Effect of Reversed Heme Orientation on Circular Dichroism and Cooperative Oxygen Binding of Human Adult Hemoglobin[†]

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ABSTRACT: We found that recombinant human adult hemoglobin (rHb A) expressed in *Escherichia coli* showed heterogeneity of components with the intensity of a positive CD band at 260 nm and that it could be resolved into three components (SP-1, SP-2, and SP-3) by SP-Sepharose column chromatography. ^{1}H NMR revealed that SP-1 is identical with native Hb A, while SP-2 and SP-3 largely contain the reversed heme isomer in both the α and β subunits, with contents of ~50 and >80% in SP-2 and SP-3, respectively. Rotation of the heme 180° about the 5,15-meso axis (reversed heme) causes an interexchange of the methyl groups at positions 2 and 7 with the vinyl groups at positions 8 and 3, respectively. To examine the effect of the modification of the heme–protein contact on the structure and function of Hb A, we compared the ^{1}H NMR, CD, and oxygen binding properties of the three components with those of native Hb A. Native Hb A exhibits a distinct positive CD band in both the near-UV and Soret regions, but rHb A with reversed heme exhibits a very weak positive CD band at 260 nm and a prominent negative CD band in the Soret region. Cooperativity, as measured by Hill's *n* value, decreased from 3.18 (SP-1) to 2.94 (SP-2) to 2.63 (SP-3) with an increase in the reversed heme orientation. The effect of an allosteric effector, inositol hexaphosphate (IHP), on the oxygen binding properties was also reduced in rHb A with reversed heme. These results indicate that changes in the heme–globin contact exert a discernible influence on CD spectra and cooperative oxygen binding.

We have studied the circular dichroism (CD)[1] spectra of human adult hemoglobin (Hb A) as a probe for elucidating the relationship between its structure and function (*1–7*). Natural mutant hemoglobins (abnormal Hbs) have provided us with valuable information about amino acid residues which are critical for physiological function (*8*). Engineered mutants of Hb A with the desired substitutions are expected to enable us to understand a more detailed molecular basis of allosteric properties. An expression system of Hb A in

Escherichia coli harboring a plasmid (pHE7) developed by Ho's group (*9*) can produce sufficient amounts of soluble recombinant Hb A (rHb A) required for biochemical and biophysical studies. We expressed the rHb A in *E. coli* using the same system and purified it from the lysate through three successive steps of Sepharose fast-flow column chromatography. As reported previously, rHb A was eluted into two major peaks at the final purification step (*10*). We found that ellipticities of the positive CD band at 260 nm of the two peaks were greatly different from each other; the first peak gave the same CD value as that of native Hb A, while the second peak exhibited a CD value of one-half of the first one. Moreover, the latter fractions of the second peak showed a further decrease in the CD band at 260 nm (approximately $^1/_4$–$^1/_5$ of native Hb A). By modifying the slope of the pH gradient for SP-Sepharose column chromatography, we were able to resolve rHb A into three components with different CD spectra (SP-1, SP-2, and SP-3). The ^{1}H NMR study revealed that the SP-3 fraction contained mostly reversed heme in both the α and β subunits. As shown in Figure 1, rotation of the heme about the 5,15-meso axis (reversed heme) interexchanges the methyl groups at positions 2 and 7 with the vinyl groups at positions 8 and 3, respectively. This modulates the heme methyl and vinyl peripheral contact with globin which is considered to be important for the transmission of oxygen binding information from one subunit to another (*11–13*).

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[1] Abbreviations: CD, circular dichroism; Hb A, human adult hemoglobin; rHb, recombinant Hb; Mb, myoglobin; PEI, polyethyleneimine; TETA, triethylenetetraamine; IPTG, isopropyl β-thiogalactopyranoside; CO, carbon monoxide; IHP, inositol hexaphosphate.
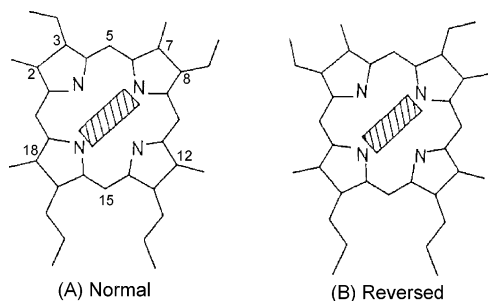
FIGURE 1: Two possible orientations of heme (protoporphyrin IX−Fe complex) relative to the proximal-His F8 plane: (A) normal found in the X-ray of Hb A (*49*) and (B) reversed with the heme rotated 180° about the 5,15-meso axis from that of panel A. The cross-hatched rectangle indicates the orientation of the proximal-His F8 imidazole plane. The nomenclature of heme approved by the IUPAC-IUB Joint Commission (*50*) was adopted to label the heme side chains; i.e., protoporphyrin IX possesses methyl groups at positions 2, 7, 12, and 18, vinyl groups at positions 3 and 8, and propionate groups at positions 13 and 17.

The heme rotational disorder in reconstituted Hb from apoglobin and hemin has been extensively characterized by ^{1}H NMR spectroscopy (*14−18*). The presence of an analogous heme disorder is also known to exist in the rHb A produced in *E. coli* (*9*). Naturally occurring heme rotational disorder was found in yellowfin tuna Mb (*19*), *Chironomus* Hb (*20*), and even in human Hb A (*14*). The changes in CD in the Soret region and oxygen binding properties were reported using reconstituted Mb with a 1:1 mixture of normal and reversed heme (*21−23*). However, there is no report on the changes of Mb carrying only the reversed heme. Furthermore, the CD and oxygen binding properties have not been examined even in reconstituted Hb A with a mixture of normal and reversed heme.

In this paper, we describe a method for separating rHb A with reversed heme from that with normal heme and comparing CD and oxygen binding properties of rHb A with reversed heme to those of Hb A with normal heme. This is the first report on the ^{1}H NMR, CD, and oxygen binding properties of Hb A with most of the heme in the reversed form in both the α and β subunits.

