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The membrane-extrinsic domain of cytochrome $b_{558/566}$ from the Archaeon *Sulfolobus acidocaldarius* performs pivoting movements with respect to the membrane surface

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Abstract The orientation of the membrane-attached cytochrome $b_{558/566}$-haem with respect to the membrane was determined by electron paramagnetic resonance spectroscopy on two-dimensionally ordered oxidised membrane fragments from *Sulfolobus acidocaldarius*. Unlike the other redox centres in the membrane, the cytochrome $b_{558/566}$-haem was found to cover a range of orientations between 25° and 90°. The described results are reminiscent of those obtained on the Rieske cluster of *bc* complexes and indicate that the membrane-extrinsic domain of cytochrome $b_{558/566}$ can perform pivoting motion between two extreme positions. Such a conformational flexibility is likely to play a role in electron transfer with its redox partners.

Key words: Archaeon; Cytochrome c_y; Cytochrome $b_{558/566}$; Electron paramagnetic resonance; *Sulfolobus*

1. Introduction

In the recently solved crystal structures of the mitochondrial cytochrome (cyt) bc_1 complex [1–4] the soluble domain of the iron sulfur subunit (the Rieske protein) was found in three distinct positions. These results were interpreted as reflecting conformational flexibility of the Rieske protein swinging back and forth between the so-called Q_o site and cyt c_1. Such a long-range conformational movement of the Rieske protein has in turn been proposed as a prerequisite for efficient electron transfer from quinol to cyt c_1 [2–5]. The conformational flexibility of the hydrophilic domain has been shown to be achieved by a tether region of amino acids linking a transmembrane anchor helix to the extrinsic carboxy-terminal domain [6]. A comparable flexibility involved in electron transfer had already been suggested by structural and biochemical studies for the two-domain protein flavo-cyt b_2 from lactate dehydrogenase and for the membrane-attached cyt b_5 from liver microsomes (reviewed in [7]). More recently, kinetic data for electron transfer involving the membrane-attached cyt c_z and cyt c_y have been interpreted to reflect conformational mobility [8,9] and a flexible region, equivalent to the linker region of the Rieske protein, is proposed to be present in several membrane-attached cyts [8,10–12]. Electron transfer involving long-range conformational movement of extramembrane domains of membrane-attached proteins may therefore be more widespread than previously thought.

We have recently shown that the conformational flexibility of the cyt *bc* complexe's Rieske protein can be conveniently monitored by electron paramagnetic resonance (EPR) spectroscopy on two-dimensionally ordered samples [13–16]. EPR on partially ordered systems has been developed in the late 1970s [17,18] and has been applied as early as 1979 to reveal conformational flexibility of the haem protein cyt b_5 [19].

Since the EPR spectral approach is significantly less time-consuming than three-dimensional structure determination, we have used this technique in order to screen prokaryotic cytoplasmic membranes for the presence of redox proteins potentially performing long-range conformational domain movement.

In this work, we report an EPR study on oriented membrane fragments from *Sulfolobus acidocaldarius* revealing multiple orientations of cyt $b_{558/566}$ with respect to the membrane. This *b*-type cyt therefore most likely employs a domain movement to optimise electron transfer with its respective redox partners.

2. Materials and methods

S. acidocaldarius cells (DSM 639) were grown, and membranes were prepared as described previously [20]. Oriented samples were obtained as described by Rutherford and Sétif [18]. The oxidised membranes were obtained by washing the membrane once in 20 mM morpholinepropanesulfonic acid (pH 7), 5 mM potassium ferricyanide, and once in unbuffered water. After this treatment and in the absence of further oxidant, cyt $b_{558/566}$ and the oxidases [20–22] were partially oxidised. EPR spectra were recorded at liquid-He temperatures on a Bruker (Karlsruhe, Germany) ESP 300E X-band spectrometer fitted with an Oxford Instruments cryostat and temperature control system. Amino acid sequences were aligned with the help of the program CLUSTALX [23] and secondary structure predictions were performed using the pSAAM package (A.R. Crofts, 1992, pSAAM for windows,

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Abbreviations: cyt, cytochrome; EPR, electron paramagnetic resonance

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a program for protein sequence analysis and modeling, University of Illinois, Urbana, IL, USA).

