Chapter 16

Sulfide Oxidation from Cyanobacteria to Humans: Sulfide–Quinone Oxidoreductase (SQR)

Yosepha Shahak
Institute of Plant Sciences, The Volcani Center, Bet-Dagan 50250, Israel

Günter Hauska*
Lehrstuhl für Botanik, Fakultät für Biologie und Vorklinische Medizin, Universität Regensburg, Germany

* Corresponding author, E-mail: guenther.hauska@biologie.uni-regensburg.de, Fax: +49 941 943 3352

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Summary

After the discovery that anoxygenic, sulfidotrophic photosynthesis can be induced in cyanobacteria, sulfide–quinone reductase (SQR) was identified and characterized in *Oscillatoria limnetica*. This was closely followed by the study of SQR in the purple bacterium *Rhodobacter capsulatus*. Subsequently the genes of the purple bacterium and of two cyanobacteria, as well as of the hyperthermophilic hydrogen bacterium *Aquifex aeolicus* were cloned, sequenced and expressed in *Escherichia coli*, and the enzymes were characterized.

Sequence analysis showed that SQR belongs to the disulfide oxidoreductase flavoprotein family, together with flavocytochrome *c* (FCC), another sulfide oxidizing enzyme. All the members of this family are characterized by two redox active cysteines which cooperate with the flavin in the redox cycle. A redox mechanism for SQR is proposed on the basis of site directed mutations of the cysteins and of other amino acid residues. Furthermore, a 3d-structural model is derived from the crystal structure of FCC.

The search into the genomes accessible in the internet documents a widely spread occurrence of SQR-genes in bacteria. From the 19 completed canobacterial genomes, five contain the gene. Phylogenetic analysis classifies these genes into at least two clades – SQR-type I and SQR-type II. However, SQR-like enzymes are not confined to prokaryotes. They occur in the mitochondria of some fungi, as well as of all animals for which the genomes have been sequenced. From these eukarytotic SQR-like proteins (SQRDL) only the one of fission yeast was isolated and was enzymatically characterized. It is involved in heavy metal tolerance, and has therefore been denoted HMT2. Since sulfide has been indentified as a gaseous transmitter substance in animals, a possible role for SQRDL signalling is considered.

Finally, phylogenetic scenarious for the descent of SQR from a common ancestor are discussed. Two observations are of special interest: (i) The mitochondrial SQRDL is of type II, although the endosymbiontic ancestor of mitochondria is considered to be a proteobacterium, which should have had a type I-SQR. (ii) The two essential cysteines among the flavoprotein family must have changed positions in the primary structure during evolution, thus constituting an example of functional plasticity within phylogenies.

I. Introduction

In the dawn of biological evolution, before cyanobacteria developed their photosynthetic water oxidation, the world was anaerobic and sulfidic (Shen et al., 2001; Anbar and Knoll, 2002). Photosynthetic bacteria largely used sulfide as the hydrogen donor for the assimilation of CO_2 and nitrogen into organic matter. Sulfidotrophy is still wide spread among extant bacteria and archaea (Blankenship et al., 1995; Brune, 1995; Friedrich, 1998; see also the chapter 15 by C. Dahl in this volume) and also some cyanobacteria that retained the ability to shift back from water to sulfide oxidation in photosynthesis under stress conditions (Garlick et al., 1977).

The first enzymatic step of sulfidotrophy was initially attributed to flavocytochrome *c*, which oxidizes sulfide with cytochrome *c* (Kusai and Yamanaka, 1973). However, further evidence (Brune and Trüper, 1986; Reinartz et al., 1999; Schütz et al., 1999) is in favour of sulfide–quinone oxidoreductase (SQR[1]), a flavoenzyme belonging to the large disulfide oxidoreductase family, like glutathione reductase or lipoamide dehydrogenase (Shahak et al., 1999; Griesbeck et al., 2000). SQR feeds electrons from sulfide into the quinone pools of the electron transport chains for energy conservation, either in photosynthesis or respiration.

Meanwhile, genes for SQR have been identified in numerous genomes, including all domains of organisms, except plants. Surprisingly, it is

Abbreviations: FCC – flavocytochrome *c* sulfide dehydrogenase; HMT2 – heavy metal tolerance factor 2; IEU – international enzyme unit (μmoles substrate reacted per mg protein and min); SQR – sulfide–quinone reductase; SQRDL – sulfide–quinone reductase like protein

[1] Unfortunately the acronym "SQR" is also in use for succinate–quinone oxidoreductase of respiratory chains.

even found in the human genome. The recent discovery that hydrogen sulfide, like NO and CO, functions as a "gasotransmitter" substance in smooth muscle relaxation and neuronal signalling (Wang, 2002; Boehning and Snyder, 2003), tempts into speculating that SQR may be involved in adjusting an appropriate sulfide level.

This review updates our earlier accounts on the structure and function of cyanobacterial and other prokaryotic SQRs (Shahak et al., 1999; Bronstein et al., 2000; Griesbeck et al., 2000) in complementation to the part that deals with sulfide oxidation in purple sulfur bacteria in chapter 15 by C. Dahl (in this volume). In addition we give a brief account on SQR-like enzymes in eukaryotes, from fungi to humans. Thus we intend to widen the view of an ancient enzyme, which is gaining new momentum by its possible involvement in cell signalling.

II. Discovery and Development of Studies

The ability of cyanobacteria to shift from oxygenic to anoxygenic photosynthesis using sulfide as hydrogen donor in place of water was an exciting discovery. It was regarded as a return to an earlier, more primitive form of photosynthesis (Padan, 1979, 1989), since it is found widely distributed among other extant phototrophic bacteria. Van Niel's ingenious generalization of photosynthesis experienced another impressive manifestation. The shift occurs under light and oxygen stress and is induced by sulfide in various cyanobacteria (Garlick et al., 1977). Further studies concentrated on the filamentous cyanobacterium *Oscillatoria limnetica* which inhabits the salty ponds of the Negev desert. The sulfide-induced cells catalyzed sulfide-dependent CO_2-fixation, as well as nitrogen fixation (Belkin et al., 1982) and hydrogen evolution (Belkin and Padan, 1978), under appropriate growth conditions. Tracking the inducible factor enabling sulfide-dependent photosynthesis led to the discovery and characterization of SQR (Shahak et al., 1987, 1992a, 1993; Belkin et al., 1988; Arieli et al., 1991, 1994), an enzyme (E.C.1.8.5.-) that feeds electrons into the quinone pool (reviewed by Shahak et al., 1999; see also Bronstein et al., 2000). Subsequently the SQR-genes from *O. limnetica* as well as from the unicellular cyanobacterium *Aphanothece halophytica* have been cloned, sequenced and were expressed in *E. coli* (Bronstein et al., 2000), in collaboration with an equivalent study on *Rhodobacter capsulatus* (Schütz et al., 1997, 1999).

