

Biological Sulfide Oxidation: Sulfide-Quinone Reductase (SQR), the Primary Reaction

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Abbreviations used are: SQR, sulfide-quinone reductase; FCC, flavocytochrome *c*; ET, electron transport; Q, quinone; UQ, ubiquinone; PQ, plastoquinone; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; bp, basepairs; NQNO, nonylhydroxyquinoline-N-oxide; HQNO, heptylhydroxyquinoline-N-oxide.

ABSTRACT

Inorganic reduced sulfur compounds as sulfide can serve as electron donors for phototrophic and lithotrophic growth of Bacteria and Archaea. For the initial step of sulfide oxidation mainly two enzymatic systems are discussed, flavocytochrome *c* and sulfide-quinone reductase. Sulfide-quinone reductase (SQR), an ancient flavoprotein, is obligatory for growth on sulfide as hydrogen donor in photo- and chemolithoautotrophic bacteria. During the last ten years it has been studied in detail for the cyanobacterium *Oscillatoria limnetica* and the proteobacterium *Rhodobacter capsulatus*. In both cases the enzyme has been purified to homogeneity, and the genes have been cloned, sequenced and expressed in *E. coli*. SQR is widely spread among Eubacteria, as documented by the occurrence of genes and/or activities, the latter even including mitochondria from lower animals. In this review latest results about SQR, its function, occurrence and expression are summarized, and implications from gene sequences and biotechnological applications are discussed.

1. INTRODUCTION: PHOTO- AND CHEMOLITHO-TROPHIC GROWTH ON SULFIDE

For more than a century, conversion of inorganic sulfur compounds by biological processes has been well established. Hydrogen sulfide, the most reduced form of inorganic sulfur, occurs in hydrothermal vents, as well as in sediments, where it is generated by sulfate-reducing bacteria [1,2]. Although hydrogen sulfide is toxic for most organisms, mainly because of the inhibition of aerobic respiration [3], it serves as electron donor for the energy generating systems of photo- and chemolithotrophic bacteria (for reviews see [4-

6]), of some archaea [7] and even of eukarya (reviewed in [8]).

During autotrophic growth of bacteria, hydrogen sulfide provides electrons for reduction of NAD^+ that is required for CO_2 fixation. The midpoint potential of the NAD^+/NADH couple at pH 7 is approximately 50 mV more negative than the midpoint potential of the $\text{S}^0/\text{H}_2\text{S}$ couple [9]. Therefore, energy is required to transport electrons from hydrogen sulfide upwards to NAD^+ (see **Fig. 1**). In all bacteria studied so far, the transport of electrons from sulfide to NAD^+ is mediated by membrane bound electron transport (ET). This is depicted in **Fig. 1** and **Fig. 2** for photosynthetic and respiratory (chemolithotrophic) proteobacteria. The electrons from sulfide enter the chain either at the level of quinone, via a sulfide:quinone oxidoreductase (SQR) - depending on the quinone species the midpoint potential of the Q/QH_2 couple is 100-300 mV more positive than the $\text{S}^0/\text{H}_2\text{S}$ couple - or at the level of *c*-type cytochromes via a sulfide:cytochrome *c* oxidoreductase (flavocytochrome *c*, FCC). *C*-type cytochromes exhibit midpoint potentials that are 500-700 mV more positive than the midpoint potential of the sulfide couple.

Phototrophic bacteria use light for the upward transport of electrons from sulfide to NAD^+ . In proteobacteria and *Chloroflexaceae* the photosystem contains a quinone-type reaction center [10]. As depicted in **Fig. 1**, the electrons are first transferred to quinone and have to be transported uphill to NAD^+ by reverse ET through the NADH dehydrogenase. This reverse transport requires an electrochemical proton potential ($\Delta\mu\text{H}^+$) across the cytoplasmic membrane that is generated by cyclic ET through the photosystem. As far as sufficient light is available, it makes no significant

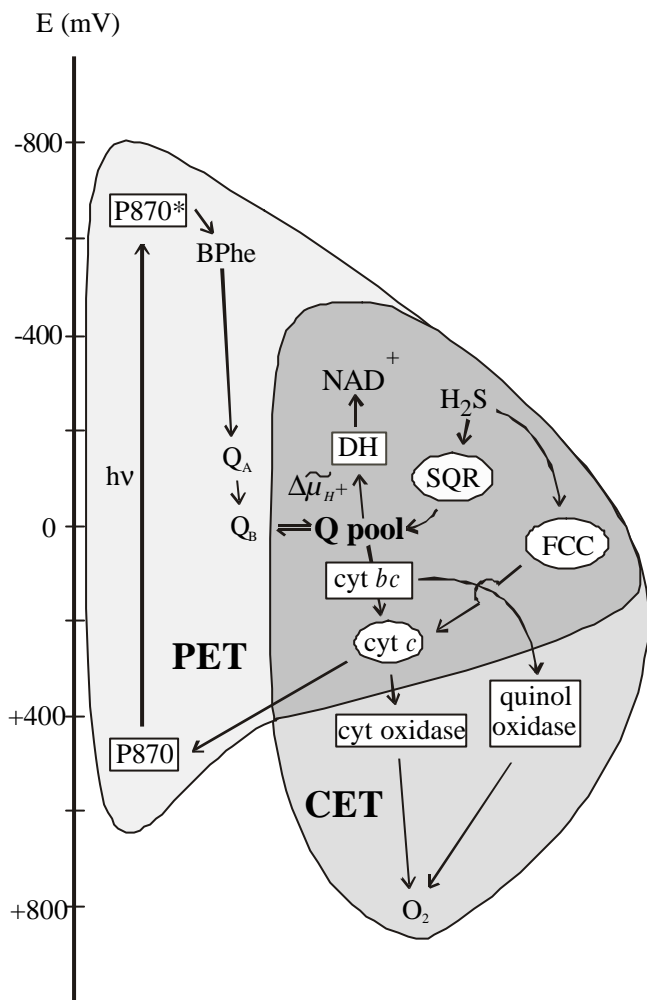


Fig. 1: Redox Scale of Photo- and Chemolithotrophic Electron Transport from Sulfide in Proteobacteria.

Abbreviations used: PET, photosynthetic electron transport; CET, chemolithotrophic electron transport; P870, special pair chlorophyll; BPhen bacteriopheophytin, Q_A and Q_B , quinone-binding sites of the reaction center; DH, NADH-dehydrogenase; *cyt bc*, cytochrome *bc* complex; *cyt c*, cytochrome *c*; *cyt oxidase*, cytochrome oxidase.

difference, whether electrons from sulfide enter the ET chain at the level of cytochrome *c* or at the level of quinone, however, at low light this may be decisive. If the electrons enter the ET chain via FCC at the level of cytochrome *c*, they have to be transported upwards to NAD^+ by reverse ET through the cytochrome *bc* complex, the quinone pool and the NADH

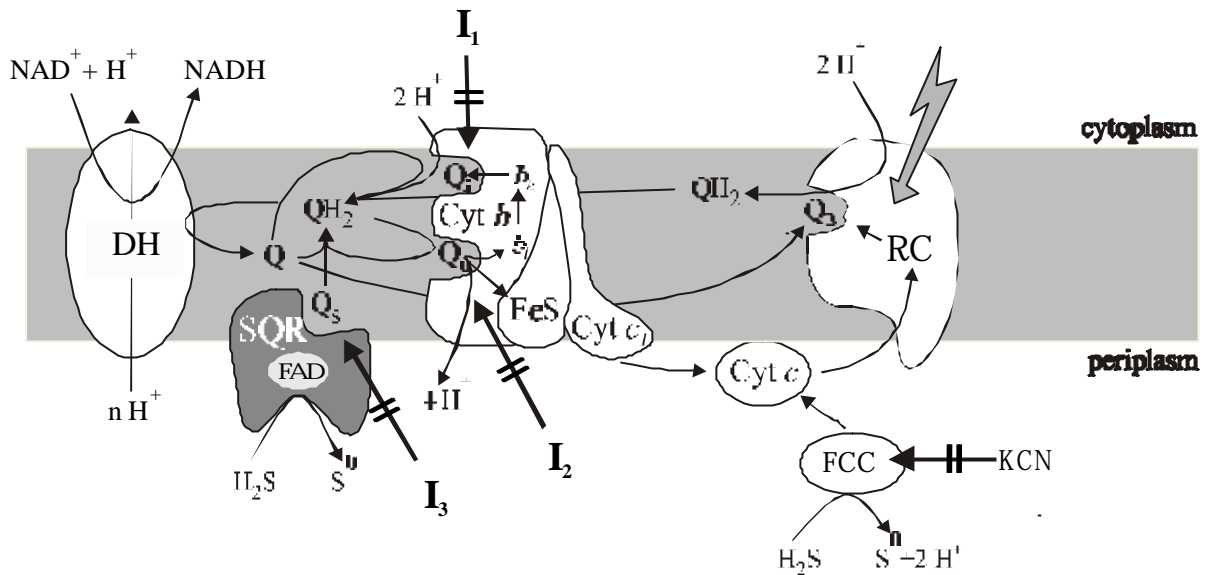
dehydrogenase (**Fig. 2A**). If the electrons enter the ET chain at the level of quinone via SQR, then the cytochrome *bc* complex contributes to the generation of $\Delta\mu H^+$ that is necessary for the upward transport from quinol to NAD^+ through the NADH dehydrogenase. Thus, sulfide oxidation by SQR provides more energy than sulfide oxidation by FCC.

Cyanobacteria do not face this problem, because they are able to transfer electrons from cytochrome *c* to NAD^+ without the help of $\Delta\mu H^+$ via a photosystem of the reaction center I-type and the ferredoxin: NAD^+ oxidoreductase [10]. This may also hold true for *Chlorobiaceae* and heliobacteria, which contain a type-I reaction center as well.

