Directed Evolution of a Yeast Arsenate Reductase into a Protein-tyrosine Phosphatase*

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Arsenic, which is ubiquitous in the environment and comes from both geochemical and anthropogenic sources, has become a worldwide public health problem. Every organism studied has intrinsic or acquired mechanisms for arsenic detoxification. In Saccharomyces cerevisiae arsenate arsenic is detoxified by Acr2p, an arsenate reductase. Acr2p is not a phosphatase but is a homologue of CDC25 phosphatases. It has the HCX_R phosphatase motif but not the glycine-rich phosphate binding motif (GXGXXG) that is found in protein-tyrosine phosphatases. Here we show that creation of a phosphate binding motif through the introduction of glycines at positions 79, 81, and 84 in Acr2p resulted in a gain of phosphotyrosine phosphatase activity and a loss of arsenate reductase activity. Arsenate likely achieved geochemical abundance only after the atmosphere became oxidizing, creating pressure for the evolution of an arsenate reductase from a protein-tyrosine phosphatase. The ease by which an arsenate reductase can be converted into a protein-tyrosine phosphatase supports this hypothesis.

Arsenic is a human carcinogen associated with increased risk of skin, kidney, lung, and bladder cancer (1). Conversely, trivalent arsenicals are used as chemotherapeutic agents against leukemia (2) and protozoan parasitic diseases such as sleeping sickness (3). The ubiquity of environmental arsenic from geological formations, fungicides, pesticides, and herbicides has provided selective pressure for the evolution of arsenic-detoxifying systems, which are found in every organism examined. In most organisms arsenate (As(V)) is reduced to arsenite (As(III)), which is removed from the cytosol by a variety of carriers or pumps (4, 5). In the yeast Saccharomyces cerevisiae there are two parallel pathways for arsenite elimination (6). Acr3p is a plasma membrane carrier protein that extrudes arsenite from the cells. Ycf1p is a vacuolar ATPase that catalyzes the sequestration of As(III)-glutathione conjugates in the vacuole.

S. cerevisiae Acr2p is the first identified eukaryotic arsenate reductase (7, 8). This 16-kDa enzyme utilizes reduced glutathione (GSH) and glutaredoxin as electron donors to reduce arsenate (As(V)) to arsenite (As(III)), the substrate of the Acr2p and Ycf1p transporters (9). It exhibits a low overall similarity to members of the rhodanese/CDC25 family (10). Rhodanases catalyze the transfer of sulfur from thiosulfate to cyanide (11). Acr2p and the CDC25 cell cycle dual specificity phosphatases (DSPs) share the protein phosphatase active site motif HCX_R (12, 13). Two other families of protein phosphatases also have an H CX_R motif, but they are structurally unrelated to the CDC25 family and may be the result of convergent evolution (12, 13). One family includes the low molecular weight protein-tyrosine phosphatases (PTPs). The other family includes a variety of PTPs that have a GXGXXG phosphate-binding loop in their active site (Fig. 1).

Because arsenate and phosphate are chemically similar oxyanions, it might be expected that the ancestors of the CDC25 phosphatases and Acr2p had an oxyanion-binding site that could accommodate either oxyanion, and indeed Acr2p is competitively inhibited by phosphate. Nonetheless, Acr2p does not exhibit phosphatase activity (9). However, Acr2p lacks the GXGXXG phosphate-binding loop of many PTPs (Fig. 1). In this study the codons for three glycine residues were introduced into the ACR2 gene to create a 79GXGXX84 sequence in the HCX_R active site. This mutagenesis transformed Acr2p into a PTP at the expense of arsenate reductase activity.

We have speculated on the origins of arsenic resistance mechanisms (14). In the primordial neutral atmosphere, arsenic would have been present in solution as As(III) so that resistance would have developed toward arsenite but not arsenate. As the atmosphere became oxidizing, arsenate would have become the predominant form of arsenic in oceans, and there would have been pressure to evolve mechanisms for resistance to the oxidized species. Because reversing the three mutations in the mutated Acr2p obviously would restore this phosphatase to an arsenate reductase, it is reasonable to assume that only a small number of mutations are necessary for an arsenate resistance enzyme to arise from the widespread phosphatases. The clear implication of these results is that the evolution of arsenate resistance is a straightforward process that builds on existing platforms of phosphatases and arsenite transporters.

