Structure-Function Relationships in Hemoglobin Kariya, Lys-40(C5)α → Glu, with High Oxygen Affinity

FUNCTIONAL ROLE OF THE SALT BRIDGE BETWEEN LYS-40α AND THE β CHAIN COOH TERMINUS*

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The ε-amino group of Lys-40α forms a salt bridge with the ε-carboxyl group of β chain in deoxyhemoglobin and is considered to impose a constraint upon hemoglobin tetramer, stabilizing the T quaternary structure. Hb Kariya, in which Lys-40α is replaced by Glu, provides a unique opportunity to investigate the functional role of this salt bridge. Hb Kariya showed oxygen binding properties characterized by a high affinity, diminished cooperativity, a reduced alkaline Bohr effect, and a decreased effect of phosphates upon oxygen affinity. In deoxyHb Kariya the reactivity of the sulfhydryl groups of cysteins-93β with 4,4'-dipyridine disulfide was profoundly enhanced, being comparable to that for normal oxyhemoglobin (oxyHb A). The Soret band spectra, UV derivative spectra, and UV oxy-minus-deoxy difference spectra indicated that oxyHb Kariya assumes a quaternary structure similar to that of oxyHb A whereas the T structure of deoxyHb Kariya is destabilized, and Hb Kariya remains predominantly in the R state upon deoxygenation. Resonance Raman scattering by deoxyHb Kariya showed that the Fe-Nε(proximal His) bond is less stretched than that of deoxyHb A. These experimental results provide structural basis for explaining the oxygen binding characteristics of Hb Kariya and further give direct evidence that the intersubunit salt bridge between Lys-40α and the β chain COOH terminus actually contributes to stabilization of the T quaternary structure, thereby playing a key role in cooperative oxygen binding by hemoglobin. The nature of another salt bridge between Asp-94β and the COOH-terminal His of β chain was also discussed in comparison with the salt bridge involving Lys-40α.

In 1983 we reported a new hemoglobin variant Kariya, Lys-40(C5)α → Glu, which was discovered in a Japanese man (1). Preliminary oxygen binding experiments showed that this variant had an increased affinity for oxygen, decreased cooperativity, and a weakened response to 2,3-diphosphoglycerate (DPG)† (1). Afterward, another case of Hb Kariya, which also showed a high oxygen affinity, was reported (2).

According to x-ray crystallographic analysis (3), the 40th position (the 5th position of helix C) of α chain is fixed at the α1-β2 contact. In deoxyhemoglobin adult hemoglobin (Hb A) the ε-amino group of Lys-40α makes up an interchain salt bridge with the ε-carboxyl group of the COOH-terminal His of the partner β chain in the α1-β2 pair (4, 5). Further, the imidazole group of the COOH-terminal His (1468) forms an intrachain salt bridge with the γ-carboxyl group of Asp-94(FG1)β (6). These salt bridges impose constraints upon deoxyhemoglobin, stabilizing the normal deoxy tertiary and quaternary structures. In the T state the side chain of the penultimate Tyr of β chain is fixed in its pocket between helices E and H. Upon oxygenation the Tyr pocket is narrowed, expelling the Tyr side chain out. The expelled Tyr, in turn, pulls the COOH-terminal His and gives rise to rupturing its salt bridges to Lys-40α and Asp-94β, shifting the allosteric equilibrium toward the R state (6). The imidazole groups of histidines-1468 undergo lowering of their pK values and release protons, contributing to 40% of the alkaline Bohr effect (6). Thus, the structural data acquired by x-ray analysis indicate that the salt bridge involving Lys-40α together with that involving Asp-94β plays a key role in cooperative oxygen binding by hemoglobin.

Several mutant hemoglobins having amino acid substitutions for His-1468 or Asp-94β are available. However, no other mutant than Hb Kariya in which Lys-40α is replaced has yet been discovered and, therefore, Hb Kariya provides a unique opportunity to investigate the functional role of the salt bridge involving Lys-40α.

