Articles

Family of Cytochrome c_7-Type Proteins from *Geobacter sulfurreducens*: Structure of One Cytochrome c_7 at 1.45 Å Resolution[†,‡]

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ABSTRACT: The structure of a cytochrome c_7 (PpcA) from *Geobacter sulfurreducens* was determined by X-ray diffraction at 1.45 Å resolution; the *R* factor is 18.2%. The protein contains a three-heme core that is surrounded by 71 amino acid residues. An unusual feature of this cytochrome is that it has 17 lysine residues, but only nine hydrophobic residues that are larger than alanine. The details of the structure are described and compared with those of cytochrome c_7 from *Desulfuromonas acetoxidans* and with cytochromes c_3. The two cytochrome c_7 molecules have sequences that are 46% identical, but the arrangements of the hemes in the two structures differ; the rms deviation of all α-carbons is 2.5 Å. These cytochromes can reduce various metal ions. The reduction site of the chromate ion in *D. acetoxidans* is occupied by a sulfate ion in the crystal structure of PpcA. We identified four additional homologues of cytochrome c_7 in the *G. sulfurreducens* genome and three polymers of c_7-type domains. Of the polymers, two have four repeats and one has nine repeats. On the basis of sequence alignments, one of the hemes in each of the cytochrome c_7-type domains does not have the bis-histidine coordination. The packing of the molecules in the crystal structure of PpcA suggests that the polymers have an elongated conformation and might form a "nanowire".

Multiheme cytochromes in the periplasm of Fe(III)-reducing bacteria (*1*, *2*) and in sulfur- and sulfate-reducing bacteria (*3*−*6*) play critical roles in the environmental processing of many metals, including radionuclides (*7*, *8*). Cytochrome c_7 from *Geobacter sulfurreducens*, designated PpcA, is a protein with 71 residues that contains three covalently bound hemes (*9*). It is one of the smallest cytochrome *c*-type molecules with the highest ratio of hemes to amino acid residues. The purified protein can reduce Fe(III), U(VI), Cr(VI), and other metal ions. The chromate binding site was identified by NMR methods (*10*) for the homologous cytochrome c_7 from *Desulfuromonas acetoxidans*, the structure of which was determined by X-ray diffraction (*11*). *G. sulfurreducens* and *D. acetoxidans* are closely related delta proteobacteria. In addition to the reduction of metal ions, both organisms have been shown to reduce elemental sulfur but not sulfate. The cytochrome c_7 is the most abundant protein in their periplasm; the level of sequence identity between the two proteins is 46% (Figure 1). However, the level of three-dimensional structural homology is less than expected from the sequence homology; the rms deviation of the α-carbons is 2.5 Å.

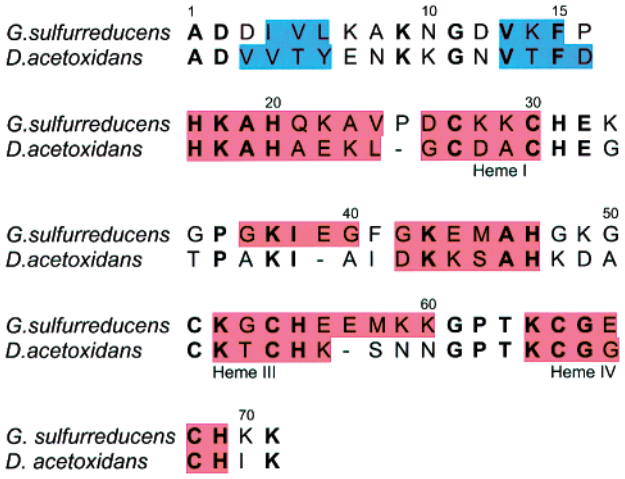

FIGURE 1: Alignment of the amino acid sequences of cytochrome c_7 from *G. sulfurreducens* and *D. acetoxidans*. Identical residues (46%) are shown in bold type. The positions of the heme attachment sites are indicated below the sequences. Protein chain segments with β-structure are shown in blue, and α-helical segments are shown in pink.

The cytochrome c_7 (PpcA) in *G. sulfurreducens* is thought to be directly responsible for the reduction of soluble metal species such as U(VI), which can enter the periplasm (*12*). Furthermore, it is believed to serve as a carrier of an

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[‡] The coordinates and structure factors have been deposited in the Protein Data Bank as entry 1OS6.

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electron from the electron donor acetate to the Fe(III) oxide reductase localized in the outer membrane of the organism (*9*).

Among the multiheme cytochromes, the cytochrome c_3 family from the sulfate-reducing bacteria is best studied. They consist of ~120 amino acid residues and four covalently linked heme groups. The hemes are numbered sequentially according to the cysteine residues to which they are attached in the amino acid sequence. Heme IV is suggested to interact with hydrogenases (*13*, *14*). While the level of sequence identity among the four-heme proteins is relatively low, ~25%, the positions and orientations of the hemes in the three-dimensional structures are maintained (*15*). The three-heme cytochrome c_7 molecules are homologous to the four-heme cytochrome c_3 molecules, but are missing heme II of the cytochrome c_3 molecules and the amino acid chain segment that keeps that heme in place. For comparison with the more extensively studied four-heme cytochromes, the cytochrome c_3 nomenclature for labeling the hemes in the cytochrome c_7 proteins is maintained.

We have determined the three-dimensional structure of one of the three-heme cytochrome c_7 molecules (PpcA) from *G. sulfurreducens* by X-ray diffraction of single crystals at 1.45 Å resolution. In this paper, we describe the unusual structural features of this protein, including structural details of a possible reduction site of chromate. Further, we compare the structure of the cytochrome c_7 from *G. sulfurreducens* with that of *D. acetoxidans*, and with cytochrome c_3 molecules. We found that the *G. sulfurreducens* genome also contains coding sequences for additional cytochrome c_7 homologues. These homologues surprisingly include several polymers of cytochrome c_7-type domains; the function or functions of the homologues are presently not known. The amino acid sequences of the homologues are analyzed in terms of the three-dimensional structure and crystal packing of PpcA.

