Cytochrome Components of Nitrate- and Sulfate-Respiring Desulfovibrio desulfuricans ATCC 27774

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Three multiheme c-type cytochromes—the tetraheme cytochrome c3 (molecular weight [MW] 13,500), a dodecaheme cytochrome c (MW 40,800), and a “split-Soret” cytochrome c (MW 51,540), which is a dimer with 2 hemes per subunit (MW 26,300)—were isolated from the soluble fraction of Desulfovibrio desulfuricans (ATCC 27774) grown under nitrate- or sulfate-respiring conditions. Two of them, the dodecaheme and the split-Soret cytochromes, showed no similarities to any of the c-type cytochromes isolated from other sulfate-reducing bacteria, while the tetraheme cytochrome c3 appeared to be analogous to the cytochrome c3 found in other sulfate-reducing bacteria. For all three multiheme c-type cytochromes isolated, the homologous proteins from nitrate- and sulfate-grown cells were indistinguishable in amino acid composition, physical properties, and spectroscopic characteristics. It therefore appears that the same c-type cytochrome components are present when D. desulfuricans ATCC 27774 cells are grown under either condition. This is in contrast to the considerable difference found in Pseudomonas perfectomarina (Liu et al., J. Bacteriol. 154:278-286, 1983), a marine denitrifier, when the cells are grown on nitrate or oxygen as the terminal electron acceptor. In addition, two spectroscopy methods capable of revealing minute structural variations in proteins provided identical information about the tetraheme cytochrome c3 from nitrate-grown and sulfate-grown cells.

Sulfate-reducing bacteria belonging to the genus Desulfovibrio are obligate anaerobes capable of performing oxidative phosphorylation coupled to the electron transport process, with sulfate and its reduction product(s) as terminal electron acceptors. A unique member of this group of organisms, Desulfovibrio desulfuricans ATCC 27774, had been shown to be capable of utilizing nitrate and its reduction product, nitrite, as terminal electron acceptors as well (24; M. P. Bryant, personal communication). Previous studies indicated that the enzymes involved in dissimilatory sulfate reduction (ATP sulfurylase, adenosine 5’-phosphosulfate reductase, trithionate reductase, thiosulfate reductase, and bisulfite reductase) are present at approximately the same level whether the cells are grown on sulfate or nitrate, while the enzymes involved in dissimilatory nitrate reduction (nitrate and nitrite reductases) are inducible, as their activities are considerably higher in nitrate-grown than in sulfate-grown cells (24). It was noted that cell mass yield was three to four times higher for cells grown on nitrate than for cells grown on sulfate. In the dissimilatory nitrate reduction performed by D. desulfuricans ATCC 27774, nitrate is reduced through nitrite to ammonia (24). This pathway is distinct from the “denitrification” process carried out by true denitrifiers, such as Pseudomonas perfectomarina, in which the final product formed is dinitrogen (N2). In our previous studies comparing the cytochrome components of the latter organism grown under denitrifying and oxygen-respiring conditions, it was observed that two cytochromes, cytochrome cd1 and the diheme cytochrome c-552, were induced only in the presence of nitrate, while a low α/β c-type cytochrome was found only in aerobically grown cells (20). The monoheme cytochrome c-551, on the other hand, was found to be present at the same level in cells grown under both conditions. Conclusions about the involvement of these cytochromes in the electron transport chains in denitrification or aerobic respiration or both were drawn from these findings. In view of this earlier investigation, we thought that a similar study on D. desulfuricans ATCC 27774 grown under nitrate-respiring and sulfate-respiring conditions might provide interesting insight into the cytochrome components specific to the two electron transport systems.

As already noted from the large number of c-type cytochromes isolated from various sulfate-reducing bacteria, the current nomenclature for these proteins appears to be inadequate to provide unambiguous classifications. It has recently been proposed that, since large variations in the polypeptide chain lengths occur even among homologous cytochromes, such as cytochrome c3, the heme content should be considered the major criterion for classification (LeGall and Fauque, in A. J. B. Zenharder, ed., Environmental Microbiology of Anaerobes, in press). Thus, former cytochrome c3 (molecular weight [MW] 13,000) becomes tetraheme cytochrome c3, former cytochrome c3 (MW 26,000) becomes octaheme cytochrome c3, and so on. We shall use this new classification method in describing the three multiheme c-type cytochromes studied in our present work.

