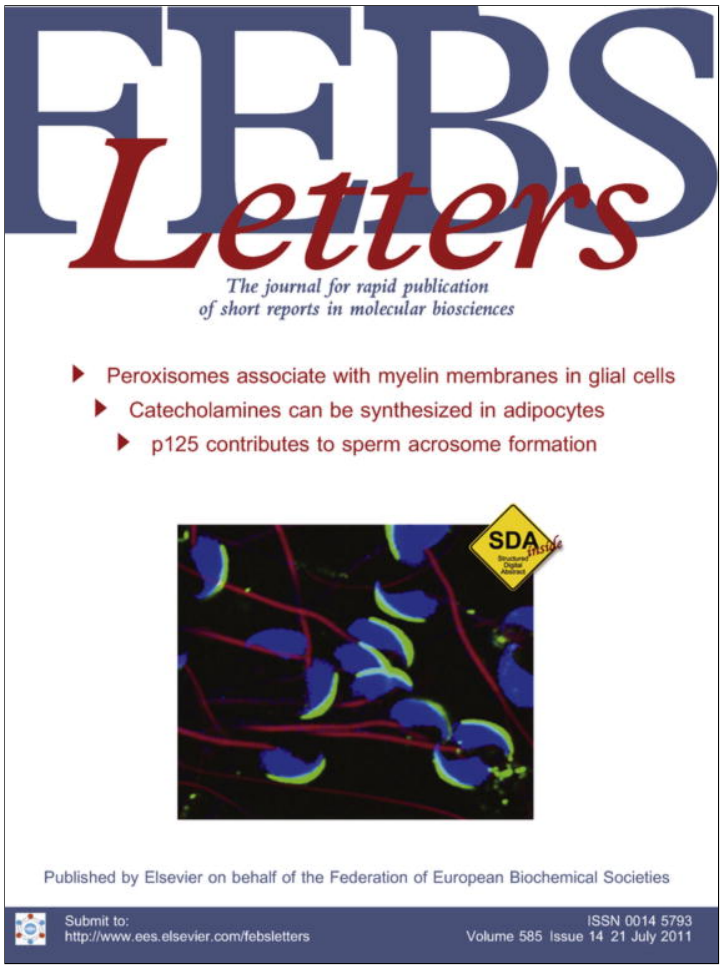

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EPR characterization of the new Qrc complex from sulfate reducing bacteria and its ability to form a supercomplex with hydrogenase and TpIc$_3$

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ABSTRACT

The Quinone-reductase complex (Qrc) is a respiratory complex with Type I cytochrome c_3:menaquinone reductase activity, recently described in sulfate-reducing bacteria. Qrc is related to the complex iron–sulfur molybdoenzyme family and to the alternative complex III. In this work we report a detailed characterization of the redox properties of the metal cofactors of Qrc using EPR spectroscopy, which allowed the determination of the reduction potentials of five out of six hemes *c*, one [3Fe–4S]$^{1+/0}$ center and the three [4Fe–4S]$^{2+/1+}$ centers. In addition, we show that Qrc forms a supercomplex with [NiFe] hydrogenase and TpIc$_3$, its physiological electron donors.

Structured summary of protein interactions:
NiFe Hydrogenase physically interacts with **Qrc** by native page (View interaction).

1. Introduction

In the main group of sulfate-reducing bacteria (SRB), of the *Deltaproteobacteria* class, most organisms are characterized by the presence of a high number of multiheme cytochromes *c*, of which the most abundant and well characterized is the tetraheme Type I cytochrome c_3 (TpIc$_3$). In addition, this group has a considerable number of membrane-associated redox complexes containing a cytochrome *c* subunit, which are responsible for quinone reduction or transmembrane electron transfer for the cytoplasmic reduction of sulfate (reviewed in [1]). Recently, a new respiratory membrane complex present in SRB was described and named Qrc (for Quinone–reductase complex) [2]. Qrc was shown to transfer electrons from the TpIc$_3$ to the menaquinone pool, with the periplasmic hydrogenases (Hases) and/or formate dehydrogenases (Fdhs) as primary electron donors. The presence of Qrc is associated with the presence of Hases and/or Fdhs lacking a membrane subunit for direct quinone reduction. Qrc is the only described respiratory complex in SRB with a quinone reductase function, and it is essential for growth on H_2 or formate, as recently shown in *D. desulfuricans* G20 [3].

