ELSEVIER

Biochimica et Biophysica Acta 1229 (1995) 356–362

BBA Biochimica et Biophysica Acta

The trinuclear iron-sulfur cluster S3 in *Bacillus subtilis* succinate:menaquinone reductase; effects of a mutation in the putative cluster ligation motif on enzyme activity and EPR properties

Cecilia Hägerhäll [a,b,*], Vladimir Sled' [a], Lars Hederstedt [b], Tomoko Ohnishi [a]

[a] *Department of Biochemistry and Biophysics, A606 Richards Building, University of Pennsylvania, Philadelphia, PA 19104, USA*
[b] *Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden*

Received 10 November 1994; accepted 18 January 1995

Abstract

Succinate:quinone reductases (SQRs) and quinol:fumarate reductases (QFRs) each contain a bi-, a tri- and a tetra-nuclear iron-sulfur cluster. The C-terminal half of the iron-sulfur protein subunit of these enzymes shows two fully conserved motifs of cysteine residues, stereotypical for ligands of [3Fe-4S] and [4Fe-4S] clusters. To analyze the functional role of the trinuclear cluster S3 in *Bacillus subtilis* SQR, a fourth cysteine residue was introduced into the putative ligation motif to that cluster. A corresponding mutation in *Escherichia coli* QFR results in a tri- to tetranuclear conversion (Manodori et al. (1992) Biochemistry 31, 2703–2731). We have found that presence of the extra cysteine in *B. subtilis* SQR does not result in cluster conversion. It does, however, affect the EPR properties of the cluster S3, whereas those of the other two clusters remain normal. The results strongly support the view that residues in the most C-terminal cysteine motif in the iron-sulfur protein subunit of SQRs and QFRs ligate the trinuclear cluster. Compared to wild-type SQR, S3 in the *B. subtilis* mutant enzyme is not sensitive to methanol and the midpoint redox potential is close to normal. The quinone reductase activity of the mutant enzyme is only 35% of normal. Thus, the architecture around cluster S3 plays a role in electron transfer to quinone or in the binding of quinone to the enzyme.

Keywords: Succinate:menaquinone oxidoreductase; Trinuclear iron-sulfur cluster; Site-directed mutagenesis; Iron-sulfur cluster; EPR; (*B. subtilis*)

1. Introduction

Succinate:quinone reductase (SQR) and quinol:fumarate reductase (QFR) are membrane-bound enzyme complexes found in aerobic and anaerobic organisms, respectively. These two enzymes can catalyze the same reactions, but in vivo the former enzyme complex catalyses the oxidation of succinate to fumarate in the citric acid cycle, and donates the electrons to quinone in the membrane, whereas the latter enzyme complex catalyses the reverse reaction. SQR and QFR are very similar in composition and most likely also in structure [1,2]. They consist of three or four protein subunits: a flavoprotein (FP) containing covalently bound FAD and an iron-sulfur protein (IP) containing three iron-sulfur clusters of [2Fe-2S], [4Fe-4S] and [3Fe-4S] type (denoted S1, S2 and S3 in SQR and FR1, FR2 and FR3 in QFR, respectively). These two water-soluble subunits are anchored to the membrane by one or two membrane-spanning polypeptides, that contain zero, one or two protoheme IX molecules depending on the organism [1,2]. In spite of this variation, and an overall lower sequence conservation than in the soluble subunits, Hägerhäll and Hederstedt [3] have recently shown that the membrane anchor polypeptides are also likely to have a common three-dimensional structural arrangement. The *Bacillus subtilis* SQR membrane anchor consists of one polypeptide, and contains two hemes with different properties, heme b_H and heme b_L [4].

The IP subunit contains three groups of cysteine-motifs, which probably are ligands to the three iron-sulfur clusters.

Abbreviations: SQR, succinate:quinone reductase; QFR, quinol:fumarate reductase; FP, flavoprotein; IP, iron-sulfur protein; NSMP, nutrient sporulation medium with phosphate; PMS, phenazine methosulfate; DCPIP, 2,6-dichlorophenolindophenol; Q_1, 2,3-dimethoxy-5-methyl-6-(3,7-dimethyl-2,6-octadienyl)-1,4-benzoquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; TTFA, thenoyltrifluoroacetone; K_i, inhibition constant.

* Corresponding author. Address (a); e-mail: Ohnishi@a1.mscf.upenn.edu; Fax: +1 215 5733748.

