

Binding of Phenylarsenoxide to Arg-tRNA Protein Transferase Is Independent of Vicinal Thiols[†]

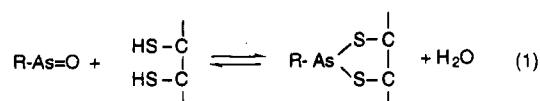
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ABSTRACT: Reversible enzyme inhibition by phenylarsenoxides is generally taken to indicate the presence of functionally important vicinal thiol groups. Arginyl aminoacyl-tRNA transferase from eukaryotes is potently inhibited by phenylarsenoxides and possesses one or more essential sulfhydryl groups [Li, J., & Pickart, C. M. (1995) *Biochemistry* 34, 139–147]. To map the putative Cys residues that mediate arsenoxide binding to the transferase from *Saccharomyces cerevisiae*, we systematically mutagenized the 15 Cys residues of the transferase, singly and in combination, to Ala (13 Cys) or Ser (2 Cys). Six mutant enzymes, encompassing all 15 Cys residues of the transferase, were characterized in detail. The results revealed that Cys-20, Cys-23, and Cys-94 and/or Cys-95 were important for activity, since mutations at these positions reduced activity by 100-fold (Cys-94 and Cys-95 were mutated simultaneously). Surprisingly, however, all of the mutant enzymes retained the ability to bind a radioiodinated phenylarsenoxide derivative, with undiminished stoichiometry and affinity. All of the mutant enzymes also remained susceptible to irreversible reaction with a bifunctional phenylarsenoxide bearing a *para*-alkyl halide substituent. Prior reaction of the enzyme with the bifunctional reagent blocked subsequent binding of the radiolabeled phenylarsenoxide, indicating that these two reagents bind at a single common site. These results indicate that high-affinity binding of trivalent arsenicals can occur by a thiol-independent mechanism.

Monosubstituted trivalent arsenicals have long been known to form addition compounds with thiols [reviewed in Webb (1966)]. Alkylarsenoxides (RAs=O) react with vicinal thiol groups to form ring structures as shown in



The crystal structure of the adduct between CH₃C₆H₄AsCl₂ and 2,3-dimercaptopropanol reveals a five-membered ring in which both sulfur atoms are chelated to the arsenic (Adams et al., 1990). Similar cyclic complexes of phenylarsenoxides with small dithiols have been identified by gas chromatographic analysis (Hannestad & Sorbo, 1980). As expected on entropic grounds, cyclic dithiolarsenites are markedly more stable than the noncyclic addition products formed from trivalent arsenicals and monothiols (Stocken & Thompson, 1946b).

On the basis of the observation that Lewisite (Cl-CH=CHAsCl₂) and arsenite (HOAs=O) reacted with thiol groups of reduced keratin in the molar ratio of 1:2, Stocken and Thompson (1946a) postulated that arsenic combined with

vicinal Cys residues of proteins to form ring structures analogous to those described above:



As expected for this mechanism, vicinal sulfhydryl reagents such as 2,3-dimercaptopropanol and DTT¹ both prevent and reverse complex formation between Lewisite and arsenoxide-sensitive enzymes, while monothiols are much less effective (Whittaker, 1947; Peters et al., 1945). The protective effects of specific dithiols correlate well with the stabilities of the corresponding cyclic dithiolarsenite adducts (Whittaker, 1947).

For the past 50 years, the ring hypothesis outlined in eq 2 has served as the paradigm for explaining enzyme inhibition by trivalent arsenicals, and inhibition has usually been taken to indicate the presence of a spatially vicinal thiol pair, except in special cases [e.g., Barber and Siegel (1983)]. For example, arsenoxides inhibit the pyruvate dehydrogenase complex by binding to the vicinal thiols of the reduced lipoamide cofactor (Stevenson et al., 1978; Adamson et al., 1984). However, vicinal thiols need not be chemically vicinal as in lipoamide. They can also be brought into spatial apposition by folding [e.g., Jauhainen et al. (1988)].

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¹ Abbreviations: BSA, bovine serum albumin; βME, β-mercaptoethanol; BrAcAn, bromoacetyl aniline; BrAcNPAsO, *para*-(bromoacetyl)amino]phenylarsenoxide; DTT, dithiothreitol; E, Arg aminoacyl-tRNA protein transferase; HEPES, (hydroxyethyl)piperazineethanesulfonate; I and ¹²⁵I-PAsO, *para*-¹²⁵I-phenylarsenoxide; MOPS, morpholinepropane sulfonate; NEM, *N*-ethylmaleimide; NPAsO, *p*-aminophenylarsenoxide; PAGE, polyacrylamide gel electrophoresis; PAsO, phenylarsenoxide; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; rcmBSA, reduced and carboxymethylated bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