Qrc comprises three subunits in the periplasm, namely a membrane-anchored hexaheme cytochrome *c* (QrcA), a periplasmic-facing large protein of the molybdopterin oxidoreductase family (QrcB), and a periplasmic iron–sulfur four cluster protein (QrcC), and also an integral membrane subunit of the NrfD family (QrcD) [2]. The three QrcBCD subunits are closely related to the bacterial respiratory enzymes of the complex iron-sulfur molybdoenzyme (CISM) family [4], although no molybdopterin is present in QrcB. The absence of Mo is also observed in the Nqo3/NuoG subunit of the respiratory complex I [5], and in the related protein of the alternative complex III (ACIII) [6–8]. ACIII, a functional substitute of the bc_1 complex, is closely related to Qrc [2,6], but has a higher number of subunits and acts reversibly of Qrc, by oxidising menaquinol [9,10].

Previous EPR studies of Qrc, allowed the detection of six low-spin hemes *c*, one [3Fe–4S]$^{1+/0}$ cluster and at least three interacting [4Fe–4S]$^{2+/1+}$ clusters [2]. Here, we report a more detailed study of the Qrc hemes and Fe–S centers through potentiometric titrations followed by EPR spectroscopy, providing a better characterization of the redox centers responsible for electron transfer in Qrc. In addition, we report that Qrc can form a supermolecular structure (supercomplex) with Hase and TpIc$_3$, its physiological electron donors.

2. Material and methods

2.1. Protein purification

Qrc was purified from *D. vulgaris* Hildenborough as previously described [2].

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2.2. Redox titration monitored by EPR

A potentiometric titration was performed inside an anaerobic chamber (95% Ar, 5% H_2) at 20 °C using 40 μM of Qrc and 70 μM of a mixture of redox mediators [2] in 100 mM MOPS pH 7, 5 mM EDTA. The redox potential spontaneously decreased slowly down to −200 mV, and sodium dithionite was used after that. Samples were transferred to EPR tubes under anaerobic conditions, capped and immediately frozen in liquid nitrogen upon removal from the chamber. EPR spectra were obtained using a Bruker EMX spectrometer equipped with an ESR-900 continuous flow of helium cryostat. Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; microwave power, 20.1 mW (for the Fe–S centers) and 2.4 mW (for the hemes); modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 10 K.

2.3. Electrophoretic techniques

Proteins were separated on 10% polyacrylamide Tricine–SDS–PAGE or 7–13% high resolution Clear Native (hr CN–PAGE) containing 0.02% (wt/vol) dodecyl maltoside (DDM), with running buffer also containing 0.02% DDM and 0.05% deoxycholic acid, as described in [11]. Gels were stained with Coomassie Blue, with heme-staining using 3,3′-dimethoxybenzidine dihydrochloride [12] or for hydrogenase activity [13]. Bands from the first-dimension CN–PAGE were excised and used for a second-dimension Tricine–SDS–PAGE, after denaturation by incubation with 5% SDS and 5% β-mercaptoethanol at 37 °C for 1 h, followed by a washing step, and thiol blockage with a 4% iodoacetamide solution.

2.4. Mass spectrometry

The supercomplex band from the CN–PAGE was subjected to in-gel tryptic digestion followed by peptide identification in a MALDI-TOF/TOF analyzer (Applied Biosystems 4800). The data were analyzed in a combined mode using Mascot search engine and NCBI database.

3. Results and discussion

3.1. EPR characterization of Qrc cofactors

EPR spectroscopy is a most useful tool to selectively characterize the redox properties of both hemes and Fe–S centers of respiratory proteins. A few detailed EPR studies on CISM enzymes have been reported, which were most informative regarding the properties of the Fe–S clusters (reviewed in [4]). These clusters provide a linear pathway to transfer electrons between the quinone pool and the catalytic site.

