The 2.0 Å Crystal Structure of Cyanide Metmyoglobin Reconstituted with 5,10,15,20-Tetrapropylhemin

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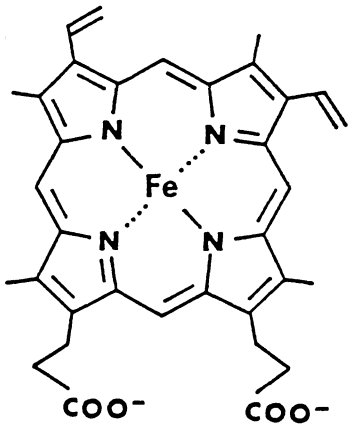
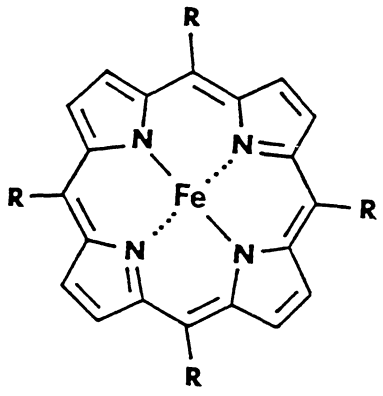
Sperm whale metmyoglobin reconstituted with 5,10,15,20-tetrapropylhemin was crystallized in the space group $P2_12_12_1$, which was different from the space group $P2_1$ of the native. The crystal structure of the reconstituted metmyoglobin was determined by the molecular replacement method. The atomic coordinates were refined to R=0.209 at 2.0 Å resolution. The overall structure is essentially the same as that of the native. The pronounced structural change is observed in the side-chain orientation of Arg 45 moving to the surface of the molecule. This conformational change may reflect a side-chain fluctuation that allows the small ligands to approach the heme pocket from the outside of the protein. The porphyrin ring does not rotate freely about the Fe–N^{ε} (His 93) bond in the crystalline state, although temperature-dependent NMR spectra suggest its free rotation in solution.

It is well-known that stereospecific interactions of the heme with globin play important roles in the functional behavior of hemoproteins. Various kinds of reconstituted myoglobins and hemoglobins have been prepared with modified protohemes to investigate the relationship between the structure and the function. The major modification has been performed mainly at the vinyl positions of protoheme, to discern whether the electronic characters or the steric effects of substituents are responsible for the oxygen-binding property.[1–3] On the other hand, 5,10,15,20-tetraalkylporphyrins do not have any substituent at the pyrrole carbons, rather they bear four alkyl groups on 5,10,15,20-positions, as shown in Fig. 1. This difference between 5,10,15,20-tetraalkylporphyrin and protoheme is expected to affect the heme-globin interactions in different ways.

Proton NMR spectra of cyanide metmyoglobin reconstituted with 5,10,15,20-tetraalkylhemin resolved the pyrrole-proton signals of porphyrins in the −5 to −15 ppm region as a singlet, a doublet, and a quartet for the methyl, ethyl, and propyl group, respectively. These peaks of ethyl- or propyl- derivative, however, coalesce into a sharp singlet at 61 °C, suggesting dynamic free rotation of the porphyrin rings about the Fe–N^{ε} (His–F8)bond.[4,5] Such a large fluctuation in amplitude of the porphyrin ring has never been appreciated. The structural perturbation in reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin is expected to be larger than that in the ethyl derivative. This paper deals with the results of structural studies of cyanide metmyoglobin reconstituted with 5,10,15,20-tetrapropylhemin (R=Pr, in Fig. 1) using X-ray crystallographic analysis. This is the first analysis of myoglobin containing 5,10,15,20-tetraalkylporphyrin.



Protoheme



5,10,15,20-Tetraalkylporphyrinatoiron(III)
R=Me, Et, and Pr

Fig. 1. Chemical structures of protoheme and 5,10,15,20-tetraalkylporphyrinatoiron(III).

Materials and Methods

Crystallization. Reconstituted sperm whale metmyoglobin was prepared as described previously[4,5] and stocked in a 10 mM (1 M=1 mol dm^{-3}) Tris-KCN buffer solution (pH 7.0). Crystals of cyanide metmyoglobin reconstituted with 5,10,15,20-tetrapropylhemin were obtained by a dialysis of 0.5 mM protein solution against a saturated ammonium sulfate solution containing 10 mM KCN. The pH of the outer solution was adjusted to 6.5—7.0 with HCl.