EXPERIMENTAL PROCEDURES

Hemoglobin. Hb A was purified from human hemolysate by preparative isoelectric focusing (*24*). The Hb concentration was determined spectrophotometrically after conversion into pyridinehemochromogen in 25% pyridine (v/v) and 0.1 M aqueous sodium hydroxide in the presence of sodium dithionite. Preparation of apoglobin and reconstitution with hemin were carried out as described previously (*14*).

Preparation of Recombinant Hemoglobin. The Hb A expression plasmid pHE7 (*9*) containing the genes for human α- and β-globin and the *E. coli* methionine aminopeptidase was kindly provided by C. Ho of Carnegie Mellon University (Pittsburgh, PA). This plasmid was transformed into *E. coli* JM109. *E. coli* cells harboring the plasmid were grown at 30 °C in a TB medium (*9*). Expression of rHb A was induced by adding isopropyl β-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. This culture was then supplemented with hemin (30 mg/L) and glucose (15 g/L), and the growth was continued for another period of 5 h at

32 °C. The cells were harvested by centrifugation and stored frozen at −80 °C until they were needed for purification.

Recombinant hemoglobin was isolated and purified according to the method of Looker et al. (*25*) with some modifications. Approximately 70 g of frozen cell paste was routinely used as the starting material for the purification of rHb A. The thawed cell paste was suspended in a lysis buffer at a concentration of 3 mL/g of cells. The cell suspension was treated with lysozyme (1 mg/g of cells) and DNase I (30 μg/mL in 10 mM $MgCl_2$ and 1 mM $MnCl_2$) on ice to disrupt the cells. The cell lysate was saturated with carbon monoxide (CO) gas and stirred overnight in a cold room. The following procedures were carried out at 4 °C or on ice unless otherwise specified. After centrifugation at 20000*g* for 45 min, the supernatant was treated with polyethylene-imine (PEI) at a final concentration of 0.3% to precipitate nucleic acids. The PEI-treated lysate was centrifuged at 20000*g* for 15 min. The supernatant was recovered, concentrated in an Amicon stirred cell concentrator, and dialyzed against 20 mM Tris-HCl and 0.1 mM triethylenetetraamine (TETA, pH 7.4) at 4 °C.

For the purification of rHb A, three successive chromatographic steps were employed (*25*). Before being loaded onto each column, the samples were always saturated with CO gas to convert the protein to the CO form which can be identified by the characteristic absorption at 569 nm. As the equilibration buffer of the column was not saturated with CO gas, Hb eluted from the column changed from the CO form to the oxy form, which exhibits its absorption peak at 576 nm, when bound to Sepharose. The first column, a Q-Sepharose fast-flow column (2.5 cm × 20 cm), was equilibrated with 20 mM Tris-HCl and 0.1 mM TETA (pH 7.4) at 4 °C. This step captured a large amount of bacterial proteins and the remaining nucleic acid while rHb A passed through the system. The rHb A fraction was collected, concentrated, and passed through a Sephadex G-25 column equilibrated with 20 mM Tris-HCl (pH 8.3) at 4 °C (Q_1 fraction). The second column, a Q-Sepharose fast-flow column (1.5 cm × 17 cm), was equilibrated with 20 mM Tris-HCl (pH 8.3) at 4 °C. After the column was loaded with the Q_1 fraction and then washed with equilibration buffer, the bound rHb A was eluted with a linear gradient (total of 300 mL) from 0 to 160 mM NaCl in equilibration buffer. Recombinant Hb A-containing fractions were pooled, concentrated, and passed through a Sephadex G-25 column equilibrated with 10 mM sodium phosphate buffer (pH 7.2) at 4 °C (Q_2 fraction). The third column, an SP-Sepharose fast-flow column (1.5 cm × 47 cm), was equilibrated with 10 mM sodium phosphate buffer (pH 7.2) at 4 °C. After the column was loaded with fraction Q_2 and then washed with equilibration buffer, a linear gradient (total volume of 400 mL) of equilibration buffer versus 10 mM sodium phosphate buffer (pH 8.0) at 4 °C was used to elute rHb A.

^{1}H NMR Measurements. These were performed at 25 °C with a Bruker Avance 400 FT NMR spectrometer operating at a ^{1}H frequency of 400 MHz. The chemical shifts of ^{1}H NMR spectra are given in parts per million relative to sodium 2,2′-dimethyl-2-silapentane-5-sulfonate (DSS), with residual H[2]HO as an internal reference. Just before the NMR measurements, all of the Hb samples were converted to the met−azido complex from the CO complex and concentrated

to approximately 1 mM (on a heme basis) with a Diaflo membrane (*14*, *15*).

CD Measurements. These were carried out with a Jasco J-820 spectropolarimeter at 25 °C using a (+)-10-camphorsulfonic acid calibration. Absorption spectra were measured with a double-beam spectrophotometer (model U-3010, Hitachi, Tokyo, Japan).

Oxygen Equilibrium Experiments. Oxygen equilibrium curves were determined by using an automatic oxygenation apparatus (*26*, *27*). Oxygen affinity (P_{50}) and cooperativity (Hill coefficient) were calculated from the best-fit stepwise Adair constants that were evaluated from the equilibrium curve by a nonlinear least-squares method (*26*, *28*). The spectrophotometer used for the apparatus was a double-beam spectrophotometer (model U-4000, Hitachi). The wavelength of the detection light was 560 nm. The hemoglobin concentration was 60 μM on a heme basis. The buffer was 0.05 M bis-Tris (pH 7.4) containing 0.1 M Cl$^-$. The temperature within the oxygenation cell was maintained at 25 ± 0.1 °C. To minimize the autoxidation of hemoglobin during measurement, an enzymatic metHb reducing system (*29*) together with catalase and superoxide dismutase (*30*, *31*) was added to each sample. The metHb content of each sample, determined after oxygen equilibrium measurement, ranged from 0.9 to 5.5% of the total Hb.

