Substitution of the Heme Binding Module in Hemoglobin α- and β-Subunits

IMPLICATION FOR DIFFERENT REGULATION MECHANISMS OF THE HEME PROXIMAL STRUCTURE BETWEEN HEMOGLOBIN AND MYOGLOBIN*

(Received for publication, December 6, 1999, and in revised form, February 7, 2000)

Kenji Inaba‡, Koichiro Ishimori‡, Kiyohiro Imai‡, and Isao Morishima‡

From the ♦Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606-8501 and the ‡Department of Physicochemical Physiology, Medical School, Osaka University, Suita Osaka 565-0871, Japan

A family of hemoproteins containing heme molecules as the prosthetic group plays a key role in various kinds of biological reactions, such as oxygen binding, electron transfer, and peroxide/oxygen activation. Most of these reactions proceed inside the heme cavity, and they are mainly modulated by the heme environmental structure (1–4). In particular, the heme proximal structure is one of the most important determinants for the regulation of heme activities. For example, significant anionic character of the proximal His-174 in cytochrome c peroxidase arising from a strong hydrogen bond between its N$_\text{H}$ and carboxylate of Asp-235 (5) is the primary cause for its extremely low redox potential and stabilization of higher oxidation states of the heme. Also, the proximal His-93 in vertebrate myoglobins is tightly constrained by a hydrogen-bonding network extending from His-93 to the heme 7-propionic acid group and the imidazole of His-97 (6), which is responsible for their considerably lower ligand affinity compared with that of another monomeric globin, leghemoglobin (7). Indeed, recent crystallographic studies on several hemoproteins together with the protein engineering studies report that the proximal ligand, its heme coordination structure, and its local environment virtually determine function and reactivity of hemoproteins (8–12). Therefore, it is a fundamental problem to understand how the heme proximal structure is regulated in hemoproteins.

In our previous paper (13), we have proposed the heme binding module, a protein continuous segment regulating the heme proximal structure in the globin family. The “module” is defined as a compact structural unit on the protein structure, which consists of 10–40 amino acid residues. Originally, the globin structure was decomposed into four modules (M1–M4), each of which corresponds to the exon on the gene structure (14). By engineering many kinds of module-substituted globin proteins and investigating their structural and functional properties, we have shown that the module can be a structural and functional unit that has advantages in producing novel artificial globin proteins, although interactions between the modules are essential for the stable structures (15–17). However, we have also succeeded in producing a novel functional globin protein by the substitution of the pseudo-module,$^1$ of which the boundaries are located at the middle of the modules (18). The βα(PM3)-subunit, where the pseudo-module PM3 is the segment from the center of the module M3 to that of module M4 (Fig. 1), of hemoglobin α-subunit was transplanted into hemoglobin β-subunit, exhibited the α-subunit-type association property, just as did the module M4-substituted β-subunit, βα(M4)-subunit (18). Moreover, NMR analysis demonstrated that the heme coordination structure of the proximal histidine in the βα(PM3)-subunit was converted into the α-subunit type (18).

* This work was supported by Grants-in-aid for Scientific Research (A) and for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture 05249102 (to K. I.) and 07280101 (to K. Ishimori) and by research fellowships of the Japan Society for the Promotion of Science for Young Scientists (to K. Inaba). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests and correspondence should be addressed.

‡ Present address: Centre for Protein Engineering, Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, U.K.

§ To whom reprint requests and correspondence should be addressed.

Tel.: 81-75-753-5921; Fax: 81-75-751-7611; E-mail: morisima@mds.moleng.kyoto-u.ac.jp
Since the change in the heme coordination structure was not observed for the \( \beta\alpha(M4)\)-subunit, it is inferred that the N-terminal half of the pseudo-module PM3, which is outside the module M4 (Fig. 1), serves to modulate the heme coordination structure. These results on the \( \beta\alpha(PM3)\)-subunit lead us to believe that the boundaries of the pseudo-module PM3, F1 and H6 sites, have some structural and functional significance.

