Heme Environmental Structure of a Novel Artificial Myoglobin with Closed Heme Pocket: Site-Specific Chemical Modification Producing Distal *N*-Tetrazolylhistidine E7 by Cyanogen Bromide and Azide Ion[†,⊥]

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Abstract: The heme environmental structure of myoglobin (Mb) chemically modified by cyanogen bromide (BrCN) followed by azide ion (N_3^-) has been resolved by X-ray crystallography at 1.7-Å resolution. In this artificial Mb, the distal histidine (His E7) was site-specifically modified; a five-membered heterocycle group, tetrazolate (CN_4^-), produced from the CN group of BrCN and N_3^- is covalently attached to the imidazole ϵ-nitrogen atom of the distal His, and the tetrazolate 2-nitrogen atom directly coordinates to the heme iron of Mb (tetrazole-Mb; Tet-Mb). The introduction of the bulky tetrazolate group in the heme pocket makes the heme plane tilt by 0.8° and the pyrrole N plane by 4.5°. The pH variation of the absorption spectrum of Tet-Mb revealed that the tetrazolate anion protonates with a $pK = 4.3$, resulting in breakage of the Fe–N(tetrazole) bond to yield a pentacoordinate heme iron and consequently a change of the iron electronic state from a ferric low-spin to a high-spin state. The ferric high-spin Tet-Mb, however, cannot combine with some potential ligands such as CN^-, N_3^-, F^-, and imidazole. This characteristic property would be explained in terms of the "closed" heme pocket of the novel artifical Mb, where the bulky tetrazolylhistidine E7 might block the entry of the external ligand into the heme pocket. Simulation for the motion of the tetrazolyl-His E7, which was based on the heme environmental structure determined by X-ray crystallographically, confirmed this suggestion.

Introduction

It has been recently of great interest to alter the electrostatic and stereochemical nature of proteins by protein engineering techniques, in order to evaluate their structure–function relationship. From this point of view, point mutation by gene technology has been applied to many proteins including several hemoproteins such as myoglobin (Mb), hemoglobin (Hb).[1-3] The mutagenesis is a site-specific method, by which one can satisfactorily replace any amino acid residue with any other one. By contrast, chemical modification of proteins has been considered as a less selective method than mutagenesis to change the protein structures and properties by gene technology. The reaction of the protein with a modifying reagent often yields undesirable byproducts as well as a desirable main product. However, if we choose a reagent that can selectively modify the special site of the protein, this method is more convenient to yield sufficient modified protein for both functional and structural characterization. Furthermore, it is much more important that the chemical modification can introduce a group other than a natural amino acid residue; in sharp contrast to that, gene technology is yet limited by the replacement with natural amino acids.

We previously reported that sperm whale Mb stoichiometrically reacts with cyangen bromide (BrCN) and have suggested that the distal histidyl (E7) imidazole is selectively modified by a cyanation with this reagent.[4,5] The BrCN-modified Mb (BrCN-Mb) has characteristic properties in its ligand-binding reaction, compared with unmodified (native) Mb. For example, the ferric form of BrCN-Mb does not bind some potential ligands of native Mb such as CN^-, F^-, H_2O, or imidazole. Carbon monoxide can bind to the ferrous heme iron of BrCN-Mb with a low affinity and at a low association rate.[6] Since the distal His E7 has been thought to play a crucial role as a "molecular door" for the ligand entry into the heme pocket,[7] the findings of characteristic properties for BrCN-Mb were noteworthy. However, in spite of characterization of its heme environmental structure with several spectroscopic methods such as optical absorption, NMR, and IR measurements,[4] there have remained some ambiguities in the modified site and structure.

