Articles

The Primary Structure of Soluble Cytochrome *c*-551 from the Phototrophic Green Sulfur Bacterium *Chlorobium limicola*, Strain Tassajara, Reveals a Novel *c*-Type Cytochrome†

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Received March 23, 1998; Revised Manuscript Received May 14, 1998

ABSTRACT: *Chlorobium limicola*, strain Tassajara, cytochrome *c*-551 is a soluble dimeric protein containing identical subunits of about 30 kDa. The amino acid sequence was determined by a combination of automated Edman degradation and mass analysis. There are 258 residues with a single heme binding site located at cysteine positions 172 and 175. In addition, there is a disulfide bridge between Cys78 and Cys109, and a free cysteine at position 219 which was found to occur as cysteic acid. The only homologue of soluble cytochrome *c*-551 is the soxA protein which is part of the thiosulfate utilization operon of *Paracoccus denitrificans*. They are 32% identical with three small gaps. This is consistent with the observation that cytochrome *c*-551 is the electron acceptor for a thiosulfate-oxidizing enzyme. On the basis of the redox potential of 135 mV, the sixth heme ligand should be a methionine. Among the seven methionine residues that are present in *c*-551, only one is conserved, two residues ahead of the heme-binding site. The far-UV circular dichroism spectrum indicates 40% α helix and 25% β secondary structure. No other known cytochrome *c* has such a mixed structure; they are either all helical or all β. Thus, *Chlorobium* soluble cytochrome *c*-551 and soxA are likely to be representative of a new class of *c*-type cytochromes.

The green sulfur bacteria are not closely related to any other family of bacteria (*1*, *2*). They contain bacteriochlorophyll *a* like that of the better known purple bacteria, but the photosynthetic reaction center is similar to photosystem I of cyanobacteria, plants, and algae (*3*). The light-harvesting bacteriochlorophylls *c*, *d*, and *e* have unique structures (*4*) and are located in organelles called chlorosome vesicles which are bound to the cytoplasmic side of the cell membrane (*5*). The principal genus is *Chlorobium*.

Soluble electron transfer proteins of *Chlorobium vibrioforme* strains Pond Mud and Larsen and *Chlorobium limicola* strain Tassajara have previously been characterized (*6*−*10*). These proteins include bacterial ferredoxin, rubredoxin, flavocytochrome *c*, cytochrome *c*-555, and cytochrome *c*-551. The same five proteins were found in all three strains and have similar properties. The amino acid sequences of the first four proteins were previously determined in one strain or another (*11*−*14*). The reaction center of strain Tassajara has been cloned and sequenced (*3*). On the basis of the gene sequence, it has also been found that strain Larsen contains a membrane-bound 24 kDa monoheme cytochrome *c*-551 which may function as the immediate electron donor for the photo-oxidized bacteriochlorophyll special pair (*15*). *Chlorobium tepidium* also contains a membrane-bound reaction center single-heme cytochrome *c*-551 of 25 kDa (*16*). Other reports suggest the presence of a membrane-bound 32 kDa cytochrome *c*-553 (*17*) which may contain four hemes such as that of purple bacteria (*18*). The cytochrome bc_1 complex from strain Tassajara has been cloned and sequenced, but was found to contain only the genes for cytochrome *b* and the Rieske iron−sulfur protein; that for cytochrome c_1 is missing (*19*). Thus, it is uncertain how many membrane-bound cytochromes may be present in *Chlorobium*.

Chlorobium cytochrome *c*-555 appears to be universally present in the green sulfur bacteria and is most closely related to algal cytochrome c_6 which mediates electron transfer between the cytochrome b_6f complex and photosystem I. We believe that soluble cytochrome *c*-555 also mediates electron transfer between the bc_1 complex and the bound reaction center cytochrome in green sulfur bacteria. Cytochrome *c*-555 functions as an electron acceptor for flavocytochrome *c*−sulfide dehydrogenase (*20*), and the two proteins form an electrostatically stabilized complex (*21*). Thus, cyto-

† This work was supported by a research grant from the FWO-Vlaanderen (6.0054.97) to J.V.B. and by a grant (GM 21277) from the National Institutes of Health to M.A.C.

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S0006-2960(98)00670-9 CCC: $15.00
Published on Web 07/09/1998

chrome *c*-555 appears to have a central role in mediating electron transfer between the various soluble and membrane-bound cytochromes.

Chlorobium soluble cytochrome *c*-551 is more restricted in its distribution; it has been found only in the three thiosulfate-utilizing strains, PM, L, and T. It is about 60 kDa in size (*7*) and is composed of identical subunits of about 32 kDa (*10*). The redox potential is 135−150 mV, and it is less water soluble than other cytochromes (it precipitates at 30−60% saturation ammonium sulfate). Cytochrome *c*-551 functions as an electron acceptor for a thiosulfate-oxidizing enzyme and mediates reduction of cytochrome *c*-555 (*20*). The interaction of the two proteins is fairly strong. Thus, they form an electrostatically stabilized complex at low ionic strengths (*21*). We have now determined the amino acid sequence of the *C. limicola*, strain Tassajara, cytochrome *c*-551 to complete the characterization of the soluble electron transfer proteins of this bacterium.

