of the $\beta$-93-labeled subunit (11). Notably the removal of the apo-$\beta^a$ subunit.
heme from the subunits fails in this case to produce nonexponential behavior of the time decay of anisotropy with the appearance of a longer correlation time corresponding to the rotation of the entire subunit, as it does for the $\beta$-3-labeled subunits. This is consistent with a high degree of compactness of the rotating cluster and with a very large amplitude of its rotation. This in turn implies little hindrance of its motion produced by the rest of the molecule and raises the possibility that in this case the cluster may be a rigid element including some 50 amino acid residues or less, depending on shape and hindrance as above discussed. It is also interesting to observe that Cys $\beta$-112 is located at the $\alpha/\beta$ interface in hemoglobin. A cluster of about 50 amino acids may include a good portion of that interface. The high mobility of the cluster would be consistent with the function of the interface in determining the conformation of the tetramer and in transmitting allosteric information between the subunits of hemoglobin.

Removal of the heme produces a drastic decrease of the helical content of the $\beta$ subunits from approximately 70% to approximately 40% (16, 21). It also produces a decrease of the short correlation time $\phi$, detectable for the $\beta$-3 AEDANS-labeled $\beta$ subunits (Table II). This may suggest that the loss of helical structure loosens the internal constraints of the molecule. However, there is some evidence for the persistence of some tertiary structure in the apo-$\beta^A$ subunits, including the behavior of the ANS complex and the partially shielded character of the tryptophans. Also the apparent average separation of the two tryptophans from an AEDANS group attached to the $\beta^A$-93 cysteine, as computed from radiationless energy transfer, is 21 $\pm$ 2 Å. Although the presence of two donor groups renders this result ambiguous, it may be significant that it is close to the average distance expected from crystallographic data for intact hemoglobin and does not suggest a major expansion or deformation of the molecule.

The rotational behavior of the derivatives obtained from hemoglobin S is essentially the same as that of the corresponding derivatives of hemoglobin A. When a difference is noticed it is easily recognized as due to a different degree of aggregation of the monomeric particles. Clearly the $\beta$-6 Glu $\rightarrow$ Val mutation does not have any effect on the degrees of freedom of internal rotation of the $\beta$ subunits of hemoglobin, as monitored by tryptophan.

The picture that begins to emerge in regard to the conformational dynamics of hemoglobin subunits in solution is that of spheres with a fluid periphery and a more rigid interior. Removal of the heme further loosens the periphery, without drastic changes of the general shape of the molecule. Subunit interactions and the penetrability of the molecular surface to ligands for the heme may be consistent with the mobility of the more superficial layer of the molecule. This image is also consistent with observations reported by Frauenfelder and Petsko (27). The functional aspects of the conformational dynamics in the hemoglobin system are now under investigation.

The peptides $\beta$ (1-55) show the presence of about 20% helical structure as compared with 80% in the crystal of hemoglobin. Also, in this case our results suggest the persistence of some amount of tertiary structure. This is shown by the partial shielding of the tryptophyl groups evident in the emission spectrum of fluorescence and by the rotational modes largely dominated by the motion of the entire polypeptide.

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Supplement to MOLECULAR DYNAMICS OF HEMOGLOBIN β SUBUNITS AS SEEN BY FLUORESCENCE SPECTROSCOPY

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Detection of internal rotation in hemoglobin subunits. If only a single rotational mode is present in a macromolecule labeled with a fluorescent group, the deconvoluted anisotropy would decay exponentially:

\[ A(t) = A_0 e^{-t/\tau} \]

where \( A_0 \) is the anisotropy at zero time, \( \tau \) is the single rotational correlation time corresponding to the entire macromolecule, and \( \lambda \) is the lifetime.

If more than one rotational mode is present, as will be the case if internal degrees of rotational freedom are present, then equation S-1 is replaced by:

\[ A(t) = \sum \left[ A_{0i} e^{-t/\tau_i^f} \right] \]

where \( A_{0i} \) and \( \tau_i^f \) are the amplitude and correlation time, respectively, corresponding to the \( i \)-th rotational mode. The amplitudes \( A_{0i} \) are taken to be normalized with respect to the sum of the entire macromolecule.

Steady state polarization measurements yield a time-averaged anisotropy:

\[ A = \frac{1}{t} \int_0^t A(t') dt' \]

and \( \lambda = \frac{1}{\sum \left( A_{0i} / \tau_i^f \right)} \)

where \( \lambda \) is the lifetime of the correlation time, \( \tau_i^f \), and \( A_{0i} \) is the amplitude of the \( i \)-th rotational mode.

For a single rotational mode, equation S-3 reduces to the familiar Perrin equation:

\[ A = A_0 / (1 + \lambda / \tau) \]

or

\[ A_0 / \lambda = (1 + \tau / \lambda)/(1 + \tau / \tau_0) \]

where \( \lambda \) is the polarization and \( \tau_0 \) is the anisotropy in the absence of rotation.

For present purposes we are primarily interested in the significance of the short correlation times observed by both dynamic and steady state measurements. The factors which may potentially contribute to a short correlation time include the free rotation of the labeled subunit, the hindered rotation of the subunit, and the internal rotation of a portion of the macromolecule containing the label.

Each of these factors may reduce the lifetime of the correlation time, \( \lambda \), and hence reduce the anisotropy decay to the extent that it is observed by the instrument. The correlation time, \( \tau_i^f \), for each rotational mode is determined by the instrument. For a spherical shape of the labeled subunit, the correlation time is determined by the instrument. For a non-spherical shape of the labeled subunit, the correlation time is determined by the instrument.