Single-crystal EPR Study of Novel Azide Complex of Cyanogen Bromide-modified Myoglobin

INFLUENCE OF SPECIFIC MODIFICATION OF THE HEME DISTAL HISTIDYL RESIDUE ON THE LIGAND-BINDING STRUCTURE

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Stable azide complex of cyanogen bromide-modified met-myoglobin (metMb) was prepared and crystallized. The principal values and eigen vectors of g-tensor were determined by single-crystal EPR spectroscopy at 77 K: \( g_{xx} = 1.50, g_{xy} = 2.32 \), and \( g_{zz} = 2.91 \). These \( g \) values were similar to those of tetrazole derivative rather than azide derivative of native metMbs, suggesting that tetrazole derivative might be formed from \( N \)-cyanimidazole of distal histidyl residue via nucleophilic attack of azide ion by 1,3-dipolar cycloaddition reaction.

The orientation of the maximal g value (\( g_{xx} \)) of the novel product was found to deviate about 13° from the heme normal of native aquometMb. Thus, the orientation of the heme plane might be altered in passing from native metMb to cyanogen bromide-mediated met-myoglobin. The present EPR results demonstrated that the modification of the histidyl residue at the heme distal side causes the changes in the stereochemical and electronic natures of the ligand binding to the heme.

The electronic and stereochemical nature of the ligand binding to myoglobin (Mb)1 and hemoglobin has been an important and interesting problem. Histidyl residue at the heme distal side has been concerned in the specific functional properties in these hemoproteins. Chemical modification of the distal His has allowed the detailed investigation of the mode of interactions among the ligand molecule, the prosthetic group, and the apoprotein moiety in these hemoproteins.

Jajczay and co-workers (1, 2) showed that the reaction of cyanogen bromide (BrCN) with Mb occurs with 1:1 stoichiometry and that this reaction could involve the modification of the imidazole group of the distal His. Later, Shiro and Morishima (3, 4) characterized the heme environmental structures and ligand binding properties of BrCN-modified Mb by NMR, IR, and optical absorption spectroscopic measurements. BrCN modification of other hemoproteins were also attempted without definitive results (3). Thus, metMb was an exceptional case where only the distal His is specifically modified.

Furthermore, Shiro and Morishima (3) also found that the BrCN-modified metMb does combine with exogenous azide ion to afford a characteristic \(^1\)H NMR spectrum that is quite different from that of native MbN\(_2\). However, the structural characterization of the heme environment in the azide complex of BrCN-modified metMb (BrCN-MbN\(_2\)) has remained uncertain. In order to understand further the electronic and stereochemical properties of this unique Mb complex, we have carried out powder and single-crystal EPR measurements. The results of these measurements and a possible structural characterization of the heme environment are described in this paper.

MATERIALS AND METHODS

Sperm whale myoglobin (Type II) was obtained from Sigma and cyanogen bromide from Wako Pure Chemical, Ltd. The reaction of Mb with BrCN was proceeded by an addition of a stoichiometric amount of BrCN to metMb solution at pH 7.0 as described previously (1). All chemicals were used without further purification.

The BrCN-modified metMb reacted with excess sodium azide to form a stable azide complex as reported previously (3). The azide complex was crystallized from 75% saturated ammonium sulfate/phosphate solution at pH 6.0, as described for native metMb by Kendrew and Parriah (5). The crystals were monoclinic and belonged to a space group of \( P_{2}1 \). The unit cell dimensions of the crystals were \( a = 64.5 \text{ Å}, b = 30.7 \text{ Å}, \text{ and } c = 34.8 \text{ Å}, \) and \( \beta = 106.5° \). Thus, these crystals were isomorphous with native metMb crystals (so called type A crystals (5)). Single-crystals in which BrCN-MbN\(_2\) and native aquometMb were cocry stallized together were also prepared in order to determine the crystallographic axes accurately by using EPR signals associated with ferric high-spin species of aquometMb, although the three crystallographic axes \((a, b, \text{ and } c)\) could be readily identified by visual examination, where \( c^* \) is perpendicular to \( a \) and \( b \) axes by definition.

