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AMMONIA-FORMING CYTOCHROME *c* NITRITE REDUCTASE FROM *SULFUROSPIRILLUM DELEYIANUM* IS A TETRAHEME PROTEIN: NEW ASPECTS OF THE MOLECULAR COMPOSITION AND SPECTROSCOPIC PROPERTIES

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SUMMARY: Ammonia-forming cytochrome *c* nitrite reductase from *Sulfurospirillum deleyianum* contains four covalently bound heme *c* groups/55 kDa subunit as determined by atomic absorption spectroscopy and the pyridine Fe(II)-hemochrome technique. Nitrite reductase was isolated from the membrane fraction as a monomer (M_r 55±2 kDa) and as a heterooligomeric complex. Both the monomeric and the complex form of the enzyme exhibited a high specific activity, with up to 1050 μmol NO_2^- min^{-1} mg^{-1}. The complex was built from four 55 kDa units and contained a 22 kDa c-type cytochrome which was absent in the monomeric form. EPR spectra of the complex displayed a prominent feature at *g* 4.83 (baseline crossing). This resonance, which was not observed in the spectra of the monomeric nitrite reductase, was assigned to the 22 kDa c-type cytochrome subunit. Identical results were obtained for the enzyme from *Wolinella succinogenes* which had been reinvestigated for comparison.

Dissimilatory nitrate reduction coupled to electron transport phosphorylation proceeds *via* two different pathways: (i) in denitrification, nitrate is reduced *via* nitrite and nitric oxide to nitrous oxide and dinitrogen, whereas (ii) in nitrate ammonification, nitrate is reduced *via* nitrite to ammonia (1,2). Ammonia-forming cytochrome *c* nitrite reductases were isolated from various bacteria including *Wolinella succinogenes* and *Sulfurospirillum deleyianum* (3-9). It was concluded that these proteins constitute a group of homologous enzymes with six covalently bound heme groups (9,10). Most cytochrome *c* nitrite reductases appeared to be monomeric with

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the tendency to aggregate *in vitro* (5,6). Blackmore et al. (11) described an unusual signal at $g \approx 4.8$ in the EPR spectrum of membranous cytochrome *c* nitrite reductase of *W. succinogenes* that was not observed by Liu et al. (12). Darwin et al. (13) reported on a 50.58 kDa sequence for the enzyme from *E. coli*, carrying four Cys-X-Y-Cys-His motifs and deduced a tetraheme structure (13). Furthermore, a hydrophilic, pentaheme cytochrome *c* (M_r 20714) was found in the DNA sequence (14). Here we present biochemical and spectroscopic evidence that cytochrome *c* nitrite reductase from *both S. deleyianum* and *W. succinogenes* are tetraheme proteins, and a heterooligomeric structure is derived for the membranous enzyme.

Materials and Methods

Purification of cytochrome c nitrite reductase. *S. deleyianum* (DSM 6946^T) was cultivated according to (9). Cytochrome *c* nitrite reductase was purified from solubilized membranes (1% (w/v) Triton X-100, 50 mM Tris/HCl, pH 8.3) and the cytoplasmic fraction by chromatography on Whatman DE 52 cellulose, hydroxylapatite and Superdex 200 (Pharmacia). The purity of the enzyme was followed by SDS-PAGE, UV/VIS spectra and activity measurement. For comparison, we also purified the membranous enzyme from *W. succinogenes* by the procedure outlined above.

Analytical methods. Specific activity was assayed according to (6); the reaction was quenched after 5 min, and ammonia was measured (15). Protein was determined with bicinchoninic acid (16), or with the BioRad DC-test system. Heme was quantitated spectrophotometrically, with cytochrome *c* as a reference (ϵ_{550} = 27.6 $mM^{-1}cm^{-1}$ (17), and by the pyridine Fe(II)-hemochrome technique (18); iron was determined by atomic absorption spectroscopy. SDS-PAGE was carried out according to Laemmli (19); gels were stained with silver for proteins (20), and with tetramethylbenzidine for c-type cytochromes (21). Cross-linking experiments were conducted with dimethyladipimidate (22). EPR spectra were measured and evaluated as described (23); microwave frequency 9.241 GHz, 100 kHz modulation frequency, 1.0 mT modulation amplitude, microwave power 2.0 mW, 10 K.