MATERIALS AND METHODS

Crystallization. Protein production and purification were performed with a slight modification of the procedure described previously (*16*); a further gel filtration purification step was added. After a large number of crystallization trials, crystals were obtained at a protein concentration of 72 mg/mL from high concentrations of ammonium sulfate (pH adjusted to 6.0 or 7.0 with ammonium hydroxide) only in the presence of deoxycholic acid. Crystals suitable for X-ray diffraction were obtained from 3.5 M ammonium sulfate (pH 6.0) in the presence of 0.25% deoxycholic acid. Tetragonal crystals (space group $P4_122$ or $P4_322$, unit cell dimensions of $a = b = 32.4$ Å and $c = 178.7$ Å) diffracted to a resolution of ~1.3 Å at the Structural Biology Center's 19ID beamline at the Advanced Photon Source (APS).[1]

Structure Determination and Refinement. The structure of PpcA was determined by the MAD method using three wavelength data sets (see Table 1) collected at the Fe K-absorption edge at the 19BM beamline (APS). Because of the longer wavelength at the Fe edge, MAD data were collected to a resolution of only 2.4 Å. Data were processed with HKL2000 (*17*). The structure was determined using the program CNS (*18*). Following density modification in space group $P4_322$, the electron density map was of excellent quality and was suitable for model building. The protein model was built manually into the electron density map using the program CHAIN (*19*) and refined against high-resolution data collected at the 19ID beamline (APS). Although the crystals diffract to 1.3 Å, because of the long *c*-axis, data resolved to only 1.45 Å were collected. Initial refinement of the model was carried out with CNS and in later stages with Shelx-97 (*20*). The Fourier difference map showed small residual peaks near the Fe atoms in all three hemes, which suggested anisotropic thermal motion of the Fe atoms. Anisotropic refinement of the Fe atoms lowered R_{free} by 0.5%. However, including the sulfur atoms of the cysteine residues in anisotropic refinement did not produce a significant drop in R_{free}. There are no residues in the disallowed regions of the Ramachandran plot, and 85.5% of the residues are in most favored regions. The quality of the electron density is illustrated in Figure 2. The data collection and refinement parameters are summarized in Table 1. Figures 2, 3b, and 4−8 were generated with CHAIN (*19*), and Figure 3a was generated with SETOR (*21*).

Bioinformatics Methods. Protein sequences homologous to that of cytochrome c_7 were identified by searching the *G. sulfurreducens* genome (GenBank entry NC_002939) with the sequence of the cytochrome c_7 PpcA (ORF01023) using BLAST software (*22*). The SignalP software (*23*) was used for prediction of leader sequence cleavage sites. The Genetic Computer Group Software package (Genetics Computer Group, Madison, WI) was used for alignments.

RESULTS AND DISCUSSION

The cytochrome c_7 from *G. sulfurreducens* has 71 residues and three covalently attached hemes at typical heme attachment sites of the CXXCH sequence pattern. The two axial ligands of the hemes are histidine residues; the details of their packing and the modulation of their redox properties are influenced by the residues that surround them. In addition to the cytochrome molecule, the asymmetric unit of the crystal contains a molecule of deoxycholic acid, three sulfate ions (numbered 76−78), and 95 water molecules. The presence of deoxycholic acid was required for crystallization.

Structure of the Protein

Secondary Structure. Except for a short β-sheet, the protein consists of six small helical regions; three of the helical regions contain the CXXCH motif that covalently binds the hemes and supplies the fifth ligand to the Fe atom of the hemes. These chain segments require a helical conformation for the Cys residues to provide the proper geometry for heme attachment (*24*). Two additional helical segments contain the sixth ligands to the Fe atoms of the hemes. The "outside" surfaces of the helical regions are highly polar and charged; they are largely occupied by Lys, Glu, and Gln residues.

Residues 4−6 and 13−15 form an antiparallel β-sheet; the loop between them has a β-turn formed by residues 8−11 (Figure 3). There is a change in the direction of the chain at Pro16, followed by a two-turn α-helix from residue 17 to 24. This segment contains the sixth ligands, His17 and His20, to hemes I and III, respectively. The chain changes direction

[1] Abbreviations: APS, Advanced Photon Source; MAD, multiple-wavelength anomalous dispersion.

Table 1: Summary of Data Collection and Crystallographic Parameters

Crystal Parameters				
unit cell (Å)	$a = b = 32.4$, $c = 178.4$			
space group	$P4_322$			
Data Collection				
	MAD data			
	inflection	peak	remote	high resolution
wavelength (Å)	1.7411	1.739	1.6919	1.0332
resolution range (Å)	50.0−2.30	50.0−2.30	50.0−2.30	100.0−1.45
no. of unique reflections	7494	7467	7868	30830
redundancy (last shell)	3.5 (2.4)	3.4 (2.4)	3.6 (2.6)	5.3 (1.8)
completeness (%) (last shell)	92 (64)	92 (63)	97 (81)	96 (73)
average $I/\sigma(I)$ (last shell)	43.2 (26.5)	43.3 (27.1)	34.8 (12.3)	57.7 (9.2)
R_{merge} (%) (last shell)	4.9 (7.1)	5.1 (7.3)	6.6 (11.1)	6.1 (13.4)

Phasing (resolution range of 50−2.3 Å)	inflection		peak		remote		
	fried	iso	fried	iso	fried	iso	all
phasing power	5.03	4.56	4.27	3.59	2.12	1.48	
FOM	0.64	0.64	0.62	0.60	0.49	0.42	0.91
density modification, FOM							0.96

Refinement	
resolution range (Å)	30.0−1.45
σ cutoff used in refinement	0.0
no. of reflections	27804
no. of non-hydrogen atoms refined [average B-factor (Å^2)]	
protein	535 (17.0)
heme	129 (12.8)
deoxycholic acid	28 (13.2)
sulfate	15 (27.5)
water	95 (29.7)
R factor, R_{free}[a] (all data)	0.182, 0.227
rmsd from ideal geometry	
bond lengths (Å)	0.022
bond angles (deg)	4.7

[a] The test set for the calculation of R_{free} was comprised of 10% of the total reflections selected at random and not used in refinement.

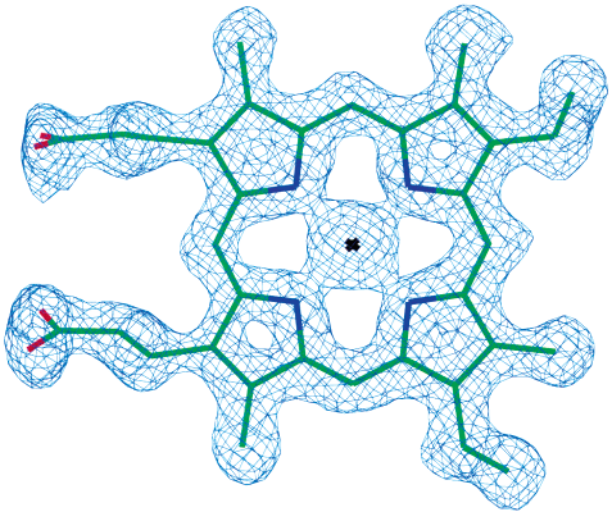

FIGURE 2: Electron density of heme III contoured at the 1σ level of a map calculated with $3F_o - 2F_c$ coefficients illustrating the quality of the structure.

at Pro25, and is followed by a 3_{10}-helix formed by residues 26−30, the attachment site of heme I. A change in chain direction occurs at Pro35, followed by a one-turn helix involving residues 36−40. There is a bend in the chain at residue Phe41, followed by what appears to be a long helical segment (Figure 3). This segment consists of an α-helix, residues 42−47, followed by a wide loop similar to a π-helix, and then by another α-helix, residues 52−59. These segments contain the sixth ligand to heme IV, His47, and the Cys51 and Cys54 of the heme III binding site. The chain changes direction at residue 60, followed by a 3_{10}-turn and an α-helix that includes residues 64−69. Cys65 and Cys68 form the binding site of heme IV. Residues 70 and 71 appear to form the third segment of the N-terminal β-sheet, though only the peptide nitrogen of residue 70 is hydrogen bonded to the carbonyl oxygen of residue 12, located at the beginning of the second β-strand segment.