EPR measurements were carried out at X-band (9.35 GHz) micro-
wave frequency by using a Varian cavity with a home-built spectrometer with 100-kHz field modulation. An immersion Dewar flask was used for measurements at 77 and 4.2 K. A home-built two-circle Teflon goniometer was used for single-crystal measurements at 77 K to rotate sample crystals in 5° or 10° steps with a reproducibility of ±1°. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken Co., Ltd., model TR9212). The magnetic field strength was determined by a nuclear magnetic resonance of protons in water.

Single crystal EPR data of the resonance positions versus angles of rotation were least squares-fitted to Schonland's equation (6) to determine the angular dependence of the square of the g values. The principal values and eigenvectors for g-tensor, with respect to the crystallographic axes, were determined according to Schonland's method (6). The signs of the nondiagonal elements of g-g-tensor were determined by measuring the angular dependence of g\(^2\) values around any direction in a single-crystal with respect to its crystallographic axes.

RESULTS

Fig. 1 illustrates powder X-band EPR spectra of BrCN-modified and native metMbs in g. Extreme at 4.2 K. These spectra were characteristic of ferric high-spin derivatives. The peak-to-peak line width of the g signal for BrCN-modified metMb was extremely larger than that of native aquometMb. No other ferric low-spin signal was observed with BrCN-modified metMb (spectrum not shown).

The BrCN-modified metMb reacted with azide ion (N\(_3\)) to afford a characteristic optical absorption spectrum which is different from that of native MbN\(_3\). The charge-transfer band at 634 nm which is derived from a ferric high-spin species diminished, suggesting an increase in a ferric low-spin species. This new azide complex of BrCN-modified metMb was surprisingly stable and was not converted back to the original ferric high-spin derivative when left standing on ice for several days. Even when the solution of BrCN-MbN\(_3\) was dialyzed against a buffer, or was gel filtrated by Sephadex G-25, the ligand free form of BrCN-modified metMb was not obtained. The iron-bound N\(_3\) could not readily be removed from BrCN-MbN\(_3\). The back reaction rate (BrCN-MbN\(_3\) + N\(_3\)) was almost zero. This nature was sharply contrasted to those of other derivatives of BrCN-modified Mb, such as its carbon monoxide (CO) complex, in which the modified site is gradually decyanated upon the CO binding to the iron. Due to an extreme stability of BrCN-MbN\(_3\), its purification and crystallization were readily achieved by the same procedures used for native myoglobin.

Fig. 2A illustrates powder X-band EPR spectra of BrCN-MbN\(_3\) and native MbN\(_3\) at 77 K, respectively. These spectra were characteristic for the ferric low-spin species. The powder EPR spectrum of the BrCN-MbN\(_3\) was very broad at 77 K, whereas the line width became narrower at 4.2 K (spectrum not shown). The principal g values determined from the powder EPR spectrum of BrCN-MbN\(_3\) (g\(_x\) = 1.50, g\(_y\) = 2.28, and g\(_z\) = 2.92) were quite different from those for MbN\(_3\) (g\(_x\) = 1.72, g\(_y\) = 2.22, and g\(_z\) = 2.80). Rather than native MbN\(_3\), these EPR characters of BrCN-MbN\(_3\) was very similar to those of imidazole, triazole, and tetrazole derivatives of native metMb as shown in Fig. 2B. The unusual g values indicated that the modification of the histidyl residue at the heme distal side causes the changes in the electronic and stereochemical natures of the azide binding to the heme.