The crystals belong to the orthorhombic space group $P2_12_12_1$ with the unit cell dimensions a=58.39(2), b=76.15(2), and c=33.86(2) Å. There are 4 molecules per unit cell, which gives the V_m value of 2.1 Å^3/dalton. The unit cell dimensions were determined by the least-squares method using the observed setting angles of 25 reflections collected on a Rigaku AFC-5 four-circle diffractometer. Native sperm whale metmyoglobin has been known to crystallize in two forms: a monoclinic form with space group $P2_1$ from an ammonium sulfate solution, and an orthorhombic form of $P2_12_12_1$ from a phosphate buffer solution.[6] Contrary to expectations, the cell dimensions of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin are similar not to those of the previous orthorhombic form, but to those of blue whale myoglobin.[6]

Data Collection and Processing. Crystals with typical dimensions of 0.4×0.4×1.2 mm were sealed in glass capillaries with the mother liquid and used for data collections. Diffraction data were collected at 10 °C on a diffractometer using a nickel-filtered Cu $K\alpha$ radiation produced by a rotating anode X-ray generator operated at 40 kV and 300 mA. The diffraction pathway was kept in vacuo with an evacuated beam tunnel extending from the vicinity of the crystal to the counter, to improve the S/N ratio of data by reducing the X-ray scattering and/or the absorption of air.

Reflection intensities were measured in a continuous omega-scan of approximately 0.8° in width at a scanning speed of 4° min^{-1}. Background counts were measured for 4 seconds at both ends of each scan. Three standard intensities were checked every 100 reflections during data collection to monitor the decay intensity and crystal slippage. Crystals were exchanged as soon as any one of the standard intensities was reduced by 10%. The intensity data were corrected for absorption using an empirical correction curve obtained by measuring the intensity of a phi-independent reflection in 36 consecutive positions of 10° phi-intervals.[7] The correction for crystal damage due to X-ray irradiation was made as a function of the elapsed time. Diffraction intensities were scaled among different data sets by the method of Rollett and Sparks.[8] The final R_{merge} value was 3.1% for 10331 unique reflections up to 2.0 Å resolution, where $R_{merge}=\sum|<F>-F_i|/\sum F_i$, and $<F>$ is the average of F_i.

Structure Determination. The structure was solved by the molecular replacement method.[9,10] The structure of native sperm whale metmyoglobin in a $P2_1$ form[11] was used as the reference molecule, and its atomic coordinates were obtained from the Protein Data Bank.[12] The reference molecule without the prosthetic group was positioned in an arbitrary $P1$ unit cell with dimensions of $a=b=c$=100 Å and $\alpha=\beta=\gamma$=90°. The structure factors were calculated in the resolution range of 10 to 6 Å, the overall temperature factor being assumed to be 25 Å^2. To determine the orientation of the reference molecule in the observed unit cell, the fast rotation function[13] was calculated using Crowther's Eulerian angles with 5° grid intervals. The lengths of the vectors to be searched were limited to within 30 Å, and origin removal was applied. The peak coordinates found in the calculation are given in Table 1 for the two kinds of reference molecules; one reference molecule consisted of all atoms of globin, the other contained only the main chain and the C^β carbon atoms. The fourth peak of the former corresponds to the secondary peak of the latter. This orientation was accepted in the next step.

Table 1. Peak Coordinates in Rotation Functions[a]

	Peak	Peak height	β	α	γ
Case 1	1	62.9	55.0	120.0	60.0
	2	57.8	5.0	50.0	−105.0
	3	57.5	40.0	140.0	165.0
	4	53.0	35.0	40.0	170.0
	5	46.9	80.0	140.0	−15.0
Case 2	1	57.9	10.0	130.0	170.0
	2	47.6	35.0	45.0	165.0
	3	39.8	60.0	120.0	−120.0

a) α, β, and γ are Crowther's Eulerian angles. As the reference molecule, all atoms were included in Case 1, and only the main chain and C^β atoms were in Case 2. The orientation of Peak 2 in Case 2, which corresponds to that of Peak 4 in Case 1, was submitted to a translation search

