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Arsenite Oxidase from *Ralstonia* sp. 22

CHARACTERIZATION OF THE ENZYME AND ITS INTERACTION WITH SOLUBLE CYTOCHROMES[S]

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We characterized the *aro* arsenite 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 c_{554}. 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, c_{552} and c_{554}, are from the parent species. Cytochrome c_{552} belongs to the *sox* and not the *aro* system. Kinetic studies furthermore established that sulfite and sulfide, 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 bioleaching of arsenic minerals (see Oremland and Stolz, Ref. 2), high amounts of soluble forms can be accumulated. These forms, arsenate (As^V)[5] and arsenite (As^{III}) are both toxic to complex life. As^V, a phosphate analog, interferes with normal phosphorylation processes by replacing phosphate, whereas As^{III} binds to sulfhydryl groups of cysteine residues in proteins, thereby inactivating them. As^{III} is considered to be 100× more toxic than As^V. As^{III} can be oxidized to As^V either chemically or microbially (3). Since the first report of bacterial As^{III} oxidation by Green (4) in 1918, an exponential number (see Refs. 5–12) of phylogenetically diverse As^{III}-oxidizing bacteria have been isolated from different environments. These bacteria can be divided into two groups: (i) chemolithoautotrophs (aerobes or anaerobes, using As^{III} as the electron donor and CO_2/HCO_3^- 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 admittedly 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 consid-

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) EU304284, EU304273, and GQ904715.

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[5] The abbreviations used are: As^V, arsenate; Aro, arsenite oxidase; 3As, *Thiomonas* sp. 3As; AMPSO, 3-((1,1-dimethyl-2-hydroxyethyl)amino)-2-hydroxypropanesulfonic acid; As^{III}, arsenite; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; CDM, chemically defined medium; cyt, cytochrome; DCPIP, 2,4-dichlorophenolindophenol; FPLC, fast protein liquid chromatography; HPLC-ICP AES, high performance liquid chromatography-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^{III} is a two-electron donating substrate, the catalytic turnover is assumed to start with the oxidation of As^{III} by the molybdenum center (which can accept up to two electrons). Several facts indicate that the catalytic cycle of As^{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^{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^{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^{-1} 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^{III} ($NaAsO_2$) or 20 mM thiosulfate ($Na_2O_3S_2$,$5H_2O$) 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 Sephacel 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^{-1} 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^{-1} 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 Sephacel 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. The c_{552} eluted from the CM column at 25 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^{-1} 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^{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 ($\epsilon_{600\ pH\ 6}$ experimentally determined at 12 mM^{-1} cm^{-1}). The reaction was initiated by addition of As^{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_{555} purified from *Aquifex aeolicus* as described in Ref. 30, or cyts 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 NaN_3, sulfite, sulfide, thiosulfate, and As^{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 As^{III} 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 cyts were estimated by gel filtration in buffer A/100 mM NaCl on Superdex 200 or 75, respectively, using apoferrin, amylase, 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′-AAGGAGGTGATCCAGCCGCA-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) (GenBank™ accession no. EF523515), oligonucleotides were designed that amplify a DNA stretch of 3508 bp using PCR, covering the *aroA*, *aroB*, and *aroC* (cyt c_{554}) genes of S22, in three overlapping fragments. For fragment 1 (1190 bp), forward primer 5′-CGTCCGAAAGCTACTTGG-3′ and reverse primer 5′-GCAGTGAACATCAGCTTCC-3′ were used; for fragment 2 (1246 bp), forward primer 5′-TTCTCCTGTTTCGACCACG-3′ was used; for reverse primer, 5′- CCTTTAGCGTGTTGGCG-3′ was used; and for fragment 3 (1241 bp), forward primer 5′-ACGCATCCGCCTATCTC-3′ and reverse primer 5′-CATTAAGCGCGAACCCG-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 GenBank™ 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 As^{III} oxidation activity from bacteria was followed by visualizing the As^{III} 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 As^{III} and As^{V} 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 $[M + 2H]^{2+}/2$ 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 Carry 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 As^{III} (1.33 mM). A pure culture was obtained by successive isolation of colonies at 25 ± 2 °C on As^{III}-supplemented CDM. Isolate S22 showed As^{III} oxidase activity when grown on CDM supplemented with 1.33 mM As^{III}. HPLC-ICP AES experiments demonstrated the progressive disappearance of As^{III} in parallel to the appearance of As^{V} in the supernatant of the strain S22 culture (data not shown). These findings suggested the oxidation of As^{III} 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

