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Three-dimensional structure of cyanomet-sulfmyoglobin C

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ABSTRACT **The atomic structure of horse heart cyanomet-sulfmyoglobin C has been established by x-ray crystallographic techniques to a resolution of 2.0 Å with an *R* value of 0.129. The protoheme IX prosthetic group of this thermodynamically stable sulfmyoglobin derivative has been converted to a chlorin in which the pyrrole ring bearing the 4-vinyl group is saturated and possesses an exocyclic thiolene ring. This study provides the three-dimensional structure of a protein with an iron-chlorin prosthetic group. The overall conformation of the surrounding polypeptide chain of the modified protein is very similar to that of the native protein. However, the addition of the sulfur atom has caused a distortion of the prosthetic group from that in the native protein to result in the repositioning of the side chains of some residues in the heme pocket.**

Sulfhemoglobin (SHb) was first observed in 1863 by Hoppe-Seyler (1, 2), who noted a green product formed from the reaction of oxyhemoglobin (HbO_2) with H_2S. In 1938, Michel (3) prepared SHb and the smaller, related protein sulfmyoglobin (SMb) by a route different from Hoppe-Seyler and determined by chemical analysis that the product possessed one additional sulfur atom per heme group. The ability of oxidized and reduced forms of the sulfheme proteins to bind exogenous ligands established that the sulfur atom does not occupy the distal coordination site of the heme iron (4–7). However, SHb binds dioxygen only at elevated pressure (8), and its presence is one cause of clinical cyanosis (9–11).

Subsequent studies of SMb indicated that the additional sulfur atom is incorporated into the heme group through the addition of H_2S across the β—β bond of one of the pyrrole rings to convert the heme prosthetic group to a chlorin (12–14). NMR studies have established that there are at least three forms of SMb. The form initially produced, S_AMb, is converted in acidic solution to S_BMb or, in basic medium, to S_CMb (15, 16). S_CMb is the most stable of these derivatives and has been suggested by subsequent NMR studies to possess a prosthetic group with a structure similar to that shown in Fig. 1 (17–19). Additional NMR experiments have yielded a spatial configuration for the heme substituents that is consistent with this model and that predicts the absolute configuration of the resulting chiral center about carbon atom C2C (20). Despite this considerable progress in structural characterization of SMb derivatives, the absence of a three-dimensional structure for the prosthetic group and its protein environment precludes unambiguous determination of whether the unusual functional properties of this form of Mb originate from structural rearrangement of the protein that accompanies heme conversion to a chlorin or whether these properties originate solely from the electronic differences between the heme and chlorin prosthetic groups.

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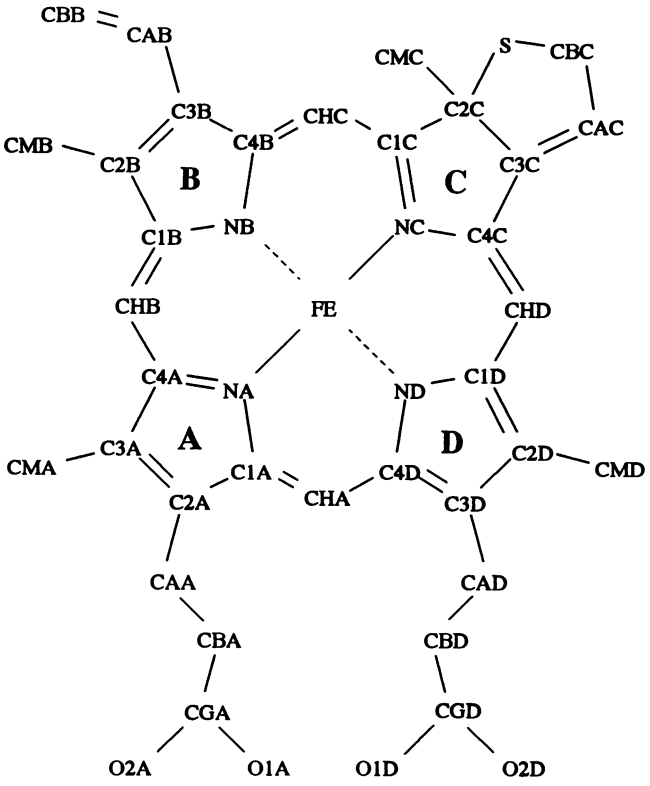