SSDI 0005-2728(95)00023-2

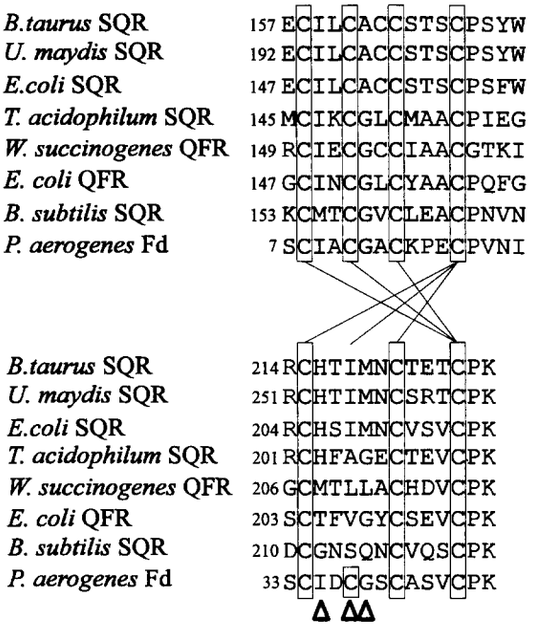

Fig. 1. Sequence comparisons of the two carboxy-terminal cysteine ligand motifs in IP of SQRs/QFRs compared to those in the *Peptococcus aerogenes* ferredoxin polypeptide. Sequence data are from *Bos taurus* [31], *Ustilago maydis* [28], *Escherichia coli* (SQR) [32], *Thermoplasma acidophilum* [33], *Wolinella succinogenes* [34], *Escherichia coli* [35], *Bacillus subtilis* [36] and *P. aerogenes* [37]. The cysteine ligands to the respective tetranuclear iron-sulfur clusters in the *P. aerogenes* protein are boxed and connected with lines. Arrowheads indicate the position of mutations in SQR/QFR which are discussed in the text.

B. subtilis and *Escherichia coli* mutant IPs, truncated before and after the second group of cysteines respectively, retain the [2Fe-2S] cluster but have lost the other two clusters [5–7]. Thus, IP can be described as consisting of two domains. The N-terminal part harbours the [2Fe-2S] cluster, ligated by a CxxxxCxxC.....C motif, resembling a plant-type ferredoxin. The ligation of this cluster has been studied in detail in *E. coli* QFR, where the four cysteines were individually changed to serine [8]. The mutants retained all iron-sulfur clusters, as well as enzymatic activity. However, the midpoint potential of FR1 was lower than in the wild-type in the C57 → S, the C62 → S and the C77 → S mutants, but higher than in wild-type in the C65 → S mutant. This third cysteine in the N-terminal motif is not absolutely conserved, *E. coli* SQR has an aspartate in this position.

The C-terminal part of the IP seemingly ligates the [4Fe-4S] and [3Fe-4S] clusters and contains cysteine-motifs characteristic for bacterial ferredoxins (Fig. 1). Assuming a ferredoxin-like structure (cf. [9]), these two clusters are located close to each other. Attempts have been made to identify the ligands to FR3 in *E. coli* QFR by site-specific mutagenesis of cysteines in the proposed ligation motif. However, the mutants lost both FR2 and FR3, but retained FR1 [10].

When a fourth cysteine residue was introduced into the putative FR3 binding motif of *E. coli* QFR (V207 → C substitution; see Fig. 1), a [3Fe-4S] to [4Fe-4S] cluster conversion occurred, i.e., a mutant enzyme with two low potential [4Fe-4S] clusters was obtained [10]. This QFR mutant retained 16-17% of wild-type enzyme activity, indicating that the redox midpoint potential and/or structure of FR3 is important for enzyme function. Opposite cluster conversion, i.e., from [4Fe-4S] to [3Fe-4S], has been reported for several enzymes after replacement of a ligating cysteine by another amino acid (cf. [11]). These observations suggest that the prototypical cysteine sequence motif predominantly determines the cluster type.

In order to study the role of cluster S3 for electron transport in *B. subtilis* SQR, we constructed a mutant with an extra cysteine in the putative S3 ligation motif, analogous to the one described for *E. coli* QFR (Fig. 1). Introduction of the additional cysteine in the S3 consensus ligation motif in *B. subtilis* did not result in a tri- to tetranuclear cluster conversion, but did specifically alter the EPR properties of the S3 signal, and affected enzyme activity and stability. Novel properties of cluster S3 in wild-type and mutant *B. subtilis* SQR are reported in this paper.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth of cells

B. subtilis strain ICD2 (*trp*C2, *ade*, *met*, *ΔsdhCAB*::*ble*) as well as plasmid pBSD1400, containing the wild-type *B. subtilis sdh* operon, are described elsewhere [3]. The construction of pBSD1452 is described below. The shuttle vector pHP13 is described in [12]. *E. coli* strain XL1Blue, plasmid pBluescript KS(−) and helper phage VCS-M13 were purchased from Stratagene and *E. coli* strain ER1451 (JM107 background, *mcrA*$^-$*B*$^-$) was a gift from New England Biolabs. *E. coli* JM83 [13] was used to propagate plasmids pBSD1400 and pBSD1452. *E. coli* cells were kept on LA-plates [14] containing tetracycline (15 μg/ml), chloramphenicol (12.5 μg/ml) or ampicillin (50 μg/ml) when indicated. *B. subtilis* cells were kept on Tryptose Blood Agar Base plates (Difco) containing chloramphenicol (5 μg/ml) and were checked for SQR positive phenotype on minimal salts agar plates [15] with 0.5% (w/v) sodium succinate as the sole carbon and energy source.