Excitingly, a new intron has been discovered near the F1 site on the gene of the Artemia hemoglobin (19), which strongly suggests that the F1 site actually has genetic or evolutionary meanings. Accordingly, the N-terminal half of the pseudo-module PM3 is supposed to be a fundamental segment on globin structure and is referred to as the heme binding module (Fig. 1).

Structural significance of this segment was also confirmed by studies on Mb(\( \beta\alpha\))-globin, in which the heme binding module of myoglobin was replaced by that of hemoglobin \( \alpha\)-subunit (13). On the basis of a variety of spectroscopic data including absorption, NMR, and resonance Raman spectra, heme electronic state and heme coordination structure of the proximal histidine in the Mb(\( \beta\alpha\))-globin were quite similar to those in the \( \alpha\)-subunit, not to those in myoglobin. This structural conversion in the Mb(\( \beta\alpha\))-globin clearly demonstrated that the heme binding structure is regulated by the heme binding module.

In the present study, to further examine the structural regulation by the heme binding module in hemoglobin, we have synthesized a novel chimeric globin, \( \beta\alpha\)-subunit, where the heme binding module of hemoglobin \( \beta\)-subunit was replaced by that of \( \alpha\)-subunit (Fig. 1), and investigated its heme environmental structure in detail. Unfortunately, the isolated \( \beta\alpha\)-subunit was so unstable that its oxy and deoxy forms were hardly characterized to confirm the structural conversion by the module substitution. In the presence of native \( \alpha\)-subunit, however, the \( \beta\alpha\)-subunit was stabilized by forming \( \alpha\beta\alpha\beta\) tetramers, affording similar NMR and resonance Raman spectra to those of the \( \beta\)-subunit in tetrameric Hb A. In the results, we did not find the structural conversion by the module substitution for the \( \beta\alpha\)-subunit in the complex with native \( \alpha\)-subunit, which implies that the structural regulation mechanism for hemoglobin subunits in the tetramer is different from that for myoglobin. In contrast to monomeric myoglobin, it is likely that the heme binding structure of tetrameric hemoglobin is closely coupled to the subunit interactions rather than to amino acid sequence of the heme binding module.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vector**—To construct the gene of the \( \beta\alpha\)-subunit, \( KpnI\) (GGTACC) and \( SacI\) (GAGCTC) sites were introduced at the boundaries of the heme binding module in the \( \beta\)-and \( \alpha\)-subunits, both, by polymerase chain reaction (Fig. 1), accompanied with the mutation Arg(G6) to Glu.\(^2\) The small \( KpnI\)-\( SacI\) fragment of the \( \alpha\)-subunit amplified by polymerase chain reaction was ligated with the digested \( T7\) expression vector encoding the \( \beta\)-subunit. Methionine was substituted for valine at the N terminus to initiate the peptide elongation for the globins. Construction of the desired expression vector was verified by the double-stranded DNA sequence analysis (373 DNA sequencer, Applied Biosystems).

\(^2\) The abbreviation used is: HBM, heme binding module.

\(^3\) The x-ray crystallographic study of hemoglobin, the side-chain of Arg(G6) is located on the protein surface of the \( \beta\)-subunit, and it does not directly contribute to the heme contact. The mutation at the G6 position, as found in the abnormal hemoglobin, Hb Sherwood Forest, has minor effects on the stability of the globin structure (69), although its oxygen affinity is slightly higher than that of normal hemoglobin (70). In fact, Glu(G6) is also found in other globins such as mammalian myoglobin. Therefore, it is unlikely that the mutation, Arg(G6) to Glu, in the \( \beta\alpha\)-subunit is a serious problem for the evaluation of structural significance of the heme binding module in hemoglobin.

**FIG. 1.** Boundaries of the original module (M1–M4), pseudo-module (PM1–PM3), and HBM, residues with well defined functional roles in hemoglobin subunits (64), and \( \beta\alpha\)-subunit synthesized in this study. Restriction enzyme sites used in this preparation are noted in parentheses. Black boxes represent the residues contributing to the heme contact and subunit contact.