Chemolithotrophic bacteria gain all energy from inorganic substrates by dark redox reactions. Respiratory ET from the H_2S/S^0 to O_2/H_2O , comprising a redox potential difference of about 1 V, serves as the “proton pump” and drives the upward ET from sulfide to NAD^+ (**Fig. 1**). This again is more efficient with SQR than with FCC, which is important at low oxygen pressures, when the oxidases become limiting (**Fig. 2B**). Higher efficiency of energy conservation by sulfide oxidation via SQR over FCC certainly is critical under the mixotrophic and changing conditions in the natural habitat.

Both enzymatic sulfide oxidizing systems, the sulfide:cytochrome *c* oxidoreductase named flavocytochrome *c* (FCC) and sulfide:quinone oxidoreductase (SQR), were isolated from bacteria. FCC is a soluble periplasmic enzyme (**Fig. 2**). It consists of a FAD-binding subunit and a cytochrome *c* subunit. The enzyme is well studied with respect to its catalytic mechanism [11-15], and the three-dimensional structure of FCC from *Allochromatium vinosum* has been solved to a resolution of 2.53Å [16,17]. Although the enzyme was

A phototrophic ET



B chemotrophic ET

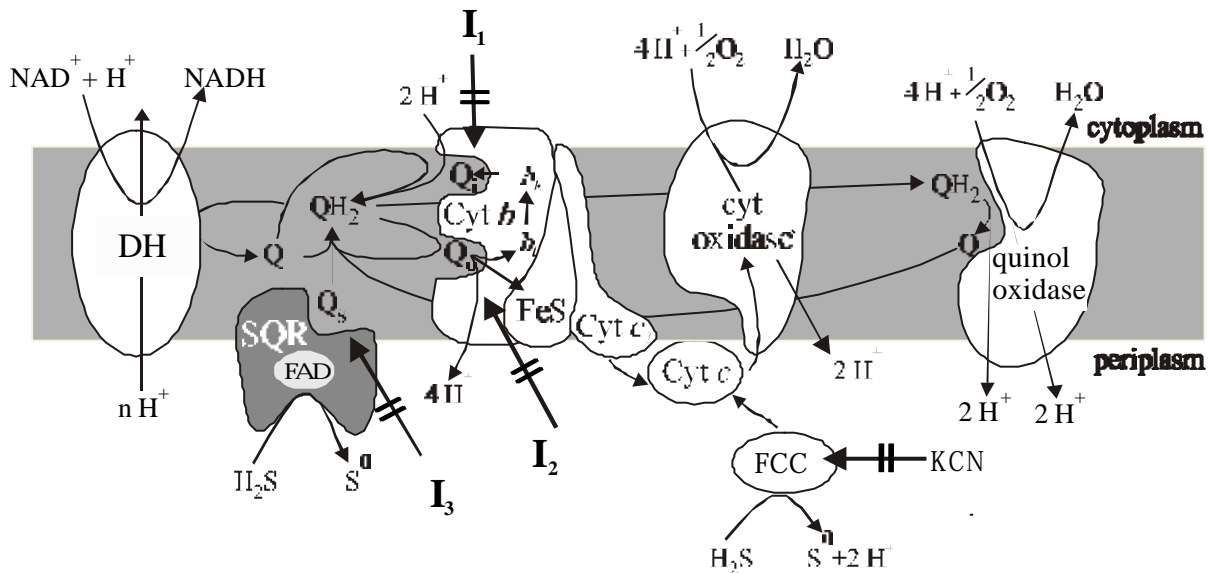


Fig. 2: Topography of Electron Transport from Sulfide in Cytoplasmic Membranes of Proteobacteria. A, phototrophic electron transport and B, chemotrophic electron transport. Sites of inhibition of the cytochrome *bc* complex and of SQR by quinone-analogues are designated as I₁, I₂ and I₃. Inhibitors acting at I₁ are antimycin A, aurachin C, NQNO and HQNO, at I₂ myxothiazol, stigmatellin and at I₃ myxothiazol, antimycin A, stigmatellin, aurachin C, NQNO, HQNO and KCN. (DH, NADH-dehydrogenase; Cyt *b*, FeS and Cyt *c*₁ stand for the cytochrome *b*, Rieske iron sulfur center and cytochrome *c*₁ subunits of the cytochrome *bc* complex; *b*_h and *b*_l, high and low potential heme of cytochrome *b*; Q_i and Q_o, quinone-reduction and quinone-oxidation sites of the *bc* complex; Q_s, quinone-binding site of SQR; Q_B, quinone-reduction site of the photosystem; RC, photosynthetic reaction center; cyt *c*, cytochrome *c*).

found in a variety of chemotrophic and phototrophic sulfide-oxidizing bacteria, it is not obligatory in sulfide oxidation. FCC does not occur in a variety of sulfide-oxidizing bacteria, and the enzyme seems to be confined to species that are also able to oxidize thiosulfate [4,6]. Moreover, mutational inactivation of FCC did not have any significant effect on the sulfide oxidizing ability of the phototrophic sulfur bacterium *A. vinosum* [18].

During the last twenty years evidence has accumulated for the second sulfide oxidation pathway. The enzyme that catalyzes the sulfide-dependent reduction of quinones, the sulfide:quinone oxidoreductase (E.C.1.8.5.1; SQR), is a single polypeptide with an apparent molecular mass of 55 kDa [19,20]. It is a membrane-bound enzyme (**Fig. 2**) that belongs to the glutathion reductase family of flavoproteins. SQR activity has been found to be widely distributed among prokaryotes (see **Fig. 5**), and protein sequence comparison leads to the conclusion that SQR is a phylogenetically very old enzyme, that was acquired early in evolution, as reviewed recently [21].

In the present account, this review will be updated, with some emphasis on SQR from *R. capsulatus*. After a view on the biochemical properties of the enzyme in the membrane and of the isolated protein, characteristics evident from peptide sequence comparison of SQRs are summarized, and some mechanistic aspects of the catalytic reaction are discussed. Finally we focus on some aspects of the regulation of SQR expression and give a glimpse on possible applications of sulfide-oxidizing bacteria.

2. DISCOVERY OF SQR

First evidence for the participation of quinones in sulfide oxidation came from the work of Knaff and Buchanan [22]. They found that the sulfide-dependent photoreduction of NADP^+ in membranes of the green sulfur bacterium *Chlorobium thiosulfatophilum* (Tassarjara) was sensitive towards inhibitors of cytochrome *bc* and *b₆f* complexes, which are membrane-bound quinol:cytochrome *c* oxidoreductases. As shown in **Fig. 2**, these inhibitors are quinone analogues that compete with the quinone substrate for the quinone-binding sites of these complexes (for reviews see [23,24]). More than ten years later, Brune and Trüper [25] found that membrane vesicles of the purple "non-sulfur" bacterium *Rhodobacter sulfidophilus* photoreduce NAD^+ with sulfide as the electron donor in a reaction sensitive towards quinone-analogues, and they demonstrated that sulfide oxidation in the dark leads to the reduction of quinones within the membranes. On the basis of their observations they suggested that the electrons from sulfide enter the membrane-bound photosynthetic ET chain at the level of quinone.

The cyanobacterium *Oscillatoria limnetica* switches from oxygenic plant type photosynthesis to anoxygenic photosynthesis in presence of sulfide [26]. Sulfide-dependent formation of a proton gradient across the thylakoid membranes of *O. limnetica* was observed [27], which was again blocked by inhibitors of *bc* and *b₆f* complexes.

Furthermore, Arieli et al. [28] established an assay for measuring sulfide-quinone oxidoreductase activity by monitoring the reduction of externally added quinones in a dual wavelength spectrophotometer. They found that in presence of thylakoid membranes from sulfide-induced *O. limnetica* cells externally added quinone becomes rapidly reduced by sulfide in the dark. This reaction was sensitive towards quinone analogues (**Tab.**

1). From their results they proposed the following reaction scheme for the sulfide-dependent ET in membranes. Sulfide becomes oxidized by a membrane-bound SQR, and the electrons are transferred to quinone at the quinone-binding site of SQR. Subsequently, the cytochrome *b₆f* complex reoxidizes quinol and reduces plastocyanine that transfers the electrons to photosystem I. As a result, a proton gradient is formed across the membrane by the Q-cycle mechanism of the *b₆f* complex (for a review of the Q-cycle see [24]).

Participation of the energy gaining Q-cycle mechanism in the sulfide-dependent ET pathway via SQR was first monitored in membranes of the purple "non-sulfur" bacterium *R. capsulatus* by time resolved measurements of the sulfide-dependent reduction of membrane-bound *b*- and *c*-type cytochromes [29]. Up to now, membrane-bound SQR activity and subsequent oxidation of quinol by the Q-cycle mechanism was found in a variety of phototrophic and chemotrophic bacteria (see **Fig. 5**) [18,21,28-34]. In **Fig. 2** models of the sulfide oxidation pathway as evident from measurements with membranes from phototrophic and chemotrophic proteobacteria are shown.

3. PROPERTIES OF SQR

3.1. PROPERTIES OF SQR BOUND TO THE MEMBRANE

Properties of the best studied SQRs in membranes are summarized in **Tab. 1**. Where determined, K_m values of SQRs for sulfide and externally added quinone substrates are in the micromolar range. The highest specific SQR-activity was found in membranes of the hyperthermophilic, chemotrophic bacterium *Aquifex aeolicus*. At room temperature, it was more than tenfold higher than the activities measured in membranes from proteobacteria as

well as from the cyanobacterium *O. limnetica* and the green sulfur bacterium *Chlorobium*. Close to the optimal growth temperature of *Aquifex* (85°C [35]), the specific activity was even more than 50 times higher. It is so far unknown, whether this high activity is due to a high amount of enzyme in membranes or to a high turnover rate. However, this high activity suggests that hydrogen sulfide, besides hydrogen, is a main electron source for this bacterium that was isolated from marine hydrothermal vents [35].