The translation search was performed by the R-factor map procedure.[14,15] In this step, porphyrin with the Fe atom (R=H, in Fig. 1) was added to the whole globin as the prosthetic group. The reference molecule was located in the orientation obtained in the previous step. The structure factors were calculated separately for each symmetry operation of the space group $P2_12_12_1$. These partial structure factors were merged using relative phase angles calculated by placing the reference molecule in various positions. The R factor at 6 Å resolution was calculated at 1 Å grids over the range of 0—1/2 lattice along the three crystallographic axes. Some short intermolecular contacts were found at the position of the lowest R value, 0.51. After a packing analysis was carried out to check intermolecular contacts, a rigid body refinement was performed to improve the results obtained from the coarse grid searches of rotation and translation about several reasonable positions. Three rotational and three translational parameters were refined to minimize the R value by the simplex method,[16] which is well-suited for searching the minimum point around the initial parameters over a wide range. The lowest R factor after this refinement was 0.38 for 186 large reflections in the resolution range of 8 to 5 Å, while R factors of the others were more than 0.41. The new position was obtained by improving the initial parameters by −3.7, 12.6, and 0.2° in rotation of the Eulerin angles and −0.2, 1.1, and 0.0 Å in translation along the three crystallographic axes. Successive rigid body refinements resulted in R values of 0.34 for 351 large reflections of 4.5—4.0 Å resolution, and 0.32 for 266 large reflections between 3.15 and 3.0 Å resolution. The position of the heme iron atom was also confirmed by the

anomalous difference Patterson map based on the intensity data which were measured using the synchrotron radiation (λ=1.74 Å) at Tsukuba Photon Factory.

Structure Refinement. Refinement of the structure was carried out by a restrained least-squares technique according to the Hendrickson-Konnert method.[17] The resolution in the refinement was gradually improved from 3 Å to 2 Å. A hundred and twelve refinement cycles were done to the convergence at 2.0 Å resolution. The temperature factors were refined independently for the individual atoms after 12 cycles. Forty-three water molecules were found in the $2F_o-F_c$ electron density map calculated after 26 cycles and included as the oxygen atoms in the following refinement. After the 20th, 38th, 48th, and 78th cycles, the refined model was modified by fitting it to the respective $2F_o-F_c$ maps with the color graphics display system. The four propyl groups of 5,10,15,20-tetrapropylhemin were not introduced into the refinement because no remarkable peaks were found in their corresponding positions on electron density maps. The final crystallographic R-factor was 0.209 for 4926 reflections with F_o larger than $3\sigma(F_o)$ at the resolution of 5.5 to 2.0 Å. The rms deviations from their ideal values are 0.013 Å for bond distances, 0.031 Å for angle distances, and 0.037 Å for planar 1-4 distances.

Results and Discussion

Heme and Its Environment. The electron density of the heme in reconstituted metmyoglobin is not so clear, as shown in Fig. 2, and the cyano group coordinating to the iron atom does not appear on the Fourier map, although the electronic absorption spectra indicate the presence of CN^- anion.[4,5] The temperature factors of the atoms in the porphyrin ring are 17 to 20 Å^2, which are slightly larger than the mean value of the overall molecular structure (15 Å^2), indicating disorder and/or vibration. Surprisingly, pyrrole B, located at the bottom of the heme pocket, has larger thermal factors (19—20 Å^2) than any other pyrrole, and the corresponding electron density becomes much lower. On the contrary, pyrrole D, located at the entrance to the heme pocket, represents the smallest thermal motion. Myoglobin, as is well-known, has some interior spaces, or cavities, in the vicinity of the heme.[18] These facts suggest that the cavity at the bottom of the heme pocket is much wider than that at the entrance which is reduced by two imidazole rings of His 64 (E7) and His 97 (FG2), and that the bottom part of the porphyrin ring, such as pyrrole B, swings up and down in the direction normal to the ring, in addition to the oscillatory movement of the heme about the Fe-N$^\varepsilon$ (His 93) bond. However, the free rotation of the heme about the Fe-N$^\varepsilon$ (His 93) bond, which was suggested by proton NMR spectra,[4,5] is not observed in the crystalline state.

