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5-Thio-2-nitrobenzoate as a Circular Dichroism Marker to Detect the Tense Structure of Bovine Hemoglobin[1]

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A characteristic, strong CD absorption appeared at 312 nm, when a 5-thio-2-nitrobenzoate (TNB) group was anchored at Cys^{93}-S(βF9) of bovine hemoglobin (Hb) through -SS- linkage. The Hill coefficient, n, = 2.7 of the TNB-modified Hb was practically identical with that of native Hb, demonstrating that the marker-anchoring was made successfully, without destroying the cooperativity function. The new 312-nm CD absorption disappears concurrently with the conversion from the tense to the relaxed quaternary structure of Hb, clearly indicating that TNB is an excellent CD marker to detect the tense structure of deoxy-Hb.

INTRODUCTION

The mechanism of hemoglobin (Hb) cooperativity is of the considerable significance and interest to organic and bioorganic chemists, and the detailed process involved in the tertiary and/or quaternary structure change has been sought through various physicochemical approaches, including NMR and ESR (*1–3*). For example, either (a) ionization of a group having a pK_a of 7.4 or (b) oxygenation of heme was found to induce a remarkable change in ^{19}F chemical shift of $^{19}F_3C$-CO-CH_2S-Cys^{93}, which was attributed either to a direct charge effect or to the expected conformational change (*1*). Spin-labeled studies suggest that the conformational change of Hb associated with the ligand binding based on the lineshape change of the ESR signal is due to the extent of immobilization of the spin-labels. To gain further insight into the conformational aspects of interaction, a more suitable approach for direct detection the rapid conformation change around the subunit contact area is necessary.

Circular dichroism (CD) (*3*) is intrinsically most sensitive to molecular asymmetry and, therefore, is very appropriate for the detection of the quaternary-structure change at the $\alpha_1\beta_2$ contact (*5*) of hemoglobin. The authors now wish to report that a characteristic, strong CD absorption appeared at 312 nm when 5-thio-2-nitrobenzoate (TNB) was anchored at Cys^{93}-S(βF9) (*4*) of bovine deoxy-Hb through an -SS- linkage (**3a**):

[1] Preliminary results of this paper were presented at the 31th Symposium on Protein Structure in Tokyo, Japan, Oct. 1980, preprint p. 25.

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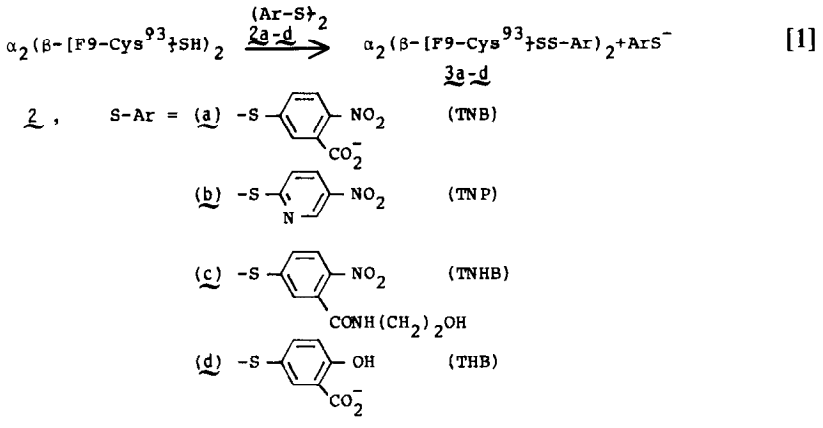
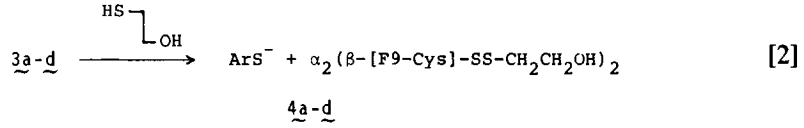


This 5-thio-2-nitrobenzoate group provides an excellent CD marker for the change of molecular asymmetry connected with the tense $\rightleftharpoons$ relaxed transition of Hb.