3. Results and discussion

S. acidocaldarius is an obligate aerobic, hyperthermoacidophilic Crenarchaeon [24]. Three different *a*-type and two different *b*-type but no *c*-type cyts have been detected in the membranes of this organism. Two of the *a*-type cyts were attributed to the SoxABCD quinol oxidase complex [22,25] whereas the third *a*-type cyt and a cyt b_{562} were attributed to the SoxM terminal oxidase complex [26]. The function of the second *b*-type cyt (named $b_{558/566}$ after its absorption maxima) remains poorly understood although the protein has been characterised in detail [21]. Cyt $b_{558/566}$ is a highly glycosylated monohaem protein of 64.2 kDa apparent mass and a redox midpoint potential of +400 mV (at pH 6.5).

In its native state, the cyt $b_{558/566}$ is attached to the membrane and biochemical purification requires detergent treatments. Secondary structure predictions based on the amino acid sequence deduced from the gene suggest single transmembrane stretches at each end and an extended hydrophilic domain representing the major part of the protein ([21]; and see below).

Since the EPR spectral parameters of both oxidases and of the cyt $b_{558/566}$ are known [21,27,28] we have tried to analyse the orientations of the respective haems with respect to the membrane in partially ordered samples.

Fig. 1 shows EPR spectra recorded on oriented ferricyanide-oxidised membranes of *S. acidocaldarius* in the range −40° to +110° angle between the magnetic field and the plane of the membrane fragments. As can be seen from the spectra, the samples were well ordered since the g_z peaks of the *a*-haems at $g = 3.06$, 2.94 and 2.8, attributed to the SoxABCD and SoxM oxidases [27,28], show unique and well defined orientations. In contrast to these cases, the $g_z = 3.13$ of $b_{558/566}$ (identified by comparison to the spectrum recorded on the isolated protein [21]) shows multiple maxima. A polar plot evaluation of the $g_z = 3.13$ signal amplitude (closed squares in Fig. 2) indicates two distinct maxima at 0° and 65° with respect to the membrane. In between these two maxima, the signal amplitude decreases by only 35%, whereas nicely separated lobes would be expected for both centres [29,30]. This indicates that either the dried membrane samples are poorly oriented or that the observed maxima correspond to the limiting directions in a range of orientations that the g_z vector can point at. The first possibility can be dismissed considering the high anisotropy of all other paramagnetic species observed in these samples (e.g. g_z peaks of the *a*-haems at $g = 3.06$, 2.94 and 2.8, attributed to the SoxABCD and SoxM oxidases). Polar plot evaluation of the $g_z = 2.8$ signal amplitude (open circles in Fig. 2) is shown as one example. We therefore conclude that there must be populations of cyt $b_{558/566}$ which point their g_z direction at angles intermediate to 0° and 65°. The $b_{558/566}$-cyt can therefore apparently wobble between two extreme positions. These two limits correspond to haem orientations of 25° and 90° with respect to the membrane, considering that the g_z direction in low-spin haems is oriented nearly perpendicular to the porphyrin plane [31].

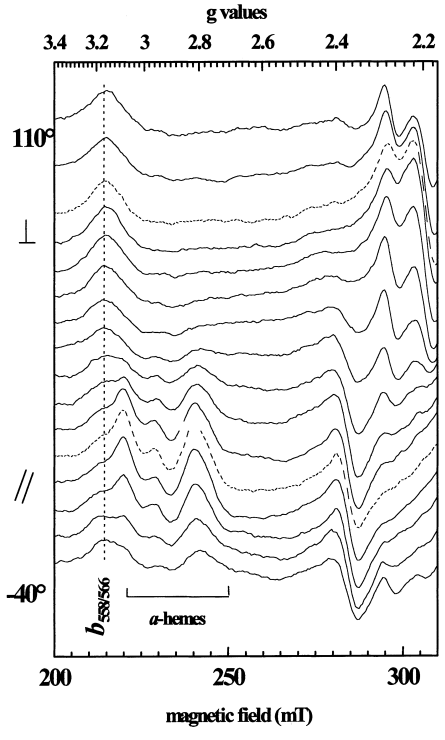

Fig. 1. EPR data obtained on ordered membrane fragments from *S. acidocaldarius*. Oriented oxidised membranes were prepared on mylar sheets as detailed in Section 2. EPR spectra in the region of low-spin g_z signals were taken in a range of orientations. Angles are given between the plane of the multilayers and the direction of the magnetic field.