To generate samples for the EPR study, we titrated Qrc in the presence of redox mediators, inside an anaerobic chamber containing 5% H_2. It was observed that the redox potential of the sample slowly decreased without addition of any reductant down to ∼−200 mV. This decrease was due to trace amounts of Hase in the Qrc sample (see below), which slowly reduced the complex, but this decrease was slow enough to allow us to take several samples at defined redox potentials. The Qrc hemes were reported to give rise to three EPR signals with g_{max} of 3.21, 2.93 and 2.82 (Fig. 1A), which by spectral simulation account for 3, 2 and 1 hemes, respectively [2]. The intensity of these peaks was followed

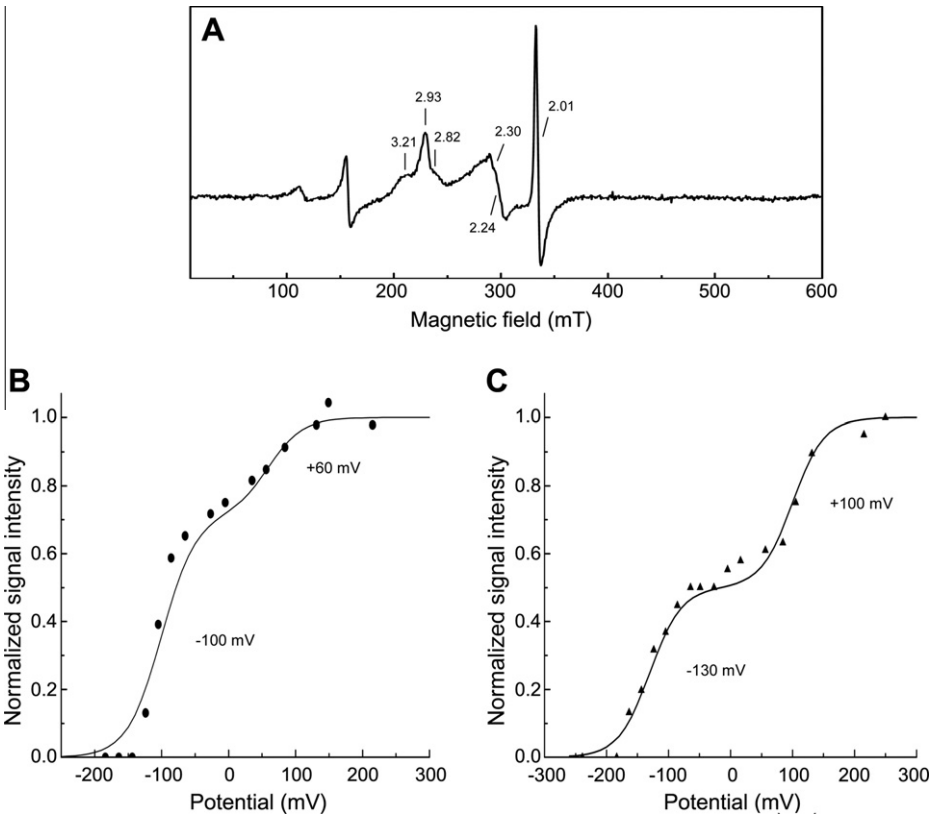

Fig. 1. Potentiometric titration of the Qrc hemes *c*. (A) EPR spectrum of the oxidized sample where the *g*-values of several heme signals are identified. Redox titration curves of the peaks at *g*-values 3.21 (B) and 2.93 (C). The points represent the experimental data and the solid line represents the best theoretical fit using Nernst equation(s), with the E_m values depicted.

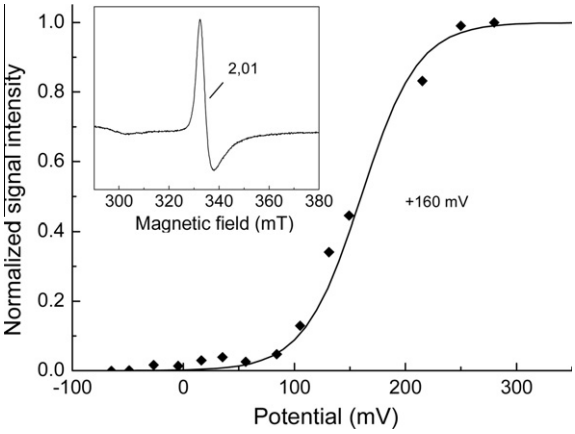

Fig. 2. Potentiometric titration of the Qrc $[3Fe–4S]^{1+/0}$ center monitored at *g*-value 2.01. Inset, EPR spectrum of the $[3Fe–4S]^{1+/0}$ center in the oxidized sample.