In the present study, therefore, we intended to investigate detailed structure-function relationships in Hb Kariya. For this purpose we measured oxygen equilibrium curves under a variety of solution conditions, reaction rate of the sulfhydryl groups of cysteins-93(F9)/3 with 4-PDS in both oxy and deoxy forms, visible and Soret region absorption spectra for various derivatives, UV region derivative spectra and oxy-minus-deoxy difference spectra, and resonance Raman spectra of hemoglobin. The results obtained from these experiments are fully consistent with those expected from the Perutz' (6) stereocmological model for cooperative effects in hemoglobin.

The abbreviations used are: DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Hb A, human adult hemoglobin; 4-PDS, 4,4'-dipyridine disulfide; NES-Hb, N-ethylsuccinimide Hb.

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EXPERIMENTAL PROCEDURES

Materials

Hb Kariya was isolated from carrier's whole hemolysate and purified by isoelectric focusing (7, 8). Hb A separated from Hb Kariya was used as control. These hemoglobin samples were prepared in Cary 118C Varian Associates. During determination the electric signals from the oxygen electrode amplifier and the spectrophotometer were acquired at real time by a microcomputer, model PDP-11/03, Digital Equipment Corp. (13). The oxygenation data were analyzed as previously described (14). Overall oxygen affinity, cooperativity in oxygen binding, and the magnitude of the Bohr effect were expressed in terms of partial pressure of oxygen at half-oxygen saturation, $P_{50}$, the Hill coefficient, $n_{max}$, i.e. the maximum slope of the Hill plot, and the Bohr coefficient, $\delta H^+$ (= $\Delta \log P_{50}/\Delta pH$), respectively.

Methods

Oxygen Equilibrium—Oxygen equilibrium curves were determined with an automatic oxygenation apparatus of Imai and co-workers (10–12). The spectrophotometer used for the apparatus was a model Cary 118C, Kyoto. Bis-Tris and inositol hexaphosphate (IHP) were purchased from Sigma. DPG was a product of Behring Diagnostics. 4-PDS was purchased from Aldrich. All other chemicals were analytical grade.

Hemoglobin Content—Visible range absorption spectra of each hemoglobin sample used for oxygen equilibrium measurement was recorded on a double-beam spectrophotometer (model 320L, Hitachi Co., Tokyo) immediately before and after oxygen equilibrium measurement. MetHb content was calculated from absorbance readings at 560, 576, and 630 nm by using the millimolar absorption coefficient values at different pH values (15). In most cases, the MetHb contents after measurement were less than 10% for both the Hbs Kariya and A. Under acidic conditions, however, they were as large as 13%.

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RESULTS

Oxygen Equilibrium—Fig. 1 shows Hill plots of oxygen binding by Hbs Kariya and A at different pH values in the presence and absence of 2 mM IHP. Compared with the plots for Hb A, the plots for Hb Kariya are shifted toward the left and less steep. Shifts of the plots upon pH changes or addition of IHP are smaller for Hb Kariya than for Hb A.

The log $P_{50}$ and $n_{max}$ values obtained from these equilibrium curves are listed in Table I and plotted against pH in Fig. 2. The overall oxygen affinity of Hb Kariya is higher than that of Hb A over all the pH conditions examined. The ratio of their $P_{50}$ values is 8.5 at pH 7.45 and becomes smaller with increase in pH. Cooperativity of Hb Kariya is greatly diminished: its $n_{max}$ values are close to unity, showing a maximal value of 1.48 compared with 2.86 for Hb A at pH 7.55. The alkaline Bohr effect of Hb Kariya is reduced: the Bohr coefficient for Hb Kariya obtained from the slope of the log $P_{50}$ versus pH plot is −0.38, being three-quarters that for Hb A ($6H^+ = −0.51$). The effect of phosphates on oxygen affinity is smaller for Hb Kariya than for Hb A. The ratios of $P_{50}$ in the presence of 2 mM DPG at pH 7.45, 2 mM IHP at pH 7.55, 2 mM IHP at pH 6.95, and 0.1 M Pi at pH 6.95 are 1.4, 4.2, 5.1, and 1.3, respectively, for Hb Kariya compared with 2.3, 9.7, and 1.7, respectively, for Hb A. Due to the different responses to phosphates, the ratio of oxygen affinities for the two hemoglobins becomes larger on the addition of phosphate, being 16-fold in the presence of 2 mM IHP at pH values 7.55 and 6.95.

