Arsenite Oxidase, an Ancient Bioenergetic Enzyme

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Operons coding for the enzyme arsenite oxidase have been detected in the genomes from Archaea and Bacteria by Blast searches using the amino acid sequences of the respective enzyme characterized in two different β-proteobacteria as templates. Sequence analyses show that in all these species, arsenite oxidase is transported over the cytoplasmic membrane via the tat system and most probably remains membrane attached by an N-terminal transmembrane helix of the Rieske subunit. The biochemical and biophysical data obtained for arsenite oxidase in the green filamentous bacterium *Chloroflexus aurantiacus* allow a structural model of the enzyme's membrane association to be proposed. Phylogenies for the two constituent subunits (i.e., the molybdopterin-containing and the Rieske subunit) of the heterodimeric enzyme and their respective homologs in DMSO-reductase, formate dehydrogenase, nitrate reductase, and the Rieske/cyt*b* complexes were calculated from multiple sequence alignments. The obtained phylogenetic trees indicate an early origin of arsenite oxidase before the divergence of Archaea and Bacteria. Evolutionary implications of these phylogenies are discussed.

Introduction

In 1992, a periplasmic soluble enzyme capable of oxidising arsenite (As^{III}) was purified from the β-proteobacterium *Alcaligenes faecalis* (Anderson, Williams, and Hille 1992). Expression of this so-called arsenite oxidase was induced by the presence of As^{III} in the growth medium, and the purified enzyme was found to contain a molybdopterin cofactor as well as a [3Fe-4S] and a Rieske [2Fe-2S] cluster as spectroscopically distinguishable redox centers (Anderson, Williams, and Hille 1992). In vitro assays indicated that arsenite oxidase transfers the electrons obtained from the oxidation of arsenite towards the soluble periplasmic electron carriers cytochrome *c* and/or amicyanin. The recently reported three-dimensional (3D) structure of this enzyme (Ellis et al. 2001) showed that arsenite oxidase is a heterodimeric enzyme containing a small and a large subunit. The small subunit belongs to the structural class of the Rieske proteins (Carrell et al. 1997) found in Rieske/cyt*b* complexes (formerly called cytochrome *bc* or cytochrome *bc*–type complexes [see Schütz et al. 2000]), in dioxygenases (Schmidt and Shaw 2001), and in phytoene desaturases. The large catalytic subunit harbors the molybdopterin cofactor and the [3Fe-4S] cluster and is structurally related to the family of the molybdopterin enzymes (see Jormakka et al. 2002). Thus, arsenite oxidase from *Alcaligenes* is composed of two subunits that have homologues in enzyme families differing significantly from each other with respect to both redox cofactors and function. Whereas for the enzyme families containing the homologs of the two arsenite oxidase subunits numerous representatives are known, only one further enzyme (also from a β-proteobacterium) related to the *Alcaligenes* arsenite oxidase has been described so far (Muller et al. 2003).

Is arsenite oxidase from *Alcaligenes faecalis* therefore a relatively recent evolutionary invention built by associating redox subunits from existing electron transfer complexes into a new enzyme with a detoxifying activity? In order to detect potential further members of a putative arsenite oxidase family, we have performed a survey of fully sequenced genomes accessible in the databases. This search came up with several further examples, both bacterial and archaeal, of enzymes almost certainly belonging to this new family. A phylogenetic analysis of both subunits argues for an ancient evolutionary origin of the arsenite oxidase family. The phylogenetic relationship of its Rieske subunit to that of the Rieske/cyt*b* complexes was analyzed and the results are discussed in the light of the evolutionary histories of both enzymes.

Materials and Methods

Sequence Analyses

Database searches were performed using BLASTP (Altschul et al. 1990) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments on the molybdenum proteins were performed using ClustalX (Thomson et al. 1997). For the case of the Rieske subunits, a structural alignment based on the available 3D structures was produced first (Lebrun et al., unpublished data) and the thus recognized structural elements were subsequently aligned using ClustalX. Phylogenies were calculated using the parsimony (phylip) and neighbor-joining methods (Clustal) and phylogenetic trees were drawn using the program TreeView developed by R.D.M. Page (http://taxonomy.zoology.gla.ac.uk/rod/ treeview).

Bacterial cultures and sample preparation

Chloroflexus aurantiacus was grown photosynthetically and membrane fragments were prepared as described previously (van Vliet, Nitschke, and Rutherford 1991;

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Brugna 1997). Oriented membrane samples were obtained by partial dehydration as described by Rutherford and Sétif (1990).

EPR experiments

EPR spectra were recorded on a Bruker ESP300e X-band spectrometer fitted with an Oxford Instruments liquid helium cryostat (temperature, 20°K; microwave frequency, 9.42 GHz; microwave power, 6.7 mW; modulation amplitude, 1.6 mT).