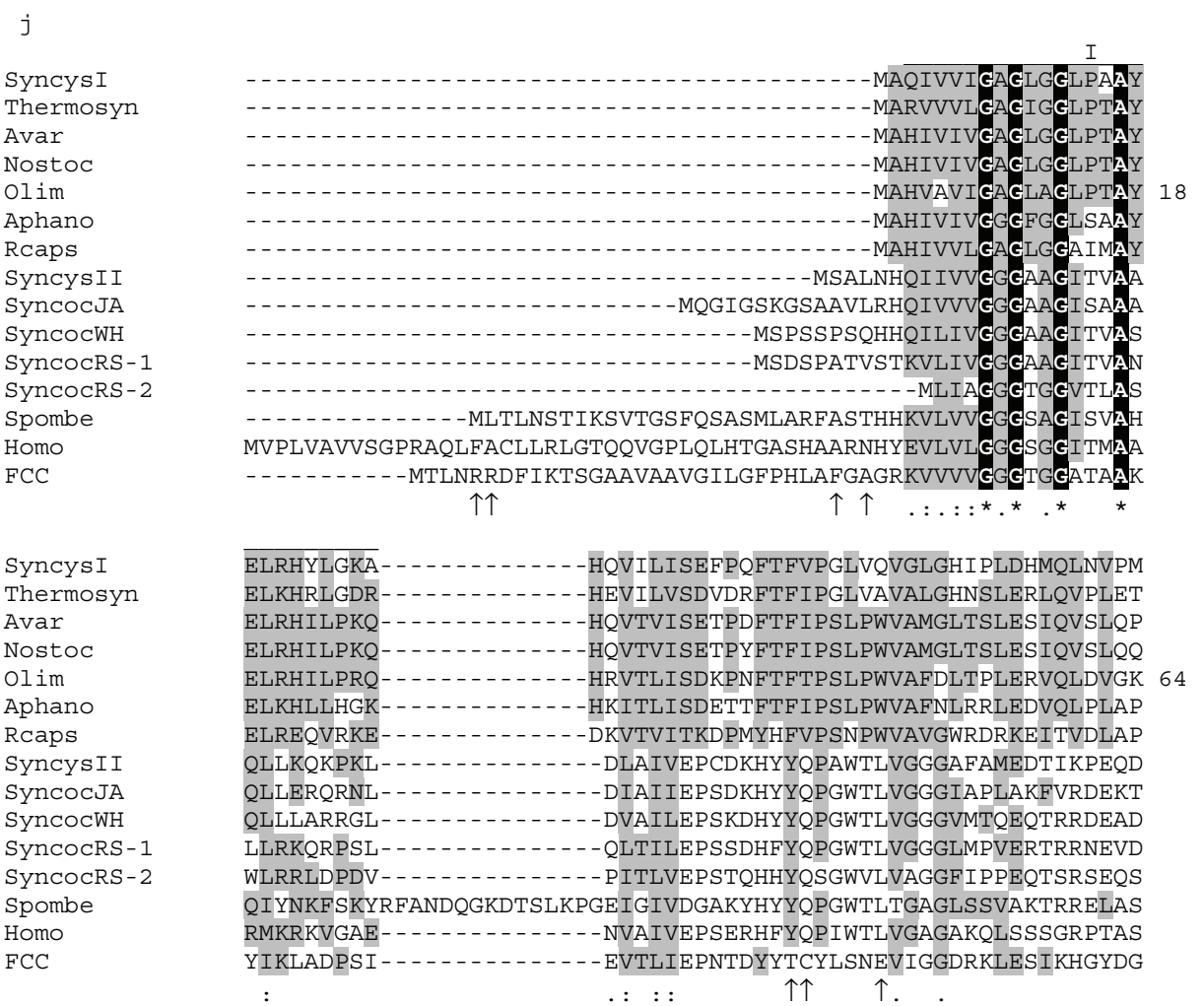
SQR activity has been detected in the membranes of several addional photosynthetic (*Chlorobium limicola* – Shahak et al., 1992b; *Allochromatium vinosum* – Reinartz et al., 1998) as well as chemosynthetic bacteria (*Paracoccus denitrificans* – Schütz et al., 1998; *Aquifex aeolicus* – Nübel et al., 2000). Most remarkably, however, SQR activity was also found in the mitochondria of invertebrates (Grieshaber and Völkel, 1998; Parrino et al., 2000) and vertebrates (Furne et al., 2001; Yong and Searcy, 2001). Moreover, a SQR-like enzyme from the mitochondria of the fission yeast *Schizosaccharomycis pombe* has been cloned and sequenced, and was expressed in *E. coli* as a His-tag protein, that after purification by Ni-chelate chromatography has been characterized in detail. It is known as HMT2, since its gene was detected by compensating a mutant defect in heavy metal tolerance (Vande Weghe and Ow, 1999, 2001).

A search through current databases reveals that the genes for SQR and SQR-like proteins (SQRDL) are present in the genomes throughout all domains of life, by far exceeding the phemomenon of sulfidotrophy. A comprehensive phylogenetic analysis of SQR-genes by Theissen et al. (2003) will be discussed below.

III. Characterization

A. Occurrence and Comparison of SQR-Genes

In the early study of Garlick et al. (1977) 11 out of 21 cyanobacterial strains where found to be capable of facultative anoxygenic photosynthesis with sulfide as the electron donor (reviewed by Padan, 1979). The phenomenon included strains of rather different habitats (aerobic, anaerobic, marine and fresh water) and of filamentous as well as unicellular types. At present, an inspection of the NCBI-website with 405 completed plus 644 unfinished microbial genome projects yields 19 completed cyanobacterial genomes and 25 in progress. Among these 34 cyanobacteria 10 strains contain a total of 12 SQR-genes. They are



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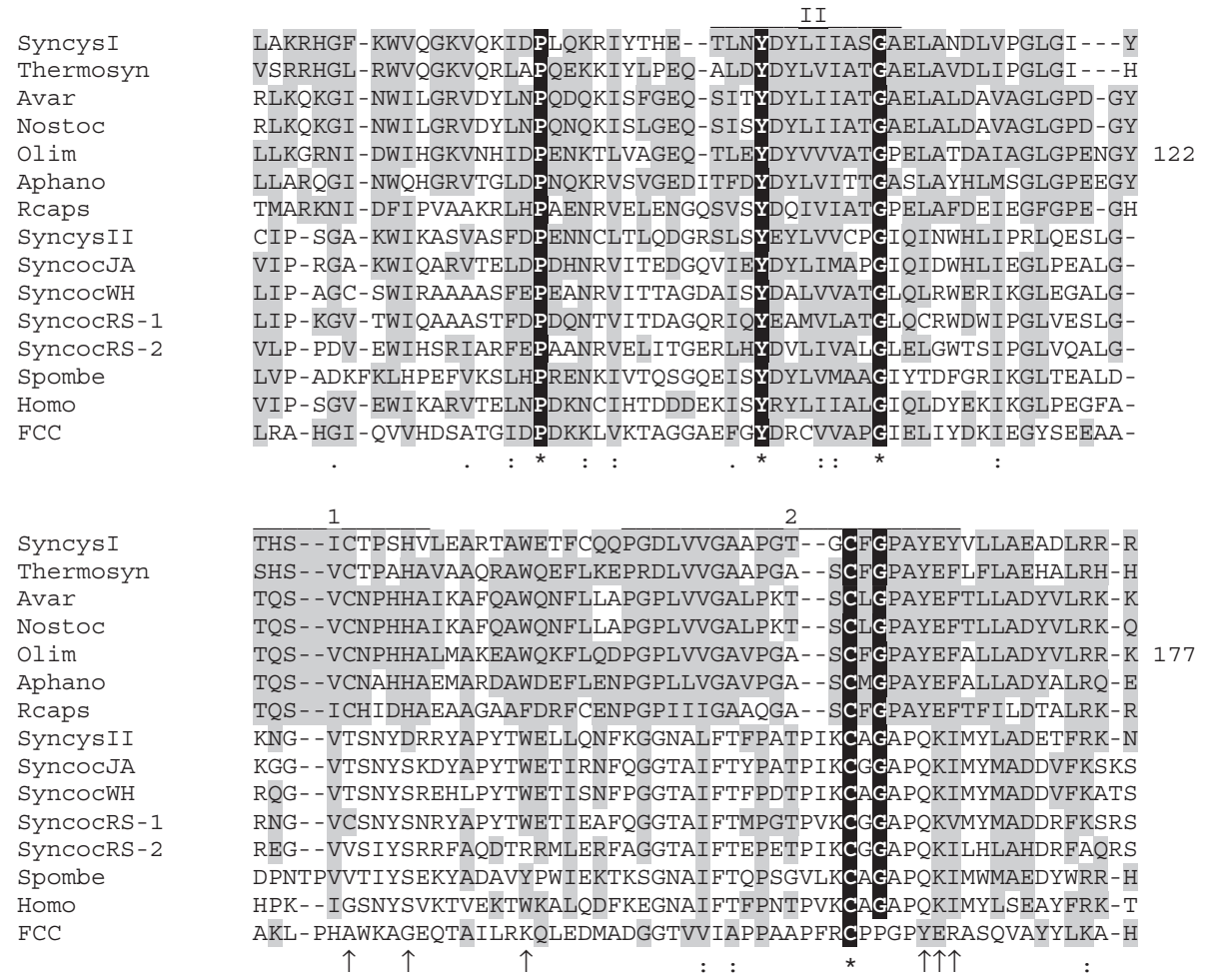