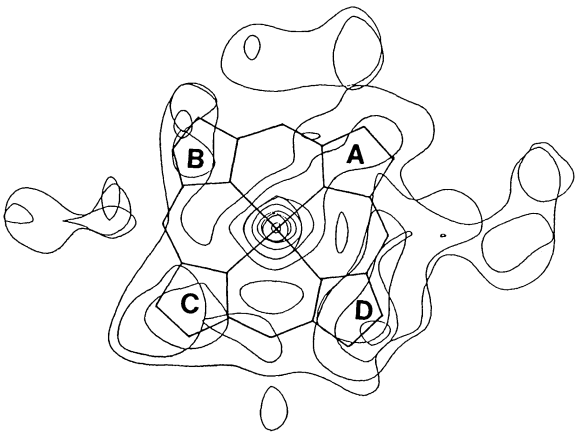


Fig. 2. $2F_o-F_c$ electron density map of the heme. The −2—2 sections parallel to the mean heme plane at intervals of 1 Å are superposed. The heme plane is located on the 0 section. Contours are drawn at arbitrary levels. The four pyrrole rings of porphyrin are labelled by the symbols A, B, C, and D.

The distance between the iron atom and N$^\varepsilon$ of His 93 (F8) is 2.01 Å, which is comparable to 2.13 Å in the native. The iron atom is displaced by 0.2 Å from the mean plane of the porphyrin ring, and by 0.1 Å from the imidazole plane of His 93 (F8). The angle between the Fe-N$^\varepsilon$ (His 93) bond and the axis normal to the mean pyrrole plane is 11.0°, which is comparable with 3.9° in the native. The imidazole ring of His 93 (F8) makes an angle of 35° to the plane composed of the iron atom and two nitrogens of pyrroles B and D. Since the corresponding angle in native myoglobin is 19°, the porphyrin ring in reconstituted metmyoglobin is rotated by 16° about the Fe-N$^\varepsilon$ (His 93) bond.

Rotation of the Heme Group. Temperature-dependent NMR spectroscopy suggested that the heme group of reconstituted myoglobin with 5,10,15,20-tetrapropylhemin freely rotates about the Fe-N$^\varepsilon$ (His 93) bond in solution at high temperature.[4,5] However, this free rotation has not been observed in the crystalline state. In order to check for the possibility of such rotation, the energy barrier for the rotational movement of the heme group about the Fe-N$^\varepsilon$ (His 98) bond was elucidated by calculating the conformational energy with the MM2′ program.[19] The sixteen amino acid residues around the heme were used in the calculation and fixed during energy minimization. The positions of the four propyl groups attached to the heme group were estimated from the standard geometry of 5,10,15,20-tetrapropylporphyrin.[20] All hydrogen atoms were located at the ideal positions. In order to estimate the energy barrier of the heme rotation, the heme group was rotated at 10° steps from 0° to 90° about the Fe-N$^\varepsilon$ (His 93) bond, considering the four-fold rotation symmetry of 5,10,15,20-tetrapropylporphyrin, and used in energy minimization.

The resulting energy variation is shown in Fig. 3 as a function of the His 93C$^\varepsilon$-His 93N$^\varepsilon$-Heme Fe-Heme CCD torsional angle, ϕ. The curve of reconstituted myoglobin with 5,10,15,20-tetrapropylhemin has a