TABLE 1

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

	H. arsenicoxydans	*Ralstonia* sp. 22
MIC As^{III}	6 mM	30 mM
MIC As^{V}	200 mM	200 mM

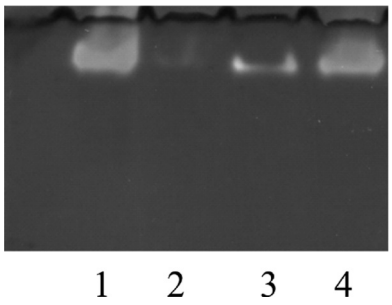

FIGURE 1. **Polyacrylamide gel electrophoresis and in-gel Aro activity staining of subcellular fractions of S22.** *Lane 1*, spheroplasts; *lane 2*, periplasm; *lane 3*, membrane fraction after French press treatment; *lane 4*, soluble fraction after French press treatment.

after 3 days at 30 °C, was determined for As^{III} and As^{V}. Interestingly, S22 showed resistance up to 30 mM As^{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^{III}, as already observed for *Thiomonas* 3As (3As) (24), but features a 20× enhanced activity when grown with 5 mM As^{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.[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 retained 95% activity after heating to 60 °C for 5 min (data not shown). This property allowed us to routinely measure Aro activity at 37 °C with a better signal to noise ratio (see "Experimental Procedures").

[6] S. Duval and B. Schoepp-Cothenet, unpublished data.

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
	mg	*μm DCPIP/min*	*μmol/min/mg*	*-fold*	*%*
Cell extract	266.6	10.5	0.0392	1	100
DEAE-Sephacel	11.9	9.6	0.802	20.5	91
MonoQ	1.368	5.7	4.189	106.9	55
Gel filtration	0.684	3.9	5.7	145.4	38

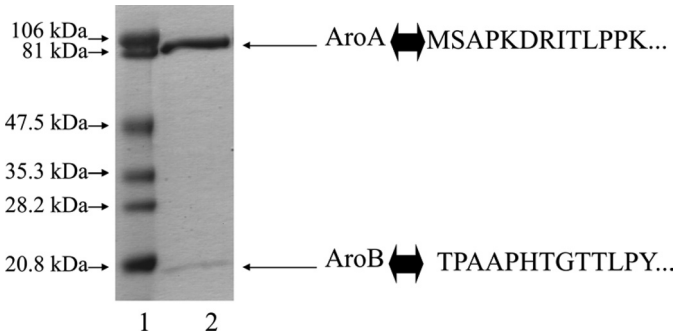

FIGURE 2. **Coomassie Blue-stained SDS-PAGE on purified Aro from S22.** *Lane 1*, molecular weight standards; *lane 2*, purified enzyme. Identification of subunits was confirmed by N-terminal sequencing.

Two anion-exchange chromatographic steps and one gel filtration chromatography step were applied to achieve the purification of the enzyme. This protocol led to a 145-fold final enrichment of the enzyme with a yield of 38% (Table 2). As shown in Fig. 2, the obtained pure enzyme consists of two subunits with apparent molecular masses of 97 kDa and 16 kDa. They correspond to AroA and AroB, respectively, as confirmed by N-terminal sequencing (Fig. 2). The native molecular mass of the isolated enzyme, based on gel filtration chromatography, was found to be 110 kDa (data not shown) suggesting a $\alpha_1\beta_1$ configuration, similar to what was reported for the *A. faecalis* enzyme (22).