RESULTS AND DISCUSSION

Bovine oxyhemoglobin **1** was treated with a modifying reagent (5.9 mM), 5,5′-dithiobis(2-nitrobenzoic acid), 6,6′-dithiobis(3-nitropyridine) (*6*), 5,5′-dithiobis[*N*-(2-hydroxyethyl)-2-nitrobenzamide],[3] or 5,5′-dithio-bis(2-hydroxybenzoic acid) (**2a–d**) according to the reported procedures (*4*). Since **2d** was significantly less reactive toward the disulfide exchange reaction than **2a–c**, the longer reaction time and the higher pH were required to the complete modification of the cysteine residue in the preparation of **3d** (see Experimental Procedures). Unreacted **2** and each arylthiolate anion formed by the disulfide exchange reaction (Eq. [1]) were removed by gel filtration (Sephadex G-25) followed by dialysis against 0.1 M phosphate buffer (0.1 M KCl) at pH 7.0, and the modified hemoglobin **3a–d**, having an aryl-S group connected with $Cys^{93}S$ in the β-chain through -SS- linkage, was isolated. Treatment of purified **3a–c** with 2-mercaptoethanol liberated arylthiolate anion[4]



which amounted to 0.97 (**3a**), 1.15 (**3b**), and 0.78 (**3c**) mole/β-chain, respectively (Table I), revealing that the cysteine modification was carried out successfully.[5]

[3] **2c** was prepared according to the reported procedure (*7*).

[4] The absorptivity ε(mM) for the thiolate anion of **2a**, **2b**, and **2c** was 9.02, 5.88, and 10.2, respectively. The reported ε (mM) value for thiolate of **2a** is 8.86 (*4*).

[5] Bovine hemoglobin has one cysteine residue at F9 position per single β-chain. α-Chain has no cysteine residue (*9*).

TABLE 1

INDUCED CD ABSORPTION AND OXYGEN BINDING PROPERTIES OF MODIFIED BOVINE HEMOGLOBINS (**3a–c**)

	CD-λ_{max} (nm)[a]	Label/β-chain	n	P_{50} (nmHg)
TNB-Hb (**3a**)	312	0.97	2.7[b]	6.3[b]
TNP-Hb (**3b**)	—	1.15	2.7[b]	15.1[b]
TNHB-Hb (**3c**)	316 (weak)	0.78	—	—
native Hb (**1**)	—	—	2.8[c]	8.3[c]

[a] Electronic absorption maxima of **2a**, **2b**, and **2c** are 325, 318, and 325 nm, respectively.

[b] In 0.1 M phosphate, 0.1 M KCl, pH 7.0, 25°C.

[c] In 0.05 M Tris–HCl 0.1 M KCl, pH 7.0, 25°C.

Zone electrophoresis of both native Hb and TNB-Hb showed sharp single peaks, demonstrating that each protein is homogeneous (*4*, *20*).

Completion of the modification of the cysteine residue with **2d** was ascertained by the observation that no further reaction of isolated **3d** with **2a**, a known SH reagent for hemoglobin (*4*), took place:

$$\mathbf{3d} \xrightarrow[\mathbf{2a}]{} \not\longrightarrow \qquad [3]$$

Hill's coefficients (n) of TNB-Hb (**3a**) and TNP-Hb (**3b**) were observed to be 2.7 and 2.7, respectively; being practically identical with $n = 2.8$ for native Hb (Table 1). Therefore, each probe was anchored without destroying the cooperativity, and the practically negligible change in the P_{50} value observed demonstrates that the

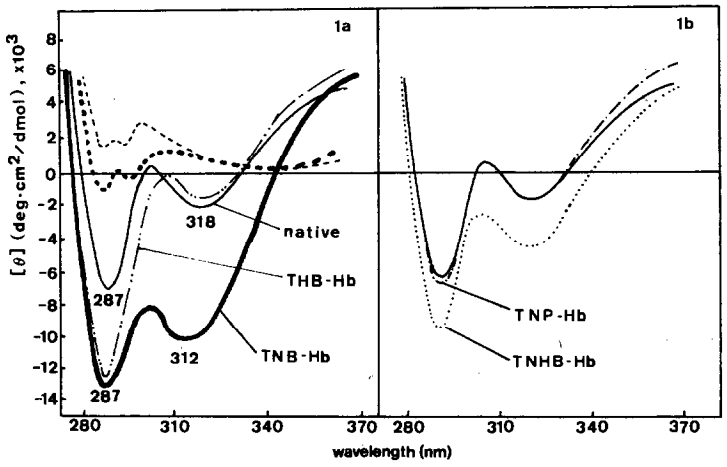


FIG. 1. Circular dichroism spectra of (a), deoxy-TNB (——), deoxy-THB (–··–), deoxy-native (——), oxy-TNB (– – –), oxy-native (– – –) bovine hemoglobin; and (b), deoxy-TNP (–·–) and deoxy-TNHB (···) bovine hemoglobin in 0.1 M phosphate buffer, 0.1 M KCl, pH 7.0.