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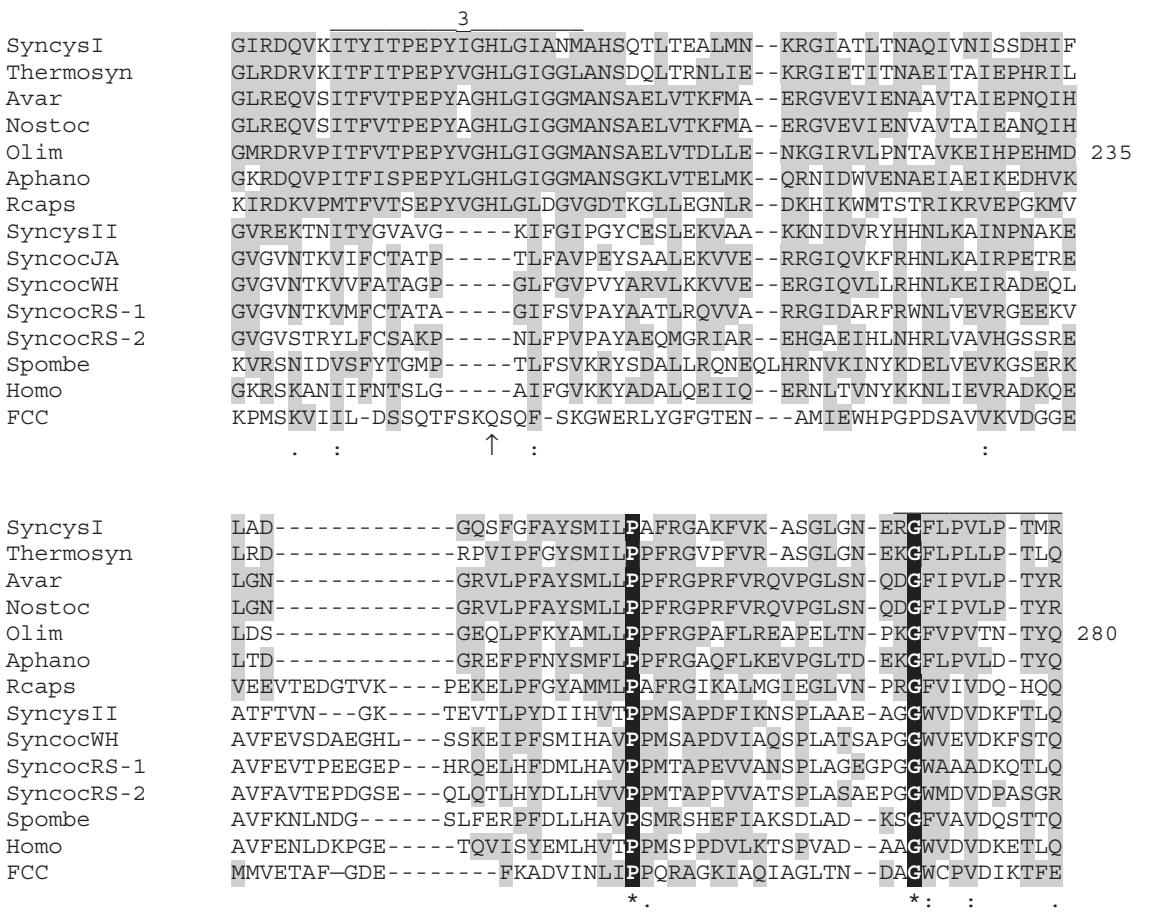

Fig. 1. (continued) Multiple alignment of SQR-sequences and comparison to FCC. The alignment shows the Clustal format obtained via the program T-Coffee (Notredame et al., 2000); the amino acid residues are given in single letter code; residues conserved in all types of SQR are underlayed in black, those which are addionally shared by FCC are indicated by a star at the bottom of the alignment; similar residues are indicated in two ways: at the bottom by single or double dots for low and high similarity in all sequences according to T-Coffee, and by shading the residues of high similarity which are typical for the cyanobacteria, but partially also extend into the other sequences; homologous peptide regions are lined and numbered on the top, I-III indicating FAD-binding in the disulfide oxidoreductase family, 1–6 indicating homology within SQR-type I (Griesbeck et al., 2000); arrows at the bottom indicate positions addressed in the text; abbreviations on the left stand for: SyncysI – *Synechocystis* PCC 6803 SQR-type I (accession number NP_942192), Thermosyn – *Thermosynechococcus elongates* (acc.no. NP_681079), Avar – *Anabaena variabilis* (acc.no. ABA22985), Nostoc – *Nostoc* PCC 7120 (acc.no. NP_488552), Olim – *Oscillatoria limnetica* (acc.no. AAF72962), Aphano – *Aphanotece halophytica* (acc.no. AAF72963), Rcaps – *Rhodobacter capsulatus* (acc.no. CAA66112), SyncysII – *Synechocystis* PCC 6803 SQR-type II (accession number NP_440916), SyncocJA – *Synechococcus* strain JA-3-3Ab (acc. no. ABD00861), SyncocWH – *Synechococcus* strain WH 5701 (acc.no. EAQ74835), SyncocRS – *Synechococcus* strain RS 9917 (acc.no. EAQ69368), Spombe – *Schizosaccharomyces pombe* (acc.no. CAA21882), Homo – *homo sapiens* (acc.no. AAH16836.1), FCC – flavocytocrome *c* from *Allochromatium vinosum* preprotein (acc.no. AAB86576); numbers at the right edge of the alignment blocks refer to the position in the sequence of *O. limnetica*, the count starting at the N-terminal M.

aligned in Fig. 1, together with the sequences of the SQR from *R. capsulatus*, of the mitochondrial SQRDL proteins from fission yeast and man, and of FCC from *Allochromatium vinosum*. In previous alignments of fewer sequences we had defined certain peptide regions that are significant for SQR-proteins (Bronstein et al., 2000; Griesbeck et al., 2000; Griesbeck et al., 2002). These are pointed out again by horizontal lines above of the alignment blocks in Fig. 1. Three FAD-binding regions which are characteristic for all members in the disulfide oxidoreductase family

are numbered with I to III, six additional regions specific for SQRs are numbered 1–6. Obviously, while the FAD-binding peptides are similar throughout the alignment of the 15 sequences, the other peptides are not. These peptides and overall similarities divide the SQR-sequences into two groups, which are represented by the upper and the lower half of the blocks in Fig. 1. The upper part contains six cyanobacterial SQRs plus the sequence of the purple bacterium *R. capsulatus*, while the lower part has closer resemblance to FCC and joins five cyanobacterial sequences with the mitochondrial SQRDL of fission yeast and humans. It is remarkable that both types are found among cyanobacteria. *O. limnetica*, *Thermosynchococcus elongatus*, *Aphanotece halophytica*, *Anabaena variabilis* and *Nostoc* PCC 7120 contain type I, while all the strains of *Synechococcus* contain type II. Two cyanobacteria contain two SQR-genes. *Synechococcus* strain RS 9917 has two versions of type II, and interestingly *Synechocystis* PCC 6803 contains one gene for type I, as well as one for type II.

