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Kinetics of the reconstitution of hemoglobin from semihemoglobins α and β with heme

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Abstract. Kinetics of the reconstitution of hemoglobin from semihemoglobins α and β with hemin dicyanide have been investigated using three kinds of stopped-flow technique (Soret absorption, fluorescence quenching of tryptophan, and Soret CD). The semihemoglobins α and β are occupied by heme in the α and β chains, respectively, the other chain being heme-free. Based on the kinetic results, the following scheme for the reconstitution is proposed; First, hemin dicyanide enters the pocket-like site of the apo chains. Second, in semihemoglobin α, the CN-ligand in the fifth coordination position of iron is replaced by the imidazole ring of the proximal His immediately after the heme insertion. In contrast, semihemoglobin β changes its conformation after the heme insertion, and this is followed by the ligand replacement. Finally, the partial structure changes induced by the ligand replacement propagate onto the whole molecule and the final conformation is attained. The results indicate that semihemoglobin α retains a more rigid and organized structure, and more closely approaches its final structure than does semihemoglobin β.

Key words: Hemoglobin – Semihemoglobin – Reconstitution – Stopped-flow

Introduction

Normal adult human hemoglobin is a tetrameric protein with two α and β chains, each of which carries a protoheme. In vivo, α and β polypeptide chains are synthesized separately, bound to heme and assembled with each other to form the hemoglobin tetramer ($\alpha_2\beta_2$) (Bunn et al. 1977). The events confer a characteristic three-dimensional structure on the hemoglobin molecule. However, the mechanism of the post-translational modification has not been fully elucidated; when the chains assemble with each other, which chain combines preferentially with heme and how is its final conformation attained, etc.

Apohemoglobin, which is prepared in vitro from hemoglobin, combines with heme to reconstitute the hemoglobin molecule. The kinetics of the reconstitution process have been described by many laboratories (Chu and Bucci 1979; Gibson and Antonini 1963; Rose and Olson 1983). However, it is difficult to describe the kinetics exactly, because the reconstitution consists of various processes; that is, there are many kinds of intermediates. In a previous report (Kawamura-Konishi and Suzuki 1985), we analyzed the reconstitution process on the assumption that the α chain had a greater affinity for hemin than the β chain in apohemoglobin. Another effective approach is to investigate the kinetics of heme binding to the intermediate species.

Semihemoglobins α and β are each composed of a pair of α and β chains but contain only a single heme on the α and β chain, respectively, with the other chain being heme-free. They are prepared from the corresponding isolated chains and apohemoglobin, and combine with protoheme to reconstitute hemoglobin (Cassoly 1981). They are considered as a good model for intermediate species in the assembly. Recently, Park and McDonald (1989) studied the kinetics for heme binding to semihemoglobin α, in which the Soret absorption stopped-flow technique was used. The complexity of the process requires multiple probes for the detection of the conformational change. In this paper, we have investigated the kinetics of the reconstitution process of hemoglobin from semihemoglobins α and β with hemin dicyanide using stopped-flow apparatuses with three kinds of detection system: (*1*) Soret absorption that reflects the ligand state and environment of the heme, (*2*) fluorescence quenching of tryptophan in the protein resulting from energy transfer to the heme moiety (Hochstrasser and Negus 1984), and (*3*) Soret CD that arises mainly from the interaction between the heme and the surrounding aromatic residues (Hsu and Woody 1971). On the basis of the results, we attempt to describe the pathway of the reconstitution and suggest that semihemoglobin α has a more rigid and orga-

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nized structure and more closely approaches its final structure than does semihemoglobin β.

Materials and methods

1. Materials

Hemin was purchased from Sigma (bovine, type I). All reactions were carried out in 0.1 M borate–NaOH buffer containing 10 mM KCN and 0.1 M KCl. The buffer was kept at pH 9.1, and was fully saturated with CO gas. Under the conditions used, hemin exists as a monomeric dicyanide form (Shack and Clark 1947).

Semihemoglobins α and β were prepared as previously reported (Kawamura-Konishi and Suzuki 1985) with some modification; the semihemoglobin α was purified by chromatography on a 1 × 50 cm column of CM Toyopearl (10 mM phosphate buffer, pH 7.0) with a linear gradient of 0–0.2 M KCl. The purified semihemoglobins consisted mainly of dimers (Cassoly 1981; Yip et al. 1977), and were characterized electrophoretically on cellulose acetate. Concentrations of the semihemoglobins, which are expressed on a heme basis, were determined by the use of the pyridine-hemochromogen method (Riggs 1981). The purified semihemoglobins were stored as the carbonmonoxy forms at −80 °C until used.