2.2. Mutagenesis

In order to obtain single stranded DNA for mutagenesis, a *Sac*I-*Eco*RV fragment containing the *sdhB* gene, encoding the IP of *B. subtilis* SQR, was cloned in plasmid pBluescript KS(−). The resulting plasmid, pFESC 1, was transformed into *E. coli* XL1-blue. XL1-blue/pFESC1

cells were infected with VCS-M13 interference resistant helper phage and single stranded DNA was isolated as recommended by the supplier of the phage (Stratagene). The *sdhB*52 mutation (corresponding to a SQ → CG substitution in the C-terminal region of IP, Fig. 1) was introduced using the phosphorylated oligonucleotide 5′CACAGTTTCCGCAGTTGCGCC3′ (MIP2) and the mutagenesis method described by Vandeyar et al. [16]. The method is based on the selective methylation of the mutant strand by the incorporation of 5-methyl-dCTP, followed by restriction enzyme digestion (*Msp*I and *Hha*I) that nicks only the non-methylated strand. The parent strand is subsequently removed by treatment with exonuclease III. The plasmid containing the mutation is recovered by transformation of a *mcrA*$^-$*B*$^-$ *E. coli* strain (ER1451), i.e., a strain that does not restrict methylated DNA. After DNA sequence analysis the *Bsi*WI-*Eco*RV fragment containing the mutation was exchanged for that of the wild-type in plasmid pBSD1400. The resulting plasmid containing the *B. subtilis sdhCAB* operon with the *sdhB*52 mutation was named pBSD1452.

2.3. General genetic techniques

Restriction enzymes and T4-DNA ligase were from Boehringer-Mannheim and were used as recommended. Agarose gelelectrophoresis were done as described [14] DNA fragments were isolated from agarose using Gene Clean (Bio101, La Jolla). DNA sequence analyses were done by the dideoxy chain-termination method with the Sequenase version II kit (USB) and [α-^{35}S]dATP. *E. coli* cells were transformed by electroporation. Plasmids were introduced into *B. subtilis* cells by protoplast transformation as described by Chang and Cohen [17].

2.4. Redox-titrations and EPR-spectroscopy

Redox titrations were performed at room temperature (25° C) in a closed vessel flushed with oxygen-free argon and equipped with an Ag/AgCl-platinum combination electrode and a pH-electrode. The potential of the reference electrode was calibrated versus a saturated solution of quinhydrone (285 mV vs. NHE at pH 7). The following mediators, dissolved in DMSO, were used at 25 μM final concentration: 5,5′-indigo disulfonate, 5,5′,7-indigo trisulfonate, 5,5′,7,7′-indigo tetrasulfonate, 2-hydroxy-1,4-naphthoquinone, 1,2-naphthoquinone 4-sulfonate and 1,4-naphthoquinone 2-sulfonate. In total, less than 0.15% DMSO was added. Titrations were carried out by stepwise addition of an anaerobic dithionite solution, and samples were transferred anaerobically to EPR-tubes and frozen in a cold mixture of isopentane:methylcyclohexane (5:1) and stored in liquid nitrogen until used. Alternatively, membranes or isolated SQR were poised with different ratios of succinate/fumarate at 20 mM total concentration under argon. After mixing, the samples were allowed to equilibrate for 2 min at room temperature prior to freezing.

EPR-spectra were recorded using an X-band Bruker ESP-300E EPR spectrometer equipped with an Oxford instruments ESR-9 helium flow cryostat.

2.5. Miscellaneous methods

B. subtilis cells for membrane preparation were grown in batches of 1 liter in nutrient sporulation medium with phosphate (NSMP, [18]) supplemented with chloramphenicol (5 mg/l) in indented 6 liter E-flasks at 37° C, aerated at 200 rpm, and harvested 1 h after the end of exponential growth phase. Membranes were prepared and SQR was isolated as described previously [4]. Heme content was determined from the pyridine hemochromogen difference spectrum using the extinction coefficient 29 $mM^{-1}\,cm^{-1}$ (558 nm minus 570 nm, [19]). Protein was determined with the Lowry procedure using bovine serum albumin as standard. Covalently bound FAD and SQR antigen were quantitated as previously described [4].