FIG. 1. The standard Protein Data Bank nomenclature for protoheme IX is used to illustrate the postulated structure of the modified heme group found in S_CMb, having one additional sulfur atom per heme.

The increased structural flexibility exhibited by hydroporphyrin ring systems relative to porphyrin systems renders hydroporphyrins more amenable to functional regulation through protein control of structural distortion (21, 22), an issue of particular relevance to photosynthetic proteins (23–25). The three-dimensional structures of several iron-containing hydroporphyrins have been reported in recent years (22, 26–28), although to our knowledge no three-dimensional structure for a protein with an iron hydroporphyrin prosthetic group has been described previously. To address the specific issues concerning the structural basis for the unusual functional properties of SMb and to address the more general issues concerning the structures of iron chlorins and their susceptibility to structural deformation upon interaction with a protein, we report the three-dimensional structure of S_CMb as determined by x-ray diffraction analysis.†

Abbreviations: SMb, sulfmyoglobin; metMb, metmyoglobin.

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†The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1YMC).

Table 1. Angles between the normals to the least-squares planes of the pyrrole rings for the horse heart native (upper value) and S_CMb (lower value) heme groups

Ring	Ring B	Ring C	Ring D
A	9.6 15.3	1.7 17.0	15.4 17.2
B	0	8.1 15.8	5.8 7.3
C	—	0	13.9 9.7

At this point in the structure elucidation, the heme model used for refinement against the S_CMb data was identical to that used for the native structure. The large discrepancy in angles involving pyrrole group C is a clear indication that this ring is the site of chemical modification.

MATERIALS AND METHODS

Horse heart S_CMb-CN was prepared from horse heart metmyoglobin (metMb) (Sigma) as described by Chatfield *et al.* (29) for preparation of S_CMb from sperm whale Mb. Bright green crystals of S_CMb were grown following the published protocol for the native protein (30). S_CMb crystals were found to be isomorphic with native crystals and are monoclinic, space group $P2_1$, with unit cell dimensions a = 64.5, b = 28.9, c = 35.7 Å and β = 107.0° and with one molecule per asymmetric unit. The 7946 reflections between 6 and 2.0 Å resolution were collected from a single crystal on a CAD4-F11 diffractometer with Ni-filtered CuKα radiation and were processed in a manner similar to that described for the native metMb (31).

RESULTS AND DISCUSSION

Structural refinement was carried out with a restrained-parameter least-squares procedure (32). The initial range of data used in the refinement was from 6 to 2.8 Å resolution, which was expanded toward the end of refinement to 6–2.0 Å resolution, including all 5369 (67.6% of the total) reflections with $F_o \geq 2\sigma(F)$. The starting coordinates for S_CMb refinement were those from the 1.9-Å-resolution determination of native horse heart metMb (31), with all solvent molecules stripped from the model. Refinement proceeded with periodic inspection and side-chain fitting using electron density maps calculated with the coefficients $(F_o - F_c)$ and $(2F_o - F_c)$. A total of 173 difference peaks with significant electron density and appropriate hydrogen bonding geometry were assigned as water molecules. As in native horse heart metMb, a water molecule was initially refined at the sixth coordination site of the heme iron. However, near the end of refinement, an observed long Fe–O distance of 2.5 Å and a low thermal parameter for this group clearly indicated that this ligand was a cyanide group as expected for S_CMb. The conventional R value for observed data after the last cycle of refinement using the native heme model was 0.133.