2. Static measurements

Fluorescence spectra were measured using a Hitachi-850 fluorescence spectrophotometer, calibrated with rhodamine B. The excitation wavelength was 296 nm with a band path of 5 nm. Emission spectra were obtained with a band path of 15 nm. Absorption spectra were measured using a spectrophotometer from Union-Giken, type SM-401, or one from Japan Spectroscopic Co. Ltd., type Ubest-50, with a cell of 10 mm path length. CD spectra were obtained using a Union-Giken Dichrograph III-J with a cell of 10 mm path length.

3. Kinetic measurements

Stopped-flow absorption, stopped-flow fluorescence and rapid-scan spectra were measured with a Unisoku-Scientific-Instruments rapid-scan spectrophotometer model USP-516 with a cell of 10 mm or 2 mm path length. In the case of fluorescence experiments, the excitation wavelength was 296 nm and emission with a wavelength longer than 300 nm was collected using a cut-off filter. Stopped-flow CD was measured with a Union-Giken stopped-flow apparatus, RA-411, with a cell of 10 mm path length. All the stopped-flow experiments were performed by mixing semihemoglobins and hemin dicyanide in a 1:1 ratio at 15±0.1 °C or 5±0.1 °C. In some cases with semihemoglobin β, slow changes in the spectra and in the absorption at a fixed wavelength were monitored using a Union-Giken SM-401 spectrophotometer by manual mixing in a standard quartz cell.

4. Analysis of stopped-flow traces of absorption change in the rapid phase

Data for the decrease in the absorbance at 422 nm and 420 nm for semihemoglobins α and β, respectively, were analyzed using the following equations, since in previous papers (Kawamura-Konishi and Suzuki 1985 and Kawamura-Konishi et al. 1988) we have shown that the binding of hemin to apomyoglobin and apohemoglobin can be analysed as a simple second-order reaction.

The simple second-order reaction, where X and Y combine to produce Z, can be described as

$$X + Y \xrightarrow{k} Z \tag{1}$$

where k is the second-order rate constant. From this equation, (2) is derived as

$$d[Z]/dt = k \cdot [X] \cdot [Y]. \tag{2}$$

By solving the differential equation under the conditions expressed by (3) and (4), we get (5).

$$\text{at } t,\ [X] = [Y] = C \tag{3}$$

$$\text{at } t = 0,\ [X] = Co \text{ and } [Z] = 0 \tag{4}$$

$$C/Co = 1/(Co \cdot k \cdot t + 1) \tag{5}$$

where Co is the initial concentration. Then, the absorbance at time t, A, is expressed as

$$A = (Ao - A_f)/(Co \cdot k \cdot t + 1) + A_f \tag{6}$$

where A_o and A_f are the absorbance at $t = 0$ and ∞, respectively. The values of k and Ao were determined by fitting the experimental time courses to (6) by use of a least squares method, the Simplex method (Nelder and Mead 1965).

Results

1. Optical absorption, fluorescence, and CD spectra of semihemoglobins α and β before and after mixing with hemin

Figure 1 shows the optical absorption spectra of semihemoglobins α and β, and of hemin dicyanide in the Soret region. The spectra of semihemoglobins α and β showed a peak at about 418 and 420 nm, respectively. After the addition of hemin dicyanide, the peak position of the spectra changed to 420 nm for semihemoglobin α and 422 nm for semihemoglobin β. The spectral changes are shown as the difference spectra between the spectrum of mixed semihemoglobin and hemin dicyanide and the sum of the spectra of the unmixed semihemoglobin and hemin dicyanide.

Figure 2 shows fluorescence spectra, excited at 296 nm, of semihemoglobins α and β before and after mixing with equimolar amounts of hemin dicyanide, compared with those of apomyoglobin and apohemoglobin. It was found that the fluorescence of semihemoglobins α and β was almost quenched after the mixing, as was that of apomyoglobin and apohemoglobin.

Figure 3 shows Soret CD spectra of semihemoglobins α and β before and after mixing with equimolar amounts of hemin dicyanide, and the difference spectra between the spectra before and after the mixing. The semihemoglobin α has a positive peak in the spectrum at about 419 nm, which was at a longer wavelength, 423 nm, after the mixing. In the spectrum of semihemoglobin β before the mixing, there is a very weak CD band with a positive peak at about 420 nm and a trough at about 431 nm. After the addition of hemin dicyanide, the intensity of the spectrum of semihemoglobin β increased and its peak position was 427 nm. The difference CD spectra for semihemoglobins α and β had peaks at about 427 nm and 429 nm, respectively.

Semihemoglobins α and β were titrated with hemin dicyanide by measuring the change in the Soret absorbance, fluorescence, and Soret CD. The titration curves reached their end points at a ratio of 1 hemin dicyanide to 1 heme-containing subunit (data not shown). Therefore, the following experiments for the reconstitution reaction were performed by mixing hemin dicyanide and semihemoglobin at a 1:1 ratio.