MATERIALS AND METHODS

Cell Growth and Acr2p Expression—Cells of Escherichia coli were grown in a low phosphate medium (15) or Luria-Bertani medium (16) at the indicated temperatures supplemented with 50–125 μg/ml ampicillin as appropriate. The phenotype of Acr2p and mutants was determined in E. coli strains W3110 (wild type) or WC3110 (ΔarsC) as described previously (9). Overnight cultures were diluted 100-fold in low phosphate medium containing various concentrations of sodium arsenate and 0.2% arabinose. Growth (Δopt) was measured after 48 h of growth at 20 °C. Expression of Acr2p and mutant proteins was determined by immunoblot analysis using anti-His tag antibody as described previously (12).

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† The abbreviations used are: DSP, dual specificity phosphatase; PTP, protein-tyrosine phosphatase; MOPS, 4-morpholinopropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; FDP, fluorescein diphosphate; pNPP, p-nitrophenyl phosphate.

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Oligonucleotide-directed Mutagenesis—Mutations in ACR2 were introduced by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis procedure (Stratagene). Plasmid pGEM-T-ACR2 was used for creating the single glycine mutants. For S79G/N81G and S79G/P84G double mutants, plasmids pGEM-T-ACR2_S79G and pGEM-T-ACR2_P84G were used as templates, respectively, with the same primers used to construct S79G. For N81G/P84G, pGEM-T-ACR2_N81G was used as a template with the primers used to create P84G. For the Acr2p triple mutant, P84G was used as the template with the same primers used to create S79G/N81G. Each mutation was confirmed by sequencing the entire gene using a CEQ2000 DNA sequencer (Beckman Coulter).

The mutagenic oligonucleotides used for both strands and the respective primers used to introduce (underlined) were as follows: S79G, 5′-CAT TGT ACT GGG GCC AAG AAT AGG GGA CAA AAA GTA GC-3′ and 5′-GC TAC TTT TGG TCC CCT ATT CTT GCC CCC AGT ACA ATG-3′; N81G, 5′-CAT TGT ACT GGG TAC AAG GTG AGG GGA CAA AAA GTA GC-3′ and 5′-GC AGC TAC TTT TGG TCC CCT ATT CTT GCC CCC AGT ACA ATG-3′; S79G/N81G, 5′-CAT TGT ACT GGG GCC AAG AAT AGG GGA CAA AAA GTA GC-3′ and 5′-GC AGC TAC TTT TGG TCC CCT ATT CTT GCC CCC AGT ACA ATG-3′.

Purification and Enzymatic Assays of Acr2p and Mutant Proteins—Acr2p and derivatives were purified and assayed for arsenate reductase activity from cultures of E. coli strain TOP10 bearing pBAD constructs with wild type and mutant ACR2 genes as described previously (9). Phosphatase activity was assayed at 37 °C with 5 μM wild type or mutant proteins using the indicated amounts of p-nitrophenyl phosphate (pNPP) in 0.1 M MOPS/MES buffer, pH 6.5 (17). The assay was initiated by the addition of pNPP, and the rate of hydrolysis was measured from the increase in absorbance at 405 nm. Each value was corrected for non-enzymatic pNPP hydrolysis. Enzymes were preincubated with inhibitors for 5 min at 37 °C prior to initiation of the reaction. The data were analyzed with SigmaPlot 2000 using an extinction coefficient for p-

Phosphatase Activity of Purified Acr2p and Glycine Mutants—Wild type Acr2p and each of the glycine mutants were purified by nickel affinity and gel filtration chromatography (9) and were examined for phosphatase activity in vitro using pNPP as a substrate (17). Only the triple mutant, Acr2ptp, exhibited time-dependent hydrolysis of pNPP (Fig. 3). It is interesting to note that Acr2ptp gained phosphatase activity at the expense of its native arsenate reductase activity (data not shown). To demonstrate that the active site HCX_RX motif of Acr2ptp is required for phosphatase activity, C76A, C76S, and R82A derivatives were constructed. None of the purified mutant proteins exhibited phosphatase activity (data not shown). These results indicate that Acr2ptp utilizes the active site cysteine and arginine residues of the HCX_R motif for pNPP hydrolysis.