The oxygen affinity of Hb Kariya at pH 9.0 is comparable to that of isolated chains of Hb A (21). Values of the Adair constant ($K_a$) for Hb Kariya evaluated by a nonlinear least squares curve-fitting method (14, 20) were within a range of 4.0–7.3 mmHg$^{-1}$ above pH 7 and in the absence of added
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TABLE I
Values of \( P_{50} \), \( n_{\text{max}} \), and the four Adair constants for Hb Kariya and Hb A

| Conditions* | Hb Kariya | Hb A |
|-------------|-----------|------|
| pH          | \( P_{50} \) | \( n_{\text{max}} \) | \( K_{1} \) | \( K_{2} \) | \( K_{3} \) | \( K_{4} \) | \( P_{50} \) | \( n_{\text{max}} \) | \( K_{1} \) | \( K_{2} \) | \( K_{3} \) | \( K_{4} \) |
| 9.0         | 0.35      | 1.23 | 1.82 | 4.9 | 1.6 | 5.3 | 1.30 | 2.42 | 0.18 | 0.23 | 2.0 | 4.8 |
| 8.5         | 0.41      | 1.22 | 2.5 | 2.1 | 2.3 | 4.3 | 1.48 | 2.57 | 0.16 | 0.19 | 1.3 | 6.4 |
| 7.55        | 0.44      | 1.20 | 1.8 | 2.5 | 1.8 | 4.0 | 2.53 | 2.66 | 0.065 | 0.42 | 7.5 |
| 7.45        | 0.53      | 1.48 | 2.8 | 2.6 | 0.65 | 7.3 | 3.70 | 2.79 | 0.062 | 0.073 | 0.27 | 6.1 |
| 6.95        | 0.52      | 1.26 | 1.2 | 3.2 | 1.1 | 4.0 | 4.42 | 2.88 | 0.056 | 0.049 | 0.16 | 8.3 |
| 6.44        | 0.86      | 1.47 | 0.48 | 3.9 | 0.28 | 4.2 | 7.1 | 2.84 | 0.028 | 0.055 | 0.052 | 6.8 |
| 5.85        | 1.27      | 1.33 | 0.44 | 1.2 | 0.41 | 2.0 | 9.4 | 2.72 | 0.037 | 0.022 | 0.087 | 2.9 |
| 7.55 2 mM DPG | 1.62      | 1.32 | 0.29 | 0.77 | 0.46 | 1.5 | 13.4 | 2.61 | 0.013 | 0.063 | 0.0062 | 7.9 |
| 7.55 2 mM IHP | 0.73      | 1.33 | 0.52 | 3.1 | 0.61 | 3.6 | 10.3 | 2.95 | 0.026 | 0.010 | 0.18 | 2.7 |
| 6.95 2 mM IHP | 4.37      | 1.78 | 0.060 | 0.18 | 0.39 | 0.53 | 69 | 2.12 | 0.0051 | 0.0077 | 0.013 | 0.013 | 0.90 |
| 6.95 0.1 M Pi | 1.10      | 1.44 | 0.65 | 1.8 | 0.29 | 3.1 | 13.4 | 2.61 | 0.010 | 0.069 | 0.022 | 3.3 |

*Other experimental conditions: Hb concentration, 60 \( \mu \)M on a heme basis; 25 °C; in 0.05 M bis-Tris, pH ±7.45, or 0.05 M Tris, pH ±7.55, containing 0.1 M Cl\(^-\). These buffers were not used in the presence of 0.1 M potassium phosphate.

**Fig. 2. Log \( P_{50} \) and \( n_{\text{max}} \) values as a function of pH.** The data in Table I are plotted. Open and closed symbols stand for Hb Kariya and Hb A, respectively. O and ●, phosphate-free; △ and ▲, +2 mM DPG; ▽ and ▼, +2 mM IHP; □ and ■, in 0.1 M potassium phosphate. The Bohr coefficient, \( \Delta H^+ \), was evaluated from the maximal slope of the Bohr plots (log \( P_{50} \) versus pH), giving \( \Delta H^+ = -0.51 \) for Hb A.