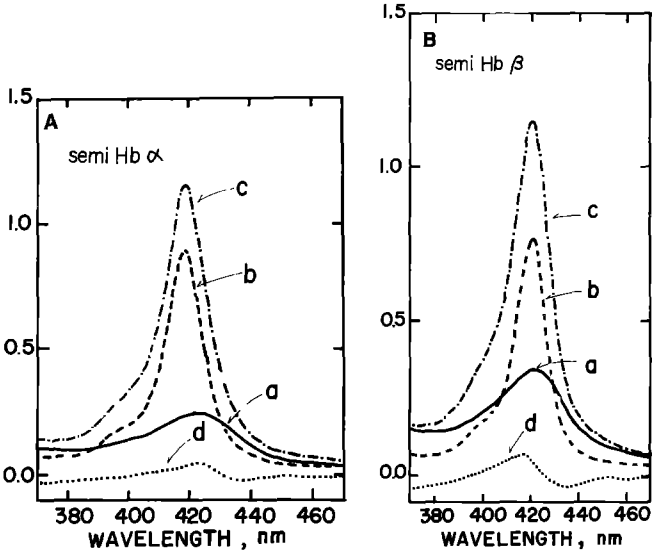


Fig. 1 A, B. Optical absorption spectrum of hemin dicyanide (spectra a) and spectra of semihemoglobins before (spectra b) and after (spectra c) mixing with equimolar amounts of hemin dicyanide, and the difference spectra (spectra d = c-a-b). (**A**) Semihemoglobin α at a concentration of 3.17 μM. (**B**) Semihemoglobin β at a concentration of 2.79 μM

2. *Kinetic measurements*

Figure 4 shows spectral changes in the absorption of semihemoglobins α and β after mixing with equimolar amounts of hemin dicyanide at 15 °C. The spectra of the corresponding semihemoglobin and hemin dicyanide were subtracted from them to reveal differences in the spectra clearly. It was found that the Soret absorption band was rapidly shifted to longer wavelengths, and then gradually shifted to shorter wavelengths. These results indicate the existence of an intermediate during the reconstitution.

To estimate rate constants for the formation and disappearance of the intermediate, time courses of the absorption change were monitored using a stopped-flow apparatus. A rapid decrease in the absorption at 422 nm (15 °C) was observed for semihemoglobin α, as in Fig. 5A. In the case of semihemoglobin β, the rapid decrease occurred mostly within the dead time (3 ms) at 15 °C (data not shown), so we measured the absorption decrease at a lower temperature, 5 °C (Fig. 5C). The time courses observed could not be explained by the first-order reaction. We then tried to analyze the time courses quantitatively using a second-order rate law (see Materials and methods section). The dotted lines in Fig. 5A, represent the theoretical time course calculated with the obtained values, and this is in a good agreement with the experimental data. The reversible reaction as $X+Y \rightleftharpoons Z$ could not explain the time courses (data not shown). The results indicate the validity of the assumption that the decrease in the absorption reflected the bimolecular reaction. The values of the second-order rate constant were estimated to be $(2.34 \pm 0.62) \times 10^7\ M^{-1}\ s^{-1}$ for semihemoglobin α (Fig. 5A inset) and $(5.19 \pm 0.63) \times 10^7\ M^{-1}\ s^{-1}$ at 5 °C for semihemoglobin β (Fig. 5C inset).

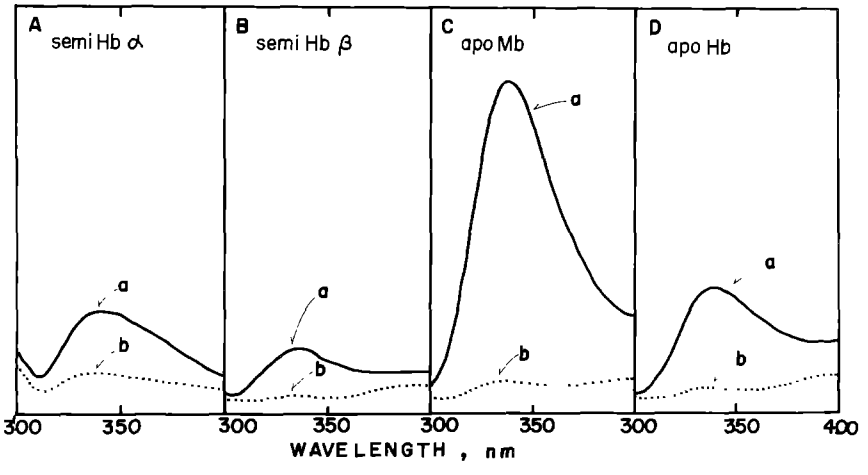


Fig. 2. Fluorescence spectra of semihemoglobins α (**A**) and β (**B**), apomyoglobin (**C**), and apohemoglobin (**D**) before (spectra a) and after (spectra b) mixing with equimolar amounts of hemin dicyanide. Concentrations of globins were 4.0 μM. The ordinate shows arbitrary unit of intensity