TABLE 2

DISTANCE FROM Cys^{93}-S(βF9) TO THE HETEROATOM CENTER OF SELECTED SIDE RESIDUES IN THE $\alpha_1\beta_2$ CONTACT REGION OF HEMOGLOBIN AND THEIR CHANGES ON OXYGENATION

		$R_{(tense)}$[a]	$R_{(relaxed)}$[a]	ΔR[b]
$-NH_3^+$	Lys^{40}-N(αC5)	3.5_4	11.2	+7.7 Å
	His^{146}-N(βHC3)	4.7	7.4_6	+2.7 Å
		4.2	8.1_6	+3.9
	His^{92}-N(βF8)	9.6_3	9.2_1	−0.4 Å
		11.0_3	10.6_5	−0.4
OH	Tyr^{145}-O(βHC2)	5.8_2	4.2_5	−0.6 Å
	Tyr^{42}-O(αC7)	10.0_4	14.3_4	+4.3 Å

[a] Calculated from the reported X-ray coordinates: $R_{(tense)}$, deoxy-Hb; $R_{(relaxed)}$, met-Hb; Ref. (*13*).

[b] $\Delta R = R_{(relaxed)} - R_{(tense)}$.

tertiary and quaternary structures of native Hb was not seriously affected by the introduction of the present CD marker.

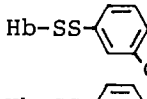
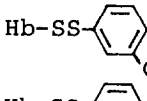
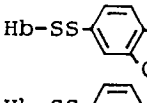
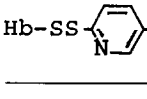
Figure 1 shows the CD spectra obtained. Apparently, deoxy-TNB-Hb (**3a**, anionic, uv_{max} = 325 nm) shows a new CD absorption in the negative sign at 312 nm ($[\theta] = -1.0 \times 10^4$ deg · cm^2 dmol^{-1}) in addition to the 287-nm absorption, the so-called "tyrosine band" due to Tyr-αC7 or Tyr-αCl (*10*) that is seen for both native deoxy-Hb and TNB-Hb (Fig. 1a). Aromatic side chains (Tyr, Phe, Trp) were excluded from the possible origin of the new 312-nm CD band, since they absorb light at shorter wavelengths. Heme shows only very weak CD absorptions due to the π-π^* N band (heme) in the region 300–350 nm (*11*), as seen in native-Hb (Fig. 1). No characteristic CD band at 312 nm, but only the 287-nm band with the slightly increased ellipticity was observed for THB-Hb (**3d**) (Table 3). These observations, together with the corresponding uv maxima of **2d** (302 nm) and **2a** (325 nm), strongly indicate that the new induced CD band at 312 nm observed for TNB-Hb (**3a**) results from the S-ϕ-NO_2 chromophore, and the CD absorption due to the S-ϕ-OH chromophore (expected at ca 290 nm) is superimposed upon the major CD band. The observed substituent effect clearly indicates that the new CD-induced absorption is due to the aryl-sulfenyl chromophore.

In contrast, oxy-TNB-Hb exhibited a CD spectrum (Fig. 1a, ---) not much different from that of native Hb (Fig. 1a, ---). Moreover, met-TNB-Hb showed a CD spectrum almost identical with that of native met-Hb, neither of which showed any characteristic induced CD absorption in the region 280–350 nm. It is concluded, therefore, that the TNB chromophore is an excellent and very sensitive CD reporter group that tells the existence of the deoxy T state.

