Purification and Characterization of Acr2p, the *Saccharomyces cerevisiae* Arsenate Reductase*

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In *Saccharomyces cerevisiae*, expression of the *ACR2* and *ACR3* genes confers arsenical resistance. Acr2p is the first identified eukaryotic arsenate reductase. It reduces arsenate to arsenite, which is then extruded from cells by Acr3p. In this study, we demonstrate that *ACR2* complemented the arsenate-sensitive phenotype of an *arsC* deletion in *Escherichia coli*. *ACR2* was cloned into a bacterial expression vector and expressed in *E. coli* as a C-terminally histidine-tagged protein that was purified by sequential metal chelate affinity and gel filtration chromatography. Acr2p purified as a homodimer of 34 kDa. The purified protein was shown to catalyze the reduction of arsenate to arsenite. Enzymatic activity as a function of arsenate concentration exhibited an apparent positive cooperativity with an apparent Hill coefficient of 2.7. Activity required GSH and glutaredoxin as the source of reducing equivalents. Thioredoxin was unable to support arsenate reduction. However, glutaredoxins from both *S. cerevisiae* and *E. coli* were able to serve as reductants. Analysis of *grx* mutants lacking one or both cysteine residues in the Cys-Pro-Tyr-Cys active site demonstrated that only the N-terminal cysteine residue is essential for arsenate reductase activity. This suggests that during the catalytic cycle, Acr2p forms a mixed disulfide with GSH before being reduced by glutaredoxin to regenerate the active Acr2p reductase.

All organisms are constantly exposed to geochemical and anthropomorphic arsenic (1). Arsenic is a human carcinogen (2) and is frequently present in high concentrations in drinking water (3). Despite the health hazards of arsenic, no specific human arsenic detoxification genes have been identified. Recently, a gene cluster, *ACR1*, *ACR2*, and *ACR3*, on *Saccharomyces cerevisiae* chromosome XVI, was shown to confer resistance to arsenate (*As(V)*) and arsenite (*As(III)*), the first such eukaryotic genes to be identified (4). Acr3p catalyzes extrusion of the arsenite from cells, thus conferring resistance (5). However, to confer resistance to arsenate, cells must first reduce it to arsenite. Although arsenite is nonenzymatically reduced by GSH, the process is too slow to be biologically significant (6), necessitating enzymatic mechanisms for reduction. Several bacterial arsenate reductases have been identified (7, 8), but until recently, there were no known eukaryotic arsenate reductases.

The *S. cerevisiae* *ACR2* gene was shown to be required for high level arsenate resistance (4), and disruption of *ACR2* resulted in arsenate sensitivity (9). The product of the *ACR2* gene, the 130-residue Acr2p, is a member of a family of proteins that are totally unrelated to any bacterial arsenate reductases. Two *S. cerevisiae* homologues of Acr2p are YGR203W, a 148-residue protein of unknown function (GenBank™ accession number S0003435), and YMR036C (GenBank™ accession number S0004639), a member of the Cdc25A family of protein phosphatases (10). These three proteins have the consensus sequence HCX3R, which corresponds to the phosphatase active site (11). This suggests that some commonality may exist in the enzymatic mechanism of an arsenate reductase and a phosphatase, both of which have oxyanionic substrates.

We have previously shown that *ACR2*-disrupted yeast cells are sensitive to arsenate but resistant to arsenite (9), the same phenotype an *arsC* deletion produces in *E. coli* (8). Native Acr2p produced in *E. coli* was found exclusively in inclusion bodies. In contrast, a maltose-binding protein-Acr2p chimera was soluble and exhibited a low level of arsenate reductase activity when supplemented with yeast cytosol. However, the activity was low, and the source of reducing equivalents unknown.

In this study, we demonstrate that the *S. cerevisiae ACR2* gene conferred arsenate resistance in an arsenate-sensitive strain *E. coli*. Conditions were established to isolate and purify a six-histidine-tagged Acr2p from *E. coli* cytosol. The enzyme was shown to have the mass of a homodimer. The source of reducing equivalents was identified as GSH and glutaredoxin (Grx). Acr2p exhibited arsenate reductase activity when the *S. cerevisiae* glutaredoxin Grx1p and GSH were supplied as electron donors. The *S. cerevisiae* thioredoxin (Trx) was unable to substitute for Grx. In addition, any of the three *E. coli* glutaredoxins supported Acr2p-catalyzed arsenate reduction. Glutaredoxins have the active site Cys-Pro-Tyr-Cys. The N-terminal cysteine is required for both protein disulfide reduction and reduction of mixed protein-glutathione disulfides (12). The other cysteine residue is required for the former activity but not for the latter. Mutation of the codon for the C-terminal cysteine of the *E. coli* glutaredoxin Grx2 had no effect on Acr2p activity. In contrast, a cysteine-to-serine substitution in the N-terminal residue rendered Grx2 incapable of serving as a reductant to Acr2p-catalyzed arsenate reduction. These results indicate that a mixed Acr2p-SG disulfide is formed during the
Yeast ArsC Family—Cloning and Characterization of a Bacterial ArsC Homolog