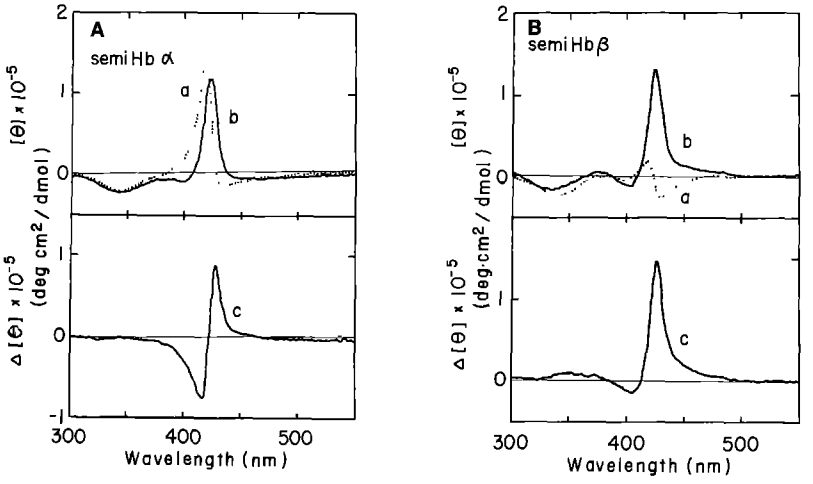


Fig. 3. CD spectra of semihemoglobins α (**A**) and β (**B**) before (spectra a) and after (spectra b) mixing with equimolar amounts of hemin dicyanide, and the difference CD spectra between them (spectra c = b-a). Each spectrum was the average of eight runs. Concentrations of semihemoglobins α and β were 6.35 μM and 6.95 μM, respectively

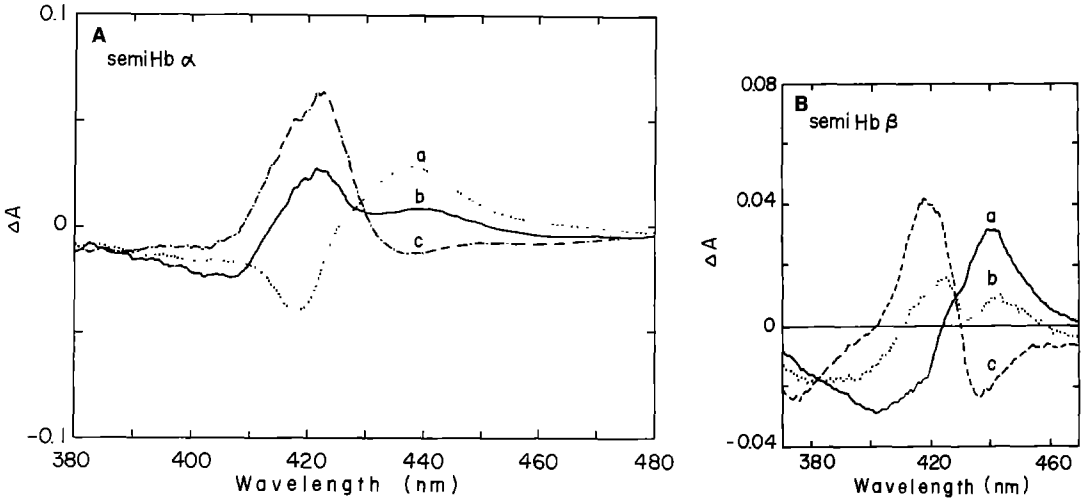


Fig. 4 A, B. Absorption spectra of semihemoglobins after mixing with equimolar amounts of hemin dicyanide at 15 °C. From each spectrum, the spectra of the corresponding semihemoglobin and hemin dicyanide were subtracted. (**A**) Semihemoglobin α. Concentration was 3 μM after the mixing. The spectra were obtained at the following times after the mixing. trace a, 200 ms; trace b, 30 s; trace c, 300 s. (**B**) Semihemoglobin β. Concentration was 2 μM after the mixing. The spectra were obtained at the following times: trace a, 60–225 s; trace b, 310–475 s; trace c, 1800–1965 s after the mixing