Results

Searching Sequenced Genomes for Arsenite Oxidase-Type Enzymes

In order to retrieve open reading frames (ORFs) potentially coding for components of an arsenite oxidase, the following search criteria were used:

(1) The sequence of the Rieske protein. A number of Rieske proteins encoded in operon contexts not corresponding to that of Rieske/cyt*b* complexes have been noticed previously in several prokaryotic genomes, and the question concerning their putative function has been raised (Schütz et al. 2000). The ensemble of these "second" Rieske genes has been subjected to multiple alignments with the sequence of the respective protein from *Alcaligenes faecalis* and the individual sequences were classed by alignment score. Three sequences (from the green filamentous bacterium *Chloroflexus aurantiacus* as well as from the two Crenarchaeota *Sulfolobus tokodaii* and *Aeropyrum pernix*) stood out by alignment scores more than one order of magnitude above those of the other cases.

(2) The sequence of the molybdopterin subunit. Unlike the structurally related proteins from assimilatory and dissimilatory nitrate reductases as well as formate dehydrogenases, the cubane cluster in the arsenite oxidases from the two β-proteobacteria is a [3Fe-4S] cluster. Since electron transport from arsenite to the soluble carriers involves redox midpoint potentials significantly more positive than the redox reactions entailed by oxidation of formate or reduction of nitrate, the replacement of a [4Fe-4S] cluster by the (intrinsically higher potential) [3Fe-4S] cluster in arsenite oxidase is likely to be related to the specific functional requirement of the latter enzyme and therefore to represent a distinguishing property. In BLAST searches using the sequence of the *Alcaligenes* molybdopterin subunit, sequences containing the motif of the [4Fe-4S] cluster consequently were discarded. Remarkably, all top BLAST hits (i.e., the sequences from *C. aurantiacus*, *S. tokodaii*, and *A. pernix*) bind a [3Fe-4S] cluster according to sequence signatures (fig. 1*a*).

(3) Although the genetic organization of the *Alcaligenes* enzyme is not known, the requirement for concerted expression in response to the presence of arsenite suggested an organization as an operon. The recent sequencing of the arsenite oxidase genes in the β-proteobacterium *Cenibacterium arsenoxidans* strain ULPAs1 (Muller et al. 2002) confirmed that the large and small subunits are operon-encoded with the Rieske gene upstream of that of the catalytic subunit. The Rieske and the molybdopterin subunits in *C. aurantiacus*, *S. tokodaii*, and *A. pernix* were therefore analyzed for genomic proximity and were indeed found to be directly adjacent in the genome and to show a conserved order with the Rieske protein upstream of the molybdopterin subunit just as in *C. arsenoxidans* (Muller et al. 2002). Interspecies comparisons of the ORFs flanking these two genes did not detect other conserved genes possibly belonging to the respective operon.

We therefore consider these three cases (i.e., the respective genes from *C. aurantiacus*, *A. pernix*, and *S. tokodaii*) as almost certainly representing arsenite oxidase–type enzymes in the respective organisms.

Biophysical Characterization of the Arsenite Oxidase from *Chloroflexus aurantiacus*

The electron paramagnetic resonance (EPR) spectrum of a Rieske iron sulfur cluster in membranes from phototrophically grown *Chloroflexus aurantiacus* was first reported almost 20 years ago (Zannoni and Ingledew 1985), and this observation was subsequently confirmed by several groups (Wynn et al. 1987; Brugna 1997). Notwithstanding its low concentration as compared with all cytochromes observable in the same samples (Wynn et al. 1987; Brugna 1997), the *Chloroflexus* Rieske protein has been considered to represent the respective subunit of a Rieske/cyt*b* complex. Despite sustained efforts, however, no enzyme corresponding to an integral Rieske/cyt*b* complex could be purified (Brugna 1997; Yanyushin 2002). The failure to isolate this enzyme is rationalized by the results of the *Chloroflexus* genome sequencing project, which shows that the operon encoding a typical Rieske/cyt*b* complex is not present in the *Chloroflexus* genome. A search of the genome for Rieske protein–related genes yielded the respective subunit from arsenite oxidase as the one and only significant hit. Arsenite oxidase in *Chloroflexus* has thus unwittingly been studied by several groups for almost two decades. The respective results are summarized in the following:

(1) Localization. Zannoni and Ingledew (1985), Wynn et al. (1987), and Brugna (1997) detected the Rieske EPR spectrum in membrane fragments. In order to determine whether the presence of this signal in membrane samples was due to unspecific membrane association of an otherwise soluble Rieske protein, we also examined the soluble supernatant by EPR. No corresponding signal could be detected even in highly concentrated samples of the soluble fraction (data not shown). EPR spectra obtained on partially ordered membranes show well-defined orientations of the Rieske center's g_y and g_x directions. It is noteworthy that the orientation of the g_x signal (45° with respect to the plane of the membrane) strongly differs from what has been reported for the Rieske subunit in Rieske/

