Arsenic Oxidase from *Ralstonia* sp. 22

**CHARACTERIZATION OF THE ENZYME AND ITS INTERACTION WITH SOLUBLE CYTOCHROMES**

We characterized the *aro* arsenic oxidation system in the novel strain *Ralstonia* sp. 22, a β-proteobacterium isolated from soil samples of the Salsigne mine in southern France. The inducible *aro* system consists of a heterodimeric membrane-associated enzyme reacting with a dedicated soluble cytochrome c₅₅₄. Our biochemical results suggest that the weak association of the enzyme to the membrane probably arises from a still unknown interaction partner. Analysis of the phylogeny of the *aro* gene cluster revealed that it results from a lateral gene transfer from a species closely related to *Achromobacter* sp. SY8. This constitutes the first clear cut case of such a transfer in the *Aro* phylogeny. The biochemical study of the enzyme demonstrates that it can accommodate *in vitro* various cytochromes, two of which, c₅₅₂ and c₅₅₄, are from the parent species. Cytochrome c₅₅₂ belongs to the *sox* and not the *aro* system. Kinetic studies furthermore established that sulfite and sulfadate, substrates of the *sox* system, are both inhibitors of *Aro* activity. These results reinforce the idea that sulfur and arsenic metabolism are linked.

Arsenic is most commonly found in an insoluble, and thereby not toxic, form associated with more than 200 rock and mineral species. However, in natural environments such as geothermal springs and in sites contaminated by industries (1) or by anaerobes, using As(III) as the electron donor and CO₂/HCO₃⁻ as the sole carbon source) or (ii) heterotrophs (growing in the presence of organic matter) (for recent reviews, see Refs. 13 and 14).

Apart from the two cases of Ectothiorhodospiraceae, *Alkalilimnicola ehrlichii* str. MLHE-1 (15) and PHS-1 (16), the enzyme identified as responsible for As(III) oxidation has been shown to be As(III) oxidase. Whereas Aox was the name first given to the gene cluster coding for the enzyme (17), it presents a drawback in denoting the molybdopterin subunit as AoxB in conflict with the general dimethyl sulfoxide reductase superfamily (to which the enzyme belongs; see below) nomenclature and in which the catalytic molybdopterin subunit invariably is called A. The name Aro, which was introduced later (18), is admitted similar to a denomination already in use since the 1970s for aromatic amino acid synthesis enzymes (19) but has the advantages of i) following the dimethyl sulfoxide reductase superfamily nomenclature and ii) explicitly matching the name of As(V) reductase. Aso, introduced by Silver (13), is used only scarcely. We have, therefore, chosen to use *Aro* in the following text.

*Aro* can be found either in the periplasm (20, 21) or associated to the cytoplasmic membrane (5, 13, 22–24). AroA (90–100 kDa) carries a molybdopterin cofactor together with a [3Fe-4S] center and characterizes the enzyme as a member of the dimethyl sulfoxide reductase superfamily. AroB (14 kDa), is a member of the Rieske proteins superfamily by virtue of its [2Fe-2S] center (see 25 and 66, accompanying article) and is inductively coupled plasma atomic emission spectrophotometry; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; NT-14, *Hydrogenophaga* NT-14; NT-26, Rhizobium NT-26; S22, *Ralstonia* sp. 22; SY8, *Achromobacter* sp. SY8; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
ERED to be responsible for a possible membrane attachment (23, 24). Only scant data have, so far, been reported on the enzymology of Aro. Because As$^{\text{III}}$ is a two-electron donating substrate, the catalytic turnover is assumed to start with the oxidation of As$^{\text{III}}$ by the molybdenum center (which can accept up to two electrons). Several facts indicate that the catalytic cycle of As$^{\text{III}}$ oxidation results in most cases in the reduction of a soluble cyt. First, cytochromes (cyts) have been copurified with the enzyme (21, 22). Secondly, cyt-encoding genes are often present in the aro gene clusters (10, 24, 26–28). And finally, the As$^{\text{III}}$ oxidation process in *Ochrobactrum tritici* requires the cyt encoded in the *aro* operon (28). No detailed studies have been presented, however, addressing the electron transfer reaction between Aro and cyt. The only enzymatic data presently available on Aro have been obtained using 2,4 dichlorophenolindolphenol (DCPIP) or azurin, two nonphysiological electron acceptors of the *Alcaligenes faecalis* enzyme (22). Enzymatic properties of Aro deduced from these studies, therefore, do not necessarily reflect the physiological reaction. In this paper, we describe the purification and characterization of Aro from the novel strain *Ralstonia* sp. 22 (S22). This β-proteobacterium has been isolated from soil samples of the Salsigne mine in southern France. In addition to the enzyme, we also purified two cyts among them is the likely physiological electron acceptor of Aro, cyt $c_{554}$, and we characterized the reaction of Aro with both cyts. The presented results therefore are the first detailed enzymatic data on an Aro reacting with cyts. Moreover, the use of cyts in the activity assays allowed us to screen the sensitivity of purified Aro toward sulfur compounds. The observed inhibitory effects support the idea that arsenic and sulfur metabolisms are functionally related. As stated above, a number of Aro enzymes have been studied in the past with respect to specific properties. However, in none of these cases, a complete characterization of the enzyme determining biochemical, biophysical (see Ref. 66, accompanying article), and enzymatic parameters has been obtained, hampering a comprehensive understanding of the enzyme and its comparison with other members of the dimethyl sulfoxide reductase superfamily. The present work fills in these gaps by presenting an exhaustive description of Aro in S22.