MATERIALS AND METHODS

Chemicals and biochemicals. Trizma base, sodium lauryl sulfate, Coomassie brilliant blue R, streptomycin sulfate, DNase I, and horse heart cytochrome c were purchased from Sigma Chemical Company. Acrylamide, N,N’-methylene-bisacrylamide, and N,N,N’,N’-tetramethylethylenediamine were products of Eastman Kodak Company. Sodium dithionite and bromphenol blue were from Fisher Scientific Company. Protein MW markers used in sodium dodecyl sulfate (SDS) gel electrophoresis were obtained from Bio-Rad Laboratories. All other reagents were of the highest purity commercially available.

Chromatographic materials. Ultrogel AcA-44 was a product of LKB Instruments, Inc. DEAE-Bio-Gel A, CM-Bio-

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Gel A, and Bio-Gel HTP were purchased from Bio-Rad Laboratories. These materials were treated before use as directed by the manufacturer.

**Organism.** *Desulfovibrio desulfuricans* ATCC 27774, originally isolated by M. P. Bryant of the University of Illinois, was used in the present study. The compositions of the media used for growing nitrate-respiring and sulfate-respiring cells have been described previously (21, 24).

**MW determination.** The MW of the native protein was determined by the sedimentation equilibrium method (26) with a Beckman Spincso model E ultracentrifuge equipped with Schlieren optics and an automatic photoelectric scanning system. Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (27).

**Heme content.** The alkaline pyridine hemochromogen method was used for the estimation of heme content. The purified cytochrome was placed in a solution containing 0.075 M NaOH and 25% pyridine. After reduction of the cytochrome with sodium dithionite, the \( A_{570} \) was measured. Heme content was then calculated based on an extinction coefficient of 29.1 \( \mu \)M\(^{-1}\) cm\(^{-1}\) previously reported for heme c (8).

**Amino acid analysis.** Amino acid analyses were performed with a Beckman model 120C automatic amino acid analyzer on hydrolyzed protein samples. Salt-free protein samples were hydrolyzed in evacuated, sealed tubes in 6 N HCl for 24, 48, and 72 h at 110°C. Cystine and cysteine were determined as cysteic acid by the oxidation of protein samples with performic acid prior to acid hydrolysis (13).

**Spectroscopic measurements.** A Varian DMS 90 recording spectrophotometer was used for the absorbance measurement and the routine recording of UV-visible absorption spectra. High-resolution proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker CXP 300 spectrometer (300 MHz) equipped with an Aspect 2000 computer. All chemical shifts are quoted in parts per million (ppm) from the internal standard sodium 3-trimethylsilyl-\( [2,2,3,3-^2] \) propionate; positive values refer to low field shifts. For NMR measurements, the cytochrome samples were desalted and lyophilized three times from \( ^2 \)H\(_2\)O and dissolved to the required concentration (1 to 2 mM). Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker ER-200 apparatus equipped with an Oxford Instruments continuous-flow helium cryostat interfaced with a Nicolet 1180 computer.

**Electrophoresis.** Analytical polyacrylamide disc gel electrophoresis was performed by the method of Brewer and Ashworth (2). SDS-PAGE was based on the procedure of Weber and Osborn (27).

**Protein determination.** The modified Biuret method developed by Zamenhof and Chargaff (31) was used for protein determination, with horse heart cytochrome c as the standard.