All SQR enzymes were sensitive towards quinone-analogues at micromolar or nanomolar concentrations (**Tab. 1**; in **Fig. 2** this inhibition is represented by the site I_3 , while I_1 and I_2 reflect the two Q-binding sites on the *bc*-complexes [24]). Sensitivity towards these inhibitors is a characteristic feature of quinone-binding enzymes. Except for the enzyme from *Paracoccus denitrificans*, aurachin C was the most potent inhibitor of sulfide-dependent reduction of externally added quinone by SQR. In contrast to the enzyme from proteobacteria and green sulfur bacteria, SQR of the cyanobacterium *O. limnetica* was insensitive towards the inhibitors myxothiazol and antimycin A. Both are inhibitors of ubiquinol-oxidizing cytochrome *bc₁* complexes of proteobacteria and mitochondria, but do not inhibit the plastoquinol-oxidizing *b₆f* complexes present in thylakoids of cyanobacteria and plants (for reviews see [23,24]). The menaquinol-oxidizing *bc* complex of *Chlorobiaceae* combines properties of both [36] and is antimycin A sensitive [31]. This suggests that the different sensitivities of SQRs towards quinone-analogues reflect differences of the quinone substrates naturally occurring in membranes.

Cyanide is a potent inhibitor of many flavoproteins in micromolar concentrations [37] and inhibits sulfide oxidation by FCC

Tab. 1: SQR-activity in bacterial membranes, substrate affinity and sensitivity towards inhibitors. K_m values for sulfide-quinone reductase (SQR), average specific activity of sulfide-dependent quinone reduction at 20°C and the efficiency of inhibitors in membranes of *Aquifex aeolicus* [34], *Paracoccus denitrificans* [32], *Rhodobacter capsulatus* [29], *Allochromatium vinosum* [18], *Oscillatoria limnetica* [19,28] and *Chlorobium limicola* f. *thiosulfatophilum* [30]. For *A. vinosum* membranes, the quinone substrate was duroquinone [18]. I_{50} -values give the micromolar concentrations of the inhibitors for 50 % inhibition (unit, μmol quinone reduced min^{-1} ; d-UQ, decyl-ubiquinone; PQ-1, plastoquinone-1; none, no inhibition; nd, not determined).

	<i>A. aeolicus</i>	<i>P. denitrificans</i>	<i>R. capsulatus</i>	<i>A. vinosum</i>	<i>O. limnetica</i>	<i>C. limicola</i>
K_m (μM)						
H ₂ S	11	26	5	nd	8	nd
Quinone	5 (d-UQ)	3 (d-UQ)	2 (d-UQ)	nd	32 (PQ-1)	<20 (PQ-1)
Specific activity [unit (mg protein) ⁻¹]	3.5 15 (70°C)	0.3	0.1	0.14	0.02	nd
I_{50} (μM)						
Myxothiazole	43	22	43	4	none	6
Stigmatellin	20	20	2.5	nd	6.7	0.005
HQNO	12	nd	nd	0.5	nd	nd
Antimycin A	10	15	50	~10	none	0.096
NQNO	6	nd	2.4	nd	0.14	0.8
Aurachin C	0.014 ¹	28 ²	0.23 ¹	nd	0.050 ¹	0.012 ¹
KCN	none	none	120	500	12	10

¹ 2-Methyl-3-(3,7,11-trimethyl-2,6,10-dodekatrienyl)-1-hydroxy-chinolon

² 2-Methyl-3-n-dodecyl-1-hydroxy-chinolon

(**Fig. 2**) both in phototrophic [38] and chemotrophic bacteria [33]. Cyanide was also found to inhibit sulfide-reduction of quinones by SQR in membranes of phototrophic bacteria (**Tab. 1**). In contrast, cyanide did not inhibit SQR-activity in membranes of the chemotrophic bacteria *A. aeolicus* and *P. denitrificans* suggesting differences of SQR enzymes in phototrophic and chemotrophic bacteria. The structural basis for this difference remains unknown and needs to be investigated.

3.2. SUBCELLULAR LOCALIZATION OF SQR

After cell disruption almost all SQR-activity was found in the membrane fraction in all systems that have been investigated until now. Except for the enzyme from *R. capsulatus*, all SQR enzymes are tightly bound to the membrane and were solubilized from membranes only by detergent treatment [18,19,34].

In contrast, SQR from *R. capsulatus* was detached from the membrane by treatment with

2 M sodium bromide [20,39]. At high concentrations, sodium bromide acts as a chaotropic agent removing proteins from membranes that are peripherally bound by hydrophobic interactions. This raised the question, whether the enzyme faces the cytoplasmic or the periplasmic side of the membrane in *R. capsulatus*. Most of the sulfide-oxidizing bacteria deposit sulfur, the product of sulfide oxidation, outside the cells [4] or store the sulfur globules in the periplasmic space [40]. Since *R. capsulatus* deposits sulfur outside the cells as well [41], it seems reasonable that the SQR is attached to the periplasmic surface of the cytoplasmic membrane. Recently, a translational fusion of SQR with the alkaline phosphatase of *E. coli* was constructed and expressed in *R. capsulatus*. It was found that cells expressing the fusion protein exhibited phosphatase activity [42]. Alkaline phosphatase is only active, if it is translocated to the periplasm [43]. This result clearly indicated periplasmic localization of SQR in *R. capsulatus* and suggests similar orientation of the sulfide oxidation site in other bacteria.

Generally, export of proteins across the cytoplasmic membrane of bacteria occurs via two distinct pathways, the sec-dependent and the sec-independent pathway [44]. In both pathways, the proteins that become translocated exhibit a characteristic N-terminal signal peptide for translocation. Surprisingly, no signal peptide was found in the peptide sequence of SQR (see section 4.), although the enzyme becomes translocated. Deletion studies with the alkaline phosphatase fusion protein indicated that the C-terminal 38 amino acid residues are essential for translocation [42]. So far, it is unknown, whether the C-terminus functions as signal peptide for translocation or deletion of the C-terminus hinders translocation as a result from incorrect protein folding. In this context, it is worth mentioning that the

FAD-binding protein dihydroliponamide dehydrogenase from the cyanobacterium *Synechocystis* is also localized at the periplasmic surface of the membrane without exhibiting the characteristic sequences for translocation [45]. The fact that no significant homology exists in the C-terminal region between SQR and dihydroliponamide dehydrogenase argues against a function of the C-terminus as a signal peptide. Possibly, a surface-exposed pattern is necessary for translocation of membrane-bound flavoproteins by a so far unknown mechanism.

Cyanobacteria also deposit the sulfur from oxidation of sulfide outside the cells as well [46,47]. Within cyanobacteria, however, SQR-activity was found in thylakoid membranes [28,48], the intracellular photosynthetic membranes of cyanobacteria [49]. Arieli et al. [28] suggested that the sulfide oxidation site of SQR is located at the cytoplasmic surface of thylakoid membranes in *O. limnetica*. This would require transport of sulfur through the cytoplasm and across the cytoplasmic membrane. However, the thylakoid lumen corresponds phylogenetically to the periplasmic space of bacteria, and, in analogy to the orientation in bacteria, localization of the sulfide oxidation site at the luminal side seems more plausible. If there is no continuity between the thylakoid lumen and the periplasmic space - continuities between the cytoplasmic and the thylakoid membranes are still a matter of debate [49] - , a more complex machinery for export of sulfur would be necessary. For a variety of cyanobacteria, contact points between the plasma and thylakoid membrane have been noted. Concentration of SQR at these contact points would make transport of sulfur through the cytoplasm unnecessary. In order to clear up, whether cyanobacteria form an outgroup within the sulfide-oxidizing bacteria with respect to the topography of SQR in membranes,

localization of the sulfide binding site in cyanobacteria has to be investigated.

3.3. ISOLATED SQR ENZYMES

At present, only two SQR enzymes have been purified to homogeneity. The first enzyme catalyzing sulfide-dependent reduction of quinone was isolated from *O. limnetica* [19] and named sulfide-quinone reductase (SQR; E.C.1.5.5.1). Three years later SQR from *R. capsulatus* was isolated [20]. The SQRs were identified as single polypeptides with apparent molecular masses of 55-57 kDa in SDS-PAGE. Fluorescence spectra of the enzymes revealed excitation and emission maxima typical for flavoproteins (**Tab. 2**). This fluorescence is quenched by sulfide. Peptide sequencing of the N-termini revealed the characteristics of the FAD-binding $\beta\alpha\beta$ -fold of many flavoproteins (see section 4.4.). The K_m values of the purified enzymes for sulfide and quinone were similar to that found for the membrane bound enzymes. The sensitivities of the isolated enzymes towards quinone-analogues were slightly increased, possibly due to increased accessibility of the quinone-binding site. Another reason might be that with purified enzyme samples less inhibitor is withdrawn by membranes or other quinone-binding enzymes present in membrane suspensions. As it was found for the membrane-bound enzymes, SQR purified from *O. limnetica* was insensitive towards myxothiazole and antimycin A [30], in contrast to the enzyme purified from *R. capsulatus* [50]. From these results it was suggested that the quinone-binding site is formed by SQR alone and no additional protein is required for activity or for substrate binding.

SQR of *R. capsulatus* was heterologously expressed in *E. coli* [20], and it was shown that the enzyme catalyzed the electron transfer from sulfide to oxygen via the Q pool and quinol oxidase [51]. After cell disruption and

differential centrifugation, the main portion of SQR-activity was found in the membrane fraction. SQR isolated from *E. coli* and SQR isolated from *R. capsulatus* behaved identical with respect to their substrate affinity and their fluorescence spectra (**Tab. 2**). Fluorescence of the heterologously expressed SQR was also quenched by sulfide. The extent of quenching depended on the sulfide concentration, and fluorescence was recovered by reoxidation with quinone.

A substantial portion of SQR expressed in *E. coli* was found in inclusion bodies. Reconstitution of the denatured protein purified from inclusion bodies revealed that FAD is the sole prosthetic group of SQR and it is required for enzyme activity (Griesbeck, unpublished results). Besides in membranes and in inclusion bodies, a decent part of SQR was also found in the soluble fraction of *E. coli* after cell disruption and differential centrifugation [20]. Although the portion of SQR in the soluble fraction did not exceed 30 - 50 %, there were significantly higher amounts than found during purification of SQR from *R. capsulatus*. Similar results were recently reported by Shibata et al. [51]. The reason for this phenomenon is unknown. Possibly differences in membrane lipid composition between both species influence the interaction of SQR with the membranes.