Trivalent arsenicals have been applied to the study of diverse enzyme systems, including the pyruvate dehydrogenase complex (Stevenson et al., 1978), lecithin-cholesterol acyltransferase (Jauhainen et al., 1988), tyrosyl phosphatases [e.g., Zhang et al. (1992)], hexose transport systems (Frost & Lane, 1985; Hoffman & Lane, 1992), thioredoxin (Brown et al., 1987), and the ubiquitin-dependent protein degradation pathway (Klemperer & Pickart, 1989; Berleth et al., 1992a). Strong and specific interactions between arsenoxides and proteins have been demonstrated using arsenical affinity chromatography and radioactive aromatic arsenoxides (Zhou et al., 1991; Hoffman & Lane, 1992; Berleth et al., 1992a; Kalef et al., 1993). In a few cases the vicinal thiols believed to mediate these interactions have been identified. Examples include lipoamide in the pyruvate dehydrogenase complex (Stevenson et al., 1978); Cys-31 and Cys-184 in lecithin-cholesterol acyl transferase (Jauhainen et al., 1988); the redox-active Cys pair in the active site of thioredoxin (Brown et al., 1987); and adjacent Cys residues in the reduced nicotinic acetylcholine receptor (Dou et al., 1994). For the majority of proteins that bind arsenoxides, however, the actual ligands of the arsenic remain unidentified [e.g., Hoffman and Lane (1992) and Kalef et al. (1993)].

We have been using phenylarsenoxides to study the mechanism of Arg-tRNA protein transferase. This enzyme catalyzes the transfer of Arg from Arg-tRNA to α -amino groups of proteins that initiate with Glu or Asp (Soffer, 1970). Its specific role is to make its targets susceptible to ubiquitin conjugation and thus to degradation by the 26S protease (Bachmair et al., 1986). Both the mammalian and the yeast transferase are inhibited by phenylarsenoxides in a DTT-reversible fashion (Klemperer & Pickart, 1989; Berleth et al., 1992b; Li & Pickart, 1995). We have interpreted this inhibition to indicate the existence of vicinal thiols that function in transferase catalysis or regulation.

In previous efforts to map the arsenoxide binding site, we identified Cys-315 of the yeast transferase as the nucleophile that reacts irreversibly with a bound bifunctional phenylarsenoxide (Li & Pickart, 1995). Here we describe the use of Cys to Ala scanning mutagenesis to identify the putative vicinal thiols that mediate arsenoxide binding. Unexpectedly, our results strongly suggest that binding of phenylarsenoxides to the transferase is not mediated by vicinal thiols.

EXPERIMENTAL PROCEDURES

Materials. The following were purchased from the indicated sources: L-[2,3-³H]Arg (53 Ci/mmol; Dupont-NEN); crude *Escherichia coli* tRNA (Sigma); crude mixture of *E. coli* aminoacyl-tRNA synthetases (Sigma); NEM (Sigma); protein A-Sepharose (Sigma); PAsO (Aldrich); and carrier-free Na¹²⁵I (Amersham). RcmBSA was synthesized as described (Evans & Wilkinson, 1985). BrAcAn, NPAsO, and BrAcNPAsO were synthesized as described (Berleth et al., 1992a).

Enzyme Purification. Recombinant Arg-tRNA transferase was induced in *E. coli* strain BL21(DE3)/pLysS (Moffatt & Studier, 1987) harboring the vector pET3d-ATT; as described previously, the wild type enzyme bears a Ser-to-Ala mutation at residue two (Li & Pickart, 1995). Wild type and mutant enzymes were purified by the published procedure (Li & Pickart, 1995). Enzyme concentrations were based on measured protein concentrations, assuming a molecular mass of 58 kDa.

Transferase Assay. Incorporation of [³H]Arg into rcmBSA was monitored as the appearance of radioactivity insoluble in TCA, as described (Li & Pickart, 1995). [³H]Arg tRNA was first generated in a preincubation containing: 50 mM Tris-HCl (24% base), 5 mM MgCl₂, 2 mM ATP, 10 mM phosphocreatine, 0.6 unit/mL each of creatine phosphokinase and inorganic pyrophosphatase, 1.5 mg/mL *E. coli* tRNA, 30 μ Ci/mL [³H]Arg, and 130 units/mL *E. coli* aminoacyl-tRNA synthetases (pH 7.3). After 4 min at 37 °C, this mixture was transferred to ice. To initiate an assay, 4 μ L of this mixture was added to 4 μ L of a second solution containing the transferase and rcmBSA (1 mg/mL final concentration). After 5 min at 37 °C, the incubation was quenched with 8 μ L of 8 M urea and spotted onto Whatman #2 filter paper. Filters were processed as described (Klemperer & Pickart, 1989), and acid-insoluble radioactivity was determined by liquid scintillation counting. Data were corrected by subtracting a blank obtained by omitting the transferase.

Production and Purification of Polyclonal Antibodies. Inclusion bodies containing the transferase were purified by a previously described method (Lin & Cheng, 1991) and subjected to 10% SDS-PAGE. The transferase band (58 kDa) was visualized by staining with 0.05% Coomassie blue (in water), excised, homogenized, and used to raise antiserum against the transferase in rabbits essentially as described (Haas & Bright, 1985). Antibodies were purified by affinity chromatography, using a column of purified transferase immobilized on activated CH-Sepharose from Pharmacia-LKB Biotech (Haas & Bright, 1985).