The rate of pNPP hydrolysis by Acr2ptp as a function of pNPP concentration was determined (Fig. 4A). The V_max was calculated to be 1.88 μmol/min/mg of protein. The K_m was 1.0 nmol/min/mg of protein. The K_m is similar to the reported values for other PTPs such as the human PTP1B (21) and the Yersinia Ptp (22). The turnover number (k_cat) for Acr2ptp with pNPP as a substrate is 3.0 × 10^-4 s^-1, and the catalytic efficiency (k_cat/K_m) is 0.17 M^-1 s^-1.

FDP has been used as an alternate substrate to determine phosphatase activity (18). Acr2ptp has a K_m of 0.15 μM for FDP (Fig. 4B), which is 1200-fold greater affinity than for pNPP. The V_max was 0.47 μmol/min/mg of protein. The k_cat/K_m values were 0.13 s^-1 and 0.87 × 10^6 M^-1 s^-1, respectively. Thus FDP is a much better substrate for Acr2ptp than pNPP. PTP1B also exhibits higher affinity for FDP with a K_m of 10 μM (23).

Inhibitors of Acr2ptp Activity—We have shown previously that phosphate is a competitive inhibitor of Acr2p arsenate reductase activity (9). Arsenate also has been shown to be a competitive inhibitor of PTPs (22, 24). Arsenate competitively inhibited the phosphatase activity of Acr2ptp with a K_i of 1.5 mM (Fig. 5A). Similar K_i values have been obtained for the Yersinia Ptp (22) and human PTP1B (24). Neither sodium arsenite (As(III)) nor sodium sulfate inhibited Acr2ptp activity (data not shown). Sodium phosphate also competitively inhibited Acr2ptp activity with a K_i of 3 mM (Fig. 5B). Sodium orthovanadate, which inhibits other PTPs (25, 26), competitively inhibited Acr2ptp phosphatase activity with a K_i of 120 μM (Fig. 5C) in comparison with a K_i of ~1 μM for PTP1B (21). Recently, it has been shown that Sb(V) in the form of sodium stibogluconate is a potent inhibitor of PTPs such as SHP-1.
However, Sb(V) did not inhibit the DSP mitogen-activated protein kinase phosphatase 1 (26). The effect of Sb(V) in the form of potassium antimonite on the genetically engineered enzyme was examined. Antimonate competitively inhibited Acr2ptp activity with a $K_i$ of 0.5 mM (Fig. 5D). In contrast, antimonite (Sb(III)) did not inhibit Acr2ptp activity (data not shown). This result suggests that Acr2ptp is more similar to PTPs than to DSPs.

**Acr2ptp Dephosphorylates Phosphotyrosine**—Using the phosphotyrosine-containing peptide LCK505 as a PTP substrate (18), the ability of Acr2ptp to dephosphorylate phosphotyrosine was determined by hydrolysis of 3 mM pNPP. The data with single and double glycine mutants were superimposable with those of wild type Acr2p. $\triangledown$, Acr2ptp; $\square$, wild type Acr2p; $\bigcirc$, no protein.
was examined (Fig. 6). Dephosphorylation of the tyrosine residue alters the absorbance and fluorescence spectra of the peptide, producing a blue shift and a reduction in intensity (19). Acr2ptp-catalyzed hydrolysis of LCK505 produced an increase in absorbance at 282 nm (Fig. 6A) and an increase in tyrosine fluorescence with an associated red shift in the $\lambda_{\text{max}}$ (Fig. 6B). Dephosphorylation of LCK505 with calf intestinal phosphatase produced a similar fluorescence change (Fig. 6B).