MATERIALS AND METHODS

Purification and Derivatization of Cytochrome c-551

Initial steps in the purification of cytochrome *c*-551 were from Meyer et al. (*7*). However, we have found that it is extremely difficult to obtain completely pure protein which is required for an unambiguous sequence determination. Therefore, the final purification of cytochrome *c*-551 was carried out by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and high-performance liquid chromatography (HPLC) on separate samples.

SDS−PAGE was performed in a Hoefer Mighty Small SE250 electophoresis unit on 16.5% T, 6% C polyacrylamide gels (10 cm × 10 cm) according to Schägger and Von Jagow (*22*) except that no stacking gel and nonreducing conditions were used. The protein band containing the cytochrome was excised and stored in 10% aqueous glycerol at −20 °C until it was used further. A sufficient amount of protein (60 μg of total protein per lane) was loaded onto the gel to allow, due to its light brown color, visual identification of the cytochrome, avoiding the use of protein-staining procedures. Gel slices were washed in 1 mL of 50% acetonitrile containing 10 mM HCl. After 1 h, the solvent was removed and the slices were partially dried for a few minutes in a Speedvac concentrator (Savant, Hicksville, NY). Heme was removed from the cytochrome by rehydration of the gel slices for 10 min in 200 μL of 8 M urea/100 mM HCl containing 1 mg of $HgCl_2$. Excess solvent was removed, and the gel slices were incubated for 20 h at 37 °C followed by addition of 1 mL of 50% acetonitrile containing 0.25 M Tris-HCl and 5 mM EDTA. After 1 h on a whirlmixer, the buffer was removed and the slices were dried as described above. Reduction and carboxymethylation were carried out in a 0.2 M Tris-HCl, 8 M guanidine hydrochloride, 5 mM EDTA buffer (pH 8) previously flushed with nitrogen. The reduction was started by adding 200 μL of buffer containing a 5 μg/μL DTT[1] solution to the gel slices. The remaining buffer was removed after 10 min, and the gel slices were incubated at 37 °C for 1 h. After partial drying as described above and cooling for 5 min in an ice bath, 200 μL of iodoacetic acid, dissolved to 5 μg/μL in buffer, was added. Excess buffer was removed, and gel slices were left in the dark for 3 h at 37 °C.

Reverse phase HPLC purification of a separate sample of cytochrome *c*-551 was carried out on a butyl C4 (2 mm × 100 mm, 5 μm particle size, 120 Å pore size) column (YMC, Kyoto, Japan) using the same HPLC system and solvents as described below. The protein was eluted with a linear gradient from 5 to 100% solvent B over the course of 30 min. Removal of heme from the HPLC-purified holocytochrome (6 nmol) was carried out by redissolving the lyophilized protein in 50% aqueous acetonitrile containing 0.1% TFA and 1.5 μg/μL $HgCl_2$. The solution was left on a whirlmixer platform at room temperature for 6 h. Reagents were removed by gel filtration on a Pharmacia column packed with Bio-Gel P6 (Bio-Rad, Eke, Belgium) previously equilibrated in 5% acetonitrile/95% water containing 0.1% TFA. Reduction and carboxymethylation were performed according to Jeno et al. (*23*) except that iodoacetamide was replaced by iodoacetic acid, followed by gel filtration as described above.

Enzymatic and Chemical Cleavage

In Situ Gel Digestion. Gel slices containing carboxymethylated apocytochrome were separately digested with the endoproteinases Lys-C and Glu-C. The slices were equilibrated prior to digestion for 1 h in the incubation buffer containing 50% acetonitrile followed by drying for a few minutes in a Speedvac apparatus. Digestion with Lys-C was performed by adding 100 μL of 50 mM Tris-HCl, 5 mM EDTA buffer (pH 8) containing 10 ng/μL enzyme to five partially dried gel slices. After complete rehydration, 300 μL of buffer was further added and the digest was incubated for 20 h at 37 °C. Glu-C endoproteinase digestion was carried out as described for the Lys-C digestion except that a 200 mM phosphate buffer (pH 7.8) and 2 μg of enzyme were used. The supernatant was removed after the digestion, and gel slices were washed once with 300 μL of 40% aqueous acetonitrile containing 0.1% TFA. The supernatant and extract were pooled and concentrated to 50 μL.

