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Spin-Labeled Hemoglobin Subunits*

Shun-ichi Ohnishi, Toyozo Maeda, Tadanao Ito, Kun-Joo Hwang, and Itiro Tyuma

ABSTRACT: The isolated subunits from human adult hemoglobin and their mercurated derivatives in carbon monoxy form have been spin labeled with *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide. The electron spin resonance spectra of the labeled subunits and their various mixtures have been measured at room temperatures. The sedimentation coefficients, the number of the bound labels, and the number of the reactive SH groups have also been determined. The spectra of all the labeled subunits are similar and are very different from that of the spin-labeled tetramer $\alpha_2\beta_2$. The spin label attached to the β93 SH group in the isolated β chains gives a different spectrum from that of the same label attached to the same SH group in the tetramer, indicating that the conformation of the β chains in the isolated form differs from that in the $\alpha_2\beta_2$ tetramer. When the labeled β chains are mixed with native α chains, the spectrum of the former changes very rapidly (within 1 sec) into the spectrum indistinguishable from that of the tetramer. The normally functioning conformation of the β chains is achieved only when they combine with the α chains of different composition.

The idea that the functional properties of hemoglobin are based upon the conformation or conformational changes in the constituent subunits seems to be receiving more and more supporting evidence from various studies (Muirhead and Perutz, 1963; Perutz and Mazzarella, 1963; Perutz *et al.*, 1964). Better understanding of these properties can be achieved by investigating the structures of the isolated subunits and the interactions between these subunits. Bucci and Fronticelli (1965) succeeded in isolating the α and β chains of human hemoglobin after treatment with PMB[1] and the Rome group studied several properties of each subunit and of reconstituted hemoglobins (Bucci *et al.*, 1965; Antonini *et al.*, 1965, 1966). Their method of mercury removal was not satisfactory, however, and Tyuma *et al.* (1966) have presented an improved method for the complete removal using SH Sephadex and mercaptoethanol. The latter authors have also investigated some physicochemical and physiological properties of the subunits thus obtained (Tyuma *et al.*, 1966; Beychok *et al.*, 1967).

The spin-label technique has been successfully applied to the study of conformational changes in hemoglobins (Boeyens and McConnell, 1966; Ohnishi *et al.*, 1966; Ogawa and McConnell, 1967). This technique is unique, compared with other usual physicochemical methods, in that the label reports its *local* environment or changes in the environment through the correlation time of molecular tumbling. In the present investigation, we used this technique to elucidate conformational properties of the subunits of human hemoglobin and of the reconstituted hemoglobin.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMB, *p*-mercuribenzoate; α_{PMB} and β_{PMB}, mercurated α and β chains; α and β, demercurated α and β chains; $\alpha_2\beta_2$ or Hb A, parent human adult hemoglobin tetramer; the superscript * indicates that the protein has been spin labeled.

Experimental Section

Human adult hemoglobin was separated into the constituent α and β chains according to the procedure described by Tyuma *et al.* (1966). All the isolated subunits, both mercurated and demercurated, as well as the parent tetramer were used as carbon monoxy derivatives. The hemoglobins were dissolved in 0.1 M phosphate buffer at pH 7.3 and their concentrations were expressed either as w/v % or as molar concentration a on heme basis. The free radical used for spin labeling was a six-membered nitroxide maleimide (*N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide) kindly donated by Dr. W. Landgraf of Varian Associates. The reagent was added to the hemoglobin solutions in a molar ratio of about three radicals per chain and allowed to react for up to 6 hr at 5 or 10°, depending on the reactivities of the subunits. The residual nitroxide radicals were removed either by dialysis or by gel filtration with Sephadex G-10. All the samples were stored at 5° and used within 5 days after isolation.

Electron spin resonance spectra were recorded at room temperatures on a Model P-10S spectrometer of Japan Electron Optics Laboratory Co. Ltd. The spin-labeled oxyhemoglobins showed essentially the same spectra as those of the labeled carbon monoxy hemoglobins.