PCR Mutagenesis. All single-site mutagenesis was performed by a two-step PCR method that permits a mutation to be generated with a single unique primer (Li & Pickart, 1995). The plasmid pET3d-ATT was the template for both PCR reactions. Flanking primers were the same as those used previously (Li & Pickart, 1995). The mutant primer and the flanking primer closest to the mutation site were used in the first PCR reaction (to minimize the size of the first PCR product). This first PCR product was gel-purified and used as one of the primers in the second PCR reaction. The other flanking primer served as the second primer. The final ~1550 bp product was digested with *Nco*I and *Bgl*II and cloned into plasmid pET3d. To make multisite mutants, the template vector contained a previously-mutated transferase gene. In each mutant plasmid, the presence of expected mutation(s) was verified by DNA sequencing (fmol Kit, Promega).

Primers for PCR mutagenesis were as follows (underlined bases induce mutations): Mut-1 (C315A): 5'-ACTGGATGTGGCTCACAGTAAATATA-3'; Mut-2 (C23A): TTACCGTGAGCATAACCCACACTTTG; Mut-3 (C94S,C95A): GTATAACCTACTAGCATTCCTCAACGGG; Mut-4 (C193A): AAGGGCGTATCAGCTAGGAATCGCTTA; Mut-5 (C233A): TCATAGTAGTAGGCTTCGTGCACAGGAC; Mut-6 (C298A): TATTGAGGACGCTCCGAAGATGAAT; Mut-7 (Δ 481-503): GGGAGATCTCAAATACGTTTCTTGAGA; Mut-8 (C56A): GCCAATTGTGGCGTTTCAATCTTTA; Mut-9 (C54A): ATCCCATATTAGCATGCGATCATACG; Mut-10 (C116A): GCGGCTGATGGCTTTTTTCAATTCCT; Mut-11 (C130A): AGCGGCTGGGGCGTAGTCTACTT; Mut-12 (C382A): TGTGGGAGGGGCTGCGTTTAAATCTG; Mut-13 (C20S): ATACCCACTCTTTGCGGCAGG; Mut-14 (C481A): AAGAAA-

CGTATTGCTGACGTGAT-ACGG; Mut-15 (C492A): GGCTTCGAAACTGCTATGAAAGCCGTCA; Δ N (Δ 1-30): GGGAGATCTCCATGGATCAGTTGTTCC.

Preparation of 125 I-PAsO. This reagent was synthesized as described by Hoffman and Lane (1992), with the following modifications. (1) The reaction was scaled down to utilize 0.5–1 mCi of Na^{125}I , maintaining the same specific radioactivity. (2) The product was purified only through the ether extraction step, since HPLC analysis showed that the purity at this stage was >90%. ^{125}I -PAsO was stored in the dark at -20°C .

Immunoprecipitation assay. Purified transferase (1.4 μM) was incubated with various concentrations of ^{125}I -PAsO in 50 mM MOPS (pH 7.2) and 2 mM EDTA at 37°C for 4 min (10 μL volume). Affinity-purified anti-transferase antibodies (4 μL of a 1.2 mg/mL stock) were added, and incubation continued at 5°C for 30 min. Protein A-Sepharose (40 μL of 25% v/v suspension in dilution buffer) was then added, and the suspension was rotated at 25°C for 20 min. (Dilution buffer consisted of phosphate-buffered saline supplemented with 2 mg/mL BSA, 5 mM EDTA, 1% Triton-X-100, 0.1 mM PMSF, and 0.02% sodium azide.) The suspension was then diluted with 400 μL of dilution buffer and vortexed. The resin was pelleted in a microfuge and washed three times with 400 μL of dilution buffer. The washed pellet was counted in a gamma counter. Data were corrected by subtracting a blank obtained by omitting enzyme from the assay.

Mass Spectrometry. Electrospray ionization mass spectrometry was carried out at the Harvard Microchemistry facility (Chicz et al., 1993). For this experiment the transferase was purified in the absence of Triton-X-100 and glycerol.

Pantothenate Assay. The transferase was tested for the presence of pantotheine by the procedure of Pugh and Wakil (1965). Bacterium *Lactobacillus plantarum* (ATCC 8014) was grown at 37°C for 20 h in 5 mL of pantothenate medium (Bacto AOAC USP) containing 20 ng/mL pantothenic acid. The cells were pelleted, washed once with 5 mL of 0.85% (w/v) NaCl, resuspended in 5 mL of 0.85% NaCl, and diluted 25-fold in 0.85% NaCl. An aliquot of 50 μL was used to inoculate a 5 mL aliquot of pantothenate assay medium that had been mixed with the sample being assayed. (This mixture was autoclaved 15 min prior to inoculation.) After incubation at 37°C for 24 h, growth was assessed by determination of absorbance at 600 nm. Samples to be assayed for pantothenate were first treated with alkali, then neutralized and treated with alkaline phosphatase (Pugh & Wakil, 1965). When assayed by this procedure, 1.5 nmol of CoA gave the expected positive result ($A_{600} = 0.41$, vs 0.014 for no addition). The result obtained with the transferase (2.9 nmol, $A_{600} = 0.01$, average of duplicate determinations) was indistinguishable from the negative control. The absence of inhibitory species in the transferase-derived sample was shown by the strong positive signal obtained when this sample was mixed with 0.23 nmol of pantothenic acid ($A_{600} = 0.56$).