**Strains and plasmids**

| Strains/plasmid | Genotype | Ref. or source |
|-----------------|----------|---------------|
| **Bacterial strains**<br>JM109 | recA1 supE44 endA1 hisD171 gyrA96 relA1 thi Δlac-proAB F' [traD36 proA+ BALC1 Δlac Z15] | 13 |
| W3111 | K12 F’ Δ(in(rrnD-rrnE)) | 33 |
| W3110 | K12 F’ Δ(in(rrnD-rrnE)) ΔarsC | This study |
| TOP10 | F’ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 lacX74 deoR recA1 araD139 Δ(araA-leu1)7697 galU galK trpE endA1 supE44 | Invitrogen |
| **Yeast strains**<br>W303–1B | Mat-a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1 | 34 |
| RMI | Mat-a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1 Δ1CR2: HIS3 | 9 |
| **Plasmids**<br>PYES2.0 | Multicopy, shuttle vector, Ap’, URA3, gal1 | Invitrogen |
| pGEM-T | Multicopy E. coli cloning vector, Ap’ | Promega |
| pT28b | E. coli cloning and expression vector, Km’ | Novagen |
| pLD55 | E. coli replicative and conjugative plasmid, tetAR, Ap’ | 17 |
| pBAD/Myc-His A/C | E. coli cloning and expression vectors, Ap’ | This study |
| pBAD-ACR2 | pGEM-T-ACR2 was digested with NcoI and HindIII and inserted into the NcoI-HindIII site of pBAD/Myc-His A | This study |
| pET-ArsC | ArsC gene from E. coli was cloned into the NdeI-HindIII sites of PET28a | 8 |
| pYES-ArsC | pET-ArsC was digested with NdeI and NotI. The NdeI site was made blunt, and the resulting fragment was inserted into pYES2.0 with a blunt BamHI end and a cohesive NotI end | This study |
| pLD55-arsC | pLD55 with 900-bp SalI-BamHI insert containing 5’ and 3’ ends of ArsC with internal 400 bp deleted | This study |
| pBAD-YGRX1 | pGEM-T-YGRX1 was digested with NcoI and EcoRI and inserted into the NcoI-EcoRI sites of pBAD/Myc-His C | This study |
| pACR2–1 | 393-bp ACR2 gene was cloned by PCR from strain W303–1B into pET28b in frame with the C-terminal six-histidine tag | 9 |
| pACR2–2 | 496-bp ACR2–6His was cloned by PCR from pACR2–1 into pMAL-C2 in-frame with coding sequence for the N terminus of the mature form of the maltose-binding protein | 9 |
| pACR2–3 | 496-bp ACR2–6His was cloned from pACR2–2 by directional cloning | 9 |
| pGEM-YGR | 2.4-kilobase fragment containing YGR203W cloned into pGEM-T | This study |
| yEP352 | E. coli-S. cerevisiae shuttle vector, Ap’, URA3 | S. Ackerman |
| yEP-PYGR | YGR203W cloned as 1257-bp HindIII-SphI fragment inserted into HindIII-SphI-digested yEP352 | This study |
| pBAD-YTRX1 | pGEM-T-YTRX1 was digested with NcoI and HindIII and inserted into the NcoI and HindIII sites of pBAD-Myc-His A | This study |
| pBAD-YTRR1 | pGEM-T-YTRR1 was digested with NcoI and EcoRI and inserted into the NcoI and EcoRI sites of pBAD-Myc-His C | This study |
| pUC18-LEU2–8 | BglII fragment containing LEU2 of yEP13 was cloned into the BamHI site of pUC18 | S. Ackerman |

** plausible scenario** The resulting Trr1 protein from *S. cerevisiae* strain W303–1B genomic DNA was amplified by PCR to introduce a NcoI site at the 3’ end and an EcoRI site at the 3’ end. The forward primer was 5’-CCATGGTATCGCTGGAG-3’, and the reverse primer was 5’-GAATTCCATTGCAAAGATGGTTCTAAAC-3’. A 30-cycle PCR (94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute) was run with yeast genomic DNA. The 596-bp amplified product was cloned into pGEM-T. The resulting construct was digested with NcoI and HindIII and inserted into the NcoI-HindIII sites of pBAD/Myc-His A in frame with the C-terminal Myc epitope and a six-histidine-residue tag, creating plasmid pBAD-YTRR1.

The thioredoxin gene TRX1 from *S. cerevisiae* strain W303–1B genomic DNA was amplified by PCR to introduce a NcoI site at the 5’ end and an HindIII site at the 3’ end. The forward primer was 5’-CCAAGCTTGTTAATATGCGCACTTCTCAAAACTGC-3’, and the reverse primer was 5’-ACGCGTCTAATCTATTGAAATTCG-3’. A 30-cycle PCR (94 °C for 0.5 minute, 55 °C for 0.5 minute, and 72 °C for 1 minute) was run with yeast genomic DNA. The 318-bp amplified product was cloned into pGEM-T, and the resulting plasmid was digested with NcoI and HindIII and inserted into the NcoI and HindIII sites of pBAD/Myc-His A, in-frame with the C-terminal Myc epitope and a six-histidine-residue tag, creating plasmid pBAD-YTRX1.