The majority of the 19 completed cyanobacterial genomes lacks any SQR gene, however. Only five contain it – *Synechocystis* PCC 6803 (type I + type II), *Anabaena variabilis* (type I), *Thermosynechocooccus elongatus* (type I), *Nostoc* PCC 7120 (type I), and *Synechococcus* strain JA-3-3AB (type II). Seven other genomes of the genus *Synechococcus* lack the gene. It has also not been detected in the genomes of *Gloeobacter, Trichodesmium*, or in any *Prochlorophyta* so far. A gene for SQR is also absent from green plants, and from bakers yeast *Saccharomyces cerevisiae*. Nevertheless, it is present as a preprotein gene targeting for mitochondria in the genomes of fission yeast and other fungi, and in all animal genomes available so far in the NCBI-website (see also Vande Weghe and Ow, 1999). Noteworthy, the SQR gene is also found in the genome for the diatom *Thalassiosira pseudonana*.

Fifteen amino acid residues are fully conserved among both SQR-types in the alignment of Fig. 1, twelve of them are also found in FCC. They are underlayed in black in Fig. 1, and are shown in the 3d-structural model of Fig. 3 as well. Most significant are the two conserved cyteines, at positions 159 and 346 in the sequence of *O. limnetica*, which form a redox active disulfide bridge close to the isoalloxazine ring of FAD (see Fig. 2). A few further interesting observations are highlighted by arrows on the bottom of the alignment blocks (Fig. 1) and are listed below according to the position numbers in the sequence of *O. limnetica*:

- At position −3 lies the putative start of the mature SQRDL protein for *S.pombe* (Vande Weghe and Ow, 1999) and possibly also for *homo*, after cleavage of the mitochondrial targeting N-terminal extensions (Von Heijne et al., 1989). At position −1 lies the aminoterminus of mature FCC, the preprotein carrying the double-arginine signal (at −26 and −27), for export into the periplasmic space (Berks, 1996; see below).
- At positions 41, 47, 139 and 166 aromatic/hydrophobic residues are found in all SQRs, unlike the charged/hydrophilic residues that are present in the FCC-sequence. Thus they may be involved in binding the quinone which is not the substrate for FCC.
- At position 42 an additional cysteine is present in FCC which covalently binds the FCC via the 8-methyl group of the isoalloxazine ring (Chen et al., 1994).
- The presence of a cysteine at position 127 is characteristic for type I-SQR with its high substrate affinities (see Fig. 1 and text above). However, a Cys is also found in the first sequence of *Synechococcus* RS 9917, which clearly belongs to type II, as judged by overall homology and the presence of an aspartate instead of valine at position 291. It would be interesting to test the sulfide affinity of this SQR.
- An aspartate instead of valine at position 291 is not characteristic for type II-SQR only. It is also found in FCC, and even in all other members of the disulfide oxidoreductase family (see Griesbeck et al., 2002). In the crystal structures available position 291 is close to the ribityl moiety of FAD (see V291 in Fig. 3).
- Y164E165 might represent the active site base in type I (Fig. 2). The two residues are replaced by QK in type II, yet are found in FCC. This is intriguing in view of the low affinity of type II for sulfide, while high affinity of both, type I SQR and FCC (12.5 μM; Cusanovich et al., 1991).
- The histidines at positions 131 and 196 have been considered to contribute to quinone binding, as suggested from inspection of quinone binding sites in 3d-structures of membrane proteins (Rich and Fisher, 1999). However, the histidines are not conserved in type II. Furthermore, site-directed mutation did not

affect the affinity for quinone. Rather the affinity for sulfide was decreased (Griesbeck et al., 2002).

B. Properties of the Protein

1. Isolation

SQR was solubilized from thylakoids of sulfide-induced cells of *O. limnetica* and *A. halophytica* by mild detergent treatment and was purified by ammonium sulfate fractionation and HPLC (Arieli et al., 1994; Bronstein et al., 2000). A similar procedure was developed for the solubilization and purification of the enzyme from chromatophores of induced cells of the purple bacterium *R. capsulatus* (Schütz et al., 1997). Later the SQR enzymes were isolated as expression proteins from *E. coli* membranes, with or without His-tags (Vande Weghe and Ow, 1999; Bronstein et al., 2000; Griesbeck et al., 2002). A particularly convenient isolation protocol was developed for the isolation of the expression protein of the hyperthermophilic hydrogen bacterium *Aquifex aeolicus* from *E. coli* membranes, since this SQR could be purified to homogeneity in a single heat step after solubilization (Schödl, 2003).

2. FAD – the Prosthetic Group

One FAD is bound non-covalently to each protomer, as suggested by the FAD-binding motifs found in the primary structure (see Fig. 2), especially by the N-terminal βαβ-fold (Wieringa et al., 1986). Accordingly, partial reconstitution of activity of the denatured enzyme was achieved with FAD only (Griesbeck, 2001). The redox potential E_0' for FAD/$FADH_2$ is −60 mV (n = 2), which is about 160 mV more positive than for free flav in (Thauer et al., 1977). This has only been determined for SQR from *Aquifex aeolicus* so far (Schödl, 2003), but is expected to be similar for the SQRs from *O. limnetica, A. halophytica* and *R. capsulatus* which belong to type I (see below).

The optical spectra of the purified SQRs are characteristic for a flavoprotein, with absorption/excitation peaks at 280, 375 and 460 nm and an emission maximum at 520 nm. The fluorescence of SQR is quenched by sulfide at micromolar concentrations, and is recovered by the addition of quinone (Schütz et al., 1997; Griesbeck et al., 2002). On this basis a sensitive microsensor for sulfide may be developed. However, our efforts to do so with the thermostable SQR from *Aq. aeolicus* have failed so far (Schödl, 2003).

3. Enzymatic Activity and Inhibitors

The activity of SQR-type I is characterized by its high affinities for both substrates, with K_m-values in the μM range. Membranes, isolated enzymes and expression proteins have been studied extensively for the cyanobacterium *O. limnetica* (Arieli et al., 1991, 1994), the purple bacterium *R. capsulatus* (Schütz et al, 1997; Griesbeck, 2001) and the hydrogen-oxidizing bacterium *Aq. aeolicus* (Nübel et al., 2000; Schödl, 2003). Data are also available for membranes of the α-proteobacterium *Paracoccus denitrificans* (Schütz et al., 1998), the green sulfur bacterium *Chlorobium limicola* (Shahak et al., 1992b) and the purple sulfur bacterium *Allochromatium vinosum* (Reinartz et al., 1998), as well as for the isolated enzyme from the cyanobacterium *A. halophytica* (Bronstein et al., 2000). The specific activities in the membranes of these organisms, as compiled by Griesbeck et al. (2000), range from 0.02 IEU for *O. limnetica* to 3.5 IEU for *Aq. aeolicus*, reflecting different amounts and possibly also different turnover numbers for the various SQR-type I enzymes. Highly effective as inhibitors are quinone analogs, like stigmatellin and the akyl-hydroxyquinoline-N-oxides, which are known to block also the quinone interaction sites of the cytochrome complexes in photosynthesis and respiration. In this context it is remarkable that antimycin A and myxothiazole are ineffective on SQR-activity of the cyanobacterium *O. limnetica*, which operates with plastoquinone, together with the cytochrome *b6f*-complex. Both are efficient inhibitors, in the μM range, of the SQRs operating with ubiquinone and of cytochrome *bc*1-complexes. Antimycin as well as myxothiazole are well known to inhibit quinol/quinone-interaction with the *bc*1-complexes, but are ineffective with the *b6f*-complex. Thus there must be something common with respect to the structure of the quinone interaction sites of SQR and the cytochrome complexes.

