Acr2p detoxifies arsenate reduction to arsenic in *Saccharomyces cerevisiae*. This reductase has been shown to require glutathione and glutaredoxin, suggesting that thiol chemistry might be involved in the reaction mechanism. Acr2p has a HCC(X)₅R motif, the signature sequence of the phosphate binding loop of the dual-specific and protein-tyrosine phosphatase family. In Acr2p, these are residues His-75, Cys-76, and Arg-82, respectively. Acr2p has another sequence, ¹¹¹¹HCR, that is absent in phosphatases. Acr2p also has a third cysteine residue at position 106. Each of these cysteine residues was changed individually to serine residues, whereas the histidine and arginine residues were altered to alanines. Cells of *Escherichia coli* heterologously expressing the majority of the mutant ACR2 genes retained wild type resistance to arsenate, and the purified altered Acr2p proteins exhibited normal enzymatic properties. In contrast, cells expressing either the C76S or R82A mutations lost resistance to arsenate, and the purified proteins were inactive. These results suggest that Acr2p utilizes a phosphatase-like Cys(X)₂Arg motif as the catalytic center to reduce arsenate to arsenite.

Three unrelated families of arsenate reductases have been identified, two in prokaryotes and one in eukaryotes (1). One group is typified by the ArsC enzyme of *Escherichia coli* plasmid R773 (2, 3). A second group includes the ArsC enzyme of *Staphylococcus aureus* plasmid pI258 (4), which is homologous to low molecular weight acid phosphatases (5). Both the pI258 and R773 ArsCs utilize cysteine residues in their reactions. The thiolate group of Cys-12 in the R773 ArsC is required for catalysis (6). The pI258 arsenate reductase has been suggested to form a disulfide bond between Cys-82 and Cys-89 during the reduction of arsenate to arsenite (7). Both prokaryotic reductases utilize small thiol transfer proteins. The R773 ArsC enzyme uses glutaredoxin (2, 8), and the pI258 reductase uses thioredoxin (9).

The first identified member of the third family is the 130-residue Acr2p of the eukaryote *Saccharomyces cerevisiae*. The ACR2 gene was shown to be required for high level arsenate resistance (10), and disruption of this gene resulted in arsenate sensitivity (11). We have recently shown that Acr2p purified from *E. coli* is a homodimer that catalyzes arsenate reduction in *vitro* and utilizes GSH and glutaredoxin (Grx) as the source of reducing equivalents (12). The *S. cerevisiae* ACR2 gene can replace the unrelated R773 arsC gene in vivo in *E. coli*, facilitating structure-function analysis.

Acr2p is a member of the Cdc25A superfamily of protein phosphotyrosyl phosphatases (PTPases) and is unrelated to the prokaryotic arsenate reductases. Two other Acr2p homologues in *S. cerevisiae* are YGR203W, a 148-residue protein of unknown function (GenBank™ accession number S0003435), and YMR036C (GenBank™ accession number S0004639), a PT-Pase (13). Each of these three proteins has the consensus sequence HCC(X)₅RS(T), which corresponds to the PTPase active site (14). Although the histidine residue is conserved in PTPases and dual-specific phosphatases (ds-PTPases), it is not conserved in low molecular weight acid phosphatases (15). The histidine residue has been suggested to function in stabilizing the active site thiolate anion in PTPases and ds-PTPases but is not essential (16). Comparison of the three-dimensional structure of PTPases and low molecular weight acid phosphatases reveals no structural homology outside the active site sequence, C(X)₅RS(T). The active site loop forms a phosphate binding pocket where the phosphate would be positioned between the cysteine and arginine residues (15). Analysis of mutagenesis data and the crystal structure of the *E. coli* ArsC also identifies an anion binding pocket composed of three arginine residues, Arg-60, Arg-94, and Arg-107, which stabilize arsenate in the binding site for attack by the active site residue Cys-12.

Substitution of the invariant cysteine residue with serine in the phosphatase signature motif C(X)₅R of several enzymes abolished hydrolytic activity *in vitro* and function *in vivo* (17). Initial binding of phosphotyrosine substrates in the phosphate binding pocket of PTPases requires the coordination of two oxygen atoms present on the phosphoryl group with the guanidium side chain of the conserved active site arginine residue. Replacing the arginine residue with alanine increased the *Kₘ* × ~30-fold and decreased the *Kₖcat* by ~8000-fold (18). Similar mutations in an equivalent arginine residue of a receptor-like PTPase, the late airway response PTPase, resulted in the complete loss of enzymatic activity (19).