Digestion in Solution. HPLC-purified holocytochrome *c*-551 (2 nmol) was digested with Lys-C (E/S = 1/30, w/w) in 100 μL of 10 mM Tris-HCl buffer containing 0.6 M guanidine-HCl and 1 mM EDTA, for 4 h at 37 °C. Carboxymethylated apoprotein (1.5 nmol) was dissolved in 10 mM Tris-HCl buffer containing 2.7 M urea, 3 mM $CaCl_2$, 1 mM EDTA, 10 mM DTT, and 20 mM methylamine (pH 7.6) and digested with Arg-C (E/S = 1/50, w/w), for 7 h at 37 °C. Asp-N digestion was performed in a 0.2 M phosphate buffer containing 0.8 M urea (pH 7.8) (E/S = 1/60, w/w) for 20 h at room temperature. CNBr cleavage of holocytochrome was carried out in 40% acetonitrile containing 0.1% TFA for 20 h at room temperature using a 5000-fold molar excess of CNBr. Endoproteinase V8 subdigestions of peptides K8, K10, K13, and CN7 were carried out in a 50 mM ammonium bicarbonate buffer (pH 7.8) at room temperature. Peptides K10, K13, and CN7 were incubated for 4 h (E/S = 1/10, w/w), and peptide K8 was digested for 2 h (E/S = 1/45, w/w).

[1] Abbreviations: *c*-551, cytochrome *c*-551; DTT, dithiothreitol; ESMS, electrospray ionization mass spectrometry; LCMS, liquid chromatography−electrospray ionization mass spectrometry; TFA, trifluoroacetic acid.

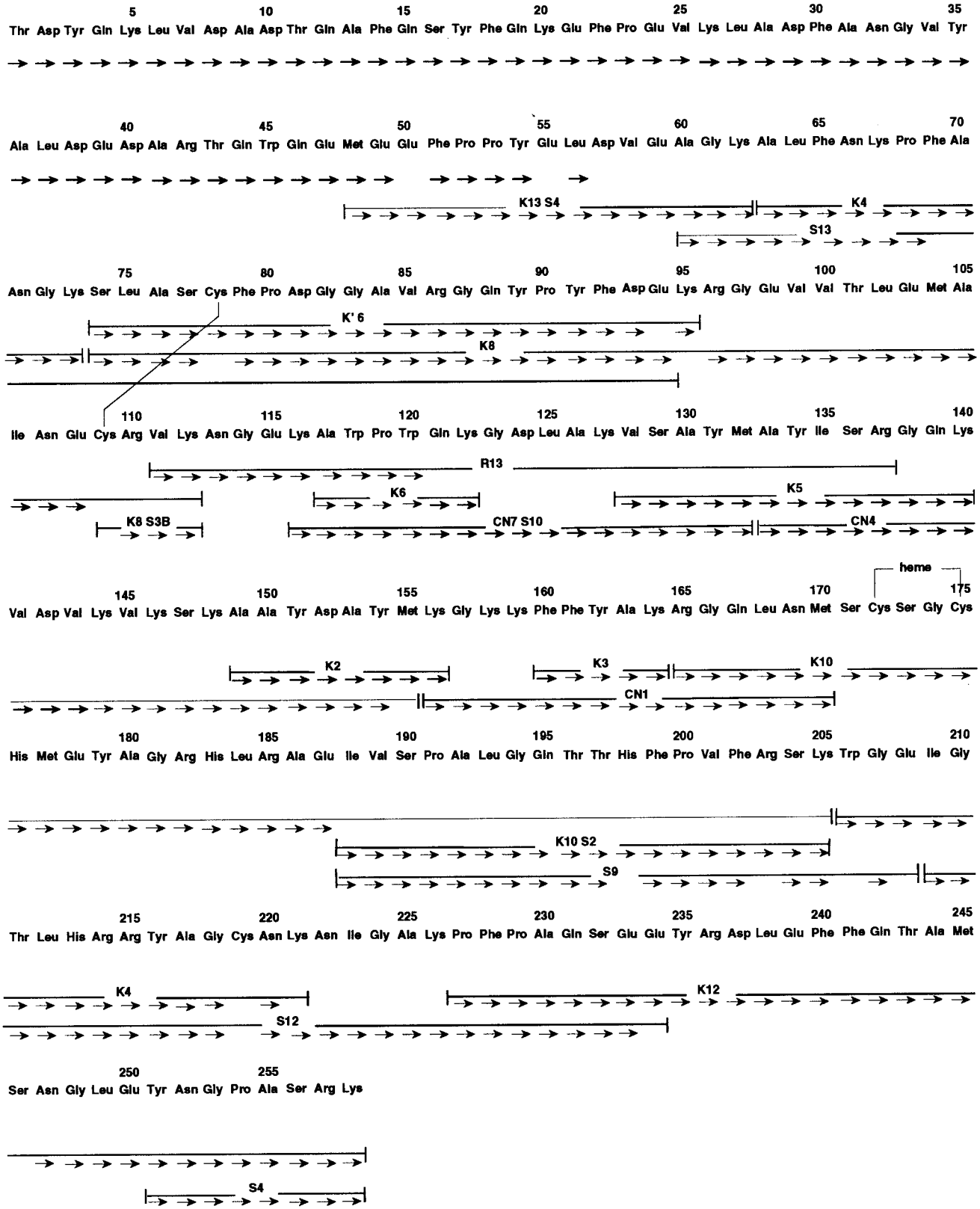

FIGURE 1: Amino acid sequence of *C. limicola c*-551. Peptides obtained after digestion with Lys-C endoproteinase on apoprotein and carboxymethylated apoprotein are represented by K and K′, respectively. Those from Glu-C and Arg-C endoproteinases and chemical cleavages on carboxymethylated apoprotein are indicated by S, R, and CN, respectively.