The thioredoxin reductase (Trx) gene TRII from *S. cerevisiae* strain W303–1B genomic DNA was amplified by PCR to introduce a NcoI site at the 5’ end and an EcoRI site at the 3’ end. The forward primer was 5’-CCAGCTGGTACCAAAAAATGTT-3’, and the reverse primer was 5’-GAATTCATTGGAAGTAGTTAGTAC-3’. A 30-cycle PCR (94 °C for 1 minute, 55 °C for 0.5 minute, and 72 °C for 1 minute) was run with yeast genomic DNA. The 966-bp amplified product was cloned into pGEM-T, and the resulting plasmid was digested with NcoI and EcoRI and inserted into the NcoI-EcoRI sites of pBAD/Myc-His C, in frame with the C-terminal Myc epitope and a six-histidine-residue tag, creating plasmid pBAD-ACR2.

**catalytic cycle. This report provides the first characterization of the enzymatic activity of a eukaryotic arsenate reductase.**

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and media**—Strains and plasmids used in this study are described in Table I. Cells of *E. coli* were grown in a low phosphate medium (8) or Luria-Bertani medium (13) at the indicated temperatures supplemented with 10 µg/ml tetracycline or 50 or 125 µg/ml ampicillin, as appropriate. *S. cerevisiae* strains were grown at 30 °C in complete yeast extract-peptone-dextrose (14) medium supplemented with 2% glucose. Alternatively, the minimal (14) medium with 2% glucose or galactose supplemented with auxotrophic requirements was used.

**DNA Manipulations**—All nucleic acid modifying enzymes and restriction enzymes were obtained from Life Technologies, Inc. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described (13). Either Qiaprep Spin mini-prep kit or Qiaquick gel extraction kit (Qiagen) was used to prepare plasmid DNA for restriction enzyme digestion, sequencing, and recovery DNA fragments from low melting point agarose gels. The sequence of each polymerase chain reaction (PCR) product was confirmed by DNA sequencing of the entire gene. Sequencing was performed using a Pharmacia Biotech Cy5 labeled autosequence kit and an ALExpress apparatus by the method of Sanger et al. (15)."
were grown on plates containing 15 marsC and allowed to grow for an additional 24 h. The resulting plasmid was digested with HindIII, and the 4.9-kilobase pair fragment was isolated and transformed into yeast strains producing the YGR203W-disrupted strain. Verification of the YGR203W disruption was confirmed by PCR using a forward primer 5′-CTCTAGTGGCTGCTTCC-3′ that hybridizes with a region 614 bp upstream of YGR203W and a reverse primer 5′-AAGTTTACCCCAACGATCGGGTAG-3′ that hybridizes with the 3′ end of YGR203W.

Disruption of arsC in E. coli and cloning of arsC into a Yeast-E. coli Shuttle Vector—Disruption of the chromosomal arsC gene that confers arsenate resistance in E. coli was carried out by allelic replacement (17). Chromosomal DNA from E. coli strain W3110 was amplified as a 400-bp fragment by PCR using a forward primer, 5′-GTCGACCTGCGTACTAAACG-3′, that hybridizes with a region 386 bp upstream of the arsC gene and a reverse primer, 5′-GAGCCTTGAGTTCTGAGTATC-3′, that hybridizes with a region that includes the first 14 bp from the 3′ end of arsC. The primers added a SalI and a HindIII site at the 5′ and 3′ ends of the fragment, respectively. Using the same genomic DNA, a second PCR fragment of 400 bp was cloned using a forward primer, 5′-AAGGTTGCCGTAATAAACGGGGCTAC-3′, that hybridizes with a region that includes the last 12 bp from the 3′ end of arsC and a reverse primer, 5′-GAGCTCTTCTCTGTAGCTTTGCAGTC-3′, that hybridizes to a region 388 bp downstream of arsC. The second set of primers added a HindIII and a BamHI site at the 5′ and 3′ ends of the fragment, respectively. A 30-cycle PCR (94 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min) was run with E. coli genomic DNA. The respective products were cloned into pGEM-T. The first plasmid was digested with SalI and HindIII, and the second plasmid was digested with BamHI and HindIII. The fragments were then co-ligated into plasmid pLD55 that had been digested with BamHI and SalI, creating plasmid pLD55-arsC, in which 400 bp of the 426-bp arsC gene had been deleted. To replace the wild type chromosomal arsC gene with the deletion, plasmid pLD55-arsC was transformed into E. coli strain W3110. The transformants were grown on plates containing 15 µg/ml of tetracycline and 2.5 mM sodium selenite to select for integrants harboring the plasmid-encoded tetA genes. Tetracycline-resistant colonies were then grown on tetracycline-sensitive-selective agar plates for selection of plasmid-containing colonies. 