Acr2p has a PTPase-like HCC(X)₅R motif corresponding to residues His-75, Cys-76, and Arg-82. At the C terminus of Acr2p, there is another HCR motif corresponding to residues His-118, Cys-119, and Arg-120 that is not present in PTPases. In addition, Acr2p has a third cysteine at position 106. To examine the role of these residues in Acr2p catalysis, those residues were changed to serines or alanines by site-directed

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*This work was supported by Grant GM52216 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: Grx, glutaredoxin; PTPase, protein-tyrosine phosphatase; ds-PTPase, dual-specific protein-tyrosine phosphatase.

² P. Martin, J. Shi, S. Demel, B. Edwards, and B. P. Rosen, unpublished results.
mutagenesis. The effects of the mutations were determined in vivo by expression in an arsenate-sensitive strain of E. coli. Altered proteins were purified, and their catalytic activity was measured. Substitution of His-75, His-118, Cys-106, Cys-119, or Arg-120 had no effect on arsenate resistance in vivo or on arsenate reductase activity in vitro. In contrast, the C76A, C119S, and R82A mutants lost both arsenate resistance in vivo and reductase activity in vitro. These results indicate that the PTPase-like Cx(3)R motif of Acr2p may be part of the active site of this arsenate reductase. We propose that the two types of enzymes possess a common oxygen binding site and that the first step in both the phosphate and reductase reactions utilizes a conserved active site cysteine thiolate.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—Strains and plasmids used in this study are described in Table I. E. coli cells were grown in a low phosphate medium (3) or Luria-Bertani medium (20) at the indicated temperatures supplemented with 50 or 125 μg/ml ampicillin as appropriate. S. cerevisiae strains were grown at 30 °C in a basal salts medium (21).

DNA Manipulations—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 (20). Either a Qiagen prep miniprep kit or Qiaquick gel extraction kit (Qiagen) was used to prepare plasmid DNA for restriction enzyme digestion, sequencing, and recovering DNA fragments from agarose gels. The sequence of each mutant gene product was confirmed by DNA sequencing of the entire gene using an Applied Biosystems 373A DNA sequencer. The sequence of each mutant gene product was determined. Substitution of His-75, His-118, Cys-106, Cys-119, Arg-82, and Arg-120 had no effect on arsenate resistance measured. Substitution of His-75, His-118, Cys-106, Cys-119, Arg-82, and Arg-120 had no effect on arsenate resistance measured.

Yeast Arsenate Reductase

TABLE I

| Strains/Plasmid | Genotype | Ref. or source |
|-----------------|----------|---------------|
| Strains/Plasmid | Genotype | Ref. or source |
| TOP10 | F- | Inw-hsdRMS-mcrBC Phi80lacZAM15 lacX74 depR recA1 araD199 Invitrogen ΔaraA-leu7697 galU galK rpsL endA1 supG |
| W3110 | MC10615 | (30) |
| WC3110 | MC10615 | (12) |
| XL1-blue | MC10615 | (11) |
| RM1 | Yeast strain; Mat-a ade2-1 his3-11,15 leu2-3,112 | (12) |
| Plasmids | | |
| pGEM-T | Multicopy E. coli cloning vector, ap’ | Promega |
| pYES2.0 | Multicopy shuttle vector, Ap’, URA3, gal1 | Invitrogen |
| pBAD/Myc-HisA/C | E. coli cloning and expression vector, ap’ | Invitrogen |
| pGEM-T-ACR2 | ACR2 gene with NcoI at the 5’ and HindIII at the 3’ ends was cloned by PCR and then cloned into pGEM-T-B by NcoI/HindIII digestion. | (11) |
| pBAD-ACR2 | pGEM-T-ACR2 was digested with NcoI and HindIII and inserted into the NcoI/HindIII site of pBAD/Myc-HisA | (12) |
| pBAD-YGRX1 | pGEM-T-ACR2 was digested with NcoI and EcoRI and inserted into the NcoI/EcoRI sites of pBAD/Myc-HisC | (12) |
| pBAD-ACR2_C76S | ACR2 codon for Cys-76 changed to Ser codon | This study |
| pBAD-ACR2_C76A | ACR2 codon for Cys-76 changed to Ala codon | This study |
| pBAD-ACR2_C76GS | ACR2 codon for Cys-76 changed to Ser codon | This study |
| pBAD-ACR2_C76AS | ACR2 codon for Cys-76 changed to Ala codon | This study |
| pBAD-ACR2_H118K | ACR2 codon for His-118 changed to Lys codon | This study |
| pBAD-ACR2_H118A | ACR2 codon for His-118 changed to Ala codon | This study |
| pBAD-ACR2_H118S | ACR2 codon for His-118 changed to Ser codon | This study |
| pBAD-ACR2_H118AS | ACR2 codon for His-118 changed to Ser codon | This study |
| pBAD-ACR2_C76S/C119S | pBAD-ACR2_C76S was digested with NcoI and Pmel1. The NcoI site was made blunt. The fragment was then cloned into blunt EcoRI site of pYES2.0 | This study |
| pBAD-ACR2_C76A/C119S | pBAD-ACR2_C76A was digested with NcoI and Pmel1. The NcoI site was made blunt. This fragment was then cloned into blunt EcoRI site of pYES2.0 | This study |