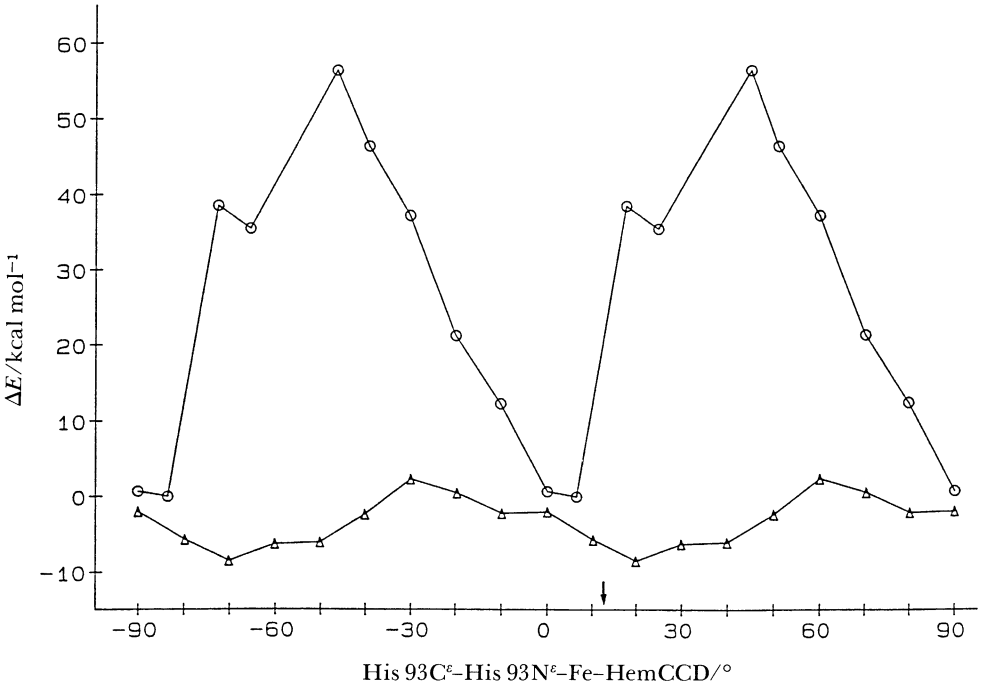


Fig. 3. The variation of the potential energy with rotational angle (ϕ) about His 93C^{ε}–His 93N^{ε}–Fe–Hem CCD for reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin (circles) and porphyrin (triangles). The potential energy is subtracted by the minimum comformation energy of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin. The arrow indicates the observed rotational angle for reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin.

minimum at ϕ value close to the observed angle of 12.5° and a maximum between −40° and −50°. In these position, the propyl groups of the heme must pass through the narrow spaces composed of Leu 72 (E15) and Phe 138 (H15), and Tyr 103 (G4) and Ile 107 (G8), which are paired above and below the heme plane, respectively; there are short interatomic distances of Leu 72$C^{\delta 1}$···Phe 138C^{ζ} (3.75 Å), Tyr 103O···Ile 107N (3.16 Å) and Tyr 103O···Ile 107$C^{\gamma 2}$ (3.27 Å). Moreover, Lys 42 (C7) and Phe43 (CD1) also prevent the heme group from rotating. Thus, the energy barrier is so high that the heme group could not freely rotate in the crystalline state.

The potential energy curve of reconstituted metmyoglobin with porphyrin (R=H, in Fig. 1) is also plotted in Fig. 3. In this case, the minimum is at ϕ=20 which is different by about 10° from that of reconstituted myoglobin with 5,10,15,20-tetrapropylhemin. Since the energy barrier at ϕ=30° is 11 kcal mol^{-1}, which is much less than that of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin, the porphyrin ring might freely rotate about the Fe–N^{ε} (His 93) bond even in the crystalline state. Actually the weak and flat electron density of heme has been observed in the crystal structure of reconstituted metmyoglobin with porphyrin, suggesting rotation and/or vibration of the heme group.[21]

Structural Comparison with Native Metmyoglobin. The overall structure of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin is essentially the same as that of the native. Before quantifying the structural differences between the two proteins, a least-square superposing procedure based on the positions of the C^{α} atoms was performed, to get the best fit between them. The resulting superposition of the C^{α}-tracings of the two molecules is illustrated in Fig. 4, and the positional deviations of the amino acid residues are plotted in Fig. 5. The root mean square deviation between the corresponding C^{α} atoms is 0.6 Å. These two figures show that the most pronounced change occurs at the C-terminal region (0.9—3.7 Å), and the next at the N-terminal (1.9 Å). The other residues that move more than 0.77 Å are located at the CD-loop and the E-helix regions: Lys 47 (CD5), His 48 (CD6), and Asp 60 (E3). These regions construct the entrance and/or exit channel through which small diatomic or triatomic ligands move from the outside of the protein to the distal heme pocket.[22]

The mean temperature factors of the backbone atoms (N, C^{α}, C, and O) in each amino acid residue are also plotted in Fig. 5, with the corresponding positional deviations. This figure shows a measure of the flexibility of the polypeptide chain by the relationship between the average temperature factor