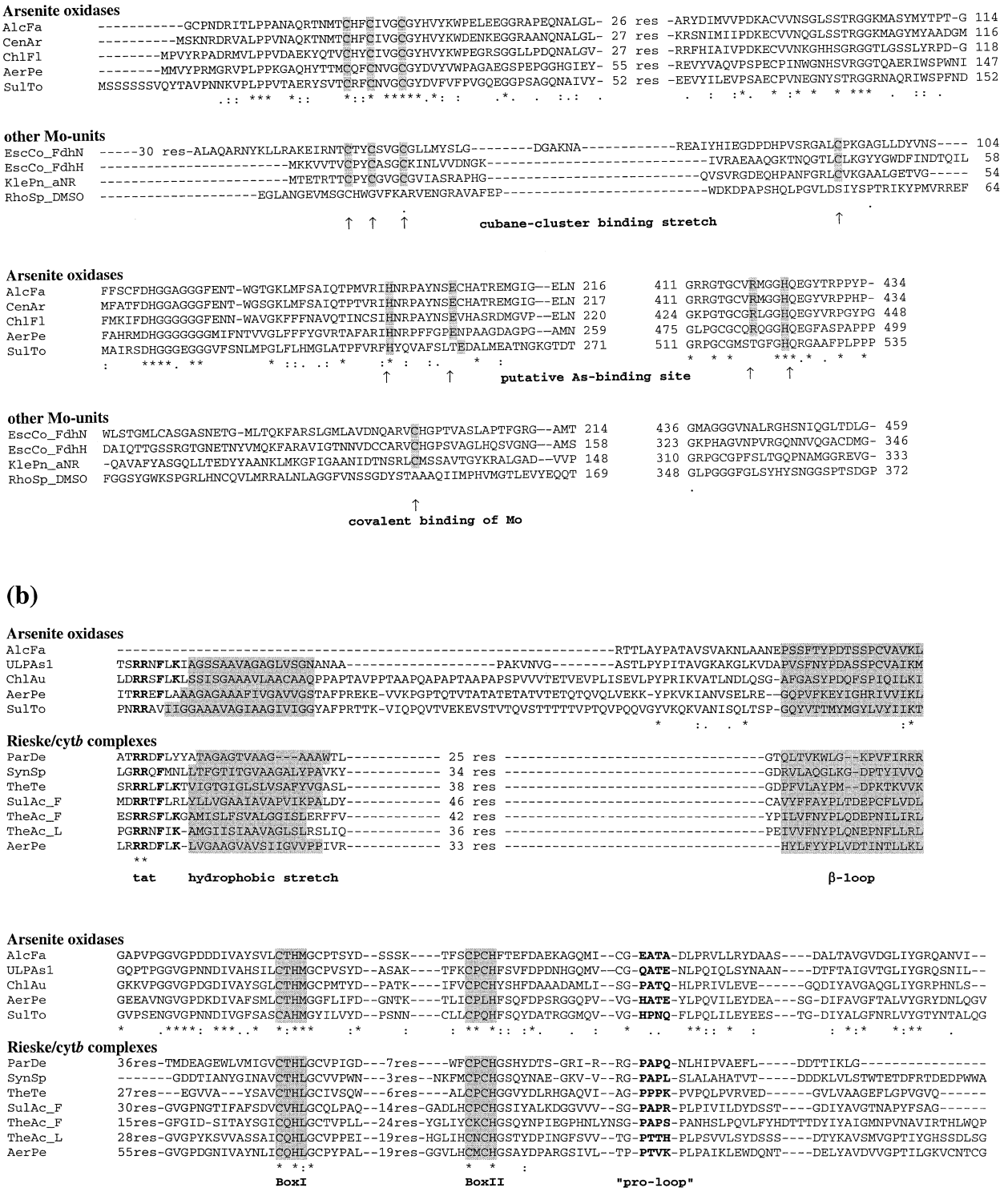

FIG. 1.—Multiple sequence alignments of (*a*) the molybdenum protein in arsenite oxidases, assimilatory nitrate reductase (aNR), formate dehydrogenase-H and -N (FdhH and FdhN) and DMSO-reductase (DMSO) as well as (*b*) the Rieske proteins in arsenite oxidases and Rieske/cyt*b* complexes. Database entries for the considered proteins are given in the legend of figure 2. The alignment used the Blossom matrix with gap opening and gap extension penalties of 10 and 0.1, respectively. Alignment of the Rieske subunit was guided by structural overlay of the available structures as mentioned in the text. Residues involved in binding of redox cofactors and the substrate arsenite, as well as the β-loop structure and the N-terminal hydrophobic stretch for the case of the Rieske proteins, are indicated by gray shading. The tat signal sequence and the "proline-loop" structural element are denoted by bold letters. Conserved residues between the arsenite oxidase proteins and between all representatives of each protein family are indicated.

cyt*b* complexes (Brugna, Albouy, and Nitschke 1998; Brugna et al. 1999; Schoepp et al. 1999; Brugna et al. 2000). These data demonstrate that the *Chloroflexus* arsenite oxidase is membrane attached in a defined geometry (see also *Discussion*).