PCR was run with 30-cycle PCR (94 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min) for an additional 24 h at 70 °C, 6 h at 0 °C, or 10 h at 20 °C. Samples (1 ml) of each was harvested by centrifugation at 3,000 × g for 10 min. The cell pellets were suspended in 0.1 ml of SDS sample buffer and incubated for 10 min in a boiling water bath. The remainder of each culture was harvested and washed once with Buffer A (10 mM Tris-HCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5, containing 20 mM imidazole, 0.5 mM NaCl, 10 mM β-mercaptoethanol, and 20% glycerol) and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Diphosphopyridine nucleotide (2.5 µl) was added immediately after lysis. The lysate was diluted to 6 ml with Buffer B and centrifuged at 100,000 × g for 1 h at 4 °C. The pellet was suspended in 6 ml of Buffer B. Portions of each of the inclusion bodies were mixed with SDS sample buffer and incubated at 37 °C for 10 min. Samples were analyzed by SDS-PAGE (18) on 15% polyacrylamide gels. The proteins were transferred overnight onto a nitrocellulose membrane at 25 V and probed with a monoclonal antibody to the six-histidine tag (CLONTECH) using an anti-mouse whole IgG (Sigma) as the secondary antibody.

Disruption of Arsenate Reductase Activity—Arsenate reductase activity was assayed using a coupled assay as described previously (19). E. coli strain TOP10 bearing pBAD-YTRR1 was grown in 2 liters of LB medium containing 50 µg/ml ampicillin with shaking at 37 °C. At an A600 of 0.5, L(+)-arabinose was added to a final concentration of 0.02% as inducer, and the culture was grown for an additional 3 h at 37 °C, 6 h at 30 °C, or 10 h at 20 °C. A sample (1 ml) of each was harvested by centrifugation at 3,000 × g for 10 min. The cell pellets were suspended in 0.1 ml of SDS sample buffer and incubated for 10 min in a boiling water bath. The remainder of each culture was harvested and washed once with Buffer A (10 mM Tris-HCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5, containing 20 mM imidazole, 0.5 mM NaCl, 10 mM β-mercaptoethanol, and 20% glycerol) and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Disphosphopyridine nucleotide (2.5 µl) was added immediately after lysis. The lysate was diluted to 6 ml with Buffer B and centrifuged at 100,000 × g for 1 h at 4 °C. The pellet was suspended in 6 ml of Buffer B. Portions of each of the inclusion bodies were mixed with SDS sample buffer and incubated at 37 °C for 10 min. Samples were analyzed by SDS-PAGE (18) on 15% polyacrylamide gels. The proteins were transferred overnight onto a nitrocellulose membrane at 25 V and probed with a monoclonal antibody to the six-histidine tag (CLONTECH) using an anti-mouse whole IgG (Sigma) as the secondary antibody.

Immunoblotting was performed using an enhanced chemiluminescence kit (NE-N Life Science, Inc.) and exposed on x-ray film at room temperature according to the directions provided by CLONTECH.

Protein Purification—E. coli glutaredoxins were purified as described previously (19). For purification of the S. cerevisiae Grx1p, E. coli strain TOP10 bearing pBAD-YTRR1 was grown in 2 liters of LB medium containing 50 µg/ml ampicillin with shaking at 37 °C. At an A600 of 0.5, L(+)-arabinose was added to a final concentration of 0.02% as inducer, and the culture was grown for an additional 3 h at 37 °C. The cells were washed once with Buffer A. The cells were suspended in Buffer B at a ratio of 5 ml of buffer/g of wet cells and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Disphosphopyridine nucleotide (2.5 µl) of wet cells was added to the lysate immediately after lysis. The lysate was centrifuged at 100,000 × g for 60 min at 4 °C, and the supernatant solution was loaded at a flow rate of 15 ml/min onto a 7-ml Ni2+-NTA column pre-equilibrated with Buffer B. The column was then washed with 350 ml of Buffer B followed by elution with 125 ml of Buffer C (50 mM MOPS, pH 7.5, containing 200 mM imidazole, 0.5 mM NaCl, 10 mM β-mercaptoethanol, and 20% glycerol). Fractions containing Grx1p were identified by SDS-PAGE (18), pooled, and concentrated using a Millipore Ultrafree-15 Biomax-5k centrifugal filter (Millipore) at 2000 × g. Trx1p and Trr1p with C-terminal histidine tags were purified by Ni2+-NTA chromatography by essentially the same procedure as Grx1p.