The final product of sulfide oxidation in sulfidotrophic organisms is either sulfate or sulfur (see Blankenship et al., 1995; and the chapter by C. Dahl in this volume), while it is thiosulfate in mitochondria

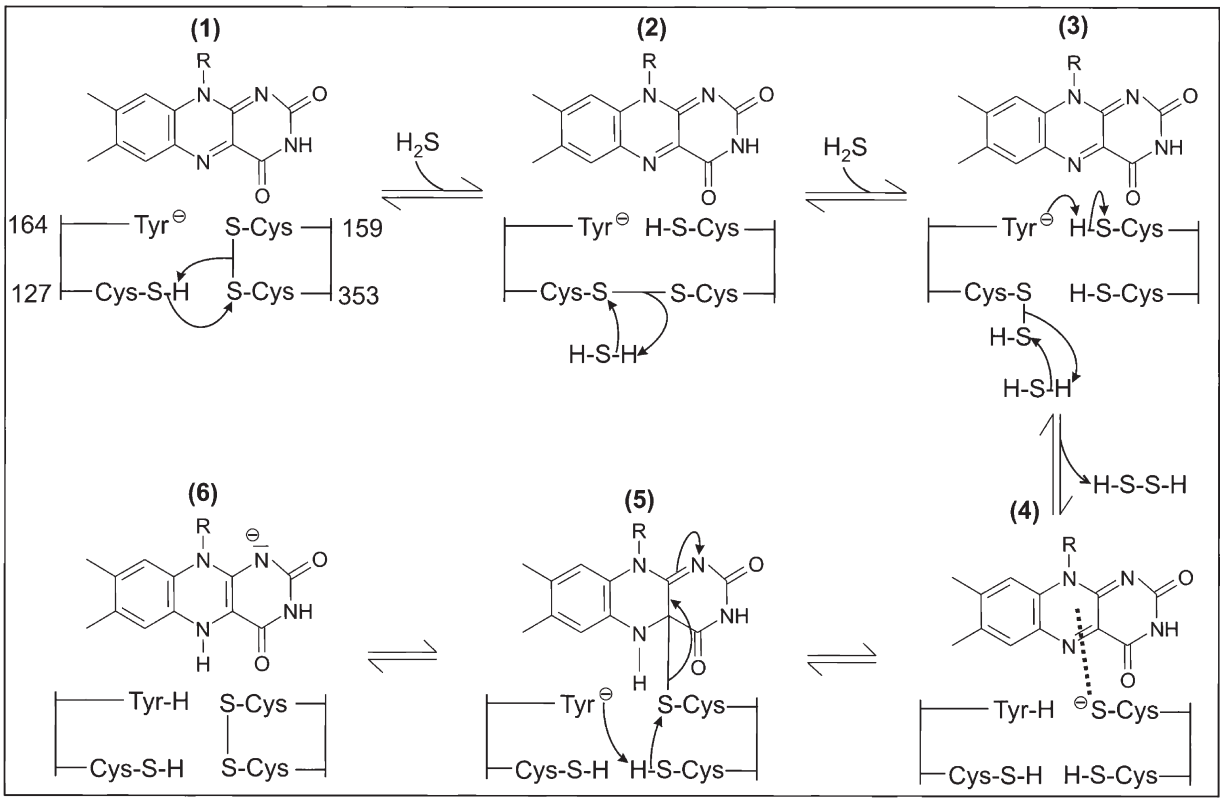

Fig. 2. Proposed mechanism of the reductive half-reaction of SQR. The proposal is a modified version of scheme 1 in Griesbeck et al.(2002), and is based on site-directed Cys/Ser-mutants; see text for explanation; the numbers correspond to the positions in the sequence of *R. capsulatus* (Fig. 1).

(Grieshaber and Völkel, 1998; Furne et al., 2001). For the SQR-reaction proper, the product is elemental sulfur. However, since this is an insoluble compound and is deposited outside the cell, the immediate products are soluble polysulfides (see contribution by C. Dahl), as documented for *R. capsulatus* (Griesbeck et al., 2002).

The only SQR of type II that was studied as an isolated expression protein is HMT2 from fission yeast (Vande Weghe and Ow, 1999). In view of its low affinity to sulfide, with K_m-values in the mM range we were initially reluctant to call it SQR (Griesbeck et al., 2000). However, since type II-SQR lacks the third conserved Cys (Fig. 1), it is possible that the enzyme cooperates with another SH-carrying cofactor *in vivo*. In this respect conserved cysteines in neighbouring ORFs of several SQR-genes have been considered (Theissen et al., 2003). In some cases these ORFs have been fused with the SQR-gene. Furthermore, work by H. Shibata and S. Kobayashi, Meji University/Japan, in collaboration with David W. Ow/USDA – unfortunately still unpublished – showed that the affinity of HMT2 as well as of SQR-type II from the cyanobacterium *Synechocystis* PCC 6803 (see below) towards the substrates could be substantially increased by adding mercaptoethanol or cyanide to the assay mixture (personal communication).

A word of caution, on the other hand, regarding the activity measurement is appropriate in this context: Quinones are directly reduced by sulfide, and this background rate increases with pH, the concentrations of the reaction partners, and – often not considered – with the presence of certain detergents. Thus, at substrate concentrations in the mM range, if detergent has been used to solubilize the SQR and is introduced together with SQR it may stimulate the reaction, thus mimicking

