

## The small subunit AroB of arsenite oxidase: lessons on the [2Fe-2S]-Rieske protein superfamily

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Running title: EPR and Redox properties of the arsenite oxidase Rieske subunit

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**Here we describe the characterization of the [2Fe-2S] clusters of the arsenite oxidases from *Rhizobium* sp. NT-26 and *Ralstonia* sp. 22. Both reduced Rieske proteins feature EPR signals similar to their homologues from Rieske/cytb complexes, with *g* values at 2.027, 1.88 and 1.77. Redox titrations in a range of pH values showed that both [2Fe-2S] centers have constant  $E_m$  values up to pH 8 at around + 210 mV. Above this pH value, the  $E_m$  values of both centers are pH-dependent similar to what is observed for the Rieske/cytb complexes. The redox properties of these two proteins together with the low  $E_m$  value (+160 mV) of the *Alcaligenes faecalis* arsenite oxidase Rieske (confirmed herein), are in line with the structural determinants observed in the primary sequences which have previously been deduced from the study of Rieske/cytb complexes. Since the published  $E_m$  value of the *Chloroflexus aurantiacus* Rieske (+100 mV) is in conflict with this sequence analysis, we re-analyzed membrane samples of this organism and obtain a new value (+200 mV). Arsenite oxidase activity was affected by quinols and quinol analogs, which is similar to what is found with the Rieske/cytb complexes. Together, these results show that the Rieske protein of arsenite oxidase shares numerous properties with its counterpart in the Rieske/cytb complex. However, two cysteine residues, strictly conserved in the Rieske/cytb-Rieske and considered to be crucial for its function, are not conserved in the arsenite oxidase counterpart. We discuss the role of these residues.**

Since the first report of bacterial arsenite<sup>1</sup> (As<sup>III</sup>) oxidation by Green in 1918 (1), numerous As<sup>III</sup>-oxidizing bacteria have been isolated from various environments. Apart from

<sup>1</sup> Abbreviations used are :

the two cases of *Ectothiorhodospiraceae*, *Alkalilimnicola ehrlichii* str. MLHE-1 (2) and PHS-1 (3), the enzyme responsible for this redox conversion has been shown to be arsenite oxidase (Aro; but also found in the literature as Aox or Aso; refs.4,5), encoded by the *aroA* and *aroB* genes. The three-dimensional structure of Aro has been solved (6). The structure of the large AroA (or AoxB or AsoA) subunit (90-100 kDa), bearing a molybdopterin cofactor together with a [3Fe-4S] cluster, establishes the enzyme as a member of the large DMSO reductase superfamily of molybdenum-containing enzymes (6). Corresponding structural similarities demonstrate that the small AroB (or AoxA or AsoB) subunit (14 kDa), harboring a [2Fe-2S] cluster, belongs to the superfamily of Rieske proteins. Aro thus illustrates the “Janus Head” principle that many bioenergetic enzymes are built from a very limited number of basic units, which we have termed a “redox enzyme construction kit” (7).

The first member of the family of Rieske proteins was identified and characterized in 1964 in Helmut Beinert's laboratory (8) as a component of mitochondrial complex III. It stood out from other known iron sulfur proteins by its extraordinarily high reduction potential (HP). Over the ensuing 40 years, Rieske proteins have been shown to form a very large superfamily encompassing subunits of the Rieske/cytb complexes (*i.e.* complexes homologous to complex III) (see 9,10), domains or subunits of dioxygenases (for review see 11,12) as well as sulredoxin from *Sulfolobus tokodaii* (13), in addition to the above cited AroB proteins. The Rieske centres are thus present in highly diverse prokaryotic systems. The Rieske/cytb complex performs electron transfer from membrane-diffusing quinols to small soluble or membrane-attached redox proteins and thereby participates in the generation of a proton-motive potential. By transferring electrons from As<sup>III</sup> to a soluble

cytochrome in the periplasm, Aro has been demonstrated to sustain chemolithoautotrophy in an aerobic respiration process. Although its precise integration into bioenergetic chains still needs to be established, Aro has therefore been proposed to bypass the Rieske/cytb complex in prokaryotic aerobic heterotrophic bioenergetic chains (14). The dioxygenases are soluble cytoplasmic enzyme systems involved in the catabolism of aromatic compounds. They consist of two or three components that transfer electrons from reduced nucleotides (NADH) via flavin and [2Fe-2S] redox centers to a terminal oxygenase. In these systems, [2Fe-2S] centers can be found in one or both components (15).

Phylogenetic analysis of Aro and the Rieske/cytb complexes strongly suggests that both systems existed in the common ancestor of Bacteria and Archaea (16) and that the emergence of these two very divergent enzymes from fundamental ancestral "building units" represents a very early event in the evolution of life (7). The substantially different enzymatic reactions of the two systems subsequently must have involved adaptation of the building blocks to specific functional requirements. Among these adaptations are the properties of the Rieske cluster.

As established crystallographically, all Rieske proteins contain a [2Fe-2S] cluster in which one of the iron atoms is coordinated by two histidines (His) (6,17-20) rather than the two cysteines (Cys) of "common" [2Fe-2S] ferredoxins. As documented for the Rieske/cytb complexes and dioxygenases, these His ligands are responsible for i) an unusual EPR spectrum characterized by a low  $g_{av}$ -value of around 1.91 as compared to around 1.94 for most "classical" ferredoxins (21) and ii) a reduction potential at least 400 mV more positive than that of the "classical" ferredoxins (for reviews see refs. 11,22) a property that set the Rieske cluster apart at its discovery in 1964. Detailed structural determinants for these properties have been deduced from a comparison of the Rieske/cytb and the dioxygenase groups and several residues close to the cluster have been proposed to distinguish the two groups. The fact that the Rieske components of dioxygenases lack the disulfide bond near the cluster whereas this bridge is universally present in the subunit of Rieske/cytb complexes has led to the proposal that the dioxygenase cases be referred to as "Rieske-type" rather than "Rieske" centers. Such clusters have reduction potentials between -150 mV and -50 mV whereas genuine Rieske proteins (*i.e.* those in Rieske/cytb complexes)

have reduction potentials in the range +100 mV to +380 mV (see 11). As we will show below, the case of the AroB protein demonstrates that the distinction between "true" Rieske and Rieske-type proteins is not so clear cut.

In comparison to the proteins from dioxygenases and Rieske/cytb complexes, very little is known about the Rieske subunit in Aro. The spectrum recorded on the enzyme from *Alcaligenes faecalis* suggested the EPR properties of the Aro-Rieske subunit was very similar to the protein in Rieske/cytb-complexes (23). The spectra of both Rieske clusters actually are so similar that the  $g = 1.90$  signal recorded on membranes of *Chloroflexus aurantiacus* was first interpreted as arising from a Rieske/cytb complex (24). The genome sequence, however, reveal that *C. aurantiacus* doesn't possess a Rieske/cytb complex and that this signal therefore must be due to Aro (16). The high sequence similarity between Aro- and Rieske/cytb- Rieskes (16) thus correlates with the similar EPR properties of their [2Fe-2S] centers. The potentiometric results obtained on the Aro of *A. faecalis* (25) and *C. aurantiacus* (24) moreover suggest that the redox properties of the Aro-Rieske cluster resemble those of the centre in Rieske/cytb-complexes. In fact, redox titrations on the [2Fe-2S] center of *A. faecalis* and *C. aurantiacus* enzymes yield values of  $E_m = +130$  mV (at pH 6) and  $E_m = +100$  mV (at pH 7), respectively.