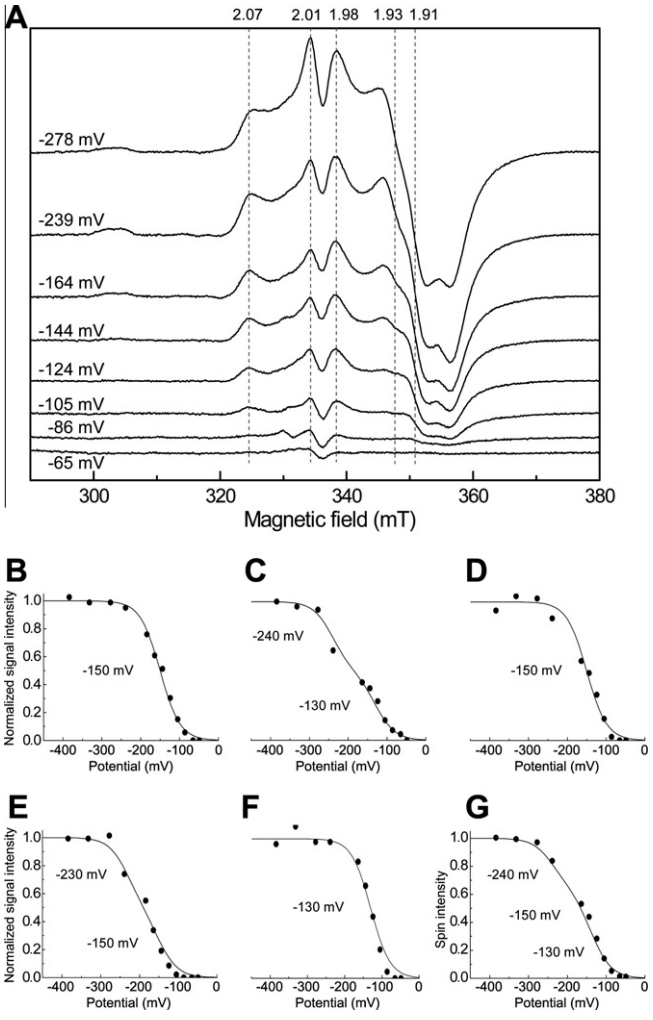

Fig. 3. Potentiometric titration of the $[4Fe–4S]^{2+/1+}$ centers of the Qrc complex. EPR spectra obtained during the redox titration (A) and the respective redox titration curves followed at *g*-values of 2.07, 2.01, 1.98, 1.93 and 1.91 (B, C, D, E and F, respectively), and total signal intensity (G). The E_m value(s) used in the Nernst fittings are depicted in the plots. No further changes are observed after −280 mV.

along the redox titration. The data points for the signal with g_{max} = 3.21 were simulated by the sum of three Nernst equations with midpoint redox potentials (E_m) of +60 and −100 mV in a 1:2 ratio approximately (Fig. 1B), in accordance with the fact that this signal corresponds to 3 hemes. The data points for the signal with g_{max} = 2.93 could be simulated with two different redox potentials with 50% contribution each, with E_m values of +100 and −130 mV (Fig. 1C). The low intensity of the signal with g_{max} = 2.82, due to one heme, together with partial overlap with the 2.93 signal, did not allow the determination of a satisfactory redox titration curve.

Table 1
Midpoint potentials of the Qrc cofactors obtained in the EPR titration.

	E_m (mV)		
Hemes *c*	*g* = 3.21 +60 −100 −100	*g* = 2.93 +100 −130	*g* = 2.82 ND
$[3Fe–4S]^{1+/0}$	*g* = 2.01 +160		
$[4Fe–4S]^{2+/1+}$	*g* = 2.07 −150	*g* = 2.01 −130 −240	*g* = 1.98 −150
	g = 1.93 −150 −230	*g* = 1.91 −130	

ND: not determined.