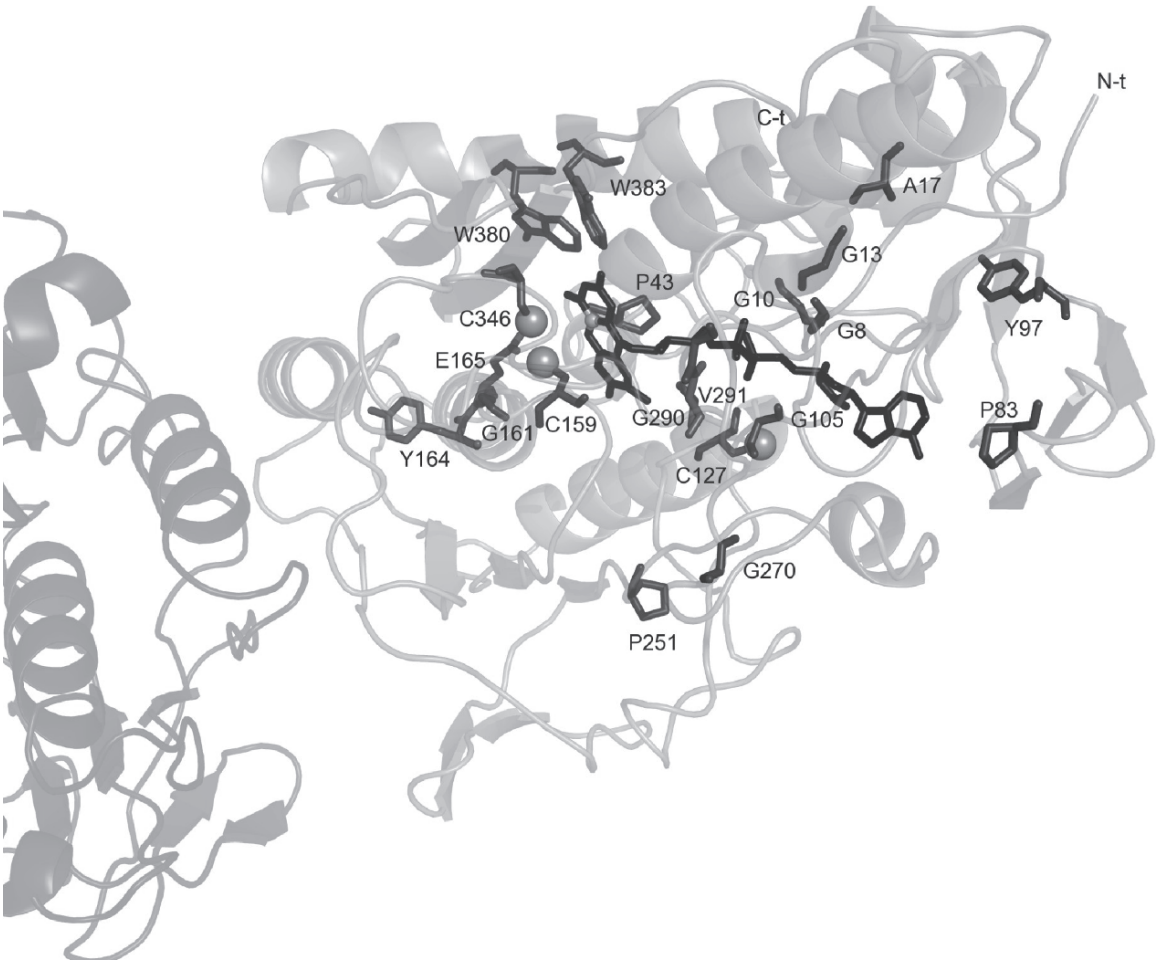

Fig. 3. A structural model. The model was obtained by threading the sequence of the SQR from *O. limnetica* into the crystal structure of FCC from *Allochromatium vinosum* (Chen et al., 1994; NCBI protein data base PDB: 1FCD), using the LOOPP server which is available online at http://cbsuapps.tc.cornell.edu/loopp.aspx (Teodorescu et al., 2004); locations of residues conserved and/or discussed for the alignment of Fig. 1 are indicated using the numbering of the sequence from *O. limnetica*; N-t and C-t stand for N- and C-terminus, respectively; the sulphur atoms of the three conserved cysteines are shown as spheres, the C4a-atom of the isoalloazine ring is indicated by a small sphere; on the left, part of the second protomer folding which forms the dimeric structure of SQR is shown in darker grey.

the enzyme. In contrast to the enzyme this artefact is heat stable (G. Hauska, unpublished).

4. Site-Directed Mutants

The three cysteines C127, C159 and C346, which are strictly conserved in SQRs of type I, and several other conserved residues have been changed in site-directed mutants of *R. capsulatus*, and the effect on activity has been tested in expression proteins (Griesbeck et al., 2002). All three cysteines are essential for the SQR activity, and for the quenching of FAD-fluorescence by sulfide. For the other members of the disulfide oxidoreductase flavoprotein family, only two cysteines are essential for activity (Williams, 1992), as documented by mutations of the gene for lipoamide dehydrogenase (Hopkins and Williams, 1995). Perhaps the third cysteine in SQR-type I is making up for the second sulfur atom in the substrates of disulfide oxidoreductases, allowing an efficient oxidation–reduction reaction.

Based on the results of site-directed mutagenesis with *R. capsulatus* a mechanism has been suggested (Griesbeck et al., 2002), which very likely also applies to the enzyme from cyanobacteria.

5. A Proposed Mechanism

The redox cycle of the disulfide oxidoreductases like glutathione reductase or lipoamide dehydrogenase involves a thiolate-FAD charge transfer complex and a covalent C4a-thioadduct of the flavin moiety (Williams, 1992). In analogy Fig. 2 shows our proposal for the reaction mechanism of the reductive interaction of sulfide and flavin in SQR-type I, with the participation of the three essential cysteines. The numbering of the essential residues in Fig. 2 corresponds to the SQR from *R. capsulatus*. In addition to the coordinated action of the cysteines an active site base is involved, which subtracts a proton from the SH-group in the cysteine vicinal to the flavin ring. Originally we considered E165 for this role. However, mutation of Y164 to leucine led to a substantially higher decrease in activity and affinity (C. Griesbeck and G. Hauska, unpublished).

The oxidized state cotains a disulfide bridge between C159 and C353 (1), or between C127 and C353. The reductive half reaction is considered to start with the breaking of the disulfide bridge between C127 and C353 by sulfide (2). The formed persulfide is split by a second sulfide molecule to form free persulfide, which undergoes chemical disproportionation to H_2S and polysulfides (not shown). At the same time a proton is taken from the vicinal C153 (3). The resulting thiolate forms a charge transfer complex to the flavin (4), followed by covalent attachment to the C4a-position which is accompanied by proton transfer from the active site base to N5 of the flavin ring (5). The C4a-adduct is in equilibrium with the anion of the reduced flavin (6), which is reoxidized by quinone (not shown).

A more extensive discussion and the description of further mutations can be found in Griesbeck et al. (2002). However, one more mutant should be considered. As pointed out above, SQR-type I differs from type II, FCC and the other members of the disulfide oxidoreductase family not only by its third conserved Cys, but also in the third FAD-binding region. At position 291/300 for the sequences of *O. limnetica/R. capsulatus*, respectively, a valine takes this place in SQR-types I instead of the aspartate found in all other cases. This may be another reason for the low substrate affinities of HMT2 from *S.pombe* (see above). Indeed a V300D-mutant of the SQR-gene from *R. capsulatus* showed drastically increased K_m-values for sulfide as well as for ubiquinone (Griesbeck et al., 2002). It is of note, however, that FCC which has an aspartate at this site (Fig. 1), has a high affinity for sulfide with a K_m-value of 12.5 µM (Cusanovich et al., 1991), in spite of having the aspartate in this position.

6. Structural Aspects

The purified and functional SQRs consist of a single protein which migrates as a band of about 55 kDa in SDS-PAGE. From gel filtration the size was estimated to range from 67 to 80 kDa, which was taken to suggest that the active enzyme is composed as a single polypeptide, surrounded by 20–46 detergent molecules (Arieli et al., 1994). Alternatively, however, since the actual M is only about 47 kDa when calculated from the primary structures (Fig. 1), the functional state of SQR may well be dimeric, as it is the case for the other members of the disulfide oxidoreductase flavoprotein family (Williams, 1992). Indeed, ultrafiltration experiments of SQR from *R. capsulatus* are in favour of a functional state that is larger than the monomer (Schütz et al., 1997).

In Fig. 3 a structural model for SQR from *O. limnetica*, which was obtained by threading the sequence into the crystal structure of FCC (Chen et al., 1994) is depicted. Noteworthy, a similar model for the human SQRDL can be found in the website http.//www.expasy.org/, via the link ModBase, where structures for all the proteins in the human genomes are suggested. In our model for SQR the location of three conserved cysteines and of the other residues pointed out for the alignment in Fig. 1 are shown with respect to the FAD in extended structure. The distance from the sulfur atom of the vicinal cysteine (C159 in SQR and C161 in FCC) to the C4a-atom in the isoalloxazine ring is 3.71 Å. By analogy to the blue light receptor phototropin, a flavoprotein with FMN (Fedorov et al., 2003), this distance should shorten to about 1.8 Å upon formation of the covalent thio-adduct, pulling the C4a-atom out of the ring plane.