To ascertain whether the structural determinants for properties of the center from Rieske/cytb-complexes also apply to the Aro-Rieske cluster, we have studied in the present work two additional enzymes from *Rhizobium* sp. NT-26 (NT-26; 26) and *Ralstonia* sp. 22 (S22; accompanying paper) which are members of the  $\alpha$ - and  $\beta$ - *Proteobacteria*, respectively. We have determined the electron paramagnetic resonance (EPR) power saturation and redox properties of their [2Fe-2S] clusters and analyzed the results in light of primary sequences and molecular structures. We have also re-examined the properties of the *C. aurantiacus* Rieske center by repeating selected experiments at higher protein concentrations. Since Aro- and Rieske/cytb- Rieskes are closely related, we have tested the ability of Aro-Rieskes to react with quinones or analogues. Altogether, our results establish common redox properties of the Aro-[2Fe-2S] group. Re-examination of the ever-increasing number of Rieske/cytb-Rieske sequences and redox properties provide new insights into the

structural determinants of the Rieske-clusters' properties.

## Experimental Procedures

*Growth of bacteria.* NT-26 and S22 and *A. faecalis* were grown aerobically and heterotrophically at 28°C in the presence of 5 mM As<sup>III</sup> as described previously (23,26,27). *C. aurantiacus* was grown photosynthetically at 55°C as already described (28) in the additional presence of 2 mM As<sup>III</sup>.

*Preparation of the Aro samples for EPR.* Titrations were performed on enriched enzymes from NT-26 and S22. Cells of NT-26 and of S22 or *A. faecalis* were resuspended in 2-(N-morpholino) ethanesulfonic acid (MES) 50 mM pH 5.5 and in Tricine 50 mM pH 8, respectively. Cells were broken by two passages through a French press cell and obtained samples were centrifuged for 10 min at 12000 g. The resulting supernatants were centrifuged again for 1h30 at 250000 g. In the case of S22 and *A. faecalis*, the soluble fraction obtained after this second centrifugation step was applied to a diethyl amino ethyl (DEAE)-sepharose column as described in the accompanying paper. The total Aro fraction thus obtained, dialyzed against MES 15 mM/ Tricine 15 mM/AMPSO 15 mM, was used for titration. For NT-26, the soluble fraction retrieved from the second centrifugation step was precipitated as described previously (26) by ammonium sulfate at 40% saturation. The soluble fraction obtained after a centrifugation for 20 min at 20000g was dialyzed overnight against MES 15 mM/ Tricine 15 mM/AMPSO 15 mM. pH values were adjusted by adding NaOH.

The titration of the Rieske center from *C. aurantiacus* was performed on membrane samples. Cells were suspended in 50 mM 3-[morpholino]propanesulfonic acid (MOPS) at pH 7.0 and broken by passing twice through a French press cell. Unbroken cells were eliminated by centrifugation at 12000 g for 10 min, and the "membrane-fraction" was retrieved as the pellet from a subsequent ultracentrifugation for 1h30 at 250000 g.

*Aro activity assays.* For activity assays, the enzymes from S22 and NT-26 were purified as described elsewhere (26,27). Aro activity was measured optically in 50 mM Tricine pH8 at 37°C, using 100 μM sodium As<sup>III</sup> as electron donor and 30 μM cytochrome *c* as electron acceptor. This protocol introduces several modifications to that published by Anderson et

al (23) and used in subsequent studies (*e.g.* by Santini (26)). As detailed in the accompanying paper cytochromes now replace 2,4-dichlorophenolindophenol (DCPIP) in a pH8 buffer instead of a pH6 buffer and the assays are conducted at 37°C instead of 20°C. *Aquifex aeolicus* cytochrome *c*<sub>555</sub> and horse heart cytochrome *c* were used for S22 and NT-26 enzymes, respectively. 100 μM Dibromothymoquinone (DBMIB), 100 μM 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) or 200 μM stigmatellin were added to the enzyme for inhibition studies.

*EPR experiments.* Redox titrations were performed on membrane samples or purified samples at 15 °C as described by Dutton (29) in the presence of the following redox mediators at 100 μM: ferrocen, 1,4 *p*-benzoquinone, 2,5-dimethyl-*p*-benzoquinone, 2-hydroxy 1,2-naphthoquinone, 1,4-naphthoquinone. Reductive titrations were carried out using sodium dithionite, and oxidative titrations were carried out using ferricyanide. EPR spectra were recorded on a Bruker ElexSys X-band spectrometer fitted with an Oxford Instruments liquid-Helium cryostat and temperature control system.

*Amino acid sequences analysis.* Amino acid sequences were aligned with the help of the program CLUSTAL (30) using the Blossom matrix and refined based on the available structures as detailed in (10).

## RESULTS

As outlined in the Introduction, all Rieske clusters are characterized by reduction potentials well above those of "classical" [2Fe-2S] ferredoxins. However, among these clusters, three subgroups can be distinguished with respect to their E<sub>m</sub>-values. The group including the dioxygenase, have reduction potentials below 0 mV. The second group, made up of Rieske proteins found in Rieske/cyt<sub>b</sub> complexes from Ubiquinone-, Plastoquinone- or Caldariellaquinone- (UQ, PQ, CQ, respectively) oxidizing organisms, titrate at 330 ± 60 mV. The third group corresponds to Rieske proteins found in Rieske/cyt<sub>b</sub> complexes from Menaquinone (MK)-oxidizing organisms and the respective clusters titrate at 160 ± 50 mV. The values published for the *A. faecalis* and *C. aurantiacus* Aro-Rieske centres suggest that they belong to the last of these groups. In this work we have characterized two additional Aro-Rieske

proteins, purified from NT-26 and S22, and can now draw reliable conclusions on how to classify this new ensemble of Rieske proteins.

*Reduction potentials of the Aro-Rieske from NT-26 and S22 at pH8-* Experiments were first carried out at pH 8 because of the higher stability and activity of the enzymes at this pH (see accompanying paper). The EPR spectra recorded at 15 K on ascorbate-reduced purified Aro from NT-26 and S22 featured a derivative-shaped  $g_y$  signal at  $g = 1.88$ , a  $g_x$  trough at  $g = 1.77$  and a  $g_z$  peak (partially obscured by a wide radical signal) at  $g = 2.027$  (Figure 1A, spectra a and b). The differing amplitude ratios of the  $g_x$  and  $g_y$  features of spectra a and b suggested that the NT-26 and S22 [2Fe-2S] clusters may have differing relaxation properties. Power saturation curves measured on the  $g_x$  and  $g_y$  lines quantitatively confirmed this. In the case of the S22 cluster, the  $g_x$  feature saturated more readily than the  $g_y$  feature, a situation which is typical also for the cluster in Rieske/cytb-complexes (31). In the case of the NT-26 cluster, however, the situation was reversed (Figure 1B).