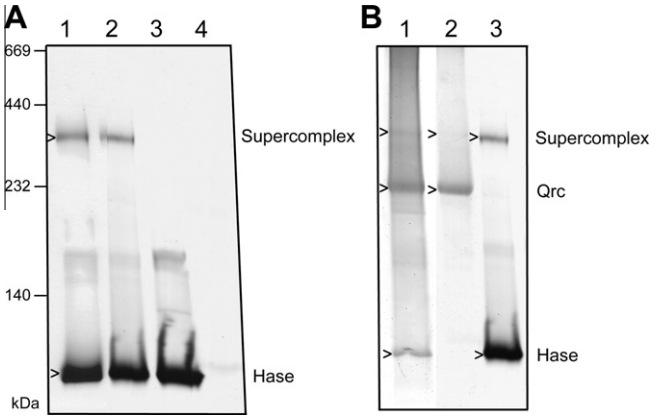

Fig. 4. (A) High resolution CN–PAGE stained for H_2 uptake activity of the Qrc-containing fraction from the first chromatographic step (1), of the Qrc-containing fraction from the second chromatographic step (2), of the Hase-containing fraction from the IMAC column (3); and of the purified Qrc (4). (B) Hr CN–PAGE of the Qrc fraction from the second chromatographic step stained by Coomassie Blue (1), by heme-staining (2) and by H_2 uptake activity (3).

In the oxidized state a strong EPR signal at $g \sim 2.01$ due to a $[3Fe–4S]^{1+/0}$ center is also observed (Fig. 2, inset), as described in other CISM and related complexes such as NarGHI nitrate reductase [14], FrdABCD fumarate reductase [15], or ACIII [16]. This signal titrates with E_m value of +160 mV (Fig. 2), which is close to the +140 mV or +180 mV redox potential determined for the $[3Fe–4S]^{1+/0}$ cluster of ACIII and NarGHI, respectively [14,16]. In Qrc, the $[3Fe–4S]^{1+/0}$ center is close to the menaquinone binding site [2].

At least three $[4Fe–4S]^{2+/1+}$ clusters were reported in *D. vulgaris* Qrc [2]. These clusters give rise to a complex EPR signal due to magnetic interactions, as reported for other four cluster proteins [17]. Five signals with *g*-values of 2.07, 2.01, 1.98, 1.93 and 1.91 (Fig. 3A) were monitored in the redox titration. The data points obtained from signals *g* 2.07, 1.98 and 1.91 can be fitted with a single component, with E_m = −150 mV for the first two and −130 mV for the latter. For both signals with *g* 2.01 and 1.93, two components have to be considered with E_m of −130 and −240 mV for *g* = 2.01

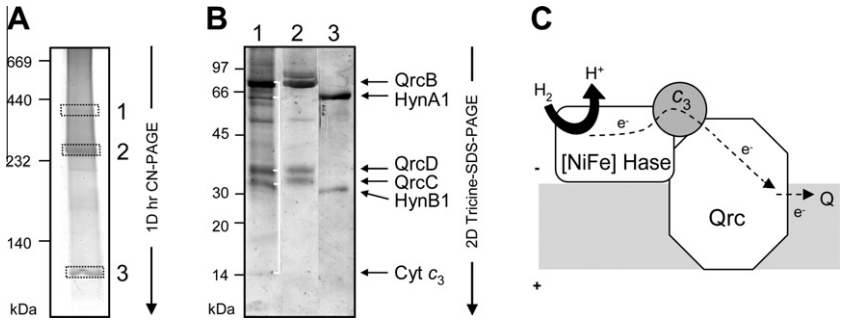

Fig. 5. Hr CN–PAGE of (A) a supercomplex-containing fraction, and the 2D Tricine–SDS–PAGE (B) of the respective bands: supercomplex (1), Qrc (2) and Hase (3). Gel A was stained with Coomassie and Gel B with silver nitrate. (C) Schematic representation of the supercomplex and its electron transfer pathway (dashed arrows). Hyn, [NiFe] Hydrogenase; Q, menaquinone.

and −150 mV and −230 mV for $g = 1.93$ (Fig. 3). These data suggest the presence of three clusters, two of which with E_m of −130 to −150 mV and the third with a lower E_m of −240 mV. This is supported by a plot of the total signal intensity versus the potential, which can be fitted with these same three potentials (Fig. 3G). Thus, one of the Qrc $[4Fe–4S]^{2+/1+}$ clusters has a lower potential than the other two, a similar situation to what is described for CISM enzymes [17]. The midpoint potentials of the Qrc cofactors have values that are suitable for electron transfer from the low redox potential TpIc_3 (−325 to −170 mV) to the menaquinone ($E_m = -70$ mV). All the potentiometric results are summarized in Table 1.