For purification of Acr2p, cells of E. coli strain TOP10 bearing pBAD-Acr2 were grown in 4 liters of LB medium containing 50 µg/ml ampicillin with shaking at 37 °C. At an A600 of 0.5, L(+)-arabinose was added to a final concentration of 0.02% as inducer, and the culture was grown for an additional 10 h at 20 °C. The cells were washed once with Buffer A, suspended in Buffer B at a ratio of 5 ml of buffer/g of wet cells, and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Disphosphopyridine nucleotide (2.5 µl) of wet cells was added to the lysate immediately after lysis. The lysate was centrifuged at 100,000 × g for 60 min at 4 °C, and the supernatant solution was loaded at a flow rate of 0.5 ml/min onto a 1.5-cm diameter column filled to 75 cm with Sepharyl S-100 (Amersham Pharmacia Biotech) pre-equilibrated with Buffer D (50 mM MOPS, pH 6.5, containing 0.5 mM NaCl, 10 mM β-mercaptoethanol, 20% glycerol, and 0.5 mM EDTA), eluted with the same buffer, pooled, and concentrated. Purified proteins were stored at −70 °C until use. Protein concentrations were determined from the absorbance at 280 nm using the following extinction coefficients for yeast proteins: Acr2p, 14,300 M−1 cm−1; Grx1p, 5360 M−1 cm−1; Trx1p, 9700 M−1 cm−1; Trr1p, 32380 M−1 cm−1. Extinction coefficients were calculated by the method of Gill and von Hippel (20). The extinction coefficients for E. coli glutaredoxins were described previously (19).

Yeast Arsenate Reductase—Arsenate reductase activity was assayed using a coupled assay as described previously (19). The assay buffer contained 50 mM MOPS, 50 mM MES, pH 6.5, 0.1 mg/ml bovine serum albumin, 0.4 mM NADPH, 15 mM yeast glutathione reductase (Calbiochem), 1 mM GSH, and 5 µM Acr2p. Reduction of 2-hydroxyethyldisulfide was used to ensure functioning of the coupling system. Sodium arsenate and glutaredoxins were added as indicated. Reductase activity was monitored at 340 nm and expressed as nmol of NADPH consumed.
Yeast Arsenate Reductase

oxidized per mg of Acr2p using a molar extinction coefficient of 6200 for NADPH. The data were analyzed using SigmaPlot v. 5.0.

RESULTS

Heterologous Expression of Arsenate Reductase Genes—The S. cerevisiae ACR2 and E. coli arsC genes each confer arsenate resistance in their respective organisms. Acr2p and ArsC are totally unrelated, with no sequence similarity, and it was not known whether either yeast or E. coli has cofactors that would allow function of the heterologous reductase. It was of interest, therefore, to examine whether arsC could complement a S. cerevisiae strain in which ACR2 was disrupted and whether ACR2 could complement the arsenate-sensitive phenotype of an E. coli arsC disruption. The arsenate-sensitive S. cerevisiae strain RM1 was transformed with pYES-ArsC, which carries a wild type arsC gene under control of the GAL1 promoter. In presence of 2% galactose, arsC conferred arsenate resistance (Fig. 1A). When galactose was replaced by glucose, there was no resistance (data not shown). In the reciprocal experiment, expression of ACR2 from plasmid pBAD-ACR2 complemented the arsenate-sensitive phenotype of E. coli strain WC3110 (Fig. 1B). Thus, Acr2p can function as an arsenate reductase in vivo in E. coli. Importantly, the results indicate that E. coli contains cofactors that support Acr2p activity.

In the chromosome of S. cerevisiae there is the gene for an ACR2 homologue, YGR203W. YGR203W under control of its native promoter on plasmid yEP-PYGR was unable to complement the ACR2 disruption, indicating that the YGR203W gene product is not an arsenate reductase (Fig. 1A). In support of this conclusion, the single YGR203W disruption was as resistant to arsenate as the wild type, and the double YGR203W-ACR2 disruption was no more sensitive to arsenate than the single ACR2 disruption (data not shown).

Expression of Acr2p in E. coli—Expression of ACR2 in E. coli resulted in the formation of inclusion bodies with little or no soluble Acr2p, and only expression of a malE-ACR2 fusion from plasmid pACR2-2 resulted in production of a soluble derivative of Acr2p (9). In this study, ACR2 was expressed from the arabinose promoter as a fusion with C-terminal sequences for the myc epitope and six histidine codons. When cultures of E. coli TOP10 pBAD-ACR2 were grown at 37 °C after induction with arabinose, all of the expressed Acr2p was found in inclusion bodies (data not shown). When the cells were induced at 30 °C, a small amount of Acr2p was found in the soluble fraction. Induction at 20 °C resulted in approximately half of the protein remaining soluble. Thus, for subsequent purification of Acr2p, cells were induced at 20 °C for 10 h.

Purification of Acr2p—Acr2p with the Myc epitope and six-histidine tag was purified by a combination of metal chelate affinity and size exclusion chromatography, as described under “Experimental Procedures.” Approximately 5 mg of purified Acr2p could be obtained per liter of cells. From the intensity of the Coomassie Blue staining of samples separated by SDS-PAGE, Acr2p was judged to be greater than 95% homogeneous.