On the other hand, we recently succeeded in making a single crystal of the azide (N_3^-) complex of BrCN-Mb and studied its microstructure in the vicinity of the heme by the single-crystal EPR technique.[8] On the basis of the EPR results, we predicted that the *N*-cyanoimidazole moiety of the distal E7 residue in BrCN-Mb could react with N_3^- to form a tetrazolylhistidine (see Figure 1) by a 1,3 dipolar cycloaddition reaction and that the tetrazolate group might coordinate to the heme iron. In the present study, we have analyzed by X-ray crystallography the heme environmental structure of Mb modified by both BrCN and N_3^- and directly demonstrated the site-specific chemical modification of the distal His E7 to the *N*-tetrazolylhistidine and the coordination of the tetrazole to the heme iron. Furthermore, we note that the acid form of the modified protein is in a ferric high-spin state, but cannot combine with external ligands such as CN^-, N_3^-, F^-,

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[⊥] Abbreviations: Mb, myoglobin; Tet-Mb, tetrazole-Mb; rms, root-mean-square; F_0 and F_c, observed and calculated amplitudes of structural factors; NMR, nuclear magnetic resonance; IR, infrared; EPR, electron paramagnetic resonance.

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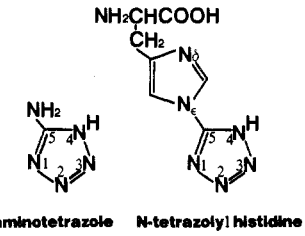

Figure 1. Model structures of 5-aminotetrazole and *N*-tetrazolylhistidine.

and imidazole. On the basis of the heme environmental structure obtained in the present work, these properties are discussed with relevance to the action of the distal His of Mb in the ligand-binding reaction.

Materials and Methods

Crystallization and X-ray Intensity Measurement. Sperm whale myoglobin (type II) was purchased from Sigma, and cyanogen bromide and sodium azide from Wako Pure Chemicals, Ltd. Preparation and crystallization of the azide complex of BrCN-Mb have been described previously.[4-6,8] The modified Mb crystal belongs to the monoclinic system. The space group of this crystal is $P2_1$, and its unit cell parameters were determined as a = 64.79 (1) Å, b = 30.817 (5) Å, c = 34.778 (6) Å, and β = 105.57 (2)° by the least-squares refinement method with 24 reflections. (The values in parentheses represent the standard deviation of each parameter in units of last significant digit.) The difference between these parameters and those of the native met-Mb crystal[9] was less than 0.5%. Therefore, we concluded that our crystal was isomorphous with native met-Mb crystal.

X-ray intensity data were collected on an automated four-circle diffractometer (Rigaku AFC-IV) with graphite-monochromatized Cu Kα radiation and the ω-scan mode to a maximum of 1.7-Å resolution. A crystal of 2 × 3 × 0.4 mm was installed into a glass capillary with a 10-μm-thick wall and 2-mm diameter. In order to avoid scaling errors between measured crystals, we used only one crystal for all measurements. X-ray damage to the crystals was checked by monitoring 3 standard reflections after every measurement of 100 Bragg spot intensities. Deterioration of the standard reflections was less than 10% on the average. Structure factor amplitudes were calculated after corrections of the X-ray damage, Lorentz, polarization, and empirical absorption[10] by using the computer program system of UNICS III.[11] The amplitudes of 13 821 reflections over 2σ (90% of total) were used for further analyses.

Structure Determination and Refinement. The PROTEIN program system, which was kindly provided by Dr. Steigemann,[12] was used for the calculations of R value, structure factor, and electron density maps. The R value ($R = \sum|F_o - F_c|/\sum F_o$) was calculated to be 34% between the F_o of modified Mb and F_c of native (unmodified) met-Mb,[9] showing that the structure of modified Mb, except for the heme distal side, is presumably similar to that of native met-Mb. The atomic coordinates of the distal E7 residue and solvent molecules in modified Mb were, thus, determined on the basis of the so-called "omit map".[13] The omit map was computed on the basis of the observed amplitudes of the structure factor of modified Mb and the structure factors calculated from the coordinates of native met-Mb[9] from which the distal His E7 side-chain atoms, a ligand H_2O, and all solvent water molecules were omitted. In the omit map, we found two positions with very high electron density, one at the distal side of the heme pocket and the other at the protein surface. The former was assigned to the *N*-tetrazolylimidazole moiety of the distal E7 residue, consistent with the prediction from our previous EPR study.[8] The latter was readily assigned to a sulfate ion (SO_4^{2-}) from its tetrahedral shape and its similar location to one reported for native met-Mb as a SO_4^{2-} binding site.[9] In addition to these two positions with high electron density, we could find 58 distributed peaks with a small spherical shape and a significant electron density at the protein surface and assigned them to be solvent water molecules.