Unlabeled SH groups in the spin-labeled hemoglobins were determined by the spectrophotometric titration method described by Benesch and Benesch (1962).

Ultracentrifugal analyses were carried out at 20–30° with 0.5% hemoglobin solutions in 0.1 M phosphate buffer at pH 7.3 and the sedimentation coefficients were corrected to 20° in water. A Hitachi Model UCA-1 analyzer was used. In agreement with the results of previous authors (Bucci *et al.*, 1965; Tyuma *et al.*, 1966) we obtained the sedimentation coefficients for the native hemoglobins as follows: α = 2.5 S, α_{PMB} = 1.8 S, β = 4.4 S, β_{PMB} = 2.7 S, and $\alpha_2\beta_2$ = 4.2 S.

Results and Discussion

Electron Spin Resonance Spectra of Spin-Labeled Subunits. The spin-labeled subunits, α*, β*, α_{PMB}*, and β_{PMB}*, gave the electron spin resonance spectra shown in Figures 1 and 2. It is noted that the spectra are similar. These spectra, on the other hand, are markedly different from the very broad spectrum of the spin-labeled tetramer, $(\alpha_2\beta_2)$*, indicated in Figure 1C, which agrees well with the corresponding spectrum reported by Boyens and McConnell (1966). These spectral shapes remained essentially unchanged during the course of the labeling, *i.e.*, independent of the extent of labeling.

The concentrations of the radicals or labels bound to the subunits were estimated from the electron spin resonance absorption intensities and the numbers of labels per heme were calculated. Unlabeled SH groups in the spin-labeled α, β, and $\alpha_2\beta_2$ were titrated with PMB and the numbers of the groups per heme were obtained. These figures are plotted against the reaction time in Figure 3 and some are listed in Table I together

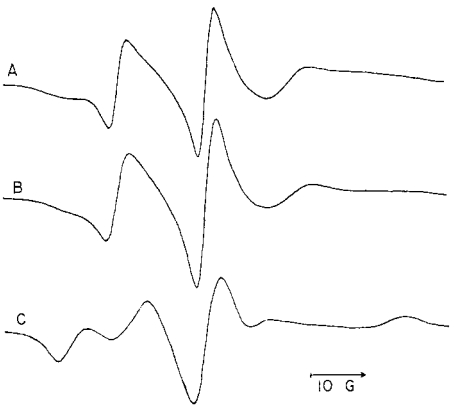

FIGURE 1: Electron spin resonance spectra of the spin-labeled native hemoglobin subunits. All spectra are taken at room temperatures in 0.1 M phosphate buffer at pH 7.3. (A) α chains (α*), 0.2%, 0.56 label/heme. (B) β chains (β*), 1.8%, 1.1 labels/heme. (C) $\alpha_2\beta_2$ tetramer ($(\alpha_2\beta_2)$*), 1.1%, 0.39 label/heme.

with the sedimentation coefficients of the subunits. In the table is also given a rough estimation of the numbers of the radicals bound to the SH group and to other residues in α*, β*, and $(\alpha_2\beta_2)$*.

These results show that the nitroxide maleimide label has different reactivities to different residues in various subunits. The label binds most quickly to the SH group of the isolated β chains and to that of β chains in the $\alpha_2\beta_2$ tetramer. The assignment of the SH group involved in the reaction of $\alpha_2\beta_2$ tetramers with the label to the β93 SH group has been well established (Guidotti and Konigsberg, 1964; Boyens and McConnell, 1966). The same argument may be applied to the reaction of the isolated β chains, since the labeled β* chains still contain approximately one reactive SH group per chain and can recombine quickly with the α chains to form tetramers as described in the next section. The labels in α_{PMB}* and β_{PMB}*, however, must have been attached to residues other than SH groups, probably to the ϵ-amino groups of lysine residues with much less reactivity. Thus, most of the labels bound to β* and $(\alpha_2\beta_2)$* were those bound to the SH group and only about 5% of them