The complete DNA sequence of the *aroA* and *aroB* genes has been obtained. The deduced amino acid sequence of AroA displays 99, 81, 72, and 71% identity with AroA from SY8, *A. faecalis*, *H. arsenicoxydans*, and *Burkholderia multivorans*, respectively (supplemental Fig. S1). The deduced amino acid sequence of AroB displays 99, 69, 57, and 54% identity with SY8, *A. faecalis*, *B. multivorans*, and *H. arsenicoxydans* AroB, respectively (supplemental Fig. S2).

Purification and Characterization of Two Cyts Able to React with Aro—During the first DEAE chromatographic step, we detected the presence of *c*-type cyts in the unretained fraction. Because *in vitro* enzymatic experiments revealed that purified Aro was able to reduce this fraction in the presence of As^{III}, we decided to purify its constituent heme proteins. We isolated three cyts using a CM-52 cation exchange column and a gel filtration column successively and named them cyts c_{551}, c_{552}, and c_{554} according to their respective α-bands in the reduced state. In contrast to cyt c_{551}, cytochromes c_{552} and c_{554} were

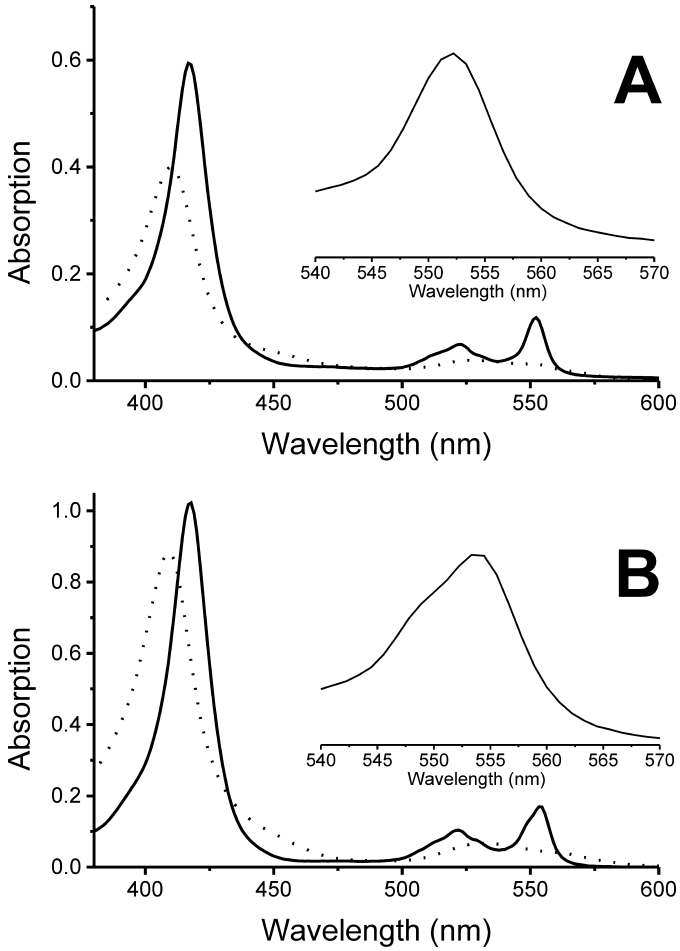

FIGURE 3. **Spectra of c_{552} and c_{554} cytochromes enzymatically reduced by purified Aro.** Oxidized cyts (*black lines*) were incubated for 3 min with the enzyme (*dotted lines*); c_{552} (*A*) and c_{554} (*B*). *Insets* show the α-bands of cytochrome c_{552} and c_{554} absorbing at 552 and 554 nm, respectively.