Reactivity of Sulfhydryl Groups—The reactions with 4-PDS phosphate (Table I). These \( K_i \) values essentially agree with those of Hb A which range between 4.8 and 8.3 mmHg\(^{-1} \) (10). In contrast, the \( K_i \) values for Hb Kariya were more than 10 times larger than those for Hb A under all the solution conditions used (Table I).

In oxyform, no difference in the rate constant was detected. In contrast, in deoxyform, the rate constant for Hb Kariya was 43 times larger than that for Hb A. This striking difference occurred from a profound reduction of the reaction rate for Hb A compared with a slight reduction for Hb Kariya upon deoxygenation. Thus, the rate constant for deoxyHb Kariya is comparable to that for oxyHb Kariya.

Absorption Spectra—Absorption spectra were recorded at 20 °C for hemoglobin preparations diluted in 0.05 M bis-Tris, pH 7.4, containing 0.1 M Cl\(^-\). No difference between Hb Kariya and Hb A was detected in the visible range (450-650 nm) absorption spectra for oxy, deoxy, aquomet, and cyanomet derivatives.

At pH 7.4, the Soret peak for deoxyHb Kariya was 8% lower than that for deoxyHb A while the Soret peak for oxy form was of the same height for the two hemoglobin. The nature of the Soret band for deoxy form was further investigated by difference spectrum measurements. Fig. 3 shows pH 6.4 minus pH 9.1 and IHP-added minus IHP-free difference Soret band spectra for deoxyHbs Kariya and A. In both the cases, Hb Kariya showed a distinct difference peak at 430 nm whereas the spectra for Hb A were of poor feature.

**UV Derivative Spectra**—Fig. 4 shows the first derivative of UV region absorption spectra for oxy- and deoxyHbs Kariya and A. In Hb A, the magnitude of the fine structure appearing around 290 nm is halved upon deoxygenation. In Hb Kariya,
however, the magnitude of the fine structure for oxy form, which is identical to that for oxy-Hb A, was only decreased to 70% upon deoxygenation.

**UV Difference Spectra**—A UV region oxy-minus-deoxy difference spectrum of Hb Kariya is presented in Fig. 5 where those of Hb A, Hb Hiroshima, and des-(His, Tyr) Hb are also shown for comparison. These spectra have fine structures around 290 nm. The magnitude of the fine structure is remarkably decreased in the abnormal and modified hemoglobins having decreased cooperativity.

**Resonance Raman Scattering**—Fig. 6 shows low frequency Raman spectra of deoxyHbs Kariya and A at pH 7.4 in the absence of added phosphate. No significant difference between the two hemoglobins was observed in the Raman lines for porphyrin vibrational modes at or above 300 cm⁻¹. Interestingly, however, the Raman line at 214 cm⁻¹ for deoxyHb A, which was assigned to the Fe-Nt(proximal His) bond stretching mode and was proved to be characteristic of the T quaternary structure (19) was shifted to 219 cm⁻¹ in deoxyHb Kariya. At pH 6.95 in the presence of 2 mM IHP, the low frequency region Raman lines other than the line originating from the Fe-Nt(proximal His) stretching remained essentially identical with those shown in Fig. 6 for both deoxyHbs Kariya and A, but the Fe-Nt(proximal His) stretching line for deoxyHb Kariya was shifted down to 216 cm⁻¹, becoming closer to the corresponding line for deoxyHb A at 213 cm⁻¹.

**DISCUSSION**

**Meanings of Experimental Results**—The enhancement in reactivity of the Cys-938–SH groups associated with oxygenation is accounted for in terms of a screening effect by the two salt bridges of His-1468 which restrict the access of -SH reagents to the -SH groups in the T state but not in the R state (6, 23). The much enhanced reactivity of deoxyHb Kariya which is almost the same as that of oxyHb A (Table II) indicates that the conformation around the β chain COOH terminus of deoxyHb Kariya resembles that of oxyHb A, i.e. the R state.