RESULTS

Separation of rHb A into Three Components by SP-Sepharose Column Chromatography. Under the original conditions (*25*) where a linear gradient of 10 mM sodium phosphate buffer (pH 6.8) versus 20 mM sodium phosphate buffer (pH 8.3) was used to elute the Hb on an SP-Sepharose column, the expressed rHb A usually gave an elution profile of absorbance at 576 nm with two major peaks. However, examination by CD at 260 nm has revealed that the two major peaks consist of more than two Hb components. After several attempts, we chose a linear gradient of 10 mM sodium phosphate buffer (pH 7.2) at 4 °C versus 10 mM sodium phosphate buffer (pH 8.0) at 4 °C to resolve the Hb components efficiently. Under this condition, rHb A usually exhibited an elution profile with two major peaks and a shoulder or a small peak.

Figure 2 shows an example of the elution profile from SP-Sepharose column chromatography. When the eluate was monitored by absorbance at 576 nm, rHb A was eluted at two major peaks and a small peak. The intensity of the positive CD band at 260 nm that is known to be derived from the L band of the heme group changed greatly among these peaks: high ellipticity in the first peak, gradually decreased ellipticity in the second peak, and very low ellipticity in the last small peak. To normalize the ellipticity at 260 nm, we chose to normalize the absorbance at 576 nm (A_{576}) as a measure of ellipticity/[heme]. In the inset of the figure, the ratio of the intensity of the positive CD band at 260 nm and absorbance at 576 nm (CD_{260}/A_{576}) is plotted as a dotted line together with the elution profile of absorbance at 576 nm, clearly indicating that the expressed rHb A consists of at least three components. On the basis of the CD_{260}/A_{576} ratio, the fractions were divided into three as shown in the inset. The first fraction exhibited a high ellipticity ratio ($CD_{260}/A_{576} \sim 50$). On the other hand, the
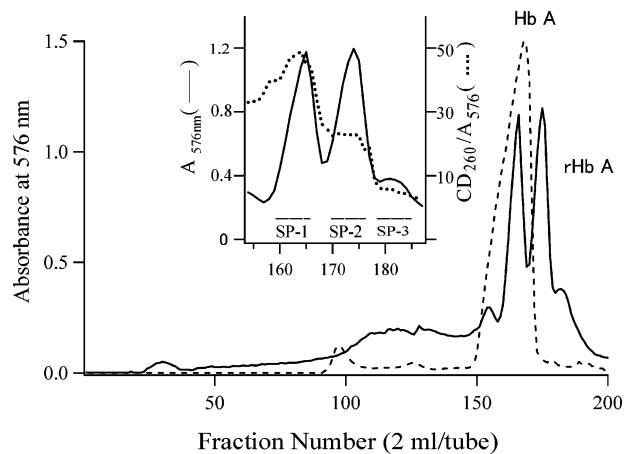

FIGURE 2: Elution profile of rHb A through an SP-Sepharose column in comparison with that of native Hb A. The SP-Sepharose column was equilibrated with 0.01 M sodium phosphate buffer (pH 7.2), and Hb was eluted with a pH gradient between pH 7.2 (0.01 M sodium phosphate buffer) and pH 8.0 (0.01 M sodium phosphate buffer): (—) absorbance at 576 nm of rHb A, (- - -) that of native Hb A, and (···, inset) ratio between the CD band intensity at 260 nm and the absorbance at 576 nm (CD_{260}/A_{576}) of rHb A. The fractions with CD_{260}/A_{576} values of 45−50 were collected as SP-1. The CD_{260}/A_{576} values of the fractions collected as SP-2 were $\sim$25 and those of the fractions collected as SP-3 less than 10. Native Hb A was purified from hemolysate via the same procedure that was used for rHb A.

Table 1: Masses (daltons) of the α and β Subunits in SP-1, SP-2, and SP-3 Fractions and Native Hb A[a]

hemoglobin	α subunit	β subunit
SP-1	15 125.7	15 866.0
SP-2	15 125.5	15 865.8
SP-3	15 125.6	15 865.9
Hb A (theoretical)	15 125.9	15 866.3

[a] The mass of each subunit was determined with an ESI-FT ion cyclotron resonance mass spectrometer, after removal of the heme, and was based on the most abundant ion in the resolved isotopic cluster.

second fraction gave a halved ellipticity ratio ($CD_{260}/A_{576} \sim$ 25). The third fraction exhibited a markedly diminished CD band at 260 nm ($CD_{260}/A_{576} < 10$). These three fractions were designated SP-1, SP-2, and SP-3, respectively, and all three were confirmed to be tetramers consisting of two α and two β subunits by Sephadex G-75 gel filtration and by SDS−PAGE. As shown in Table 1, the masses of all these fractions were consistent with those from native Hb A, indicating that there is neither amino acid substitution nor post-translational modification in the SP-1, SP-2, and SP-3 fractions. Although Shen et al. (*9*) reported that some fractions that eluted via Mono-S column chromatography contained an extra-Met residue at the N-termini even though the expression plasmid contained the methionine aminopeptidase gene, the three fractions presented here (SP-1, SP-2, and SP-3) had no extra-Met residue on the basis of the mass spectroscopic analysis (Table 1). They cultured the transformed *E. coli* at 30 °C before and after induction with IPTG. We cultured the transformed *E. coli* at 30 °C before induction but at 32 °C after induction as recommended by C. Ho to remove Met more efficiently (personal communication). There are several small rHb fractions before the three main components (Figure 2). It is possible that these fractions contain rHb A with an extra-Met residue at the N-termini.
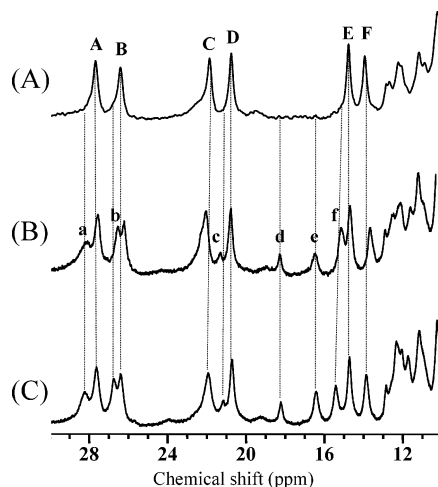
FIGURE 3: Hyperfine-shifted region of the 400 MHz ^{1}H NMR spectra of the met−azido derivatives of native Hb A (A), Hb A reconstituted from apoglobin and hemin (B), and rHb A before SP-Sepharose column chromatography (Q$_2$ fraction) (C), in a 90% H$_2$O/10% ^{2}H$_2$O mixture at pH 7.0 and 25 °C. Resolved signals A−F and a−f have been assigned as follows: heme 12-CH$_3$ (A), 2-CH$_3$ (C), and 18-CH$_3$ (E) for the normally oriented heme of the α subunit, heme 12-CH$_3$ (B), 2-CH$_3$ (D), and 18-CH$_3$ (F) for the normally oriented heme of the β subunit, heme 18-CH$_3$ (a), 8-vinyl C$_α$H (c), and 12-CH$_3$ (e) for the reversed heme of the α subunit, and heme 18-CH$_3$ (b), 8-vinyl C$_α$H (d), and 12-CH$_3$ (f) for the reversed heme of the β subunit (*14, 32−35*).