**Protein Preparation**—The prepared gene encoding the \( \beta\alpha\)-subunit was transformed into an Escherichia coli strain (BL21), which was grown at 37 °C overnight in 2x TY (tryptone-yeast extract) culture medium containing ampicillin (100 \( \mu\)g/ml). The expressed subunit was purified as previously reported for recombinant Hb (20, 21). We confirmed the correct expression of the desired subunits by fast atom bombardment mass spectroscopy (data not shown) (22), and no additional mutations were detected. We also synthesized the wild-type \( \beta\)-subunit,\(^4\) which has methionine at the N terminus instead of valine as the reference and confirmed that the structural properties of the wild-type \( \beta\)-subunit are virtually the same as those of native \( \beta\)-subunit isolated from human red blood cell.

The carbon monoxide derivative was prepared by adding minimal amounts of sodium dithionite to the ferric globins under CO atmosphere. The globin solution was deoxygenated by repeated evacuation and flushing with \( N_2\) gas under gentle shaking, and complete deoxygenation was achieved by the addition of minimal amount of sodium dithionite.

**Gel Chromatogram**—Gel filtration measurements were performed by using a Sephacryl S-200 high resolution column (0.8-cm diameter × 62-cm length) at 4 °C. The buffer used for the chromatography was 50 mM Tris-HCl in the presence of 0.1 M NaCl and 1 mM Na2EDTA at pH 7.4, and the flow rate was 7 ml/h. The eluted fractions were monitored by absorption at the Soret band (420 nm). Tetramer-dimer dissociation constants of the samples were determined by the concentration dependence of the elution volume in the column over the range from 0.5 to 800 \( \mu\)M (23, 24). The dependence of the centroid elution volume \( V_e\) versus protein concentration (\( C_p\)), Equation 1, allows us to determine the dimer-tetramer equilibrium constants for the samples.

\[
V_e = \sum V_j (m_j)/C_T
\]  
(Eq. 1)

where \( V_j\) is elution volumes for the individual species pertaining to the various aggregates (\( m\)-mers), and the \( m_j\) term represents molar concentration for the respective species.

**Spectral Measurements**—\(^1\)H NMR spectra at 500 MHz were recorded

\(^4\) Wild-type subunit represents the protein expressed in E. coli; a methionine residue is located at the N terminus. “Native” subunit corresponds to the protein purified from human red blood cell. In the wild-type \( \beta\)-subunit, we confirmed that the mutation of Val to Met does not seriously perturb the globular structure and heme environmental structure of the \( \beta\)-subunit. The CD and NMR spectra for the wild-type \( \beta\)-subunit were virtually the same as those of the native \( \beta\)-subunit.
RESULTS

Association Properties—Before analyzing the heme environmental structure of the βα(HBM)-subunit, we investigated its association property with native hemoglobin subunits, since the heme environmental structure of globin proteins is very sensitive to the subunit assembly (33, 34). Fig. 2A represents gel chromatograms of the carbonmonoxy chimeric subunit in the presence and absence of the native subunits. Under the condition employed here, the mixture of the native α- and β-subunits forms a tetramer, whereas the isolated α-subunit remains a monomer (23). The β-subunit is in equilibrium between monomers and tetramers (23). As shown in Fig. 2A, the position of the elution peak for the βα(HBM)-subunit corresponds to that of the α-subunit, indicative of its monomeric structure. In the chromatogram for the mixture of the βα(HBM)- and β-subunits, a single broad peak was observed, but this elution pattern can be simulated by simple addition of those of the isolated subunits. On the other hand, the mixture of the βα(HBM)- and α-subunits showed a single broad peak that cannot be reproduced by the simple addition (Fig. 2A). This broad peak was significantly shifted to the lower elution volume from that of the isolated βα(HBM)- or α-subunit. These elution patterns, therefore, demonstrate that the βα(HBM)-subunit was preferentially associated with the α-subunit not with the β-subunit.