**EXPERIMENTAL PROCEDURES**

**S22 Isolation and Growth Conditions**—The strain S22 was isolated from arsenic-contaminated soil collected near the gold mine of Salsigne, Aude, France. Soil samples were inoculated at 25 ± 2 °C into a liquid chemically defined medium (CDM) (described by Muller et al. (17) for *Herminiimonas arsenicoxydans*), supplemented with 1.33 mM As$^{\text{III}}$ to develop enriched cultures. A pure culture was obtained by successive isolation of colonies at 25 ± 2 °C on CDM, solidified by addition of 20 g liter$^{-1}$ of agar-agar (Difco).

S22 was grown aerobically at 28 °C in 5-liter bottles of CDM. When included in the medium, 5 mM As$^{\text{III}}$ (NaAsO$_2$) or 20 mM thiosulfate (Na$_2$S$_2$O$_3$·5H$_2$O) were added. The final pH was ~7. Cultures were harvested during the late exponential phase.

**Preparation of Spheroplast and Periplasmic Fractions**—Spheroplasts were prepared as published previously (29) with some modifications. Bacteria were incubated for 1 h at 30 °C (instead of 30 min) with lysozyme 1 mg$^{-1}$ ml$^{-1}$ (instead of 0.5 mg$^{-1}$ ml$^{-1}$). After incubation, cells were centrifuged at 4000 × g for 30 min, and spheroplasts were retrieved in the pellet, whereas the supernatant constitutes the periplasmic fraction. Spheroplasts were resuspended in 100 mM phosphate buffer at pH 7.4 for subsequent characterization.

**Purification of the Aro**—Cells were suspended in 50 mM Tricine at pH 8 (buffer A) and broken by passing twice through a French press. Unbroken cells were eliminated by centrifugation at 10,000 × g, and a subsequent ultracentrifugation (280,000 × g) separated the “total soluble fraction” (in the supernatant) from the “membrane fraction” (in the pellet). Enzyme purification was performed from the total soluble fraction at 4 °C. The sample, once oxidized with ferricyanide, was loaded on a DEAE Sephadex column equilibrated with buffer A. Aro eluted at 50 mM NaCl from this column. The sample was then dialyzed to eliminate NaCl and subsequently loaded on a monoQ DEAE column (fast protein liquid chromatography (FPLC) system) equilibrated with buffer A. This second DEAE was eluted at 1 ml min$^{-1}$ with a 0–100 mM NaCl gradient and Aro eluted at ~30 mM NaCl. The Aro fraction was then concentrated by centrifugation in Amicon Ultra-5 concentrators. The sample was then loaded onto a Superdex 200 gel filtration column (FPLC system), which was equilibrated with buffer A/NaCl 100 mM and eluted at 0.4 ml min$^{-1}$. Only freshly purified enzyme was used for enzymatic analyses.