In contrast to rHb A, native Hb A was eluted as a main peak via an SP-Sepharose column under the same conditions shown in Figure 2. Examination of the peak fractions by the CD ellipticity at 260 nm revealed that only the last two or three fraction tubes in the peak gave an intermediate intensity (CD$_{260}$/A$_{576}$ ∼ 25) similar to that of SP-2, whereas more than 90% of the fractions exhibited high levels of intensity (CD$_{260}$/A$_{576}$ ∼ 50).

Characterization of the Three Components of rHb A by ^{1}H NMR. It is well-known that the free heme is not optically active and can exhibit CD when incorporated into a chiral macromolecule. The variation of CD in the rHb A fractions suggested the possibility that it arose from a difference in the orientation of the heme group incorporated into the pocket of the globin. An early ^{1}H NMR study suggested the possible occurrence of a disordered heme in the rHb A expressed in *E. coli* (*10*). To examine the heme orientation, we measured the paramagnetic ^{1}H NMR spectra of SP-1, SP-2, and SP-3. The heme orientations in the individual subunits of Hb A are sensitively reflected in the heme methyl proton shifts of the paramagnetic met−azido form of Hb (*14, 15*). Therefore, the ^{1}H NMR spectra of the three components were measured just after the conversion of CO-rHb A to the met−azido form by photodissociation of CO in the presence of ferricyanide and sodium azide.

Figure 3 shows the hyperfine-shifted portions of the 400 MHz ^{1}H NMR spectra in the met−azido form of native Hb A (A), Hb A reconstituted from apoglobin and hemin (B), and partially purified rHb A (the fraction after the second Q-Sepharose column chromatography, Q$_2$ fraction) (C). As reported by La Mar et al. (*14*), in native Hb A two sets of heme methyl proton signals are resolved below 12 ppm, each set arising from the α or β subunits (Figure 3A). The set of peaks A, C, and E and that of peaks B, D, and F were assigned to heme methyl protons of the α and β subunits,
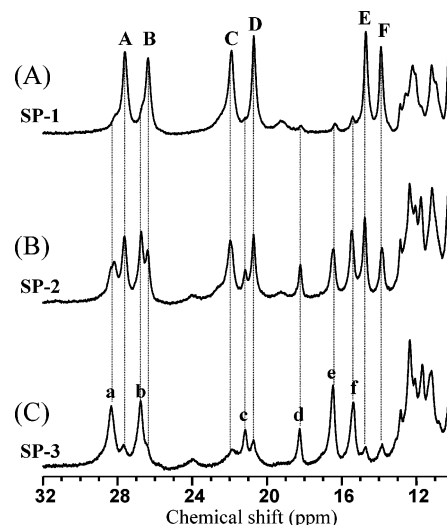

FIGURE 4: Hyperfine-shifted region of the 400 MHz ^{1}H NMR spectra of the met−azido derivatives of the SP-1 (A), SP-2 (B), and SP-3 (C) fractions of rHb A separated via SP-Sepharose column chromatography in a 90% H$_2$O/10% ^{2}H$_2$O mixture at pH 7.0 and 25 °C. Peak labeling is as described in the legend of Figure 3.

respectively (*14, 32*). The individual methyl protons of Figure 3A were further assigned to 12-CH$_3$ (A and B), 2-CH$_3$ (C and D), and 18-CH$_3$ (E and F) in each subunit (*14, 33*). ^{1}H NMR studies have revealed that in addition to native Hb A, the same metastable intermediate with heme rotated 180° about the 5,15-meso axis (reversed heme) is formed when apoglobin is reconstituted with either oxidized or reduced heme (*14, 15*). This reconstituted Hb A with "reversed" heme gives different proton signals (*14*); that is, in addition to the six peaks (A−F) in met−azido Hb A, six extra peaks (labeled a−f) appear, as shown in Figure 3B. From isotope labeling studies, it is known that peaks a and b are identified with 18-CH$_3$ and peaks e and f with 12-CH$_3$ of the α and β subunits with reversed heme (*34*). Peaks c and d were assigned to the 8-vinyl C$_α$H group of reversed heme of the α and β subunits by reconstitution with the 8-vinyl-perdeuterated hemin (*34, 35*). The ^{1}H NMR spectrum of partially purified rHb A (Q$_2$ fraction) (Figure 3C) bears a close resemblance to the hyperfine shift patterns exhibited by reconstituted Hb A (Figure 3B), implying that the rHb A expressed in *E. coli* contains the heme orientational heterogeneity.

Figure 4 shows the ^{1}H NMR spectra of the met−azido forms of SP-1 (A), SP-2 (B), and SP-3 (C). The spectrum of SP-1 (A) resembled that of native Hb A (Figure 3A), indicating that rHb A in this fraction had mostly the normally oriented heme. On the other hand, the spectra of SP-2 (B) and SP-3 (C) clearly exhibited peaks a−f, demonstrating that reversed heme coexisted with the normally oriented heme in these proteins. There are distinct differences in the hyperfine shifts between SP-2 and SP-3. These fractions exhibit increases in the intensities of peaks a−f with decreases in those of peaks A−F, and peaks a−f become more prominent in SP-3 than in SP-2. This implies that rHb A in SP-3 contains the reversed heme more abundantly than that in SP-2.