However, the dissociation constant between the βα(HBM)- and α-subunits is much larger than that between native α- and β-subunits. Fig. 2B delineates the centroid elution volumes of the samples as a function of protein concentration, which clearly shows that the complex of the βα(HBM)- and α-subunit is in equilibrium between heterodimeric αβ(HBM)- and heterotetrameric αβ(βα(HBM)₂). The fitting curve of the complex shifts to the right side from that of Hb A (Fig. 2B), revealing that the dissociation into the dimers was enhanced for the complex. The tetramer-dimer dissociation constants, KD values, were estimated as 1.4 and 40 μM for Hb A and the complex of the βα(HBM)- and α-subunits, respectively.

Subunit Interface Structure—To investigate the subunit interface structure of heterotetrameric αβ[βα(HBM)₂], we measured the 1H NMR spectra in the hydrogen-bonded proton region for the carbonmonoxy and deoxy forms (Figs. 3, A and B). In the downfield region from 10 to 15 ppm, three exchangeable proton signals were observed for carbonmonoxy Hb A, which have been assigned to the hydrogen bonds in the subunit interface, as described in the figure legends (35–37). The αβ[βα(HBM)₂] tetramer also exhibited three exchangeable resonance peaks at almost the same positions as Hb A (Fig. 3A), and the spectral feature in this region was quite similar to that of Hb A. Dissociation of the ligands from the heme iron induces the structural rearrangements on the αβ₂ subunit interface in Hb A (35, 36), which is clearly reflected in the downfield region of the NMR spectra (Fig. 3B). Additional
exchangeable proton resonances were observed at 13.9 and 11.0 ppm for deoxy Hb A (Fig. 3B), assignable to the hydrogen bonds of \( ^{\alpha}\text{Tyr}(C7), ^{\beta}\text{Asp}(G1) \) (35) and \( ^{\alpha}\text{Asp}(G1), ^{\beta}\text{Trp}(C3) \), respectively (38). The deoxy-\( ^{\alpha}\beta[^{\beta}\text{HBMM}]_2 \) tetramer also afforded these additional peaks, although the peak positions slightly moved from the positions for Hb A (Fig. 3B). Thus, the spectral features of the \( ^{\alpha}_{\alpha}[^{\beta}\text{HBMM}]_2 \) tetramer were almost identical with those of Hb A in the carbonmonoxy and deoxy states, implying that the structural rearrangements on the \( ^{\alpha}_{\beta}[^{\beta}_{\alpha}\text{subunit interface}} \) induced by deoxygenation in the \( ^{\alpha}_{\beta}[^{\beta}_{\alpha}\text{HBMM}]_2 \) tetramer correspond to those in native Hb A.

Heme Environmental Structure in Deoxy and Carbonmonoxy Forms—By using a combination of spectroscopic methods, we examined structural effects of the module substitution on the heme environmental structure. NMR spectra in the hydrogen-bonded proton region for deoxy globins. Proton resonance exchangeable proton resonances were observed at 13.9 and 11.0 ppm for deoxy Hb A (Fig. 3B), assignable to the hydrogen bonds of \( ^{\alpha}\text{Tyr}(C7), ^{\beta}\text{Asp}(G1) \) (35) and \( ^{\alpha}\text{Asp}(G1), ^{\beta}\text{Trp}(C3) \), respectively (38). The deoxy-\( ^{\alpha}_{\beta}[^{\alpha}\text{HBMM}]_2 \) tetramer also afforded these additional peaks, although the peak positions slightly moved from the positions for Hb A (Fig. 3B). Thus, the spectral features of the \( ^{\alpha}_{\alpha}[^{\beta}\text{HBMM}]_2 \) tetramer were almost identical with those of Hb A in the carbonmonoxy and deoxy states, implying that the structural rearrangements on the \( ^{\alpha}_{\beta}[^{\beta}\text{subunit interface}} \) induced by deoxygenation in the \( ^{\alpha}_{\beta}[^{\beta}\text{HBMM}]_2 \) tetramer correspond to those in native Hb A.