DISCUSSION

Structural studies suggest that there are three distinct and unrelated groups of arsenate reductases (4). One group includes the *E. coli* plasmid R773-encoded ArsC, which has a unique fold (27) and does not have phosphatase activity. A second group includes arsenate reductases found in many Gram-positive bacteria. Even though they are termed ArsC enzymes, their three-dimensional structures are unrelated to the *E. coli* enzyme (28, 29). These enzymes belong to the low molecular weight PTP family and catalyze a low rate of concentrations of inhibitors. The inhibitors were sodium arsenate (A), sodium phosphate (B), sodium orthovanadate (C), and potassium antimonate (D). Solid lines represent best fits of the data using SigmaPlot. The $K_i$ was calculated from the intersection of the lines from the two pNPP concentrations (dashed lines).

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$^2$ R. Mukhopadhyay, Y. Zhou, and B. P. Rosen, unpublished results.
hydrolysis of pNPP (28) but have not been shown to have PTP activity. The third group includes the eukaryotic arsenate reductases, such as Acr2p, which has been predicted to have a three-dimensional structure related to rhodanese and CDC25 DSPs (10, 13). Although Acr2p has an HCXR active site similar to that of CDC25 (12), it does not exhibit measurable phosphatase activity (9).

In the absence of a three-dimensional structure of Acr2p, our goal was to trace the evolutionary ancestry of this unique arsenate reductase from yeast by strategic mutagenesis. Toward this goal we first aligned Acr2p with the catalytic domains of a variety of PTPs (Fig. 1). The absence of a GXGXXG motif in Acr2p was obvious. Introduction of all three glycines was required to transform the enzyme into a phosphatase. No single or double glycine mutation was sufficient. Both Cys76 and Arg82 of the HCXR sequence of wild type Acr2p are required for arsenate reduction (12). Mutagenesis of those residues in the mutant Acr2ptp abolished the acquired phosphatase activity, indicating that the transfomed enzyme also utilizes the same catalytic residues and implying mechanistic similarities between arsenate reductases and phosphatases. Acr2ptp utilizes not only pNPP and FDP as substrates but also dephosphorylates the phosphotyrosine residue in the synthetic peptide LCX505. Moreover, PTP inhibitors such as arsenate, antimonate, phosphate, and orthovanadate also inhibit Acr2ptp activity with reasonable K<sub>i</sub> values. Thus the acquired PTP activity of the genetically engineered enzyme has many of the key properties of classical PTPs.

We hypothesized that the common ancestor of Acr2p and CDC25 could form either a thiol phosphate or a thiol arsenate intermediate. In all phosphatases, an aspartate that is 30 residues upstream of the HCXR active site functions as a general acid base. For example, in mammalian PTP1, Asp<sup>101</sup> protonates the leaving group phenolic oxygen to facilitate the removal of the tyrosine substrate from the enzyme-substrate complex (30). In the next step Asp<sup>101</sup> acts as a general base, abstracting a proton from a water molecule and facilitating hydrolysis of the phosphoenzyme intermediate (31). Acr2p has a corresponding residue, Asp<sup>34</sup>, which is 40 residues upstream of the active site HCXR<sup>82</sup>. We predict that the cysteine-phosphate intermediate is positioned for hydrolysis using water as a nucleophile activated by Asp<sup>34</sup>.

We propose that the common ancestor of the rhodanese/CDC25 family and Acr2p was a PTP that had a GXGXXG phosphate-binding loop. The arsenate reductase lineage arose when larger residues were substituted at positions corresponding to 79, 81, and 84 of the present-day Acr2p. This resulted in a loss of flexibility in the catalytic loop that prevented the approach of Asp<sup>34</sup>. Although the enzyme still could form thiol intermediates with phosphate or arsenate, hydrolysis was lost, and gradual acquisition of arsenate reductase activity occurred under the evolutionary pressure provided by the appearance of environmental arsenate as the atmosphere became oxidizing.

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