Expression of Wild Type and Mutant ACR2 Genes—Cultures of E. coli WC3110 or Top10 bearing the indicated plasmids were grown in LB medium at 37 °C to an A600 of 0.5, at which point expression of ACR2 was induced by the addition of the 0.02% arabinose for strain Top10 or 0.2% arabinose for strain WC3110. To determine the ability of ACR2 mutants to be expressed, 2 ml of cells of E. coli WC3110 bearing ACR2 genes in pBAD-Myc-HisA were induced at 20 °C for 4 h with 0.2% arabinose and centrifuged, and the pellets were suspended in 0.2 ml of SDS sample buffer. Samples were prepared by boiling SDS sample buffer for 10 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis (23). Immunological blotting with antibody to a six-histidine tag was performed as described previously (22). To examine the solubility of mutant proteins, ACR2 genes in pBAD-Myc-HisA were expressed in E. coli TOP10. Cells were induced at 20 °C for 10 h, and production of cytosolic-soluble Acr2p was determined as described previously (12).

Purification of Acr2p and Arsenate Reductase Activity—Acr2p was purified from cultures of E. coli strain Top10 bearing pBAD constructs with wild type or mutant ACR2 genes. Purification and assay of arsenate reductase activity was as described previously (12). The data were analyzed using a SigmaPlot 2000. The concentration of purified Acr2p was 34739.
wild type to arsenate. Arg-82 was also modified to lysine, another C76A sensitive phenotype. Cells bearing ACR2

ACR2

ACR2

bearing plasmids with wild type and mutant arsC

types) or WC3110 (/H9004

ACR2R120A

Cells bearing plasmid pBAD-

ACR2R82A

were as resistant as the cells with vector only. However, these mutant genes were characterized phenotypically (Fig. 2).

RESULTS

Arsenate Resistance Phenotype of Cells with ACR2 mutations—Acr2p residues Cys-76, Cys-106, and Cys-119 were individually changed to alanine or serine residues, producing the C76A, C76S, C106S, and C119S mutants. Cells bearing the mutated ACR2 genes were characterized phenotypically for arsenate resistance in E. coli strain WC3110 in which the chromosomal \( \alpha r s C \) gene was disrupted (Fig. 1). Cells expressing the wild type ACR2 gene could grow in medium containing 1 mM sodium arsenate. Cells bearing ACR2C76S were as sensitive to arsenate as the WC3110 cells bearing just vector plasmid pBAD-Myc-HisA. In contrast, cells expressing either pBAD-ACR2C106S or pBAD-ACR2C119S were as resistant to sodium arsenate as cells with the wild type ACR2 gene. A double mutant, pBAD-ACR2C106S/C119S, exhibited an arsenate-sensitive phenotype. Cells bearing ACR2C76A were as sensitive to arsenate as cells bearing ACR2C76S (data not shown). However, expression of the C106S/C119S protein was extremely poor in strain WC3110, and no soluble mutant reductase was observed in strain Top10, suggesting that this mutant could not fold properly (data not shown).