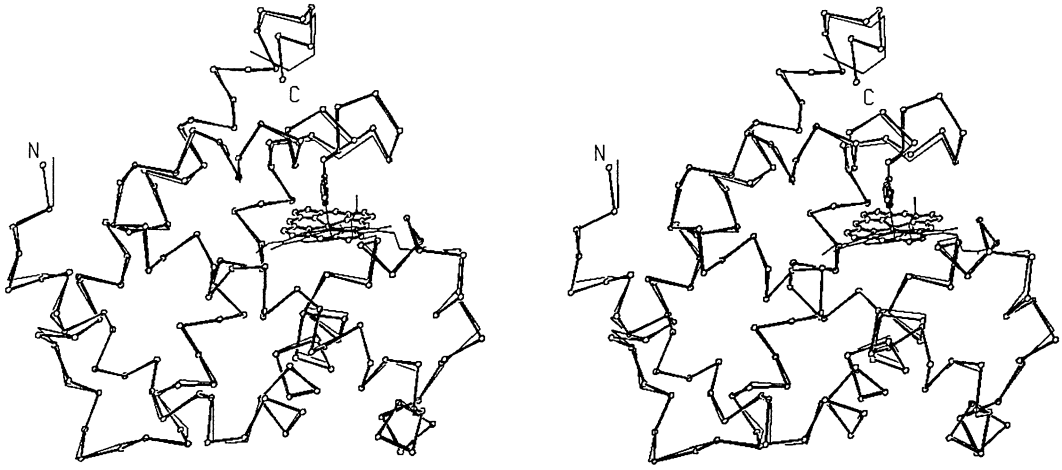


Fig. 4. The conformations of the C^{α} backbone, the heme moiety, and the heme linkage to His 93 in reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin (thick lines) and native metmyoglobin (thin lines). The amino and carboxyl termini are labelled by N and C.

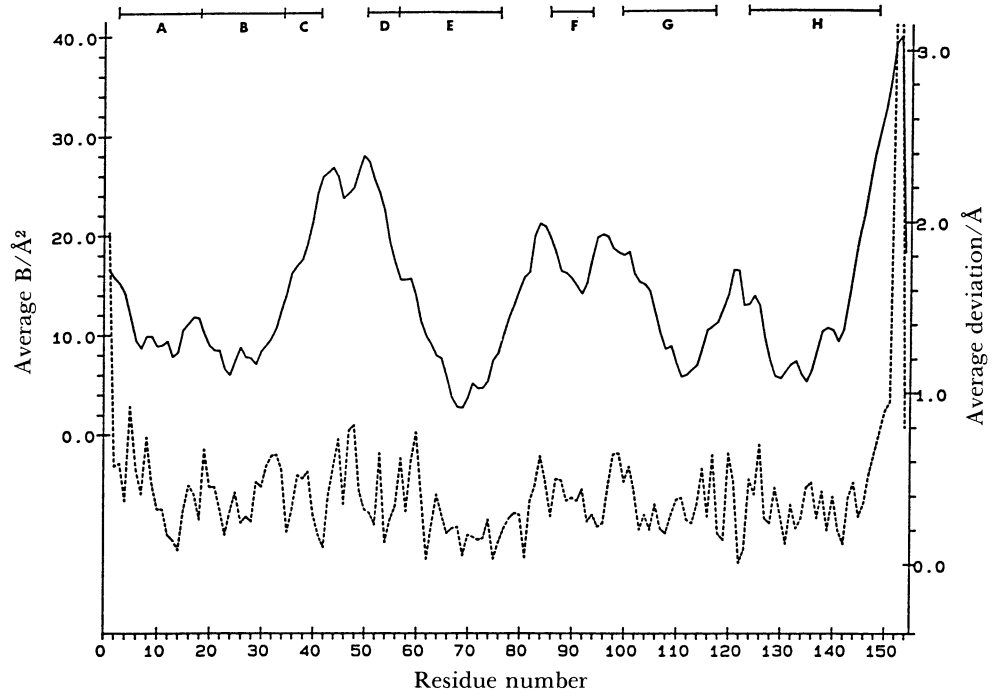


Fig. 5. Plots of the mean temperature factors for each amino acid residue of the main chain in reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin (solid line) and the positional deviations in the corresponding C^{α}-atoms between reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin and native metmyoglobin (a dotted line). Bars drawn on the upper part of the graph represent the regions of alpha-helical structure.

and the posisional deviation. The average temperature factors of the overall main chain is 14.1 Å², but considerable variablity is observed along the course of the polypeptide chain. The highest thermal motion is observed in atoms of the C-terminal region. Main-chain atoms in the loops, especially in the CD-loop, have considerably high temperature factors and have been shown to be particularly mobile by temperature-dependent X-ray diffractions of myoglobin.[23] These flexible regions of a polypeptide chain might be able to move easily and to adopt different conformations depending on their circumstances, such as existence of the heme ligand.