To gain further insights into the heme coordination structure of the proximal histidine, we also measured the resonance Raman spectra of the globins in the deoxy form. Fig. 5 shows the low frequency region of the Raman spectra measured with a 1.00 cm \(^{-1} \) resolution. The stretching mode of the Fe-His bond, \( ^{\nu}(\text{Fe-His}) \), for the \( \alpha \) - and \( \beta \) -subunits were observed at 220 and 222 cm \(^{-1} \), respectively (41). For tetrameric Hb A, the peak position of the \( ^{\nu}(\text{Fe-His}) \) was shifted by 7 cm \(^{-1} \) to the lower wave number from that of the isolated \( \beta \)-subunit, indicating that the structural strain is imposed on the Fe-His bond by association of the \( \alpha \) - and \( \beta \)-subunits (42). In the complex of the \( \beta[^{\alpha}\text{HBMM}]_2 \) and \( ^{\alpha}_{\beta}[^{\beta}_{\alpha}\text{HBMM}]_2 \), the stretching mode of the Fe-His bond was observed at 215 cm \(^{-1} \), revealing that the Fe-His bond in deoxytetrameric hemoglobin is insensitive to the substitution of the heme binding module.

\(^{1}H\) NMR spectrum for the carbonmonoxy form is also useful for characterization of the heme environmental structure. As shown in Fig. 6, a peak from \( \gamma \)-methyl proton of Val (E11) appeared at \(-2.0 \) and \(-2.2 \) ppm for the carbonmonoxy \( \alpha \) - and \( \beta \)-subunits, respectively, which can serve as a marker for the heme environmental structure (33, 43, 44). The corresponding signals of Hb A and the \( ^{\alpha}_{\beta}[^{\beta}\text{HBMM}]_2 \) tetramer were detected at the same position (Fig. 6), suggesting that the local...
structure around the Val (E11) residue in the $\alpha_2[\beta\alpha(HBM)]_2$ tetramer is similar to that in Hb A. On the contrary, the signals between $-0.5$ and $-1.5$ ppm from other heme-surrounding residues were significantly different between Hb A and the $\alpha_2[\beta\alpha(HBM)]_2$ tetramer, indicative of some conformational changes in the heme vicinity by the module substitution. Such a conformational change seems to be enhanced for the isolated $\beta\alpha(HBM)$-subunit. The resonance peak from $\gamma_1$-methyl proton of Val (E11) was detected around $-2.0$ ppm, as was in the native subunits, but it is highly asymmetric and split (Fig. 6), suggesting the heterogeneity in its heme environmental structure.

Heme Environmental Structure in Cyanomet Form—Although the cyanomet form is not biologically active in globin proteins, the $^1$H NMR spectra afford various structural information for liganded hemoglobin (45–48). As illustrated in Fig. 7, 5-, 1-methyl, and 2-vinyl $\alpha$ proton signals have been assigned for the human hemoglobin $\alpha$- and $\beta$-subunits and tetrameric Hb A (49). Noteworthy here is that the spectral feature of the cyanomet-$\alpha_2[\beta\alpha(HBM)]_2$ tetramer was also virtually identical to that of Hb A, indicating that the heme electronic state of the $\alpha_2[\beta\alpha(HBM)]_2$ tetramer was not significantly affected by the module substitution. For the isolated $\beta\alpha(HBM)$-subunit, the resonances from the heme methyl and vinyl groups were detected at almost the same positions as those for the isolated $\beta$-subunit, but these resonance peaks were clearly split (Fig. 7). These split peaks were still observed after sufficient reconstitution time with the heme molecule (48 h). These results suggest that there are two kinds of heme local conformations in the isolated $\beta\alpha(HBM)$-subunit (47, 48), which is consistent with the heterogeneity in the heme environmental structure found for its carbonmonoxy form (Fig. 7).