Each of the two arginine residues, Arg-82 and Arg-120, were individually changed to alanine residues, producing mutants R82A and R120A. Cells of E. coli strain WC3110 expressing these mutant genes were characterized phenotypically (Fig. 2). Cells bearing plasmid pBAD-ACR2R82A were nearly as sensitive to sodium arsenate as the cells with vector only. However, cells bearing plasmid pBAD-ACR2R120A were as resistant as the wild type to arsenate. Arg-82 was also modified to lysine, another basic residue, and cells bearing plasmid pBAD-ACR2R82K were as sensitive to arsenate as the R82A mutant. However, the R82K protein was poorly expressed in WC3110, and the majority of the protein was found in inclusion bodies as determined by cellular fractionation and immunoblotting (data not shown).

Two histidine residues, His-75 and His-118, were altered individually to alanines, producing the H75A and H118A derivatives.

Cells expressing either of the altered genes exhibited arsenate resistance similar to that of the wild type (Fig. 3), demonstrating that neither of the two histidines is required for activity.

To demonstrate that the phenotype observed by heterologous expression in E. coli was a predictor of the phenotype of the mutants in yeast, a representative sensitive mutant, C76S, and a representative resistant mutant, H118A, were cloned in vector pYES2.0 and transformed into S. cerevisiae strain RM1 (\( \alpha r s C \)). As anticipated, yeast cells bearing plasmid pYES2.0-ACR2C76S were as sensitive to arsenate as cells bearing vector only, and cells bearing plasmid pYES2.0-ACR2H118A were as resistant to arsenate as the cells bearing a wild type ACR2 gene (data not shown). These results provide a measure of confidence that expression of ACR2 genes in E. coli can be used to evaluate the effects of mutagenesis of this yeast gene.

Purification of Mutant Acr2p Proteins—The steady state level of production of most of the altered Acr2p proteins was approximately the same as the wild type as demonstrated by immunoblotting using an anti-His tag monoclonal antibody (data not shown). Two exceptions were C119S/C106S and R82K, which were found exclusively in inclusion bodies. Except for those two, each of the other altered Acr2p proteins were as soluble as the wild type when produced in E. coli strain Top10 induced with 0.02% arabinose at 20 °C. Each purified mutant Acr2p protein was shown to be a homodimer by gel filtration chromatography, and each eluted from chromatographic matrices at the same position as the wild type protein. Similarly, there was no difference between the wild type and altered proteins in mobility on SDS-polyacrylamide gel electrophoresis nor was abnormal degradation of the altered proteins observed. These results indicate that the overall tertiary structure of the altered proteins was not significantly different from the wild type, although local changes cannot be ruled out.

Arsenate Reductase Activity of Wild Type and Mutant Acr2p Enzymes—Acr2p reduces arsenate to arsinite with GSH and any of several glutaredoxins serving as the source of reducing equivalents (12). As a function of arsenate concentration, Acr2p was shown to exhibit sigmoidal kinetics, although the basis for this apparent cooperativity is not known. The reductase activity of the cysteine, arginine, and histidine mutants was measured using a coupled assay (12).

Purified C76S or C76A exhibited no arsenate reductase activity over background, even when assayed at a 3-fold higher
concentration than the wild type (data not shown). Under these conditions the limit of detection of reductase activity would be 1–2% of wild type, so catalytic rates of less than 1% of wild type would not be detected. C106S and C119S exhibited similar sigmoidal kinetics as the wild type enzyme (Fig. 4) with apparent Hill coefficient ($n_{\text{app}}$) values of 2.0 and 3.2, respectively, compared with 2.1 for the wild type (Table II). The $K_{\text{app}}$ values were slightly higher in both the cysteine mutants when compared with the wild type. The $V_{\text{max}}$ of the two altered enzymes was approximately the same as the wild type.

When the activity of the two arginine mutants was examined, purified R82A exhibited no arsenate reductase activity over background, even when 3-fold more protein was used. R120A exhibited sigmoidal kinetics. The apparent Hill coefficient ($n_{\text{app}}$) was 2.3, similar to that of the wild type (Fig. 5). The $V_{\text{max}}$ was approximately the same as the wild type, and the $K_{\text{app}}$ value was slightly higher (Table II).