The peak height of the Soret band for deoxygenated derivative is reduced in hemoglobins with high oxygen affinity and
entirely or highly diminished cooperativity such as isolated $\alpha$ and $\beta$ chains (24), haptooglobin-bound hemoglobin (25), des-(His, Tyr)Hb (26), Hb McKees Rocks (27), Hb Osler (28), and Hb Toyoake (9), and the lowered Soret peak has been used as a diagnostic indicator for “deoxygenated R structure.” The reduction of the Soret peak height in deoxyHb Kariya, which is comparable in magnitude to that in deoxygenated hemoglobins given above, indicates that this hemoglobin remains predominantly in the R state upon deoxygenation. The Soret-band difference spectra in Fig. 3 indicate that, in deoxyHb Kariya, the T structure is stabilized to some extent at pH 6.4 compared at pH 9.1 and also in the presence of IHP compared with in its absence whereas deoxyHb A is predominantly in the T state irrespective of the pH and IHP conditions.

It was shown that the ligand-induced change in the fine structure around 290 nm in UV derivative spectrum of hemoglobin is attributed to perturbations of aromatic amino acid residues, most probably Trp-37(C3)β (22, 29) and/or Tyr-42(C7)α (30) which are located at the $\alpha1-\beta2$ interface and undergo environmental changes upon ligand binding. Magnitude of this fine structure is a good indicator of the quaternary state of hemoglobin since it is closely related to the first and fourth Adair constants ($K_1$ and $K_4$) which are modulated by the addition of DPG or chemical modifications of protein moiyet (22). The present derivative spectra for Hb Kariya (Fig. 4) indicate that oxyHb Kariya assumes the same quaternary structure as that of oxyHb A while a significant fraction of deoxyHb Kariya is in the R state as long as the quaternary state is observed at the $\alpha1-\beta2$ interface.

It was also shown that the magnitude of the fine structure around 290 nm in oxy-minus-deoxy difference spectrum, which is considered to originate from environmental changes for the above-mentioned aromatic residues associated with oxygenation, represents an extent of the oxygenation-induced change of quaternary structure, since it is closely related to $n_{\text{max}}$ and free energy of cooperation ($\Delta \text{G} = K_1 \ln (K_4/K_1)$) which are modulated by the addition of DPG or by chemical modification.

![Resonance Raman spectra for deoxyhemoglobin in low frequency region. Numbers attached to the spectra indicate the wave numbers in cm$^{-1}$ for prominent Raman lines. Hb concentration, 250 $\mu$M on heme basis in 0.05 M bis-Tris, pH 7.4, containing 0.1 M Cl$^{-}$; 20 °C.](image)

**TABLE III**

Comparison of properties of hemoglobins with $\beta$ chain COOH-terminal salt bridges affected