**Preparation and fractionation of cell extracts.** Unless otherwise stated, buffers used were pH 7.6 and all operations were carried out at 4°C. Nitrate-grown or sulfate-grown cells (300 g, wet weight) were mixed with 10 mM Tris hydrochloride (Tris-HCl) buffer to give a 1:1 (wt/vol) suspension and broken by passing the slurry three times through a Manton-Gaulin homogenizer at 9,000 lb/in\(^2\). A few crystals of DNase were added to lessen the viscosity of the extract, and after 10 min, the preparation was centrifuged at 13,200 \( \times \) g in a Sorvall RC-5B refrigerated centrifuge for 30 min. The pellet was discarded, and the supernatant was treated with neutralized streptomycin sulfate (0.5 mg/mg of protein). After being stirred for 15 min, the preparation was centrifuged again at 13,200 \( \times \) g for 30 min. The pellet was discarded, and the supernatant obtained was termed the crude extract. The crude extract was subjected to further centrifugation at 140,000 \( \times \) g for 2 h at 4°C. Clear supernatant containing soluble proteins was then used in the purification work described below.

As will become clear in the following sections, identical cytochromes were isolated from supernatant fractions prepared from nitrate-grown and sulfate-grown cells under nearly identical purification conditions. Therefore, a common procedure will be described for both cases. The supernatant fraction prepared was first dialyzed against 10 mM Tris-HCl for 24 h with three changes of the dialysis buffer. Dialyzed preparation was then loaded onto a DEAE-Bio-Gel A column (6 by 40 cm) equilibrated with 10 mM Tris-HCl. The same buffer was used to remove the nonadsorbed proteins, which were found to contain the tetraheme cytochrome \( c_3 \). A continuous Tris-HCl gradient (0.01 to 0.4 M) in a total volume of 4 liters was thereafter applied to elute the remaining proteins. Two cytochrome bands were resolved during the elution. The first one, eluted at 0.07 M Tris-HCl, was found to contain the dodecaheme cytochrome \( c_7 \), and the second one, eluted at 0.12 M Tris-HCl, was found to contain the so-called split-Soret cytochrome \( c \). These two fractions, along with the tetraheme cytochrome \( c_3 \) fraction described previously, were subjected to further purification as shown in Fig. 1 through 3.

**RESULTS**

Since the homologous cytochromes isolated from nitrate-grown and sulfate-grown cells exhibited nearly identical...
VOL. 170, shown is from the DEAE-Bio-Gel

Yields:

FIG. 2. Purification scheme for dodecaheme cytochrome c. The scheme shown is from the DEAE-Bio-Gel A step onwards.

FIG. 3. Purification scheme for split-Soret cytochrome c. The scheme shown is from the DEAE-Bio-Gel A step onwards.

FIG. 4. Absorption spectra of (A) tetraheme cytochrome c, (B) dodecaheme cytochrome c, and (C) split-Soret cytochrome c from D. desulfuricans ATCC 27774. Symbols: —— oxidized; ····· dithionite reduced. The protein concentration of the three cytochromes was (A) 0.0145 mg/ml, (B) 0.013 mg/ml, and (C) 0.095 mg/ml. The buffer used was 0.1 M potassium phosphate buffer, pH 7.6. For reduction of the cytochrome, a few crystals of sodium dithionite were added.

physical and chemical properties, no discrimination was made between cytochromes isolated from the two kinds of cells.

Absorption spectroscopy. All three proteins purified displayed typical c-type cytochrome UV-visible absorption spectra (Fig. 4). In the oxidized state, the absorption spectra of purified cytochromes revealed maxima at ca. 409 and 530 nm and a shoulder at approximately 350 nm. Of the three, the spectra of the split-Soret cytochrome c revealed a maximum at 280 nm that was absent from those of the tetraheme cytochrome c, and the dodecaheme cytochrome c, possibly due to their low aromatic amino acid contents (see Table 3). When the cytochromes were reduced with sodium dithionite, new absorption maxima appeared at ca. 552, 523, and 420 nm, which represent the \( \alpha \), \( \beta \), and Soret (\( \gamma \)) bands, respectively. For the split-Soret cytochrome c, a
shoulder at 415 nm for the Soret band was also observed, which led us to use the name split-Soret for this cytochrome. Each of these three cytochromes showed a characteristic absorption peak ratio (R), which is defined as the ratio of the absorbance at the α-band position in the reduced state to the A$_{280}$ in the oxidized state. The R values and the absorption maxima of the three cytochromes purified are given in Table 1.