Amino Acid and Sequence Analysis

Sequence analyses were performed on a 476A protein sequencer connected to an on-line 120A PTH amino acid analyzer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA).

Mass Spectrometric Analysis

Electrospray ionization mass spectrometry of the native protein was carried out on a Bio-Q quadrupole mass spectrometer equipped with a liquid sheath probe and a pneumatically assisted electrospray source (Micromass, Al-

trinham, U.K.). Samples were dissolved in 1% aqueous formic acid and introduced into the ion source at a flow rate of 5 μL/min. The mass spectrometer was calibrated using horse heart myoglobin at a resolution of 0.8 amu (half-bandwidth) for the ion at *m*/*z* 998.

For on-line mass analysis of peptides separated by reversed phase HPLC (LC−MS), the system setup was as described in detail elsewhere (*24*). The separations were performed on a 140A Solvent Delivery System equipped with a 1000S Photodiode Array Detector (Perkin-Elmer, Applied Biosystems Division). Peptides derived from in-gel digestions were separated on an Alltima C18 (2.1 mm × 150 mm, 5 μm particle size, 100 Å pore size) column (Alltech, Laarne, Belgium) using a linear gradient from 0 to 55% solvent B over the course of 60 min. Peptides obtained from chemical and enzymatic cleavage in solution were separated on a butyl C4 (2 mm × 100 mm, 5 μm particle size, 120 Å pore size) column (YMC) using a linear gradient from 0 to 50% solvent B over the course of 40 min. The mass spectrometer was, prior to LC−MS analysis, calibrated with a mixture of poly(ethylene glycol)s (average masses of 400 and 2000 Da) at a resolution of 2 amu (half-bandwidth) at *m*/*z* 995 of the calibration mixture. During LC−MS analysis, spectra were scanned from *m*/*z* 375 to 1800 in 6 s and the total ion current (TIC) was acquired. The TIC was subsequently transformed into the base peak intensity (BPI) chromatogram in which the most intense peak in each scan determined the intensity of the plotted signal.

RESULTS AND DISCUSSION

Sequence Determination. Because the protein was difficult to purify completely using standard liquid chromatography techniques, we started the sequence determination from electrophoretically purified cytochrome *c*-551 obtained by SDS−PAGE. To our knowledge, it is the first time that the primary structure of a *c*-type cytochrome was obtained after heme removal and cysteine modification of apoprotein starting from protein material residing in gel slices. This is also the reason the description of the methodology is reported in detail in the previous section.

The result of the complete amino acid sequence determination, obtained via a combination of chemical sequence analysis and mass spectrometry of the generated peptides, is given in Figure 1. By way of example, the separation of the Lys-C endoproteinase digest with followup by mass spectrometric analysis (LC−MS) is given in Figure 2. Overlapping sequence information was obtained via digests with Glu-C and Arg-C endoproteinases, chemical cleavage with cyanogen bromide, and Glu-C endoproteinase subdigestions of either Lys-C or CNBr peptides. The disulfide bridge between Cys78 and Cys109 was found to exist upon sequence analysis of fraction K8S3 which appeared to consist of two fragments starting at positions 74 and 109. Residue 219 was found to be cysteic acid upon digestion of the carboxymethylated apoprotein with Asp-N endoproteinase. An 18-residue peptide (219−236) indeed was shown to have cysteic acid at its N terminus, a known cleavage site for this proteinase (*25*). The correctness of the C-terminal residue of the protein was proven by sequence and mass analysis of peptides S4 and K12. The mass data for all the peptides generated are given in Tables 1 and 2.