Deoxycholic Acid. The part of the chain between residues 35 and 42 has only a few contacts with the rest of the protein and has a large solvent accessible opening. A molecule of deoxycholic acid is located in this channel (Figure 4). On the basis of previous small-angle X-ray scattering results (*16*), the deoxycholic acid does not appear to change the overall structure of the molecule. The calculated radius of gyration using cytochrome c_7 coordinates with deoxycholic acid is 12.1 Å and without deoxycholic acid is 12.2 Å. These values are in good agreement with the measured radius of gyration of 12.3 Å obtained in the absence of deoxycholic acid. Therefore, the relative positions of the hemes observed in our structure are not expected to be caused by the binding of deoxycholic acid. The hydrophobic portion of the deoxycholic acid interacts with the hemes and with residue Phe41. Its carboxyl group is surrounded by Lys29, -33, -37, and

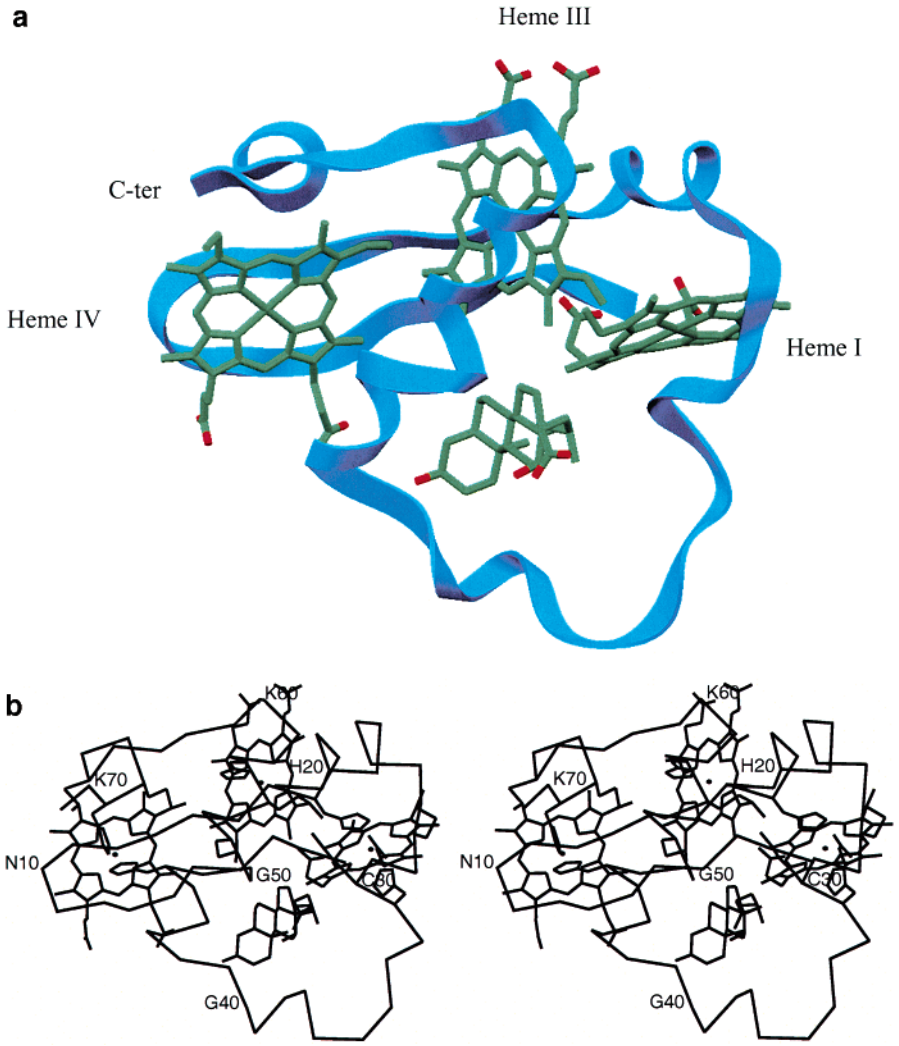

FIGURE 3: Structure of cytochrome c_7 PpcA. The molecule has a helical face (front) and a β-sheet face (back). (a) The polypeptide backbone is shown in blue; hemes and deoxycholic acid are shown in green with their oxygen atoms in red. (b) Stereoview of the molecule in the α-carbon trace of the protein. Cysteine, histidine side chains, and the hemes are also shown.

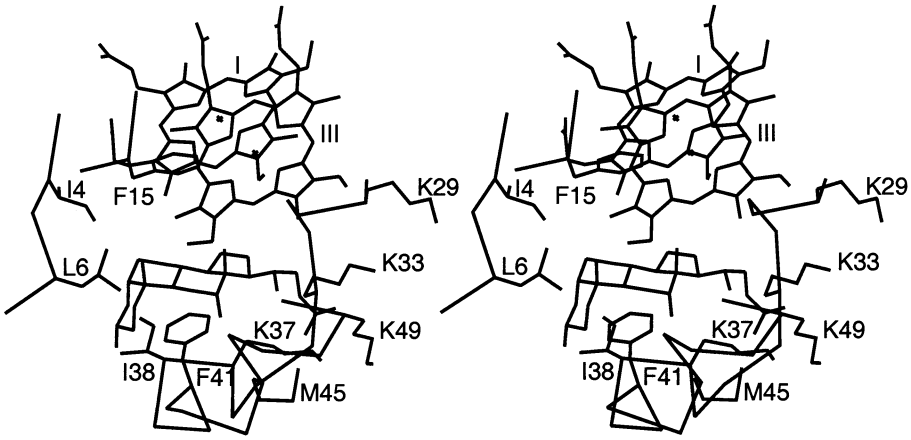

FIGURE 4: Stereoview showing the surroundings of the deoxycholic acid. It is located in a hydrophobic opening that consists of residues Ile4, Leu6, Phe15, Ile38, Phe41, and Met45 and hemes I and III. The hydroxyl groups of the deoxycholic acid interact with water molecules (not shown), whereas the carboxylic group interacts with Lys33, Lys37, and Lys49; Lys29 is also in the vicinity.