SQR enzyme activity measurements were performed aerobically at 30° C, with excess substrates, using dichlorophenolindophenol (DCPIP), and phenazine methosulfate (PMS) or 2,3-dimethoxy-5-methyl-6-(3,7-dimethyl-2,6-octadienyl)-1,4-benzoquinone (Q_1) as primary electron acceptors as indicated [4]. The extinction coefficient 20.7 $mM^{-1}cm^{-1}$ at 600 nm was used for DCPIP. The inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) was used at 20 μM (for redox titrations), and at various concentrations to determine K_i from Dixon plots.

Optical spectroscopy was conducted with a Shimadzu UV3000 spectrophotometer at room temperature, using a cuvette with a 1 cm light path.

3. Results and discussion

3.1. Construction of mutant SQR with a SQ → CG substitution in the IP subunit

The serine residue in the putative S3 cysteine ligation motif in the *B. subtilis* IP subunit (S214) was changed to a cysteine by site-directed mutagenesis. In order to mimic the sequence of *Peptococcus aerogenes* ferredoxin and *E. coli* QFR, the neighboring glutamine (Q215) was simultaneously changed to a small amino acid, glycine (Fig. 1). The presence of only the desired mutation was confirmed by DNA sequence analysis. Plasmids pBSD1400 (encoding wild-type SQR), pBSD1452 (encoding SQR with mutated IP), and pHP13 (vector without *sdh* genes) respectively were introduced into *B. subtilis* strain ICD2, that has the entire *sdh* operon deleted from the chromosome. SQR is overproduced about three-fold in ICD2/pBSD1400 compared to normal *B. subtilis* strains with one copy of the *sdh* operon in the chromosome.

The mutant SQR supported growth of *B. subtilis* ICD2/pBSD1452 on minimal medium containing succinate as the sole carbon and energy source, growth conditions that require a functional SQR. ICD2/pBSD1400 and ICD2/pHP13 were used as positive and negative controls for growth under the same conditions. There was no clear difference in the growth rate of strains ICD2/pBSD1452 and ICD2/pBSD1400 in liquid media, i.e., minimal succinate or NSMP. This demonstrates that the SQR with mutant IP is functional.

3.2. Mutant SQR is enzymatically impaired and less stable than wild-type

Membranes were isolated from *B. subtilis* ICD2/pBSD1400, ICD2/pBSD1452, and from ICD2/pHP13. A summary of properties of these membranes is presented in Table 1. The content of SQR in ICD2/pBSD1452 membranes was significantly lower than in ICD2/pBSD1400 membranes, whereas the content of cytochrome b_{558} (membrane anchor subunit) was elevated in the former membranes. However, the content of FP in whole cell lysates was similar in strains ICD2/pBSD1400 and ICD2/pBSD1452 (not shown). This indicates that there is no significant difference in expression level of the *sdh* operon from pBSD1452 compared to pBSD1400, and suggests that the mutation affects the stability of SQR in vivo. ICD2/pHP13 did not, as expected, contain any SQR subunits in membranes or in whole cell lysates.

The mutant SQR showed lower enzyme activity than wild-type enzyme as assayed in vitro using DCPIP and PMS or DCPIP and Q_1 as electron acceptors (Table 1). The Q_1 reductase activity of the mutant enzyme was found to be sensitive to the inhibitor HQNO with the same apparent K_i as the wild-type enzyme (0.4 μM, not shown). Optical spectra of membranes showed that heme also in mutant SQR is reducible with succinate, but the extent of reduction was difficult to assess due to the presence of excess cytochrome *b* subunit in these membranes. As in the case of wild-type, enzyme activity with PMS increased when detergent was added to membranes containing mutant SQR, but in contrast to wild-type the activity subsequently declined rapidly ([4], not shown). Due to the decreased stability in the presence of detergent, regardless of the redox state of the enzyme, the mutated enzyme could not be purified.

In succinate dehydrogenase, i.e., preparations consisting of only the FP and IP subunits, iron-sulfur cluster S3 is particularly sensitive to oxidative damage. It has been demonstrated that reconstitution of SQR from succinate dehydrogenase and membrane subunits requires an intact S3, indicating that this cluster interacts with the membrane anchor [1,2]. The decreased stability of the *B. subtilis* SQR with mutant IP compared to the wild-type enzyme, bothin vivo and in vitro, indicates impared interaction between the FP/IP part of the enzyme and the membrane anchor. In the analogous *E. coli* QFR mutant, the mutation was reported not to affect enzyme stability [10].