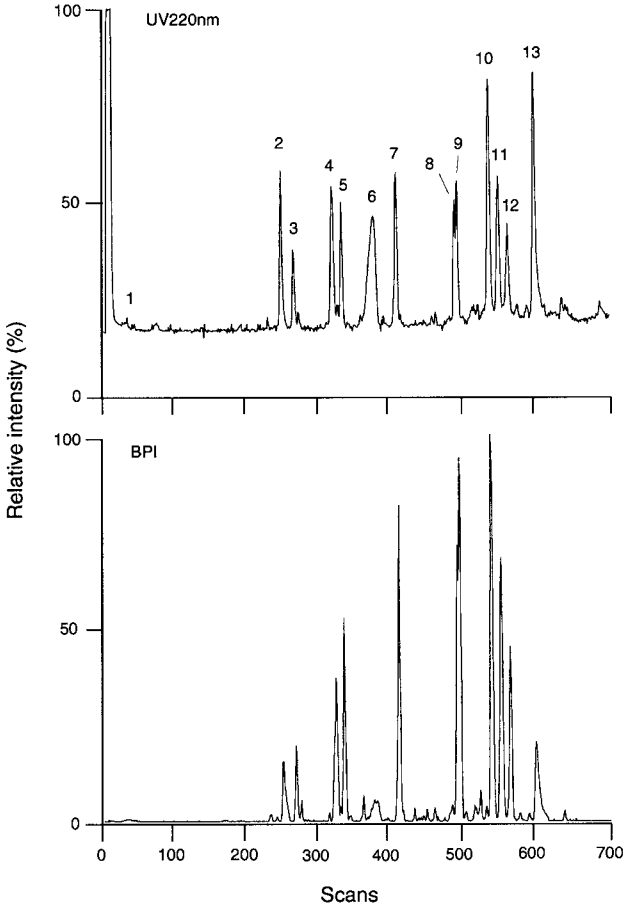

FIGURE 2: Liquid chromatography−mass spectrometry analysis of peptides obtained after digestion with Lys-C endoproteinase. The *Y*-axes represent the relative absorbance measured during chromatography and the base peak intensities measured during mass spectrometry (BPI). The *X*-axis gives the total number of mass spectra scanned during chromatographic analysis. Chromatographic conditions are given in Materials and Methods.

The correctness of the complete sequence is also corroborated by the fact that the measured mass of the protein is 29 903.8 Da (Figure 3), which is consistent with the sum of the masses of the amino acids supplemented with a covalently bound single heme, a cysteic acid residue instead a free cysteine at position 219, and a disulfide bridge (29 906.5 Da). Peptide K9, on the basis of its molecular mass, appears to contain asparagine at position 81. It is very likely, therefore, that this is the residue in the native protein, and that the aspartic acid seen in peptides K8 and K′6 has originated because of deamidation during the purification procedure or storage of the protein. The net charge of the protein, including the two heme propionates and with asparagine at position 81, is +2.

Comparison to Single-Heme Cytochromes c. Although *C. limicola* cytochrome *c*-551 contains only one covalently bound heme group, it is not at all homologous to the well-documented class I monoheme cytochromes. Not only is the total length of the polypeptide chain (258 amino acids) more than twice that of the class I proteins, but the position of the heme binding region Cys-X-X-Cys-His, at positions 172−176, is also very unusual; in class I cytochromes, this region occurs near the N-terminal region of the polypeptide chain (*26*). The occurrence of a disulfide bridge is another remarkable feature of *C. limicola c*-551. In the nearly 100

Table 1: Molecular Masses of Peptides Obtained after Digestion of the Apoprotein (K) or Carboxymethylated Apoprotein (K′) with Lys-C Endoproteinase[a]

peptide			measured mass (Da)	calculated[b] mass (Da)	position
K1		A	653.4	653.7	1−5
		B	502.1	502.6	123−127
		C	459.4	459.5	141−144
K2		A	931.9	932.1	149−156
		B	747.7	747.8	21−26
K3			674.9	674.8	160−164
K4[f]		A	1909.1	1909.1	206−221
		B	1206.2	1206.4	63−73
K5			1474.2	1473.7	128−140
K6			814.8	815.0	117−122
K7			1760.6	1760.4	6−20
K8[c]			4353.4	4352.9	74−112
K9[c,d]			4352.4	4351.9	74−112
K10[e]			5190.5	5190.0	165−205
K11			4164.9	4164.6	222−258
K12			3681.8	3681.0	227−258
K13			4148.8	4149.0	27−62
K′8			2465.7	2465.6	74−95
K′9			2005.5	2005.3	96−112
K′10			4690.5	4690.6	165−205
K8	S1		576.6	576.5	104−108
	S2		902.0	901.8	96−103
	S3[c]	A	2910.3	2910.7	74−95
		B			109−112
K10	S1		1071.2	1072.2	179−187
	S2		1985.2	1985.3	188−205
	S3[e]		2167.8	2168.6	165−178
K13	S1		1032.9	1033.1	40−47
	S2		1397.4	1397.5	27−39
	S3		1497.5	1497.6	48−59
	S4		1753.6	1753.9	48−62
	S5		2412.7	2412.6	27−47

[a] Peptides K8, K10, and K13 were subdigested with Glu-C endoproteinase (K8 S, K10 S, and K13 S). [b] The average mass was calculated using the Biopolymer Analysis Software (Micromass). [c] Peptides are linked to each other by an intact disulfide bridge at positions 78 and 109. [d] The peptide contained an asparagine residue at position 81. [e] Peptide containing Hg^{2+} (200 Da) at heme cysteines after removal of the heme. [f] The mass includes the presence of cysteic acid at position 219 (+48 Da vs free cysteine).