-49. The deoxycholic acid moiety appears to stabilize this part of the molecule, which may otherwise be too flexible and would have multiple conformations. The position of the deoxycholic acid in the molecule might explain why the protein could not be crystallized in its absence. The deoxycholic acid is not present in the periplasm of *G. sulfurreducens*; however, the cytochrome c_7 protein in its native environment might bind a small molecule that is important for its function in the cell, in the same position that the deoxycholic acid is observed in the crystal.

Hydrogen Bonds. A characteristic of this cytochrome c_7 is that there are very few side chain−side chain or side

Table 2: Side Chain−Side Chain and Side Chain−Main Chain Hydrogen Bonds and Hydrogen Bonds Formed by His Residues

		distance (Å)
Lys7 NZ	Asp12 OD1	2.68
Asp3 OD1	His17 N	2.95
Glu67 OE1	Thr63 N	3.18
Glu67 OE1	Lys64 N	3.16
heme I O2A	Ala1 N	2.74
heme IV O2A	Lys9 N	2.68
His20 ND1	Pro16 O	2.77
His55 ND1	Pro62 O	2.81
His69 ND1	Gly11 O	2.83
His17 ND1	Wat129	2.92
His31 ND1	sulfate76 O2	2.80
His47 ND1	sulfate77 O1	2.83

chain−main chain hydrogen bonds (Table 2). The only side chain−side chain hydrogen bond is between Lys7 and Asp12. In addition to the hydrogen bonds formed by the His residues to carbonyl oxygens, Asp3 forms a hydrogen bond with the peptide nitrogen of His17 and Glu67 forms hydrogen bonds with peptide nitrogens of Thr63 and Lys64. Except for the His residues, none of the above residues are conserved. Peptide nitrogens of Ala1 and Lys9 form hydrogen bonds with the propionic acid moieties of hemes I and IV, respectively. In addition, a charge−charge interaction occurs between Lys60 and the propionic acid moiety of heme III.

Large Hydrophobic Residues. The core of this molecule is formed by the three hemes; this could be the reason there are relatively few hydrophobic residues larger than Ala in the structure. At seven homologous positions, hydrophobic residues occur in both *G. sulfurreducens* and *D. acetoxidans* cytochrome c_7. A characteristic of the β-pleated sheet segment is that every second residue is hydrophobic; Ile4, Leu6, Val13, and Phe15 form the signature for the short β-pleated sheet segment. Ile4 and Leu6 are close to the propionic acid groups of hemes I and IV, respectively. Val13 and Val24 are close to heme III, and Phe15 is close to hemes I and III. Phe41 is located close to heme IV; Ile38 is on the surface. Two Met residues, Met45 and Met58, are not conserved. Met45 is on the outside surface of a helical region and is disordered, while Met58 is in van der Waals contact with heme III (CAC and CMD atoms).

Long Charged and Polar Residues. The most striking feature of this molecule is that 17 of 71 residues (24%) are Lys residues, while six are Glu residues. All but four of the Lys residues are disordered. Lys64 appears to have two conformations after its CG atom. Two of the four Lys residues with relatively low *B*-factors have specific interactions in the crystal lattice; Lys28 and Lys43 are on the surface and interact with sulfate ions. Only two of the Lys residues form hydrogen bonds within the molecule. Lys7 interacts with Asp12, and Lys60 interacts with the propionic acid moiety of heme III. Of the six Glu residues in the structure, only three are ordered; two residues, Glu32 and Glu39, form hydrogen bonds with the sulfate ions, and the third, Glu67, hydrogen bonds with backbone nitrogen atoms of residues 63 and 64. The only glutamine residue in the structure, Gln21, is also disordered. It appears that the main role of the long charged residues is to provide the surface charge of the molecule.

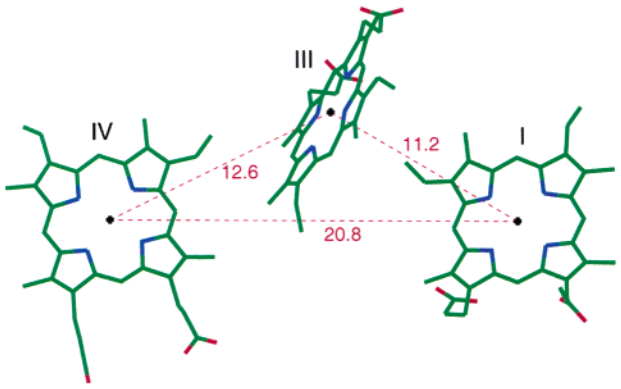

FIGURE 5: Arrangement of the hemes in PpcA. Hemes I and IV are separated mainly along the *a*-axis of the crystal, and they are related by a pseudo-2-fold axis (173° rotation) which is approximately parallel to the *c*-axis of the crystal. The distances between iron atoms of the hemes are given in angstroms.

Table 3: Comparisons of Relative Heme Geometries of *G. sulfurreducens* (*G.s*) and *D. acetoxidans* (*D.a*) Cytochrome c_7 Molecules and Average Values for Six Cytochrome c_3 Molecules Taken from Refs *25*−*30*

	G.s c_7	*D.a* c_7	c_3
Heme Fe−Fe Distances (Å)			
heme I−heme III	11.2	11.5	11.1
heme III−heme IV	12.6	12.6	12.1
heme I−heme IV	20.8	19.3	17.7
Angle between Mean Heme Planes (deg)			
heme I−heme III	86	81	83
heme III−heme IV	69	77	84
heme I−heme IV	35	8	21

Hemes

Arrangement of the Hemes. The three hemes form the core of the molecule. Heme III is located between heme I and heme IV; a local pseudo-2-fold axis relates heme I and heme IV (Figure 5). The arrangements of the hemes are such that hemes I and III are in direct van der Waals contact with each other (the CBC atom from heme I and the C2C atom from heme III) and heme IV is in van der Waals contact with the coordinating His residue of heme III (the CE1 atom of His55 and the CBC atom from heme IV).

The iron−iron distances are 11.2 Å between heme I and heme III, 12.6 Å between heme III and heme IV, and 20.8 Å between heme I and heme IV (Figure 5 and Table 3). The angles between the mean planes of the hemes are 86° between heme I and heme III, 69° between heme III and heme IV, and 35° between heme I and heme IV (Table 3).

Heme I has the largest accessible surface area (225 Å^2 or 29.3%), followed by heme IV (189 Å^2 or 24%), and heme III is the least exposed (144 Å^2 or 19%). For heme I, the CMA and CMB atoms are the most exposed and the O1A, O2A, and CAD atoms are less exposed. In heme III, the O1A, O2A, and O2D atoms are the most exposed and the CMA atom is less exposed. For heme IV, the CMA and CMB atoms are the most exposed and the O1A, O2D, and CAB atoms are less exposed.