**Purification of Cytochromes**—The fraction containing almost all the soluble cyts eluted during washing of the DEAE Sephadex used for the Aro purification. This “DEAE-cyt fraction,” was then loaded on a CM-52 column equilibrated with buffer A. Because binding of the cyt $c_{552}$ on the CM column depends on its oxidation state, we systematically oxidized the fraction before loading. Cyts $c_{551}$ and $c_{554}$ eluted together during washing of the CM. After this step, the cyt $c_{552}$ eluted at 1 ml min$^{-1}$ from 20 mM NaCl. Both cyt fractions were then separately concentrated by centrifugation in an Amicon Ultra-5 concentrator and loaded separately onto a Superdex 75 gel filtration column (FPLC system), which was equilibrated with buffer A/NaCl 100 mM and eluted at 0.4 ml min$^{-1}$. Pure cyts $c_{554}$ and $c_{552}$ were obtained after this step. The enriched cyt $c_{551}$ obtained from this step was not further purified.

**Aro Activity Assays**—Aro activity was routinely measured optically in 50 mM MES, pH 6, at 37 °C, using 200 μM sodium As$^{\text{III}}$ as an electron donor, 150 μM DCPIP as an electron acceptor, and 20 μM phenazine methosulfate as an electron mediator between Aro and DCPIP. The activity was followed as the reduction of DCPIP, i.e. decreasing absorption monitored at 600 nm ($\epsilon_{600}$=16 experimentally determined at 12 mm$^{-1}$ cm$^{-1}$). The reaction was initiated by addition of As$^{\text{III}}$. In the specific enzymatic studies, sulfite (sodium sulfite) and thiosulfate (sodium thiosulfate) were tested as electron donors, whereas azurin from *Pseudomonas aeruginosa* and cyt c from bovine heart (commercially available) and cyt $c_{554}$ purified from *Aquifex aeolicus* as described in Ref. 30, or cyt $c_{552}$ and $c_{554}$ purified from S22 (see above) were tested as electron acceptors. The pH optimum was assayed by using mixed buffers MES/MOPS/Tricine/AMPSO/CAPS at 15 mM each. Finally Na$_2$S$_2$, sulfite, sulfate, thiosulfate, and As$^{\text{V}}$ were tested as potential.
inhibitors. In these cases, the kinetics were followed as reduction of cyt, i.e., increasing absorption monitored at the α band maximum. Aro activity was also detected on native polyacrylamide gels. Total soluble fraction and membrane fraction from French press treatment on one side or periplasm and spheroplasts from lysozyme treatment on the other side were analyzed by native gel electrophoresis. Equivalent samples (~30 μg of total proteins) from each cell-breaking treatment were loaded on the gel. The electrophoresis was done on a native 10% polyacrylamide Laemmli gel system (31) containing 0.1% Triton X-100. The gel was then equilibrated in 50 mM MES, pH 6, for 15 min and subsequently incubated for 30 min in the dark in the same buffer supplemented with 300 μM DCPIP and 100 μM phenazine methosulfate. Addition of 200 μM sodium AsIII allowed the detection of the Aro band by its destaining activity.

Biochemical Protein Analyses—Protein concentrations were determined by the BCA method using bovine serum albumin as standard. The subunit composition was determined by SDS-PAGE following the procedure of Laemmli (31) on a 5–15% gradient polyacrylamide gel. The cyt composition of the “total soluble fraction” (see above) was analyzed by electrophoresis following the procedure of Judd (32) on a 18% polyacrylamide gel. The molecular weight of native Aro and cyt was estimated by gel filtration in buffer A/100 mM NaCl on Superdex 200 or 75, respectively, using apoferrin, amyrase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and bovine heart cyt as molecular weight standards.

DNA Works and Sequencing—DNA manipulations were carried out according to standard protocols as described by Sambrook et al. (33). Total DNA of strain S22 was isolated using the Wizard Genomic DNA purification kit (Promega). 16S rDNA fragments were amplified by PCR on DNA extract using the eubacterial universal primers specific for 16S rDNA (P8, 5’-AGAGATTTGATCCTGGCTCAG-3’ and Pc1544, 5’-AAGGAGGTGATCCAGCAGCAGCA-3’). The amplified 16S rDNA fragment was purified via phenol extraction and 2-propanol precipitation and sequenced. Based on the aro operon of Achromobacter sp. SY8 (SY8) (GenBankTM accession no. EF523515), oligonucleotides were designed that amplify a DNA stretch of 3508 bp using PCR, covering the aroA, aroB, and aroC (cyt c55) genes of S22, in three overlapping fragments. For fragment 1 (1190 bp), forward primer 5’-CGTCCGAAAGCTTCTTGGG-3’ and reverse primer 5’-GGAGTGAACATTGCCCTCC-3’ were used; for fragment 2 (1246 bp), forward primer 5’-TTCTCCTGGTCCAGCAG-3’ was used for reverse primer, 5’-CCTTACCAGTGTTGGC-3’ was used; and for fragment 3 (1241 bp), forward primer 5’-ACGCATCCGCTATCTC-3’ and reverse primer 5’-CATTAGCCGG-GAACCCG-3’ were used. PCR amplification was done using Pfu DNA polymerase (Promega) on DNA extract, and the amplified DNA fragments were cloned into vector pSTBlue-1 using the Perfectly Blunt cloning kit (Novagen). Then, DNA sequencing of the vector inserts was performed (GATC Biotech) using the T7 and SP6 primers, and the resulting sequences were assembled into the complete 3508-bp sequence.