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A cyt_Cnec   MKQFVIAALMLGAAASAHAVDAAKAQEIANKNACMGCHQV 40
  cyt_Ctai   MKQFVIAALMLGAAASAQAVDAAKAQEIANKNACMGCHQV 40
  cyt_Cmet   MKQFVIAAALLAAAASAHAVDAAKAQEIANKNACMGCHQV 40
  c552_S22   -------------------VDVAKAQQIAQKLACMGCHAV 21
                               **.****:**:* ****** *
  cyt_Cnec   DKKLVGPSYKEVAAKYKGDKNALATLTKKVKSGGSGVWGP 80
  cyt_Ctai   DKKLVGPSYKEVAAKYKGDKNALATLSKKVKSGGSGVWGP 80
  cyt_Cmet   DKKLVGPAYKDVAAKYKGDKTALAKLTAKVKNGGSGVWGP 80
  c552_S22   DKKLVGPSYQDVAAKYKGNKGAAATLVAKVKAG------- 54
             *******:*::*******:* * *.*  *** *
  cyt_Cnec   VPMPANAALNDADLKTVVEWVLAGAPAK 108
  cyt_Ctai   VPMPANAALSDADLKTVVEWVLAGAPAK 108
  cyt_Cmet   VPMPANAAVSDADLKTVVEWVLAGAPAK 108
  c552_S22   ----------------------------

B cyt_Bmul   MSAAPMKSFRQVARRVVQGAVAASLVGAWAASAHAQAGDG 40
  cyt_Bcen   MTGMQMK------RWMVQGAAAAALVAGTLGAAHADVGDG 34
  c554_S22   ---------------------------APDAAVNA--- 8
  cyt_SY8    MRTIKLL--------VSLGLASTSLACLAAPDAAVNA--- 29
                                              * .:.
  cyt_Bmul   LRVARSNACLGCHAVDRKLVGPSFKDIAARYKGDPQAVAK 80
  cyt_Bcen   LKVARSNACMGCHAVDRKLVGPSFKDIAARYKSDPQAVAK 74
  c554_S22   --ILSKNKCLACHAVDRKVVGPSYKDVGAKFKDDPNAVTL 46
  cyt_SY8    --ILSKNNCLACHAVDRKVVGPSYKDVGAKFKDDPNAVTL 67
               :  .* *:.*******:****:**:.*::*.**:**:
  cyt_Bmul   LSKKVKDGGSGVWGAIPMPAHPRMSDADLRSVVEWVLAGA 12
  cyt_Bcen   LSKKVKDGGSGVWGAIPMPAHPRMSDADVRSVVEWVLAGA 11
  c554_S22   LVGKIKNGSAT---------------------------- 57
  cyt_SY8    LVGKIKNGSAGTWGPVPMPPNPGISPDEAKQVVNWILSGA 10
             *  *:*:*.:
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FIGURE 4. **Sequence alignment of cytochromes purified from S22.** The N-terminal sequence of purified cyt c_{552} (c552_S22; *A*) is compared with the sequences of cyts from *Cupriavidus necator* H16 (cyt_Cnec; GenBank™ accession no. YP_727996), *C. taiwanensis* (cyt_Ctai; GenBank™ accession no. YP_002007005) and *C. metallidurans* CH34 (cyt_Cmet; GenBank™ accession no. YP_585565), whereas the N-terminal sequence of c_{554} (c554_S22; *B*) is compared with the sequences of cyts from *Achromobacter* sp. SY8 (cyt_SY8; GenBank™ accession no. ABP63661), *Burkholderia multivorans* (cyt_Bmul; GenBank™ accession no. YP_001578392) and *B. cenocepacia* (cyt_Bcen; GenBank™ accession no. YP_002229329).

both able to react with isolated Aro (Fig. 3) and were therefore further characterized.

The N-terminal sequence (54 residues) of cyt c_{552} (Fig. 4*A*) revealed a high identity (75%) with cyts of *Cupriavidus necator* (formerly *Ralstonia eutropha*) H16, *Cupriavidus taiwanensis* and *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans*) CH34 (for which genome sequence are available) encoded by genes located in the *sox* cluster. The N-terminal sequence (56 residues) of cyt c_{554} (Fig. 4*B*) 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 cyt gene present in the arsenite oxidase *aox* operon (10) of SY8 (99%) and shares only 45 and 43% identity with cyts $c_{551/552}$ of *Burkholderia multivorans* and *Burkholderia cenocepacia*, respectively. Between each other, the two cyts c_{554} and c_{552} from S22 are <40% identical.