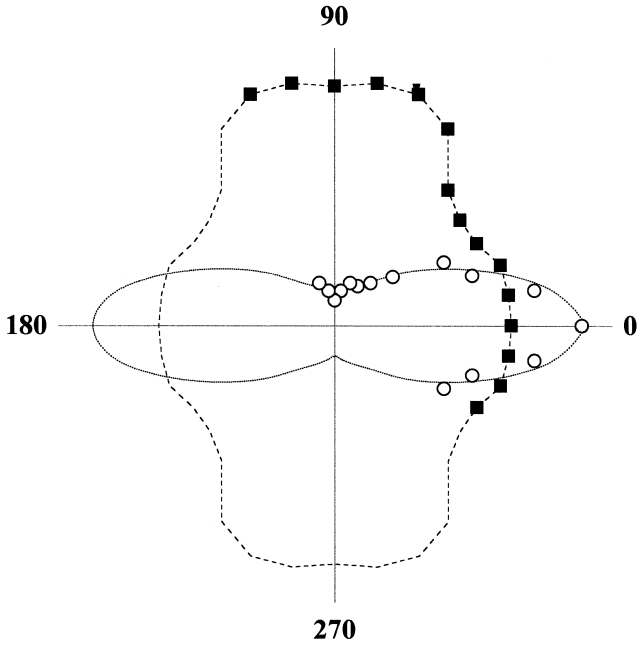

Fig. 2. Polar plot evaluation of EPR spectra obtained on ordered membrane fragments from *S. acidocaldarius*. The closed squares correspond to signal amplitudes of the $g = 3.13$ peak whereas the open circles denote the signal amplitude of the $g = 2.8$ peak measured on spectra shown on Fig. 1.

3.1. A structural model for cyt $b_{558/566}$

The gene encoding cyt $b_{558/566}$ has been detected using ami-

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solfa      1 MSIKRKSKYTLGVLLLASFLAIIMGLANVPMAQTSPQIPVYKVVGNADLS
acido      1 MSLKIKSKITIGVLLIIFLLSIIFTLENVSLAQTSPQISVYKVVGSADLS
             **:* *** *:****:  :*:**: * **.:*******.******.****

solfa     50 NPGSASYWSQIPWINISLTANIPMAPTSGLTHYLLVKAVWNGSWIIILER
acido     50 NPGSAGYWSQIPWTNISLTANIPMAPTSGLTHYLLVKAAWNGSWIFILEE
             *****.******* ***********************.******:***.

solfa    100 WYAPEPAFGAWSAAAAALYPPASGPGLFRQIMLTPGTTYTIEKNYTNYFS
acido    100 WQAPEPAFNAWSTAVAGIYPNASGPGLFRMIELTPGTTYSLERNYTNYVS
             * ******.***:*.*.:** ******** * *******::*:*****.*

solfa    150 IVNGNIIQGRLVLNYSGILLPAPNDTQITVLSNGTIILWHSPRPIEDLLY
acido    150 IINGKEETGRIVFNYSGITLPAPNNTEITVMSNGTILLWHSPRPVEDLLY
             *:**:   **:*:***** *****:*:***:*****:*******:*****

solfa    200 SDGMFYGYYTNNTWYYPDRAAIMWYMGSVIPPTKDGMNIGGKVPGQAFDG
acido    200 NDGMFYGYYVNSTWYYPDRAAIMWYLGSGVP-TKDDMNIGGKYPGQQFDG
             .********.*.*************:** :* ***.****** *** ***