Oxygen Binding Property—To evaluate effects of the module substitution on the oxygen affinity and cooperativity for the oxygen binding, oxygen equilibrium curves were measured for the complex of the $\beta\alpha(HBM)$- and $\alpha$-subunits. Fig. 8 represents the oxygen equilibrium curves expressed by the Hill plot. The
Heme Binding Module in Hemoglobin

The heme environmental structure in the isolated heme binding module. The rapid autooxidation of the complex of the Mb(HBM)-subunit was, therefore, seriously perturbed upon the substitution of the heme binding module induced different structural effects on Hb A. As shown in Figs. 4 and 5, the NMR and resonance Raman peaks of the complex were observed at almost the same positions as those of Hb A. Since the hyperfine shift of the proximal histidyl N-H proton and the Fe-His vibrational mode in the deoxy state depend on the bond strength between the heme iron and proximal histidine (34, 41, 42), such similar spectral features indicate that the heme coordination structure of the proximal histidine in the complex of the Mb(HBM)- and α-subunits was identical with that of Hb A. In addition to the heme coordination structure, the αg[βα(HBM)]3 tetramer exhibited almost the same heme electronic state as Hb A. Chemical shifts of the resonances from the heme peripheral groups were quite common between αg[βα(HBM)]3 tetramer and Hb A both in deoxy and cyanomet derivatives (Figs. 4 and 7), suggestive of their identical heme electronic states. Thus, the substitution of the heme binding module did not significantly affect the heme environment of Hb A, and the heme environmental structure of the Mb(HBM)-subunit bound to the α-subunit was equivalent to that of the β-subunit bound to the α-subunit.

This finding is interesting in that the substitution of the heme binding module induced different structural effects on Hb A and myoglobin. The heme proximal structure of the Mbα(HBM)-globin was quite similar to that of the α-subunit (13), demonstrating that the substitution of the heme binding module is sufficient to convert the heme proximal structure to the α-subunit-type in myoglobin. However, such a structural conversion was not encountered for the complex of the βα(HBM)- and α-subunits. These different structural effects by the module substitution strongly suggest that regulation mechanism of the heme environmental structure is different in Hb A and myoglobin. One of the key features for the different structural regulations in these two globins is that the hemoglobin subunits, α- and β-subunits, form a α2β2 heterotetramer unlike myoglobin. The heme proximal structure of monomeric myoglobin can simply be determined by the amino acid sequence of the heme binding module. In the β-subunit of the tetrameric Hb A, on the other hand, the subunit association with the α-subunit significantly affects the heme proximal structure. Comparisons of the spectroscopic properties and x-ray structures between the isolated and α-subunit-bound β-subunits clearly show some structural difference around the heme proximal structure (34, 53). By binding the α-subunit, the subunit interface of the βα(HBM)-subunit would be rearranged to that of the β-subunit.
in tetrameric Hb A, which leads to the β-subunit-like heme proximal structure in the β(HBM)-subunit. Based on the fact that the heme binding module includes both the residues contributing to the α1-β2 subunit contact and to the heme contact (Fig. 1), it is likely that the heme proximal structure constituted by the heme binding module is affected by the α1-β2 subunit interactions. Although the limited structural information of the isolated β(HBM)-subunit prevents us from drawing the confirmative conclusion, it could be safely said that there are some interplays between the subunit interactions and the heme proximal structure in tetrameric Hb A.

Interestingly, it can be inferred that the interplays in Hb A are deeply related to its cooperative oxygen binding property. On the basis of the Perutz model (54, 55), the movement of the proximal histidine induced by the ligand binding or dissociation is linked to structural rearrangement on the subunit interface to control high (R) and low (T) oxygen affinity states. Notably, deletion of the bond between the proximal histidine side chain and the polypeptide prevented the quaternary structure in native Hb A is closely coupled to the cooperativity (56). This result strongly supports that the heme side chain and the polypeptide prevented the quaternary structure is linked to structural rearrangement on the subunit interface. As encountered for deoxy Hb A, two additional peaks explained by the structural rearrangement on its subunit interface. As found for the deoxy Hb A, two additional peaks attributable to the heme proximal structure in tetrameric Hb A.