In summary, the following results were established by studying the SQR expression protein:

- SQR is a single peptide;
- electron transfer from sulfide to membrane-intrinsic quinones and quinone-binding are mediated by SQR alone, and no additional protein is necessary;
- attachment of SQR to membranes is not mediated by an additional protein;
- FAD is the sole prosthetic group of SQR.

Tab. 2: Properties of purified SQR proteins. Comparison of the SQR proteins purified from *Oscillatoria limnetica*, *Rhodobacter capsulatus* and *Rhodobacter* SQR expressed and purified from *E. coli*. Data were taken from [19,20,39,48,50] and Griesbeck (unpublished data). I_{50} -values give the concentration of inhibitor for 50% inhibition of activity. (unit, $\mu\text{mol quinone reduced min}^{-1}$; nd, not determined; PQ-1, plastoquinone-1; d-UQ, decyl-ubiquinon).

	<i>O. limnetica</i>	<i>R. capsulatus</i>	<i>R. capsulatus</i> (expr. in <i>E. coli</i>)
Apparent size in SDS-PAGE (kDa)	57	55	55
Theoretical molecular mass (kDa)	48	47	47
Florescence maxima (nm)			
Excitation	280 / 373 / 461	280 / 375 / 458	280 / 375 / 475
Emission	527	518	525
<i>Specific activity</i> [unit (mg protein) ⁻¹]	1.9	3.5	10-15
K_m (μM)			
H ₂ S	8	2	2
Quinone	32 (PQ-1)	2 (d-UQ)	2 (d-UQ)
pH optimum	nd	6.3	6.5
I₅₀ (μM)			
Stigmatellin	1.4	1.2	
Myxothiazole	none	13	
Antimycin A	none	32	
KCN	12	500	

4. SQR GENES AND PROTEIN SEQUENCES

4.1. CLONING OF THE *SQR* GENE FROM *R. CAPSULATUS*

The first *sqr* gene to be cloned was the gene from *R. capsulatus* [20,42]. By use of PCR amplified probes obtained with oligonucleotides deduced from tryptic peptides of the purified enzyme, a 3.5-kb fragment of genomic DNA from *R. capsulatus* was isolated and sequenced. This fragment contains the *sqr* gene, a second complete and one partial open reading frame in same orientation upstream the *sqr* gene, and the 3'-end of an open reading frame in opposite orientation downstream.

The *sqr* gene encodes a protein of 427 amino acid residues with a theoretical molecular weight of 47 kDa and a net charge of +1. As evident from N-terminal peptide sequencing, the first amino acid residue of the native protein is alanine (A_{Rc2} in **Fig. 4**). Five bp upstream the codon for M_{Rc1} a nucleotide sequence was found that matches well with ribosome-binding sites of *R. capsulatus*.

In order to test whether the isolated *sqr* gene is the only gene encoding sulfide oxidizing activity in *R. capsulatus*, mutants of *R. capsulatus* were constructed with insertions and deletions in the *sqr* gene [42]. These strains were unable to grow photoauto-

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R.caps. -----MAHIVVLGAGLGGAIMAYELREQVRKEDK 29
S.putre. -----MKKIIVIGAGLGGVSVAFELKQRLPSDCE 29
T.ferro. -----MAHVVLGAGTGGMPAAYEMKEALGSGHE 29
A.aeol. -----MAKHVVVIGGGVGGIATAYNLRNLMP-DLK 29
O.lim. -----MAHVAVIGAGLAGLPTAYELRHILPRQHR 29
A.7120 -----MAHIVIVGAGLGGGLPTAYELRHILPKQHQ 29
A.halo. -----MAHIVIVGGGFGGLSAAVELKHLHLHGKHK 29
 FCC MTLNRRDFIKTSGAAVAAVGILGFPHLAFGAGRKVVVVGGTGGATAAKYIKLADP-SIE 59
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R.caps. VTVITKDPMYHFVPSNPWVAVGWRDRKEITVDLAPTMARKNIDFIPVAAKRLHPAENRVE 89
S.putre. IGVISEGTDFQFVPSNPWVALGERTREDISLPIGTYLAKRKISYSGEGVTHIDPLNKKLT 89
T.ferro. VTLISANDYFQFVPSNPWVGWGERDDIAFP IRHYVERKGIHFIAQSAEQIDAEAQNIT 89
A.aeol. ITLISDRPYFGFTPAFPHLAMGWRKFEDISVPLAPLLPKFNIEFINEKAESIDPDANTVT 89
O.lim. VTLISDKPNFTFTPSLPWVAFDLTPLERVQLDVGKLLKGRNIDWIHGKVNHDIPENKTSV 89
A.7120 VTVISETPYFTFIPSLPWVAMGLTSLESIQVSLQQRLLKQGINWILGRVDYLNPNQNKIS 89
A.halo. ITLISDETTFTFIPSLPWVAFNLRLEDVQLPLAPLLARQGINWQHGRVTGLDPNQKRVS 89
 FCC VTLIEPNTDYTYCYLSNEVIGGDRKLESIKHGYDG-LRAHGIQVVHDSATGIDPDKKLVK 118
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. . ** :: : ** . * . * * : * : : *

R.caps. LENGQSVSYDQI VIATGPELAFDEIEGFGP---EGHTQSICHIDHAEAAAGAFDRFCENP 146
S.putre. LGDGTNNYDYLVICTGPKLAFENVP GSGP---EGFTQSICTIDHAEKAYECFQQLLAKP 146
T.ferro. LADGNTVHYDYLMIATGPKLAFENVP GSDPH---EGPVQSICTVDHAERAFAEYQALLREP 147
A.aeol. TQSGKKIEYDYLVIATGPKLVFG-AEQQ----EENSTSICTAEHALETQKKLQELYANP 143
O.lim. AGE-QTLEYDYVVVATGPELATDAIAGLGPE--NGYTQSV CNPHHALMAKEAWQKFLQDP 146
A.7120 LGE-QSISYDYLIIATGAELALDAVAGLGPD---GYTQSV CNPHHAIKAFQAWQNFL LAP 145
A.halo. VGEDITFDYDYLVIITGASLAYHLMSGLGPE--EGYTQSV CNAHHAEMARDAWDEFLENP 147
 FCC TAGGAEFGYDRCVVAPGIELIYDKIEGYSEEA AAKLPHAWKAGEQTAILRKQLEDMADGG 178
 ** :: . * . * * : : : :

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-----3-----↓-----

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R.caps. GPIIIGAAQGASCFCGPAYEFTFILD TALRKRKIRDKVP-MTFVTSEPYVGHGLDGVGDT 205
S.putre. GPVVVGALQGASCFCGPAYEYVLSLES LLRKHKVRQHIP-ITFVTSEPYVGHMGLGGVGDS 205
T.ferro. GPIVIGAMAGASCFCGPAYEYAMIVASDLK KRGMRDKIPSFTFITSEPYIGHLGIQGVGDS 207
A.aeol. GPVVIGAI PGVSCFCGPAYEFALMLHYELK KRGI RYKVP-MTFITSEPYLGHFGVGGIGAS 202
O.lim. GPLVVGAVPGASCFCGPAYEFALLADYV LRKGM RDRVP-ITFVTPEPYVGHGLGIGMANS 205
A.7120 GPLVVGALPKTSCLGPAYEFTLLADYV LRKQGLREQVS-ITFVTPEPYAGHLGIGMANS 204
A.halo. GPLLVGAVPGASCMGPAYEFALLADYALRQ EGKRDQVP-ITFISPEPYLGHGLGIGMANS 206
 FCC TVVIAPPAAPFRCPGPPYERASQVAYYLKAHKPKSKVII LDSSQTFQSKQSQFSKG----W 234
 : : . * . ** . * : : : : : . . : . :

: : : : . . . * * : * . * :

R.caps. KGLLEGNLRDKHIKWMTS--TRIKRVEPGKMVVEEVTEDGTVKPEKELPFGYAMMLPAFR 263
S.putre. KSLMEHEFRERSIKWICN--AKTTHFESGIAYVDELD RKGEVEFQHTL PFSLAMFLPPFK 263
T.ferro. KGILT KGLKEEGIEAYTN--CKVTKVEDNKMYVTQVDEKGETIKEMVLPVKFGMMIPAFK 265
A.aeol. KRLVEDLFAERNIDWIAN--VAVKAIEPDKVIYEDLNGN-----THEVPAKFTMFMPSFQ 255
O.lim. AELVTDLLENK GIRVLPN--TAVKEIHPEHMDLDSG-----EQLPFKYAMLLPPFR 254
A.7120 AELVTKFMAERGVEVIEN--VAVTAIEANQIHLGNG-----RVLPFAYSMLLPPFR 253
A.halo. GKLVTELMKQRNIDWVEN--AEIAEIKEDHVKLT DG-----REFPFNYSMFLPPFR 255
 FCC ERLYGFGTENAMIEWHPGPD SAVVKVDGGEMMVETA FG-----DEFKADV INLIPPQR 287
 : : : . . . : * . :