solfa    250 VTFNDTGGSLVQPGGAANIWMWVSGATWNNATYDPAFKVNLWQNTSLTGL
acido    250 ITFKDAGGSLAQSGGSANIWMWVSGATWNNSTYDPAFKSNIWQNESLTGL
             :**:*:****.*.**:**************:******* *:*** *****

solfa    300 PYIDPDNHGFAVPLYTNNTNMYEVDTAGIWYTPVTTSGLNGSLFFIWTGA
acido    300 SYVDSGNHGFAVPLYTNNTNMYEVDTAGIWYTPVASEGLNGSLFFIWTGA
             .*:*..*****************************::.*************

solfa    350 TYQNGYWTVEFARPLAVPSAYAKWMPNITVGKTYYVAFAVWQGKLGETLF
acido    350 KYENGSWVVEFARPLSVPLDYQPFMPNITVGKTYYVAFAVWQGRLGETLF
             .*:** *.*******:**  *  :*******************:******

solfa    400 DKSITSNFLTLELVTTPPTSTTTSTISSTSVTTTTSITTVTSIPSTTIYV
acido    400 DKSITSSFLSLELVTTPPTSTTTSTSPVTTISS--------AIPPVTLYV
             ******.**:*************** . *::::        :**..*:**

solfa    450 TIVGVVIAIIALIILYVVFRR
acido    450 TIIGVVVALVALVILYVVFRR
             **:***:*::**:********
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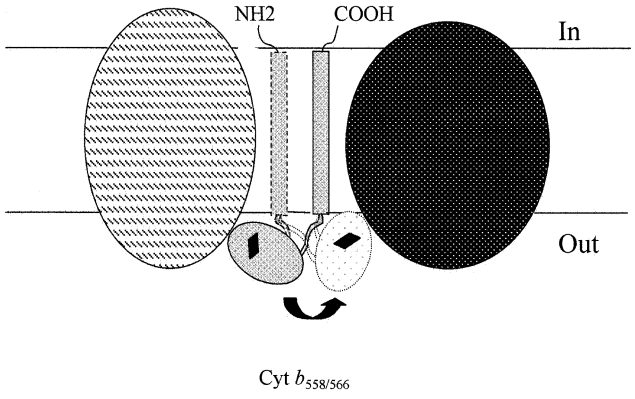
Fig. 3. Sequence alignment of the $b_{558/566}$-cyts from *S. solfataricus* and from *S. acidocaldarius*. Sequences of the proteins from *S. solfataricus* (solfa) and from *S. acidocaldarius* (acido) are aligned. Identical residues are marked by asterisks and homologous residues are indicated by double dots. Identified hydrophobic spans are underlined. Methionine residues corresponding to putative N-termini are shown in bold characters.

no acid sequences from internal (protease-cleaved) fragments of the mature protein and has been sequenced [21]. Unfortunately, the N-terminal sequence of the mature protein from *S. acidocaldarius* could not be obtained and two different methionines are therefore possible candidates for the N-terminal residue (as discussed in [21]). The sequence of a homologous cyt in the phylogenetically close organism *Solfolobus solfataricus* has been retrieved from the genome and is aligned to the *S. acidocaldarius* cyt in Fig. 3. As can be seen from the figure, cyt $b_{558/566}$ is strongly conserved between these two species. Both methionine residues (bold characters in Fig. 3) are conserved in *S. solfataricus*.

Secondary structure prediction indicates two putative membrane-spanning regions, one close to the N-terminus and another close to the C-terminus of the protein (underlined in Fig. 3). The remaining bulk of the protein (flanked by these putative membrane helices) is predicted to be essentially hydrophilic, i.e. to represent a domain which is exposed to the aqueous phase. The fact that this part of the protein is strongly glycosylated [21] furthermore indicates that this domain is situated on the outer face of the cytoplasmic membrane (*Sulfolobus* does not contain a periplasmic space). Since, as mentioned above, the N-terminus is not known, it cannot be decided at present whether the transmembrane region close to the N-terminus is a genuine transmembrane helix of the mature protein or whether it represents a membrane-targeting presequence which may be cleaved off in the mature cyt $b_{558/566}$. No clear evidence of a classic (Bacteria-type) signal peptide and cleavage site can be seen. However, little is known concerning maturation mechanisms in Archaea and to our knowledge, it is not clear whether the described rules hold for the Archaeal domain.