His Residues that Coordinate the Hemes. The hydrogen bonds formed by the His residues are listed in Table 2; the angles that the planes of the His residues form with each other are described in Table 4. His17 and His31 form the

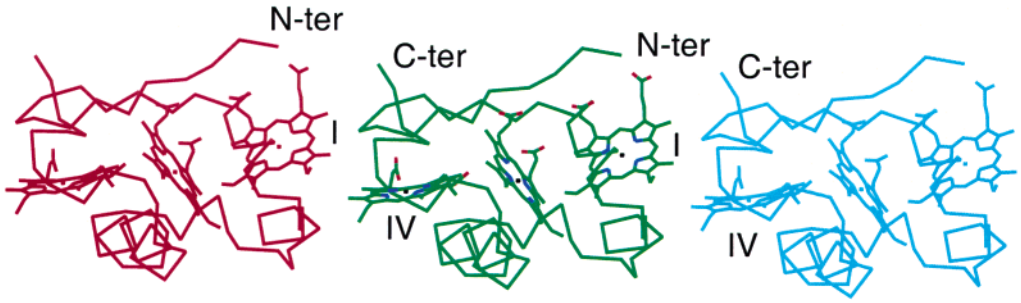

FIGURE 6: Packing of cytochrome c_7 molecules in a one-dimensional array along the *a*- and *b*-axes of the crystal, which can serve as a model for the polymers of c_7-type domains. The intermolecular Fe−Fe distance of 12.8 Å is similar to the intramolecular Fe−Fe distances. The distance between the N- and C-termini of the neighboring molecules is 9 Å.

Table 4: Comparison of the Angles between Histidine Ring Planes Made by the Axial Ligands of Each Heme in *G. sulfurreducens* (*G.s*) and *D. acetoxidans* (*D.a*) Cytochrome c_7 Molecules

		G.s	*D.a*
His17 (17)[a]	His31 (30)	57°	79°
His20 (20)	His55 (53)	22°	31°
His47 (45)	His69 (66)	89°	83°

[a] The corresponding His residue number in *D. acetoxidans* c_7 is given in parentheses.

axial ligands of heme I. The histidine planes make a 57° angle with each other. The ND1 atom of His17 forms a hydrogen bond with a water molecule. The ND1 atom of His31 forms a hydrogen bond with sulfate ion 76. His20 and His55 form the axial ligands of heme III. The planes of the His side chains are nearly parallel to each other. Phe15 is nearly parallel (23°) to His20; the closest distance between them is 3.4 Å. Further, the ring of Pro62 is approximately parallel (12°) to the ring of His55. The ND1 atom of His20 forms a hydrogen bond with the carbonyl oxygen of Pro16. The ND1 atom of His55 forms a hydrogen bond with the carbonyl oxygen of Pro62. A similar hydrogen bond present between the fifth axial ligand and the carbonyl oxygen of a Pro residue was found to be conserved in mitochrondrial-type cytochrome *c* molecules (*3*). His47 and His69 form the axial ligands of heme IV. The planes of these His residues are perpendicular to each other. The ND1 atom of His47 forms a hydrogen bond with sulfate ion 77. The ND1 atom of His69 forms a hydrogen bond with the carbonyl oxygen of Gly11. The His rings and the heme planes are generally close to perpendicular with each other, between 82° and 90°; His17 and heme I, however, make an angle of 62°.

Description of the Crystal

Crystal Packing. Close packing within the unit cell is observed between molecules related by a 2-fold axis perpendicular to the (110) plane at $z = {}^1/_8$. Molecules in the neighboring unit cell, related by translation along the *x*- or *y*-axis, are also involved in close packing. A larger surface area of 1942 Å^2 is buried between the 2-fold related molecules, compared to 458 Å^2 between molecules related by translation. The β-sheet at the N-terminus of the molecule is extended by the 2-fold axis perpendicular to the sheet. Sulfate ion 76 is buried within this interface. Both the side chain carboxyl and the main chain nitrogen atoms of Asp2 of one molecule are within hydrogen bonding distance of the propionic acid moiety of heme IV from the 2-fold related molecule. There is no close contact between the hemes, but the deoxycholate molecules are within van der Waals distance of each other. Heme I and heme IV are in the proximity of each other between molecules related by translation along the *x* or *y*-axes in neighboring unit cells. Sulfate ion 77 is found at this interface. A packing diagram showing three molecules of cytochrome c_7 in the crystal structure is presented in Figure 4. The closest approach is between the CMB atoms (5.6 Å), and between the C2B atoms on the porphyrin rings (7.0 Å) of heme I and heme IV from neighboring molecules. These distances are comparable with contact distances within the same molecule of heme I and heme III of 4.5 and 5.9 Å between the C3C and C2C atoms and between the CAC and CMC atoms, respectively, and of heme III and heme IV of 4.4 and 5.9 Å between the CMB and CMD atoms and between the C2B and CHD atoms, respectively. The interheme Fe−Fe distances between neighboring molecules in the unit cell are only ~1 Å longer than the ones within a molecule.

Sulfate Ions. Sulfate ions form three of the crystal contacts between neighboring molecules. Sulfate ion 76 is completely buried. The oxygen atoms of sulfate ion 76 form hydrogen bonds with the ND1 atom of His31 and the peptide nitrogen of Glu32 of one molecule and the peptide nitrogens of residues 36, 38, and 39 and the OE1 atom of Glu39 (2.88 Å) of a symmetry-related molecule. Oxygen atoms of sulfate ion 77 form hydrogen bonds with the ND1 atom of His47 and the NZ atom of Lys43 (3.2 Å). It also forms a hydrogen bond with residues of a neighboring molecule, the OE1 atom of Glu32 (2.65 Å), and is 3.2 Å from the NZ atom of Lys28. Sulfate ion 78 forms hydrogen bonds (3.1 Å) with the backbone nitrogens of residues 43 and 44; in addition, it is 3.4 and 3.7 Å from the NZ atom of Lys71 and the N-terminal nitrogen of a neighboring molecule in the crystal, respectively. Sulfate ions 77 and 78 are also in contact with the solvent.