RESULTS

^{125}I -PAsO as a Probe of the Enzyme–Arsenoxide Interaction. In order to monitor directly the interaction between the transferase and phenylarsenoxide, we synthesized ^{125}I -

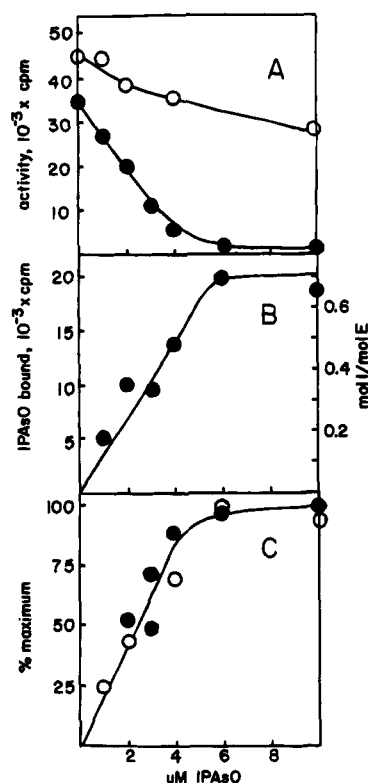


FIGURE 1: Binding of ^{125}I -PAsO to the transferase and effect on activity. Wild type transferase (1.4 μM) was incubated with the indicated concentration of ^{125}I -PAsO (2094 cpm/pmol) for 4 min at 37°C in 50 mM MOPS (pH 7.2) and 2 mM EDTA. (A) Reversible inhibition. Replicate aliquots of the incubation were diluted 10-fold into 50 mM MOPS (pH 7.2) alone (filled circles) or 50 mM MOPS supplemented with 10 mM DTT (open circles); aliquots of 2 μL were assayed for transferase activity. (B) Binding (immunoprecipitation assay). A 10 μL aliquot of the incubation was analyzed for transferase-bound ^{125}I -PAsO by immunoprecipitation (Experimental Procedures). (C) Relationship between binding and inhibition. Each parameter is expressed relative to its maximum value, as a function of ^{125}I -PAsO concentration: filled circles, binding; open circles, inhibition. The lines in each panel have no theoretical basis.

PAsO by the method of Hoffman and Lane (1992) and developed an immunoprecipitation assay to quantitate binding of this ligand to the transferase under native conditions (Experimental Procedures). The relationship between ^{125}I -PAsO binding and enzyme activity is shown in Figure 1. As expected, the transferase (1.4 μM) was strongly inhibited by ^{125}I -PAsO, with $K_{0.5} \sim 2.2 \mu\text{M}$ (filled circles, Figure 1A). Inhibition was reversed by DTT (open circles, Figure 1A). The concentration dependence of inhibition was similar to that seen previously with PAsO ($K_{0.5} \sim 1.2 \mu\text{M}$ at 1.5 μM enzyme; Li & Pickart, 1995), indicating that the iodo moiety did not impede the interaction between enzyme and arsenoxide. This was the expected result, based on the lack of effect of other *para*-substitutions (Li & Pickart, 1995). Thus, the radiolabeled phenylarsenoxide was a suitable probe for studying phenylarsenoxide binding to the transferase.

Quantitative binding studies were first carried out by the immunoprecipitation assay. ^{125}I -PAsO binding showed a saturating concentration dependence (Figure 1B). Moreover, there was a linear relationship between binding and inhibition (Figure 1C), with a stoichiometry of ~ 0.7 mol I/mol E at a saturating concentration of 6 μM (Figure 1B). These data suggest that the transferase has a single phenylarsenoxide binding site. The observed stoichiometry of 0.7 (rather than