**MW, subunit structure, and heme content.** The MW of the tetraheme cytochrome $c_3$ was determined by both SDS-PAGE and the sedimentation equilibrium method to be ca. 13,500. It is therefore clear that the cytochrome exists in the monomeric form. Heme content was determined by the pyridine hemochromogen method to be 4.36 heme $c$ groups per cytochrome molecule. Therefore, the tetraheme cytochrome $c_3$ from *D. desulfuricans* ATCC 27774 appears to be similar to the well-characterized tetraheme cytochrome $c_3$ found in other sulfate-reducing bacteria.

A minimal MW of 40,800 was obtained for dodecaheme cytochrome $c$ by SDS-PAGE, with samples treated extensively with 5% SDS and 5% 2-mercaptoethanol. Removal of the covalently bonded heme $c$ groups by mercuric chloride, which was previously shown by others to decrease the MW of the octaheme cytochrome $c_3$ to half of the original MW (9), prior to the treatment for SDS-PAGE did not have any effect on the minimal MW determined. Heme content of the dodecaheme cytochrome $c$ was estimated by the pyridine hemochromogen method to be 12.15 heme $c$ groups per 40,800 MW. The cytochrome therefore was called the dodecaheme cytochrome $c$ (MW 40,800).

The MW of the native split-Soret cytochrome $c$ was determined by the sedimentation equilibrium method to be 51,540; minimal MW determined by SDS-PAGE was 26,300, and only one protein band was observed. These results indicate that the split-Soret cytochrome $c$ is a dimer consisting of two equal-sized (if not identical) subunits. The number of heme $c$ groups per molecule was calculated from the alkaline pyridine hemochromogen data to be 3.61 heme $c$ groups per 51,540 MW. It was therefore deduced that the split-Soret cytochrome $c$ is a tetraheme protein containing 2 heme $c$ groups per subunit. Our unpublished data on the determination of heme redox potentials also indicate the presence of two distinct heme species.

**Table 1. Absorption maxima and purity indexes (R) of the three multiheme c-type cytochromes purified from *D. desulfuricans* ATCC 27774**

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Oxidized state</th>
<th>Reduced state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraheme cytochrome $c_3$</td>
<td>532, 409.2, 350, —*</td>
<td>551.7, 523.2, 418.5, —</td>
</tr>
<tr>
<td>Dodecaheme cytochrome $c$</td>
<td>530, 409.5, 351, —</td>
<td>551.7, 523.0, 418.5</td>
</tr>
<tr>
<td>Split-Soret cytochrome $c$</td>
<td>533, 407.7, 350, 280</td>
<td>552.2, 523.0, 424.0, 415b</td>
</tr>
</tbody>
</table>

* — Absence of absorption maximum.
b Shoulder at the Soret absorption band.

Data obtained from the MW determinations and heme content analyses for the three cytochromes purified are given in Table 2.

**Amino acid composition.** Nearly identical data were obtained for the homologous cytochromes isolated from nitrate-grown and sulfate-grown cells. These results are shown in Table 3, together with data for the hexaheme nitrite reductase present in the same organism (21). It is clear that these four multiheme c-type cytochromes had distinct amino acid compositions, which suggests that they are products of different genes and are probably not related in terms of their biosynthesis. It was noted that even though the performic acid oxidation method for cysteine determination may lead to underestimation, the number of cysteine residues obtained was high enough to account for 4, 12, and 4 covalently bonded heme $c$ groups in the case of the tetraheme cytochrome $c_3$, the dodecaheme cytochrome $c$, and the split-Soret cytochrome $c$, respectively. Although the number of histidine residues was enough for the histidine-heme-histidine ligand arrangement for the four heme $c$ groups in the tetraheme cytochrome $c_3$, insufficient histidine residues were observed to account for the same type of ligand arrangement for all heme $c$ groups in either the dodecaheme cytochrome $c$ or the split-Soret cytochrome $c$. The exact ligand arrangements for the heme $c$ groups in the latter two cytochromes will be investigated further. The considerably higher aromatic amino acid content of the split-Soret cytochrome $c$ was also observed.