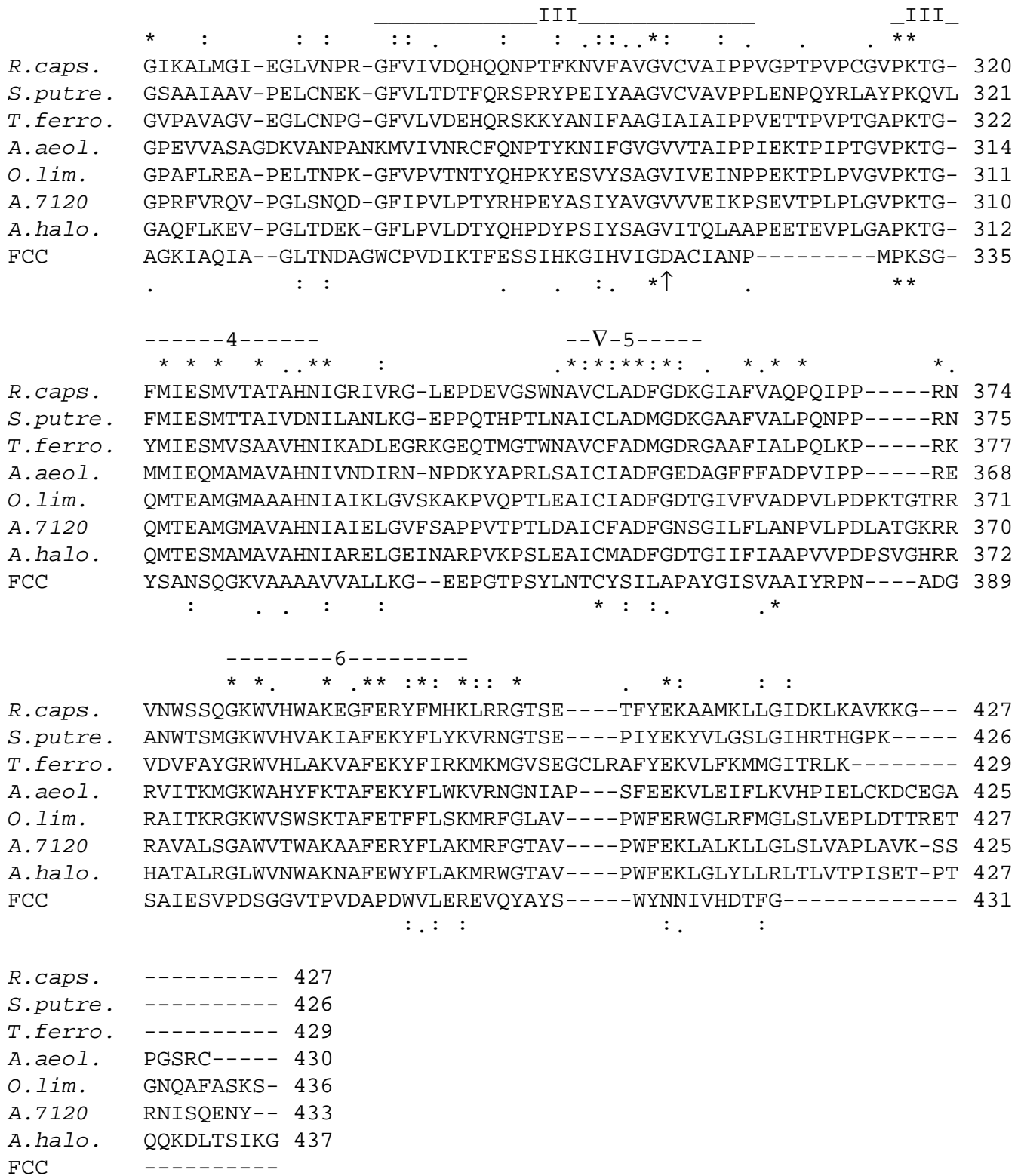


Fig. 3: Alignment of SQR Sequences and FCC. SQR sequences are from *Rhodobacter capsulatus* (*R. caps.*) [20], *Shewanella putrefaciens* (*S. putre.*) [48], *Thiobacillus ferrooxidans* (*T. ferro*) [34], *Aquifex aeolicus* (*A. aeol.*) [35], *Oscillatoria limnetica* (*O. lim.*) [48], *Anabaena* ATCC 7120 (*A. 7120*) (Kazusa DNA Research Institute, <http://www.kazusa.or.jp>) and *Aphonathece halophytica* (*A. halo.*) [48]. The sequence of flavocytochrome *c* (FCC) is from *Allochromatium vinosum* [17]. The FAD-binding domains are indicated by continuous lines

and roman numbers, the SQR-fingerprints are indicated by broken lines and arabic numbers. The three cysteines that are conserved among SQR-sequences are indicated by open triangles. Histidines that are possibly involved in quinone-binding are signed by arrows above the sequences. The aspartate that is conserved among all members of the glutathion reductase family of flavoproteins but is missing in SQR is indicated by an arrow below the sequences. Positions of identical amino acid residues are marked by *, positions of similar amino acid residues with colons (high similarity) and with dots (lower similarity). These positions for the SQR sequences are given above the alignment and for SQRs and FCC below the alignment.

trophically on sulfide, did not deposit any sulfur outside the cells under heterotrophic conditions and did not consume any sulfide in liquid medium. In addition, no SQR protein was detected in western blot analysis with membranes. This defect was complemented by the *sqr* gene on an autonomously replicating plasmid. Overexpression of SQR in *R. capsulatus* significantly increased the sulfide-oxidation capability and increased SQR-activity in membranes 6-7fold. These results clearly indicate that SQR is the only sulfide-oxidizing enzyme in *R. capsulatus* and it is absolutely required for sulfide-dependent growth.

The *sqr* gene of *R. capsulatus* is not part of an operon. It is separated by 164 nucleotides from the upstream open reading frame, and a sequence motif was found within this region that matches well with transcription start initiation sites and regulatory sequences of *R. capsulatus*. In addition, insertional inactivation of the upstream ORF in *R. capsulatus* did not alter SQR expression, and SQR was expressed in *Rhodobacter* from an autonomously replicating plasmid lacking the upstream ORF [42]. Because of significant peptide sequence similarity of the deduced amino acid sequences to subunits of the ribose transporter in *Archaeoglobus fulgidus* it seems likely that the reading frames upstream *sqr* are part of an operon encoding the ribose transporter in *R. capsulatus*. The peptide sequence deduced

from the 3'-end of the sequence downstream *sqr* revealed significant similarity to the C-terminal EAL motif pattern of many regulatory proteins [42]. Since SQR in *R. capsulatus* is an inducible protein (see section 6.), participation of this downstream sequence in the regulation of SQR expression should be tested.

4.2. CLONING OF CYANOBACTERIAL SQR GENES

Recently, the *sqr* genes from the cyanobacteria *O. limnetica* and *Aphanathece halophytica* have been isolated and sequenced [48]. The two genes encode proteins of 436 and 437 amino acid residues in size with a net charge of 0 and -14, respectively. Interestingly, downstream both *sqr* genes, open reading frames encoding small proteins with significant similarity to transcription factors of bacteria and cyanobacteria were found in the same direction as *sqr*. In *A. halophytica*, the *sqr* gene and the downstream ORF are only separated by 3 nucleotides suggesting cotranscription of both genes. In contrast, the two ORFs in *O. limnetica* are separated by approximately 180 nucleotides. Since SQR is an inducible protein in both cyanobacterial systems and regulatory proteins are often located in the vicinity of their targets of regulation, these small proteins may play a role in the regulation of SQR. The two small cyanobacterial proteins do not share any significant sequence similarity with the

Tab. 3: Identity and similarity of SQR and FCC. Percentage values for identity (upper value) and similarity (lower value) are summarized. Values were obtained using BLASTP 2.0.9 (<http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi>).

<i>R. capsulatus</i>	100							
	100							
<i>S. putrefaciens</i>	46	100						
	64	100						
<i>T. ferrooxidans</i>	46	49	100					
	56	65	100					
<i>A. aeolicus</i>	38	39	40	100				
	57	53	66	100				
<i>O. limnetica</i>	42	42	38	44	100			
	61	56	66	61	100			
<i>Anabaena 7120</i>	42	41	39	40	64	100		
	61	57	58	57	79	100		
<i>A. halophytica</i>	40	42	37	40	58	59	100	
	59	59	55	56	74	75	100	
<i>FCC (A. vinosum)</i>	23	24	23	24	25	22	25	100
	38	36	36	40	38	36	38	100

sequence deduced from the 3'-end of the ORF downstream *sqr* in *R. capsulatus*. Therefore, if all these proteins are involved in regulation of SQR expression, the regulatory mechanism in cyanobacteria should be different from the mechanism in *R. capsulatus*.

4.3 COMPARISON OF BACTERIAL SQR SEQUENCES

Besides the above mentioned *sqr* genes, open reading frames encoding proteins with high similarity to SQR sequences have been identified in sequence databases for *A. aeolicus* [34,35], *Thiobacillus ferrooxidans* [34,48], *Shewanella putrefaciens* [48] and *Anabaena* ATCC7120 (Kazusa DNA Research Institute, <http://www.kazusa.or.jp>). The overall similarity among the SQR sequences is in the range from 53 to 79 % (identity 37-64 %, see **Tab. 3**). The highest similarity was found between the three

cyanobacterial sequences. For none of the SQR sequences membrane anchoring or membrane spanning domains were found by secondary structure prediction analysis. This is less surprising with respect to SQR of *R. capsulatus*, because this enzyme is peripherally bound to the membrane (see section 3.2.), but it is remarkable with respect to the cyanobacterial enzymes and the SQR of *A. aeolicus* that are all tightly membrane-bound proteins. However, secondary structure prediction analysis methods are approximations, and the results in some cases differ from the real folding pattern of proteins. Therefore, additional structural data are necessary to elucidate the way SQR interacts with membranes.

4.4. FAD-BINDING

Although the overall similarity between SQR and FCC is significantly lower than similarity among the different SQRs (22-25 % and 38-64 % identity, respectively, see **Tab. 3**), all SQR sequences as well as FCC exhibit the three FAD-binding domains which are characteristic among proteins that belong to the glutathion reductase family of flavoproteins (indicated by continuous lines and roman numbers in **Fig. 3**). These are the N-terminal $\beta\alpha\beta$ -fold [52], a second motif close to the N-terminus [20] and a motif close to the C-terminus [53].

Within the N-terminal $\beta\alpha\beta$ -fold, all SQRs as well as FCC contain the expected fingerprint residues of the ADP-binding site characterizing many NAD(P)/FAD containing proteins [52]. The function of some of the residues in binding FAD is known from the three-dimensional structure of several enzymes that belong to the glutathion reductase family of flavoproteins, e.g. glutathion reductase [54] and FCC [16,17]. In the following section we summarize structural details as evident from sequence comparisons of SQR with glutathion reductase and FCC. The addressed amino acid residues are given with their position in the sequence of the SQR from *R. capsulatus* (Rc) and FCC from *Allochromatium vinosum* (FCC).