For a crystallographic refinement of modified Mb structure, we used the restrained-parameter least-squares technique of Hendrickson and Konnert,[14] in which stereochemical information of amino acid residues, such as bond length and angle, torsional angle, and so on, are included in the least-squares formula as well as the observed structure factor amplitudes. For the structure refinement of the distal E7 residue, a model of *N*-tetrazolylhistidine was constructed de novo on the basis of the crystal structure of 5-aminotetrazole[15] and histidine, as shown in Figure 1. The bond length between the heme iron and its axial ligand atoms (tetrazole 2-nitrogen and the ϵ-nitrogen of the proximal His F8) was restrained to 2.13 Å,[9] while the torsional angle formed by any two planes (the heme, the tetrazole, the proximal imidazole, or the distal imidazole) was not restrained. The starting structure for the refinement was composed of the coordinates of met-Mb lacking the distal His E7, any ligand or solvent molecules, and of those determined from the omit map. One refinement step included (i) four least-squares refinement cycles with isotropic temperature factors, (ii) calculation of new electron density maps with $2F_o - F_c$ and $F_o - F_c$ Fourier coefficients by using the refined coordinates, (iii) rearrangement of some amino acid side chains including the N- and C-termini on these maps, (iv) elimination of solvent molecules assigned wrongly whose isotropic temperature factors increased above 50 Å^2, and (v) new assignment of solvent molecules. After three refinement steps (12 cycles of least-squares refinement), the crystal structure consisted of the protein molecule, one SO_4^{2-}, and 232 solvent waters. The structure thus obtained was idealized by using the computer program of Hendrickson and Konnert,[14] and the final R value in the present study was 24%.

Absorption Spectral Measurement. The absorption spectrum was measured with a Hitachi U-3210 spectrometer by using a cell with a 1-cm path length. The pH titration was performed by directly adding 0.1 N HCl or 0.1 N NaOH to the protein solution. The pH value was measured with a Horiba pH meter equipped with a glass electrode.

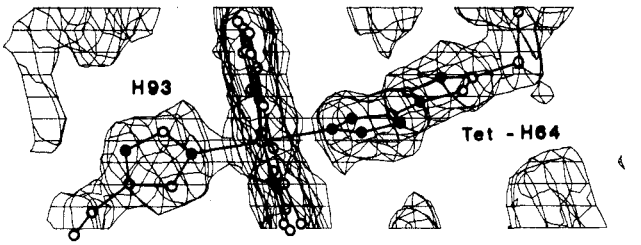

Figure 2. *N*-tetrazolyl modification of the distal His E7. The $2F_o - F_c$ electron density map is represented around the heme, and the corresponding structure was superimposed on this map; the proximal histidine F8 (H93), the heme, and the distal *N*-tetrazolylhistidine E7 (Tet-H64). The large open circle represents the heme iron, while the small open and closed circles show carbon and nitrogen atoms, respectively.