In order to gain further insights into the heme environmental structure of BrCN-MbN\(_3\), we determined more precise principal g values and eigenvectors by measuring single-crystal EPR spectra of BrCN-MbN\(_3\). The angular variations of g\(^2\) values for the single-crystals are shown in Fig. 3. The principal values and eigenvectors of g-tensor for this new complex were determined according to the method of Schonland (6) and the stereographic diagram (Fig. 4), together with the results of MbN\(_3\) and aquometMb for comparison (7). It should be pointed out that the direction of the maximal g value (g\(_z\) = 2.91) for BrCN-MbN\(_3\) deviates significantly about 13° from the heme normal of the native aquometMb and from that of native azide derivative, respectively. To confirm this deviation, we tried to compare the single-crystal EPR spectra between BrCN-MbN\(_3\) and native aquometMb. However, it was technically quite difficult to distinguish any possible difference in the direction of the z axis between these.
Single-crystal EPR of Azide Complex of BrCN-Mb

FIG. 3. Angular variations of $g^2$ values in BrCN-MbN$_5$ single-crystals at 77 K. Top, in the $ab$ plane; center, in the $bc^*$ plane; and bottom, in the $ac^*$ plane. Site 1 (●) and site 2 (○) in the unit cell of the BrCN-MbN$_5$ single-crystal are shown.

TABLE I

| Complex       | Principal $g$ values | Angle (°) to      | Ref. |
|---------------|----------------------|-------------------|------|
| BrCN-MbN$_5$ | $g_\alpha = 1.50$   | 103 67 27         | This paper |
|               | $g_\beta = 2.32$    | 83 153 63         |      |
|               | $g_\gamma = 2.91$   | 15 78 81          |      |
| MbN$_5$      | $g_\alpha = 1.12$   | 109 83 21         | 7    |
|               | $g_\beta = 2.22$    | 67 152 75         |      |
|               | $g_\gamma = 2.80$   | 31 63 76          |      |
| AquometMb    | $g_\alpha (g_1) = 2.00$ | 28 69 73         | 8    |

species from separate EPR measurements, because minor differences in mounting crystals on the sample holder were unavoidable. Therefore, to circumvent this technical difficulty, single-crystals containing both native metMb and BrCN-MbN$_5$ were prepared. Such a mixed crystal was isomorphous with native aquometMb crystals and had a space group of P2$_1$ (5). To check whether BrCN-MbN$_5$ and native aquometMb were present in the same crystal, we measured the optical absorption spectrum of the solution after dissolving this crystal in 0.5 M phosphate buffer at pH 7.0 at room temperature.

When a magnetic field was applied along the direction of the $x$ axis ($g_\alpha = 2.00$) corresponding with native aquometMb crystal, a $g$ value of ~2.89 with considerably broad signal was obtained for BrCN-MbN$_5$. In truth, the $g$ value was found to change only 0.02, when a magnetic field was applied along the direction inclined at an angle of about ±10° to the exact $x$ axis ($g_\alpha$) for BrCN-MbN$_5$ crystal. Whereas the line shape of the signal in the vicinity of the $x$ direction ($g_\alpha$) of this crystal was broad, its $g$ value was rather sensitive to an angular variation than in the case for the $z$ direction. Therefore, we rotated the magnetic field along the great circle including both the crystallographic $c^*$ axis and the $g_\alpha$ direction of BrCN-MbN$_5$ as illustrated in the inset circle of Fig. 5. The angular variations of $g^2$ value both in the high-spin aquometMb and low-spin BrCN-MbN$_5$ in mixed crystal are shown in Fig. 5.