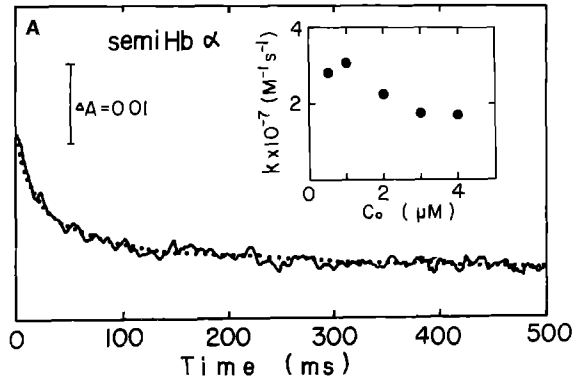
On the other hand, the time course of the absorption increase at 15 °C (Fig. 5B and 5D) obeyed first-order kinetics. The first-order rate constants were determined at different concentrations, and the mean values were found to be $(1.34 \pm 0.10) \times 10^{-2}$ s^{-1} for semihemoglobin α (Fig. 5B inset) and $(2.99 \pm 0.16) \times 10^{-3}$ s^{-1} for semihemoglobin β (Fig. 5D inset).

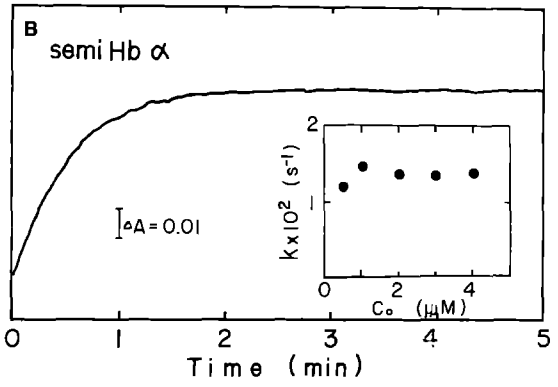
The time courses of fluorescence quenching of semihemoglobins α and β after mixing with hemin dicyanide were measured with a stopped-flow apparatus. Though traces of the quenching were obtained at 15 °C for semihemoglobin α and at 5 °C for semihemoglobin β (data not shown), accurate rate constants could not be determined. This was because the intensities of the quenching were very small, as judged by comparing those for apomyoglobin and apohemoglobin (Fig. 2), so that the signal-to-noise ratio of the stopped-flow traces was very low. However, the apparent rates of the quenching were almost comparable to the corresponding rates of the traces for the decrease in the absorption (Fig. 5A, C). In the case of semihemglobin α, we observed slow quenching at 15 °C (Fig. 6), which followed a first-order rate law with a rate constant of $(1.14 \pm 0.18) \times 10^{-2}$ s^{-1} (Fig. 6 inset).

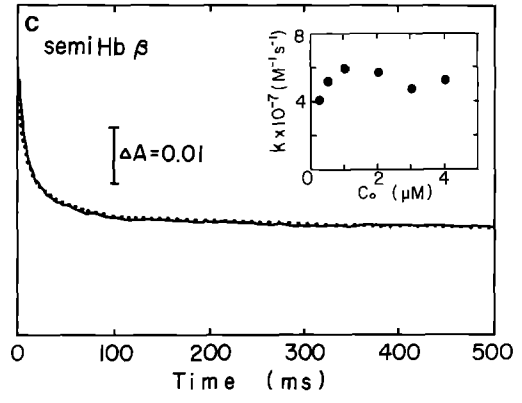
Figure 7 shows time courses of the Soret CD change of semihemoglobins α and β after mixing with equimolar amounts of hemin dicyanide at 15 °C. The increase in the Soret CD for semihemiglobin α obeyed first-order kinetics, with a rate constant estimated to be $(4.99 \pm 0.27) \times 10^{-3}$ s^{-1} (Fig. 7A inset). In the case of semihemoglobin β, there were rapid and slow phases (Fig. 7B). The rate constants were roughly estimated as 20–40 s^{-1} for the rapid phase and $(1-3) \times 10^{-3}$ s^{-1} for the slow phase. More accurate values could not be obtained because of the poor signal-to-noise ratio.

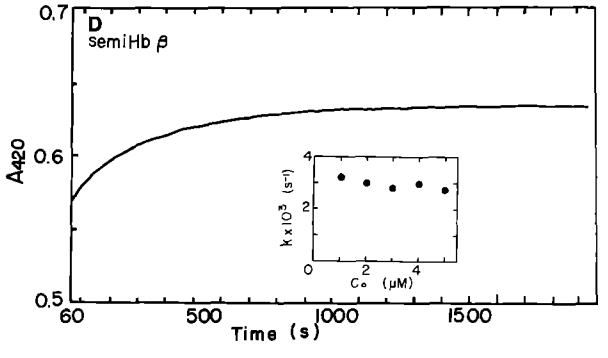
Discussion

On the basis of the above results, a plausible mechanism for the reconstitution of hemoglobin upon heme binding was proposed as follows (Table 1).