Two global structures are therefore in line with the presently available data.

1. Cyt $b_{558/566}$ contains a large hydrophobic domain exposed to the aqueous phase anchored to the membrane by two transmembrane helices flanking the soluble domain on both the N- and the C-terminal ends of the sequence. A corresponding structure has not been reported for membrane-attached cyts so far.
2. The N-terminal hydrophobic stretch (illustrated in dashed lines in Scheme 1) is removed in the mature protein and cyt $b_{558/566}$ consists of a soluble (haem-binding) domain anchored to the membrane via a C-terminal helix as is for example the case for cyt *f*.



Scheme 1. Schematic representation of cyt $b_{558/566}$'s position in the membrane. The protein can be integrated in the membrane by the N-terminus, the C-terminus (if the N-terminal helix is cleaved as a signal peptide) or both of them. As discussed in the text, this cyt could either function as an electron carrier within the membrane-bound respiratory system or as an intermediate between a pseudo-periplasmic redox metabolism and the membranous electron transfer system.

Both structural models are schematically depicted in Scheme 1.

3.2. Functional significance of cyt $b_{558/566}$'s domain movement

The functional role of cyt $b_{558/566}$ is only poorly understood. It has been suggested that it could either represent an electron carrier within the respiratory system [32] or an intermediate between pseudoperiplasmic metabolism and the respiratory system [21]. The above described results are indicative for positional flexibility of the haem-carrying soluble domain in cyt $b_{558/566}$ and are strongly suggestive of a conformational movement involved in electron shuttling from a donating towards an accepting redox protein. It has been demonstrated that the respiratory system of *S. acidocaldarius* is constituted by two terminal oxidases: a SoxM complex – a supercomplex of a Rieske–*b* complex (i.e. a *bc* complex without *c*-type subunit) and an oxidase – and a SoxABCD complex. The similarity of cyt $b_{558/566}$ to intermediate electron carriers has been discussed [32]. Intriguingly, in the supercomplex between the cyt bc_1 complex and the aa_3 oxidase from *Paracoccus denitrificans* [11], the membrane-attached cyt c_{552} has been found to fulfil such a function and a flexible head-group has been postulated for this cyt. An equivalent function is attributed to the flexible head-group of cyt c_y from *Rhodobacter capsulatus* during electron transfer from the cyt bc_1 complex to the photosynthetic reaction centre and cyt oxidase [33]. A supercomplex between a cyt *bc* complex and a cyt oxidase fused to an exposed cyt domain has furthermore been described in *Bacillus* PS3 [34]. It is of note, however, that synthesis of cyt $b_{558/566}$ in *S. acidocaldarius* can be almost completely repressed under appropriate conditions [35] showing that this cyt does not appear to be an indispensable component of the *S. acidocaldarius* respiratory chain.

3.3. Domain flexibility, an ancient mechanism for electron transport?

Apart from the eukaryotic examples cyt b_5 and flavo-cyt b_2, the possibility of domain flexibility in membrane-attached cyts has been discussed for proteo- and green sulfur bacteria [8–11]. Using the above described technique, we have observed 'swinging' cyts in organisms covering almost the complete phylogenetic tree of the Bacteria (unpublished results). Notably, a member of this class of cyts was found in the hyperthermophile *Aquifex aeolicus*, a species representing one of the earliest branches of the Bacteria (manuscript in preparation). The existence of such a cyt in an Archaeon suggests that the common ancestor of Bacteria and Archaea/Eukarya already employed this mechanism for electron shuttling between membrane-integral redox complexes. This appears reasonable considering that the earliest organisms most probably were devoid of an outer membrane (as is still the case in numerous Archaea and for example the Gram-positive bacteria).

Membrane-attached flexible cyts can therefore be assumed to represent the evolutionary precursors of their soluble homologues and only the invention of the outer membrane allowed for abolition of the membrane anchor domain and thus for complete rotational freedom in formation of electron transfer complexes involving these redox carriers.

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