The molecular masses, determined by MALDI-TOF mass spectrometry, of cyts c_{552} and c_{554} are 9615 ± 2 and 9648 ± 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 c_{552} and c_{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 c_{552} and c_{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 cyts c_{552} and c_{554} at pH 8 were determined at +230 ± 5 mV and +250 ± 5 mV, respectively (data not shown), close to the value determined for cyt c_{552} in NT-26 (27).

The fact that the *aroC* gene in the *aro* operon of S22 codes for cyt c_{554} strongly argued in favor of c_{554}, rather than c_{552}, 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 cyt c_{552} 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. 5*A*) 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 c_{552} and c_{554} (Fig. 5*A*, *lanes 5* and *6*), due to their very similar molecular masses, as mentioned above. We therefore spectroscopically quantified each of the cyts con-

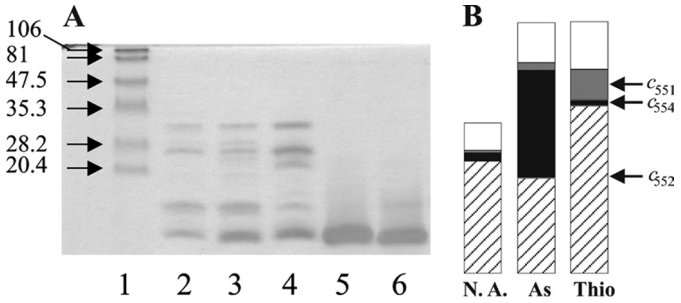

FIGURE 5. *A*, 3,3′,5,5′-tetramethyl-benzidine- stained SDS-PAGE of cytochromes present in the S22 periplasm under different growing conditions. *Lane 1*, molecular weight standards; *lane 2*, cyts expressed without any addition; *lane 3*, cyts expressed in the presence of 5 mM As^{III}; *lane 4*, cyts expressed in the presence of 20 mM thiosulfate; *lane 5*, purified cyt c_{554}; *lane 6*, purified cyt c_{552}. *B*, schematic representation of cyt content of the DEAE-cyt fraction under different growing conditions. Lane *N.A.*, cyt content without any addition; lane *As*, cyt content with 5 mM As^{III}; lane *Thio*, cyt content with 20 mM thiosulfate. Note that the DEAE-cyt fraction represent only part of the total cyt fraction (see "Experimental Procedures") and that ~20% of cyt content in this DEAE-cyt fraction remains unidentified under each of the growth conditions.

tained in the DEAE-cyt fraction, under the tested growth conditions (Fig. 5*B*). In the absence of As^{III} or thiosulfate, cyt c_{552} represents 75% of the DEAE-cyt fraction content (again spectroscopically quantified), whereas cyt c_{554} amounts to ~5% and c_{554} accounts for <2%. In the presence of 5 mM As^{III}, the c_{552} fraction fell to 39% of the DEAE-cyt fraction content, whereas the c_{554} complement increased to at least 43%. The relative increase of cyt c_{554} quantity in response to As^{III} therefore approximately parallels that observed for Aro activity (see above). In the presence of 20 mM thiosulfate, the contribution of c_{552} fell to 67% in relative cyt content but increased in absolute quantity as compared with the control conditions (Fig. 5*A*, *lane 4 versus lane 2*, and Fig. 5*B*, *Thio versus N.A.*). The proportion of c_{552} decreased due to the increase of c_{551} from 2 to 12%. Altogether, these results established not only that the accumulation of c_{551} is linked to the As^{III} oxidation process but also suggested that production of c_{552} and c_{551} is linked to the thiosulfate oxidation pathway.