Results and Discussion

Molecular properties and heme content of cytochrome c nitrite reductase. The molecular parameters of cytochrome *c* nitrite reductase from the soluble and the membrane fraction of *S. deleyianum* and *W. succinogenes* are summarized in Table 1. From solubilized membranes a high M_r form ($\approx$ 77%) and a low M_r form ($\approx$ 23%) were separated. The soluble, and both the low and the high M_r forms of the membranous nitrite reductase migrated on SDS-PAGE as one single band (M_r 55$\pm$2 kDa, Fig. 1); note the presence of a second band at $\approx$ 22 kDa in the high M_r enzyme. Bovine liver catalase (M_r 58.1 kDa/subunit) served as reference (24,25). For all three forms of nitrite reductase a M_r = 53$\pm$2 kDa was observed on SDS-PAGE in the absence of dithiothreitol. SDS-PAGE followed by heme-staining revealed a c-type cytochrome (M_r 22$\pm$2 kDa) in the high M_r nitrite reductase only (Fig. 1). Gel filtration under nondenaturing conditions gave a M_r = 54$\pm$2 kDa for the soluble and the low M_r membranous enzyme (with or without detergent). For the complex we found a M_r = 305$\pm$20 kDa (Triton X-100) *vs*

Table 1. Molecular parameters of membranous cytochrome *c* nitrite reductase from *Sulfurospirillum deleyianum* and *Wolinella sucinogenes*

	M_r[a] [kDa]	heme/M_r[b]	Fe/M_r[c]	ϵ_{553} [mM^{-1} cm^{-1}]	Activity [U]
Sulfurospirillum deleyianum					
Monomer	55±2	3.9±0.2	3.5±0.3	98	1050
Complex	245±15	17.8±1.3	16.5±2.1	397	970
Wolinella succinogenes					
Monomer	55±2	3.4±0.2	3.2±0.4	89	850
Complex	245±15	13.6±2.1	13.6±2.4	361	810

[a] SDS-PAGE and gel filtration (complex in 0.7% OcGlc); [b] Pyridine Fe(II)-hemochrome technique (n=4); [c] Atomic absorption spectroscopy (n=4); for the monomer isolated from the soluble fraction of *S. deleyianum* we find M_r 55±2 kDa, 3.5± 0.1 heme, 2.9±0.4 Fe, ϵ_{553} 85 mM^{-1} cm^{-1}, specific activity 1070 U, 1U = 1 μmol NO_2^- mg^{-1} min^{-1}.

245±15 kDa (octyl ß-D-glucopyranoside). This form of the enzyme is a heterooligomer built from four 55 kDa subunits and the 22 kDa c-type cytochrome. SDS was necessary and sufficient to dissociate the complex into its components. Dithiothreitol had no influence on the dissociation of the complex indicating that disulfide bridges were not involved; this would also explain why the 22 kDa c-type cytochrome component is partially lost during the purification of the

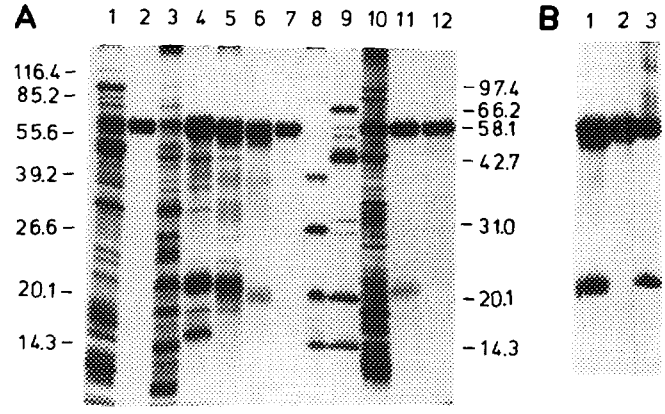

Figure 1. Silver (A) and Heme (B) stained SDS-PAGE of cytochrome *c* nitrite reductase *S. deleyianum*: Lane 1A) soluble fraction; 2A) soluble monomer; 3A) membrane fraction; 4A) membranous nitrite reductase, after DE 52 chromatography; 5A) as 4A, after hydroxylapatite chromatography; 6A) pure membranous complex; 7A) pure membranous monomer; 1B) pure membranous complex; 2B) pure membranous monomer. ***W. succinogenes***: Lane 10A) membrane fraction; 11A) pure membranous complex; 12A) pure membranous monomer; 3B) pure membranous complex. Lanes 8,9: Boehringer-Combithek and BioRad marker proteins.