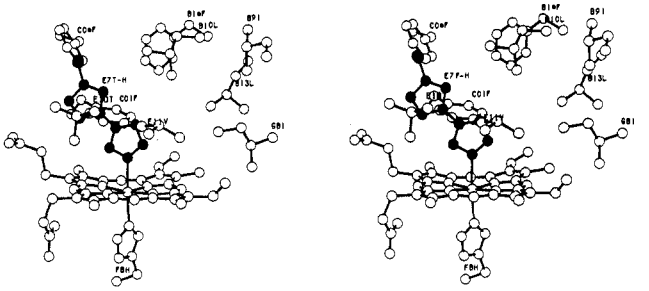

Figure 3. Stereodrawing of the heme pocket of Tet-Mb viewed from the distal side. The *N*-tetrazolyl-His E7 (E7T-H; closed circles) is surrounded by the heme and the side chains of Ile B9 (B9I), Leu B10 (B10L), Leu B13 (B13L), Phe CD1 (CD1F), Phe CD4 (CD4F), Thr E10 (E10T), Val E11 (E11V), and Ile G8 (G8I).

Results and Discussion

Heme Environmental Structure of Tetrazole-Mb and Site Specificity of Modification. In Figure 2 is illustrated the final $2F_o - F_c$ electron density map in the heme vicinity of modified Mb. The corresponding molecular structure is superposed on this map, showing the fit to be excellent for the heme, the imidazole moiety of the proximal His F8, and the *N*-tetrazolylimidazole moiety of the distal E7 residue. The tetrazole group is covalently

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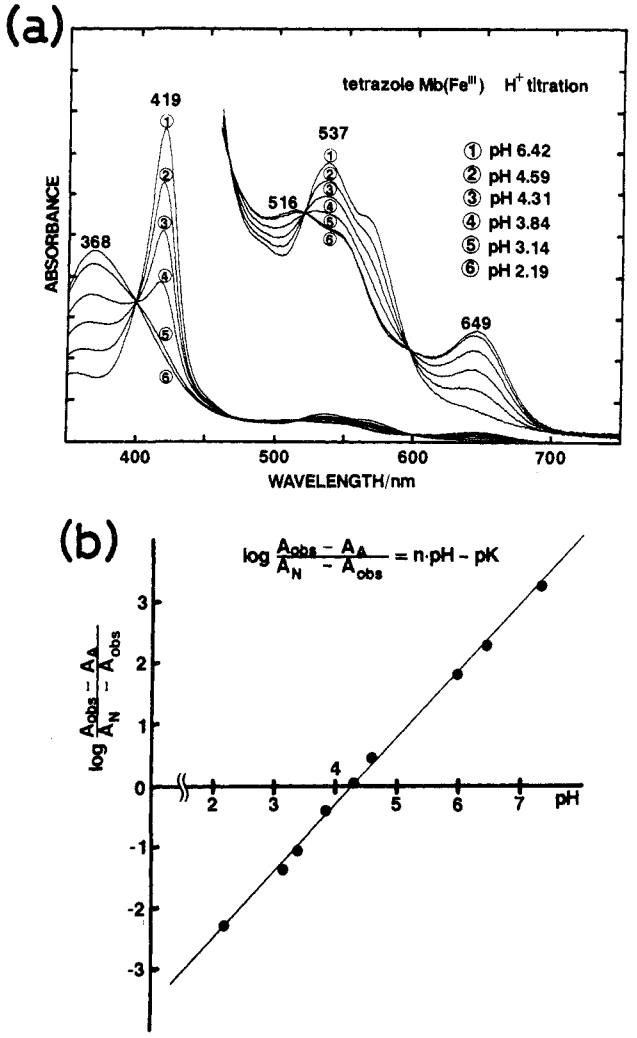

Figure 4. (a) Optical absorption spectral change of ferric Tet-Mb with pH changing from 7 to 2. (b) Analysis of the spectral change (a) according to the equation represented in the figure. A_A and A_N represent the absorbances at 419 nm for acid and neutral forms, respectively. A_{obs} is absorbance at 419 nm at the observed pH. n is the number of protons responsible for this spectral change. The analysis showed that the spectral change corresponds to one proton uptake (slope of 1 in the plot) with pK = 4.3 (intercept at y = 0).

attached to the imidazole of the distal His E7 through a bond between the tetrazole 5-carbon and the imidazole ϵ-nitrogen with a bond length of 1.4 Å. On the basis of the direct evidence obtained by the present X-ray crystallographic analysis, we hereafter denoted the modified Mb as tetrazole-Mb (Tet-Mb).