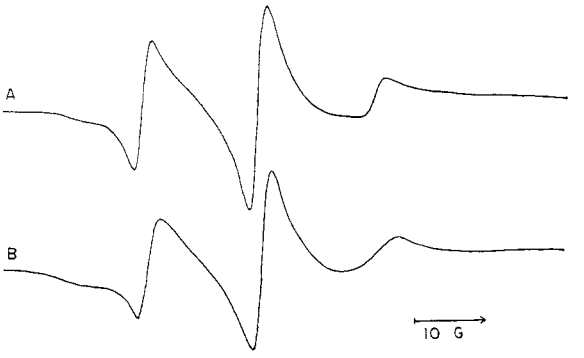

FIGURE 2: Electron spin resonance spectra of the spin-labeled mercurated hemoglobin subunits. All spectra are taken at room temperatures in 0.1 M phosphate buffer at pH 7.3. (A) Mercurated α chains (α_{PMB}*), 1.4%, 0.16 label/heme. (B) Mercurated β chains (β_{PMB}*), 1.6%, 0.12 label/heme.

TABLE I: Sedimentation Coefficients and Contents of Reactive SH and Label Groups of Spin-Labeled Subunits.

Subunits	Reaction Condn °C	Reaction Condn hr	$s_{20,w}$ (S)	SH/Heme	Labels/Heme
α^*	10	6	2.3	0.65	0.56 (0.35 to SH, 0.21 to NH_2)[a]
β^*	5	3	2.2	0.96	1.1 (1.05 to SH, 0.04 to NH_2)[a]
α_{PMB}^*	10	4	1.9	0.00	0.16
β_{PMB}^*	10	4	2.7	0.00	0.12
$(\alpha_2\beta_2)^*$	5	3	4.4	0.13	0.39 (0.38 to SH, 0.02 to NH_2)[a]

[a] Figures in parentheses are listed only for reference.

to the amino groups. SH groups in α chains were less reactive than that of β chains, so that only 0.35 radical attached to the SH group after 6 hr at 10°, during which period 0.21 radical bound to the amino groups. The number of radicals attached to α^* chains did not increase even after further reaction for 21 hr.

The spin labeling did not significantly affect the sedimentation coefficient of α, α_{PMB}, β_{PMB}, and $\alpha_2\beta_2$. That of β, on the other hand, was decreased from 4.4 to 2.2 S by the labeling, indicating dissociation of the native tetramer to almost monomers.

The results suggest that the same label bound to the same β93 SH group gives different electron spin resonance spectra depending on whether the host protein is the isolated β chains or the associated $\alpha_2\beta_2$ tetramers. The very broad spectrum of the label in $\alpha_2\beta_2$ (Figure 1C) corresponds to a strong immobilization and implies that the label is trapped in the hydrophobic pocket, excluded from free water molecules (Ohnishi *et al.*, 1966). The same label in the isolated β chains is not strongly immobilized, since the spectrum is narrower, and it can now have some contact with solvent molecules. This indicates a different environment of the β93 label in the isolated β chains from that in the $\alpha_2\beta_2$ tetramers and suggests that these two proteins have different conformations. Conformational distinctions between hemoglobins A ($\alpha_2\beta_2$) and H (β_4) have been implicated by

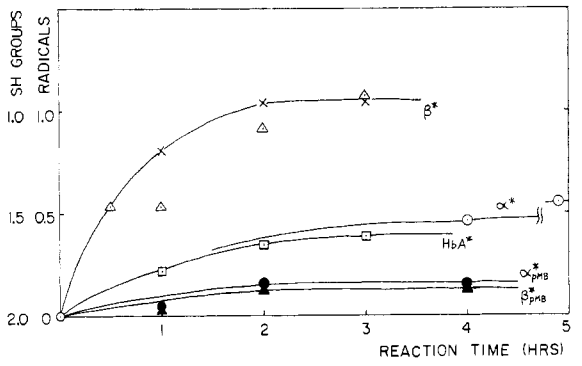

FIGURE 3: Number of the label per heme attached to hemoglobins against the reaction time. For the β^* chains is also shown the number of the reactive SH groups per heme (×). ○, ●, △, ▲, and □ are for α^*, α_{PMB}^*, β^*, β_{PMB}^*, and $(\alpha_2\beta_2)^*$, respectively. An extra circle in the curve of α^* is the value for 6-hr reaction.