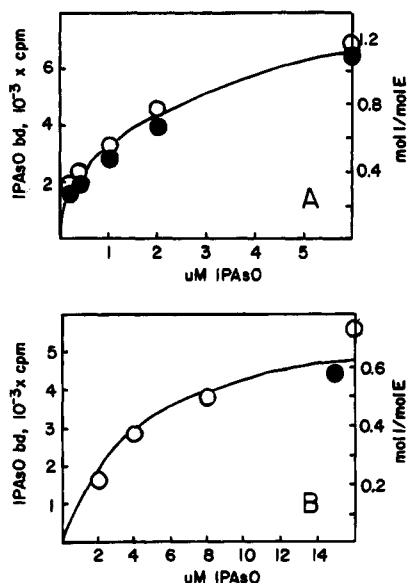


FIGURE 2: Binding of ^{125}I -PAsO to the transferase (SDS-PAGE assay). (A) Native conditions. Fully active mutant enzyme, 0.25 μM , was incubated with the indicated concentration of ^{125}I -PAsO (2370 cpm/pmol) in 50 mM HEPES (pH 7.5) and 0.8 mg/mL rcmbSA at 37 $^{\circ}\text{C}$ (10 μL volume; filled circles, Mut-6 = C298A; open circles, Mut-5 = C233A). After 5 min, the incubation was quenched with 5 μL of 2 \times concentrated sample buffer (lacking βME), boiled for 1 min, and loaded onto a 10% SDS gel. The gel was dried and autoradiographed. The band corresponding to the transferase was excised and counted (left axis). The calculated stoichiometry is shown on the right axis. Other experiments showed that rcmbSA did not affect ^{125}I -PAsO binding and that 6 μM IPAsO was saturating. (B) Denaturing conditions. Fully active transferase enzyme Mut-1 (C315A, 1.3 μM) was incubated with indicated concentrations of ^{125}I -PAsO (458 cpm/pmol) in 40 mM MOPS (pH 7.2) and 8 M urea at 37 $^{\circ}\text{C}$ (13 μL volume). After 10 min, the incubation was quenched with an equal volume of SDS-PAGE sample buffer (lacking βME), boiled for 1 min, and loaded onto a 10% SDS gel. The gel was dried and autoradiographed. The band corresponding to the transferase was excised and counted. The filled circle shows data from an incubation containing 32 μM ^{125}I -PAsO. The lines in each panel have no theoretical basis.

1.0) probably reflects partial loss of the labeled reagent during the extensive washing required by this assay (Experimental Procedures) and perhaps the presence of some inactive oxidized enzyme, as indicated by the finding that DTT stimulated activity by 29% in the absence of ^{125}I -PAsO (Figure 1A).

^{125}I -PAsO binding was also monitored using an SDS-PAGE assay (Hoffman & Lane, 1992). Here the incubation containing transferase and ^{125}I -PAsO was quenched in sample buffer lacking mercaptoethanol and electrophoresed; the transferase band was excised and counted. At 6 μM ^{125}I -PAsO, this assay yielded a stoichiometry of ~ 1 mol/mol E (Figure 2A), comparable to that obtained in the immunoprecipitation assay (Figure 1B). The observed retention of bound ^{125}I -PAsO during SDS-PAGE and immunoprecipitation requires that the rate constant for dissociation of the E·I complex is extremely slow. Overall, the results of both assays indicate that the transferase is completely inactivated upon binding of 1 mol/mol E. Since the two assays gave comparable results (Figure 1B vs 2A), most subsequent studies employed the simpler SDS-PAGE assay.

Cys to Ala (Ser) Scanning Mutagenesis To Identify Arsenoxide-Binding Residues. Although the extremely slow dissociation kinetics of the E·I complex encouraged us to

attempt identification of the arsenoxide-binding residues by protein chemical approaches, an approach utilizing radiolabeled NEM was unsuccessful (below). We therefore took a mutagenesis approach. There are 15 Cys residues in the transferase sequence, including two closely spaced pairs, Cys-20/Cys-23 and Cys-94/Cys-95 (Balzi et al., 1990). Cys residues separated by zero to two intervening amino acids have been shown, or suggested, to serve as arsenoxide binding sites in other proteins [e.g., Hoffman and Lane (1992), Dou et al. (1994), and Brown et al. (1989)]. Arsenoxide binding to closely spaced Cys residues of the transferase could also be consistent with the observed persistence of arsenoxide binding in 8 M urea (below). Thus, we initially mutated these two pairs of Cys residues. Since the resulting mutant enzymes still bound phenylarsenoxide (below), we conducted a more systematic analysis.

Each of the 15 Cys residues of the transferase was mutated singly to Ala, with the following exceptions: Cys-20 and Cys-95 were mutated to Ser (to minimize the number of base pair changes); and Cys-94 and Cys-95 were mutated simultaneously to Ser and Ala, respectively (Table 1). All 14 of these minimally mutated enzymes were expressed in a soluble form, and only three showed altered activity: the specific activities of the C20A, C23A, and C94S,C95A enzymes were reduced by $\sim 99\%$ (Table 1; Table 2 below). (Although these three mutant enzymes retained detectable activity, as described below, they will be referred to as "inactive" for the sake of simplicity.) The three or four functionally important Cys residues (C20, C23, and C94 or C95) are all located toward the N-terminus of the protein, implying that this region may be important for structure or catalytic function.

Enzymes 4Mut and MutC bore four and five mutated Cys residues, respectively (Table 1). Both mutant enzymes were soluble and exhibited activities that were only slightly reduced relative to the wild type enzyme (Table 1; Table 2 below). In two cases where seven or more Cys residues were mutated, the enzyme became insoluble (7Mut and MutN, Table 1). This may be due to gross changes in structure caused by the more numerous mutations. Deleting 30 residues from the N-terminus or 15 residues from the C-terminus also resulted in loss of solubility (Table 1). The requirement for the extreme N- and C-termini of the transferase for solubility rules out approaches to the structure-function relationship that involve N- and C-terminal deletion mapping.

Interaction of ^{125}I -PAsO with Mutant Enzymes: Cys Residues Do Not Mediate Binding. If the interaction between the transferase and phenylarsenoxides involves vicinal thiols, eliminating even one of the corresponding Cys residues should abolish binding. We purified six mutant enzymes in which one or several Cys residues were mutated to Ala (or Ser), with the combination of mutation sites in the six proteins encompassing all 15 Cys residues. The binding of ^{125}I -PAsO to each mutant enzyme was then assessed.