In an interesting contrast to the strong, induced CD observed for anionic TNB-Hb, neutral TNP-Hb (**3b**) or TNHB-Hb (**3c**) exhibited practically none or only a weak enhanced CD absorption at 316 nm, respectively. These observations strongly indicate that the remarkable ellipticity induced by TNB-Hb is connected with the electrostatic interaction (TNB-CO_2^-)— (a certain cationic residue, most

TABLE 3

INDUCED CD ABSORPTION OF MODIFIED BOVINE HEMOGLOBIN

Hb derivatives	Induced CD-$\lambda_{max}([\theta]^a)$		uv λ_{max} (disulfide)
	Tyrosine band	New band ascribed to probe chromophore	
native Hb	287(− 7.4)		
Hb-SS-  (TNB-Hb)	287(−13.0)	312(−10.0)	325
Hb-SS-  (THB-Hb)	287(−12.6)	[b]	302
Hb-SS-  (TNHB-Hb)	287(−10.3)	316(− 5.0)	325
Hb-SS-  (TNP-Hb)	287(− 8.0)	316(− 3.1)	318

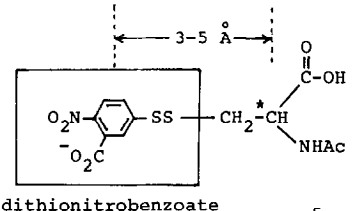
Note. In 0.1 M phosphate, 0.1 M KCl, pH 7.0, 25°C.

[a] $\times 10^{-3}$ deg cm^2 dmol^{-1}.

[b] New CD band was blueshifted and superimposed on the 287-nm band.

probably Lys^{40}-NH_3^+ (αC5)) at the $\alpha_1\beta_2$ interface (cf. Table 2), and His^{146}-imidazolium (βHC3) is less likely.[6] This ammonium group comes close to Cys^{93}-S bearing the marker moiety in the tense form and moves far away from Cys^{93}-S in the relaxed form, based on the reported X-ray results (see Table 2).

Since the peptide bond of Cys^{93} is also the asymmetric element which keeps the closest distance to the TNB group, it may be argued that simple Cotton effect from Cys^{93} is responsible for the observed induced CD absorption. To clarify this possibility, we prepared **5** as a model for the induced Cotton effect.



dithionitrobenzoate chromophore **5**

λ_{max}(uv) = 318 nm, $\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}

λ_{max}(CD) = 308 nm $[\theta] = -1.7 \times 10^3$ deg · cm^2 dmol^{-1} in EtOH

The observed molar ellipticity of **5**, with $[\theta] = -1.7 \times 10^3$ deg · cm^2 dmol^{-1}, was too small to account for the remarkably large $[\theta]$ value (-1.0×10^4

[6] Preliminary experiments at pH 9.5 did not show appreciable change in shape or magnitude of the 312-nm induced CD band.

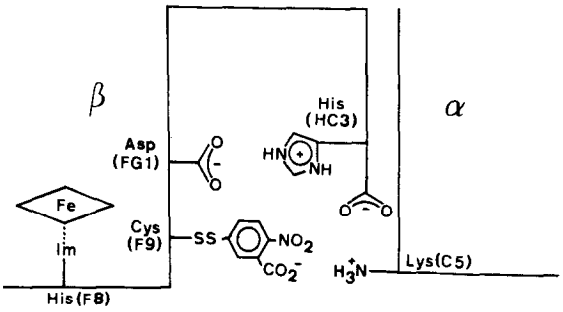


FIG. 2. Schematic description of the local structure in the vicinity of the CD marker (TNB) in the deoxy-TNB-Hb.

deg · cm^2 dmol^{-1}) presently observed for TNB-Hb, clearly demonstrating that the new induced CD band is mostly due to the rest of the protein. The imidazole ring of His^{146} (βHC3), and the benzene ring of Tyr^{145} (βHC2) located closest to the sulfur center of Cys^{93} (3.9 and 3.5 Å, respectively) seem to give induced CD absorption. According to the calculation by Hsu and Woody (*12*), the imidazole ring at 3.6 Å from the heme center brings about a large rotational strength of 0.417 DBM, or $[\theta] = 3 \times 10^4$ deg · cm^2 dmol^{-1}, which is in the same order of magnitude with the induced Soret Cotton effect presently observed. It is apparent, therefore, that coupling between a π-electron system (His or Tyr) and a chromophore having an allowed π-π^* transition (CD marker) in close proximity induces the relatively large rotational strength observed. In other words, the major portion of the magnetic dipole effect in the TNB chromophore comes from certain conjugated electron system(s) asymmetrically located in the vicinity of the TNB moiety (most probably His^{146} (βHC3)). It is true that the observed CD spectrum of deoxy-TNB-Hb is given approximately by the sum of the observed CD spectrum of deoxy-native Hb and the expected tail portion of the newly appeared CD band centered at 312 nm, which is obtained by assuming a symmetric, smooth shape. However, a small increment is also visible in the tyrosine band ($\leq 2 \times 10^3$ deg · cm^2 dmol^{-1}). This small increment of ellipticity may be caused by the weak interactions between the TNB chromophore and tyrosine residue(s). To test for the possible interaction, we mixed *p*-cresol and DTNB (**2a**) in CH_3CN or DMF, and measured the uv spectrum. No appreciable increment was observed at concentrations up to 2.1×10^{-3} M for *p*-cresol and 1.5×10^{-3} M for DTNB. Although the concentration is low, the present observations strongly suggest that the direct interaction (e.g., charge-transfer type) of *p*-cresol and DTNB is weak. Therefore, the origin of the ellipticity increment in the tyrosine band is still uncertain.