TABLE II: Sedimentation Coefficients of Equimolar Mixture of Spin-Labeled Subunits.

Mixture	$s_{20,w}$ (S)
$\alpha + \beta^*$	3.8
$\alpha^* + \beta$	4.1
$\alpha_{PMB}^* + \beta$	4.2
$\alpha_{PMB} + \beta^*$	2.2
$\alpha^* + \beta_{PMB}$	3.5

Perutz and Mazzarella (1963). The labels attached to the amino and SH groups in the isolated subunits showed essentially similar spectra.

Mixing of Different Subunits. The spin-labeled subunits were mixed with different partners in various ratios. Some electron spin resonance spectra of the resulting solutions are reproduced in Figures 4–6 and the sedimentation coefficients of the mixture are collected in Table II.

$\alpha + \beta^*$. Addition of native α chains to the solution of β^* chains changed the latter's spectrum to a very broad one as shown in Figure 4. As the relative concentration of α chains increased, the very broad component increased proportionally at the expense of the initial sharp

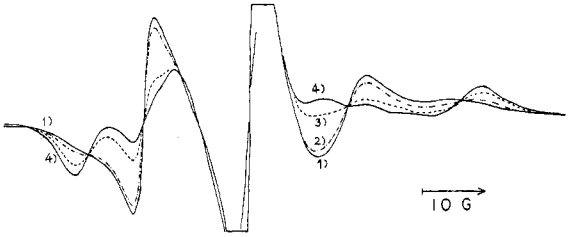

FIGURE 4: Change in electron spin resonance spectrum of the spin-labeled β^* chains on addition of native α chains. The molar ratio (heme basis) of α to β^* in the solution is (1) 0.0, (2) 0.33, (3) 0.66, and (4) 1.0. The millimolar concentration of hemoglobin after mixing (heme basis) is (1) 0.54, (2) 0.72, (3) 0.90, and (4) 1.1. The β^* chains have 1.1 bound labels/heme. A half-spectrum on the higher field side is recorded at 1.6 times higher gain to show the change more clearly.

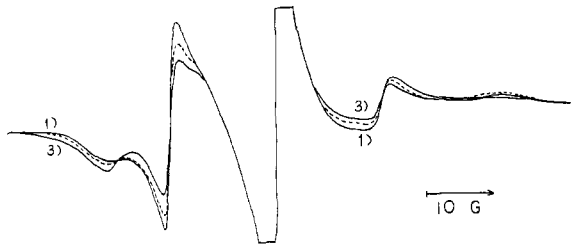

FIGURE 5: Electron spin resonance spectra of the spin-labeled α^* chains mixed with native β chains in various molar ratios. The ratio β/α^* is 0.0 for curve 1, 0.6 for curve 2, and 1.0 for curve 3. The concentration of hemoglobin after mixing is 0.96 mM (heme basis). The α^* chains have 0.53 label/heme.

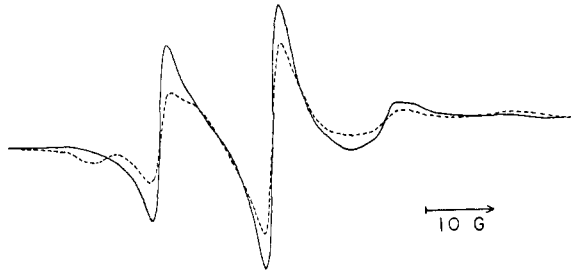

FIGURE 6: Electron spin resonance spectrum of an equimolar mixture of the spin-labeled mercurated α_{PMB}* chains and native β chains. Concentration is 1.2 mM (heme basis). The α_{PMB}* chains have 0.16 label/heme.