Table 2 shows the relative population of normally oriented and reversed hemes in these SP fractions roughly estimated from the area of peaks A, B, E, and F and peaks a, b, e, and f. In both of the α and β subunits of rHb A, ∼90% of the

Table 2: Relative Populations (percent) of Heme Orientations in the Three Fractions of rHb A (SP-1, SP-2, and SP-3) Estimated by ^{1}H NMR Spectroscopy[a]

hemoglobin fraction	α subunit		β subunit		tetramer	
	normal	reversed	normal	reversed	normal	reversed
SP-1	88	12	88	12	88	12
SP-2	59	41	39	61	49	51
SP-3	17	83	11	89	14	86

[a] The percentages of normally oriented and reversed hemes in the SP fractions were roughly estimated from the area of peaks A, B, E, and F and peaks a, b, e, and f of the ^{1}H NMR spectra in Figure 4.
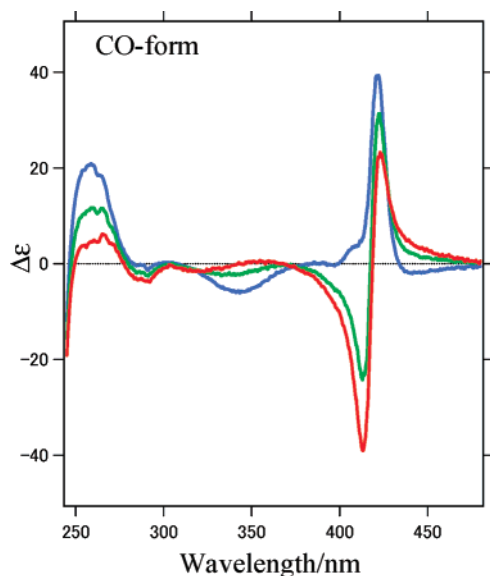

FIGURE 5: CD spectra of SP-1 (blue), SP-2 (green), and SP-3 (red) in the CO form. CD spectra of CO-Hb (50 μM in heme) in 0.05 M phosphate buffer (pH 7.0) were measured in a cell with a light path length of 2 mm. The scan speed was 50 nm/min, and 20 scans were averaged.

heme groups in SP-1 were normal whereas more than 80% of the heme groups in SP-3 were reversed. In addition, the SP-2 fraction contained an ~1:1 mixture of the two heme orientations in both subunits of the protein. Shen et al. (*10*) showed that in rHb A the degree of heme orientational heterogeneity in the α subunit was considerably smaller than that in the β subunit. The study presented here, however, reveals that both the α and β subunits of the protein were similar to each other in terms of the degree of heme orientational heterogeneity.

CD Spectra of rHb A Containing Reversed Heme. Figure 5 shows the CD spectra of the three rHb A components in the CO form from 245 to 480 nm. They exhibited different spectra both in the L band near 260 nm and in the B (Soret) regions. SP-1 showed a broad positive CD band near 260 nm and a prominent positive band at 422 nm, which were identical with those of native Hb A. SP-2 exhibited a halved band near 260 nm and a complex CD with a positive band at 423 nm and a negative band at 413 nm. In the spectrum of SP-3, a further decrease in the CD band near 260 nm was observed and the negative CD band became dominant in the Soret region. These results clearly indicate that rHb A with reversed heme displays a CD spectrum which is obviously different from that of native Hb A in both the near-UV and Soret regions.
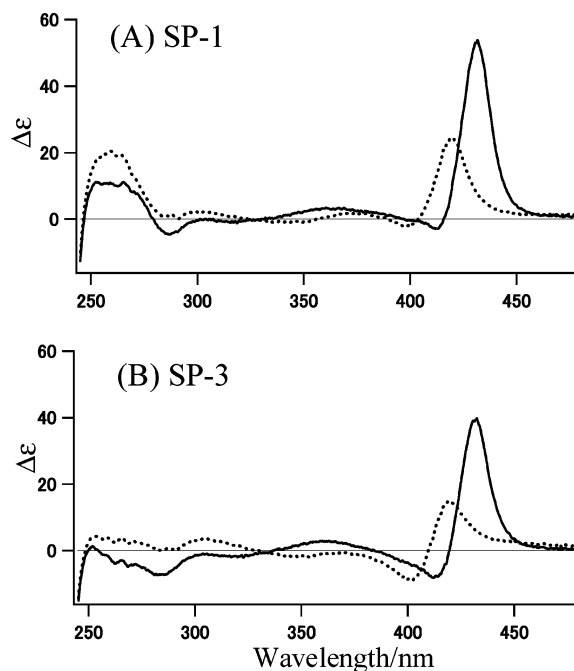

FIGURE 6: CD spectra of SP-1 (A) and SP-3 (B) in the deoxy (—) and oxy (···) forms. The experimental conditions were the same as those described in the legend of Figure 5.

The absorption spectrum of SP-3 was also different from that of SP-1. The absorption peaks of SP-1 in the CO form were observed at 419, 538, and 569 nm, exactly the same as those of native Hb A. On the other hand, SP-3 exhibited peaks at 417, 537, and 567 nm, shifted by 1−2 nm toward the blue. Except for these differences, the two absorption spectra were similar to each other.

Figure 6 shows the CD spectra of the SP-1 (A) and SP-3 (B) fractions in oxy and deoxy forms. The magnitudes of the CD bands near 260 nm of SP-3 in both the oxy and deoxy forms were very low in comparison with those of SP-1. Although changes in the CD spectra in the Soret region were less distinct than those of the CO form (Figure 5), both oxy and deoxy forms of SP-3 also exhibited an increase in the magnitude of the negative CD band with a decrease in the magnitude of the positive CD band.

As described above, the content of reversed heme was estimated from analysis of the ^{1}H NMR spectra to be 10% in SP-1 and 80% in SP-3. The CD spectrum of rHb A containing fully reversed heme was extracted by subtracting the contribution of rHb A with normal heme from the spectrum of SP-3.