(2) Redox potentials of cofactors. Zannoni and Ingledew (1985) reported a redox midpoint potential (at pH 7) for the *Chloroflexus* Rieske protein of +100 mV. A similar value was more recently obtained by one of the authors of this work (Brugna 1997). The EPR signal of a [3Fe-4S] center was furthermore observed in membrane fragments (Zannoni and Ingledew 1985; Brugna, unpublished data) and its redox midpoint potential was determined to +60 mV (Zannoni and Ingledew 1985). This value is similar to the E_m value of the (two-electron) transition from arsenite (As^{III}) to arsenate (As^{V}) and thus compatible with the observed center corresponding to the [3Fe-4S] cluster of the arsenite oxidase enzyme.

(3) Implication of arsenite oxidase in photosynthetic electron transfer. The reduced Rieske protein in *Chloroflexus* membrane fragments was shown to be reoxidized by short illumination (Zannoni and Ingledew 1985), suggesting that the enzyme is able to transfer its reducing equivalents towards the photosynthetic reaction center, probably via the membrane associated small copper protein auracyanin.

Multiple Sequence Alignments and Phylogenetic Analyses

Figure 1 shows multiple sequence alignments of the catalytic large and the Rieske subunits. Included in the multiple alignments are selected sequences from the homologous proteins contained in DMSO-reductase, assimilatory nitrate reductase, formate dehydrogenase (fig. 1*a*) as well as Rieske subunits from Rieske/cyt*b*-type enzymes (fig. 1*b*). The alignment of the Rieske proteins relies on exploiting the available three-dimensional coordinates for a structure-guided superposition of structurally conserved features in the N-terminal half of the protein, which is characterized by low conservation of primary sequence (a detailed description of this method will be published elsewhere). Residues involved in cofactor binding as well as the twin arginine translocation (tat) signal sequence and the subsequent hydrophobic stretch in the case of the Rieske protein are highlighted in the multiple alignments.

The obtained multiple sequence alignments (including further representatives of the various groups as indicated in the legend of figure 2) were used to create phylogenetic trees based on neighbor-joining and parsimony methods. Resulting phylogenies are shown as unrooted trees in figure 2*a* and *b* for the molybdopterin and the Rieske subunits, respectively.

Discussion

General Features of Amino Acid Sequences

In figure 1*a*, selected regions of arsenite oxidase catalytic (large) subunits' amino acid sequences are compared with those of the structurally related molybdopterin subunits from formate dehydrogenases, assimilatory nitrate reductases, and DMSO-reductases. Whereas significant sequence homologies between the arsenite oxidase sequences are present, residues conserved among all members of the molybdopterin family considered in figure 1*a* basically are limited to the cofactor binding stretches.

As judged by the replacement of a cysteine by a serine residue in the respective terminal sequence motif (fig. 1*a*), the cubane cluster in all arsenite oxidases is most probably a [3Fe-4S] cluster rather than a [4Fe-4S] center as is the case in nitrate reductase and formate dehydrogenase (Jormakka et al. 2002). This is in line with the relatively high redox midpoint potential of this cluster as required by function (discussed above) and as measured in *Chloroflexus* (+60 mV). Two of the four residues suggested to be involved in arsenite binding (Ellis et al. 2001) are also fully conserved (fig. 1*a*). A further feature is the absence of the cysteine/selenocysteine residue covalently binding the molybdenum atom in nitrate reductases and formate dehydrogenases. The lack of this covalent bond in arsenite oxidases has been proposed to play a crucial role in the As tolerance of the molybdenum center in this specific enzyme (Ellis et al. 2001).