Location of the Chromate Binding Site

Sulfate (SO_4^{2-}) ion 77 in the crystal structure (Figure 7) is located in approximately the same position as the chromium(III) found in the *D. acetoxidans* cytochrome c_7, in which the insoluble chromium(III) was generated by the reduction of chromate (CrO_4^{2-}) ion; its position was determined by NMR techniques (*10*). The sulfate ion is isostructural with the chromate ion; therefore, its binding to the molecule emulates the binding of the chromate ion. The sulfur atom is 7.1 and 16.5 Å from the iron atoms of heme IV and heme III, respectively; these separations are comparable to the reported chromium(III)−iron distances of 7.9

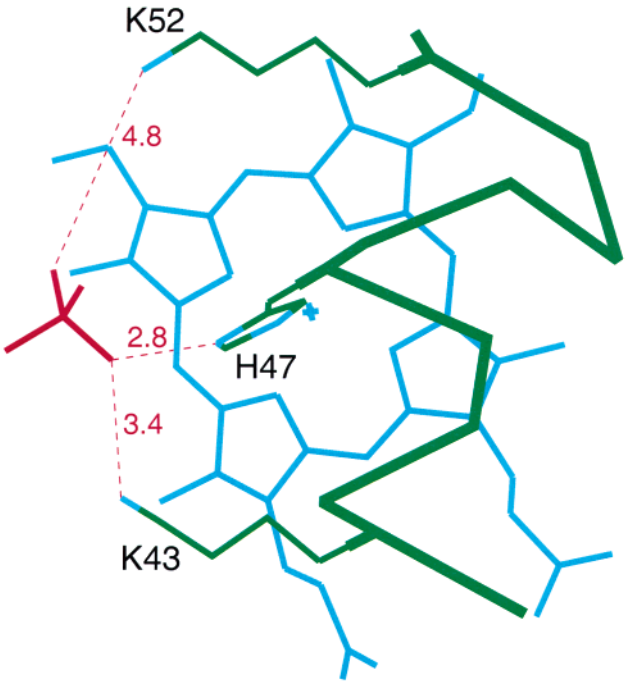

FIGURE 7: Binding site of sulfate ion 77 near heme IV of PpcA. Heme IV is shown in blue, and the sulfate ion is shown in red. Contacts between sulfate oxygen atoms and side chains of H47, K43, and K52 are shown with dashed lines with the distances in angstroms. The sulfate ion mimics the probable mode of binding of the chromate ion in *D. acetoxidans* cytochrome c_7 (*10*) at this site.

$\pm$ 0.4 and 16.3 $\pm$ 0.4 Å, respectively, determined by NMR. His45 and Lys41, -42, -46, and -50 were identified (*10*) as being close to the chromate binding site in *D. acetoxidans* cytochrome c_7. Residues His47, Lys43, and Lys52 in *G. sulfurreducens* cytochrome c_7 are close to the oxygen atoms of the sulfate ion in the crystal. These residues are homologous to the *D. acetoxidans* His45, Lys41, and Lys50 residues, respectively. The Lys residues, Lys42 and Lys46, in *D. acetoxidans* are replaced with Glu and Gly, respectively, in *G. sulfurreducens* cytochrome c_7 (see Figure 1).

Comparison of the G. sulfurreducens and D. acetoxidans Cytochrome c_7 Structures

The relative positions of hemes III and IV are the same in the two structures, but the relative location of heme I is quite different. Figure 8 shows the overlapped structures of the molecules. The iron−iron distance between heme III and heme IV is 12.6 Å in both molecules. The distances between hemes I and III and between hemes I and IV are 11.2 and 20.8 Å in *G. sulfurreducens* cytochrome c_7 and 11.5 and 19.3 Å in *D. acetoxidans* cytochrome c_7, respectively (*11*) (see Table 3). The change in heme distances also affects the angles between the His residues that coordinate the hemes (see Table 4).

There are 71 residues in *G. sulfurreducens* cytochrome c_7 compared to 68 in *D. acetoxidans* cytochrome c_7. Thirty-three residues are identical between the two molecules, 12 of which are Cys and His residues that form the heme binding sites (Figure 1). In the *G. sulfurreducens* cytochrome c_7, there is one insertion before the binding site of heme I, one between the binding site of heme I and the sixth axial ligand of heme IV, and one between binding sites of hemes III and IV. The relative positions of heme I compared to heme III and heme IV differ in the two structures probably because of the insertions before and after the binding site of heme I; therefore, some of the secondary structure elements are the same, but they are displaced. As discussed above, on the basis of small-angle X-ray scattering results (*16*), the inclusion of the deoxycholate molecule in the *G. sulfurreducens* cytochrome c_7 crystal structure does not change the distances between the hemes. For comparison of the two structures, we use the numbering for the *G. sulfurreducens* cytochrome c_7.

The secondary structures of residues Ile4−Phe15 are the same in both molecules, but a hydrogen-bonded loop present between two β-strand segments is shifted by ~1.5 Å in one versus the other. Of the 12 residues in this segment, four amino acid residues are the same, two in the loop region and two in the second β-segment. The latter includes the conserved Phe15, a residue that is also conserved in the cytochrome c_3 molecules (*25*). The pattern of hydrophobic residues in the short β-segments is maintained.

Segments of residues His17−Val24 in the two molecules are both helical, but their orientations differ. This chain segment contains His17 and His20 that coordinate heme I and heme III, respectively. Since the relative positions of hemes I and III differ in the two structures, the angle that the plane of His17, the sixth ligand of heme I, makes with heme I differs in the two structures. The relative positions of heme I and the segment of residues Cys27−His31, the covalent binding site of heme I, are the same, but chain segments before and after differ in conformation. Segments

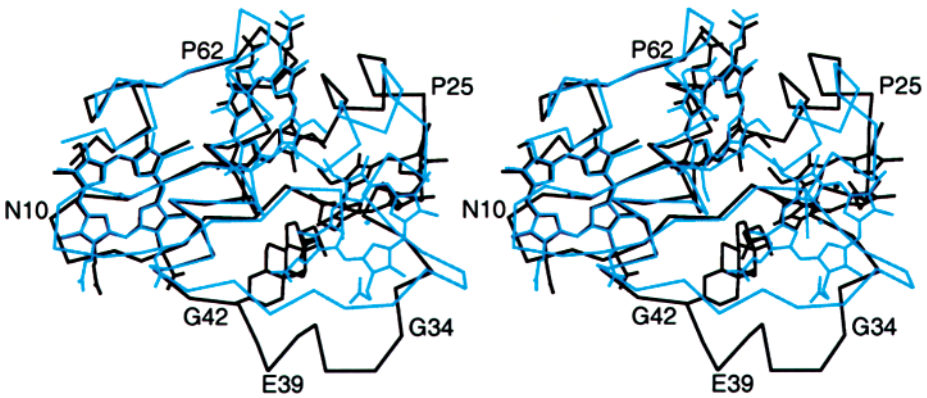

FIGURE 8: Stereoview of the cytochrome c_7 molecules from *G. sulfurreducens* (black) and *D. acetoxidans* (blue) overlapped using hemes III and IV. The rms deviation for all α-carbons is 2.5 Å.