1. First step: hemin dicyanide enters a hydrophobic region of the apo chain

In the first step, hemin dicyanide enters a hydrophobic region of the apo chain, the so called 'heme pocket'. This step is the second-order reaction, which was detected by the decrease in the absorption (Fig. 5A, C). The rate constant obtained was different for semihemoglobin α and semihemoglobin β (2×10^7 M^{-1} s^{-1} at 15 °C for the former and 5×10^7 M^{-1} s^{-1} at 5 °C for the latter). When hemin enters the heme pocket, the interaction between the hemin and surrounding hydrophobic residues in the pocket causes a red-shift in the absorption maxima (Fig. 4). Similar spectral behavior was observed when heme derivatives were incorporated into hydrophobic phases such as liposomes (Rose and Olson 1983; Cannon et al. 1984). In this step, the fluorescence of Trp in the vicinity of the heme pocket may be quenched.

Table 1. Rate constants obtained by different probes (at pH 9.1 and 15 °C). F, A and CD are fluorescence, absorption and CD, respectively. Their subscripts represent wavelengths of the measurements

	Semihemoglobin α		Semihemoglobin β	
Step	Probe	Rate constant	Probe	Rate constant
1	A_{422}, F	2×10^7 M^{-1} s^{-1}	A_{420}, F	5×10^7 M^{-1} s^{-1} *
2	–	–	CD_{429}	20–40 s^{-1}
3	A_{422}, F, CD_{427}	$(5-10) \times 10^{-3}$ s^{-1}	A_{420}, CD_{429}	$(1-3) \times 10^{-3}$ s^{-1}

* Measured at 5 °C

Fig. 5A–D. Time courses of absorption changes after mixing semihemoglobins and equimolar amounts of hemin dicyanide. **(A)** Rapid phase for semihemoglobin α at 422 nm and 15 °C. Concentration was 2 μM after the mixing. Dotted line shows the best fit to a second-order reaction with a rate constant of 2.28×10^7 M^{-1} s^{-1}. Inset: Relationship between the concentration and the second-order rate constant obtained from absorption stopped-flow traces. The average value of the rate constant was $(2.34 \pm 0.62) \times 10^7$ M^{-1} s^{-1}. **(B)** Slow phase for semihemoglobin α at 422 nm and 15 °C. Concentration was 2 μM after the mixing. Inset: Relationship between the concentration and the first-order rate constant obtained from the absorption stopped-flow traces. The average value of the rate constant was $(1.34 \pm 0.10) \times 10^{-2}$ s^{-1}. **(C)** Rapid phase for semihemoglobin β at 420 nm and 5 °C. Concentration was 2 μM after the mixing. Dotted line shows the best fit to a second-order reaction with a rate constant of 5.77×10^7 M^{-1} s^{-1}. Inset: Relationship between the concentration and the second-order rate constant obtained from the absorption stopped-flow traces. The average value of the rate constant was $(5.19 \pm 0.63) \times 10^7$ M^{-1} s^{-1}. **(D)** Slow phase for semihemoglobin β at 420 nm and 15 °C. Concentration was 2 μM after the mixing. Inset: Relationship between the concentration and the first-order rate constant obtained from the absorption stopped-flow traces. The average value of the rate constant was $(2.99 \pm 0.16) \times 10^{-3}$ s^{-1}