Table 2: Molecular Masses of Peptides Obtained after Digestion of the Carboxymethylated Apoprotein with Arg-C (R) and Glu-C (S) Endoproteinase[a]

peptide	measured M_r (Da)	calculated M_r (Da)	position
S1	576.5	576.6	104−108
S2	651.7	651.7	51−55
S3[b]	694.7	694.7	235−239
S4	893.0	892.0	251−258
S5	1072.0	1072.2	179−187
S6a	1033.5	1033.1	40−47
S7	1115.8	1115.2	116−124
S8	1245.0	1244.4	240−250
S9	2357.5	2357.7	188−208
S10	1625.5	1624.8	25−39
S11[b]	2899.5	2899.1	1−24
S12[b,c]	2906.6	2906.2	209−234
S13[b]	4254.0	4252.7	60−98
R1	425.2	424.5	183−185
R3	1302.1	1302.4	87−96
R4	1960.0	1960.2	166−182
R5	1284.0	1283.5	204−214
R6	3256.4	3255.9	138−165
R8	1621.7	1621.9	97−110
R9	1970.2	1970.3	186−203
R10[b]	2376.8	2376.7	183−203
R11[b]	2906.5	2906.4	87−110
R12	2347.8	2347.5	237−257
R13	3098.2	3097.6	111−137
R14[b]	4877.9	4877.3	215−257
R15[c]	2392.4	2391.6	216−236
R16	4934.9	4934.5	43−86
R17	4849.2	4848.3	1−42
R18[b]	9765.8	9764.8	1−86

[a] Peptides are numbered following the order of their chromatographic elution (figure not shown). [b] Peptide contains noncleaved Arg− or Glu−X peptide bond. [c] The mass includes the presence of cysteic acid at postion 219 (+48 Da vs free cysteine).

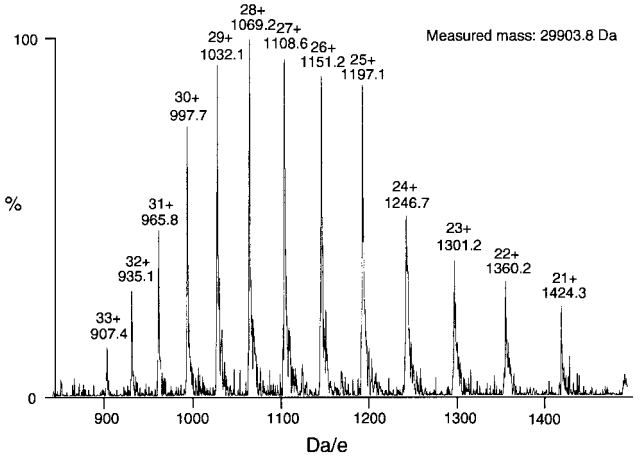

FIGURE 3: Electrospray ionization mass spectrum of native *C. limicola* *c*-551. The number at the top of each peak represents the number of positive charges for the particular *m*/*z* peak.

class I cytochromes sequenced, only *Pseudomonas* cytochrome c_5 contains a disulfide bridge; however, there are only two residues between the cysteines, whereas there are 29 in the *c*-551. Moreover, the disulfide bridges are located on different sides of the heme binding sequence (*27*). We recently solved the primary structure of the oxygen binding heme protein (SHP) from *Rhodobacter sphaeroides* and found that this 112-residue protein also contains a disulfide bridge downstream of the single Cys-X-X-Cys-His heme binding site similar to that of cytochrome c_5, but with seven residues between the disulfide cysteines (*28*). Clearly, this protein is not related to the *Chlorobium* *c*-551 either.

The *Chlorobium* soluble cytochrome *c*-551 has only one homologue, the incomplete soxA gene product, which is part of the thiosulfate utilization operon of *Paracoccus denitrificans* (*29*, *30*). The 165 C-terminal residues of soxA are 32% identical to those of cytochrome *c*-551, and there are only two-residue and two single-residue gaps as shown in Figure 4. It is significant that these two proteins are related because *Chlorobium* cytochrome *c*-551 was reported to be the electron acceptor for a thiosulfate-oxidizing enzyme (*20*).

The other five genes identified in the thiosulfate utilization operon of *Paracoccus* are soxB, a presumed 5′-nucleotidase, soxC, a sulfite oxidase, soxD, a diheme cytochrome c_4c_2 hybrid protein, soxE, a diheme cytochrome c_2c_4 hybrid protein (in which the order of the two domains is reversed), and soxF, an incomplete homologue of flavocytochrome *c*−sulfide dehydrogenase (FCSD) (*29*, *30*). SoxE is probably the heme subunit and soxF the flavoprotein subunit of the sulfide dehydrogenase. FCSD has either a diheme or a monoheme cytochrome c_4 subunit in the photosynthetic sulfur bacteria in which it is usually found (*14*, *31*, *32*). The *Paracoccus* FCSD homologue is unusual in two respects; the heme subunit appears to be a fusion of a cytochrome c_2