Figure 3 shows the *N*-tetrazolylimidazole moiety of the distal E7 residue in the heme pocket of Tet-Mb. The tetrazole group is coordinated to the heme iron as a sixth internal ligand through the nitrogen atom at the 2-position. The iron–nitrogen distance is 2.1 Å, which is in agreement with bond distances observed for Fe(III)–N coordination.[16] The Fe–N bond is perpendicular to the heme plane (coincident with the heme normal) and is symmetric to the bond of Fe–N(proximal His), which is also perpendicular to the heme plane. The tetrazole plane is almost perpendicular to the heme plane, passing through the heme iron atom and across the methine (meso) carbon between pyrroles III and IV. (Numbering of the pyrrole rings refers to that of met-Mb.[9] The orientation of the tetrazole group relative to the imidazole group of the proximal His F8 is such that the two planes form an angle of 45°.

Since we could not determine X-ray crystallographically whether a proton is present or not at the 4-nitrogen position of

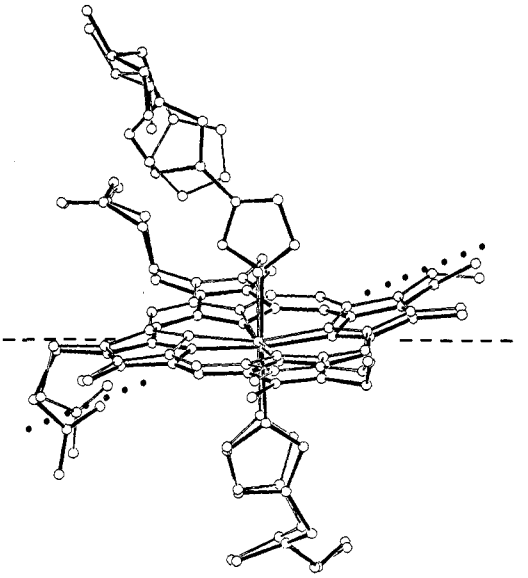

Figure 5. Structural comparison between Tet-Mb and met-Mb in the heme environment. The heme plane rotates around the broken line, while the nitrogen plane rotates around the dotted line.

the tetrazole group (see Figure 1) coordinating to the heme iron, we tried to determine spectrophotometrically its protonated (neutral) and deprotonated (anionic) state. Figure 4a shows the pH-dependent change in the optical absorption spectrum of Tet-Mb in solution. The spectrum of Tet-Mb at neutral pH has absorption maxima at 419 and 537 nm and is characteristic of the heme in a ferric low-spin state. When the pH of the solution was lowered, the spectrum changed to a new one with isosbestic points at 399, 474, 519, and 596 nm. The resultant spectrum has absorption maxima at 368, 516 and 649 nm, characteristic of a pentacoordinate heme iron in a ferric high-spin state: a low absorptivity at the blue-shifted and broad Soret band.[4,17] [This form gave a broad EPR signal at g = 6 (H. Hori, private communication)]. This change was reversible with respect to lowering or rising pH and corresponded to a single proton uptake with a pK value of 4.3 (Figure 4b). On the basis of this spectral change, we concluded that a deprotonated form of tetrazole, tetrazolate, coordinates to the heme iron of Tet-Mb in the neutral pH region and that the protonation of the tetrazolate anion at acidic pH results in dissociation of the internal ligand, probably tetrazole, from the ferric heme iron.