C. Cellular Location

The SQR-enzymes are usually isolated by detergent treatment and are therefore considered to

be membrane bound. For chromatophores of *R. capsulatus* also the chaotropic agent NaBr sufficed to detach the enzyme (Schütz et al., 1997). It was concluded that in this case the enzyme is bound peripherally to the membrane, although by hydrophobic interaction. The enzymes of *O. limnetica* (Arieli et al., 1994), *Allochromatioum vinosum* (Reinartz et al., 1998) and *Aquifex aeolicus* (Nübel et al., 2000) are bound more tightly, and an integral insertion into the membrane has been suggested (Shahak et al., 1999). In view of the other similarities within SQR-type I, and since solubilization with NaBr has not been tried out systematically, further investigation is required to establish such a profound difference firmly.

Another problematic aspect with respect to function in membrane bound form is the dimeric state of functional isolates (see above), in particular since the dimer is central symmetric (Fig. 3). Such a structure on a membrane surface implies that only one of the two active centers is functional at a given time, with a possible switching between the two centers.

It was demonstrated by PhoA-fusion constructs that SQR of *R. capsulatus* faces the periplasmic space (Schütz et al., 1999), in line with the deposition of elemental sulfur outside the cells (Hansen and Van Gemerden, 1972), as it is known for many of the sulfide-oxidizing photosynthetic bacteria (Brune, 1995). However, no N-terminal extension as export signal into the periplasmic space is present in the bacterial SQR-squences, neither for the Sec-dependent nor for the Sec-independent pathway (Berks, 1996). The latter is characterized by a double RR-motiv, which is found-for FCC only (Fig. 1). Translocation of SQR depends on the C-terminal region and obviously uses a third pathway (Schütz et al., 1999), as discussed more extensively by Griesbeck et al. (2000).

In spite of the notion that cyanobacteria deposite elemental sulfur outside the cell (Garlick et al., 1977), we initially concluded that SQR in *O. limnetica* is inserted into the thylakoid membranes with its sulfide oxidation site facing the cytoplasm (Arieli et al., 1991, Shahak et al., 1999). This would require transport of elemental sulfur or at least of polysulfides through the cytoplasm to the outside. Alternatively, if in accordance with the location of SQR in purple bacteria the sulfide oxidation by the cyanobacterial SQR faces the intrathylakoid space, contact sites between the cytoplasmic and the thylakoid membranes would be required (Gantt, 1994). Further investigations should clarify this point.

The eukaryotic typeII-SQRDLs are directed into mitochondria by N-terminal signal sequences, as can be seen for fission yeast and *homo* in Fig. 1. Whether sulfide oxidation by analogy to the bacteria faces the intramembrane space remains to be established.

IV. Physiological Considerations

A. Bacteria

1. Sulfidotrophy and Sulfide Tolerance

Cyanobacteria thrive in two H_2S-rich ecosystems – in the "thiobios" of marine benthic mats (Fenchel and Riedl, 1970), and in sulfidic hot springs (Castenholtz, 1977), as reviewed extensively by Padan (1979, 1989). Other strains are not sulfidotrophic but rather sulfide tolerant – they can use sulfide for photosynthetic CO_2-fixation but do not grow on sulfide as the sole hydrogen donor (Garlick et al., 1977). Significantly the various strains of cyanobacteria which can shift from oxygenic to anoxygenic photosynthesis can fix CO_2 optimally with sulfide of a rather wide concentration range. At super optimal concentrations sulfide becomes inhibitory. The optima were found at 0.1, 0.7 and 3.5 mM for *Lyngbya* 7140, *Aphanotece halophytica* and *O. limnetica*, respectively. Since sulfide is rather toxic, the major susceptible target being the respiratory cytochrome oxidase (Grieshaber and Völkel, 1998), SQR in addition to energy conservation obviously serves for sulfide detoxification.

B. Fungi and Animals

1. Detoxification

The role of SQR in sulfide detoxification certainly holds also for mitochondrial SQRDL, although ATP formation with sulfide as the electron donor has been observed in mitochondria (Grieshaber and Völkel, 1998; Parrino et al., 2000; Yong and Searcy, 2001). Noteworthy, the colon mucosa of vertebrates is specialized for the mitochondrial oxidation of the sulfide produced by the anaero-

bic metabolism of enterobacteria (Furne et al., 2001), possibly via SQRDL. Sulfide has been implicated in the etiology of ulcerative colitis, and recently it has been identified as the "first inorganic substrate" for mitochondria of human colon cells (Goubern et al., 2007).

A more general role of detoxification by SQRDL could be linked to the turnover of FeS-centers. The biosynthesis of FeS-centers in eukaryotes is a mitochondrial process, which was inherited from the parent bacterial endosymbiont (Mühlenhoff and Lill, 2000). Nothing is known though about the fate of sulfide which is liberated during degradation of FeS-centers. Mitochondria containing a large number of FeS-centers, possibly liberating toxic amounts of sulfide during FeS-turnover, should benefit from the detoxifying function of SQRDL. However, since SQRDL is confined to animals and some fungi, this protective mechanism cannot be universal.

2. Heavy Metal Tolerance

The SQRDL of the fission yeast *Saccharomyces pombe* is denoted HMT2 for its subtle role in heavy metal tolerance (Vande Weghe and Ow, 1999, 2001). Resistance requires the complexation of heavy metals in the cytoplasm by phytochelatins and sulfide in a defined stoichiometry, suitable for efficient uptake into the vacuole by the ABC-transporter HMT1. If sulfide concentrations become too high heavy metal sulfide precipitates in the cytoplasm, and becomes lethal. The major role of HMT2 in this system is adjusting the optimal level of sulfide by oxidation with ubiquinone in the mitochondria.

3. A Role in Sulfide Signalling

It has been put forward recently that besides its toxicity, at low concentrations sulfide plays a role in animal signallig. This double role is shared with NO and CO. Sulfide is therefore recognized as the third "gasotransmitter" (Wang, 2002; Boehning and Snyder, 2003). Besides its local formation by the intestinal microflora sulfide originates from pyridoxal phosphate dependent cysteine metabolism in our body. It causes swelling of blood vessels by smooth muscle relaxation, much like and in cooperation with NO. Sulfide may be involved in long term potentiation of the central nervous system as well. Efficient signalling requires well controlled adjustment of the signal transmitters. Thus it is conceivable that sulfide oxidation by the SQRDL enzymes in mitochondria plays a role in the fine tuning of the sulfide level, reminiscent of the function of HMT2 in fungal heavy metal tolerance. Such a central role would explain why SQRDL proteins are ubiquitous in animals, but are absent from plants.

V. Phylogenetic Aspects

A. Cladograms

An extensive phylogenetic analysis of SQR-genes was provided by Theissen et al., in 2003. From 37 SQR-genes the peptide regions containing the two essential cysteines C159 and C346 (peptide 2 and 5 in Fig. 2), plus the FAD-binding peptide III, with the distinctive GV or GD-pair (see above) from 37 SQR-genes were taken for the calculation of phylogenetic distances. In the resulting cladograms three major groups of SQR-genes could be discerned. Types I and II were well separated and correspond to the two types specified in Fig. 1. Type III, which is prevailing in archaea and green sulfur bacteria, was not as well defined. Type I spreaded over the proteo- and cyanobacteria, but also included the phylogenetically distant bacterium *Aquifex aeolicus*. Type II comprised *Chloroflexus*, Bacilli and the eukaryotic, mitochondrial SQRDL proteins, and surprisingly also one SQR from a cyanobacterium (SyncysII in Fig. 1). The relationship between the mitochondrial and this special cyanobacterial protein had previously been recognized by Vande Weghe and Ow (1999). It was put forward that SQR represents an ancient enzyme originating from a common ancestor which enabled photo- and/or chemosulfidotrophy in the archaic, sulfidic environment. Furthermore, SQR should probably have entered the eukaryotic world by the monophyletic event of endosymbiosis creating the mitochondria. The alternative event of a later horizontal gene transfer could not be excluded, however.