Reaction of the porphyrin to form a chlorin by saturation of a β—β bond of one of its pyrrole rings would be expected to alter significantly the geometry of the heme group and the reacted pyrrole ring in particular. An examination of the angles between the normals to the pyrrole ring planes of both proteins (Table 1) showed that the largest discrepancies between native and S_CMb heme groups rested in angles involving pyrrole ring C. Furthermore, the only significant difference electron density peaks found in the heme cavity were in proximity to this same pyrrole ring. To investigate the nature of the chemical modification, all substituents and both C^β atoms C2C and C3C were removed from the model of pyrrole group C and three cycles of refinement were carried out. Inspection of the subsequent difference electron density map (Fig. 2) allowed an unambiguous assignment of the positions of all atoms present. The position of the sulfur atom was apparent as a dense peak on the distal side of the heme group located ≈1.8 Å from carbon atom C2C. In addition, the position of methyl group CMC is evident as a finger of electron density protruding into the proximal side of the heme pocket. In the native protein, the terminal bond of the vinyl group on pyrrole ring C is directed 45° out of the ring plane. However, in S_CMb this group is now bonded to the sulfur as the electron density of the vinyl group extends from pyrrole carbon atom C3C to the sulfur atom and is completely contained within the S–C2C–C3C plane. Our analysis thus confirms the structure proposed for S_CMb in which a modified heme group is formed by attack of H_2S at the distal side of the heme group to result in addition of the elements of H_2S to the β—β bond of pyrrole C to form a chlorin substituted with an exocyclic thiolene ring.

To complete the restrained-parameter least-squares refinement, standard molecular mechanics programs (MMP2; ref. 34) were used to generate a model of a chlorin group fused to a thiolene ring system. This model was found to be satisfactory in that the generated bond lengths and angles involving the sulfur atom, as well as the angles about the strained bridgehead carbon atom C2C (Fig. 1), were comparable to those found in small molecular structures (35–37). The model was fit to the observed electron density, and five additional

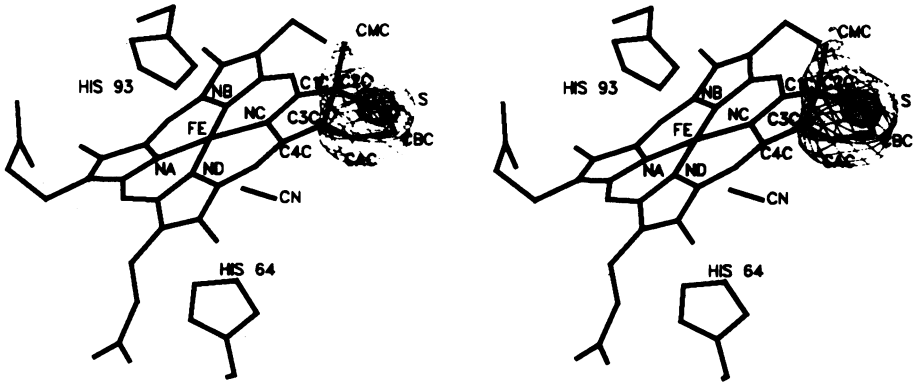

FIG. 2. Difference electron density map of S_CMb about heme pyrrole ring C contoured at the 4.0σ and 9.0σ levels after three cycles of refinement with the C2C and C3C carbon atoms and their substituents removed from the model. Wire frame model of the remainder of the chlorin group is also drawn. The heme iron atom, pyrrole nitrogen atoms, and residues about the reaction site are labeled. The position of the sulfur atom that has been chemically added to the heme group is evident as the area of higher electron density, as is the position of the displaced methyl group CMC, which can be seen pointing into the proximal side of the heme pocket.