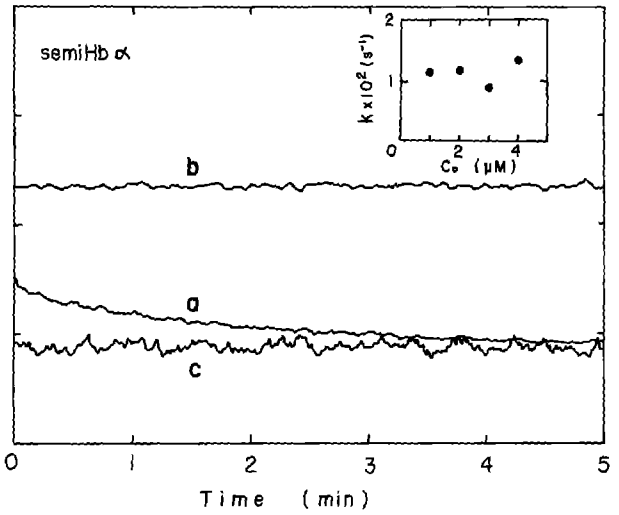


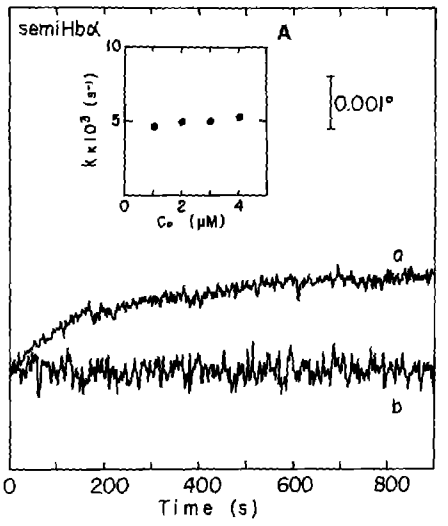
Fig. 6. Time courses of fluorescence quenching after mixing semihemoglobin α and hemin dicyanide. Concentration was 3 μM after mixing at 15 °C. Trace a, time course at 0–5 min after the mixing; trace b, initial level taken by mixing semihemoglobin α and buffer; trace c, final level of the time course. The ordinate shows arbitrary units of intensity. Inset: Relationship between the concentration and the first-order rate constant obtained from the fluorescence stopped-flow traces. The average value of the rate constant was $(1.14 \pm 0.18) \times 10^{-2}\ s^{-1}$

2. Second step: the partial structure is changed

The second step was observed in semihemoglobin β, but not in semihemoglobin α. An apparent first-order rate constant of 20–40 s^{-1} was obtained from the data for the Soret CD stopped-flow experiment (Fig. 7B). The Soret CD of hemoglobin is due to coupling between the $\pi-\pi^*$ transitions of the porphyrin ring and the electronic and magnetic dipole transitions of the surrounding aromatic amino acid residues (Hsu and Woody 1971). It can be presumed that in the second step the tertiary structure of semihemoglobin β is changed at least partly, and this induces the rearrangement of the interaction between the heme and aromatic side-chains. Such a structural change may not occur in semihemoglobin α. The difference between the two proteins will be discussed later.

3. Third step: the ligand replacement of CN^- *by the proximal* His

This step was monitored by Soret absorption changes, whose rate constant was $1 \times 10^{-2}\ s^{-1}$ for semihemoglobin α (Fig. 5B) and $3 \times 10^{-3}\ s^{-1}$ for semihemoglobin β (Fig. 5D), respectively. It is most likely that this process is the ligand replacement of CN^- by the imidazole ring of the proximal His in the fifth coordination position of iron, since (a) there is a difference in the Soret absorption between hemin dicyanide and hemin monocyanide bound to the imidazole ring (Yoshikawa et al. 1985), (b) two phases were observed in the Soret absorption change and one of them was already attributed to the first step. The small values of the rate constant would be attributed to the strong affinity of CN^-.



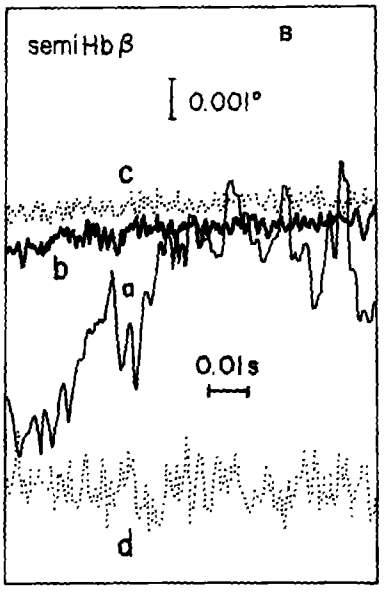


Fig. 7A,B. Time courses of CD increase after mixing of semihemoglobins and equimolar amounts of hemin dicyanide at 15 °C. **(A)** Time course of semihemoglobin α at 427 nm (trace a). Trace b was the initial level taken by mixing semihemoglobin α and buffer. Concentration was 2 μM after the mixing. Response time for trace a was 1 s. Inset: Relationship between the concentration and the first-order rate constant obtained from the CD stopped-flow traces. The average value of the rate constant was $(4.99 \pm 0.27) \times 10^{-3}\ s^{-1}$. **(B)** Time courses of semihemoglobin β at 429 nm. Traces a and b were obtained at 0–100 ms, and 0–300 s, respectively, after the mixing; trace c, final level of the time course; trace d, the initial level taken by mixing semihemoglobin β and buffer. Each trace was the average of 16 runs. Concentration was 5 μM after the mixing. Response times for traces a and b were 1 ms and 1 s, respectively

In the third step, the slow phase of fluorescence quenching was observed in semihemoglobin α (Fig. 6) but not in semihemoglobin β, whereas the fluorescence quenching in the first step was observed in the two proteins. Human hemoglobin contains Trp on the A helix segment (Trp A12) in both the α and the β chains, and an additional Trp exists on the C helix segment in the β chain (Trp C3) (Perutz 1987). It can be considered that the fluorescence quenching observed in the third step for semihemoglobin α accounts for Trp C3 in the β chain.

This idea is supported by our previous observation (Kawamura-Konishi et al. 1988) in the reconsitution reaction of myoglobin from apomyoglobin and hemin dicyanide that the fluorescence of two Trp in the A helix segment was quenched only in the first step. Consequently, the ligand replacement of CN^- by the proximal His induces the conformational change, including the C helix segment in the β chain of semihemoglobin α.