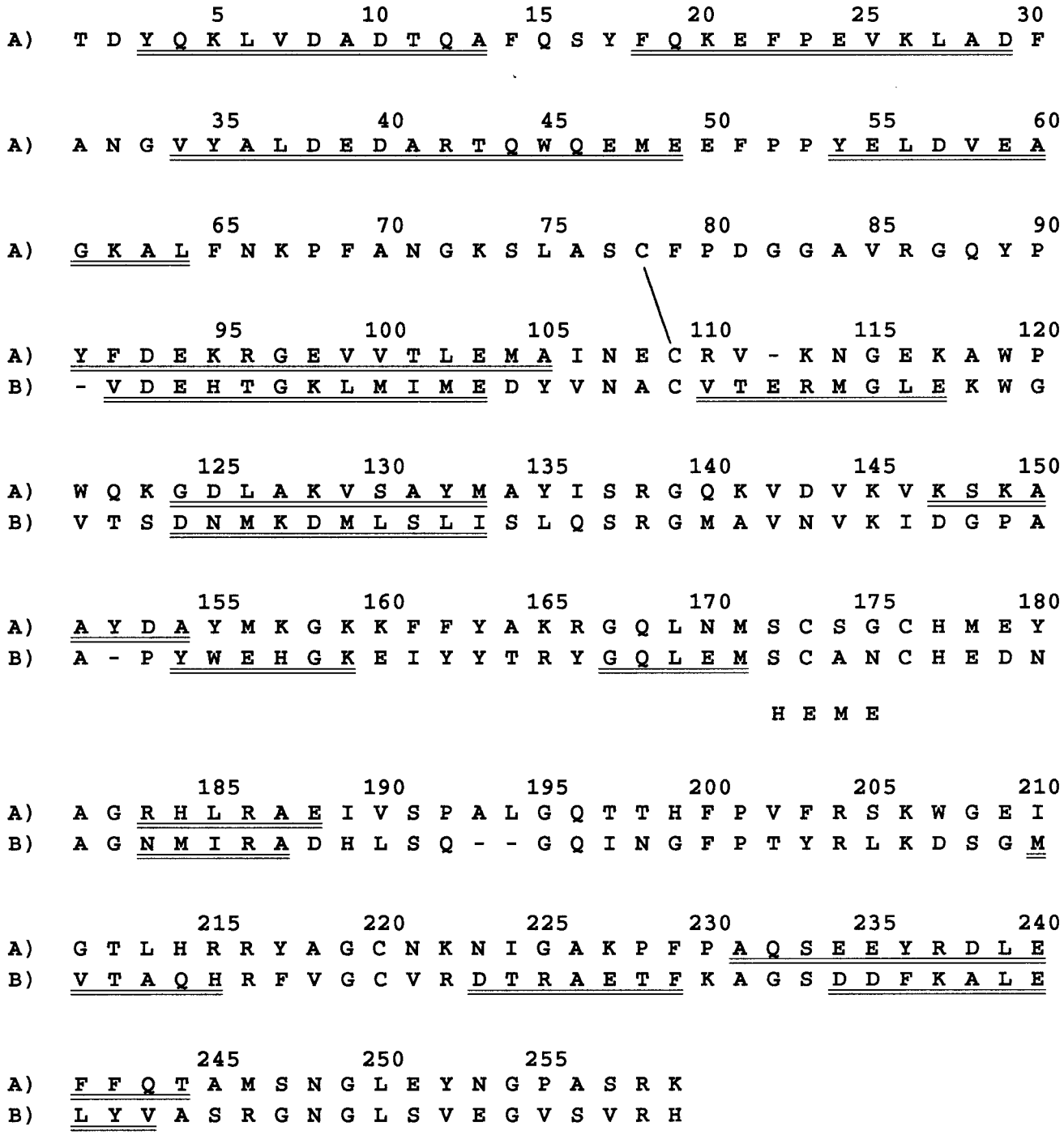

FIGURE 4: Alignment of the amino acid sequence of *C. limicola c*-551 (A) with that of the incomplete *P. denitrificans* soxA gene product (B) (*28*). α helices determined by the Chou−Fasman prediction method are indicated by double underlines.

and a monoheme cytochrome c_4, and there is a hairpin separating the cytochrome and flavoprotein genes, suggesting that they might not be synthesized in equal abundance. The soxC gene is also unusual in that sulfite oxidase normally has a cytochrome b_5 domain fused to the N terminus of the molybdopterin domain (*33*), but the b_5 is lacking in this instance. Perhaps one of the hybrid *c*-type cytochromes such as soxD serves as an electron acceptor for this enzyme. It may be significant that both cytochrome *c*-551 (soxA) and FCSD (soxEF) are part of the thiosulfate utilization operon in *Paracoccus* because the presence of these proteins has been loosely correlated with the use of thiosulfate in *Chlorobium*. The actual roles of these proteins remain to be determined.