In conclusion, the tense structure of bovine Hb was conveniently detected by TNB (Fig. 2), an excellent CD marker, via the induced Cotton effect, in which the strong molecular asymmetry at the $\alpha_1\beta_2$ interface induced the large rotational strength.

EXPERIMENTAL PROCEDURES

General. NMR spectra were recorded on a PMX-60 or JEOL 100-H spectrome-

ter. Electronic spectra were measured with a Union SM-401 high-sensitivity spectrophotometer, the cell chamber of which was thermostated at 25.0 ± 0.1°C by circulating thermostated water. CD spectra of native or modified Hb of (0.6–30) × 10^{-6}M concentration were recorded with a Jobin–Yvon Mark III-J dichrograph at room temperature, and data collection was made with a Union CD450 data processor. The CD-50 apparatus manufactured by Toyo was used for preparative electrophoresis.

Material. The following commercially available reagents were used. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, **2a**) (Nakarai Co.): mp 245–246°C (dec) (lit. 237–238°C) (*15*); ν_{max}(KBr) 1690, 1530, 1410, 1360, 1280, 1140, 840, 740, 710 cm^{-1}. 6,6′-Dithiobis(3-nitropyridine) (DTNP, **2b**) (Nakarai Co.): mp 155–156°C (lit. 150–151°C) (*16*); ν_{max}(KBr) 1580, 1550, 1500, 1440, 1330, 1090, 840, 740 cm^{-1}.

5,5′-Dithiobis(*N*-(2-hydroxethyl)-2-nitrobenzamide)(DTNHB, **2c**) was prepared from **2a** according to the reported procedure (*7*): mp 229–230°C (lit. 220–250°C) (*7*); ν_{max}(KBr) 3260, 1630, 1560, 1510, 1350, 1050, 830 cm^{-1}. *Anal.* Calcd for $C_{18}H_{18}N_4O_8S_2$: C, 44.78; H, 3.76; S, 13.22. Found: C, 45.09, H, 3.86; S, 13.20.

5,5′-Dithiobis(2-hydroxybenzoic acid)(DTHB, **2d**). To a mixture of 2-hydroxy-5-mercaptobenzoic acid (*8*) (0.30 g, 1.76 nmol) and triethylamine (0.20 g, 2.0 mmol) in 20 ml EtOH–H_2O (1 : 1, v/v) was added an ethanolic solution of I_2 (0.6 g/20 ml) dropwise at 20°C, until the iodine color no longer disappeared. Evaporation of the solvent *in vacuo,* followed by the addition of 10 ml H_2O, afforded an aqueous solution of **2d** · triethylamine salt. After the filtration of a small amount of insoluble material, the filtrate was acidified with 1 N HCl. The resultant white precipitates were collected by suction filtration, washed with H_2O (2 × 10 ml), and dried *in vacuo* to give 0.14 g (50%) **2d**: mp 208–209°(dec); ν_{max}(KBr), 3030, 1660, 1590, 1420, 1200, 820, 780, 670 cm^{-1}; ^1H NMR($CDCl_3$-Me_2SO-d_6 = 1 : 1, v/v) δ 7.0 (d, 1H, J = 8 Hz), 7.6 (dd, 1H, J = 8, 2.4 Hz), 7.95 (d, 1H, J = 2.4 Hz); MS, *m/e* (rel. intensity) 340 (M + 2, 4), 339 (M + 1, 7), 338 (M, 40), 320 (M − H_2O, 17), 152 (35), 86 (100). The concentration of Hb was determined, after conversion to methemoglobin cyanide, by using the molar extinction coefficient of 11.5 × 10^3 at 540 nm. All hemoglobin concentrations are given on a heme basis throughout the paper.