We have compared the heme environmental structure with that of native met-Mb (Figure 5). When the tetrazole group binds to the iron, the heme plane shifts by 0.2 (1) Å on average in the direction of the proximal side. In association with the heme shift, the heme plane tilts by 0.8 (6)° around the line joining pyrrole nitrogen II and IV, while the pyrrole nitrogen plane tilts by 4.5 (15)°. (The values in parentheses represent the standard deviation of each parameter in units of last significant digit.) The movement and tilt of the heme are mainly caused by the steric constraint due to the short contacts between the tetrazole 1-nitrogen atom and two pyrrole nitrogen atoms of III and IV (see Figure 3). A previous EPR study[8] claimed that the tilting angle of the heme or nitrogen plane is 13°, which is larger than that in the present result. This discrepancy is presumably ascribed to the assumption of the EPR analysis that the eigenvectors g_{xx} and g_{yy} are fixed on the heme plane. It has been reported for some hemoproteins in a ferric low-spin state that the direction of g_{zz} is inclined by ~7° from the heme normal.[18]

The distal structure of Tet-Mb is compared with those of imidazole complexes of Mb and hemoglobin (Hb),[19,20] which have

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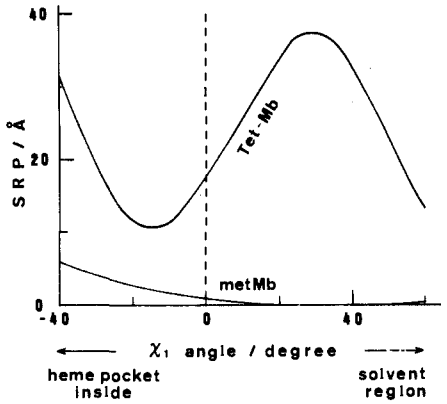

Figure 6. Rotational stability of the distal *N*-tetrazolyl-His E7 around its C_α–C_β bond. The indicated value, steric restriction parameter (SRP), was calculated at each rotational angle around the C_α–C_β bond (χ_1 angle) of the distal E7 residue for Tet-Mb (thick line) or native met-Mb (thin line) as follows. When the side chain of the distal His E7 was rotated stepwise, the distance between the atoms of the tetrazolylimidazole E7 and those of the surrounding residues (see Figure 3) was computed. If the distance obtained was shorter than that of van der Waals contact (chosen as 3.8 Å in this calculation), the difference between them was summed for all atomic pairs. This value serves as an indicator of the steric restriction of the surrounding residues to the tetrazolylimidazole; a large value represents large steric hindrance.

the five-membered heterocycle group as an external iron-coordinated ligand. In the structural comparison among these derivatives, the most remarkable difference is the location of the imidazole of the distal E7 residue. In the case of imidazole-Mb and -Hb, the imidazole group of His E7 rotates by approximately 50° around the C_α–C_β bond and eventually moves by more than 2 Å toward the solvent region. By contrast, the imidazole moiety of the distal E7 residue in Tet-Mb is essentially unaltered in position from that of native met-Mb (see Figure 5). It is fixed in its position by the covalently attached tetrazolate, which directly coordinates to the heme iron. In consequence of this situation, the side chains of the amino acids in the heme distal side, such as Val E11, Phe CD1, Thr E10, Leu B10, and Arg CD3, do not move upon the modification of the distal His E7 (see Figure 3; Arg CD3 is not shown in this figure).