Fig. 1. ACR2 confers arsenate resistance in E. coli, and arsC confers arsenate resistance in S. cerevisiae. A, arsenate resistance in S. cerevisiae; filled circles, W303–1B (wild type) pYES2.0; open inverted triangles, RM1 pYES-ArsC; closed inverted triangles, RM1 pACR2–3; open circles, RM1 pYES2.0; open squares, RM1 yEP352; filled squares, RM1 yEP-PYGR. B, arsenate resistance in E. coli: filled circles, W3110 (wild type); open circles, WC3110 pBAD/Myc-HisA; filled inverted triangles, WC3110 pBAD-ACR2.

Fig. 2. Acr2p purification and aggregation state. Inset; purification was performed as described under “Experimental Procedures.” Samples at each step were analyzed by SDS-PAGE on a 15% polyacrylamide gel. Lane 1, standard markers are ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa); lane 2, 100 μg of cytosol; lane 3, 50 μg of flow through from nickel affinity chromatography; lane 4, 100 μg of pooled nickel resin fractions; lane 5, 100 μg of pooled Acr2p-containing fractions from Sephacryl S-100 chromatography. The mass of Acr2p was determined from its elution position (arrow) on Sephacryl S-100 chromatography. The elution positions of the standard proteins are indicated for albumin (66 kDa), ovalbumin (46 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (14.3 kDa).
mined by gel filtration chromatography using a Sephacryl S-100 column (Fig. 2). From the nucleotide sequence of the ACR2 gene with the C-terminal myc-epitope and six-histidine tag, the gene product has a predicted mass of 16,882 Da. From its elution position from the Sephacryl column, a mass of approximately 34 kDa was determined, consistent with an Acr2p homodimer. In contrast, the bacterial ArsC reductase is a functional monomer.

**GSH and Grx Serve as Electron Donors for Acr2p-catalyzed Arsenate Reduction**—Previously a MalE-Acr2p chimera was shown to reduce radioactive arsenate to arsenite (9). Reduction required supplementation with yeast cysteol, indicating the requirement for a cofactor or cofactors. The E. coli ArsC enzyme utilizes GSH and Grx as sources of reducing potential (21), and the *Staphylococcus aureus* ArsC enzyme uses Trx as an electron donor (7). Complementation of ArsC function in *E. coli* by the unrelated Acr2p indicated that similar cofactors might be utilized by Acr2p. Therefore, the gene for the *S. cerevisiae* Grx1p was cloned and expressed in *E. coli*, and the protein product was purified. The coupled assay used for measuring *E. coli* ArsC activity was adapted for measuring Acr2p activity. In this assay, NADPH oxidation is coupled to reduction of GSSG by glutathione reductase, and the resulting GSH serves as electron donor for arsenate reduction. In the presence of purified Acr2p, yeast Grx1, and arsenate, oxidation of NADPH was observed, reflecting reduction of arsenate to arsenite (Fig. 3A). Reductase activity required each component. In the absence of any, there was only a low basal rate of NADPH oxidation (Fig. 3B). For unexplained reasons, the rate of NADPH oxidation was considerably slower if arsenate was added as last; in subsequent assays, the reaction was initiated by addition of Grx. To examine whether Trx could function as electron donor for Acr2p arsenate reductase activity, the genes for Trx1 and Trr1 from *S. cerevisiae* were expressed in *E. coli*, and the proteins were purified. Using a coupled assay (22), no Acr2p activity was observed with Trx1 and Trr1 in place of Grx1 and glutathione reductase (data not shown). Thus, Trx1 is unable to serve as an electron donor to Acr2p for the reduction of arsenate.

**Kinetic Parameters of Acr2p-catalyzed Arsenate Reduction**—The rate of arsenate reduction as a function of arsenate concentration was determined (Fig. 4). The data were best fit with a sigmoidal curve, and transformation of the data as a Hill plot gave a linear fit (Fig. 4, inset). The apparent Hill coefficient \( \alpha_{\text{app}} \) was calculated to be 2.7, indicating strong positive cooperativity (23). The \( K_{\text{app}} \) for sodium arsenate was 35 mM. The \( V_{\max} \) was usually in the range of 0.3–0.4 \( \mu \text{mol/min/mg} \) of purified Acr2p protein. These values are quite similar to those reported for the *E. coli* ArsC enzyme (19).

**Other Properties of Acr2p-catalyzed Arsenate Reduction**—Acr2p activity exhibited a broad pH optimum, from about pH 4.5 to 6.5, declining sharply above and below those pH values (data not shown). The enzyme appeared to be highly specific for arsenate. When arsenate was replaced by 100 mM sodium phosphate, sodium nitrate, or sodium sulfate, no NADPH oxidation was observed (data not shown). These results suggest that Acr2p does not reduce other oxyanions at rates equivalent to arsenate reduction. Because antimonite salts are used as antiparasitic agents, with Sb(V) most likely reduced to Sb(III) to form the active species of the drug (24), the question of whether Acr2p can reduce Sb(V) is of interest. Due to the limited solubility of potassium antimonite, it was not possible to assay concentrations greater than 4 mM, but, at 4 mM potassium antimonite, no NADPH oxidation was observed. However, this result does not rule out reduction of antimonite by Acr2p at rates too low to be detected by the coupled assay. If a radioisotope of Sb becomes available, this question can be explored in more detail by the more sensitive direct measurement of reduction.