Both the histidine mutants H75A and H118A were purified, and their arsenate reductase activity was determined (Fig. 6). The sigmoidal kinetics of H118A was similar to the wild type protein with an apparent Hill coefficient ($n_{\text{app}}$) of 2.1. H75A exhibited sigmoidal kinetics, although the $n_{\text{app}}$ was only 1.3. The $K_{\text{app}}$ of H118A was the same as the wild type, but the H75A $K_{\text{app}}$ was approximately 4-fold higher (Table II). The $V_{\text{max}}$ for each of the histidine mutants was approximately the same as the wild type.

**DISCUSSION**

Acr2p is a 30-kDa homodimeric yeast enzyme that catalyzes the reduction of As(V) to As(III) (12). Both GSH and Grx are

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**TABLE II**

| Protein | $V_{\text{max}}^{a}$ | $K_{\text{app}}^{b}$ | $n_{\text{app}}$ |
|---------|----------------------|-------------------|----------------|
| WT      | 337                  | 31                | 2.1            |
| C106S   | 343                  | 47                | 2.03           |
| C119S   | 317                  | 58                | 3.18           |
| H75A    | 340                  | 117               | 1.3            |
| H118A   | 340                  | 32                | 2.1            |
| R120A   | 389                  | 49                | 2.3            |

* Calculated per mg of Acr2p.
* $K_{\text{app}}$ for sodium arsenate at 5 $\mu$M Acr2p and 1 $\mu$M Grx1p.
required for catalysis in vitro, and the data suggested the formation of a mixed disulfide between Acr2p and glutathione. These results imply that at least one cysteine residue in the enzyme is involved in catalysis. Acr2p exhibits sequence similarity with members of the Cdc25A family of PTPases, and Cys-76 in Acr2p can be aligned with the catalytic Cys-430 of the human Cdc25A (13). PTPases possess a catalytic domain with a highly conserved HC(X)5R signature motif (25). Mutational and chemical modification experiments suggest that the invariant cysteine residue in this signature motif is essential for enzymatic activity (18) and is directly involved in the formation of a covalent phosphoenzyme intermediate (26, 27). Similarly, the conserved arginine residue is essential and has been suggested to participate both in substrate binding and in stabilization of the transition state (18).

In this study Acr2p residues Cys-76 and Arg-82 in the conserved HC(X)5R motif were individually changed to serine and alanine, respectively. In vivo, cells expressing the altered proteins exhibited an arsenate-sensitive phenotype. In vitro, the purified C76A, C76S, and R82A mutant proteins were inactive. Thus, the data indicate that both Cys-76 and Arg-82 are required for catalysis.

On the other hand, the conserved histidine residue at the start of the signature sequence of PTPases does not appear to have an essential role in catalysis. Similarly, in Acr2p, the \( V_{\text{max}} \) of the H75A mutant of Acr2p was the same as the wild type enzyme, but the apparent \( K_m \) was increased 4-fold (Table II). Although His-75 is not required for catalysis, it could play a less direct role. In PTPases the conserved histidine has been suggested to have a structural role. For example, a H445N mutant of Cdc25B, an isoform of Cdc25A, exhibited catalytic activity similar to that of the wild type enzyme but had an increased \( K_m \) (25). In the human PTP1B, the equivalent residue, His-214, helps to maintain the conformation of the catalytic Cys-215 and the structure of the phosphate binding loop (28). The conserved histidine may also affect protonation of the lytic Cys-215 and the structure of the phosphate binding loop (28).

These data strongly imply that the C76(X)5R82 motif in the yeast Acr2p arsenate reductase is involved in catalysis. Thus, Acr2p shares an active site motif with phosphatases, yet it does not exhibit phosphatase activity. It seems reasonable that the initial step in the two enzymatic reactions could utilize a common oxanion binding site. In both cases, attack by the thiolate of the conserved cysteine residue would result in the formation of a thiolate or a thiol arsenate bond. Considering that Acr2p is a homologue of PTPases and that the pI258 ArsC is a homologue of the low molecular weight acid protein-tyrosine phosphatases, it may be that all arsenate reductases are evolutionarily descended from phosphatases, retaining remnants of the ancestral active site.

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The Phosphatase C(X)$_5$R Motif Is Required for Catalytic Activity of the Saccharomyces cerevisiae Acr2p Arsenate Reductase
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J. Biol. Chem. 2001, 276:34738-34742.
doi: 10.1074/jbc.M103354200 originally published online July 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103354200

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