Sequence Analyses—ClustalX (34) was used to obtain multiple sequence alignments of proteins or 16S rDNA. Phylogenetic trees were reconstructed from these alignments using the NJ algorithm implemented in ClustalX or using the parsimony method (PHYLYP package). The nucleotide sequences described in this study have been deposited in GenBankTM with the following accession numbers: EU304284 (S22 16S rRNA), EU304273 (partial aoxB gene), and GQ904715 (total 3508-bp aro cluster).

**Determination of Arsenic Speciation in the Growth Medium and Minimal Inhibitory Concentration—Qualitative AsIII oxidation activity from bacteria was followed by visualizing the AsIII concentration in the growth medium by a colorimetric method as described by Simeonova et al. (35). When needed, arsenic species were more precisely quantified by HPLC-ICP AES as described by Weeger et al. (36). Minimal inhibitory concentration of AsIII and AsV were determined by following the procedure described by Lim and Cooksey (37).

Protein Identification Techniques—Intact protein mass analyses were performed on a MALDI-TOF mass spectrometer UltraflexII from Bruker Daltonik. External calibration was made on the singly charged ion [M + H]+ at 16,951.56 of apomyoglobin, at 12,361.96 of cyt, and the doubly charged ion [M + 2H]+/2 at 8476.28 of apomyoglobin. N-terminal sequence determination was performed by Edman degradation using an automatic sequencer model Procise 494 from Applied Biosystems on bands from blotted protein onto 0.2 μm polyvinylidene fluoride membrane stained with Ponceau Red.

Optical spectra were recorded on a Cary 5E spectrophotometer. Redox titrations were performed on purified cytochromes at 15 °C as described by Dutton (38) in the presence of the following redox mediators at 10 μM: 1,4-p-benzoquinone, 2,5-dimethyl-p-benzoquinone, and 2-hydroxy-1,2-naphthoquinone. Reductive titrations were carried out using sodium dithionite, and oxidative titrations were carried out using ferricyanide.

**RESULTS**

Isolation and Phylogenetic Characterization of the Novel Strain Ralstonia sp. 22—A soil sample contaminated by gold mine wastes rich in arsenic (Salsigne, Aude, France) was cultured on CDM supplemented with AsIII (1.33 mM). A pure culture was obtained by successive isolation of colonies at 25 ± 2 °C on AsIII-supplemented CDM. Isolate S22 showed AsIII oxidase activity when grown on CDM supplemented with 1.33 mM AsIII. HPLC-ICP AES experiments demonstrated the progressive disappearance of AsIII in parallel to the appearance of AsV in the supernatant of the strain S22 culture (data not shown). These findings suggested the oxidation of AsIII by strain S22.

Phylogenetic analyses based on the 16S rDNA (1,455 bp) sequence indicated that the strain belongs to the class of the β-proteobacteria and that the nearest phylogenetic relatives are members of the Burkholderiaceae family in the order Burkholderiales. Analysis of binary similarity data showed that the 16S rRNA sequence of strain S22 displays 97% identity to sequences of representatives of Ralstonia genera (solanacearum species), suggesting that the strain S22 is a new species member of the genus Ralstonia, a group of bacteria frequently found in soils.