membranous enzyme. Blackmore et al. (26) reported on a 360 kDa nitrite reductase complex from *W. succinogenes* and claimed that it was composed of a putative 120 kDa hydrophobic protein bound to 4-6 nitrite reductase molecules; a 22 kDa c-type cytochrome component was not mentioned. We obtained the high M_r form of nitrite reductase from both microorganisms, but never observed the 120 kDa hydrophobic protein (26). The heterooligomeric nature of cytochrome *c* nitrite reductase was confirmed by cross-linking experiments which gave five distinct bands on SDS-PAGE including a band at M_r 78±2 kDa from the cross-linking product between a 55 kDa component and the 22 kDa c-type cytochrome (data not shown). As a control, the homotetramer of bovine liver catalase (25) yielded four distinct bands on SDS-PAGE. These results were confirmed by a Western blot with antiserum raised against the soluble monomeric enzyme from *S. deleyianum*; the antiserum reacted with the monomeric and the complex membranous enzyme (data not shown). In the pyridine Fe(II)-hemochrome assay, we obtained ≈ 4 heme/M_r 55 kDa (monomer) and ≈ 18 heme/M_r 245 kDa (complex) in agreement with the Fe content (Table 1), and the sequence data for the *E. coli* enzyme (13).

Spectroscopic properties. Cytochrome *c* nitrite reductase (as isolated) exhibited absorption maxima at 280, 409, and 534 nm, with a shoulder at 615 nm *vs* 420.5, 523.5 and 553.3 nm

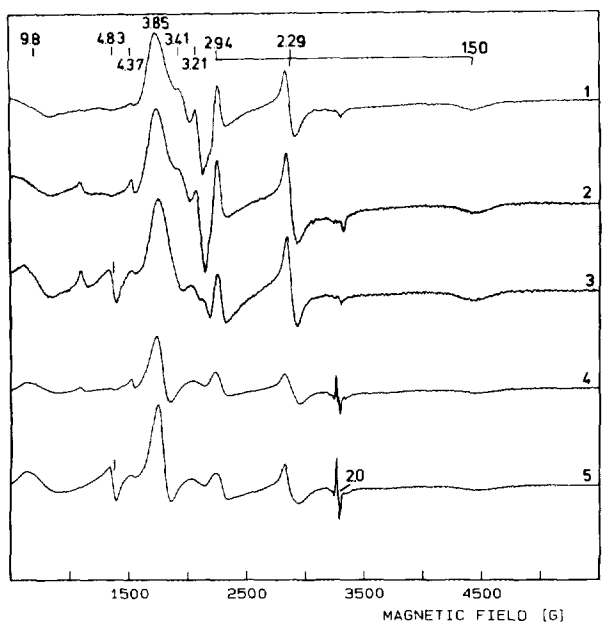

Figure 2. X-band EPR spectra of cytochrome *c* nitrite reductase. Enzymes as isolated, in 0.05 % Triton X-100, 50 mM KP_i, pH 7.5.
S. deleyianum: 1) soluble monomer, 155 μM; 2) membranous monomer, 145 μM; 3) membranous complex, 55μM. *W. succinogenes*: 4) membranous monomer, 84 μM; 5) membranous complex, 57 μM.

(dithionite-reduced). The molar absorption coefficient ϵ_{553} of the monomer from *S. deleyianum* was ≈ 4x the coefficient ϵ_{550} of reduced horse heart cytochrome *c* (17), but was slightly smaller than ϵ_{552} of the tetraheme cytochrome c_3 from *Desulfovibrio desulfuricans* (27). The EPR spectra (Fig. 2) showed the dominant signal at *g* 3.85 (9,11,12, 28-31), and low-spin Fe(III) resonances at *g* 2.94, 2.29 and ≈ 1.50 (11,31). Depending on the sample there was a minor signal around *g* 6.1 from high-spin Fe(III) heme which became more intense in the SDS-treated enzyme (11). The characteristic features at *g* 9.8 and 3.8 were also reported for the tetraheme cytochrome c_{554} from *Nitrosomonas europaea* (33), and assigned to an exchange-coupled pair of low and high-spin heme centers. Most important, EPR spectra of the cytochrome *c* nitrite reductase complex displayed a significant resonance around *g* 4.83 (baseline crossing) which was absent in the spectra of the monomeric enzyme of both *S. deleyianum* and *W. succinogenes*. We attribute this resonance to the 22 kDa cytochrome *c* component of the nitrite reductase complex. In agreement with this assignment, the soluble monomeric enzyme from *E. coli* (33) as well as from *W. succinogenes* prepared by Liu et al. (6) did not show the EPR signal at *g* 4.83 (12).

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