EPR spectra were next recorded under non-saturating conditions. The very particular EPR spectral parameters, specific to Rieske clusters, allows their characterization even in non-purified samples. Since the final yield of enzyme purification was only in the range of 30 % (26, accompanying paper), we performed EPR titrations on partially purified but substantially more concentrated enzyme samples (see *Experimental Procedures* section) in order to obtain stronger signals. In both cases (NT-26 and S22), the observed signals (not shown) were identical to those seen with purified enzymes. Since the AroB spectrum was readily observed in samples reduced by ascorbate, the only further center susceptible to contribute to the spectrum is the Rieske center of the Rieske/cytb complexes in NT-26 and S22. However, the Rieske protein of the Rieske/cytb complex is membrane-attached, anchored by its uncleaved twin arginine translocation signal sequence and cannot be extracted from the membrane without detergent treatment (see 32). It therefore appeared unlikely that this protein might contribute to the spectrum in samples obtained without detergent extraction (see *Experimental Procedures*).

Redox titrations of the Aro-Rieske from NT-26 and S22 at pH 8, evaluated from the size of the  $g_y$  feature, are presented on Figure S1. The determined  $E_m$  values are similar with  $+225 \pm 5$  mV and  $+190 \pm 5$  mV for NT-26 and S22,

respectively. These values are substantially higher than those reported for the Aro Rieske clusters from *A. faecalis* and *C. aurantiacus*.

These latter  $E_m$ -values were obtained at lower pH values. All Rieske-type clusters, however, have been demonstrated to show pH dependent redox properties. The Rieske/cytb-subunit from neutrophilic organisms features  $E_m$ -values which are pH-independent up to pH 8 and pH-dependent above this value with a slope of  $-60$  mV/pH. Furthermore, in several cases it has been shown that the pH dependence can attain a slope of  $-120$  mV/pH at pH values above 9. The Rieske/cytb-Rieskes from acidophilic organisms and the dioxygenase ferredoxins show the same overall pH-dependence only shifted by 2 pH units to lower and to higher pH values, respectively (22,33,34). Since the  $E_m$  values reported at low pH values for *A. faecalis* and *C. aurantiacus* are lower than those determined for S22 and NT-26, the mentioned type of pH-dependency cannot account for the difference between our values and those published. However, very peculiar pH-dependences in Aro-Rieskes *a priori* cannot be excluded. We therefore next determined the redox potentials of the NT-26 and S22 Aro-Rieske clusters in the range between pH 6 and 9.5.

*pH-dependence of reduction potentials of NT-26 and S22 Aro-Rieske clusters-* The dependence of Aro Rieske  $E_m$ -values versus pH is depicted in Figure 2. The  $E_m$  values obtained at pH 6, 7 and 8 suggest that the reduction potential is independent of pH up to pH 7.8 at a value of  $+225 \pm 5$  mV and  $+215 \pm 5$  mV for NT-26 and S22, respectively. The pH-dependence of the Aro-Rieske from S22 in the alkaline region can be interpreted as a decrease with a homogeneous slope of  $-60$  mV/pH from 7.8 to 9.5. In the case of the NT-26 Aro-Rieske, the cluster's  $E_m$  versus pH-dependence requires consideration of slopes of  $-60$  mV/pH from 7.8 to 8.8 and of  $-120$  mV/pH at very high pHs. These results agree with previous observation on neutrophilic Rieske/cytb-Rieskes (reviewed in (11)) featuring two  $pK_{a,ox}$  values around 7.6 and 9.2.

The pH-dependence of  $E_m$  thus does not provide an explanation for the difference between our results and the previously published values. While the  $E_m$ -values published for *A. faecalis* and *C. aurantiacus* are comparable to those for MK-oxidizing Rieske/cytb-Rieske centers, the values obtained for the NT-26 and S22 Aro-Rieskes are intermediate between those of MK-oxidizing and of UQ- PQ- or CQ-

oxidizing Rieske/cytb-Rieske proteins (22). Extensive studies of Rieske/cytb complexes have established that selected residues involved in a complex hydrogen-bonding network near the cluster environment, modulate its redox properties. Taking into account the close structural relationship between Aro- and Rieske/cytb-Rieske centers, we therefore inspected the Aro-Rieske sequences to determine whether these hydrogen-bonding residues may account for the difference in  $E_m$  between *A. faecalis* and *C. aurantiacus* on the one hand and NT-26 and S22 on the other hand.

*Sequence analysis of Aro-Rieske proteins-* Aro-Rieske sequences from NT-26, *A. faecalis* and *C. aurantiacus* are available in protein data banks. Only a fragment of the S22 AroA subunit sequence was available (ABY19329) at the onset of this work. We made an effort to obtain the entire sequence of the S22 Aro-Rieske (GQ904715) and as discussed in the accompanying paper, the sequence obtained was astonishingly close to that of *Achromobacter* sp. SY8 (99% identity). A comparison was made of all four Aro-Rieske sequences to those of other Rieske proteins.

Rieske proteins (*i.e.* Aro-Rieske, Rieske/cytb-Rieske) are composed of two distinct structural domains (10). The first, essentially represented by the N-terminal half of the primary sequence has only limited sequence similarity among Rieske proteins. On the other hand, the second domain, corresponding to the [2Fe-2S] cluster-binding domain, features strong similarities in proteins from all three domains of life allowing us to produce reliable multiple sequence alignments. An alignment corresponding to the cluster-binding domain is shown in Figure 3. Sequences from S22 (GQ904715), NT-26 (AAR05655), *A. faecalis* (AAQ19839), *C. aurantiacus* fl.10 (YP\_001634828), *Aeropyrum pernix* (NP\_148694) and *Pyrobaculum calidifontis* (YP\_001056257) Aro-Rieskes are compared to sequences from *Rhodobacter sphaeroides* (YP\_354476), *Thermus thermophilus* (AAB91482) and *Sulfolobus acidocaldarius* (CAA88318) Rieske/cytb-Rieskes, from *Pseudomonas Putida* Naphtalene DiOxygenase (NDO) (P0A110) and *Burkholderia* sp. LB400 Biphenyl dioxygenase Ferredoxin (BphF) (AAB63428).

As exemplified by the *R. sphaeroides* or *T. thermophilus* sequences, in addition to the amino acids ligating the [2Fe-2S] centre, two

residues are strictly conserved in the cluster binding domain of this protein, *i.e.* Cys 134 and Cys 151 (*R. sphaeroides* numbering; marked with grey arrows in Figure 3), and these form a disulfide bond (17-20, 35). These residues have been implicated in the stability, the increase of  $E_m$  value, and the catalytic activity of the protein (see 35-38). By contrast, these Cys are absent in dioxygenase ferredoxins, a fact which had been considered to be a crucial parameter setting these latter centers apart from those of the Rieske/cytb complexes and which gave rise to the name “Rieske-type” clusters. Figure 3 shows that the S22- but not the NT-26- protein possesses these Cys. An examination of all available Aro-sequences also revealed that none of the  $\alpha$ -*Proteobacteria* (the phylum to which NT-26 belongs) and Archaea, feature these Cys residues (not all shown here but presented in SI of Duval *et al.* (39)). Aro-Rieskes from NT-26 and S22, however, have similar reduction potentials and stabilities (see preceding paragraph) similar to those of other AroBs (27,40). This suggests that, at least in Aro, the disulfide bond in fact does not modulate the properties of the iron-sulfur cluster. Since these Cys residues are conserved in the Aros from both *A. faecalis* and *C. aurantiacus*, they furthermore cannot be the origin of the relatively low  $E_m$  values seen in these proteins.