3.3. Properties of cluster S3 in mutant and wild-type SQR

The iron-sulfur clusters in membranes containing wild-type and mutant SQR were studied by EPR spectroscopy. The low temperature EPR signals in *B. subtilis* membranes are dominated by those from SQR [20]. This was confirmed with membranes from ICD2/pHP13 that does not contain SQR. In ICD2/pBSD1452, containing SQR with the SQ → CG mutation in IP, we found the S3 signal in oxidized membranes, with an altered EPR spectral lineshape and power saturation behavior compared to that in the wild-type (Fig. 2, Table 2). The S1 and S2 signals were normal. In dithionite reduced membranes we did not detect any conversion of the trinuclear cluster to tetranuclear. We conclude that cluster S3 remained trinuclear in spite of the introduction of a cysteine residue in the cluster ligation motif, but that the S3 environment specifically was affected by the mutation.

The observed redox midpoint potential of S3 in the wild-type and the mutant SQR differed only by 30 mV, and was in good correlation with data previously reported for the wild-type ([21], Table 2).

The S3 signal of *B. subtilis* wild-type SQR is much broader than those of *E. coli* SQR and QFR and also shows much faster spin relaxation (Sled' et al., unpub-

Table 1
Properties of *B. subtilis* membranes containing wild-type SQR, or SQR with mutant IP subunit, and of membranes without SQR

Membranes from:	nmol heme [a]/ mg protein	nmol FP [b]/ mg protein	nmol FAD [c]/ mg protein	mol heme/ mol FP	Turnover no. [d] PMS, (s^{-1})	Turnover no. [d] Q_1, (s^{-1})
ICD2/pBSD1400	2.54	1.32	1.28	1.98	46	16
ICD2/pBSD1452	3.05	0.73	0.57	4.17	18	7
ICD2/pHP13	0.15	n.d.	n.d.	–	n.d.	n.d.

[a] Protoheme IX determined as pyridine hemochromogen [19].
[b] Determined as antigen by rocket immunoelectrophoreses [4].
[c] Determined as covalently bound FAD [4].
[d] Assayed at 30° C, and calculated as mol DCPIP reduced per mol covalently bound FAD per s.
n.d., not detectable.

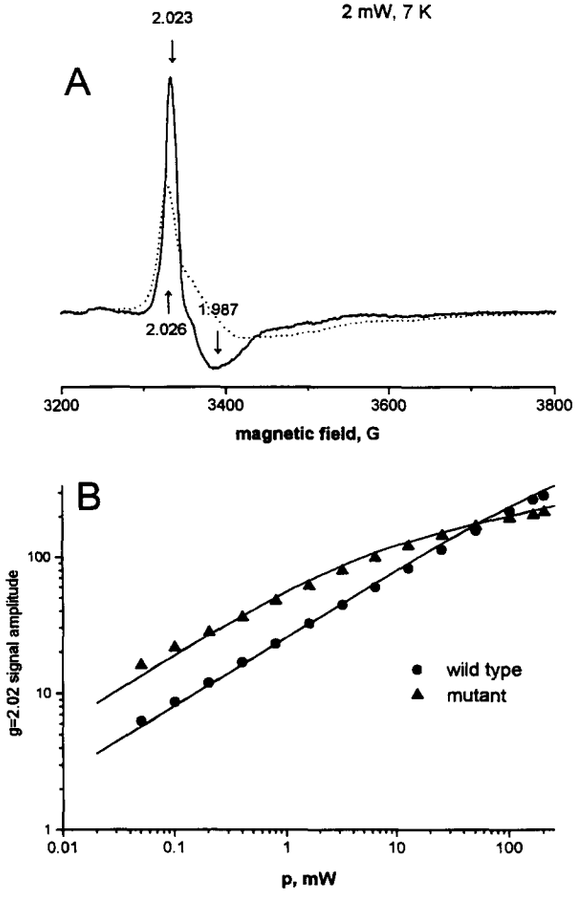

Fig. 2. (A) EPR spectra of *B. subtilis* wild-type SQR (dotted line) and SQR with mutant IP subunit (solid line) in membranes oxidized by 20 mM fumarate. The ICD2/pBSD1400 (wild-type) membrane preparation contained 46 μM SQR and the ICD2/pBSD1452 (mutant) membrane preparation contained 21 μM SQR. The signal amplitude has been normalized to correspond to the same concentration of SQR. Peak positions are indicated with arrows. EPR conditions were: microwave frequency, 9.4455 GHz; modulation amplitude, 10 G; microwave power, 2 mW; time constant, 0.164 s; scan rate, 6G/s; sample temperature, 7 K. (B) Power saturation behavior of the $g = 2.02$ EPR signal in oxidized ICD2/pBSD1400 and ICD2/pBSD1452 membranes.

lished data, [22]). The S3 signal of the *B. subtilis* mutant SQR was found to behave more like the *E. coli* wild-type counterparts (Fig. 2).