3.2. Qrc forms a supercomplex with the [NiFe] hydrogenase

During the purification of Qrc, we observed that it was difficult to separate it from the [NiFe] Hase, as they eluted together in the first two ion-exchange columns, and also in a subsequent gel filtration. We could only separate these two proteins using a metal chelating IMAC column (Supplementary Fig. 1). This suggested the presence of a strong interaction between Qrc and Hase, which could result in the formation of a supercomplex. Analysis of the protein fractions by high resolution clear native gels (hr CN–PAGE) [11] stained for H_2 uptake activity, indeed revealed the presence of a high molecular mass band, besides the band for the isolated Hase at 87 ± 9 kDa (Fig. 4A). This band, with an estimated molecular mass of 360 ± 10 kDa, was found in fractions containing both Qrc and the Hase, but was no longer detected in the fractions from the IMAC column, which can separate the two proteins. The band for the isolated Qrc complex is present at 250 ± 24 kDa and stains with Coomassie and for hemes (Fig. 4B), but does not stain for H_2 uptake activity, while the supercomplex band is very well detected by Hase activity staining (which is more sensitive), but stains weakly for hemes.

To define the composition of the supercomplex, a two dimension Tricine–SDS–PAGE was performed on the 360 kDa band (Fig. 5B). Several subunits from the Qrc complex were identified, as well as the large subunit of the Hase, by comparison with the 2D gel of the isolated Qrc and Hase native gel bands. Furthermore, the 360 kDa band was excised from the hr CN–PAGE gel, digested with trypsin and analyzed by mass spectrometry. Two proteins were clearly identified to be present: the large subunit of Qrc (QrcB) and the large subunit of the soluble periplasmic [NiFe] Hase (HynA1) (Supplementary Table 1), showing that this high molecular mass band is indeed formed by a supercomplex between Qrc and the [NiFe] Hase. The identification of this Hase was also confirmed by Western blot (Supplementary Fig. 1).

The electron transfer between Hase and Qrc was shown to be functional only in the presence of the TpIc_3 cytochrome [2], the Hase physiological electron acceptor. Like the Hase, the TpIc_3 was detected in the Qrc fraction from the second Q-Sepharose column as a heme-staining band of 15 kDa (Supplementary Fig. 1). Additionally, a tenuous band of this molecular mass was also observed in the 2D gel of the supercomplex, but was absent in the Qrc and Hase controls of the 2D gel (Fig. 5B). Moreover, the molecular weight difference between the supercomplex band and the sum of the Hase plus the Qrc native bands, estimated from several CN gels, is about 22 kDa, which likely corresponds to the TpIc_3. Altogether, these results indicate that the 360 kDa supercomplex is constituted of three proteins: Qrc, [NiFe] Hase and TpIc_3. The formation of organized respiratory enzyme complexes in supermolecular structures has been well described in mitochondria [18] (sometimes called respirasomes), and also in bacteria [19].

In summary, the redox studies reported here provide the first detailed characterization of a new bacterial respiratory complex, and support the function of Qrc in electron transfer between the low redox potential TpIc_3 and menaquinone. The assembly of the [NiFe] Hase, TpIc_3 and Qrc in a supermolecular structure is likely to optimize the oxidation of H_2, a key step in energy metabolism of SRB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.054.