Although the structural changes of the main chain

are small, except for the amino and carboxyl termini, a number of pronounced changes occur at the side groups. The side chain of Arg 45 (CD3) dramatically moves toward the outside of the protein. The guanidino group of Arg 45 (CD3) in native metmyoglobin forms two hydrogen bonds: to the carboxyl groups of Asp 60 (E3) and 6-propionic acid of protoheme. In reconstituted metmyoglobin, the latter hydrogen bond is ruptured because of the lack of a carboxyl group of the heme, and the guanidino group is rotated by ca. 170° about the C^{α}–C^{β} bond, to shift its terminal nitrogen atom by 6 Å. As a result, the side chain of Arg 45 (CD3) is no longer stabilized by the hydrogen bond to Asp 60 (E3). Structural differences between phenyl-ligated metmyoglobin and native metmyoglobin have been observed in the side-chain orientation of Arg 45 (CD3), His 64 (E7), and Val 68 (E11) around the distal His 64 (E7), suggesting that these amino acid residues may play an important role in forming the pathway through which small ligands have been presumed to enter the distal heme pocket from the outside of the protein.[22] In reconstituted metmyoglobin, however, only the side group of Arg 45 (CD3) swings out, and those of His 64 (E7) and Val 168 (E11) do not significantly move.

The other drastic changes are observed mainly at the side chains located at the surface of the protein. The maximum deviations in each side chain of Glu 83 (EF6), Lys 96 (FG1), Lys 98 (FG3), and Lys 147 (H24) are all more than 4.0 Å. Among these residues, only Lys 96 (FG1) is situated in the vicinity of the heme pocket. Crystallographic study of sperm whale metmyoglobin reconstituted with 2,4-diisopropyldeuterioheme or 2-isopropyl-4-vinyldeuterioheme has elucidated that the introduction of a bulky isopropyl group to the 2-position of the heme forces the phenyl group of Phe 138 (H15) to rotate around the C^{α}–C^{β} and C^{β}–C^{γ} bonds, resulting in the average deviation of 4 Å by the phenyl atoms.[24] In reconstituted metmyoglobin with 5,10,15,20,-tetrapropylhemin, this rotation of the phenyl group does not occur, although the porphyrin ring has propyl groups.

The superposition of the heme moieties of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin and native metmyoglobin is illustrated in Fig. 6. The iron atoms are deviated by 0.8 Å from each other. The porphyrin ring is rotated by 33° within the plane of the ring, and the dihedral angle between these porphyrin planes is 13°. Among four carbons to which the propyl groups are attached, the CDA atom between the pyrrole rings D and A in Fig. 6 is positioned only 1.2 Å from the C-6 position of protoheme in the native. Consequently, it is expected that the propyl group bonded to the CDA atom might be located at the position of 6-propionic acid. On the other hand, the position of the 7-propionic acid is not occupied by any propyl group of 5,10,15,20-tetrapropylporphyrin.

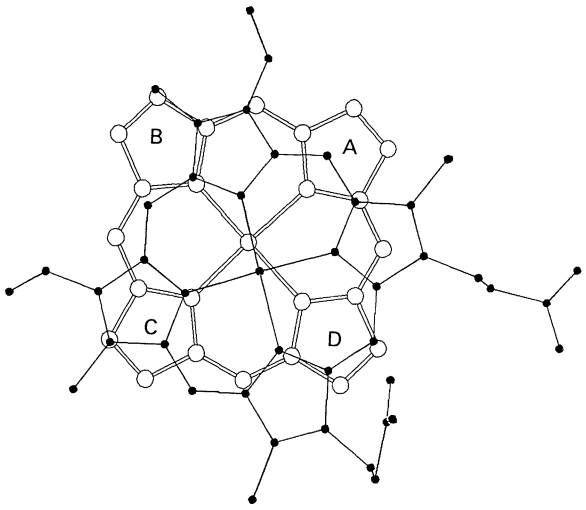


Fig. 6. Superposition of the heme moieties of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin (large opened circles) and native metmyoglobin (small full circles). The four pyrrole rings of reconstituted metmyoglobin with 5,10,15,20-tetraproylhemin are labelled by the symbols A, B, C, and D.