The minimal inhibitory concentration value, defined as the ion concentration that inhibited confluent growth on plates
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TABLE 1

Comparison of S22 to H. arsenicoxydans for As\textsuperscript{III} and As\textsuperscript{V} Minimal Inhibitory Concentrations

|          | H. arsenicoxydans | Ralstonia sp. 22 |
|----------|-------------------|-----------------|
| MIC As\textsuperscript{III} | 6 mM             | 30 mM          |
| MIC As\textsuperscript{V}     | 200 mM            | 200 mM         |

TABLE 2

Purification of Aro from S22

All kinetics were performed using DCPIP as electron acceptor.

| Purification step | Total proteins | Total activity | Specific activity | Purification Yield |
|-------------------|----------------|----------------|-------------------|--------------------|
| Cell extract      | 266.6 g        | 10.5 μmol/min/mg | 0.0392 mg⁻¹/min/mg | 100                |
| DEAE-Sephacel     | 11.9 mg        | 9.6 μmol/min/mg  | 0.802 mg⁻¹/min/mg  | 20.5               |
| MonoQ             | 1.368 mg       | 5.7 μmol/min/mg  | 5.7 mg⁻¹/min/mg    | 106.9              |
| Gel filtration    | 0.684 mg       | 3.9 μmol/min/mg  | 5.7 mg⁻¹/min/mg    | 145.4              |

FIGURE 1. Polyacrylamide gel electrophoresis and in-gel Aro activity staining of subcellular fractions of S22. Lane 1, spheroplasts; lane 2, periplasm; lane 3, membrane fraction after French press treatment; lane 4, soluble fraction after French press treatment.

FIGURE 2. Coomassie Blue-stained SDS-PAGE on purified Aro from S22. Lane 1, molecular weight standards; lane 2, purified enzyme. Identification of subunits was confirmed by N-terminal sequencing.

after 3 days at 30 °C, was determined for As\textsuperscript{III} and As\textsuperscript{V}. Interestingly, S22 showed resistance up to 30 mM As\textsuperscript{III}, which is a 5× higher resistance level than that exhibited by the arsenic oxidizing bacterium H. arsenicoxydans (17) (Table 1) and comparable to the recently characterized SY8, Pseudomonas sp. TS44 and O. tritici bacteria (13, 23, and 50 mM, respectively) (10, 28). The Aro enzyme from S22 subsequently was further characterized.

Inducibility and Cellular Localization of the Aro—In contrast to A. faecalis (22), H. arsenicoxydans (36), and Rhizobium NT-26 (NT-26) (27), S22 shows a basic, although weak, activity when grown in the absence of As\textsuperscript{III}, as already observed for Thiomonas 3As (3As) (24), but features a 20× enhanced activity when grown with 5 mM As\textsuperscript{III} (data not shown).

Because conflicting results have been published previously concerning the localization of the Aro enzyme, we addressed this question for the S22 enzyme after treatment of the bacteria with French press or lysozyme. As found previously for A. faecalis (22), H. arsenicoxydans (17), Chloroflexus aurantiacus (23), and 3As (24), the Aro of S22 is membrane-associated as evidenced by the detection of the major part of the activity in the fraction of spheroplasts on native gel (Fig. 1). However, as already observed for A. faecalis (22), the enzyme could be released to the soluble fraction by changing the cell rupture method (Fig. 1). As previously published (20), the Aro from NT-26 is dominantly retrieved from the soluble fraction, even after lysozyme treatment (data not shown), whereas the Aro from H. arsenicoxydans, even after French press treatment, remained up to 65% membrane-associated.\textsuperscript{6} The “localization” of the enzyme, therefore, seems to depend not only on the breaking conditions but also on the specific organism.

Purification and Preliminary Characterization of Aro—As almost all the Aro from S22 is found in the soluble fraction after French press treatment, this fraction was used for purification. As first noticed for A. faecalis (22), Aro is particularly thermostable in all organisms tested so far (39). The enzyme from S22

\textsuperscript{6} S. Duval and B. Schoepp-Cothenet, unpublished data.
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both able to react with isolated Aro (Fig. 3) and were therefore further characterized.

The N-terminal sequence (54 residues) of cyt\(_{552}\) (Fig. 4A) revealed a high identity (75%) with cyt552 from Cupriavidus necator (formerly Ralstonia eutrophica) H16, C. taiwanensis and Cupriavidus metallidurans (formerly Ralstonia metallidurans) CH34 (for which genome sequence are available) genomes are encoded by genes located in the sox cluster. The N-terminal sequence of cyt\(_{554}\) (Fig. 4B) revealed this protein to be the product of the aroC gene located in the S22 aro cluster. Its sequence is astonishingly similar to that translated from the gene present in the arsenite oxidase aox operon (10) of SY8 (99%) and shares only 45 and 43% identity with cyt\(_{552}\) homologs of Burkholderia multivorans and Burkholderia cenocepacia, respectively. Between each other, the two cyt\(_{552}\) and cyt\(_{554}\) from S22 are <40% identical.