The overall structure of the IP subunit in various organisms seems similar, but the midpoint potential of the respective iron-sulfur clusters varies depending on how the enzyme operates in vivo. In SQRs interacting with ubiquinone, the E_m of S3 is higher than that in QFRs or in SQRs interacting with low-potential quinones. S1 has a high E_m (higher than S3) in SQRs interacting with low-potential quinones, an intermediate E_m (lower than S3) in SQRs interacting with ubiquinone and a low E_m in QFRs. Cluster S2 generally has a very low midpoint potential and may not participate in electron transfer [1,2]. The aerobic, Gram-positive bacterium *B. subtilis*, contains only menaquinone ($E_m = -74$ mV, [23,24]) and has an S1 with $E_m = +80$ mV and a S3 with $E_m = -27$ mV ([21], this work). In *B. subtilis*, the membrane anchor subunit of SQR contains two *b* hemes with $E_m = +13$ mV (b_H) and -132 mV (b_L). In contrast, *E. coli* SQR, that operates with ubiquinone ($E_m = +112$ mV, [23]) contains only heme b_H, and *E. coli* QFR, that in vivo operates with menaquinone, does not contain any heme [1,2]. The role of cluster S3 in electron transfer to quinone, and thus the properties of the cluster, may therefore be different in these succinate:quinone oxidoreductases.

3.4. Cluster S3 in wild-type SQR is sensitive to methanol

It was noted that the S3 signal, but not the S1 or S2 signal in wild-type *B. subtilis* SQR was extremely sensitive to methanol. Within minutes in the presence of 0.2% to 1% (v/v) methanol the lineshape and power saturation behavior of the S3 signal dramatically changed, from broad to narrow and from extremely fast to slow relaxing, whereas the midpoint potential of the cluster was little affected (Table 2, Fig. 3). This effect of methanol on S3 was seen both with membranes and with the isolated enzyme. Interestingly, ethanol of corresponding concentra-

Table 2
Properties of cluster S3 in *B. subtilis* membranes containing wild-type SQR or SQR with mutant IP subunit, in the absence and presence of 1% (v/v) methanol, as determined by EPR spectroscopy

Properties	ICD2/pBSD1400 membranes (wild-type SQR)		ICD2/pBSD 1452 membranes (SQ → CG mutant SQR)	
		+ methanol		+ methanol
$g_{z,y,x}$ [a]	2.017, 1.978, 1.920	2.014, 2.005, 2.001	2.014, 1.992, 1.955	2.014, 1.992, 1.955
$L_{z,y,x}$ (G) [a]	8, 34, 70	4.8, 16, 26	4.9, 24, 48	4.9, 24, 48
$P_{1/2}$ (mW)	> 300	1.2 ± 0.1	7.1 ± 1.2	7.1 ± 1.2
E_m (mV) [b]	-27.5 ± 4.2	-15.8 ± 8.0	-57.4 ± 4.2	-40 ± 6.9

[a] $g_{z,y,x}$ and $L_{z,y,x}$ were obtained from simulations of the EPR spectra under non-saturated conditions, assuming no hyperfine interaction and gaussian lineshapes, as described in [38].

[b] Determined from reductive titrations with dithionite at room temperature in the presence of dyes. EPR spectra were recorded at 6 K, 20 mW, and at 10 K, 10 mW, in the presence and absence of methanol, respectively (other EPR conditions were as described in Fig. 2). Essentially the same midpoint potentials were obtained in titrations using the succinate/fumarate couple to poise the potential of the system.

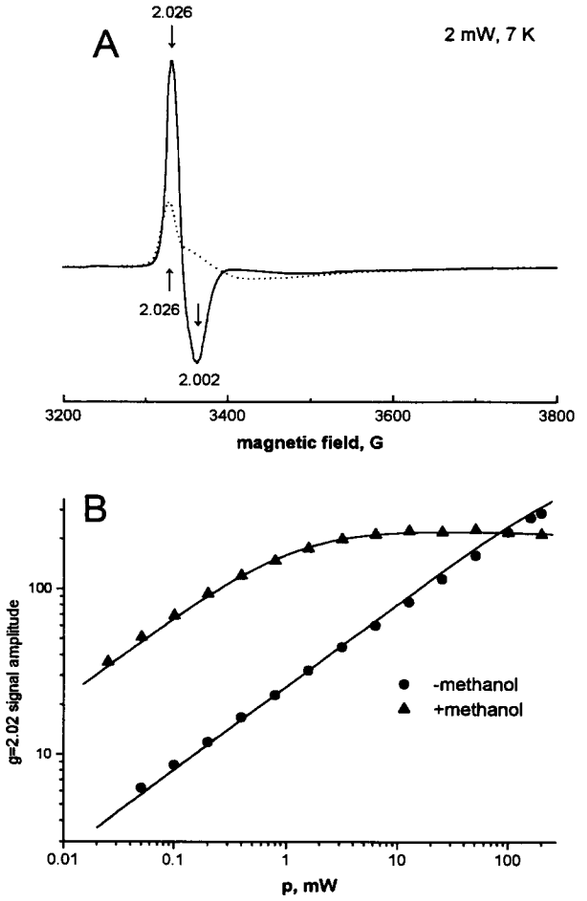

Fig. 3. (A) EPR spectra of oxidized *B. subtilis* ICD2/pBSD1400 membranes, which contain wild-type SQR, in the presence of 1% (v/v) methanol (solid line) and without additions (dotted line). Peak positions are indicated with arrows. EPR conditions were the same as in Fig. 2. (B) Power saturation behavior of the $g = 2.026$ signal in oxidized ICD2/pBSD1400 membranes in the presence and absence of 1% (v/v) methanol.