Figure 1*b* shows the multiple sequence alignment of Rieske proteins from arsenite oxidases and from Rieske/cyt*b* complexes. Structurally conserved regions comprise the transmembrane helix, the β-loop structure (Lebrun et al., unpublished data), and the cluster binding C-terminal domain. It is noteworthy that the subunits of arsenite oxidases in *A. pernix* and *S. tokodaii* lack the cluster stabilizing disulfide bond (fig. 1*b*), which, according to standard nomenclature (Schmidt and Shaw 2001), would qualify these subunits as Rieske-type rather than genuine Rieske proteins. The Rieske-type proteins so far were only found in bacterial dioxygenases. The fact that the presence/absence of the disulfide bond is obviously not related to the classification into different enzymes, to our mind, renders the distinction between Rieske and Rieske-type proteins obsolete and the term Rieske-type should therefore be avoided in future work. The prominent structural feature of the "proline-loop," well-studied in Rieske/cyt*b* complexes (Carrell et al. 1997), is structurally conserved in the arsenite oxidase Rieske protein from *A. faecalis* (Ellis et al. 2001). Ironically, in the majority of Rieske proteins from arsenite oxidases, not a single proline is present in the respective sequence stretch and, again, it may be necessary for the sake of clarity to coin a new term for this conserved structural element of Rieske proteins.

The presently available sequences suggest that the first cluster-binding box in the Rieske proteins might serve as a marker sequence distinguishing between representatives of the arsenite oxidase and the Rieske/cyt*b* complex families. In all arsenite oxidases, this sequence reads CXHMG, whereas the methionine residue never occurs in Rieske/cyt*b* complexes.

Most (All?) Arsenite Oxidases Are Membrane-Attached Periplasmic Enzymes

The Rieske subunits of all arsenite oxidases detected in the genomes as well as of those from the two