As shown in Table 2, each of the six mutant enzymes still bound ^{125}I -PAsO with the same stoichiometry as the wild type enzyme (0.7–1.0 mol/mol E). Since the concentration of ^{125}I -PAsO used in these experiments, 20 μM , was only ~ 8 -fold higher than $K_{0.5}$ for the wild type enzyme (Figure 1A), none of the mutant enzymes had a strongly reduced affinity compared to wild type (see also Table 4 below, in which a lower ligand concentration was used). These results

Table 1: Summary of Activities of Mutant Transferase Enzymes

name	mutation site(s)	activity ^a
Mut-1	C315A	+++
Mut-2	C23A	—
Mut-13	C20S	—
Mut-213	C20S,C23A	—
Mut-3	C94S,C95A	—
Mut-4	C193A	+++
Mut-5	C233A	+++
Mut-6	C298A	+++
Mut-8	C56A ^b	+++
Mut-9	C64A ^b	+++
Mut-10	C116A	+++
Mut-11	C130A ^b	+++
Mut-12	C382A	+++
Mut-14	C481A ^b	+++
Mut-15	C492A ^b	+++
4Mut	C56A,C64A,C130A,C193A	++
7Mut	C56A,C64A,C116A,C130A,C193A,C481A,C492A	NS ^c
MutN	C20S,C23A,C56A,C64A,C94S,C95A,C116A,C130A,C193A,C233A	NS
MutC	C298A,C315A,C382A,C481A,C492A	++
ΔN	Δ1-30	NS
Mut-7	Δ481-503	NS

^a “+++” indicates 80–100% of wild type specific activity; “++” indicates 30–80% of wild type specific activity; “—” indicates <2% of wild type specific activity. See also Table 2. ^b Specific activities estimated from assays of crude lysates of recombinant *E. coli* cells. The concentration of the transferase is readily estimated by Coomassie staining (Li & Pickart, 1995). ^c NS, not soluble (hence activities of these enzymes could not be determined).

Table 2: Binding of ¹²⁵I-PAsO to Mutant Enzymes^a

name	mutation(s)	sp act. (% of wt)	pmol E	¹²⁵ I-PAsO bound		stoichiometry (mol I/mol E)
				cpm	pmol	
Mut-2	C23A	~1%	18.8	6322	13.3	0.71
Mut-13	C20S	~1%	20.0	7188	15.2	0.76
Mut-3	C94S,C95A	~1%	19.3	6175	12.9	0.67
Mut-5	C233A	100%	20.0	6678	14.2	0.71
Mut-10	C116A	100%	12.0	5744	12.0	1.0
4Mut	C56A,C64A,C130A,C193A	30%	29.3	11363	24.0	0.82
MutC	C298A,C315A,C382A,C481A,C492A	70%	19.5	6774	14.2	0.73

^a To monitor binding, purified transferase (~1.4 μM) was incubated with 20 μM ¹²⁵I-PAsO (473 cpm/pmol) in 40 mM HEPES (50% base, pH 7.5), 160 mM KCl, 0.4 mM βME, 0.16 mM PMSF, and 0.8 mM EDTA at 37 °C for 6 min (15 μL volume). After quenching with an equal volume of SDS–PAGE sample buffer (lacking βME), the mixture was loaded onto a 10% SDS gel. The gel was stained with Coomassie, dried, and autoradiographed. The band corresponding to the transferase–¹²⁵I-PAsO complex was excised and counted.

Table 3: Irreversible Inactivation of Mutant Enzymes by Bifunctional Arsenoxide

name	mutation(s)	rel act. ^a (% control)	observed activity (cpm) ^b		% inactivation by BrAcNPAsO
			–BrAcNPAsO	+BrAcNPAsO	
Mut-2 ^c	C23A	0.6	17 162	1587	95
Mut-3 ^c	C94S,C95A	0.6	17 652	2218	88
Mut-13 ^c	C20S	0.6	17 351	457	97
4Mut ^c	C56A,C64A,C130A,C193A	140	87 163	9728	89
MutC ^d	C298A,C315A,C382A,C481A,C492A	106	65 899	7568	89
Mut-10 ^d	C116A	81	50 038	4758	90
Mut-5 ^e	C233A	72	26 004	1582	94

^a The specific activities of 4Mut, MutC, Mut-10, and Mut-5 were averaged. The specific activities in the assays without BrAcNPAsO are expressed relative to this average. ^b Transferase activity expressed as [³H]Arg incorporation into rcmBSA (Experimental Procedures). ^{c,d} Enzyme (6.9 μM) was incubated with 16 μM BrAcNPAsO in 50 mM HEPES (pH 7.5), 0.5 mM βME, 0.35 M KCl, 1 mM EDTA, and 0.2 mM PMSF. After 5 min at 37 °C, the reaction was quenched with 4 mM DTT, either without dilution, or by making a 50-fold dilution^d into 50 mM HEPES (pH 7.5), 1 mM EDTA, 0.1 M KCl, and 4 mM DTT. An aliquot of 2 μL was then withdrawn for assay (Experimental Procedures). ^e Enzyme (1.6 μM) was incubated with 6.8 μM BrAcNPAsO at 37 °C in 50 mM HEPES (pH 7.5), 1 mM EDTA, 0.5 mM βME, and 0.1 M KCl. After 4 min, the incubation was quenched with 5 mM DTT and diluted 20-fold before assaying for transferase activity.

strongly suggest that Cys residues do not mediate the binding of ¹²⁵I-PAsO to the transferase.