The molecular masses, determined by MALDI-TOF mass spectrometry, of cyt\(_{552}\) and cyt\(_{554}\) are 9615 \(\pm\) 2 and 9648 \(\pm\) 2 Da, respectively. The calculated molecular masses of the cyts retrieved from the Cupriavidus genomes and from the S22 aro operon, however, are both higher, around 11,600 Da. Indeed, in both the cyt\(_{552}\) and cyt\(_{554}\) precursor sequences, a 20-amino acid stretch was predicted, using the SignalP program (40), to be a signal peptide characteristic of the Sec secretion pathway (see (41) for review). The prediction of the cleavage site was in perfect agreement with the determined N-terminal sequences VDA and APD (Fig. 4) for cyt\(_{552}\) and cyt\(_{554}\), respectively.

Both cyts have been purified in the monomeric state as judged from size exclusion chromatography (data not shown). The redox potentials \(E_m\) of cyt\(_{552}\) and cyt\(_{554}\) at pH 8 were determined at \(+230\pm 5\) mV and \(+250\pm 5\) mV, respectively (data not shown), close to the value determined for cyt\(_{552}\) in NT-26 (27).

The fact that the aroC gene in the aro operon of S22 codes for cyt554 strongly argued in favor of cyt554 rather than cyt552 being involved in As\(^{III}\) oxidation. However, both cyts were found in approximately equal amounts when purified from cells grown on 5 mM As\(^{III}\). We therefore analyzed which of the cyts was induced by the presence of As\(^{III}\). Because the cyt552 homologs in the sequenced Cupriavidus genomes are encoded by genes localized in the sulfur oxidation sox cluster, we also analyzed the cyt contents of cells grown with and without thiosulfate (known to be the regulator of the sox operon (42–45)). Cyt contents under these conditions were then compared with those obtained from cells grown with 5 mM As\(^{III}\). SDS-PAGE (Fig. 5A) detected an increased total amount of 10-kDa cyts in the cells grown either on thiosulfate or As\(^{III}\) but could not discriminate between cyt\(_{552}\) and cyt\(_{554}\) (Fig. 5A, lanes 5 and 6), due to their very similar molecular masses, as mentioned above. We therefore spectroscopically quantified each of the cyt con-
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Enzymatic Study of the AsIII Oxidation System—As detailed above, the enzyme from S22 was able to reduce cyts c552 and c554 from S22 (Fig. 3). Several further cyts were tested for their reactivity with Aro. Bovine heart cyt c or Rhodobacter sphaeroides cyt c2 were not reduced by the S22 enzyme (data not shown), whereas cyt c553 from A. aerolius and azurin from P. aeruginosa were (data not shown). Due to the limited quantities of purified c552 and c554, in-depth kinetic studies were performed using cyt c553 from A. aerolius and yielded a Km of 6 μM for AsIII (Table 3) as well as substrate inhibition at concentrations higher than 100 μM. We therefore analyzed the affinity for the reacting cyts at a concentration of 100 μM AsIII. Kinetic data, analyzed by reciprocal plots, yielded a Km value of 50 μM for cyt c553 (Table 3). With the aim to compare the efficiency of cyts and DCPIP as electron acceptors, we first measured kinetics at pH 6, i.e. the pH value determined by Anderson et al. (22) and ourselves (data not shown) to be optimal with this electron acceptor. The  \( V_{max} \) obtained using c553 at this pH value is an order of magnitude higher than the  \( V_{max} \) determined using DCPIP at the same pH. However, kinetics measured at other pH values yielded the range of pH 8–9 as optimal for electron transfer from Aro to the cyts and led to a further doubling of  \( V_{max} \). Preliminary kinetic studies with cyts c552 and c554 purified from S22 yielded Km values of 13 μM and 7 μM, respectively, at a pH of 8.5.