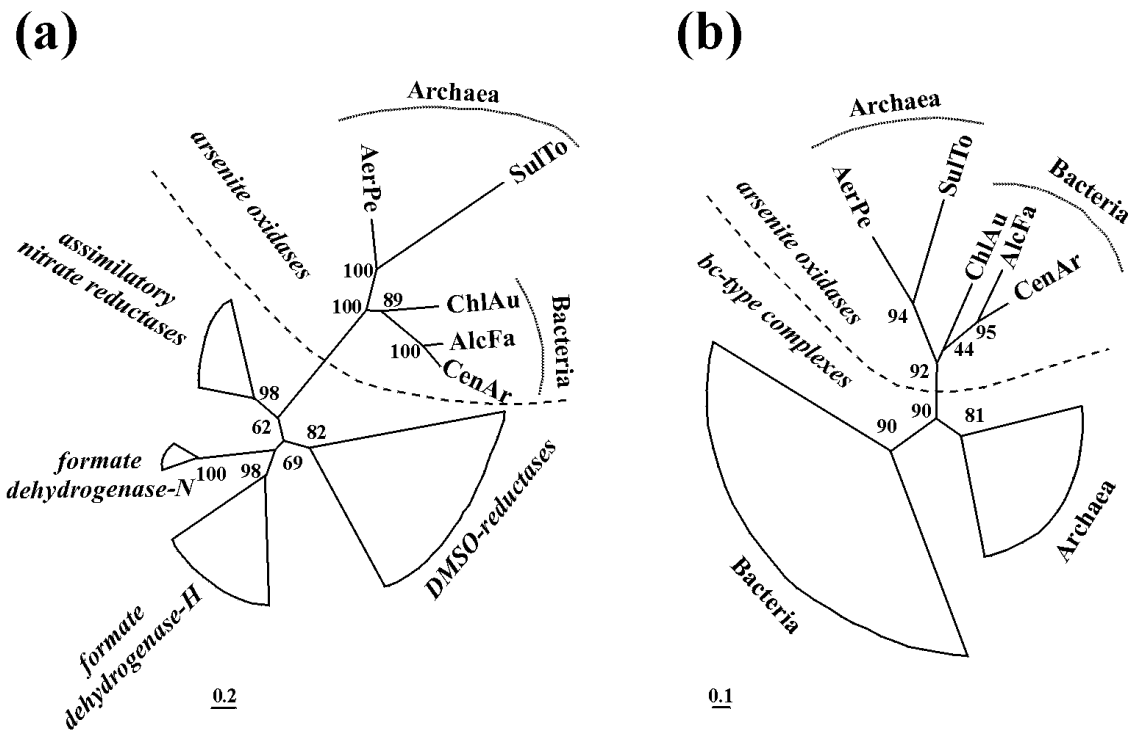

FIG. 2.—Unrooted phylogenetic trees calculated from multiple sequence alignments as shown in figure 1 of (*a*) the molybdopterin subunit from arsenite oxidases, DMSO-reductases, assimilatory nitrate reductases, and formate dehydrogenases (-N and -H), as well as (*b*) the Rieske subunit from arsenite oxidases and Rieske/cyt*b* complexes. The phylograms were constructed using the Neighbor-Joining method of Saitou and Nei as well as Kimura's correction for multiple substitutions. Numbers on nodes correspond to the frequency of occurrence of nodes in 1.000 bootstrap replicates using the Neighbor-Joining tree reconstruction. Individual branches are only specified for the arsenite oxidase subunits. The following proteins were included in the analysis: Arsenite oxidase/Rieske subunit: *Sulfolobus tokodaii* NP_378392, A. *pernix* BAA81580, *Chloroflexus aurantiacus* ORF 1492, *Alcaligenes faecalis* GI:12084496, *Cenibacterium arsenoxidans* strain ULPAs1 GI:22758843. Arsenite oxidase/Mo-subunit: *Sulfolobus tokodaii* NP_378391, A. *pernix* NP_148692, *Chloroflexus aurantiacus* ORF 1493, *Alcaligenes faecalis* gi|12084495, *Cenibacterium arsenoxidans* strain ULPAs1 GI:22758844. Assimilatory nitrate reductase: *Synechocystis* sp. PCC 6803 GI:2497967, *Klebsiella pneumoniae* GI:18314343, *Bacillus subtilis* GI:2828506. DMSO-reductase: *Rhodobacter sphaeroides* GI:9954924, *Escherichia coli* GI:1742611, *Aquifex aeolicus* GI:15606464. Formate dehydrogenase-N: *Escherichia coli* GI:16129433, *Haemophilus influenzae* GI:16273678. Formate dehydrogenase-H: *Escherichia coli* GI:119890, *Campylobacter jejuni* GI:15792825, *Yersinia pestis* GI:16120679. Rieske/cyt*b*-complexes: *Rhodobacter sphaeroides* GI:581491, *Paracoccus denitrificans* GI:77577, *Neurospora crassa* GI:136704, *Bos taurus* GI:1351360, *Allochromatium vinosum* GI:3929386, *Helicobacter pylori* GI:2314722, *Campylobacter jejuni* GI:6968619, *Aquifex aeolicus* GI:2982799, *Bacillus subtilis* GI:1168647, *Bacillus stearothermophilus* GI:2500507, *Bacillus halodurans* GI:15614235, *Chlorobium limicola* GI:2500506, *Spinacea oleracea* GI:136712, *Synechocystis* sp. PCC6803 GI:136712, *Deinococcus radiodurans* GI:15805462, *Thermus thermophilus* GI:2695699, *Sulfolobus acidocaldarius* SoxF GI:927524, *Sulfolobus acidocaldarius* SoxL GI:1419215, *Thermoplasma acidophilum* SoxF GI:16082230, *Thermoplasma acidophilum* SoxL GI:16082599, *Thermoplasma volcanium* SoxF GI:13541203, *Thermoplasma volcanium* SoxL GI:13541197.

β-proteobacteria (Mukhopadhyay et al. 2002; Muller et al. 2003) contain an N-terminal hydrophobic stretch similar to the transmembrane helix of the respective subunit in Rieske/cyt*b* complexes. The tat-recognition motif preceeding this hydrophobic stretch is also conserved between the Rieske subunits of the two different enzymes (see fig. 1). Similar to its counterparts in Rieske/cyt*b* complexes (Hinsley et al. 2001), the Rieske subunit of arsenite oxidase must therefore be translocated via the tat system (as also proposed by Muller et al. 2003 and Mukhopadhyay et al. 2002). The catalytic subunit is possibly cotranslated in this step, as is for example the case for the [NiFe] hydrogenases' small and large subunits (Vignais, Billoud, and Meyer 2001).

The presence of an N-terminal hydrophobic stretch in all arsenite oxidases for which gene sequences are available raises the question of whether this stretch is cleaved after the protein has been transported over the cytoplasmic membrane or whether the enzyme remains membrane attached in these species. Several arguments strongly favor the second model. (1) Sulfolobales do not contain a confined periplasmic compartment. Consequently, no truly soluble extracytoplasmic proteins are possible in Sulfolobales. (2) As outlined above, all experimental data suggest a membrane attachment of arsenite oxidase in *Chloroflexus*. (3) In the β-proteobacterium *C. arsenoxidans*, arsenite oxidase activity was found to be associated with membranes (Muller et al. 2003).