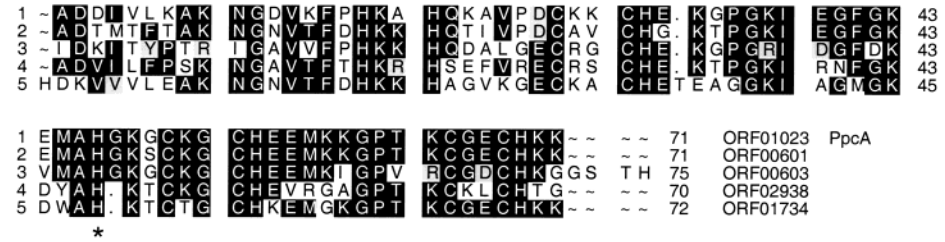

FIGURE 9: Sequence alignment of the five cytochrome c_7 molecules encoded by the *G. sulfurreducens* genome. The levels of sequence identity of proteins 2−5 with protein 1, the structure of which we have determined, are 77, 62, 65, and 57%, respectively. These sequences have the signature for binding three hemes and for residues in the secondary structure elements discussed in the text. The histidine residue that forms the sixth ligand of heme IV is marked with an asterisk. This histidine is not present in the c_7-type domains (see Figures 10 and 11).

of residues Glu32−Phe41 have different conformations; residue Glu39 is probably the one that is inserted. This segment in *G. sulfurreducens* forms the opening for the deoxycholic acid. Of the 10 residues in this segment, four are identical. Segments of residues Pro35−Ile38 have three identical residues but have different secondary structures. Residues 42−55 have similar secondary structures; seven of 14 residues are identical in this segment.

Heme III and heme IV and the polypeptide chains associated with them have similar conformations in the two structures except for the five-residue segment after the heme III binding site, residues 56−60, the location of one of the insertion sites. On the basis of the structures, Glu57 is the inserted residue. Residues 61−69 have similar structures; eight of nine residues are identical in this segment.

The relative orientations of the His residues that form the axial ligands of the hemes in the *G. sulfurreducens* and *D. acetoxidans* cytochrome c_7 molecules are shown in Table 4; the biggest difference is between the angles of His17 and His31 that coordinate heme I, the orientation of which differs in the two structures. The hydrogen bonds with water molecules of His30 and His45 in *D. acetoxidans* cytochrome c_7 are replaced with hydrogen bonds with sulfate ions for His31 and His47 in *G. sulfurreducens* cytochrome c_7.

Even though the two c_7 proteins were crystallized in different space groups, hemes are observed at the molecular contacts in the crystal packing. The molecular faces in contacts differ, and the iron−iron distances are longer in the *D. acetoxidans* cytochrome c_7 crystals (19.3 and 16.7 Å between hemes III and IV and between hemes I and IV, respectively, compared to 12.8 Å between hemes I and IV in the *G. sulfurreducens* cytochrome c_7 crystal).

Comparison of Cytochrome c_7 and c_3 Molecules

The cytochrome c_7 molecules are part of the cytochrome c_3 family, distinguished by the absence of heme II (in the c_3 nomenclature). The level of amino acid sequence homology is very low, ∼25%, among the cytochrome c_3 molecules. The level of homology is also very low when cytochrome c_7 molecules are compared with cytochrome c_3 molecules. In addition to the two Cys and two His residues that bind and coordinate each of the hemes, only Phe15 (equivalent to Phe20) in cytochrome c_3 is identical. The residues equivalent to Val13 and Val24 that are located near heme III maintain their hydrophobic character. A two-stranded antiparallel β-sheet at the N-terminus is characteristic of these molecules. As described previously, Val13 and Phe15 are the hydrophobic residues in the second strand. The loops between the two strands of the β-sheet have different lengths in the cytochrome c_3 molecules.

The distances and angles between hemes are listed in Table 3. For the two cytochrome c_7 molecules, the largest difference is between the relative positions of heme I and heme IV; the distances between the two hemes are 20.8 and 19.9 Å and the angles between the two hemes 35° and 8°, respectively. In cytochrome c_3 molecules, the distance varies from 17.3 to 18.0 Å with an average value of 17.7 Å; the angle varies between 15° and 30° with an average of 21° for six molecules (*26*−*31*). The above values indicate that the distances and angles between hemes are better maintained among the cytochrome c_3 molecules than among cytochrome c_7 molecules. It is possible that heme II, the heme that is not present in the cytochrome c_7 molecules, is required for the maintenance of heme geometry in the cytochrome c_3 molecules.

Homologues of Cytochrome c_7 in the G. sulfurreducens Genome

We found that the genome of *G. sulfurreducens* contains additional homologues of the three-heme cytochrome c_7 gene that might be expressed under certain conditions depending on the environment of the organism. To determine if the *G. sulfurreducens* genome contained genes for four-heme cytochrome c_3 molecules, BLAST searches (*22*) of the *G. sulfurreducens* genome were carried out with two different four-heme cytochrome c_3 molecules, sequences from *Desulfovibrio gigas* (Swiss-Prot entry P00133) and *Desulfovibrio desulfuricans* Norway (Swiss-Prot entry P00136). We were not able to find any genes for cytochrome c_3-like four-heme cytochromes in the genome. Therefore, our searches suggest that for *G. sulfurreducens* the three-heme cytochrome c_7 is the predominant pattern. Three-heme cytochromes have also been purified from *Geobacter metallireducens* (*2*) and from *D. acetoxidans* (*11*). These bacteria are closely related phylogenetically and are capable of reducing Fe(III) (*32*). The cytochrome c_7 molecules probably have special metal reducing properties since they appear to be characteristic of the *Geobacter* sp. that reduce U(VI) and Fe(III) ions.

The genomic sequence of *G. sulfurreducens* was searched with the sequence of the cytochrome c_7 PpcA (ORF01023) using BLAST (*22*). Four additional open reading frames (ORFs) whose sequences were 57−75% identical to the search sequence were identified (Figure 9). Two of them, ORF00601 and ORF00603, were located on contiguous gene

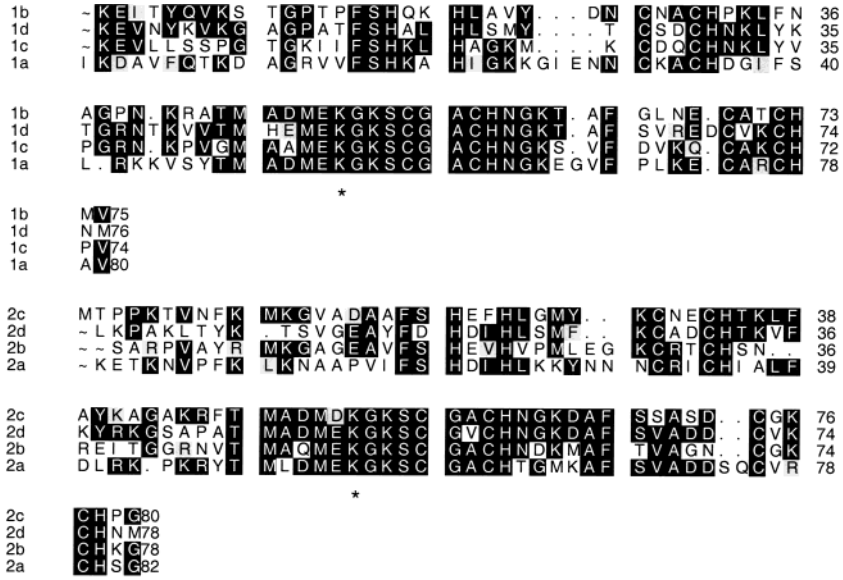

FIGURE 10: Sequence alignment of the four homologous sections (a−d) from two 12-heme cytochromes, ORF00991 (1) and ORF03300 (2), encoded by the *G. sulfurreducens* genome. Each repeat unit has the CXXCH signature for binding three hemes, but the His residue that forms the sixth ligand to heme IV in PpcA is not present; its position is marked with an asterisk.