Because no data have been reported to identify potential inhibitors of Aro, we screened the effect of selected chemicals on the Aro activity. We first tested the product of the physiological reaction, AsV, as potential inhibitor and found no effect. We observed any inhibitory effect by Na3AsO3, a well-known inhibitor of several molybdopterin enzymes (46, 47). Using cyts as electron acceptors allowed us to test sulfur compounds that otherwise would reduce DCPIP directly. Because H2S has been reported to strongly inhibit AsIII oxidation of Hydrogenobaculum whole cells (48), we tested this compound on Aro and indeed found an inhibitory effect on our enzyme. We did not perform a detailed enzymatic analysis in the presence of sulfide but observed an  \( I_{50} \) (sulfide concentration yielding 50% inhibition) of ~70 μM. A further sulfur compound, i.e. sulfite (but not thiosulfate), appeared to strongly inhibit Aro. A more detailed enzymatic analysis in the presence of sulfite showed a “mixed mode of inhibition” (data not shown), i.e. with not only an effect on affinity (Km) but also on catalysis (Km/ \( V_{max} \)), with an I50 of 10 μM. Precise kinetics parameters were obtained with the A. aerolius cyt but were always verifed using the S22 cyts.

As cyt c552 potentially participates in thiosulfate oxidation, we assayed whether the Aro featured measurable thiosulfate or sulfite oxidase activity. This was not the case (data not shown).

DISCUSSION

As mentioned in the introduction, many different aspects of the Aro enzymes have been studied in a variety of species, although an exhaustive characterization of a single case is still lacking. We took advantage of the availability of a new species of AsIII oxidizer, the β-proteobacterium S22, to perform a comprehensive study of its Aro covering its phylogenetic positioning, expression properties, biochemical and biophysical (see Ref. 66, accompanying article), as well as enzymatic parameters and its interaction with potential redox partners.

Aro from S22, a Clear Cut Case of Lateral Gene Transfer—Previous phylogenetic studies on the molybdopterin subunit of Aro (23, 49) suggested this enzyme to have evolved with its parent species. These conclusions have later been confirmed by the study of its Rieske subunit (50). Analysis of binary similarity data showed the 16S rDNA sequence of S22 to be more closely related to H. arsenicoxydans (91% identity) and B. multivorans (92%) species than to SY8 (86%) and A. faecalis (89%) species. As described above, however, both the sequences of the molyb-
The evolutionary relationship is further corroborated by the transfer in the evolutionary pathway of Aro. It is noteworthy, does not allow for such a possibility for S22. This case, there-
high homology between the SY8 and S22 sequences, however, with the phylogenetic tree of the parent species. In some of
2 in Ref. 49) pinpoints several abnormal branches as compared
detailed examination of the AroA-based phylogenetic tree (Fig.
dopterin and the Rieske subunits of Aro from S22 cluster with
FIGURE 6. Prediction of twin arginine translocation signal cleavage site in the N-terminal sequences of the Rieske subunit from Aros and Rieske-cyt b complex. Prediction has been performed using the method developed by Bendtsen et al. (51). The first residue after the cleavage site is colored in gray, whereas the observed first residue in the purified enzyme is boxed.
dredient to anchor Aro to the membrane. Cleavage sites were predicted
this signal peptide is cleaved after the protein has been trans-
(18 and Fig. 6). This observation raises the question of whether
signal has now been detected in all available AroB sequences
H. arsenicoxydans
ent in the soluble fraction even after mild treatment (18, 21).
Armobacter
Hydrogenophaga
C. aurantiacus
Achro-
coxydans, A. faecalis, Arthrobacter, and S22 cases, for which the relative abundance in the membrane and the soluble fractions varies as a function of the harshness of cell disruption (5, 17, 22 and present work).
A scenario reconciling all results obtained so far, already pro-
posed by Santini and vanden Hoven (18), consists in the attachment of AroAB to the membrane via another protein. This attachment must be of variable strength to explain the ensemble of the data and structurally specific as the Rieske protein has been observed to be oriented in a defined geometry (23) on Chloroflexus membranes. This question is reminiscent of the problem arising from the study of the AsV reductase enzyme (49). Some of the representatives of this family have been isolated as membrane-associated and others as soluble. However, in all characterized AsV reductase enzymes, a membrane-associated component, which can be variable proteins, has been identified (53–55). We can imagine a similar scenario for the Aro enzyme, with the corresponding membrane-attached partner still to be identified. To address this question, we are currently studying the case of the H. arsenicoxydans Aro, as this enzyme can indeed be easily obtained in a membrane-associated form (see “Results”).
Aro Displays Strong Selectivity toward Its Electron Transfer Partners—Whereas the Aro enzyme may be more or less tightly membrane-associated in different species, it invariably appears to reduce soluble periplasmic electron carrier proteins. To the exception of one previous study (27), nonphysiological electron acceptors have been employed in activity tests, and the physi-
logical electron acceptors have been deduced merely from genetic arguments. To place conclusions concerning the interaction of Aro with its redox partners on firm ground, we have studied coexpression profiles and electron transfer activities of soluble cys interacting with Aro in S22. The obtained results strongly suggest the physiological electron carrier of the S22 Aro to be cyt c554, i.e., the cyt present in the aro cluster. Spectroscopic quantification clearly established a strong increase of the content of cyt when cells were grown on AsIII. This result suggests that c554 gene expression is induced by AsIII. It is of note that only four species were shown to have a cyt gene co-
transcribed with the aroA and aroB genes and hence induced by AsIII: Agrobacterium tumefaciens, H. arsenicoxydans, 3As, and O. triflaci (26, 28, 56, 57).
As we have shown, cyt c554 is not the only cyt reacting with Aro in S22. Another cyt, c552, dominant in the absence of AsIII but still as abundant as c554 in the presence of AsIII, accepts electrons from Aro and therefore mediates high turnover of the enzyme. Although no equivalent study has been performed on other bacteria, this result correlates well with circumstantial observations made in other organisms. For example, the Δc552 mutant of NT-26 is still capable of autotrophic growth on AsIII (27), suggesting that at least one other carrier may accept electrons from Aro. Finally, the recent sequencing and character-
ization of the aro operon in 3As identified two cys as being co-transcribed with aroAB (57). The Aro enzyme therefore appears to often be able to interact with several different electron carriers in the same organism and the production of these carriers can either be regulated by AsIII or not. In stark contrast to this stands the clear cut and strong discrimination against a
specific subgroup of type I cyt as exemplified by horse heart or R. sphaeroides cyts. This selectivity is striking and not related to the redox potential of the carrier because all of the above cited carriers have $E_{m, pH \approx 8}$ values $\approx -240$ mV. We therefore have initiated an in-depth study of this phenomenon in a range of different Aro enzymes. The corresponding results and a structural rationalization for this selectivity, observed in all examined Aros, will be published elsewhere.$^6$