The three conserved glycine residues, G_{Rc}8, G_{Rc}10 and G_{Rc}13 in the first sequence motif of *R. capsulatus*, are likely to provide space for the phosphate groups of FAD and form hydrogen bonds to the phosphate O-atoms directly or via solvent molecules. The carboxyl-group of an acidic residue, D_{Rc}36, at the end of the second β -sheet is in contact with the hydroxyl-groups of the ribose.

Within the second motif (II), a conserved glycine (G_{Rc}106) is possibly located in a turn in the C α -chain at the end of a β -sheet. A_{Rc}104 might be in contact with the phosphate bound

to the ribityl chain of FAD. Although the two amino acid residues, Y_{Rc}98 and D_{Rc}99, are conserved among most of the pyridine nucleotide oxidoreductases, indications about their function in binding FAD were not found.

Within the third motif (IIIa,b), G_{Rc}299 is possibly conserved because of a turn in the C α -chain to accommodate the phosphate groups of FAD. The carbonyl O-atom of V_{Rc}298 could be in contact with the phosphate group near the ribityl chain via a solvent molecule. The hydrophobic and aromatic residues in position 296 and 298 are possibly conserved to form a hydrophobic environment. Surprisingly, an aspartate that interacts with the ribityl chain of the flavin moiety of FAD and that is conserved among all flavoproteins that belong to the glutathion reductase family (D_{FCC}324 in FCC) is absent in all SQR proteins (position 300 in the sequence from *R. capsulatus*, marked by \uparrow in **Fig. 3**). The reason for the absence of this aspartate in SQRs is unknown. Possibly the conformation of FAD in SQR is somewhat different. Near the benzenoid ring of FAD in FCC is a pentapeptide (PKSGY_{FCC}332-336), which is also found in SQR (PKTGF_{Rc}317-321). The peptide nitrogen atoms of glycine and the aromatic amino acid residue form hydrogen bonds to N1 and O2 of FAD, and the side chain of the lysine is close enough to form a hydrogen bond with the peptidyl oxygen atom of D_{FCC}324 (V_{Rc}300).

A cysteine residue, C_{FCC}72, which is conserved among FCCs, covalently binds FAD in FCC of *A. vinosum* [17]. This amino acid residue is absent in SQR, indicating that FAD is not covalently bound. This is in agreement with the observation, that only a part of purified SQR contains FAD (Griesbeck, unpublished results).

4.5. AMINO ACID RESIDUES THAT MIGHT BE INVOLVED IN QUINONE-BINDING

Besides the three FAD binding domains, several stretches were identified that are conserved among the SQR sequences (indicated by broken lines and arabic numbers in **Fig. 3**). Within two of these stretches there are histidines ($H_{Rc}131$ and $H_{Rc}196$) that are conserved among all SQRs [48] and might be involved in quinone-binding (indicated by \downarrow in **Fig. 3**). Based on crystallographic data of several quinone-binding proteins, Rich and Fisher proposed a structural element for quinone binding sites [55]. It is composed of a helical stretch that flanks one side of the quinone headgroup and contains a triad of close contact residues. The central residue of the triad is a histidine which forms a hydrogen-bond to one carbonyl of the quinone. The fourth residue upstream of this histidine is aliphatic, usually leucine, and is the closest of the triad to the isoprenoid side chain. Another close-contact residue three or four residues downstream the histidine makes up the contact triad and is well conserved within homologous sequences of different Q-binding proteins. In SQR, $H_{Rc}131$ is located in a putative α -helix formed by the conserved stretch [S-I/V-C-(X)₃-H-A-(X)₂-A]. $H_{Rc}196$ is also located in a conserved stretch [E-P-Y-V/L-G-H-L/F-G-L/I]. Although the two regions are somewhat different from the proposed quinone-binding motifs, they are good candidates for the quinone-binding site in SQR, especially the aliphatic and aromatic amino acid residues in close vicinity to $H_{Rc}131$ might participate in forming a hydrophobic quinone-binding pocket. These histidines are absent in FCC. Thus both of them, as well as the presence of valine ($V_{Rc}300$) instead of aspartate in FCC (s. **Fig. 3**) are significant for SQR.

4.6. AMINO ACID RESIDUES THAT MIGHT BE INVOLVED IN SULFIDE OXIDATION

In the three-dimensional structure of FCC, two cysteines ($C_{FCC}191$ and $C_{FCC}367$) are positioned close to the isoalloxazine moiety of FAD [16]. These two cysteines are linked by a disulfide bridge and are located above the pyrimidine portion of the flavin. They possibly play a redox-active role in catalysis of FCC [15]. In the SQRs, three cysteine amino acid residues ($C_{Rc}127$, $C_{Rc}159$ and $C_{Rc}353$) are well conserved (indicated by open triangles in **Fig. 3**). Two of them ($C_{Rc}159$ and $C_{Rc}353$) are in a position identical to the position of the redox-active cysteines in the peptide sequence of FCC. Preliminary results from replacements of these cysteines by site-directed mutagenesis in the SQR of *R. capsulatus* indicate that all three cysteines are essential for SQR-activity (Griesbeck, unpublished data). Binding of FAD by SQR was not impaired. These data suggest, that three cysteine residues are involved in the catalytic reaction of SQR and two of them are possibly positioned close to the pyrimidine portion of FAD.

In the sequence of SQR, glutamate $E_{Rc}165$ is in a position identical to the position of a glutamate in FCC. $E_{FCC}197$ is near the N5 position of FAD and was found to be responsible for a pK-shift of sulfite binding to FAD [16]. In analogy, a similar function of $E_{Rc}165$ in SQR seems plausible.

Besides the above mentioned residues, several stretches of conserved amino acid residues in SQR were identified (**Fig. 3**, broken lines). Although these stretches can serve as finger prints for the identification of SQR sequences, their function needs to be investigated. Within the C-terminus, there is remarkable high degree of similarity among SQR sequences. Four aromatic amino acid residues, four positively charged and two negatively charged amino acid residues are present in all sequences. This is of special interest with respect to the function of

the C-terminus in glutathion reductase and lipoamide dehydrogenase. In these homodimeric enzymes, the C-terminal region of one subunit is involved in the formation of the catalytic site of the other subunit, and some of the C-terminal amino acid residues participate in stabilisation of the dimer, e.g. by formation of a salt bridge [54]. Size exclusion experiments [20] and native gel electrophoresis ([50], Schödl and Griesbeck, unpublished results) suggest that SQR of *R. capsulatus* might be a homodimer as well. Possibly some of the C-terminal amino acid residues are also involved in dimer stabilisation and the formation of the catalytic site in SQR.

5. MECHANISM OF SULFIDE OXIDATION

At present, data about the mechanism of sulfide oxidation and quinone reduction in SQR are scarce. However, in this section we try to formulate a rough model for the mechanism of sulfide-dependent reduction of quinones by SQR on basis of available data and in analogy to enzymatic systems that have been well investigated.

Most of the proteins that belong to the glutathion reductase family of flavoproteins, e. g. glutathion reductase, lipoamide dehydrogenase or FCC, catalyze reactions by which thiol groups or sulfide become either reduced or oxidized. In all these proteins two cysteines are involved in catalysis. Thereby, they change between the reduced thiol- and the oxidized disulfide-state. [15,54,56]. Except for FCC, in these enzymes electrons are transferred from the two electron carrier NAD(P) via the two electron carrier FAD and via two cysteine residues of the enzyme to disulfide, e.g. glutathion, that becomes reduced. Within SQR, the situation is similar, although the reaction occurs in opposite direction. Electrons from sulfide reduce the

two electron carrier FAD, possibly via two cysteine residues, and the two electron carrier quinone becomes reduced.

There are some evidences in favour of the transfer of two electrons from sulfide to the two electron carrier quinone by SQR. First, the sulfur-product from oxidation of sulfide (S^{2-}) by *R. capsulatus* is zero-valent sulfur (S^0) [41], (Griesbeck, Rethmeier and Fischer, unpublished). Neither sulfide-oxidation nor formation of sulfur occurred in a mutant strain of *R. capsulatus*, in which the *sqr* gene had been deleted [42]. Second, membranes of an *E. coli* strain that expressed SQR from *R. capsulatus* catalyzed the electron transfer from sulfide to oxygen (for reduction of 1 Mol O_2 4 Mol electrons are necessary). The sulfide:oxygen ratio was 2:1, and the sulfur product was zero-valent sulfur [51]. Third, the sulfide:quinone ratio of the purified SQR was found to be 1:1 (Griesbeck, unpublished). Although a clear determination of the sulfur product of this reaction failed so far, transient formation of polysulfide was shown (Griesbeck, Rethmeier and Fischer, unpublished).

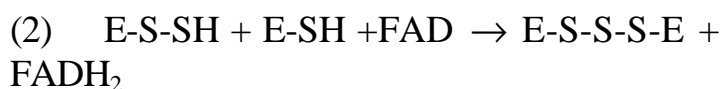
What is the sulfur-product of the reaction? Atomic sulfur is an unstable, highly reactive species, and it is very unlikely that it could be the initial product. In nature, elemental sulfur mainly occurs in polymers, rings of mostly 8 atoms (S_8) [57]. Elemental sulfur is nearly insoluble in aqueous solutions. Therefore, organisms that do not store sulfur globules in the periplasm would have the problem to transport it out of the cell prior to precipitation. This problem would not occur, if polysulfides ($H-S_n-H$) would be the product. Polysulfides are soluble in aqueous solutions. They are unstable because of disproportionation to $H_2S + S_{n-1}$ [57]. This could explain the occurrence of sulfur precipitates outside the cells, even if polysulfide is the immediate sulfur-product. Indeed, polysulfides deriving from biological

oxidation of sulfide have been detected in purple and green sulfur bacteria [58]. Formation of longer polysulfide chains as initial states for formation of S₈-sulfur would require polymerization of 8 sulfide molecules by subsequent oxidation reactions with the growing polysulfide chain bound to the enzyme. Interestingly, Klimmek et al. [59] have recently shown that the Sud protein of *Wollinella succinogenes* covalently binds polysulfide. Sud catalyzes the formation of thiocyanate from cyanide and polysulfide. Thereby, polysulfide with up to ten sulfur atoms is covalently bound to a cysteine residue of Sud.