Figure 7 shows the resultant spectra in CO (A), oxy (B), and deoxy (C) forms in comparison with the spectra of native Hb A. Native Hb A exhibited two prominent positive CD bands: one in the near-UV region and the other in the Soret region. On the other hand, the CO- and oxy-rHb A with only reversed heme almost totally lacked the positive band near 260 nm. The deoxy-rHb A showed a broad negative CD band at around 280 nm. It is known that the near-UV CD of Hb A shows a characteristic change upon the quaternary structure transition: from a small positive band in the R (relaxed) state to a distinct negative band at 287 nm in the T (tense) state. This change is ascribed to environmental alteration of aromatic residues (Trp and Tyr) in Hb A (*36*). As shown later, the SP-3 fraction exhibited significant cooperativity
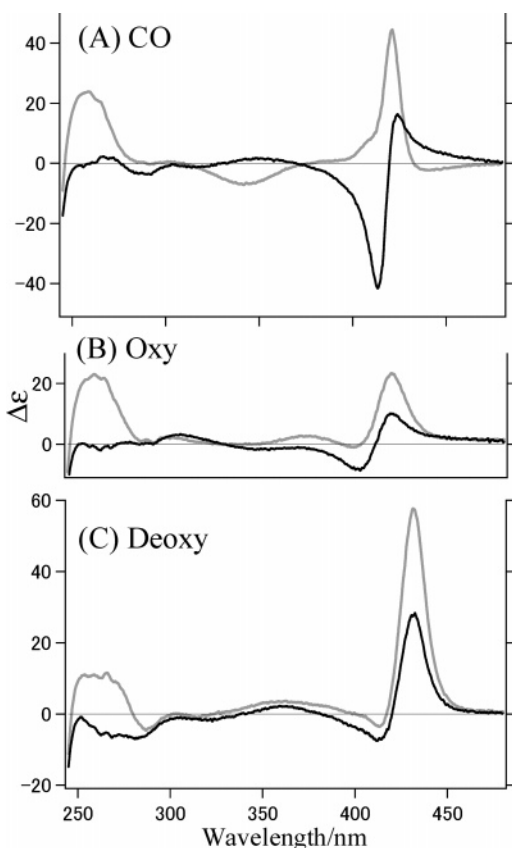
FIGURE 7: Comparison of the CD spectrum of rHb A having a fully reversed heme (black) with that of native Hb A (gray) in the CO (A), oxy (B), and deoxy (C) forms. The spectrum of rHb A having a fully reversed heme was calculated by subtraction of 20% of the normal heme contribution from the spectrum of SP-3.

in oxygen binding, indicating that this fraction was able to undergo the R → T quaternary structure transition. Therefore, it is very likely that the broad negative CD band in the deoxy form of rHb A with only reversed heme does not originate from the disorder of heme orientation but from the changes in the quaternary structure transition. In the Soret region, native Hb A gave a distinct positive CD band under any coordination state. On the other hand, rHb A with reversed heme exhibited two proximate bands of opposite sign. These two proximate bands varied depending on the coordination state: a prominent negative CD band with a small positive CD band in the CO derivative, negative and positive CD bands with similar intensity in the oxy form, and an extensive positive CD band with a small negative CD band in the deoxy form. So far, ^{1}H NMR has been used as the only spectral technique for characterization of heme rotational disorder. The results described above show that CD spectra in both the near-UV and Soret regions can possibly also be used for monitoring heme rotational disorder.

Oxygen Binding Properties. Figure 8 shows the Hill plots of oxygen binding by SP-1, SP-2, and SP-3 at pH 7.4 with and without an allosteric effector, inositol hexaphosphate (IHP), together with those of native Hb A for comparison. Table 3 summarizes the values of the O_2 binding parameters for these hemoglobins calculated from the best-fit stepwise Adair constants that were evaluated from the equilibrium curves of Figure 8. Although O_2 affinities, as measured by P_{50}, of SP-1, SP-2, and native Hb A were similar in the absence of IHP, SP-3 gave a slight increase in O_2 affinity.
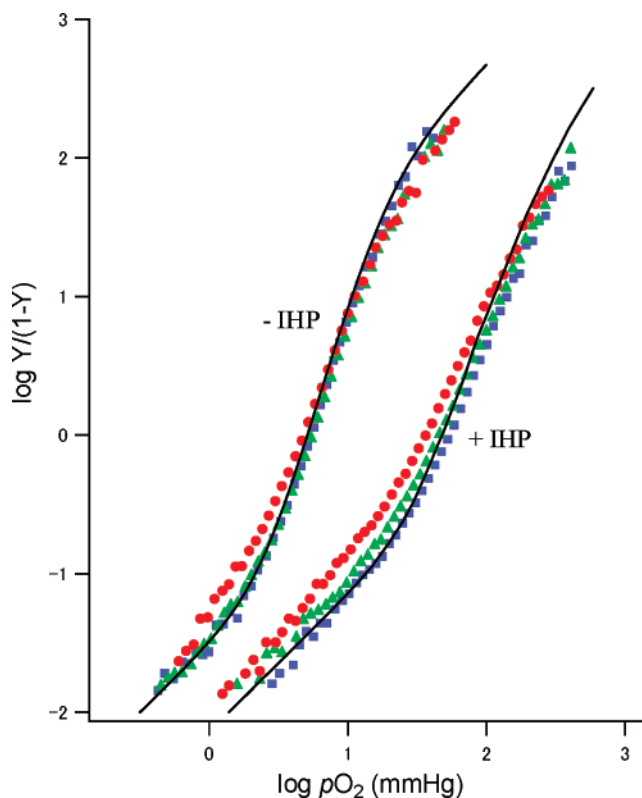

FIGURE 8: Hill plots of oxygen binding by SP-1 (blue squares), SP-2 (green triangles), SP-3 (red circles), and native Hb A (solid line) in the presence (right group of curves) and absence (left group of curves) of 2 mM IHP. Y is the fractional oxygen saturation and pO_2 the partial pressure of oxygen in millimeters of Hg. Symbols were the observed points, and the lines were calculated from the best-fit values of the four stepwise Adair constants (26, 28). The hemoglobin concentration was 60 μM on a heme basis in 0.05 M bis-Tris buffer (pH 7.4) containing 0.1 M Cl^-; the temperature was 25 °C. IHP was added to a final concentration of 2 mM.