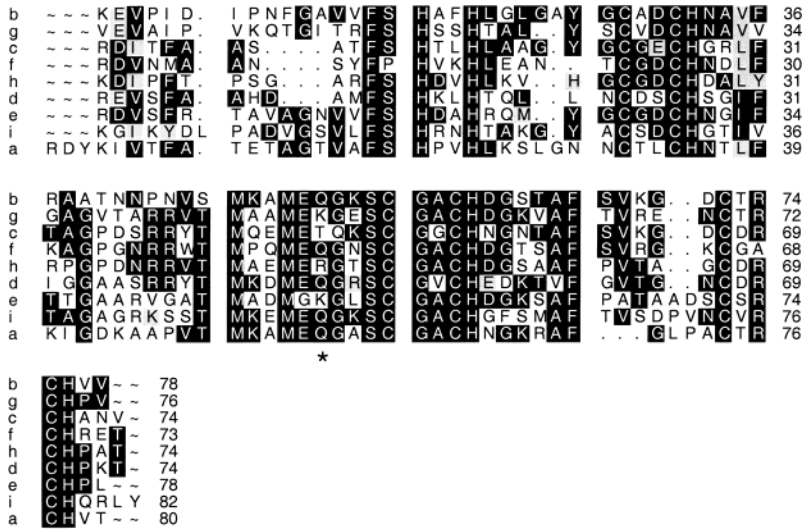

FIGURE 11: Sequence alignment of the nine homologous sections (a−i) from the 27-heme cytochromes (ORF03649) encoded by the *G. sulfurreducens* genome. Each repeat unit has the CXXCH signature for binding three hemes, but the His residue that forms the sixth ligand to heme IV in PpcA is not present; its position is marked with an asterisk.

segments. The calculated pIs of the homologues, based on the protein sequences, vary from 9.0 to 9.5. The length of the homologues varies from 70 to 75 residues. The structural signatures for the β-strand segments near the N-terminus are maintained. These are hydrophobic residues at positions 4 and 6, and Val and Phe residues that are conserved at positions 13 and 15, respectively. ORF00603 (Figure 9) has four extra residues on its C-terminal end. ORF02938 has a deletion after the sixth His ligand to heme IV. ORF01734 has an extra residue at its N-terminus and after the heme I binding site and a deletion after the sixth His ligand to heme IV. The relative positions of the hemes in the sequences of ORF02938 and ORF01734 might be different from those of the cytochrome c_7 structure that we have determined because of the deletions and insertions. Because of sequence differences and possible differences in the relative heme positions, it is expected that the above homologues would have different reduction potentials.

Polymers of Cytochrome c_7

We also identified three polymers of cytochrome c_7-type domains that represent multiples of the three-heme proteins. Two of the proteins (ORF00991 and ORF03300) have four repeats (total of 12 hemes), and one (ORF03649) has nine repeats (total of 27 hemes) of the cytochrome c_7-type domains (Figures 10 and 11). The molecular masses and calculated pIs based on the protein sequences of the two molecules with four cytochrome c_7-type repeats are 41 kDa and 9.3 and 42 kDa and 9.2, respectively. The 41 kDa protein might be attached to the membrane with a possible membrane anchor helix because SignalP (*22*) did not predict a proper leader peptide cleavage site. The protein with nine repeats of the cytochrome c_7-type domains has a molecular mass of 90 kDa and a calculated pI of 8.4.

The repeats within each of the three polycytochrome c_7-type proteins are highly homologous. The repeats are longer than that of the cytochrome c_7 PpcA; they vary from 73 to

82 residues. Insertions and deletions occur in the loop between the two β-strands, although the hydrophobic residues that anchor the β-sheet, equivalent to residues 4, 6, 13, and 15, are maintained, except the equivalent to residue 13 is Ser or Thr in some repeats. The equivalents to residue Phe15 are conserved. Insertions and deletions also occur before and after the heme I binding site, between the heme III and heme IV binding sites, and at the N- and C-termini. The insertions and deletions will probably affect the relative positioning of the hemes in the individual domains.

These proteins appear to represent a new type of cytochrome. Interestingly, in each repeat of the multicytochrome c_7-type proteins, one of the His ligands to heme IV is missing, and the homologous residue in the sequence is mostly Lys or Gln. Either the Lys or Gln residues or one of the two conserved Met residues in the vicinity could be the second axial ligand to heme IV in those domains. Since the chain segments between the heme I and heme III binding sites are longer in the cytochrome c_7-type domains from the polymers than in the cytochrome c_7 molecule, the structure of this segment cannot be predicted with confidence.

The reduction potential of the individual hemes is influenced by the extent of solvent exposure, by the proximity of charged residues, and most significantly by the properties of the residues that form the axial ligands to the iron. It is expected that the reduction potentials of the above molecules will be changed because of the different coordination of one of the hemes (*33*). The loop connecting the two β-strands near the N-terminus of the molecule is close to heme IV; therefore, the nature of the residues present in these loops, as well as the deletions and insertions in these segments, will also influence the reduction potentials of heme IV in the different domains.

The properties of the polycytochrome c_7-type molecules will also be determined by the interactions of the neighboring domains. The packing of the molecules in the crystal of the cytochrome c_7 PpcA protein suggests how the domains in the polycytochrome c_7-type proteins might be arranged (Figure 4). Molecules in the crystal form a one-dimensional array with heme I of one molecule very close to heme IV of the next molecule (12.8 Å). This compares with the intramolecular Fe−Fe distances of 11.2, 12.6, and 20.8 Å. The C-terminus of one molecule is ~9 Å from the N-terminus of the next molecule. The N- and C-termini of neighboring molecules could be connected by the addition of one or two residues or simply by changes in the conformations of these segments. If indeed the polycytochrome c_7-type molecules are linearly arranged, only domains at the two ends might interact with other macromolecules, such as hydrogenases. The linear packing of individual cytochrome c_7 molecules might also occur in the periplasm, and could suggest intermolecular electron transport signifying cooperativity between them.

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