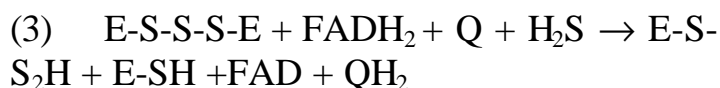
Reaction model: From the above data we suggest the following model for oxidation of sulfide by SQR. In SQR there are two cysteines that are linked by a disulfide bridge in analogy to FCC. This disulfide bridge is opened by addition of sulfide resulting in a reduced cysteine residue and a thiolgroup bound to the second cysteine.



In a second step, FAD becomes reduced and a trisulfide bridge is formed.



In a third step, the electrons from the flavin are transferred to quinone and a second sulfide molecule becomes bound to the enzyme.



After several cycles through reaction (2) and (3), the polysulfide chain is released from the enzyme by formation of the initial disulfide bridge.



In view of the results from the site-directed mutagenesis the mechanism may actually involve three cysteines.

6. REGULATION OF EXPRESSION OF SQR

6.1. EXPRESSION OF SQR IN *R. CAPSULATUS*

The "non-sulfur" purple bacterium *R. capsulatus* holds a very versatile metabolism. It lives in pools and brooks that are often polluted by waste water. There it has to adapt its metabolism to changing nutritional conditions, e. g. hydrogen sulfide. Adaptation requires *de novo* synthesis of the enzymatic systems that are required for metabolizing newly appearing substrates.

First indications that the sulfide oxidizing capability in *R. capsulatus* is an inducible process were given in the work of Wijbenga and van Gernerden [60]. They observed that after the first addition of sulfide to acetate-limited continuous cultures sulfide oxidation occurred only after an initial lag phase. After approximately 1 hour, a constant oxidation rate was reached. No lag phase was observed after a second addition of sulfide. In addition, lower growing cultures oxidized sulfide more rapidly than fast growing cells and the initial lag phase was elongated in fast growing cultures suggesting that the metabolic status of the cells influences the sulfide oxidation capability.

In our laboratory, we observed that an increase in the sulfide oxidation capability of *R. capsulatus* cultures correlated with an increase of membrane-bound SQR-activity (Meiler, Schaible, Schütz and Hauska, unpublished), and this increase was enhanced, if oxygen or CO₂ were added as electron sinks to the cultures [42,61].

In order to simplify expression studies, we used reporter gene techniques. First, the genes encoding luciferase of *Vibrio fischeri* were inserted into the genome of *R. capsulatus* under control of the *sqr*-promotor [42,50]. Addition of sulfide to heterotrophically growing cultures caused a significant increase of luciferase activity, and interestingly the level of expression of luciferase was enhanced, if

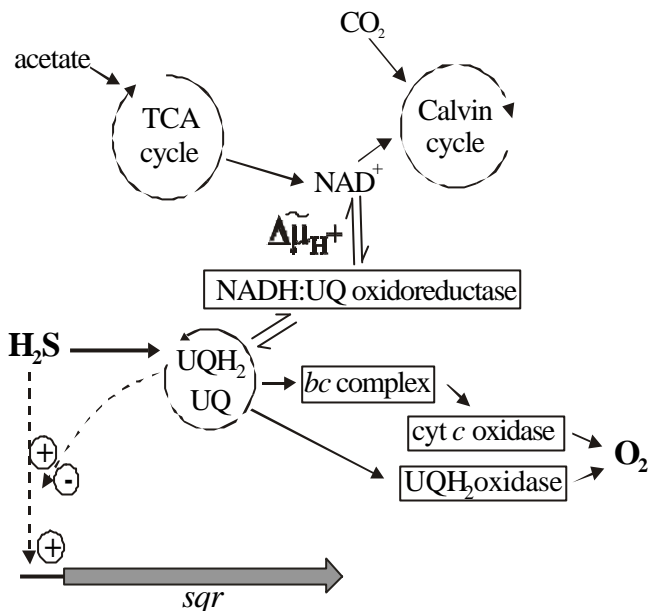


Fig. 4: Model of the Regulation of SQR-expression in *Rhodospirillum rubrum*. Regulatory effects are shown by broken arrows, electron transport steps by solid lines. (TCA cycle, tricarboxylic acid cycle; cyt, cytochrome).

oxygen was present. Since luciferase activity depends on oxygen, this result was ambiguous, however. Therefore, we changed to the oxygen insensitive phosphatase system.

An expression construct of *sqr* fused with the gene of the alkaline phosphatase of *E. coli* was set under control of the *sqr*-promotor on an

autonomously replicating plasmid and was transformed into *R. rubrum* [42,61]. Induction of heterotrophically growing cells by sulfide significantly increased phosphatase-activities of the cells, and the activities reached a constant rate approximately 5 to 7 hours after sulfide addition. After induction under oxic conditions, activities were several-fold higher than under unoxic conditions. In order to test enhancement of expression by oxygen, parallel cultures were cultivated anoxically for 25 hours. Then sterile air was added to half of the cultures. Addition of air increased phosphatase-activities 2- to 3-fold within 7

hours. If the transcription inhibitor rifampicin was added together with air, no increase of phosphatase-activity was observed.

Based on the data from our studies and on the experiments from Wijbenga and van Gernerden [60] two mechanisms are conceivable for the modulation of SQR expression:

- either there is a distinct regulator of SQR expression for each stimulating or repressing agent
- or the activity and/or concentration of one regulator protein is modulated by a signal that reflects the metabolic status of the cell.

We favor the second model of regulation of the SQR expression in *R. rubrum* as depicted in Fig. 4 for the following reason. The quinone-pool plays a central role in the ET chain in *R. rubrum* (Fig. 1). It is the connection between phototrophic and chemotrophic metabolism [62,63]. Cyclic ET is only ensured in case of an appropriate QH₂/Q ratio. An oversupply of reductants, e. g. acetate and sulfide, would result in a high QH₂/Q ratio in absence of appropriate oxidants, e. g. oxygen. As a result, the generation of a proton gradient by the cyclic photosynthetic ET in the light as well as by the ET through the cytochrome *bc* complex in the dark would be inhibited. Consequently, ATP synthesis and reduction of NAD⁺ would be attenuated. The negative control of the expression of proteins that feed electrons into the quinone-pool would prevent over-reduction of the pool. However, this model needs to be tested by further experimentation.

6.2. EXPRESSION OF SQR IN CYANOBACTERIA

Cyanobacteria are exceptional in the world of phototrophic prokaryotes. They are the only bacteria that perform plant-type photosynthesis, using two photosystems in

series and water as the electron donor. However, some species can facultatively shift to bacteria-type, anoxygenic photosynthesis in which only photosystem I is involved (see reviews [46,47]). Efficient anoxygenic photosynthesis depends on sulfide-induced *de novo* protein synthesis. After incubation in the presence of sulfide and light, the cyanobacteria *O. limnetica* and *A. halophytica* shift to anoxygenic photosynthesis within 2-3 hours [64]. The shift was found to be accompanied by synthesis of SQR in membranes [19,48]. Besides SQR, induction of several small periplasmic and membrane-bound proteins was observed in *O. limnetica* [65]. These proteins were not found to be induced by dithionite suggesting that sulfide is the sole stimulus. Up to now, neither additional information about modulation of expression of proteins by sulfide nor information about the function of the small sulfide-induced proteins is available.

7. OCCURENCE OF SQR

7.1. SQR IN PROKARYOTES

The occurrence of SQR in either photo- or chemolithotrophic Eubacteria known so far is shown in **Fig. 5**. This occurrence has been documented by SQR activity and/or identification of genes encoding proteins with high similarity to known SQR sequences (see section 4.). Both SQR-activity and the responsible gene sequence are known for the cyanobacteria *O. limnetica* and *A. halophytica* [48], the purple non-sulfur bacterium *R. capsulatus* [20] (α -subgroup within the proteobacteria) and for *A. aeolicus* [34]. This SQR sequence exhibits high identity to the proteobacterial and cyanobacterial SQRs. Three additional gene sequences have been found in representatives of cyanobacteria (*Anabaena* ATCC7120) and the γ -subgroup of proteobacteria (*S. putrefaciens* and *T. ferrooxidans*). For members of the latter group

SQR activity has been demonstrated without knowing the corresponding gene, i.e. for *A. vinosum* [18] and *Thiobacillus* sp. W5 [33], as well as for one additional representative of the α -proteobacteria, *Paracoccus denitrificans* [32]. Beyond that, SQR activity has been found in green sulfur bacteria (*Chlorobium limicola* f. *thiosulfatophilum* [30] and *C. tepidum* [31]) and Chloroflexaceae (*C. aurantiacus*) [21]. This far distribution among Eubacteria and the occurrence of SQR in *A. aeolicus*, a member of the Aquificaceae, the most deeply branching family within the bacterial domain [66], leads to the conclusion that SQR could be an evolutionary old enzyme that was invented during the early periods of life in a sulfidic atmosphere [67]. Therefore, occurrence of SQR also in *Archaea* [7] is predictable, but investigations have not been carried out so far.

Even in mitochondria of eukaryotes good evidences for SQR activity have been found [21,68,69]. This is not surprising because of the prokaryotic origin of mitochondria [67].