Soret CD increase was observed in semihemoglobins α and β (Fig. 7). The Soret CD of hemoglobin is ascribed to the interaction between the heme and the surrounding aromatic amino acid residues (Hsu and Woody 1971). The interaction is provided not only by the heme binding to each chain but als by $\alpha 1\beta 1$ contacts (Kawamura et al. 1982; Kawamura and Nakamura 1983; Mawatari et al. 1983). It is therefore plausible that the ligation of heme to the proximal His would cause significant conformational changes of the chains, resulting in an increase in the $\alpha 1\beta 1$ interaction. At this stage, reconstituted hemoglobin achieves its final conformation.

Freshly reconstituted hemoglobin with protoheme is known to be a mixture of isomers possessing the two heme orientations differing by a 180 rotation about the α, γ-meso axis, the result being defined as either the "native" or the "disordered" heme orientation (La Mar et al. 1983; 1984). The heme orientation depends not only on the heme substituents but also on the chains. The proportion of native orientation relative to disordered orientation in the reconstitution reaction is 10:0 for the β chain in semihemoglobin α and 6:4 for the α chain in semihemoglobin β (Ishimori and Morishima 1988). The disordered states equilibrate with time to form the native structure which possesses a significant degree of orientationally disordered heme. The reorientation rates of the protohemin of α and β chains are about $8.3 \times 10^{-5}\ s^{-1}$ and $2.8 \times 10^{-4}\ s^{-1}$, respectively, at pH 9.1 and 25 °C (Yamamoto and La Mar 1986). In the case of metcyanide derivatives, its reorientation rate is much smaller (La Mar et al. 1984). It is shown that the reorientation process accompanies an increase in the Soret CD (Aojula et al. 1986; Light et al. 1987). We observed the Soret CD increase with rate constants of $5 \times 10^{-3}\ s^{-1}$ for semihemoglobin α and $20-40\ s^{-1}$ and $(1-3) \times 10^{-3}\ s^{-1}$ for semihemoglobin β at 15 °C. As judged from the rate constants, it is concluded that the CD changes observed are not related to the reorientation process.

Leutzinger and Beychok reported the kinetics of heme binding to isolated α and β globin chains (Leutzinger and Beychok 1981). In their results, the α and β chains combined with heme with almost the same rate constant ($3.3 \times 10^7\ M^{-1}\ s^{-1}$ for the former and $3.4 \times 10^7\ M^{-1}\ s^{-1}$ for the latter at 4 °C). These values were measured by fluorescence quenching. The value is comparable to that of semihemoglobin β ($5.19 \times 10^7\ M^{-1}\ s^{-1}$ at 5 °C as shown in Fig. 5C inset). However, the rate constant of semihemoglobin α is much smaller ($2.34 \times 10^7\ M^{-1}\ s^{-1}$ at 15 °C as shown in Fig. 5A inset). This result shows that the complementary chain having heme affects the protein conformation of the apo β chain in semihemoglobin α, but this is not the case for the apo α chain in semihemoglobin β. It is likely that heme is more easily inserted into the apo chain with the flexible conformation. In other words, semihemoglobin α would retain a more rigid and organized structure and more closely approach its final structure than semihemoglobin β. This view agrees with the following observation; (a) The second step was observed in semihemoglobin β but not in semihemoglobin α, that is, some conformational change in semihemoglobin β is required for the formation of Fe-His bonding but not in semihemoglobin α. (b) In the case of the reconsitution of hemoglobin from apohemoglobin and caffeine hemin (Kawamura-Konishi and Suzuki 1985), hemin binding to the α chain caused a conformational change of the β chain, resulting in the β chain having a greater affinity for hemin than before.

Semihemoglobins α and β already have a highly folded structure before the heme binding, judging from the results of CD spectra in the far-UV region (data not shown); (a) the far-UV CD spectra of the two proteins were almost the same as the spectrum of native hemoglobin, and (b) they were not changed on heme binding. The secondary structure of the α and β chains are similar to each other, but helix D is absent in the α chain (Perutz et al. 1968). We previously suggested (Kawamura-Konishi et al. 1988) an important role of helix D in the folding of the myoglobin molecule on the basis of the folding pathway presented by Lim and Efimov (1977). The flexible structure of apo α chain in semihemoglobin β, as suggested above, would be attributable to the absence of helix D.

It is noted that native hemoglobin is in equilibrium with $\alpha\ \beta$ dimer and $\alpha_2\beta_2$ tetramer. As there is no difference in the Soret CD and Soret absorption between them, our experimental data did not provide any information regarding the assembly. However, it seems likely that the dimers having heme on both the chains assemble to form the tetramer at or after the third step.

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