Membrane attachment as a general feature of arsenite oxidases is seemingly in conflict with the fact that the enzyme from *A. faecalis* has been isolated as a soluble complex. However, purification of the *Alcaligenes* enzyme involves a heating step to 60°C (Anderson, Williams, and Hille 1992), which may well have cleaved the soluble domain of the Rieske protein off its membrane anchor. A proteolytic lability of the hinge region between the transmembrane helix and the soluble domain has previously been observed for the homologous protein in the

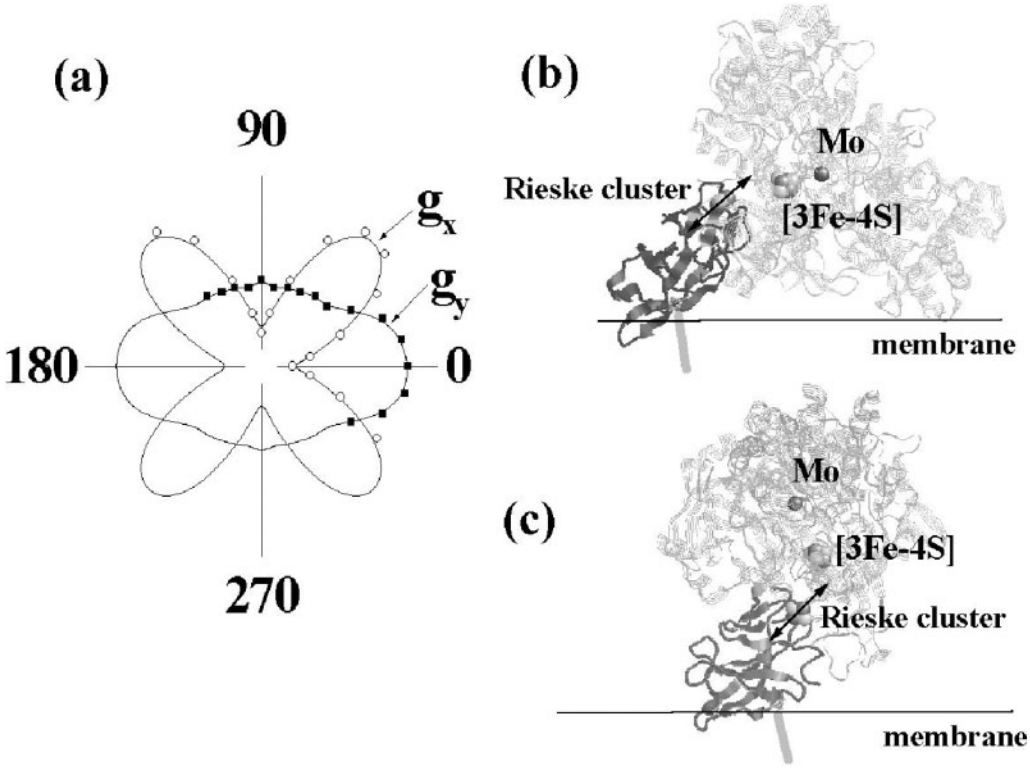

FIG. 3.—(*a*) Polar plot of the EPR signal amplitude of the g_y and g_x lines of the Rieske [2Fe-2S] center observed in ascorbate-reduced oriented membranes from *Chloroflexus aurantiacus*. (*b*) Ribbon representation (based on the coordinates of the *Alcaligenes* arsenite oxidase) of the two geometric arrangements of the enzyme with respect to the membrane that positions the g_x direction (i.e., the Fe-Fe vector; double-headed arrow) at 45° and the vector connecting the two acid-labile sulfur atoms (*b*) or the normal to the plane of the [Fe-S] cluster (*c*) parallel to the membrane plane. The upper end of the thick gray line (representing the transmembrane helix) points towards the position of the N-terminal residue observed in the structure. PDB-entry for arsenite oxidase from *Alcaligenes*: 1G8J.

Rieske/cyt*b* complexes (Carrell et al. 1997). The ensemble of these data therefore strongly suggests that arsenite oxidases are membrane-attached enzymes in all species considered in this work.

A Structural Model for the Membrane Attachment

The Rieske center's g-tensor directions determined in *Chloroflexus* (fig. 3*a*), together with the attribution of paramagnetic to molecular axes in Rieske proteins (Schoepp et al. 1999; Brugna et al. 2000) and the presence of a three-dimensional structure for arsenite oxidase (Ellis et al. 2001) allows a structural model for the enzyme's membrane association to be proposed. As shown in figure 3*a*, the Rieske center's g_x direction lies at an angle of 45° with respect to the membrane, whereas its g_y axis is parallel to the membrane. The g_x direction of Rieske centers has been demonstrated to be collinear with the Fe-Fe direction of the [2Fe-2S] cluster (Brugna et al. 2000), whereas the attribution of g_y to either the vector connecting the acid labile sulfur atoms or to the remaining orthogonal direction is still not definitively settled. Taking this g_z/g_y uncertainty into account, four orientations of the enzyme with respect to the membrane plane are possible. The two that point the exposed (histidine ligated) Fe atom of the Rieske cluster towards the membrane must be discarded since they would place the large subunit within the membrane. Figure 3*b* and *c* show the remaining two possible geometries of the whole enzyme with respect to the membrane. Remarkably, both conformations place the N-terminally oriented end of the Rieske protein's chain, which has to connect up to the linker region and ultimately to the transmembrane helix, in proximity of the membrane. A definitive correlation of the g-tensor's g_z and g_y directions to molecular axes will ultimately allow the removal of the residual ambiguity shown in figure 3*b* and *c*.