Inhibitory Effects of Sulfur Compounds and Metabolic Significance Thereof—Arsenic and sulfur often coexist in the environment and share similar microbial transformations. The study of the effect of sulfur compounds on As$^{III}$ oxidation is therefore indispensable to understand how both metabolisms interact. Our work revealed inhibitory effects of both sulfite and sulfide, with apparent $I_{50}$ of 70 $\mu$M and 10 $\mu$M, respectively. Are these compounds true enzymatic inhibitors? A concentration of 60 $\mu$M sulfide has been observed to completely stop As$^{III}$ oxidation in whole cells of Hydrogenobaculum at low pH (48), whereas sulfide has been reported to strongly enhance the As$^{III}$ oxidation in Mono Lake samples at high pH (58). However, did sulfide act on the Aro directly in these cases? Sulfide has been shown to react with As$^{III}$, forming orpiment at low pH (59) and thioarsenic species at high pH (58, 60). In these three cited works, sulfide was added in equal if not higher quantities compared with As$^{III}$, allowing the product of the reaction between As$^{III}$ and sulfide to significantly modify the As$^{III}$ quantity available for Aro-mediated oxidation. The observed effects are therefore potentially nonenzymatic. In our case, sulfide shows significant inhibitory effect at concentrations where, whatever the sulfide/As$^{III}$ reaction product, free As$^{III}$ is always saturating. Our work is therefore the first to clearly establish a true inhibitory effect of sulfide on the enzymatic As$^{III}$ oxidation.

Sulfite is another sulfur compound revealed by our work to be a strong inhibitor of the Aro enzyme from S22. A conflicting result was published by Phillips (61) on A. faecalis whole cells, but we observed a similar inhibition also on the NT-26 enzyme. In both cases, the inhibition appears to be of a mixed-type inhibitory effect of sulfide on the enzymatic As$^{III}$ oxidation.

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