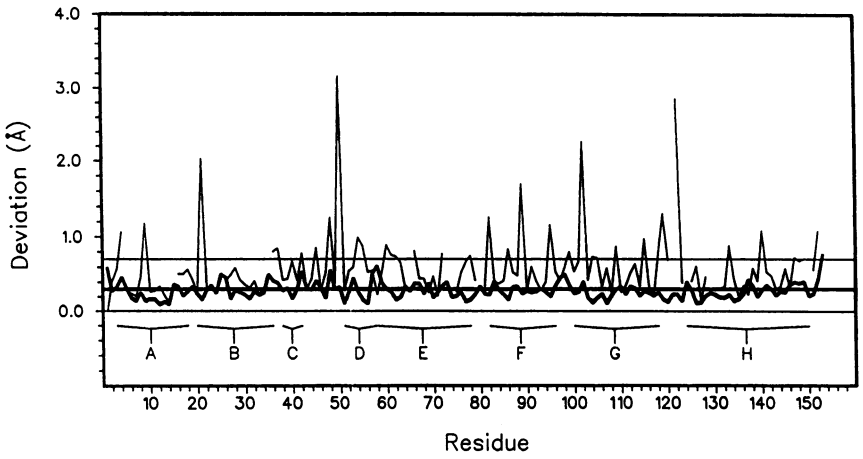

FIG. 3. Plot of observed mean positional deviations between main-chain (thick line) and side-chain (thin line) atoms of native Mb and S_CMb along the course of the polypeptide chain. The eight helical regions of this protein are labeled A–H and are indicated below the plot. The overall rms deviations observed for main-chain (0.31 Å) and side-chain (0.70 Å) atom positions are shown by horizontal lines of corresponding thickness.

cycles of refinement led to convergence to a final R value of 0.129. Inspection of an electron density map of S_CMb calculated with the coefficients $(2F_o - F_c)$ shows that all main-chain and side-chain atoms are contained in well-resolved electron density, the only exception being the C-terminal residue Gly-153. The resultant structural model also has excellent agreement with established stereochemical parameters. The overall rms deviation from ideal values for bond distances is 0.036 Å and that for angle distances is 0.06 Å.

The average deviations in both main-chain and side-chain atom positions between S_CMb and the native protein are presented in Fig. 3. The overall structures of the two proteins are very similar. No main-chain atom position has shifted by more than twice the overall rms deviation of 0.31 Å except the C-terminal residue Gly-153. Deviations for side-chain atoms of more than twice the overall rms value of 0.70 Å are found for residues Ile-21, Lys-50, Leu-89, Lys-102, and Asp-122, each of which occurs at or near flexible interhelical regions of the polypeptide chain.

Fig. 4 is a stereodiagram in which the active site structures of native Mb and S_CMb have been overlaid for comparison. Despite the addition of the exocyclic ring to the heme structure, the largest deviations in the positions of residues lining the heme cavity do not occur about the reaction site but are associated with the repositioning of other portions of the modified heme group. Table 2 provides the rms deviations for various groups of atoms of the heme group. The largest change in protein side-chain conformation within the heme cavity occurs for Leu-89, which is in contact with pyrrole ring A and has a x_1 value ≈180° away from that found for the native protein. The motion of pyrrole ring D has been transmitted through the hydrogen bond formed between its propionic acid group and Lys-45, with atoms of this side chain shifting by as much as 1.5 Å. In contrast, there are few changes associated with the sulfur atom itself, with the conformations of residues close to this atom being largely conserved. However, larger shifts are observed about the reoriented methyl group CMC, where the adjacent side-chain atoms of Ile-99 and Leu-104 have been displaced by as much as 1.5 and 1.4 Å from their positions in the native structure, respectively. It is interesting that some of the larger changes in the position of side-chain atoms lining the heme cavity are found for residue Ile-99, which had been observed by nuclear Overhauser effect experiments to conserve its orientation relative to the heme group (20).