Another residue which has been demonstrated to influence the reduction potential of the [2Fe-2S] cluster, is tyrosine (Tyr) 156 (*R. sphaeroides* numbering). This Tyr has been shown (38,41,42) to increase the  $E_m$  value of the cluster by forming a hydrogen bridge with the sulfur atom of one of the cluster ligating Cys (18-20,35). It is noteworthy that at present, none of the Aro-Rieskes examined here possesses this Tyr but they all have phenylalanine (Phe) instead. This Tyr is also preplaced by Phe in all the “Rieske-type” proteins (see Figure 3, residue marked in bold grey). Since this Phe is present in all the Aro-Rieske sequences, including those of *A. faecalis* and *C. aurantiacus*, this cannot be the reason for the lower  $E_m$  values of the two latter clusters compared to those of NT-26 and S22.

Finally, a serine (Ser-154 in the *R. sphaeroides* sequence), has been shown to be responsible for an increase of  $E_m$ -value of this cluster. Structural studies established that this Ser forms a hydrogen bridge with one of the bridging sulfur atoms. This Ser is observed in all sequences of HP Rieske/cytb-Rieskes. In all Rieske/cytb-Rieske centers having a cluster reduction potential below +160 mV (LP)

[(11,22), the hydrogen bond to this bridging sulfur atom is absent. As detailed above, the *R. sphaeroides* Rieske/cytb-Rieske (having a cluster  $E_{m7}$  value of 300 mV, see Scheme 1) possesses this Ser residue whereas *T. thermophilus* (having a Rieske-cluster  $E_{m7}$  value of 160 mV, see Scheme 1) has a glycine (Gly) residue instead (for a comparative study see (33)). NDO (having a “Rieske-type” cluster with an  $E_{m7}$  value of -150 mV) features a tryptophan (Trp) at this position, and BphF (having a Rieske-type cluster with  $E_{m7} = -120$  mV) a Gly (reviewed in (11)). Among Aro-Rieske sequences, NT-26, S22 and *C. aurantiacus* also both have a Ser residue. The *A. faecalis* protein appears to be an exception with threonine (Thr) in place of Ser. While the lower reduction potential published for the *A. faecalis* Aro-Rieske cluster (25) is consistent with the observed absence of Ser, this does not hold for the *C. aurantiacus* Aro-Rieske (24). Either the structural determinants for redox properties differ in Aro-Rieske clusters from those deduced for Rieske/cytb-clusters or the reduction potential determined for the *Chloroflexus* case is incorrect. We therefore re-examined the redox properties of the Aro-Rieske cluster from *C. aurantiacus*.

*Reexamination of the C. aurantiacus Aro-Rieske cluster-* Despite significant efforts, we were unable to purify enzyme in sufficient quantity for EPR characterization. As discussed above, the EPR spectrum of the Rieske center is unique and in most cases may be studied in membrane-fragments. As reported previously (16), *C. aurantiacus* constitutes a conspicuous case, since the organism contains only one Rieske protein (*i.e.* the AroB-subunit; YP\_001634828) which is firmly associated to the membrane. The other Rieske-type protein (YP\_001634332) identified by analysis of the *C. aurantiacus* whole genome is homologous to the ferredoxin from the NDO system (15) which is soluble. We therefore performed EPR titrations on membrane fragments, as described by Zannoni and Ingledew (24), using membrane-fragment from bacteria grown in the presence of 2 mM  $As^{III}$ . When membranes were progressively reduced from +350 mV to -100 mV, two signals were found to titrate in the 1.94-1.9 region. Figure 4 depicts the evaluation of the EPR titration of the  $g_y = 1.88$  feature (open squares) but also the  $g_y = 1.94$  feature (closed squares) and the  $g_x = 1.77$  (closed triangles). Although noisy, the amplitude variation of the  $g_y = 1.88$  can be more satisfactorily fitted with two  $n=1$  Nernst

components at  $E_m = +50$  mV and  $E_m = +200$  mV (straight line) than with only one at  $E_m = +100$  mV (dotted line). The amplitude variations of the  $g_y = 1.94$  and of the  $g_x = 1.77$  signals, by contrast, were nicely fitted using only one  $n=1$  Nernst component at  $E_m = +50$  mV and  $E_m = +200$  mV, respectively. These results suggest the presence of two independent paramagnetic species. The first one, with  $g_y = 1.88$  and  $g_x = 1.77$  at  $E_m = +200$  mV, arising from the Aro-Rieske cluster (see Figure 1) and a second one having  $g_y = 1.94$ ,  $g_x = 1.88$  at  $E_m$  value of +50 mV. It therefore appears highly probable that the  $g = 1.9$  signal results from the overlap of two spectral species. This interpretation is reinforced by the observation of a shift of the  $g = 1.88$  signal at lower reduction potentials (Figure 4 insert). We therefore conclude that the Aro-Rieske from *C. aurantiacus* has a reduction potential of approximately +200 mV (Scheme 1).

This value differs substantially from that published by Zannoni and Ingledew (24) which have interpreted the  $g = 1.9$  signal as arising from a single paramagnetic center with  $E_m = +100$  mV. The growth conditions (2 mM  $As^{III}$ ) used for the present work favors *aro* gene expression (4,14,26,43). The contribution of Aro-Rieske in our spectra is therefore expected to be significantly higher than in the membranes used in the previous work. Secondly, the titration of the  $g = 1.9$  signal was previously obtained from spectra recorded at 40 K, rather than the 15 K used here. The higher measuring temperature has been shown to favor the spectral contribution of the cluster with  $E_m = +50$  mV yielding the  $g = 1.94$  signal (24). Its contribution will be decreased at the 15 K used here. Bacterial growth and EPR conditions are therefore sufficient to explain why the biphasic character of the  $g = 1.9$  titration was overlooked in the previous work.

Our EPR analysis indicates that the Aro-Rieske from *C. aurantiacus* has a reduction potential comparable to those for the NT-26 and S22 AroBs, *i.e.* in the vicinity of +200 mV and in line with the presence of the Ser residue in the cluster binding motif. Thus, in Aro, just as in the Rieske/cytb complexes, the presence of this Ser in the [2Fe-2S] cluster binding motif therefore appears to correlate well with a high (around +210 mV)  $E_m$  value (Scheme 1).

In order to compare the  $E_m$  value of the *A. faecalis* cluster obtained with the same technique, we analyzed enriched soluble samples (see *Experimental Procedures*) from this strain using EPR redox titration. Such titrations, shown

in Figure S1, determined the reduction potential of the Rieske cluster from *A. faecalis* Aro to be  $+155 \pm 15$  mV. Thus, in Aros just as in the Rieske/cytb complexes the absence of the Ser residue in the [2Fe-2S] cluster binding domain correlates with a low (well below +200 mV)  $E_m$  value (Scheme 1).