**Oxygen Binding for the Complex of the β(HBM)- and α-Subunits**—The present study on the β(HBM)-subunit also involves some information on oxygen binding mechanism of Hb A. In the oxygen equilibrium curves (Fig. 8), the complex of the β(HBM)- and α-subunits clearly exhibited cooperative oxygen binding, although its $n_{\text{max}}$ value was even lower than that of Hb A. The cooperative oxygen binding of the complex can be explained by the structural rearrangement on its subunit interface. As encountered for deoxy Hb A, two additional peaks assignable to the hydrogen-bonded protons on the functional α1-β2 interface appeared in the NMR spectra of deoxy α2β2(HBM)$\beta$ tetramer (Fig. 3), strongly suggesting its T-quantenary structure. Also, since the deoxy-α2β2(HBM)$\beta$ tetramer showed the hyperfine shift of the proximal histidyl $N\_H$ proton and the Fe-His vibrational mode at almost the same positions as Hb A (Figs. 4 and 5), the movement of the proximal histidine linked to the structural rearrangement on the α1-β2 interface is thought to be induced upon the deoxygenation, as proposed in the Perutz model (54, 55). Such a structural rearrangement for the α2β2(HBM)$\beta$ tetramer is probably due to high sequence homology between the α- and β-subunits in the heme binding module. On the basis of the previous crystallographic and site-directed mutagenesis studies, the positions of FG3–4 and G1–4 in the heme binding module are essential for the allosteric transition of Hb A (55, 58, 59), but Leu(FG3), Asp(G1), Pro(G2), and Asn(G4) are common in the α- and β-subunits. Therefore, it is plausible that the hydrogen bonds between $^{\alpha1}\text{Tyr(C7)}$ and $^{\beta2}\text{His(FG4)}$ and between $^{\alpha1}\text{Asp(G1)}$ and $^{\beta2}\text{(HBM)2Asn(G4)}$ were formed on the α1-β2(HBM)$\beta$ interface of the complex, as found for the α1-β2 interface of Hb A (see Fig. 3).

However, functional roles of the other two residues at the FG4 and G3 positions are not negligible. Some Hb natural mutants at these positions showed a remarkable decrease in the cooperativity. $n_{\text{max}}$ values of Hb Malmo ($^{\alpha1}\text{His(FG4)}$ → Glu), Hb Alberta ($^{\alpha1}\text{Glu(G3)}$ → Gly), and Hb Potomac ($^{\alpha1}\text{Glu(G3)}$ → Gly) were 1.6, 2.0, and 1.6, respectively (60, 61), which are comparable with that of the complex of the β(HBM)- and α-subunits. Also, it is to be noted that the complex of the β(HBM)- and α-subunits exhibited a much higher tetrameric dissociation constant than that of Hb A. Indeed, other Hb natural mutants, Hb Georgia ($^{\alpha1}\text{Pro(G2)}$ → Leu) and Hb Hirose ($^{\alpha1}\text{Tyr(C3)}$ → Ser), whose tetramer-dimer dissociation constants are extremely large (more than 300 times greater than that of Hb A), hardly possess the cooperative oxygen binding property (62, 63). Therefore, it can be inferred that the remarkable reduction in the cooperativity observed for the complex of the β(HBM)- and α-subunits is caused not only by change in the interactions at the FG4 and G3 positions of the β(HBM)-subunit with adjacent residues in the α-subunit but also by enhancement of the subunit dissociation.

**Summary**—As described above, the substitution of the heme binding module in hemoglobin α- and β-subunits significantly perturbed the heme environmental structure of the isolated β-subunit. The structural disorder in the β(HBM)-subunit was reduced by the association with the α-subunit, and its heme environmental structure was almost identical to that of the β-subunit bound to the α-subunit. In the results we could not confirm that the heme binding module corresponds to a structural unit regulating the heme proximal structure and heme electric state in globin proteins. In tetrameric Hb A, the subunit interactions appear to play a role in the regulation of the heme environmental structure. Such a structural regulation in Hb A would deeply be related to its cooperative oxygen binding mechanism, in which the structural change of the heme proximal side, especially the proximal histidine, is linked to the structural rearrangement on the subunit interface (55). At present, to characterize the tertiary and quaternary structure of α2β2(HBM)$\beta$ tetramer, its x-ray crystal structure analysis is in progress. Detailed structure comparison between α2β2(HBM)$\beta$ tetramer and Hb A will provide further insights into how the local folding of the heme binding module is coupled to the subunit interface structure in Hb A.