Enzymatic Study of the As^{III} Oxidation System—As detailed above, the enzyme from S22 was able to reduce cyts c_{552} and c_{554} from S22 (Fig. 3). Several further cyts were tested for their reactivity with Aro. Bovine heart cyt *c* or *Rhodobacter sphaeroides* cyt c_2 were not reduced by the S22 enzyme (data not shown), whereas cyt c_{555} from *A. aeolicus* and azurin from *P. aeruginosa* were (data not shown). Due to the limited quantities of purified c_{552} and c_{554}, in-depth kinetic studies were performed using cyt c_{555} from *A. aeolicus* and yielded a K_m of 6 μM for As^{III} (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 As^{III}. Kinetic data, analyzed by reciprocal plots, yielded a K_m value of 50 μM for cyt c_{555} (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_m obtained using c_{555} at this pH value is an order of magnitude higher than the V_m 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

TABLE 3
Kinetic properties of the Aro from S22

*V_{max} is expressed as μM $As_{oxidized}$ × min^{-1} mg^{-1}. It is noteworthy that 1 mole of As^{III} reduces 1 mole of DCPIP but 2 moles of cyt. N.D., not determined.

e^- donor	As^{III}			
K_m (μM)	6			
Inhibitors	Sulfite		Sulfide	
I_{50} (μM)	10		70	
e^- acceptor	c_{555}	c_{552}	c_{554}	DCPIP
K_m (μM)	50	13	7	N.D.
V_{max}*	152	140	140	5.7

transfer from Aro to the cyts and led to a further doubling of V_m. Preliminary kinetic studies with cyts c_{552} and c_{554} purified from S22 yielded K_m 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, As^V, as potential inhibitor and found no effect. We observed any inhibitory effect by NaN_3, a well known inhibitor of several other molybdopterin enzymes (46, 47). Using cyts as electron acceptors allowed us to test sulfur compounds that otherwise would reduce DCPIP directly. Because H_2S has been reported to strongly inhibit As^{III} 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 (K_m) but also on catalysis (K_m/V_m), with an I_{50} of 10 μM. Precise kinetics parameters were obtained with the *A. aeolicus* cyt but were always verified using the S22 cyts.

As cyt c_{552} 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 As^{III} 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-

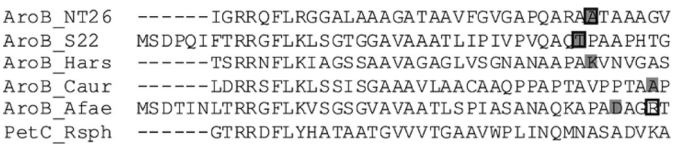

FIGURE 6. **Prediction of twin arginine translocation signal cleavage site in the N-terminal sequences of the Rieske subunit from Aros and Rieske-cyt *b* complex.** Prediction has been performed using the method developed by Bendtsen *et al.* (51). The first residue after the cleavage site is colored in *gray*, whereas the observed first residue in the purified enzyme is *boxed*.

dopterin and the Rieske subunits of Aro from S22 cluster with those of SY8 and *A. faecalis* enzymes instead of those from *B. multivorans* and *H. arsenicoxydans* species (see "Results"). This evolutionary relationship is further corroborated by the sequence that we determined for cytochrome c_{554}. The deduced identity of the AroA, AroB, and AroC sequences from S22 with the AoxB, AoxA, and AoxC sequences from SY8 were so suspiciously high (99%) that we were led to verify by 16S rDNA sequencing that our S22 strain was pure even when grown in 5 mM As^{III}. These results therefore suggest that the entire *aro* operon of S22 has been acquired from an *Achromobacter*-related species by horizontal gene transfer. A detailed examination of the AroA-based phylogenetic tree (Fig. 2 in Ref. 49) pinpoints several abnormal branches as compared with the phylogenetic tree of the parent species. In some of these cases, discrepancies may be due to misalignments. The high homology between the SY8 and S22 sequences, however, does not allow for such a possibility for S22. This case, therefore, constitutes the first unambiguous example of lateral gene transfer in the evolutionary pathway of Aro. It is noteworthy, though, that this lateral gene transfer occurred within the β-proteobacterial subclass and thus does not significantly blur the picture of an overall coevolution of the enzyme and its parent species.