A potential caveat in the interpretation of the results shown in Table 2 is that, in the mutant enzymes, ¹²⁵I-PAsO could be binding to a “new” site resulting from a mutation-induced structural change. Arguing against this possibility is the finding that the activity of each mutant enzyme, including

those with very low activity, remained sensitive to irreversible inactivation by BrAcNPAsO (Table 3). With the wild type enzyme, the *para*-alkyl halide moiety of this bifunctional reagent alkylates a nucleophile proximal to the arsenoxide binding site (Stevenson et al., 1978; Li & Pickart, 1995). Irreversible inactivation of the transferase by BrAcNPAsO is strictly dependent upon the presence of the arsenoxide

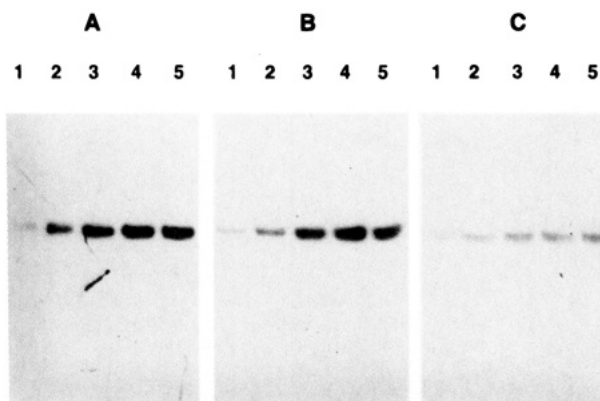


FIGURE 3: BrAcNPAsO blocks binding of ^{125}I -PAsO to the transferase (autoradiograph). Transferase (fully active enzyme Mut-6 = C298A, $6.9 \mu\text{M}$) was incubated in the absence of arsenoxide (panel A), in the presence of $16 \mu\text{M}$ PAsO (panel B), or in the presence of $16 \mu\text{M}$ BrAcNPAsO (panel C) at 37°C in 50 mM HEPES (pH 7.5), 0.36 M KCl, 0.5 mM βME , 1 mM EDTA, and 0.2 mM PMSF. After 4 min, the mixture was diluted 4-fold into 50 mM HEPES (pH 7.5), 0.1 M KCl, 1 mM EDTA, 0.2 mM PMSF, and 0.5 mM βME . An aliquot ($12 \mu\text{L}$) of the dilution was withdrawn and further incubated with different concentrations of ^{125}I -PAsO (650 cpm/pmol) at 37°C for 6 min (lane 1, $2 \mu\text{M}$; lane 2, $4 \mu\text{M}$; lane 3, $10 \mu\text{M}$; lane 4, $20 \mu\text{M}$; lane 5, $50 \mu\text{M}$). The mixture was then quenched with SDS-PAGE sample buffer (lacking βME) and loaded onto a 10% SDS-PAGE gel.

moiety and is accompanied by covalent incorporation of 1 mol BrAcNPAsO/mol E (Li & Pickart, 1995). The data shown in Table 3 indicate that the bifunctional arsenoxide bound to a catalytically relevant site in each of the mutant enzymes. To test definitively whether ^{125}I -PAsO bound to the same functionally relevant site in the inactive mutant enzymes, we tested whether prior modification of these enzymes with BrAcNPAsO blocked the binding of ^{125}I -PAsO.

Figure 3 shows the positive control for these studies. In this experiment, a fully active transferase enzyme (Mut-6, C298A, Table 1) was preincubated with a saturating concentration of BrAcNPAsO and then diluted into incubations containing increasing concentrations of ^{125}I -PAsO. Pretreatment with BrAcNPAsO almost completely blocked binding of even high concentrations of ^{125}I -PAsO (Figure 3, panel A vs C). This was due to covalent alkylation of the enzyme by BrAcNPAsO, since preincubation with PAsO failed to block binding of all but the lowest concentrations of ^{125}I -PAsO (Figure 3, panel A vs B). Moreover, covalent reaction of BrAcNPAsO required the presence of arsenoxide moiety, since pretreatment with BrAcAn failed to block ^{125}I -PAsO binding (lanes 1 vs 2, Figure 4). BrAcNPAsO reacts with the transferase at a specific site that has been partially mapped (below). The results shown in Figures 3 and 4 thus indicate that ^{125}I -PAsO binds with similar specificity to the same site. BrAcNPAsO also reacts with 1:1 stoichiometry (Li & Pickart, 1995), consistent with the stoichiometry observed for ^{125}I -PAsO (Figure 1B). Finally, the finding that modification of the transferase with BrAcNPAsO completely blocks subsequent binding of ^{125}I -PAsO excludes the unlikely possibility that the SDS-PAGE assay monitors binding at a functionally irrelevant site resulting from rearrangement in SDS.