*Effect of quinols and analogs on Aro activity-* As presented in the *Introduction*, the Rieske/cytb complex is a quinol oxidase. The quinol binds to the  $Q_o$  site, and interacts with the Rieske iron-sulfur protein. A strong hydrogen bond between the hydroxyl/phenoxy-functions on the quinol/quinone and the  $N^{\delta}$ -proton on one of the histidine ligands had been proposed long time ago (44) and was suggested to be crucial for quinol oxidation by Rieske/cytb complexes (45) with a pK-value for this  $N^{\delta}$ -proton close to 8. 3D-structures and EPR studies with stigmatellin, DBMIB and UHDBT have confirmed this interaction (22,35,46-51). All these inhibitors are quinol analogs considered to be structural mimics of quinols or semiquinones (52,53). The structurally close relationship between the AroB-subunit and its counterpart from Rieske/cytb complexes as well as the conservation of a pK-value close to 8 for Aro-Rieskes studied in this work led us to test the effects of Rieske/cytb complex inhibitors on the Aro.

We tested the effects of DBMIB, UHDBT and stigmatellin (with ethanol alone as control) on the  $As^{III}$  oxidation activity of purified Aro from S22 and from NT-26. The strongest effect was obtained with stigmatellin which yields up to 35% inhibition of cytochrome reduction (dotted line, Figure 5). As illustrated by the inset in Figure 5, stigmatellin also induces EPR spectral changes on the S22 Aro-Rieske cluster, very similar to those observed with the center in Rieske/cytb-complexes (53), *i.e.* shifts of the  $g_y$  and  $g_x$  features. These spectral changes were not observed with the NT-26 enzyme. DBMIB and UHDBT induced little if any spectral change on the EPR spectrum of the Rieske subunit from both AroBs (data not shown). We furthermore titrated the NT-26 and S22 Aro-Rieske in the presence of 200  $\mu$ M stigmatellin. The binding of this inhibitor has indeed been shown to result in a striking increase of the reduction potential of the Rieske/cytb iron-sulfur cluster (53). We did not observe an equivalent shift of the reduction potential in any of the Aro-[2Fe-2S] centers examined here(not shown).

We also tested quinol and quinone as inhibitors of the Aro activity. Addition of quinones (not shown) produces an inhibition of cytochrome reduction by Aro, comparable to that induced by stigmatellin.

## DISCUSSION

The importance of selected residues in determining the spectral, redox and catalytic properties of iron-sulfur clusters has been recognized for some time. Rieske proteins are well-suited for such analyses. Sequence and structural comparisons of Rieske proteins from various systems have highlighted a significant degree of conservation in the C-terminal part of the protein, that is, the segment including the cluster binding motif (10-12). The strong sequence diversity in this group (both from wild type and from mutated proteins) and the number of available crystal structures now allow a reliable analysis of the individual effects of residues surrounding the cluster. This kind of analysis has already been carried out on the subgroup of the Rieske/cytb proteins (38,41,42,54). The Aro subgroup of Rieske proteins has not yet been examined in this way. Our present study has determined parameters required to extend the comparative approach to this group and therefore to include a new degree of sequence variation. In fact, two residues considered crucial for the Rieske/cytb-subunits' properties turn out not to be conserved in Aro-Rieske proteins.

*Role of the Ser and Tyr residues in the vicinity of the cluster-* As shown by our EPR study on the Aro-Rieske proteins, the reduction potentials of the [2Fe-2S] cluster of Aros from NT-26, S22 and *C. aurantiacus*, are in the region of +210 mV (Scheme 1). All three proteins have a conserved Ser in the cluster binding motif (Fig. 3A). The *A. faecalis* Aro however contains a Thr in this position and also shows a substantially lower  $E_m \sim +160$  mV (our work; 25) (Fig. 6). In Aro a hydrogen bridge between Ser and one of the cluster-bridging sulfur atoms is responsible for the higher  $E_m$  values as is seen in the Rieske/cytb complexes. This Thr also forms a hydrogen bond to the cluster-bridging sulfur, as shown in Fig. 6. Studies of the Rieske/cytb (41,42) have demonstrated that replacement of Ser by Thr in the Rieske/cytb-Rieske produces only a small decrease ( $\Delta E_m = -26/28$  mV) in  $E_m$  as compared with substitutions fully eliminating the hydrogen bond, in line with the moderate

decrease of  $E_m$  in the *A. faecalis* [2Fe-2S] protein (Scheme 1).

Aro-Rieskes from NT-26, S22 and *C. aurantiacus* show the sequence CPCHGSxF in the cluster binding motif (Fig. 3), *i.e.* each contains a Phe replacing the canonical Tyr present in the *R. sphaeroides* wild-type sequence. In the large majority of Rieske/cytb-Rieskes (mutated or wild type (38,41,42,55)) studied so far featuring this CPCHGSxF motif,  $E_{m,7}$  value around +230 mV have been found, *i.e.* close to the  $E_{m,7}$  value cited above. In the case of mutagenesis, a 45-70 mV decrease compared to the wild-type protein containing the CPCHGSxY sequence results from the absence of a hydrogen bond between Phe and a cluster-ligating Cys as shown by the crystal structure (35,42). The structure of Aro demonstrates that the Phe residue in this enzyme is in exactly the same place as the Tyr residue in the Rieske/cytb complex (Fig. 6).

Ser and Tyr residues appear therefore to play equivalent roles in Aro- and Rieske/cytb-Rieskes but specific cases preclude a generalization of the “Ser- and Tyr-induce-high  $E_m$ ” rule. The PetA protein from *Aquifex aeolicus* as well as sulredoxin from *S. tokodai* feature a Gly and an Ala, respectively, in place of the Ser residue yet their  $E_m$  values have been determined around +200 mV (56,57). On the other hand PetA from *Allochromatium vinosum*, bears the CPCHGSxF sequence (see S1 from (10)) yet titrates at  $E_m = +285$  mV (see 11). At present we cannot determine whether these discrepancies are due to experimental problems (*cf.* the *C. aurantiacus* case) or whether they reflect the action of presently unidentified residues compensating the Ser and Tyr effects.

If the potential-increasing effect of the Ser residue was indeed an universal property of Rieske proteins, its presence in all basal branches of Archaea and Bacteria (Fig. 3B) presents a bioenergetic conundrum. We have pointed out previously (58,59) that the ancestral Rieske/cytb enzyme is likely to have operated in a MK-based bioenergetic electron transfer chain, using a Rieske centre with a correspondingly LP. The universality of the “Ser- induces-high  $E_m$ ” rule would therefore suggest a different adaptation of the bioenergetic chain to the reduction potential of the quinone pool in all basal branches of Archaea and Bacteria. Unfortunately, only very limited electrochemical data are presently available for these phyla and it remains for future studies to address this issue.