Is Aro a Membrane-bound Enzyme?—Muller *et al.* (17) observed a twin arginine translocation signal peptide in the *H. arsenicoxydans* AroB protein, and the presence of such a signal has now been detected in all available AroB sequences (18 and Fig. 6). This observation raises the question of whether this signal peptide is cleaved after the protein has been transported across the cytoplasmic membrane or whether it serves to anchor Aro to the membrane. Cleavage sites were predicted (via the method developed by Bendtsen *et al.* (51)) for all Aro-Rieske proteins but not for the Rieske subunit of Rieske-cyt *b* complexes (a homologous protein, see Ref. 66, accompanying article). The latter result is in line with the following. i) All Rieske/cyt *b* Rieskes characterized so far have been demonstrated to be membrane bound with their uncleaved twin arginine translocation signal peptide serving as an anchor (52). ii) The predicted cleavage sites in Aro-Rieskes correspond to the N terminus determined in the isolated enzymes. Nevertheless, three types of Aro can be found. The *C. aurantiacus* and 3As enzymes correspond to the "rather membranous type" *i.e.* retained in the membrane fraction even after harsh treatment (23, 24). The NT-26 and *Hydrogenophaga* NT-14 (NT-14) enzymes belong to the class of "rather soluble type" Aros present in the soluble fraction even after mild treatment (18, 21). Intermediate between these extreme cases are the *H. arsenicoxydans*, *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 proposed 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 As^V reductase enzyme (49). Some of the representatives of this family have been isolated as membrane-associated and others as soluble. However, in all characterized As^V 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 physiological 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 cyts interacting with Aro in S22. The obtained results strongly suggest the physiological electron carrier of the S22 Aro to be cyt c_{554}, *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 As^{III}. This result suggests that c_{554} gene expression is induced by As^{III}. 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 As^{III}: *Agrobacterium tumefaciens, H. arsenicoxydans*, 3As, and *O. tritici* (26, 28, 56, 57).

As we have shown, cyt c_{554} is not the only cyt reacting with Aro in S22. Another cyt, c_{552}, dominant in the absence of As^{III} but still as abundant as c_{554} in the presence of As^{III}, 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 Δc_{552} mutant of NT-26 is still capable of autotrophic growth on As^{III} (27), suggesting that at least one other carrier may accept electrons from Aro. Finally, the recent sequencing and characterization of the *aro* operon in 3As identified two cyts 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 As^{III} 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 $E_{m\,pH\,8}$ values ~+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 sulfide and sulfite, with apparent I_{50} of 70 μM and 10 μM, respectively. Are these compounds true enzymatic inhibitors? A concentration of 60 μM 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 inhibition, *i.e.* both competitive and noncompetitive. The competitive character, *i.e.* the effect on the K_m for As^{III} suggests that sulfite reacts at the molybdopterin center. Because dialysis restores the activity, the binding of sulfite seems to be transient. This effect appears, therefore, to be distinct from the irreversible effect described for sulfite oxidase (a molybdopterin enzyme) in presence of As^{III} (62).

An increasing number of studies find relationships between sulfur and arsenic metabolism. Interest has been focused on the interaction between As^{V} and sulfate reduction (see Refs. 63, 64 for original works). More recently, the interaction between As^{III} oxidation and sulfur metabolism was addressed (48, 58, 65), but it has not been studied at the molecular level prior to this work. Although ability of Aro to use cyt c_{552} as electron acceptor may be interpreted as a possible cross-talk between sulfur and arsenic metabolisms, this merging of electron transfer pathways is not obligatory because Aro possesses a second, dedicated, acceptor, *i.e.* cyt c_{554}. The more straightforward link between As^{III} and sulfur oxidation processes revealed by our work consists in the sensitivity of Aro to two substrates of the *sox* system, *i.e.* sulfite and sulfide. The *sox* system, well characterized in *Paracoccus pantotrophus* and coded by 15 genes, catalyzes thiosulfate-, sulfite-, sulfur-, and sulfide-dependent cyt *c* reduction (43, 44). We can therefore imagine that the *sox* system, will enhance the As^{III} oxidation rate of S22, by promoting the consumption of sulfite and sulfide.

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