Chlorobium also contains a membrane-bound reaction center cytochrome *c*-551 which contains 206 residues and a single heme in the second half (*15*, *16*). The first 96 residues of the membrane-bound cytochrome *c*-551 are markedly hydrophobic and, unlike the soluble cytochrome *c*-551, provide an obvious site of attachment to the membrane.

In some preparations of the reaction center, a 32 kDa heme protein rather than the cloned 18−23 kDa form was observed (*17*). The size alone suggests a relationship with the soluble cytochrome *c*-551. However, the 32 kDa protein is thought to have multiple hemes (*18*); the wavelength maximum is at 553 nm, and the unreliability of SDS−PAGE masses for membrane proteins provides evidence that does not support a relationship with soluble cytochrome *c*-551. The lower

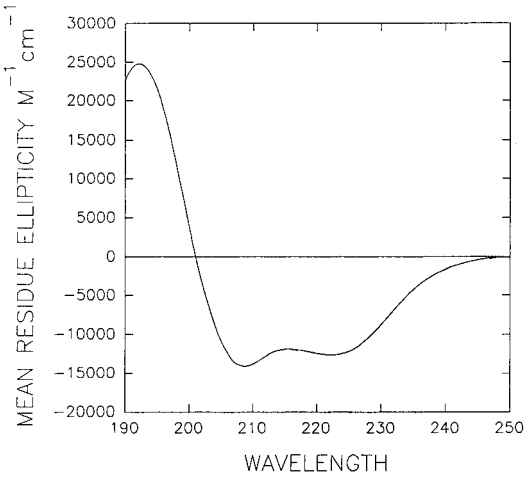

FIGURE 5: Far-UV circular dichroism spectrum of *C. limicola c*-551.

than normal solubility of the "soluble" cytochrome *c*-551 indicates that at least a portion should be loosely membrane-bound, thus complicating this issue. However, more work is necessary to establish the identity of the 32 kDa membrane cytochrome.

There is virtually no similarity between the *Chlorobium* soluble cytochrome *c*-551 and the other cytochromes from green bacteria, cytochrome *c*-555 and flavocytochrome *c*−sulfide dehydrogenase (*13*, *14*). Both cytochrome *c*-555 and the heme subunit of FCSD are small class I *c*-type cytochromes approximately 10 kDa in size. Cytochrome *c*-555 is related to *Pseudomonas* cytochrome c_5 (*34*), to *Ectothiorhodospira halophila* cytochrome *c*-551 (*35*), and to cyanobacterial and algal cytochromes c_6 (*36*). The FCSD heme subunit is most similar to that from the purple sulfur bacterium *Chromatium vinosum* (*31*, *32*) and to the cytochromes c_4 (*37*, *38*). The class I cytochromes *c* are easily recognized by the presence of consensus residues (*39*) that are present in cytochrome *c*-555 and FCSD, but which are all lacking in the soluble cytochrome *c*-551.

The redox potential of soluble cytochrome *c*-551 is 135−150 mV, suggesting that the sixth ligand is a methionine. There are seven methionines in the protein which could serve as a heme ligand, but only one is conserved in the 165-residue overlap of the two homologues . Thus, a possible sixth heme ligand may be the Met residue which is two positions ahead of the heme binding site.

We measured the secondary structure of the protein by circular dichroism as shown in Figure 5 and found 40% helix and 25% β structure. The helical content is like that of class I cytochromes, but there is normally no β structure in such proteins except in cytochrome *f* (*40*). Chou−Fasman secondary structure prediction (*41*) indicates that approximately 106 residues should be in a helical conformation, consistent with the circular dichroism measurement. A similar prediction analysis on the *Paracoccus* soxA gene product reveals that several sequence regions have the same secondary structure (see Figure 4).

Chlorobium soluble cytochrome *c*-551 is not found in many green sulfur bacteria and is notably absent in the soluble fraction of *Chlorobium limicola*, strain Gilroy Hot Springs (*42*), *Chlorobium vibrioforme*, strain Moss Landing (*43*), and *Prosthecochloris aestuarii*, strain 2K (*44*). Thus, if it is present, it must be membrane-bound or not expressed under the same conditions as in the other species. None of these three strains uses thiosulfate. There seems therefore to be a correlation between the utilization of thiosulfate and the presence of cytochrome *c*-551. This is understandable since it was shown that the cytochrome is an electron acceptor for the thiosulfate-oxidizing enzyme (*20*).

It was recently found that thiosulfate utilization genes reside on a 14 kb plasmid in strain Tassajara and can be transferred to strain Gilroy Hot Springs (*45*). The same plasmid was found in a newly isolated strain related to Tassajara, but a much larger plasmid (650 kb) was found in an unrelated thiosulfate-utilizing strain (*46*). It is likely that more than one species of green sulfur bacteria naturally may be capable of using thiosulfate. The fact that soluble cytochrome *c*-551 has been found to have a role in thiosulfate utilization and that it has only been found in the thiosulfate-utilizing strains Tassajara, Pond Mud, and Larsen suggests that it too may reside on a plasmid.

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BI9806706