**Acknowledgments**—We are indebted to Dr. Satoshi Takahashi and Dr. Teizo Kitagawa for the resonance Raman spectrometry. We are also grateful to Dr. Yoshinao Wada for the measurement of the mass spectrometry.

**REFERENCES**

1. Adachi, S., Sunohara, N., Ishimori, K., and Morishima, I. (1992) J. Biol. Chem. 267, 12614–12621
2. Uchida, T., Ishimori, K., and Morishima, I. (1997) J. Biol. Chem. 272, 30108–30114
3. Matsui, T., Ozaki, S., Liang, E., Phillips, G. N., Jr., and Watanabe, Y. (1999) J. Biol. Chem. 274, 2838–2844
4. Tanaka, M., Ishimori, I., and Morishima, I. (1999) Biochemistry 38, 14963–14973
5. Pouls, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Errickson, B., Xiong, N., Yonetani, T., and Kraut, J. (1980) J. Biol. Chem. 255, 575–583
6. Oldfield, T. J., Smerdon, S. J., Dauster, Z., Petratsos, K., Wilson, K. S., and Wilkinson, A. J. (1992) Biochemistry 31, 8732–8739
7. Smerdon, S. J., Krywda, S., and Wilkinson, A. J. (1993) Biochemistry 32, 5132–5138
8. Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., and Morishima, I. (1991) Biochem. Biophys. Res. Commun. 180, 138–144
9. Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., Egawa, T., Kitagawa, T., Makino, R., and Morishima, I. (1993) Biochemistry 32, 241–252
10. Hildebrand, D. P., Burk, D. L., Maurus, R., Ferrer, J. C., Brayer, G. D., and Mauk, A. G. (1995) Biochemistry 34, 1997–2005
11. Matsui, T., Nagano, S., Ishimori, K., Watanabe, Y., and Morishima, I. (1996) Biochemistry 35, 13118–13124
12. Pouls, T. L. (1996) J. Biol. Inorg. Chem. 1, 356–359
13. Inaba, K., Ishimori, K., and Morishima, I. (1998) J. Mol. Biol. 283, 311–327
14. Go, M. (1981) J. Biol. Chem. 256, 272–279
15. Inaba, K., Ishimori, K., Imai, K., and Morishima, I. (1998) J. Biol. Chem. 273, 30054–30060
16. Watanabe, K., Watanabe, Y., and Morishima, I. (1997) J. Biol. Chem. 272, 30054–30060
17. Ndiaye, A., M. I., W., and Trotman, C. N. A. (1996) J. Biol. Chem. 272, 30054–30060
18. Inaba, K., Ishimori, K., Imai, K., and Morishima, I. (1998) J. Biol. Chem. 273, 8080–8087
19. Jellie, A. M., Tate, W. P., and Trotman, C. N. A. (1996) J. Mol. Biol. 242, 641–647
20. Nagai, K. and Thogerson, H. C. (1984) Nature 309, 810–812
21. Nagai, K., Perutz, M. F., and Poyart, C. (1985) Proc. Natl. Acad. Sci. U. S. A.
Substitution of the Heme Binding Module in Hemoglobin α- and β-Subunits: IMPLICATION FOR DIFFERENT REGULATION MECHANISMS OF THE HEME PROXIMAL STRUCTURE BETWEEN HEMOGLOBIN AND MYOGLOBIN

Kenji Inaba, Koichiro Ishimori, Kiyohiro Imai and Isao Morishima

J. Biol. Chem. 2000, 275:12438-12445.
doi: 10.1074/jbc.275.17.12438

Access the most updated version of this article at http://www.jbc.org/content/275/17/12438

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 66 references, 16 of which can be accessed free at http://www.jbc.org/content/275/17/12438.full.html#ref-list-1