Preparation of N-Acetyl-S-(3-carboxy-4-nitrophenyl)-L-cysteine (**5**). In a 50-ml two-necked flask, 0.2 g (0.51 mmol) DTNB (**2a**) was suspended in 6 ml $CHCl_3$–ethyl acetate (1 : 1 v/v) mixture. Into the suspension was slowly introduced gaseous Cl_2 (generated from 2.4 g $KMnO_4$ and 30 ml concentrated HCl) with stirring at 0°C until **2a** was completely dissolved, and stirring was continued for additional 2 hr at 0°C. At this stage the amount of the unreacted DTNB was small (less than 10%), judging from the ir spectrum of the reaction mixture. The resultant yellow solution was concentrated to dryness *in vacuo,* to which was added a solution of 0.16 g (0.1 mmol) *N*-acetyl-L-cysteine in 10 ml acetic acid, and the mixture was stirred for additional 2 hr at room temperature. The mixture was evaporated to dryness *in vacuo.* Dissolution of the oily residue in 3 ml ethyl acetate followed by the addition of 10 ml petroleum ether (bp 30–70°C), afforded slightly yellow precipitates (**5**). Yield amounted to 0.34 g (85%). For further purification, reprecipitation of **5** from an ethanol solution by the addition of ether was successfully carried

out: mp 138–141°C (ethanol–ether) (dec); ν_{max}(KBr), 3310, 1720, 1610, 1520, 1340, 1230, 870, 820, 750, 710 cm^{-1}; ^1H NMR ($CDCl_3$-Me_2SO-d_6-D_2O=10 : 1 : 0.5, v/v) δ 2.05 (s, 3H, Ac) 3.1–3.5 (m, 2H, CH_2), 4.5–5.0 (m, 1H, CH) 7.7–8.2 (m, 3H, aromatic proton); uv (EtOH): λ_{max} 318 nm, $\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}; CD(ETOH): λ_{max} 308 nm, $[\theta] = -1.7 \times 10^3$ deg · cm^2 $dmol^{-1}$, 271 nm, $[\theta] = -2.0 \times 10^3$ deg · cm^2 $dmol^{-1}$; MS *m/e* (rel. int.) 265 (21) 230 (M^+-$CH_2CH(NHAc)CO_2H$, 100), 199 (230-S, 5), 186 (18), 166 (18), 148 (19), 122 (199-CO_2, 24).

Chemical Modification of Hb: TNB-Hb (**3a**). An aqueous solution of met-Hb(bovine, Sigma) was converted to aqueous oxy-Hb by the reported procedure (*17*), and the aqueous oxy-Hb was used for the chemical modification (*4*). To reduce met-Hb to the deoxy-Hb chemically, a solution of 100 mg Hb (bovine, Sigma) in 10 ml 0.1 M phosphate buffer (pH 7.0) was treated with 5 mg powdered $Na_2S_2O_4$. Purification of the resultant deoxy-Hb from the excess $Na_2S_2O_4$ and other inorganic salts was conducted through a column under anaerobic conditions. Thus, the top end of a Sephadex G-25 column (2 × 20 cm) was connected to a reservoir containing carefully degassed (freeze–thaw) 0.1 M phosphate buffer with Teflon tubing and connectors. The buffer reservoir was further connected to a vessel filled with argon at normal pressure. A sufficient amount (ca 300 ml) of the degassed buffer was passed through the column before sample injection. Then the deoxy-Hb sample was injected from a syringe onto the column top through Teflon tubing, and the elution was started. A 20-ml aliquot of eluant containing deoxy-Hb was collected and allowed contact with air to regenerate oxy-Hb. Chemical modification of Cys^{93}-SH proceeded at a much faster rate in the oxy-Hb than that in the deoxy-Hb.[7] Thus, to 20 ml oxy-Hb solution (ca. 230 μM) was added a solution of 14 mg (35 μmol) DTNB (**2a**) in 6 ml 0.1 M phosphate buffer (pH 7.0), and the resultant mixture was stirred for 2 hr in the dark. An excess amount of the modifying reagent and the 3-carboxy-4-nitrothiophenolate anion liberated were removed by gel filtration through Sephadex G-25 and/or successive dialysis against 0.1 M phosphate buffer (pH 7.0) at 4°C. TNP-Hb (**3b**) and TNHB-Hb (**3c**) were prepared by the similar procedure described for TNB-Hb (**3a**), by using 10 mg modifying reagent, DTNP (**2b**) or DTNHB (**2c**), respectively. For THB-Hb (**3d**), to a solution of oxy-Hb (0.1 g, 1.2×10^{-6} mol) in 10 ml phosphate buffer (0.1 M phosphate, 0.1 M KCl, pH 7.0) was added a solution of DTHB (**2d**) (26 mg, 7.7×10^{-5} mol) dissolved in 1 ml of the same buffer solution. After the pH of the mixture was adjusted to 9.5 by the addition of 0.1 N NaOH, the mixture was kept standing for 30 hr at 8°C in the dark. Then the pH of the mixture was readjusted to 8.0 by 1 M KH_2PO_4, and the excess disulfide and thiophenolate anions liberated were removed by gel filtration on Sephadex G-25 (2 × 30 cm; 0.1 M phosphate, 0.1 M KCl, pH 7.0). This modified Hb did not react with DTNB, i.e., when 8 M excess DTNB was added to the solution of modified oxy-Hb (**3d**) (pH 7.0, 25°C, 30 min), the absorbance increase at 440 nm was negligible (less than 3×10^{-3} OD), strongly indicating that the SH residues of Hb were already completely (>99.6%) modified by DTHB (**2d**).