A Bioenergetic Origin for Arsenite Oxidase

With the exception of the two β-proteobacteria, all other parent organisms of arsenite oxidases dealt with in this work dwell in thermophilic or hyperthermophilic environments on a volcanic background (volcanic hot ponds, seepages, or solfataras). These habitats are intrinsically rich in arsenics (for a discussion of the arsenic global geocycle, see Mukhopadhyay et al. 2002). It has been shown recently that in such habitats, microorganisms are able to grow on the oxidation of As^{III} when the concentrations of reduced sulfur and iron compounds become limiting (Jackson et al. 2001). Aquatic volcanic habitats are often cited as possible vestiges of where life on earth may have originated, and most of the deeply branching phyla on the tree of life indeed thrive in such

environments. The presence of arsenics in these habitats and the possibility to feed electrons from the oxidation of As^{III} into the bioenergetic chains may have provided an evolutionary driving force for the invention of this enzyme early on in the evolutionary history of bioenergetic mechanisms (see following section). The above-mentioned finding that in *Chloroflexus aurantiacus* the photosynthetic reaction center is able to oxidize the arsenite oxidase Rieske center (Zannoni and Ingledew 1985) further supports an involvement of this enzyme in bioenergetic mechanisms. Light-dependent electron transport in *Chloroflexus* can be studied in vivo by monitoring flash-induced optical absorption changes (Brugna, Nitschke, and Vermeglio, unpublished data), as has previously been done for other anoxygenic phototrophs (Albouy et al. 1997; Kramer et al. 1997). This fact makes *Chloroflexus* a species ideally suited for an in-depth investigation of the metabolic role as well as the mode of functioning of the arsenite oxidase enzyme.

Arsenite Oxidase Is a "Pre-LUCA" Enzyme

Both subunits of arsenite oxidase have homologs in functionally unrelated enzymes. The respective family relationships are mutually exclusive, that is, no other enzymes with Rieske subunits also containing molybdopterin subunits and vice versa are known so far. Thus, arsenite oxidase is an exemplary case of an enzyme put together by picking out required redox domains from a restricted set of redox protein building blocks as described recently (Baymann et al. 2003). The respective homologues of arsenite oxidase's Rieske and molybdopterin subunits can be exploited for rooting the phylogenetic tree of arsenite oxidase as depicted in figure 2*a* and *b*. Both the phylograms of the Rieske proteins and the phylograms of the molybdopterin subunits show a clear-cut diversification between arsenite oxidases on one side and the remaining enzymes on the other side (i.e., the arsenite oxidase subunits form distinct subtrees in these phylograms). The roots of these subtrees lie in between the archaeal and the bacterial representatives (fig. 2*a* and *b*), and the phylogeny of arsenite oxidase therefore corresponds to that of the parent species. This similarity of topology extends to the details of the bacterial part of arsenite oxidase's phylogenetic trees featuring *Chloroflexus* as an early branching entry in line with 16S rRNA trees (Olsen, Woese, and Overbeek 1994). As discussed in more detail in Baymann et al. 2003, this indicates that an arsenite oxidase–type enzyme was present before the divergence of Bacteria and Archaea. The evolutionary history of arsenite oxidase therefore seems to date back to the era before the existence of the last universal common ancestor (LUCA), making arsenite oxidase part of the electron transfer enzymes that appear to have been assembled from the basic redox building blocks during the very early stages of the evolution of life on earth. The finding of a pre-LUCA origin of these mostly bioenergetic enzymes is particularily intriguing in the light of a novel hypothesis on the origin of life recently put forward by Russell and Hall (1997) and Russell, Hall, and Mellersh (2003) and extended by Martin and Russell (2003). According to this scenario, the earliest "cellular" structures may have been cavities in colloidal FeS precipitates of ocean floor hydrothermal systems. A good deal of the basic metabolic processes (see Martin and Russell 2003) would have evolved in these structures, and it was not before the replacement of the inorganic walls by lipids that free-living cells came into being. According to Martin and Russell, two independent lineages would have invented fundamentally different lipids and biosynthetic pathways thereof, eventually giving birth to the archaeal and the bacterial domains of prokaryotes. One of the main elements of this model, that is, the development of full-fledged metabolic systems already within the mineral-confined, quasicellular structures, therefore predicts that many bioenergetic enzyme systems should have existed in this early phase before the Archaea/Bacteria diversification. Electron transfer utilizing arsenite as electron donor might well be among these "ancient" bioenergetic pathways.

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