tions did not cause these changes. The effect on S3 was reversible, i.e., a near normal S3 signal was restored when methanol was washed off from membranes (not shown). Cluster S3 of SQR with the SQ → CG mutation in the IP subunit was, in contrast, insensitive to methanol (Table 2). The presence of 1% (v/v) methanol did not affect succinate reductase activity in vitro using DCPIP and PMS or Q_1 as electron acceptors (not shown). The effect of methanol but not ethanol can possibly be explained such that methanol fits into a small pocket, where it affects the S3 micro environment. This pocket is then absent or perturbed in the mutant enzyme, which makes it completely insensitive to methanol.

3.5. Cluster S3 is not affected by HQNO, a powerful inhibitor of succinate:quinone oxidoreductase activity

EPR investigations of the semiquinone spin-spin interaction in mitochondrial SQR have suggested that S3 is positioned close to the quinone binding site [25,26]. Thenoyltrifluoroacetone (TTFA) and carboxins are potent inhibitors of the mitochondrial SQR. They seemingly act at the same site, blocking the reduction of quinone. Carboxin is assumed to bind to the membrane anchor subunits, but at a site which is in close proximity to the cluster S3 and in a way that blocks electron transfer to ubiquinone [1,27]. A carboxin-resistant mutant SQR of *Ustilago maydis* was recently shown to have a histidine to leucine substitution within the putative S3 ligation motif ([28], Fig. 1). Addition of TTFA to pigeon heart SQR increases the EPR signal amplitude of S3, shifts E_m of S3 by about −50 mV, and quenches the hyperfine splitting originating from the semiquinone pair [29]. *B. subtilis* SQR is not inhibited by TTFA or carboxins but is sensitive to HQNO [4,30]. We could not detect any effect of HQNO on the *B. subtilis* S3 signal lineshape or amplitude. The E_m of S3 in membrane-bound SQR was determined to −58 mV in the presence of HQNO, which is to be compared to −27 mV in the absence of HQNO (Table 2). Furthermore, the *B. subtilis* mutant enzyme has the same apparent K_i for HQNO as the wild-type. These observations add support to the view that two independent quinone binding sites are present in *B. subtilis* SQR [30]. One site is close to the negative side of the membrane, near heme b_H and S3 and the other site, where HQNO mainly acts [30], is close to the positive side of the membrane (near heme b_L).

4. Conclusion

Since a tri- to tetranuclear cluster conversion did not occur when an extra cysteine was inserted in the S3 cluster ligation motif, it is reasonable to assume that a cysteine residue in this position in *B. subtilis* IP is somehow sterically hindered to act as a ligand for a tetranuclear cluster, whereas in *E. coli* QFR this is not the case. In the *B. subtilis* IP the SQ → CG mutation is apparently sufficiently close to the trinuclear cluster to alter the EPR properties of the S3 signal, but does not significantly change the midpoint potential of the cluster. In spite of this, the enzyme activity of the mutant SQR is about 35% compared to wild-type SQR. We conclude that S3, or amino acid residues in the putative S3 ligation motif may be important for stabilizing and/or donating electrons to quinone, as also suggested by the position of a mutation conferring resistance to carboxin in *U. maydis* (Fig. 1, [28]).

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Medical Research Council and the Emil and Wera Cornells Stiftelse to L.H. and NSF grant MCB-9119300 to T.O. We thank Karin Tsiobanelis for technical assistance.

References

[1] Ackrell, B.A.C., Johnson, M.K., Gunsalus, R.P. and Cecchini, G. (1992) *in* Chemistry and Biochemistry of Flavoenzymes (Mueller, F., ed.), Vol. 3, pp. 229–297, CRC Press, Boca Raton.

[2] Hederstedt, L. and Ohnishi, T. (1992) *in* Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 163–198, Elsevier, Amsterdam.

[3] Hägerhäll, C. (1994) Doctoral thesis, ISBN 91-628-1273-4, University of Lund, Sweden.

[4] Hägerhäll, C., Aasa, R., Von Wachenfeldt, C. and Hederstedt, L. (1992) Biochemistry 31, 7411–7421.