**Table 2. Physical properties of three c-type cytochromes purified from *D. desulfuricans* ATCC 27774**

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>MW</th>
<th>Heme content</th>
<th>Subunit structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraheme cytochrome $c_3$</td>
<td>13,500</td>
<td>4.36</td>
<td>Monomer</td>
</tr>
<tr>
<td>Dodecaheme cytochrome $c$</td>
<td>40,800</td>
<td>12.15</td>
<td>Monomer</td>
</tr>
<tr>
<td>Split-Soret cytochrome $c$</td>
<td>51,540</td>
<td>3.61</td>
<td>Dimer</td>
</tr>
</tbody>
</table>

* Two equal-sized subunits of MW 26,360.

**Table 3. Amino acid composition of the four multiheme c-type cytochromes from *D. desulfuricans* ATCC 27774**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Tetraheme cytochrome $c_3$</th>
<th>Dodecaheme cytochrome $c$</th>
<th>Split-Soret cytochrome $c$ (per subunit)</th>
<th>Hexaheme nitrite reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>16</td>
<td>26</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>His</td>
<td>8</td>
<td>20</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Arg</td>
<td>0</td>
<td>13</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>Trp</td>
<td>N.D.*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>5</td>
</tr>
<tr>
<td>Asp + Asn</td>
<td>5</td>
<td>33</td>
<td>22</td>
<td>74</td>
</tr>
<tr>
<td>Thr</td>
<td>5</td>
<td>25</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>Ser</td>
<td>7</td>
<td>14</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>9</td>
<td>30</td>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>Pro</td>
<td>8</td>
<td>30</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>Gly</td>
<td>7</td>
<td>18</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td>Ala</td>
<td>9</td>
<td>36</td>
<td>33</td>
<td>61</td>
</tr>
<tr>
<td>Cys</td>
<td>8</td>
<td>26</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Val</td>
<td>10</td>
<td>15</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>Met</td>
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<td>11</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Ile</td>
<td>0</td>
<td>12</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Leu</td>
<td>7</td>
<td>21</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Tyr</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>Phe</td>
<td>1</td>
<td>5</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

* Data taken from Liu and Peck (21).

b N.D., Not determined.

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Chrome c than of the other two explains the presence of the 280-nm absorption band in the former but not in the latter two.

EPR and NMR spectroscopic data on tetraheme cytochrome c₃. The tetraheme cytochrome c₃ purified in our study revealed EPR spectroscopic features very similar to those obtained for the tetraheme cytochrome c₃ from other sulfate-reducing bacteria. As shown in Fig. 5, the spectra recorded at 10 K were quite complex and showed several superimposed signals in the $g_{\text{max}}$ region, with a broad component at 2.30 and prominent but poorly resolved features at 2.97 and 2.89. A derivative peak tentatively assigned to a $g_{\text{max}}$ of 2.31 and two broad components at very high fields ($g_{\text{min}}$ 1.56 and 1.39) were also observable. These sets of resonances were present for the spectra of tetraheme cytochromes isolated from both sulfate- and nitrate-grown cells. These results, in conjunction with EPR spectroscopic features previously reported for other tetraheme cytochromes (18), clearly indicate that the four heme c groups are not quite equivalent. The anisotropic $g$ values are characteristic of rhombic distorted heme c moieties with histidinyl axial ligands.