The structure of metmyoglobin shows that the pathway of the ligand from the surface of the molecule into the distal heme pocket is shielded by side chains of His 64 (E7) and several other residues, as shown in Fig. 7. In order to form an open channel to the surface, the imidazolyl group of His 64 (E7) has to rotate after the side chain of Arg 45 (CD3) moves toward the outside, as suggested by Nobbs.[25] This mechanism may be regarded as the "double door" mechanism. A structure analysis of reconstituted metmyoglobin reveals that only the side chain of Arg 45 (CD3) moves toward the outside of the molecule to open the "outer door" of the channel. Once the "outer door" is opened, ligands are expected to move inside through the channel in order to open the "inner door" by rotating the imidazole ring of His 64 (E7). Finally, the channel is opened completely. Crystallographic study of phenyl-ligated metmyoglobin,[22] which has a bulky ligand, reveals that the binding of the ligand to the distal position of iron atom makes the side chains of His 64 (E7) and Arg 45 (CD3) move aside and the channel open to the surface.

The guanidino group of Arg 45 (CD3) in native metmyoglobin is locked by two hydrogen bonds to the carboxyl groups of Asp 60 (E3) and 6-propionic acid of the heme. One of the four propyl groups in the porphyrin ring of reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin occupies the position of the 6-propionic acid of the heme in native metmyoglobin, resulting in a breakage of the hydrogen bond between Arg 45 (CD3) and the 6-propionic acid. In addition, the replacement of the carboxyl group by the

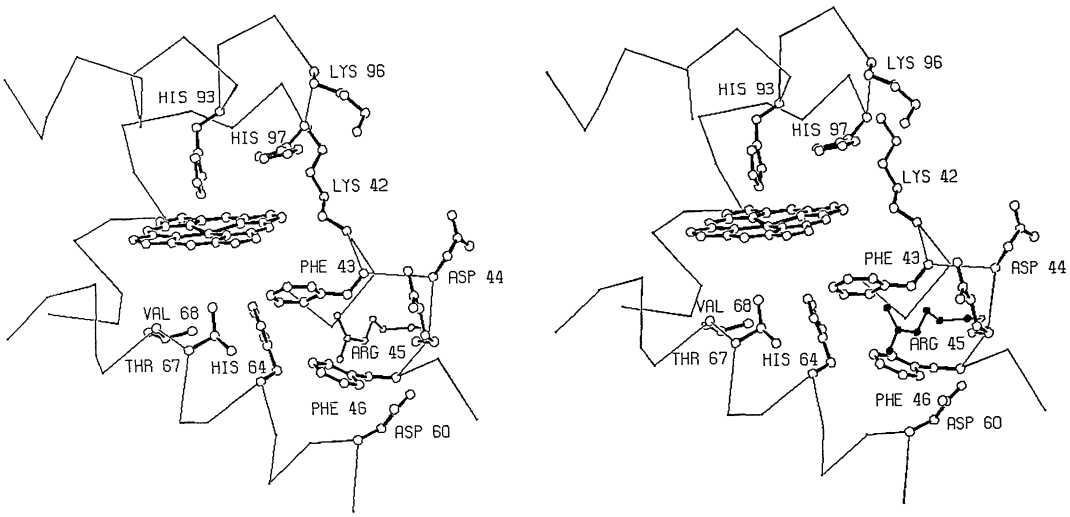


Fig. 7. Drawing of the C^{α}-tracing and the amino acid residues around the heme pocket in reconstituted metmyoglobin with 5,10,15,20-tetrapropylhemin. The pathway through which small ligands have been presumed to enter the ligand pocket lies among Arg 45 (CD3), His 64 (E7), Val 68 (E11), and the heme.[22] The guanidino group of Arg 45 (CD3) moves to the surface of the protein molecule from its position in native metmyoglobin (small full circles).

alkyl group makes the hydrophilic environment change to a hydrophobic one. As the reusult, the hydrophilic guanidino group might be turned toward the hydrophilic globin surface.

As to the opened "outer door", an alternative explanation could been made as follows. Since the amino acid residues of Arg 45 (CD3) and Asp 60 (E3), which are hydrogen-bonded to each other, are located on the surface of the molecule, they are highly influenced by a change of the environment, such as the solvent. If these are species around the protein that are more preferable to the hydrogen bond than Asp 60 (E3), the guanidino group of Arg 45 (CD3) may move outwards to form hydrogen bond with them after breakage of the previous hydrogen bonds. In reconstituted metmyoglobin, there are a large number of the potassium cations and cyano anions around the protein molecule. Such changes in the outer environment may be responsible for the opening and shutting of the "outer door". In addition, the conformational changes observed here should account for the polymorphism of myoglobin depending on the conditions for crystallization.

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