Table 3: Comparison of Oxygen Binding Properties of Three Fractions of rHb A (SP-1, SP-2, and SP-3) with Native Hb A in the Presence and Absence of 2 mM Inositol Hexaphosphate (IHP)^a

hemoglobin	oxygen affinity P_{50} (mmHg)	cooperativity, Hill coefficient, n_{max}	$P_{50}(+IHP)/P_{50}(-IHP)^c$
SP-1	5.4 (53)^b	3.18 (2.45)^b	9.8
SP-2	5.5 (47)^b	2.94 (2.34)^b	8.4
SP-3	4.7 (36)^b	2.63 (2.19)^b	7.8
Hb A	5.1 (50)^b	3.19 (2.74)^b	9.8

^{a} These parameters were calculated from the best-fit stepwise Adair constants (26, 28) that were evaluated from the equilibrium curves of Figure 8. ^{b} Values in the presence of IHP. ^{c} Ratio of P_{50} in the presence of 2 mM IHP to P_{50} in its absence.

Thus, O_2 affinity does not show any simple trend with the fractional amounts of normal to reverse heme. SP-1 showed cooperativity identical to that of Hb A, whereas cooperativity (Hill's n) decreased from 3.18 (SP-1) to 2.94 (SP-2) to 2.63 (SP-3) with an increase in the level of reverse heme orientation (Table 3). In the presence of IHP, the curve of SP-1 was nearly identical with that of native Hb A, while those of SP-2 and SP-3 were slightly different from that of native Hb A (Figure 8). As a result, the P_{50} value decreased from 53 mmHg (SP-1) to 47 mmHg (SP-2) to 36 mmHg (SP-3). The effect of IHP on O_2 affinity was evaluated by the ratio of $P_{50}(+IHP)$ to $P_{50}(-IHP)$. As shown in Table 3, the ratio of SP-1 was the same as that of Hb A, and the

ratio decreased from 9.8 (SP-1) to 8.4 (SP-2) to 7.8 (SP-3) with an increase in the level of reversed heme orientation.

The results described above indicate that reversed heme orientation in both the α and β subunits exerts a significant influence on the allosteric O_2 binding properties of hemoglobin.

Reorientation of Reversed Heme in rHb A. The CD spectra of SP-2 and SP-3 with reversed heme did not change in the CO, oxy, and deoxy forms after they stood at 4 °C for 2 weeks. However, standing at 4 °C for 2 days after being converted into the aquo−met form by ferricyanide oxidation changed their CD spectra into those of native Hb A, indicating that heme reorientation occurred. We also observed ^{1}H NMR spectra in which additional peaks (a−f) of the Q_2 fraction (Figure 3B) disappeared after oxidation to the aquo−met form followed by standing at 4 °C for 2 days (data not shown). These results demonstrate that reversed heme in SP-2 and SP-3 in the aquo−met form can reorient to normal. Previous studies have shown that when Hb is oxidized to metHb, heme readily dissociates from globin, allowing for rapid exchange of the heme and re-equilibration (*16, 37*), and also that the β subunit of metHb A has a faster rate of heme dissociation than the α subunit. However, it should be noted that this treatment sometimes causes an increase in oxygen affinity and a decrease in the cooperativity of rHb A.

DISCUSSION

In this paper, we could separate the component with reversed heme from that with normal heme in the rHb A expressed in *E. coli* by SP-Sepharose column chromatography. Mass spectroscopic analysis demonstrated that the three components had the same mass, indicating that neither amino acid replacement nor post-translational modification in the globin moiety had occurred among them. They probably have the same isoelectric point. Accordingly, the separation does not seem to be due to the interaction of a specific group of proteins with the charged groups of SP-Sepharose. Under our gradient conditions, the components are eluted over a very narrow pH range, from the component with normal heme to that with fully reversed heme, in order. Therefore, it is very likely that the heme−globin contact causes some delicate structural change through the rotation of the heme 180° about the 5,15-meso axis, which results in a minute difference in the distribution of the surface charge of the protein molecule available to the charged groups of SP-Sepharose.

Changes in the CD spectra in the Soret region and oxygen binding properties of reconstituted Mb with a 1:1 mixture of normal and reversed heme have been reported (*21−23, 38*). Although ^{1}H NMR studies have demonstrated that reconstituted Hb A also consists of a mixture of normal and reversed heme (*14, 15*), there is no detailed report on its CD and oxygen binding properties. We have found that the rHb A expressed in *E. coli* shows heterogeneity of components with the positive CD band at 260 nm, and we could successfully resolve these components into three components by SP-Sepharose column chromatography. ^{1}H NMR analysis revealed that the component with the lowest CD value at 260 nm (SP-3) has most of the heme in the reversed form. The ^{1}H NMR spectrum of the SP-3 fraction showed intense

methyl proton signals from the heme which rotated 180° about the 5,15-meso axis (Figure 4C). Using the SP-3 fraction of rHb A, we present here the CD spectra in the near-UV and Soret regions and the oxygen binding properties of rHb A having most of the heme in the reversed form.

Heme proteins contain a prosthetic group, which is a derivative of porphyrin. Because of its symmetry, the porphyrin alone is optically inactive. When the heme group is incorporated into a chiral environment such as the heme pocket of Hb A, induced heme optical activity arises from the heme−globin interaction (*39*). The CD bands near 260 nm and in the Soret region of Hb A are interpreted to derive from the L band and the B (Soret) band in the absorption spectrum ascribed to the interaction of the porphyrin π−π* transition with allowed π−π* transitions of nearby aromatic amino acid residues (*39*) and the peptide π−π* transition (*40*). However, the L band has not been characterized in detail because it overlaps in part with the UV absorption of protein aromatic residues. Native Hb A shows a distinct positive CD band in both the near-UV and Soret regions. On the other hand, rHb A with only reversed heme did not display any CD band near 260 nm and gave a prominent negative CD band in the Soret region (Figure 7). It was shown that reconstituted Mb containing reversed heme exhibited a positive CD band decreased in magnitude but not the negative CD band in the Soret region (*22, 38, 41*). This difference in CD spectra between Mb and Hb with reversed heme seems to be derived from the difference in the globin structure of the surrounding heme.