NMR spectra of the tetraheme cytochrome c₃ isolated from the nitrate-grown and sulfate-grown cells are shown in Fig. 6. The spectra are typical of low-spin ferric multiheme cytochromes which reveal several well-resolved three-proton intensity resonances at very low field. These resonances belong to the methyl groups associated with the heme c moieties and are shifted to low field by contact and pseudocontact interactions due to the paramagnetic low-spin ferric iron present at the heme cores. From 12 to 14 of the 16 heme methyl group resonances were readily detected. The remaining heme methyl resonances were in the crowded region below 10 ppm and were not readily assigned. Part of this region, which also contains the resonances due to the aromatic amino acid side chains, is shown in Fig. 6B in an expanded scale. The NMR spectroscopic features were nearly identical in the aromatic region, and the slight differences which were apparent in the very low field region were probably due to differences in solution conditions in the two samples. It is known that resonances shifted through paramagnetic interactions are extremely sensitive to modifications on the heme electron density distribution induced, for example, by a small pH change (28). A comparison of the NMR characteristics of this class of homologous proteins has previously been made (23). An obvious common feature of the NMR spectra was the large down-field chemical shifts experienced by the heme methyl group. However, striking differences are found in heme methyl resonance distribution among the cytochromes c₃ isolated from different Desulfovibrio species. These differences result from the relative orientation or the electronic properties of the four hemes. However, it is noteworthy that even when X-ray structures are known [e.g., D. vulgaris (Miyazaki) and D. desulfuricans (Norway 4)], although the geometry and orientation of the hemes are highly conserved (10, 12), the NMR data are quite different. Therefore, the NMR analysis of the two cytochrome c₃ preparations from D. desulfuricans ATCC 27774 cells grown in different metabolic conditions confirmed that this multiheme protein was conserved.

DISCUSSION

Electron transfer proteins involved in the energy-yielding metabolism of the Desulfovibrio species of sulfate-reducing bacteria have been well characterized (18). Included in this array of proteins are a variety of monoheme and multiheme c-type cytochromes.

Monoheme cytochromes (methionine-heme-histidine). A low-MW (6,000 to 9,000) cytochrome c-553 with a relatively
high redox potential (ca. −0.05 V) has been isolated from different strains of Desulfovibrio vulgaris (4, 30). The axial (fifth and sixth) ligands of the heme iron in these cytochromes have been shown to be histidine and methionine. With formate used as the source of electrons, cytochrome c-553 was shown to be reduced through the action of formate dehydrogenase from the same organism (30). From D. desulfuricans strain Norway 4, a cytochrome c-553 (c-550) with an MW of 9,200 has been isolated (6); its physiological function, however, is currently unknown.

Tetraheme cytochrome c₃ (histidine-heme-histidine). The tetraheme cytochrome c₃ has been isolated from a large number of the Desulfovibrio species and was shown in all cases to contain four heme c groups with an MW of ca. 13,000 (1, 16, 25). This type of cytochrome has bihistidinyl coordination to the heme iron. Redox potentials of the four hemes are quite low (ranging from −350 to −400 mV) and have been shown to differ between species by 40 to 80 mV (29). Tetraheme cytochrome c₃ appears to be extensively involved in the hydrogen metabolism of sulfate-reducing bacteria (18). It has been shown to be capable of mediating both the transfer of electrons deriving from the phosphoroclastic reaction to hydrogenase for the production of hydrogen, and the transfer of electrons from hydrogen/hydrogenase to bisulfite reductase for the terminal reduction of sulfate to sulfide (18). In some strains, the cytochrome c₃ appears to have an additional sulfur reductase activity (5, 7).

Octaheme cytochrome c₅ (histidine-heme-histidine). This cytochrome has been shown to have an MW of ca. 26,000 and contain eight heme c groups per molecule (18). Its distinct amino acid composition indicates that it is not merely a dimeric form of the tetraheme cytochrome c₃ (6). In D. gigas, it was shown to be involved in the coupling between hydrogenase and thiosulfate reductase (11). The octaheme cytochrome c₅ (MW 26,000) from D. desulfuricans strain Norway 4, however, has been reported to be composed of two equal-sized subunits of 13,500 subunit MW (9). The interaction between the two subunits was reported to be strong, persisting even in the presence of denaturing reagents like 8 M urea and 6 M guanidine hydrochloride. Only after treatment with mercuric chloride for the removal of heme c groups could the two subunits be separated, and they migrated as a single protein band of MW 13,500 during SDS-PAGE.