Determination of the label content. The CD label content of each modified Hb,

[7] Protein purification was best carried out for the deoxy state.

TNB-Hb (**3a**), TNP-Hb (**3b**), or TNHB-Hb (**3c**), was determined colorimetrically by measuring the absorbance at 440 nm of 3-carboxy-4-nitrobenzenethiolate, 3-nitropyridine-6-thiolate, or 3-[*N*-(2-hydroxyethyl) carboxamide]-4-nitrobenzenethiolate, which were quantitatively liberated from the purified TNB-Hb, TNP-Hb, or TNHB-Hb by the addition of a large excess amount (65 M excess) of mercaptoethanol. Thus, to the oxygenated modified Hb solution (30–40 μM) was added a stock solution of mercaptoethanol to give a final concentration of 1.3 mM at pH 8.0. The absorbance increase (ΔA) at 440 nm was measured by a Union SM-401 spectrophotometer, and the label content was calculated by using the molar extinction coefficients 8.86×10^3 (**4a**), 5.88×10^3 (**4b**), or 10.2×10^3 (**4c**).

Measurements of oxygen binding equilibrium. A 4-ml sample 10–20 μM native oxy-Hb or modified oxy-Hb (**3a–c**) was kept in a quartz cuvet of a Union oxygen meter, and deoxygenated by passing N_2 gas under cooling with ice-water bath. Satisfactory deoxygenation (conversion of oxy-Hb to deoxy-Hb was followed by electronic spectrum) required 2–4 hr, depending on the oxygen affinity of Hb. After the satisfactory deoxygenation was attained, air was introduced slowly[8] onto the solution of deoxy-Hb with stirring, and both the oxygen concentration and the optical density at 567 nm were recorded on a X-Y recorder at 25°C. The recorded equilibrium curve was analyzed by Hill's treatment (*14*). P_{50} values and Hill coefficients (n) evaluated are shown in Table 1.

Multiphase zone electrophoresis on polyacrylamide gels. Preparative gel electrophoresis was performed using the apparatus described previously (*18*). In a typical experiment 10 ml 0.1 M phosphate buffer (pH 7.0) containing about 25 mg hemoglobin and 5% (w/v) sucrose was carefully layered on top of a preparative acrylamide column, 4.0 cm in height with a 12.5 cm^2 cross-sectional area (40 ml lower gel, 10 ml upper gel). A constant current of 50 mA and 350 V was applied at 4°C. The acrylamide concentrations of the upper gel (concentrating gel) and the lower gel (separating gel) were 3.1 and 7.7%, respectively. The elution rate was 0.2 ml/min. Fractions (3 ml each) were collected, and the concentration of Hb was measured by electronic absorption of 254 and 410 nm.

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[8] The procedures are based on the reported one (*19*). An acetyl-cellulose-coated platinum electrode and an Ag/AgCl electrode, purchased from Union Giken Company, were used as anode and cathode, respectively, and 0.6 V was applied. The electrodes showed 90% O_2 response after 3 sec.

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