It was technically difficult to distinguish any possible difference in the direction of the $z$ axis between azide complexes of BrCN-modified and native metMbs by using single crystal containing both azide complexes of Mbs because of their broad line shapes and low signal intensities.
When a magnetic field was applied along the direction of the x axis \((g_x)\) of the ferric low-spin species, a \(g\) value \((g_x)\) of \(-5.80\) was observed for corresponding ferric high-spin heme. If we assumed the ferric high-spin Mb exhibits an axial EPR signal, \(g_x = 6.0\), and \(g_y = 2.0\) \((8)\), the angle between the direction of the observed \(g\) value \((g_y)\) and the heme normal \((g_z)\) in the single-crystal was \(74^\circ\). Thus, the direction of the \(z\) axis for this ferric low-spin species was found to deviate about \(16^\circ\) from the heme normal of the native aquometMb. Since the mixed crystal has a space group of \(P2_1\), and its unit cell contains two sets of ferric high-spin and/or ferric low-spin molecules, four sets of \(EPR\) signals corresponding to the two ferric high-spin species and the other two ferric low-spin species can be expected in an arbitrary crystalline plane. The direction of the magnetic field with respect to the crystallographic axes can be calculated from the two sets of the ferric high-spin EPR signal. Using this orientation of the magnetic field and principal values and eigenvectors of \(g\) tensor for BrCN-MbN\(_3\) listed in Table I, we can estimate the two sets of \(g\) value corresponding to the ferric low-spin signals. The observed \(g\) values were in good agreement with expected values.

**Fig. 6. Schematic presentations of the modified histidyl residue at the heme distal side in hemoproteins.**

**Fig. 7. Projections of \(g\)-tensor of BrCN-MbN\(_3\) onto the heme plane, viewed from the proximal side (top) and the motion of the heme plane (bottom).** The projection of the imidazole plane \((C_{\text{Im}}-C_{\text{Im}})\) of the proximal histidine \((FS)\) is indicated by the dotted line. The atomic coordinates of aquometMb are used \((Ref. 16)\).

**DISCUSSION**

**Reaction Mechanism of BrCN-Mb with Azide Ion**—The azide complex of BrCN-modified Mb was readily crystallized to form a single-crystal isomorphous with an unmodified aquometMb. Therefore, it is reasonable for us to assume no major conformational change in protein moiety on going from the aquometMb to BrCN-MbN\(_3\) except in the heme vicinity. In the present EPR study, the differences in the \(g\) values and its angular dependence are predominantly attributable to the structural differences in the heme environment between the azide complexes of BrCN-modified and native Mbs.

The first theoretical analysis of the \(g\) values in ferric low-spin azide hemoglobin was provided by Griffith \((9)\) and Kotani \((10, 11)\). Harris Loew \((12)\) made a general analysis of the \(g\) value variation to be expected for ferric low-spin hemoproteins and their model complexes as a function of axial and rhombic distortion of a strong octahedral perturbation. She demonstrated that a good criterion for increasing rhombicity is the decrease in a value of \(g_x\), with increase in the value of \(g_y\). The anisotropy in metMbN\(_3\) has been attributed mainly to the orientation of the azide ion relative to the heme plane. From an x-ray crystallographic study of metMbN\(_3\), the azide ion was inclined at \(21^\circ\) to the porphyrin plane \((13)\). Our present EPR results of BrCN-MbN\(_3\) indicate that the value of \(g_y\), is larger than that of native metMbN\(_3\), indicating a decrease in rhombic symmetry of this new azide complex. According to Harris Loew's criterion, the orientation of the azide ion might be altered toward heme normal in BrCN-MbN\(_3\). Indeed, it was found that the steric hindrance associated with the BrCN modification of the histidyl residue at the heme distal side induces changes in binding properties and profiles of the external ligand; ferric BrCN-Mb cannot bind with exogenous ligands such as cyanide ion and imidazole, and its ferrous form can combine with CO, but the modified site is gradually decyanated to convert back to the unmodified distal histidine in the CO derivative \((3)\). However, it is not the case for the azide complex of BrCN-Mb. Compared with other liganded forms of hemoproteins, BrCN-MbN\(_3\) is surprisingly stable: the iron-bound N\(_3\) cannot readily be removed from this complex. In our recent IR measurements, BrCN-MbN\(_3\) has exhibited no absorption in the region of the iron-bound N\(_3\)-stretching frequency, 2100–1900 cm\(^{-1}\)\(^4\). In addition, our preliminary \(^15\)N NMR experiments by using \(^15\)N-enriched azide ion have also indicated that the azide ion does bind to the heme iron in a different manner from native MbN\(_3\). \(^2\) The above results strongly suggest that the linear azide ion may not coordinate to heme iron by bent end-on coordination structure, but certain chemical products of cyanated histidyl residue with azide ion may bind to heme iron. Here, it is very noticeable that the powder EPR spectrum of BrCN-MbN\(_3\) is surprisingly similar to those of imidazole, triazole, and tetrazole derivatives of native metMb as shown in Fig. 2B. Then, as one possible ligand structure, we propose here a five-membered heterocyclic derivative such as tetrazole. It was reported previously that five-substituted tetr azoles are conveniently prepared from organonitriles via nucleophilic attack of azide ion by a 1,3-dipolar cycloaddition reaction \((14, 15)\). N-Cyanimidazole of the distal His, thus, might react with azide ion to form a tetrazolato-modified histidyl residue and then coordinate to heme iron as internal ligand as illustrated in Fig. 6A. This type of inhibition of hemoprotein has been reported for catalase \((2)\), in which the distal histidyl imidazole is irreversibly modified by the reaction with