*Role of the Disulfide Bond in Rieske-proteins-* The disulfide bond has long been thought to be essential for cluster stability in Rieske proteins. This conclusion is based on the loss of the cluster in mitochondria upon treatment with 2,3-dimercaptopropanol (60) and on mutation of the disulfide-Cys in the *R. capsulatus* Rieske/cytb complex (36). The structures of NDO and BphF (17,20), however, revealed that the disulfide bond is not indispensable (see Fig. 3). Subsequent mutagenesis studies with mitochondrial Rieske proteins support a more moderate role for this disulfide bond with effects only on redox properties, catalytic activity and the EPR spectrum (37,38). Our results suggest an even more modest role of this disulfide bond at least in Aro. Figures 1 and 2 demonstrate that neither the EPR spectral rhombicity nor the  $E_m$  values are significantly modified in Aro-Rieskes devoid of this disulfide bond (*e.g.* the NT-26 protein). We note that an error in the NT-26 AroB sequence is excluded since all  $\alpha$ -proteobacterial and archaeal AroB sequences lack the disulfide bond. None of the residues seen to replace the two Cys, *i.e.* Phe/Gly, in these natural cases have, however, been tested in mutagenesis studies (37,38).

Our results do suggest that the presence of a disulfide bond correlates with quinol-oxidizing activity and EPR saturation properties. Even if the effect of Rieske/cytb inhibitors on Aro is greatly lower compared to Rieske/cytb itself (*i.e.* 100%; see for example (37)), the fact that stigmatellin and quinols inhibit 35 % of the Aro activity and perturb the EPR spectrum indicate that Aro binds quinols. Whether this interaction has a physiological role is beyond the scope of this study. The fact that the EPR signal of the NT-26 enzyme is not perturbed upon addition of stigmatellin possibly relates to the absence of the disulfide bond. Loss of reactivity towards inhibitors of Rieske/cytb complexes and decrease of quinol-oxidizing activity have indeed previously been correlated with the absence of this bond (37,38). Quinone and inhibitor binding by Aro thus challenges the Rieske/cytb dogma that quinone binding is governed by the entire  $Q_o$ -site (Rieske plus cytb) and argues for an intrinsic affinity of the Rieske-protein for quinones.

The disulfide bond's Cys, together with selected residues surrounding the His ligands of the Rieske center (among them Thr130, Leu132 in the *R. sphaeroides* numbering), have also been implicated in pK variations observed in  $E_m$

vs pH-dependences (11,22,38,61). The hydrophobicity of the Leu and Thr residues have been proposed to rationalize i) why the coupling between cluster redox state and His protonation state is electrostatically more efficiently screened out in BphF as compared to Rieske/cytb complexes and ii) why this cluster has a lower  $E_m$  value (33). In the S22 Aro-Rieske, both the disulfide bond and the Thr residue are conserved while the Leu is replaced by Met. The similarity of this center's redox properties to those of the Rieske/cytb proteins thus appears logical. The simultaneous substitution of Leu and Thr in the NT-26 sequence with Lys and Pro introduces more significant changes in the chemical environment of the His. Although these changes entail only minor modifications of reduction potential, they appear to correlate with shift in pK value. These substitutions in NT-26 are concomitant with the absence of the disulfide bond, and it is presently impossible to attribute an individual role to any of these residues in shifting pK values. The observed pK shifts nevertheless fall dramatically short of reproducing the dependence observed in BphF, which is characterized by a more than 2 pH unit more alkaline pK value. We therefore conclude that either the model put forward to explain the difference between the Rieske/cytb complex and BphF does not apply to the Aro enzyme or, more likely, that further, hitherto unidentified, factors are involved.

Our results thus suggest that the disulfide bond, in Aro-Rieske, has little or no effect on the redox properties of its iron-sulfur cluster, but confirm that its presence correlates with the pK values associated with the  $E_m$  pH-dependence, with quinol-oxidizing activity and with EPR saturation properties. Absence of the disulfide bond may furthermore correlate with strong selectivity towards specific cytochromes (accompanying paper). The determining factors of Aro's selectivity for its cytochrome electron acceptors are not yet understood. We can, however, ascertain that the presence/absence of the disulfide bond in Aro-Rieske does so far correlate with cytochrome selectivity (40). Work aimed at further elucidating the structural basis for this selectivity is underway.

## CONCLUSIONS

In the past, comparisons of the properties of Rieske proteins from diverse groups of Rieske/cytb complexes, and in

particular their HP representatives on the one hand and the bacterial dioxygenases on the other have identified several residues potentially affecting the physicochemical properties of the iron-sulfur cluster. Six specific residues have been proposed to influence stability, spectroscopic parameters, reduction potential and pH dependence, *i.e.* the two residues close to the cluster-ligating His, the Ser and Tyr residues forming hydrogen bonds with the cluster and the two Cys residues forming the disulfide bond.

As we have detailed above, although our study indicates that presently unidentified residues may induce effects exclusively attributed to the Ser and Tyr positions, the role of the Ser and Tyr residues in the AroB subfamily appears to be similar to what has been observed on the remaining ensemble of Rieske proteins. Further, the properties we have observed for the subgroup of  $\alpha$ -proteobacterial AroB proteins, typified by the NT-26 enzyme, strongly argue against the dramatic influence on cluster properties hitherto ascribed to the disulfide bond.

Combined molecular phylogenies of Rieske/cytb complexes and Aro indicate that i) both enzyme families already existed in the Last Universal Common Ancestor (LUCA) of Bacteria and Archaea and ii) their Rieske subunits are related (10,16) and share a common ancestor. The results obtained with the superfamily of Rieske proteins allows us, by analyzing the distribution of evolutionary markers on the reconstructed phylogenetic tree, to deduce some of the basic properties of this ancestral Rieske protein (Fig. 3B). The fact that all Rieske subunits in Rieske/cytb complexes as well as the majority of AroBs contain the disulfide bond, qualify this as likely being in the ancestor of Rieske/cytb complexes and Aros. In the same vein, the Ser residue forming a hydrogen bond with one of the cluster-bridge sulfur atoms of the cluster is also likely to have been already present in the common ancestor. Finally, our results suggest that Aros, in addition to Rieske/cytb complexes, are able to react with quinols. This suggests that the common ancestor of Rieske proteins was likely already able to bind quinones. The absence of these traits in the bacterial dioxygenases reflects that they represent a more recently evolved subgroup, a conclusion in line with their limited occurrence in a few proteobacterial species.

## FOOTNOTES

Abbreviations used are: AMPSO: 3-((1,1-Dimethyl-2-hydroxyethyl)amino)-2-hydroxypropanesulfonic acid; Arsenite: As<sup>III</sup>; BphF: Biphenyl dioxygenase Ferredoxin; CQ: Caldariellaquinone; Cys: cysteines; DBMIB: Dibromothymoquinone; DEAE: diethyl amino ethyl ; EPR: electron paramagnetic resonance; Gly: glycine; His: histidines; HP: high reduction potential; LP: low reduction potential; NDO: Naphtalene DiOxygenase; MES: 2-(N-morpholino) ethanesulfonic acid; MK: Menaquinones; MOPS: 3-[morpholino]propanesulfonic acid; Phe: phenylalanine; PQ: Plastoquinone; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; UQ: Ubiquinone; UHDBT: 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole

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## FIGURES LEGENDS

**Figure 1.** EPR properties of the Aro Rieske center from NT-26 and S22. All spectra were recorded on isolated complex. Reduction of the Rieske center was achieved by ascorbate. Spectra a (NT-26) and b (S22) in Panel A were recorded under non-saturating conditions. Instrument settings: microwave

frequency, 9.4 GHz; modulation amplitude 3 mT, temperature 15 K; microwave power, 6.3 mW. Panel B present power saturation curves measured on the  $g_x$  (circles) and  $g_y$  (squares) values of the ascorbate-reduced Rieske center from NT-26 (closed symbols) and S22 (open symbols) at 15 K. EPR conditions were as in Panel A.