Hexaheme cytochrome c (high spin/low spin [histidine-heme-histidine] hemes). A hexaheme c-type cytochrome was isolated from D. desulfuricans ATCC 27774 and shown to contain six heme c groups per 66,000 MW (21). The six hemes appeared to have different redox potentials, as some hemes were ascorbate reducible, while others were reduced only by dithionite. This hexaheme cytochrome was characterized as the dissimilatory nitrite reductase catalyzing the six-electron reduction of nitrite to ammonia (21). The enzyme exhibited both high-spin and low-spin heme signals in EPR studies (19), which is distinct from what was found with the tetraheme cytochrome c₃ (18). A large cytochrome (MW 70,000) with a reduced α-band at 553 nm had been briefly reported to be present in a strain of D. vulgaris (30). Whether it is related to the hexaheme nitrite reductase found in D. desulfuricans ATCC 27774 remains to be clarified.

Our present work adds two new members to the c-type cytochrome family of the Desulfovibrio species. The two, a dodecaheme cytochrome c (MW 40,500) and a dimeric split-Soret cytochrome c (MW 51,540), together with the tetrahemeheme cytochrome c₃ (MW 13,500), were isolated from D. desulfuricans ATCC 27774. Because this organism is capable of growth with either nitrate or sulfate as the terminal electron acceptor during anaerobic respiration, it was anticipated that the electron transfer components—c-type cytochromes studied in the present case—would be different between cells grown on nitrate and cells grown on sulfate. This rationalization was based on the fact that the redox potentials are far more positive for the reduction of nitrate and nitrite than are those involved in dissimilatory sulfate reduction. Surprisingly, however, the same c-type cytochrome components were detected in both nitrate-grown and sulfate-grown cells. Although slight differences in the final yields of the three cytochromes purified were observed, these differences may or may not have physiological significance in view of the method (protein purification) used in the study. It seems likely that, similar to the enzymes involved in dissimilatory sulfate reduction, these cytochromes are constitutively synthesized under both growth conditions. In contrast to the present finding, however, is that the level of the hexaheme nitrite reductase was found to be three to four times higher in induced (nitrate-grown) than in uninduced (sulfate-grown) D. desulfuricans ATCC 27774 cells (24).

Based on the physical properties, spectroscopic results, and amino acid composition, the three cytochromes isolated from nitrate-grown and sulfate-grown cells appeared to be identical. Furthermore, in the case of the tetraheme cytochrome c₃, both EPR and NMR spectroscopy failed to reveal any significant difference between the proteins isolated from cells grown under the two conditions. Magnetic resonance methods, especially proton NMR, are very useful for probing structural differences between similar proteins. These methods are sensitive enough to detect minute differences which may not be apparent when other spectroscopic methods are used. As shown in Fig. 5 and 6, nearly identical EPR and NMR spectra were obtained for the tetraheme cytochrome c₃ isolated from both sources. The fact that identical c-type cytochrome components are present in nitrate-grown and sulfate-grown D. desulfuricans ATCC 27774 is in contrast to the previous findings for a facultative anaerobe, P. perfectomarina, for which differences in cytochrome components were detected between aerobically grown cells and cells actively performing denitrification (20).

It is interesting that a large number of multiheme c-type cytochromes are present in D. desulfuricans ATCC 27774 and in sulfate-reducing bacteria in general. A similar, although less dramatic, situation exists in the denitrifying bacteria. These organisms have been shown to contain at least two diheme c-type cytochromes, a cytochrome c peroxidase (4) and a diheme cytochrome c-552 (22), in addition to cytochrome cd₁, the denitrifying nitrite reductase (14, 15). The evolutionary and functional significance of multi-redox center proteins rather than multiple redox proteins is an interesting subject for investigation.

As a sulfate-reducing bacterium which constitutively synthesizes many of the electron transfer proteins involved in dissimilatory sulfate reduction, nitrate-grown D. desulfuricans ATCC 27774 offers an advantage for the preparation of those metal-containing redox proteins enriched in stable isotopes, because growing this organism on nitrate avoids the wasteful precipitation of expensive isotopes such as ⁵⁷Fe by the sulfide produced during growth on sulfate, which is required for other sulfate-reducing bacteria. This advantage has already been used in preparing ⁵⁷Fe- and ⁶⁰Ni-enriched hydrogenase from nitrate-grown D. desulfuricans ATCC 27774 (17).
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