[5] Aevarsson, A. and Hederstedt, L. (1988) FEBS Lett. 232, 298–302.

[6] Johnson, M.K., Kowal, A.T., Morningstar, J.E., Oliver, M.E., Whittaker, K., Gunsalus, R.P, Ackrell, B.A.C. and Cecchini, G. (1988) J. Biol. Chem. 263, 14732–14738.

[7] Maguire, J.J. and Hederstedt, L. (1989) FEBS Lett. 256, 195–199.

[8] Werth, M.T., Cecchini, G., Manodori, A., Ackrell, B.A.C., Schröder, I., Gunsalus, R.P. and Johnson, M.K. (1990) Proc. Natl. Acad. Sci. USA 87, 8965–8969.

[9] Adman, E.T., Sieker, L.C. and Jensen, L.H. (1976) J. Biol. Chem. 251, 3801–3806.

[10] Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R.P., Werth, M.T. and Johnson, M.K. (1992) Biochemistry 31, 2703–2712.

[11] Rothery, R.A. and Weiner, J.H. (1993) Biochemistry 32, 5855–5861.

[12] Haima, P., Bron, S. and Venema, G. (1987) Mol. Gen. Genet. 209, 335–342.

[13] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–109.

[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, 2nd Edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[15] Spizizen, J. (1958) Proc. Natl. Acad. Sci. USA 44, 1072–1078.

[16] Vandeyar, M.A., Weiner, M.P., Hutton, C.J. and Batt, C.A. (1988) Gene 65, 129–133.

[17] Chang, S. and Cohen, S.N. (1979) Mol. Gen. Genet. 168, 111–115.

[18] Fortnagel, P. and Freese, J. (1968) J. Bacteriol. 95, 1431–1438.

[19] Falk, J.E. (1964) in Biochim. Biophys. Acta Library, Vol. 2, pp. 240–241, Elsevier, Amsterdam, .

[20] Hederstedt, L., Maguire, J.J., Waring, A.J. and Ohnishi, T. (1985) J. Biol. Chem. 260, 5554–5562.

[21] Maguire, J.J., Magnusson, K. and Hederstedt, L. (1986) Biochemistry 25, 5202–5208.

[22] Morningstar, J.E., Johnson, M.K., Cecchini, G., Ackrell, B.A.C. and Kearney, E.B. (1985) J. Biol. Chem. 260, 13631–13638.

[23] Thauer, R.K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. 41, 100–180.

[24] Liebl, U., Pezennec, S., Riedel, A., Kellner, E. and Nitschke, W. (1992) J. Biol. Chem. 267, 14068–14072.

[25] Ingeldew, W.J., Salerno, J.C. and Ohnishi, T. (1976) Arch. Biochem. Biophys. 177, 176–184.

[26] Miki, T., Yu, L. and Yu, C-A. (1992) Arch. Biochem. Biophys. 293, 61–66.

[27] Ramsay, R.R., Ackrell, B.A.C., Coles, C.J., Singer, T.P., White, G.A. and Thorn, G.D. (1981) Proc. Natl. Acad. Sci. USA 78, 825–828.

[28] Keon, J.P.R., Broomfield, E., White, G.A. and Hargreaves, J.A. (1994) Biochem. Soc. Trans. 22, 234–237.

[29] Ingledew, W.J. and Ohnishi, T. (1977) Biochem. J. 164, 617–620.

[30] Smirnova, I., Hägerhäll, C., Konstantinov, A.A. and Hederstedt, L. (1995) FEBS Lett. 359, 23–26.

[31] Yao, Y., Wakabayashi, S., Matsuda, S., Matsubara, H., Yu, L. and Yu, C.-A. (1986) in Iron-sulfur Protein Research (H. Matsubara, Y. Katsube and K. Wada, eds.), pp. 240–244, Springer, Berlin,

[32] Darlison, M.G. and Guest, J.R. (1984) Biochem. J. 223, 507–517.

[33] Bach, M., Reiländer, H., Gärtner, P., Lottspeich. F. and Michel., H. (1993) Biochim. Biophys. Acta 1174, 103–107.

[34] Lauterbach, F., Körtner, C., Albracht, S.P.J., Unden, G. and Kröger, A. (1990) Arch. Microbiol. 154, 386–393.

[35] Cole, S.T., Grundström, B., Jaurin, J., Robinson, J. and Weiner, J.H. (1982) Eur. J. Biochem. 126, 211–216.

[36] Phillips, M.K., Hederstedt, L., Hasnain, S., Rutberg, L. and Guest, J.R. (1987) J. Bacteriol. 169, 864–873.

[37] Adman, E.T., Sieker, L.C. and Jensen, L.H. (1973) J. Biol. Chem. 248, 3987–3996.

[38] Blum, H. and Ohnishi, T. (1980) Biochim. Biophys. Acta 621, 9–18.