| Hemoglobin designation | Oxygen affinity$^a$ | Bohr effect$^a$ | Cooperativity$^a$ | Phosphate effect$^a$ | $K_1'$ | $K_4'$ | Cys-93$^b$ reactivity | $\Delta(\Delta \text{Abs})^c$ | Ref. |
|------------------------|---------------------|----------------|------------------|---------------------|-------|-------|---------------------|---------------------|-----|
| Hb Kariya              | Lys-40α             | ↑↑↑             | ↓↓↓              | (n = 1.4)          | ↑↑↑   |      | Normal             | (43-fold)           | 31  |
|                        | Glu                 |                 |                  |                     |       |      | Normal             | (5-fold)            | 32  |
| Hb Barcelona           | Asp-94β             | ↑↑               | ↓↓               | (n = 2.4)          | ↑↑↑   |      | Normal             | (8-fold)            | 34  |
|                        | His                 |                 |                  |                     |       |      | Normal             | (2-fold)            | 34  |
| Hb Bunbury             | Asp-94β             | ↑↑               | ↓↓               | (n = 2.4)          | ↑↑↑   |      | Normal             | (5-fold)            | 32  |
|                        | Asn                 |                 |                  |                     |       |      | Normal             | (8-fold)            | 34  |
| Hb Hiroshima           | His-146β            | ↑↑               | ↓↓               | (n = 2.2)          | ↑↑↑   |      | Normal             | (6-fold)            | 35  |
|                        | Asp                 |                 |                  |                     |       |      | Normal             | (2-fold)            | 34  |
| Hb Cowtown             | His-146β            | ↑↑               | ↓↓               | (n = 2.2)          | ↑↑↑   |      | Normal             | (6-fold)            | 32  |
|                        | Leu                 |                 |                  |                     |       |      | Normal             | (2-fold)            | 34  |
| Hb Cochin Port-Royal   | His-146β            | ↑↑               | ↓↓               | (n = 2.2)          | ↑↑↑   |      | Normal             | (8-fold)            | 34  |
|                        | Arg                 |                 |                  |                     |       |      | Normal             | (8-fold)            | 34  |
| Hb York                | His-146β            | ↑↑               | ↓↓               | (n = 2.2)          | ↑↑↑   |      | Normal             | (8-fold)            | 34  |
|                        | Pro                 |                 |                  |                     |       |      | Normal             | (2-fold)            | 34  |
| NES-Hb$^c$             | NES at              | ↑↑               | ↓↓               | (n = 1.8)          | ↑↑↑   |      | Normal             | (6-fold)            | 32  |
|                        | 93β                 |                 |                  | (n = 2.2)          | ↑↑↑   |      | Normal             | (2-fold)            | 34  |

$^a$ Under moderate ionic strength (~0.1 M) at or near physiological pH; upward and downward arrows indicate increase and decrease, respectively, of quantity.

$^b$ Computed in terms of $P_o$.

$^c$ Computed in terms of $\Delta H^\circ$ ($= \Delta \log P_o/\Delta \rho\text{H}$).

$^d$ $n$ is the Hill coefficient.

$^e$ Compared in terms of increment of $\Delta P_o$ upon addition of DPG or IHP.

$^f$ The first Adair constant.

$^g$ The fourth Adair constant.

$^h$ Rate constant in reaction of 4-PDS in deoxy-form.

$^i$ Magnitude of fine structure around 290 nm in oxy-minus-deoxy difference spectrum.

$^j$ Although a reduced $n$ value was observed for hemolysate Bunbury/A, that does not necessarily mean a small $n$ value for pure Hb Bunbury.

$^k$ Hb A treated with N-ethylmaleimide.
showing most drastic changes in oxygen affinity, cooperativity, and phosphate effect, suggesting that the salt bridge between Lys-40α and His-146β imposes a stronger constraint upon

Hb A tetramer than that between Asp-94β and His-146β. The extraordinarily enhanced reactivity of Cys-93β in deoxyHb Kariya further suggests that the former salt bridge makes a greater contribution to the screening of the Cys–SH group than the latter.

Under moderate ionic strength the imidazole groups of histidines-146β make a 40% contribution to the alkaline Bohr effect of Hb A (6, 39). In this light the less than 40% reduction of the Bohr effect in Hbs Barcelona and Cochin Port-Royal needs some structural explanations, although a tentative one has been given to Hb Barcelona (40).

When either of the two β chain COOH-terminal salt bridges is perturbed the quaternary structure change upon oxygenation is impaired as observed at the α1-β2 interface by UV difference spectra for Hbs Kariya, Barcelona, and Hiroshima and NES-Hb. Although no evaluation was attempted for the Monod-Wyman-Changeux model parameters of Hb Kariya, it is obvious that L (allosteric constant) is decreased, $K_T$ (oxygen association constant for the T state) is raised, $K_R$ (oxygen association constant for the R state) remains unchanged in Hb Kariya, just as observed in Hbs Barcelona and Cowtown and NES-Hb. The impairment of quaternary structure change described above would occur in such a way that the R structure is not affected (no change in $K_R$) whereas the T structure is destabilized (decrease in L) and the structural constraints are weakened (rise of $K_T$). These pictures are fully consistent with the proposal given by Perutz (6, 41) that these salt bridges impose constraints upon deoxyHb A tetramer which disappear in oxyHb A, thereby playing a key role in cooperative oxygen binding by hemoglobin.

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