-- S. Adachi, Y. Shi ro, and I. Morishima, unpublished results.

* Y. Shi ro, unpublished results.
aminotriazole (Fig. 6B). The above ligand structure proposed for BrCN-MbN$\textsubscript{3}$ is now being examined by $^{15}$N NMR spectroscopy and x-ray crystallography.

**Stereochemistry of BrCN-MbN$\textsubscript{3}$ (Tetrazole-Mb)—**Present powder EPR, together with optical absorption and $^{1}$H NMR (3) spectral studies, allowed us to consider that the modification of the distal His of Mb causes a drastic conformational change in the heme environments. To further clarify the structural changes, we will discuss the stereochemistry of the heme in this novel Mb complex based on the single-crystal EPR studies.

From the x-ray crystallographic study of aquometMb, the heme normal makes angles of 27°, 68°, and 75° with the a, b, and c* crystallographic axes, respectively (16). The EPR study indicates that the direction of $g_{zz}$ (or $g_{z}$) of aquometMb coincides with the heme normal (cf. Table I). The directions of the maximal $g$ values ($g_{xx}$) of ferric low-spin Mb derivatives are found to deviate about 7° from the heme normal of aquometMb (7). If the ($x,y,z$) axis system is assumed to be fixed in the framework of the heme, the heme plane orientation should incline at 7° in passing from the ferric high-spin Mb to the liganded ferric low-spin Mb.

While our present single-crystal EPR results indicate that the direction of the $g_{zz}$ of BrCN-MbN$\textsubscript{3}$ does not coincide both with that of native aquometMb and MbN$\textsubscript{3}$; the deviations observed are 13° and 16°, respectively. The stereographic diagram as illustrated in Fig. 4 indicates that the modification of the heme distal side causes the heme plane to rotate by 13° about an axis approximately coincident with a line between pyrrole nitrogens II and IV ($N_{II}-N_{IV}$). Fig. 7 illustrates the motion of the heme plane and the projections of the directions of $g_{xx}$ and $g_{xy}$ onto the heme plane, together with the projection of the proximal His(F8). The numbering of the heme is the same as that for metMb by Takano (16). The unique motion of the heme is undoubtedly caused by the steric hindrance of the bulky ligand, which binds tight to both heme iron and distal histidyl residue.

In the case of the derivative of metMb with externally added imidazole, the motion of the heme is not so extremely large as judged from the single-crystal EPR analysis (7). Although imidazole is also a bulky ligand, steric interaction between the imidazole and the distal His, involving hydrogen bond, is not so strong. In this case, the distal His can move in order to stabilize the ligand-bound conformation as demonstrated in the x-ray crystallographic study for imidazole methemoglobin (17).

In conclusion, we have presented hitherto unreported features of unique heme-ligand geometry in the azide complex of BrCN-modified Mb obtained by powder and single-crystal EPR measurements. Our present speculation should be confirmed by $^{15}$N NMR spectroscopy and x-ray crystallographic study. Further investigations are in progress.

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