**Figure 2.** The reduction potential of the Rieske center from NT-26 and S22 as a function of pH. The open and closed symbols represent data obtained with S22 and NT-26 enzyme, respectively. The data obtained on S22 Rieske are fitted by assuming two ionization equilibria with  $pK_a$ -values of 8 and 9.8 and  $E_m$  (low pH) of +210 mV whereas the data obtained on NT-26 Rieske are fitted by assuming two ionization equilibria with  $pK_a$ -values of 8 and 8.8 and  $E_m$  (low pH) of +220 mV.

**Figure 3.** Sequence comparisons of cluster-binding domain of Rieske-type proteins. In panel A, sequences of Aro-Rieske from *Ralstonia* sp. 22 (Aro\_S22; GQ904715), *Rhizobium* NT-26 (Aro\_NT26; AAR05655.1), *Alcaligenes faecalis* (Aro\_Alcf; AAQ19839.1), *Chloroflexus aurantiacus* (Aro\_Chla; YP\_001634828.1), *Pyrobaculum calidifontis* (Aro\_Pyrca; YP\_001056257.1) and *Aeropyrum pernix* K1 (AroB\_Aerpe; NP\_148694) are compared to sequences of Rieske/cytb-Rieske from *Rhodobacter sphaeroides* (PetA\_Rhosp; YP\_354476.1), *Thermus thermophilus* (PetA\_Theth; AAB91482.1) and *Sulfolobus acidocaldarius* (soxF\_Sulac; CAA88318), of NDO-Rieske from *Pseudomonas putida* (NDO\_Psepu; P0A110.1), and of BphF-Rieske from *Burkholderia* sp. LB400 (BphF\_Bursp; AAB63428.1). The reduction-potential-influencing Ser and Tyr residues (and eventual substitute) are marked by vertical black arrows. Fully conserved His and Cys residues, binding the cluster are marked in boxes. Cys residues considered to form a disulfide bond in Rieske proteins are marked by grey arrows. In panel B, a schematic phylogenetic tree, reconstructed from detailed phylogenetic analysis (22), represent the distribution of the two important phylogenetic markers, i.e. the Ser (marked Ser) and the di-sulfur bond (marked S-S) into the Rieske/cytb and Aro clades in Bacteria and Archaea.

**Figure 4.** Redox titration at pH 7.0 on membranes from *C. aurantiacus*. Signal amplitudes at various ambient potentials were measured on the  $g = 1.88$  (open squares),  $g = 1.77$  (closed triangles) and  $g = 1.94$  (closed squares) lines. The straight line through the open squares represent a theoretical curve with two  $n=1$  components with  $E_m$  of +200 mV and +50 mV, whereas the dotted line through the open squares represent a theoretical curve with one  $n=1$  component with  $E_m$  of +100 mV. The  $g = 1.77$  and  $g = 1.94$  titrations are fitted with a  $n = 1$  Nernst curve with an  $E_m$  of +200 mV and +50 mV, respectively. Inset shows the signal shift observed during the titration on the  $g = 1.88$  feature by comparing a spectrum recorded at +124 mV and -56 mV.

**Figure 5.** Effect of stigmatellin on the S22 Aro-Rieske. Panel A shows kinetics recorded in absence (straight line) or in presence of 200  $\mu$ M stigmatellin (dotted line) with isolated Aro from S22 in presence of 100  $\mu$ M  $As^{III}$  and 20  $\mu$ M cytochrome at pH 8. Panel B shows EPR spectra recorded in absence (straight line) or in presence of 200  $\mu$ M stigmatellin (dotted line) on isolated and ascorbate reduced Aro from S22. EPR conditions are as in Figure 1.

**Scheme 1.** Schematic representation of the reduction potential of the Rieske cofactor on a redox scale. Rectangles for MK (menaquinone)- and HPQ (High potential quinone)- using Rieske/cytb denote the total redox range covered by complexes from the different characterized organisms. Values for Aro-Rieske are taken from this work.

**Figure 6.** Structure of the Rieske-cluster binding pocket. The structure of crystallized Rieske/cytb-Rieske from *R. sphaeroides* is compared to the structure of crystallized Aro-Rieske from *A. faecalis* and of modeled Aro-Rieske from NT-26. The residues implicated in the determination of cluster's redox properties and discussed in the text are indicated by their initials.