References

[1] Pereira, I.A.C., Ramos, A.R., Grein, F., Marques, M.C., da Silva, S.M. and Venceslau, S.S. (2011) A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. Front. Microbiol. 2, 69. doi:10.3389/fmicb.2011.00069.
[2] Venceslau, S.S., Lino, R.R. and Pereira, I.A. (2010) The Qrc membrane complex, related to the alternative complex III, is a menaquinone reductase involved in sulfate respiration. J. Biol. Chem. 285, 22774–22783.
[3] Li, X., Luo, Q., Wofford, N.Q., Keller, K.L., McInerney, M.J., Wall, J.D. and Krumholz, L.R. (2009) A molybdopterin oxidoreductase is involved in H_2 oxidation in *Desulfovibrio desulfuricans* G20. J. Bacteriol. 191, 2675–2682.
[4] Rothery, R.A., Workun, G.J. and Weiner, J.H. (2008) The prokaryotic complex iron–sulfur molybdoenzyme family. Biochim. Biophys. Acta 1778, 1897–1929.
[5] Sazanov, L.A. and Hinchliffe, P. (2006) Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science 311, 1430–1436.

[6] Yanyushin, M.F., del Rosario, M.C., Brune, D.C. and Blankenship, R.E. (2005) New class of bacterial membrane oxidoreductases. Biochemistry 44, 10037–10045.
[7] Pereira, M.M., Refojo, P.N., Hreggvidsson, G.O., Hjorleifsdottir, S. and Teixeira, M. (2007) The alternative complex III from *Rhodothermus marinus* – a prototype of a new family of quinol:electron acceptor oxidoreductases. FEBS Lett. 581, 4831–4835.
[8] Gao, X.L., Xin, Y.Y., Bell, P.D., Wen, J.Z. and Blankenship, R.E. (2010) Structural analysis of alternative complex III in the photosynthetic electron transfer chain of *Chloroflexus aurantiacus*. Biochemistry 49, 6670–6679.
[9] Gao, X.L., Xin, Y.Y. and Blankenship, R.E. (2009) Enzymatic activity of the alternative complex III as a menaquinol: auracyanin oxidoreductase in the electron transfer chain of *Chloroflexus aurantiacus*. FEBS Lett. 583, 3275–3279.
[10] Refojo, P.N., Teixeira, M. and Pereira, M.M. (2010) The alternative complex III of *Rhodothermus marinus* and its structural and functional association with caa(3) oxygen reductase. Biochim. Biophys. Acta Bioenergetics 1797, 1477–1482.
[11] Wittig, I., Karas, M. and Schagger, H. (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol. Cell. Proteomics 6, 1215–1225.
[12] Francis Jr., R.T. and Becker, R.R. (1984) Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. Anal. Biochem. 136, 509–514.
[13] da Silva, S.M., Venceslau, S.S., Fernandes, C.L., Valente, F.M. and Pereira, I.A. (2008) Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia*. Antonie Van Leeuwenhoek 93, 381–390.
[14] Rothery, R.A., Magalon, A., Giordano, G., Guigliarelli, B., Blasco, F. and Weiner, J.H. (1998) The molybdenum cofactor of *Escherichia coli* nitrate reductase A (NarGHI). Effect of a mobAB mutation and interactions with [Fe–S] clusters. J. Biol. Chem. 273, 7462–7469.
[15] Manodori, A., Cecchini, G., Schroder, I., Gunsalus, R.P., Werth, M.T. and Johnson, M.K. (1992) [3Fe–4S] to [4Fe–4S] Cluster conversion in *Escherichia coli* fumarate reductase by site-directed mutagenesis. Biochemistry 31, 2703–2712.
[16] Pereira, M.M., Carita, J.N. and Teixeira, M. (1999) Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: a novel multihemic cytochrome bc, a new complex III. Biochemistry 38, 1268–1275.
[17] Cheng, V.W., Rothery, R.A., Bertero, M.G., Strynadka, N.C. and Weiner, J.H. (2005) Investigation of the environment surrounding iron–sulfur cluster 4 of *Escherichia coli* dimethylsulfoxide reductase. Biochemistry 44, 8068–8077.
[18] Dudkina, N.V., Kouril, R., Peters, K., Braun, H.P. and Boekema, E.J. (2010) Structure and function of mitochondrial supercomplexes. Biochim. Biophys. Acta-Bioenergetics 1797, 664–670.
[19] Prunetti, L., Infossi, P., Brugna, M., Ebel, C., Giudici-Orticoni, M.T. and Guiral, M. (2010) New Functional Sulfide Oxidase-Oxygen Reductase Supercomplex in the Membrane of the Hyperthermophilic Bacterium *Aquifex aeolicus*. J. Biol. Chem. 285, 41815–41826.