The present result directly demonstrates the formation of a novel artificial Mb having *N*-tetrazolyl-His E7 and the coordination of its tetrazolate to the heme iron as a sixth internal ligand. The formation of *N*-tetrazolyl-His in a protein is a unique reaction and has never been reported for any other protein as yet. Here, we should check whether any other amino acid residues of Mb are altered by the reaction with BrCN and N_3^-, because cyanogen bromide is known as a strong modifying reagent for proteins cleaving methionine peptides or oxidizing several amino acid residues.[21,22] However, when we extensively surveyed the electron density map of Tet-Mb, we found that the imidazole of His E7 is site-specifically modified, while all other residues are not. Under the mild conditions employed here (reaction of Mb with a stoichiometric amount of BrCN at pH 7), two Met residues at D5 and H8, which are buried in hydrophobic cores, could not react with BrCN. Moreover, although sperm whale Mb contains 12 His residues, none of these, except for the distal E7, are modified in going from met-Mb to Tet-Mb, suggesting that His E7 is located in an environment specially suited for BrCN modification. The demonstration of the site specificity of the modification allowed us to conclude that alteration of some properties of Mb is reasonably explained as due to formation of the *N*-tetrazolyl group at the imidazole moiety of the distal His E7.

Structural Explanation of Characteristic Properties of Tet-Mb. In order to gain insight into the characteristic properties of Tet-Mb, we studied the ligand binding to ferric Tet-Mb. We examined the absorption and EPR spectra of Tet-Mb in the presence or absence of some potential ligands of the ferric hemoproteins such as CN^-, N_3^-, F^-, and imidazole. At neutral pH, addition of the ligand caused no change in the spectra. The observation is reasonably consistent with the structural characteristics of Tet-Mb, where the tetrazolate occupies the sixth iron site as an internal ligand, resulting in no accessibility to these external ligands. On the other hand, as was manifested by the spectral changes (Figure 4), the tetrazole group dissociates from the heme iron to form the pentacoordinate and the high-spin state at an acidic pH, in which the iron sixth site is vacant. However, in spite of this structure, the EPR and absorption spectra of Tet-Mb at an acidic pH were unchangeable upon addition of a large amount of the ligand. Thereby, we concluded that Tet-Mb in a ferric state does not have ligand-binding ability.

The imidazole group of the distal His E7 has been postulated to act as a "molecular door" for the external ligand to enter to, or to exit from, the heme pocket.[7] This is based on the concept that Mb in solution is in an equilibrium state between a "closed conformation" and an "open conformation". In the closed conformation, the imidazole of His E7, together with Val E11 and Thr E10, blocks the entrance path for ligands into the heme pocket. However, the distal imidazole can substantially swing out and away from the heme pocket toward the solvent region by rotating around the C_α–C_β bond, so that the channel for the ligand to enter is widely open, giving the open conformation. The open conformation has recently been observed in the crystal structure for phenyl-Mb generated from the reaction of met-Mb and phenylhydrazine,[23] or for the ethyl isocyanide complex of Mb.[24]

This knowledge allows us to suggest that the introduction of the tetrazole to His E7 substantially affects the rotational ability of the distal E7 imidazole. We have tried to simulate the rotation of the tetrazolylimidazole around its C_α–C_β bond (χ_1 angle), assuming that the Fe–N(tetrazole) bond is broken without any change in the rest of the protein structure. As shown in Figure 6, when the tetrazolylimidazole moiety rotates and moves toward the solvent region (an increase in χ_1 angle), the structure became energetically unstable due to the steric constraint between the tetrazole and the heme. This is sharply contrasted to native met-Mb, in which the imidazole rotation is not energetically unfavorable. Eventually, the His E7 residue of Tet-Mb could not swing out into the solvent region without drastic changes in the protein conformation, and the channel for the external ligand to the heme pocket would be always "closed". The results from the simulation were in good agreement with the experimental results mentioned above.

In summary, we have found by X-ray crystallographic and spectral analysis that, upon the reaction of Mb with both BrCN and N_3^-, the distal His E7 residue is site-specifically modified to yield *N*-tetrazolylhistidine coordinating to the ferric heme iron, and that the modification prevents the E7 residue from rotating around its C_α–C_β bond, preventing accessibility of the heme pocket to the external ligand.

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Registry No. Fe, 7439-89-6; His, 71-00-1; N_2, 7727-37-9; BrCN, 506-68-3; N_3^-, 14343-69-2; heme, 14875-96-8.

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