β^* component, and finally, when the molar ratio of the chains became unity, only the very broad spectrum indistinguishable from that of $(\alpha_2\beta_2)^*$ tetramer was observed. Further addition of the α chains did not affect the spectrum. Since the final solution gave the sedimentation coefficient of 3.8 S, the observed reaction can be reasonably assumed as follows: $\alpha + \beta^* \rightarrow \alpha\beta^* \rightarrow \alpha_2\beta_2$. A striking similarity between the electron spin resonance spectrum of $\alpha\beta^*$ dimers and that of $(\alpha_2\beta_2)^*$ tetramers has been demonstrated in a separate study (S. Ohnishi, T. Maeda, T. Ito, K.-J. Hwang, and I. Tyuma, unpublished data).

This result indicates that the conformation of the isolated β chains, as seen by the β93 label, changes dramatically into the normally functioning conformation on being combined with the α chains. There are no stable intermediate conformations in the transition, since some isosbestic points are clearly observed in Figure 4. A preliminary measurement of the time taken for the spectral change gave the upper limit of 1 sec, when the concentrations of both the chains were 1 mM.

$\alpha^* + \beta$ and $\alpha_{PMB}^* + \beta$. The electron spin resonance spectrum changed also in these combinations but to a lesser extent (Figures 5 and 6). The narrow peak decreased by about 40% of its original height and the broad component appeared in the corresponding amount. The appearance of the broad component in $\alpha_{PMB}^* + \beta$ combination suggests that about 40% of the labels attached to the ϵ-amino groups of lysine residues become immobilized on recombination with β chains. Such labels might have been located on the α–β contact surface, and they would be strongly immobilized in the interchain crevice on combination with β chains. A similar change of the electron spin resonance spectrum in the $\alpha^* + \beta$ combination implies that the same immobilization of about 40% of the labels at the ϵ-amino groups has occurred in this case as well. On considering the sedimentation coefficients in Table II, the observed reactions may be written as follows: $\alpha^* + \beta_4 \rightarrow \alpha^*\beta \rightarrow \alpha_2^*\beta_2$ and $\alpha_{PMB}^* + \beta_4 \rightarrow \alpha_{PMB}^*\beta \rightarrow (\alpha_{PMB}^*)_2\beta_2$.

The change in the electron spin resonance spectrum took place rather slowly in these combinations, in contrast to the $\alpha + \beta^*$ combination. After mixing with β chains the peak height of the narrow component of the α_{PMB}^* spectrum decreased to a final value of 60% of the initial height in 40 min. The time for half-decrease was 4 min, when the concentration of both the chains was 0.56 mM. The $\alpha^* + \beta$ reaction showed almost the same time course. In view of the results reported by Antonini *et al.* (1966) on the association kinetics of the deoxygenated subunits, $\alpha + \beta$ and $\alpha_{PMB} + \beta_{PMB}$, the rate of $\alpha^* + \beta$ and $\alpha_{PMB}^* + \beta$ combinations seems very slow. A study to clarify this question is in progress.

$\alpha_{PMB} + \beta^*$ and $\alpha^* + \beta_{PMB}$. The electron spin resonance spectra showed essentially no change upon mixing of the chains in equimolar amounts and the sedimentation coefficients increased only slightly.

A conclusion drawn from the results of the present investigations is that the two kinds of chains are necessary for the physiological function of hemoglobin, because the conformation of the β chains is transformed into the normally functioning one on combination with the α chains. The conformation of the α chains, on the other hand, does not appear to be significantly altered by combination with the β chains.