In addition to the coordination bond between heme iron and the proximal-His F8, hemes in the subunits of the Hb tetramer are surrounded by highly conserved amino acid residues (heme contact residues) (*42*). Comparison of the amino acid sequences of the heme contact in Hb of different mammalian species shows almost all of them to be common in the α and β subunits. The invariance of the residues surrounding the heme group implies that they are essential for the structure and function of the hemoglobin molecule. Since modifications of the heme vinyl group or mutation at Val FG5 greatly reduced the cooperativity of Hb (*43−47*), the contact between Val FG5 and the vinyl group of the heme was considered to be essential for transmitting the information of oxygen binding to another subunit (*11*). Hbs reconstituted with chemically modified hemes at the positions of the 3- and 8-vinyl groups exhibited higher oxygen affinity and reduced cooperativity, suggesting that the specific interaction of the 3- and 8-vinyl groups with globin was involved in the cooperative oxygen binding of Hb A (*43, 45*).

Rotation of the heme 180° about the 5,15-meso axis interexchanges the methyl groups at positions 2 and 7 with the vinyl groups at positions 8 and 3, respectively, and this will modulate the heme methyl and vinyl peripheral contacts with globin. We could separate the rHb A with most of heme in reversed form by SP-Sepharose column chromatography. This enabled us to examine discriminatively the influence of the reversed heme orientation on the oxygen binding properties of Hb A. For the oxygen binding property of Mb containing reversed heme, the literature results appear ambiguous: one paper showed no effects on O_2 binding affinity (*22*), while another showed a significant effect (*21*). For Hb, the impact of heme orientation disorder poses a more

complex case, especially with respect to two kinds of subunits and cooperativity. In the absence of IHP, O_2 affinity, expressed as P_{50}, rose from 5.1 mmHg (Hb A) to 5.4 mmHg (SP-1) and then fell from 5.5 mmHg (SP-2) to 4.7 mmHg (SP-3). In contrast, in the presence of a potent allosteric effector, IHP, the P_{50} value of SP-1 (53 mmHg) was nearly identical to that of Hb A (50 mmHg) and decreased linearly from 53 mmHg (SP-1) to 47 mmHg (SP-2) to 36 mmHg (SP-3) (Table 3). In the absence of IHP, cooperativity, expressed as n_{max}, decreased from 3.18 (SP-1) to 2.94 (SP-2) to 2.63 (SP-3), showing a linear relationship with the normal to reverse heme ratio. These changes in cooperativity were also observed in the presence of IHP. These results indicate that changes of O_2 affinity and cooperativity show a simple trend with a fractional amount of normal to reverse heme and that the vinyl−globin (Val FG5) contact is involved in the cooperative oxygen binding of Hb A.

Mutant Hbs with impaired function exhibit very high O_2 affinity ($P_{50} < 1$ mmHg) and no cooperativity ($n = 1.0-1.3$) (*8, 48*). Although SP-3 with mostly reversed heme in both the α and β subunits shows a slightly higher O_2 affinity ($P_{50} = 4.7$ mmHg) and lower cooperativity ($n = 2.63$) than Hb A does ($P_{50} = 5.1$ mmHg, and $n = 3.19$), these parameters are much larger than those of mutant Hbs with no cooperativity. This means that Hb with mostly reverse heme in both the α and β subunits still retains $\sim 70\%$ of its cooperative oxygen binding ability, that is, the T $\rightarrow$ R quaternary structure transition upon oxygen binding.

The ^{1}H NMR spectra of dark muscle from yellowfin tuna indicated that Mb with reversed heme accounted for 40% of total Mb (*19*). Major components of monomeric Hb of the insect *Chironomus thummi thummi* have the reversed heme (*20*). Even in native Hb A, 10% of the heme is reversed (*14*). The conversion of "reversed heme" to "normally oriented heme" in the aquo−met form suggests that the normal heme orientation is thermodynamically favored in Hb A. However, the heme of tuna Mb and *Chironomus* Hb is stable in the reversed heme orientation. These results indicated that the relative stability of the two different heme orientations depends on the heme−protein contact. Ishimori and Morishima (*16*) have revealed that heme orientation in reconstituted Hb A depends upon the heme peripheral substituents at positions 3 and 8; the α subunit of apoHb preferentially binds deuteroheme, which possesses methyl groups at positions 2 and 7 and hydrogen ones at positions 3 and 8, in the reversed orientation, while the β subunit of apoHb prefers the normal orientation. Although Hb A reconstituted with protoheme IX (possessing methyl groups at positions 2 and 7 and vinyl ones at positions 3 and 8) contains two heme orientations, mesoheme (possessing methyl groups at positions 2 and 7 and ethyl ones at positions 3 and 8) is inserted predominantly in the normal heme orientation in both the α and β subunits (*16*). This preferential normal heme orientation for mesoHb suggests that apoHb can also distinguish stereochemically between the vinyl and ethyl groups at positions 3 and 8 of the heme. It is likely that the difference in specificity in the heme orientation between the α and β subunits arises from the difference in their heme environmental structures.

As shown in this paper, the in vivo biosynthesis of the rHb A in *E. coli* produced an amount of species with reversed heme comparable to the amount from the in vitro reconstitu-

tion. We employed overnight stirring of the lysate in an atmosphere of CO for the extraction, which enabled us to efficiently enhance the yield of rHb A. Under these conditions, the lysate might still contain not only free heme but also apoglobin. If so, the overnight stirring possibly facilitates the reconstitution of rHb A from apoglobin and hemin in the lysate. However, we observed that stirring of the lysate for only 1 h also produced similar molecular heterogeneity of rHb A with reversed heme comparable to that from the overnight stirring, although the total amount of rHb A obtained was reduced to half. Thus, the expression system presented here can similarly but diminutively produce rHb A without an external supply of heme. In this case, the product also showed a molecular heterogeneity comparable to that in the system with an addition of external hemin. Considering these findings together, insertion of heme into apoprotein in this expression system may resemble the in vitro reconstitution of Hb A from apoHb and heme. These results further emphasize that one must be careful to check if the expressed proteins have the correct conformation when the *E. coli* expression system contains a prosthetic group like a heme.

ACKNOWLEDGMENT

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