Other oxyanions, including arsenite, phosphate, sulfate, nitrate, antimonite, and antimonate, were examined as inhibitors of Acr2p arsenate reductase, and only arsenite, the product of the reaction, inhibited (data not shown). Addition of either 1 or 2.5 mM sodium arsenite reduced the apparent Hill coefficient from 2.6 to 1.8 and increased the \( K_{\text{app}} \) from 35 to 58 mM (for 1 mM sodium arsenite) or to 74 mM (for 2.5 mM sodium arsenite) (Fig. 5). The dose-dependent inhibition and suppression of inhibition by higher concentrations of substrate indicate that arsenite is a competitive inhibitor of Acr2p (23, 25).

Although phosphate was only a poor inhibitor of arsenate reduction, it did have the effect of abolishing the apparent positive cooperativity (Fig. 6). The \( V_{\max} \) was unchanged in the presence of 100 mM sodium phosphate, but the apparent Hill coefficient decreased from 2.6 to 1.0. The loss of cooperativity gives the appearance of activation by phosphate at low concentrations of arsenate, a well known effect of noncatalytic substrate analogues on enzyme kinetics (26). These results imply that phosphate does, in fact, bind to Acr2p.

**Role of Glutaredoxins in Arsenate Reduction**—The rate of
reduction as a function of *S. cerevisiae* Grx1p concentration at a saturating concentration of sodium arsenate was determined (Fig. 7A). Three glutaredoxins have been identified in *E. coli*, Grx1, Grx2, and Grx3 (27). Each of the three has been shown to be capable of filling that role for the *E. coli* ArsC reductase (19). An important conclusion from the observation that ACR2 complements an *arsC* deletion is that *E. coli* must have a cofactor that supports Acr2p reductase activity. For this reason, the ability of the three *E. coli* glutaredoxins to serve as electron donor for Acr2p-catalyzed arsenate reduction was examined. Activity was hyperbolic as a function of *S. cerevisiae* Grx1p and *E. coli* Grx1 and Grx3 (Fig. 7A). In contrast, the data for Grx2 were sigmoidal, with \( n_{\text{app}} = 2.8 \) (Fig. 7B). The most striking difference was the 300-fold higher apparent affinity of Acr2p for *E. coli* Grx2 than for the *S. cerevisiae* Grx1p (Table II). The turnover number \( (k_{\text{cat}}) \) of Acr2p was nearly the same regardless of which glutaredoxin was used, but the catalytic efficiency \( (k_{\text{cat}}/K_{\text{app}}) \) with *E. coli* Grx2 was approximately 2 orders of magnitude greater than that with the *S. cerevisiae* Grx1p. The *E. coli* ArsC reductase also exhibits a preference for Grx2 (19). These results suggest that Acr2p can utilize a variety of Grxs, whether from a prokaryote or eukaryote.

Grxs can reduce either intramolecular disulfides (e.g. ribonucleotide reductase) or mixed disulfides between a thiol compound and GSH (e.g. the complex between 2-hydroxyethyl-disulfide and GSH (12)). Grx1p from yeast and Grx1, Grx2, and Grx3 from *E. coli* have the conserved active site sequence Cys-Pro-Tyr-Cys. Both cysteine residues are required for protein disulfide reduction. For reducing glutathione containing mixed disulfides, however, the N-terminal cysteine is sufficient. To elucidate the role of Grx in Acr2p-catalyzed arsenate reduction, the effect of single and double cysteine-serine substitutions in *E. coli* Grx2 was examined. Grx2 with its N-terminal cysteine changed to serine (C9S) was unable to serve as the hydrogen donor for the reduction of arsenate by Acr2p (Fig. 8). The double substitution (C9S/C12S) exhibited the same phenotype as the single substitution. In contrast, Grx2 with intact N-terminal cysteine but serine substitution in the C-terminal cysteine (C12S) retained nearly complete wild type activity. Thus, for Acr2p catalysis, Cys-9 for *E. coli* Grx2 was sufficient, indicating that Acr2p forms a mixed disulfide with glutathione during the catalytic cycle, and glutaredoxin serves to regenerate the active form of the enzyme.

**DISCUSSION**

Compared with the extensive studies in prokaryotes, little is known about the mechanism of arsenical resistance in eukaryotes (28, 29). Considering that humans are constantly...
exposed to arsenic (1), a human carcinogen (2), identification of arsenic metabolizing enzymes is imperative. Recently three contiguous genes, \textit{ACR1}, \textit{ACR2}, and \textit{ACR3}, were reported to confer resistance to arsenite and arsenate in \textit{S. cerevisiae} (4, 30). \textit{ACR2} is specifically required for resistance to arsenate and not to arsenite, and preliminary data had suggested that Acr2p catalyzes arsenate reduction (9). The data in this paper clearly show that Acr2p is a specific arsenate reductase. Characterization of a eukaryotic arsenate reductase is a good first step toward the goal of identification of human arsenic detoxification mechanisms.