7.2. THE *HMT2* GENE FROM

SCHIZOSACCHAROMYCES POMBE

Recently, sulfide-quinone oxidoreductase activity was reported for the mitochondrial enzyme HMT2 from fission yeast (*Schizosaccharomyces pombe*) [70]. A cadmium-hypersensitive mutant of *S. pombe* was found to accumulate abnormally high levels of sulfide. The gene required for normal regulation of sulfide levels, *hmt2*⁺, was cloned by complementation of the cadmium-hypersensitive phenotype of the mutant. Cell fractionation and immunocytochemistry indicated that HMT2 protein is localized to mitochondria. HMT2 protein, expressed in and purified from *E. coli*, was soluble, bound FAD and catalyzed the reduction of quinone. However, the K_m values of 2 mM for the binding of sulfide and quinone by HMT2 are

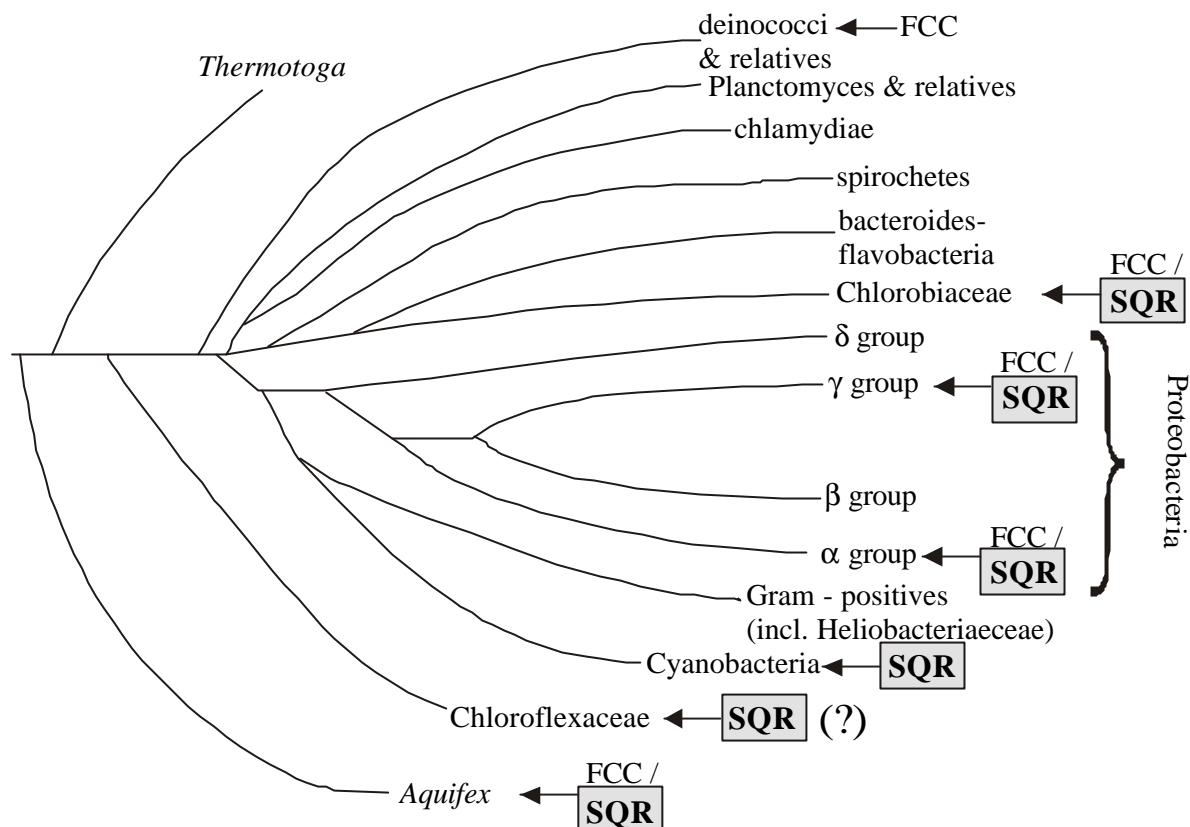


Fig. 5: Occurrence of SQR within the domain Bacteria. Phylogenetic tree of Bacteria based on 16S rRNA (modified from [4]) and the occurrence of SQR (SQR-activity and/or gene sequence) and FCC. SQR activity in Chloroflexaceae is not firmly established.

almost a thousand times higher than the K_m values of all known SQR proteins (see **Tab. 1**). A K_m for sulfide of 2 mM seems rather high because of the enormous toxicity of sulfide. Inhibition by quinone-analogue inhibitors, which would be a good indication for the existence of a specific quinone-binding site,

was not tested. In addition, sequence comparison revealed a rather low similarity to the known SQR sequences. Whereas the FAD binding domains and the two cysteines that form a disulfide bridge in FCC of *A. vinosum* are present (see **Fig. 4** and section 4.), the aspartate (D_{Av324} in FCC) which is involved in FAD binding in all flavoproteins that belong to the glutathion reductase family, and which is not present in any of the known SQR

sequences, was found in HMT2. The domains that were found as fingerprint residues of SQR, were not conserved in HMT2 [34]. Overall identity to SQR was 17-22 %, which is as low as the identity between SQR and FCC. In contrast the microbial SQR sequences exhibit an identity between 38 and 64 % (see **Tab. 3**). Therefore, the physiological role of HMT2 as SQR is questionable.

8. APPLICATIONS OF SULFIDE OXIDIZING MICROORGANISMS

As a component of anthropogenic waste water, hydrogen sulfide often causes problems because of its high toxicity even at low concentrations and its unpleasant odour. In biological sewage plants sulfide concentrations

can be found which are toxic not only to man, but also to the microorganisms clarifying the water [71]. Therefore, operation of sewage plants can be inhibited by sulfide. In addition, sulfide can be oxidized to sulfuric acid by microorganisms which inhabit biofilms inside waste water tubes. The resulting sulfuric acid leads to corrosion of metallic or concrete material.

In order to remove sulfide from liquid and gaseous effluents, biotechnological applications have been developed using photo- or chemotrophic bacteria. It can also be removed by chemical methods, such as chemical oxidation or precipitation, but these methods are expensive and may cause new problems because of the chemicals used. Bioreactors with anoxygenic phototrophic bacteria have been successfully tested for sulfide removal (for a review see [72]). The advantages of such systems are the almost complete removal of sulfide from the effluents and no need for addition of chemical oxidants. The phototrophic bacteria acting in these systems are species of the genera *Chlorobium* and *Rhodobacter*. The above mentioned species convert sulfide mainly to sulfate, therefore the sulfur is not removed completely from the system and can be reduced to sulfide by sulfate-oxidizing bacteria again. In order to circumvent this problem and to obtain elemental sulfur as the main product, it would be useful to replace these organisms by other phototrophic ones which only can oxidize sulfide to elemental sulfur, e.g. *R. capsulatus*.

From a sulfur-producing bioreactor the dominant sulfide-oxidizing bacterium has been isolated and characterized. The isolate has been shown to be a new *Thiobacillus* species and has been given the working name *Thiobacillus* sp. W5 [73]. From *Thiobacillus* sp. W5 a novel membrane-bound flavocytochrome *c* sulfide dehydrogenase has been isolated, and SQR activity has been shown to be present in membranes [33]. Studies of such bacteria may

help to explain under which conditions sulfide is oxidized only to sulfur instead of completely being oxidized to sulfate. The results can then be used for process control and optimization of sulfide removal and sulfur production [73].

Elimination of unpleasant odor from animal feeding has been tried by growing of sulfide-oxidizing phototrophic bacteria on swine feces. The level of substances responsible for the offensive odor of swine sewage could be reduced by cultivation of *R. capsulatus* on waste from animal feeding plants [74]. Another attempt is the genetic manipulation of intestinal bacteria. Hereby, expression of SQR from *R. capsulatus* in *E. coli* was studied, as well as expression of genes for quinone biosynthesis together with the *sqr* gene [51]. In both cases, sulfide oxidation to elemental sulfur by the recombinant bacteria as well as by isolated membranes was observed in laboratory scale, but only under aerobic conditions. For application of this systems inside the strictly anaerobic intestines, another terminal electron acceptor has to be found.

As a byproduct from sulfide-removing systems using phototrophic bacteria growing on waste, it is possible to produce foodstuff, medical products or biomass that can be used as fertilizers or livestock feed (reviewed in [75]).

9. OPEN QUESTIONS AND PROSPECTS

Over the last ten years, investigations about SQR have led to new insight about sulfide oxidation in microorganisms. However, many questions remain open. SQR is regarded to have evolved very early in bacterial evolution because of its occurrence in phylogenetically very distant bacteria. Thus, occurrence of SQR amongst sulfide-oxidizing Archaea would not be surprising, but remains to be examined. Occurrence of SQR within mitochondria is still a matter of investigation. Although SQR activity has been detected in mitochondria of the lugworm *Arenicola marina*, no SQR

protein has been purified up to now from Eukaryotes. HMT2 was suggested to catalyze sulfide-dependent reduction of quinones in mitochondria of *S. pombe*. However, as pointed out in section 4, it is not clear yet, whether this is the physiological function of HMT2 in yeast and in other sulfide-oxidizing Eukaryotes.

Whereas for SQR in *R. capsulatus* only an energy gaining function has been documented [42], in *A. halophytica* the role of SQR seems to be detoxification of sulfide [48]. *A. vinosum* expresses two sulfide-oxidizing enzymes, SQR and FCC [18], and no effect on the sulfide-oxidizing capability of the cells was found after inactivation of FCC. These results raise the questions, whether the major role of SQR is energy conversion or detoxification and why some bacteria express both SQR and FCC.

Although some progress has been made, experimental data about the catalytic mechanism and about the structure of SQR are scarce. What is the immediate sulfur product of the SQR-reaction, what is the exact function of the three cysteines in catalysis, which amino acid residues are involved in binding of quinone, and what is the function of the highly conserved stretches? The SQR of *R. capsulatus* functionally expressed in *E. coli* now allows the isolation of larger quantities of the native and mutated enzyme for structural and functional studies.

In the cyanobacteria *O. limnetica* and *A. halophytica* expression of SQR depends on sulfide, and in *R. capsulatus* the sulfide-dependent expression is modulated by various substrates. However, up to now neither information about the nature of the regulatory proteins and the sulfide-sensing mechanism is available nor has the stimulus for the modulation of SQR-expression in *R. capsulatus* been identified. With the translational fusion of SQR with alkaline

phosphatase of *E. coli* in *R. capsulatus*, we now have the tool in our hands to investigate the modulation of expression in more detail and to test whether the downstream ORF is involved in regulation of SQR.

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