The Fe–His-93 NE2 bond length is 2.1 Å. The heme iron is displaced just 0.1 Å from the mean plane of the pyrrole ring nitrogen atoms in the direction of the proximal histidine group. However, the distortion of the heme results in displacement of the iron atom by 0.29 Å from its position in the native structure relative to the surrounding polypeptide chain. This movement of the iron induces a similar but smaller displacement of the proximal histidine ligand. Smaller conformational changes are associated with the

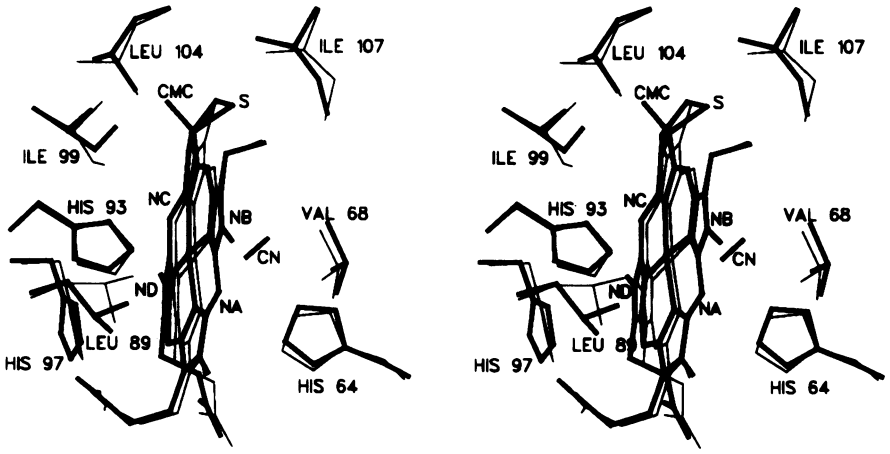

FIG. 4. Stereodiagram of superimposed structures of native Mb (thin lines) and S_CMb (thick lines) in and about the heme cavity. The four pyrrole nitrogen atoms of the heme group (center) are labeled, as are the sulfur atom and repositioned CMC methyl group of SMb.

Table 2. RMS deviations of heme groups between S_CMb and native protein

Group	rms deviation, Å	
	Heme	Globin
Pyrrole A	0.08	0.22 (5)
Pyrrole B	0.05	0.13 (5)
Pyrrole C	0.20	0.43 (5)
Pyrrole D	0.07	0.36 (5)
Fe	0.05	0.29 (1)
Porphyrin core	0.11	0.28 (21)
All atoms	0.36	0.47 (43)

Comparison in atomic positions is presented first by overlapping the porphyrin core of the heme groups and then by overlapping the main-chain atoms of the globin. Values in parentheses represent the number of atoms used in the calculation. The largest changes in heme conformation occur near heme pyrrole ring C and result in a significant repositioning of the modified heme group with respect to the globin. Concomitant shifts also occur in the positions of residues near pyrrole rings A and D.

Table 3. Conformations of diatomic heme ligands in Mb

Protein	θ	ϕ
S_CMb	137°	−10°
MbCO	141°	60°
MbCO	120°	−62°
MbO_2	115°	22°

Conformations of diatomic heme ligands in Mb are characterized by the bond angle θ = Fe–A–B and the dihedral angle ϕ between the Fe–A–B and B–Fe–NC planes, where A and B represent the ligand atoms. The observed conformation of the CN group in S_CMb is intermediate to those reported for MbCO and similar to that observed for MbO_2.

substitution of a cyanide group at the sixth coordination site of the chlorin, where atom CD2 of His-64 undergoes a shift of 0.61 Å. As indicated in Table 3, the geometry observed for the CN group is intermediate between the two disordered conformations reported for CO in MbCO (38) and is similar to that observed for O_2 in MbO_2 (39, 40).

From ligand binding studies and electron paramagnetic resonance analysis, Berzofsky *et al.* (12) inferred that the altered ligand binding properties of SMb could be explained adequately on the basis of the electronic effects of protoheme IX conversion to an iron chlorin without the necessity of invoking concomitant steric changes at the active site of the protein. The present results provide direct evidence that steric alterations arising from conversion of Mb to SMb are indeed minor and that the profound changes in ligand binding properties exhibited by SMb therefore arise almost entirely from electronic effects.

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