Although Acr2p is a member of a family that includes Cdc25a, a phosphotyrosine phosphatase, it does not catalyze hydrolysis of p-nitrophenyl phosphate and so is probably not a phosphatase (data not shown). Acr2p uses Grx and GSH as primary electron donors but cannot use Trx. Although Acr2p and the \textit{E. coli} ArsC reductase are unrelated in sequence, ArsC also uses Grx and GSH but not Trx (21). An arsenate reductase from \textit{S. aureus} that is completely unrelated to either the yeast or \textit{E. coli} enzymes uses Trx but not Grx and GSH (7). Thus, three independently evolved arsenate reductases each use thiol transfer proteins in reduction, but the preference for thioredoxin or glutaredoxin differs. Although the eukaryotic Acr2p shows superficial similarities to the prokaryotic enzymes, it is quite different in structure and kinetic behavior. The \textit{E. coli} ArsC is a monomer that exhibits a typical Michaelis-Menten hyperbolic relationship of activity with substrate concentration. In contrast, Acr2p exhibits positive cooperativity with respect to substrate concentration. Such cooperative interactions are most frequently associated with multisubunit proteins. Unlike the monomeric ArsC, Acr2p purifies as a homodimer, which may suggest interaction between subunits.

Acr2p has low affinity for arsenate, in the range of 30 mM. However, arsenate may be accumulated to high concentrations in vivo. In \textit{S. cerevisiae}, arsenate is likely accumulated by phosphate transporters (31). In the pink yeast \textit{Rhodotorula rubra}, intracellular phosphate concentrations were in the range of 15–200 mM, even at very low external phosphate concentrations (32). Sensitivity to arsenate can be observed...
only in low phosphate medium, in which cells would be expected to accumulate high concentrations of arsenate. Thus, even with low affinity for substrate, Acr2p would be able to function at a physiologically relevant range of intracellular arsenate.

The data shown in Fig. 8 are consistent with formation of a mixed disulfide between glutathione and the Acr2p during the reaction cycle. Through thiol exchange with glutaredoxin, the enzyme sulfhydril is regenerated, with concomitant formation of the glutathionylated glutaredoxin. The GrxS-SG complex is finally reduced by GSH, and the GSSG is reduced by glutathione reductase with NADPH as the ultimate source of reducing potential, as the predicted reaction scheme below depicts.

\[
\begin{align*}
\text{ES}^- \cdot \text{As(V)} & \leftrightarrow \text{ES}^- \cdot \text{As(V)} \\
\text{ES}^- \cdot \text{As(V)} + \text{GS}^- & \leftrightarrow \text{ES}-\text{SG} + \text{As(III)} \\
\text{ES}-\text{SG} + \text{Grx-S} & \leftrightarrow \text{ES}^- + \text{GS}-\text{Grx} \\
\text{GS}-\text{Grx} + \text{GS}^- & \leftrightarrow \text{Grx-S}^- + \text{GSSG} \\
\text{GSSG} + \text{NADPH} & \leftrightarrow 2 \text{GS}^- + \text{NADP}^+ 
\end{align*}
\]

**Scheme 1**

In Reaction 1, there is noncovalent binding of oxyanion to Acr2p. The effect of phosphate on the apparent positive cooperativity of Acr2p suggests that this site can recognize either arsenate or phosphate. In Reaction 2, arsenate is reduced to arsenite with one electron transferred from a protein cysteine thiolate and the second from glutathione. In Reaction 3, Grx acts as the electron donor for reduction of the Acr2p-SG mixed disulfide. This is followed by the regeneration of reduced Grx by GSH, forming oxidized glutathione (GSSG) (Reaction 4). GSSG is reduced to 2 mol of GSH by NADPH and glutathione reductase (Reaction 5). The reaction scheme proposed above for Acr2p is quite similar to that proposed for the *E. coli* ArsC arsenate reductase (19). Because the yeast and bacterial enzymes have different subunit structure and different kinetics, these similarities are probably fortuitous. Considering that these two enzymes are most likely the result of convergent evolution, it is remarkable that they demonstrate any mechanistic similarities.

Acr2p has three cysteines in its primary sequence, Cys-76, Cys-106, and Cys-119. Which cysteine residue is catalytic is not known at this time. However, Acr2p exhibits sequence similarity with members of the Cdc25A family of phosphotyrosine phosphatases, and Cys-76 in Acr2p can be aligned with the catalytic Cys-430 of the human Cdc25A (10). Even though no phosphatase activity was detected with purified Acr2p, by analogy with the phosphatase active site cysteine, we would propose that Cys-76 is the catalytic cysteine residue in arsenate reduction. In support of this proposition, mutants were constructed with each of the three cysteine codons individually changed to serine codons, and only the C76S mutant lost arsenate resistance.2 These similarities may also point to mechanistic similarities between phosphatases and arsenate reductases.

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