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Azotobacter Free-Radical Flavoprotein. Preparation and Properties of the Apoprotein*

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ABSTRACT: Precipitation with 3% trichloroacetic acid easily separates an apoprotein from a flavin mononucleotide containing flavoprotein isolated from *Azotobacter vinelandii*. When dissolved in 5% $NaHCO_3$ and stored at 3–4° the apoprotein is stable for flavin mononucleotide binding for at least 11 months. Combination of the apoprotein with flavin mononucleotide is a second-order reaction ($k = 7.9 \times 10^5$ M^{-1} sec^{-1}, pH 5.0, 30°) which proceeds more rapidly near pH 5.0 than at other pH values. Oxidized or reduced reconstituted flavoprotein has an absorption spectrum identical with that of oxidized or reduced unresolved flavoprotein. Reconstituted flavoprotein exhibits electron paramagnetic resonance when reduced with $Na_2S_2O_4$. Urea (1.65 M) inhibits flavin mononucleotide binding by apoprotein but subsequent addition of 0.16 M NaCl relieves this inhibition. Nitration of 4.1 of the 5 tyrosines/27,000 g of apoprotein eliminated *ca.* 90% of its capacity for flavin mononucleotide binding. Flavin bound to the protein protected two of these tyrosines from nitration. The association constants of the apoprotein at 30° with flavin mononucleotide, flavin–adenine dinucleotide, and riboflavin are 7.3×10^8 M^{-1} (pH 5.2), 9.1×10^5 M^{-1} (pH 7.0), and 6.4×10^5 M^{-1} (pH 5.0), respectively. As a working hypothesis these data are tentatively interpreted to mean that the flavin mononucleotide binding site of the apoprotein contains a binding site for phosphate and one binding site, probably containing two tyrosines, for the nonphosphate portion of flavin mononucleotide.

Although apoproteins from several flavoproteins have been prepared (De Luca *et al.*, 1956; Dixon and Kleppe, 1965; Mahler, 1954; Miyake *et al.*, 1965; Negelein and Brömel, 1939; Prosky *et al.*, 1964) an apoprotein has not been prepared from a flavoprotein which under aerobic conditions and neutral pH values forms a stable free radical. Recently, I have prepared such an apoprotein from the unusual flavoprotein of *Azotobacter vindlandii* (Hinkson and Bulen, 1967; Shetna *et al.*, 1964, 1966). The holoprotein reconstituted from the apoprotein and FMN[1] has the same properties as the original, including the ability to form a free radical. In addition to facilitating studies of flavin–protein interactions with respect to flavin binding, this particular apoprotein will allow studies of protein–flavin interactions responsible for free-radical stabilization. In contrast to many apoproteins from flavin-containing proteins (De Luca *et al.*, 1956; Mahler, 1954; Miyake *et al.*, 1965; Negelein and Brömel, 1939), this apoprotein is stable for several months and is easily prepared from a readily obtainable flavoprotein.

Experimental Procedure

Absorption spectra were recorded with a Cary Model 14 spectrophotometer. Spectrophotometric measurements at a single wavelength were determined with a Beckman Model DU or a Gilford Model 240 spectrophotometer. The electron paramagnetic resonance spectrum of a sample was obtained with a Varian Model 4500 spectrometer. A Branson Sonic Power 20-kc sonic oscillator equipped with the S-125 converter was used for sonic oscillation.

Riboflavin was White Label material from Eastman Organic Chemicals, Rochester, N. Y. Sigma Chemical Co., St. Louis, Mo., furnished the FMN (commercial grade) and FAD (grade III). Dr. G. Tollin of this department supplied the recrystallized lumiflavin. Tetranitromethane was a product of Aldrich Chemical Co., Inc., Milwaukee, Wis. All other chemicals were reagent grade obtained from common commercial sources. Except for growth media, deionized distilled water was used throughout.

Protein was measured either by the method of Gornall *et al.* (1949), by the method of Lowry *et al.* (1951), or from A_{280} as calibrated with the method of Lowry

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: TNM, tetranitromethane.