The occurrence in cyanobacteria has been extended since, as documented in the NCBI-website and outlined above already. Both SQR-types occur among cyanobacteria, either type I or type

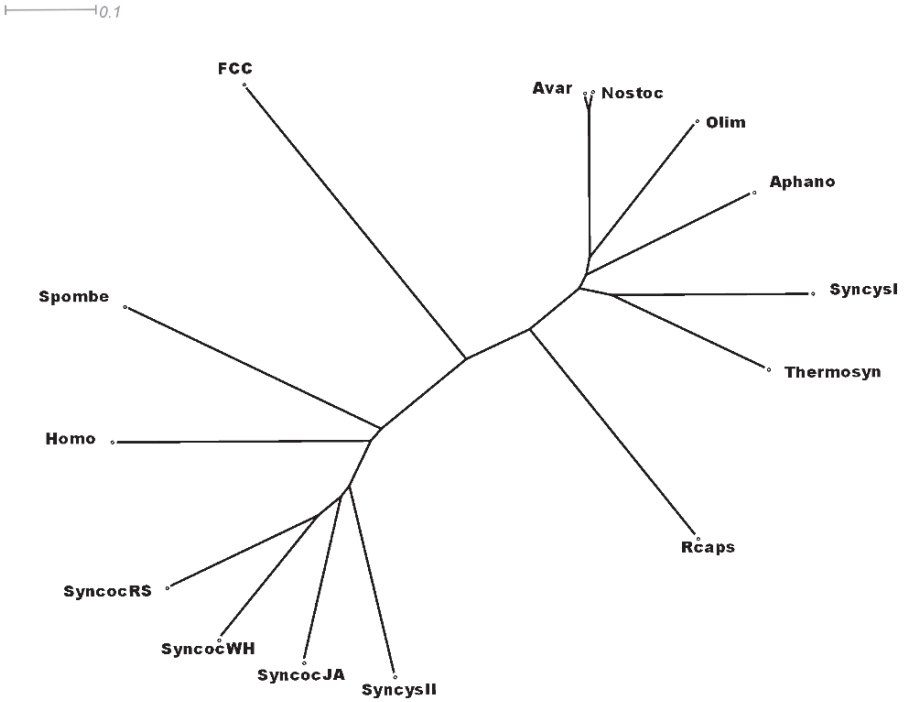

Fig. 4. Cladogram of the SQR-sequences presented in Fig. 1. The cladogram was obtained by using the SplitsTree4 implementation of the NJ-algorithm based on UncorrectedP distances. Different algorithms resulted in similar trees (Huson and Bryant 2006).

II, while both types occur in *Synechocystis* PCC 6803 (Syncys in Fig. 1). The distance cladogram of Fig. 4 shows the relatedness of the sequences presented in Fig. 1.

The split into the two groups with types I on the right and types II on the left, and FCC branching off in between the two, is obvious. Thus we support the analysis of Theissen et al. (2003), and further specify the conclusions in two aspects: (1) The closer relation of bacterial SQR-type II with the mitochondrial SQRDL enzymes and with the notion that the mitochondrial ancestor came from proteobacteria that have SQR-type I, is suggesting the acquirement of SQR by horizontal gene transfer from a Bacillus or cyanobacterium. (2) The absence of SQR from the plant kingdom, and its obligatory occurrence in all animals, may reflect the attainment of new functions during evolution. One such possible function is an involvement in animal type signalling, which may have evolved somehow from fungal heavy metal tolerance. Plants and green algae may have lost their SQR-typeII gene, but the diatoms, and possibly other orders of algae, interestingly, have retained this gene, for an unknown reason. A more comprehensive phylogenetic treatment of SQR, in particular with regard to the closer relationship between type II and FCC will be given elsewhere (R. Merkl and G. Hauska, in preparation).

B. Convergence within a Phylogeny – the Position of the Conserved Cysteines

The SQR proteins and FCC represent an additional interesting aspect within the phylogeny of the disulfide oxidoreductase family, previously noticed for FCC (Todd et al., 2002). In the crystal structures of glutathione reductase and lipoamide dehydrogenase, as well as of FCC (Mattevi et al., 1992; Chen et al., 1994) two cysteins are located as a redox active disulfide bridge, close to the isoalloxazine ring of the FAD. We concluded above, that this is also the case for SQR (Figs. 2 and 3). However, the positions are rather different in FCC and the disulfide oxidoreductases proper. While in the disulfide oxidoreductases the

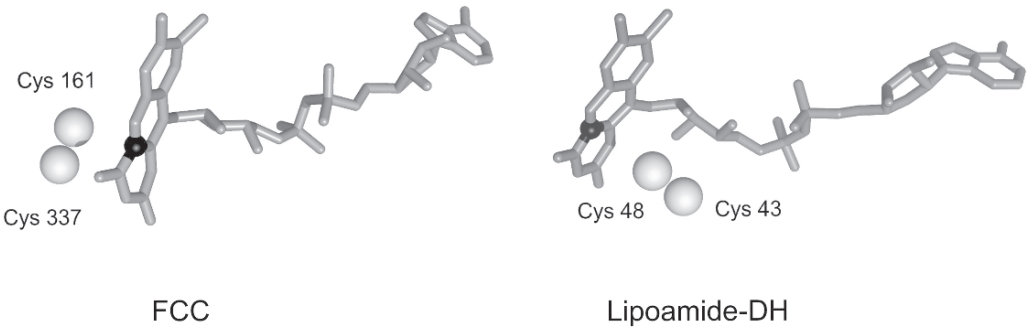

Fig. 5. Change in the spatial position of the two essential cysteines within the disulfide oxidoreductase flavoprotein family. The figure was derived from the crystal structures of FCC from *Allochromatium vinosum* (Chen et al., 1994; PDB: 1FCD) and lipoamide dehydrogenase from *Pseudomonas putida* (Mattevi et al., 1992; PDB: 1LVL). The sulfur atoms of the cysteines are shown by spheres, the C4a-position of the isoalloxazine ring in FAD is shown as a small black sphere.

two cysteines are located close together in the primary sequence, spaced by four amino acid residues, in the N-terminal region, in both FCC and SQRs one cysteine comes from the middle and one from the C-terminal region. As shown in Fig. 5 these are C48 and C53 for a bacterial lipoamide dehydrogenase, while C161 and C337 for FCC. Moreover, the spatial location of the Cys-pairs differ. In FCC the pair approaches the isoalloxazine ring from the opposite side of the ribityl-ADP moiety, while from the same side in lipoamide dehydrogenase (Fig. 5).

Such plasticity of catalytically essential positions within phylogenies is puzzling at first sight, yet known for other enzyme families as well (Todd et al., 2002). How could this be reconciled with the origin from a common ancestral enzyme? A reasonable assumption is that the ancestor contained both pairs of cysteines. Perhaps C72 in the N-terminal region of FCC (Fig. 1), a third cysteine, which is conserved in bacterial FCCs for covalent binding of FAD (Chen et al., 1994), represents a relic of the ancestral enzyme.

VI. Concluding Remark

Finally we would like to express our own surprise about the remarkable extension of the biological relevance for the SQR enzymes, hoping that our review encourages future investigations, with all the precautions for the still speculative parts of it. Own efforts focus on the human SQRDL which has been expressed in *E. coli* after engineering for codon usage (T. Schödl, unpublished). Studies on activity, as well as on expression in rat and human tissues are in progress (M. Ackermann, K. Maier, A.-L. Piña and G.Hauska, in preparation).

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