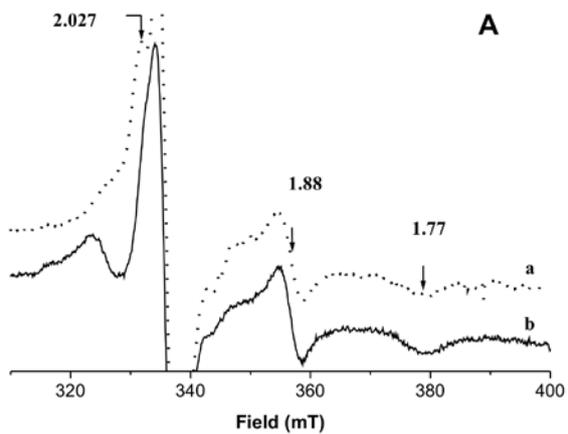


Figure 1A

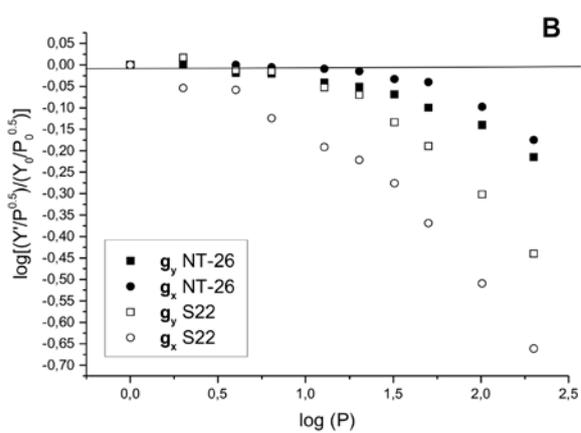


Figure 1B

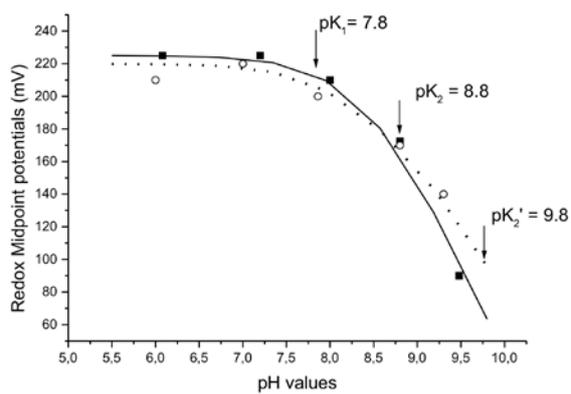
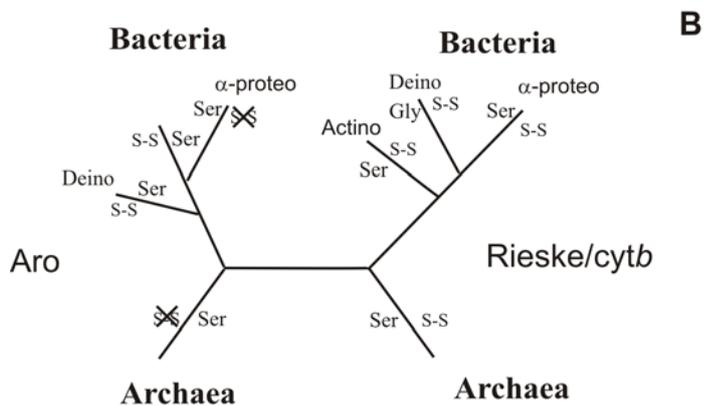
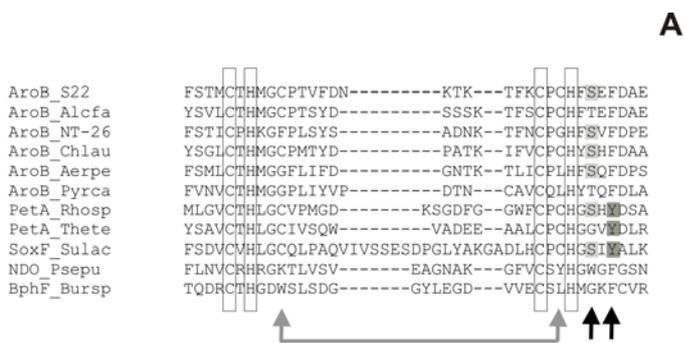
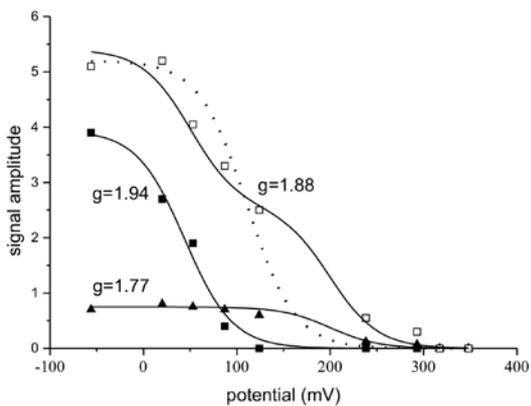


Figure 2



**Figure 3**



**Figure 4**

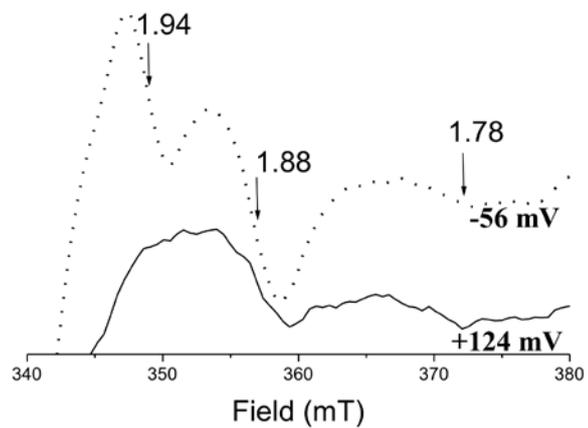


Figure 4inset

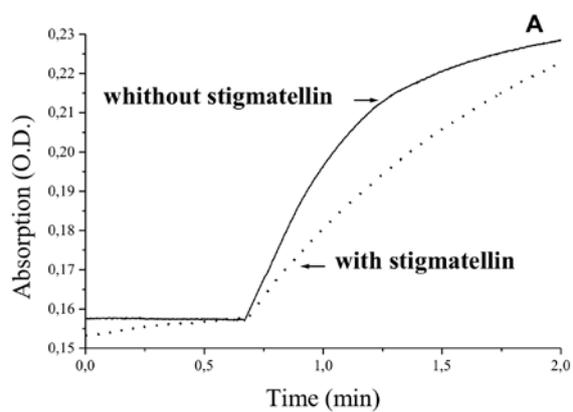


Figure 5A

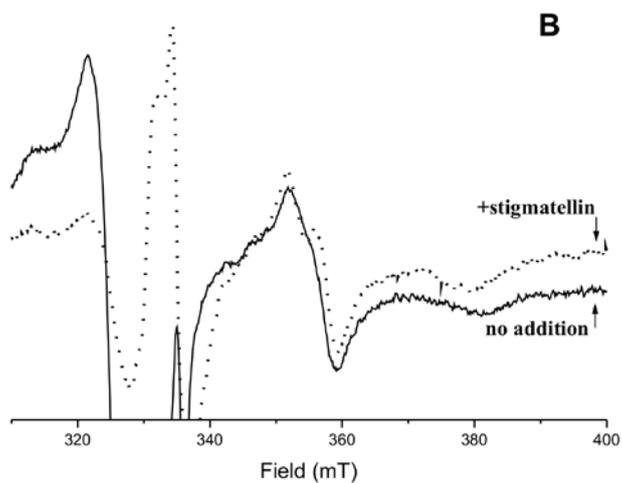
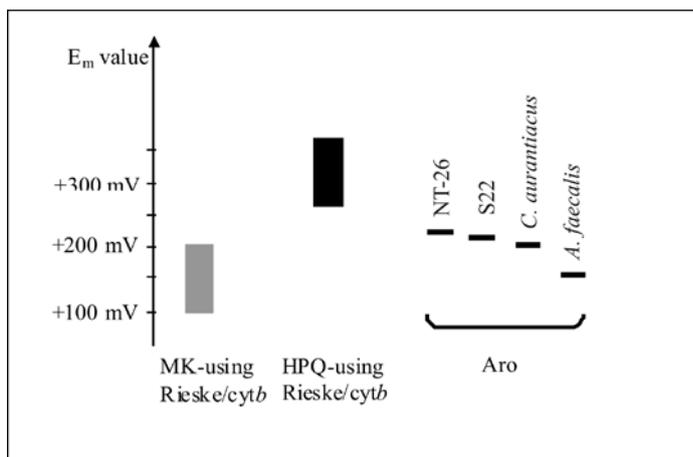
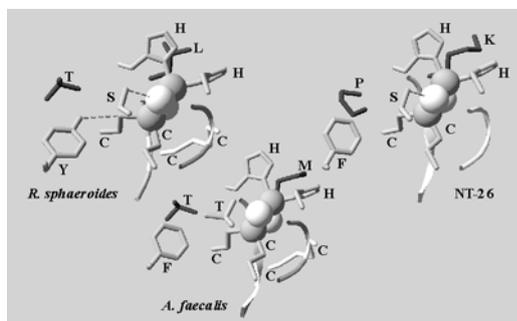


Figure 5B



**Scheme 1**



**Figure 6**