Repeating this experiment with the three inactive mutant enzymes gave results similar to those obtained with the active enzyme (Figure 5 vs Figure 3). Taken together, these results

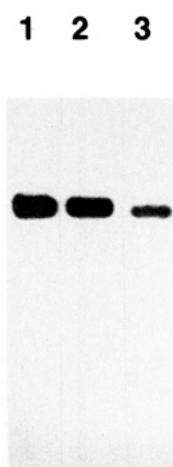


FIGURE 4: BrAcAn does not block binding of ^{125}I -PAsO to the transferase (autoradiograph). Transferase (fully active enzyme Mut-5 = C233A, $1.4 \mu\text{M}$) was incubated alone (lane 1), with $3 \mu\text{M}$ BrAcAn (lane 2), or with $3 \mu\text{M}$ BrAcNPAsO (lane 3) in 50 mM MOPS (pH 7.2) and 1 mM EDTA ($10 \mu\text{L}$ volume). After 5 min on ice, ^{125}I -PAsO (700 cpm/pmol) was added to a concentration of $5.5 \mu\text{M}$. After 5 min incubation at 37°C , incubations were quenched with SDS-PAGE sample buffer (lacking βME) and loaded onto a 10% SDS gel.

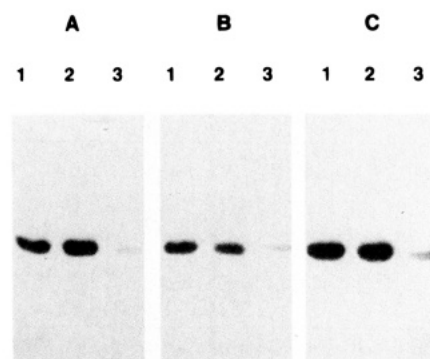


FIGURE 5: Bifunctional but not monofunctional phenylarsenoxide blocks ^{125}I -PAsO binding to inactive mutant enzymes (autoradiographs): (A) Mut-2 (C23A); (B) Mut-3 (C94S, C95A); (C) Mut-13 (C20S). The procedures were the same as described in the legend to Figure 3, except that only one (saturating) concentration of ^{125}I -PAsO ($20 \mu\text{M}$), was used in the final incubation. Lane 1, enzyme preincubated alone; lane 2, preincubated with a saturating concentration of PAsO; lane 3, preincubated with a saturating concentration of BrAcNPAsO.

indicate that arsenoxide binds at the same site in the inactive and active enzymes. This site is identical to that occupied by BrAcNPAsO. In the wild type enzyme, and by extension in the mutant enzymes, BrAcNPAsO binds in a highly specific fashion, as shown by the finding that bound BrAcNPAsO alkylates a weakly nucleophilic Glu side chain when the primary site of alkylation is eliminated by making a Cys-to-Ala mutation at position 315 (Li & Pickart, 1995). On the basis of these considerations, we conclude that arsenoxide binding is not mediated by Cys residues of the transferase (see Discussion).

The Transferase Lacks Thiol-Containing Cofactors. Given the unexpected nature of this conclusion, we considered whether the transferase might possess thiol groups not documented in its primary structure. Thiol-containing prosthetic groups can be sites of arsenoxide binding to enzymes, for example, as seen in pyruvate dehydrogenase (Stevenson et al., 1978). It is unlikely that the transferase

contains lipoamide, since this cofactor is usually associated with mitochondrial enzymes in eukaryotes and moreover has a redox function in all known cases. The transferase, in contrast, is cytosolic and catalyzes an acyl-transfer reaction. Also, the transferase sequence lacks any region with similarity to known lipoamide attachment sites (Wang et al., 1993). Another thiol-containing prosthetic group, phosphopantetheine, has been localized in the center of bacterial multifunctional peptide antibiotic synthetases (Schlumbohn et al., 1991; Stindl & Keller, 1993). Phosphopantetheine is also present in *E. coli* EntF, a protein involved in biosynthesis of the cyclic siderophore enterobactin (Rusnak et al., 1991). In actinomycin synthetase II, Ser-linked 4'-phosphopantetheine functions as a swinging arm that transfers the peptide intermediate (Stindl & Keller, 1993). In principle, a "swinging arm" could assist in transferase-catalyzed aminoacylation. However, the transferase sequence does not contain any region with similarity to known phosphopantetheine attachment sites (Rusnak et al., 1991; Wang et al., 1993). More significantly, the standard microbiological assay (Pugh & Wakil, 1965) failed to detect phosphopantetheine in the transferase (Experimental Procedures).

To confirm the above conclusions and to test for the presence of an uncharacterized prosthetic group, the molecular mass of the transferase was determined by electrospray ionization mass spectrometry. The observed molecular mass, 57 888 Da, was very close to the calculated molecular mass of 57 850 Da. The 38-Da difference may be due to an associated potassium ion, since KCl was used to elute the enzyme from the anion exchange column during the last step of purification. The estimated accuracy of the molecular mass determination was ± 100 Da. The expected minimum increment in molecular mass would be 190 for lipoic acid, 302 for phosphopantetheine, and 352 for an iron-sulfur cluster. [An iron-sulfur cluster has been implicated in arsenite binding to a molybdenum-containing redox enzyme (Barber & Siegel, 1983).] Combining the ^{125}I -PAsO binding data for the mutant enzymes (Table 3) with the mass spectrometric data, we conclude that PAsO does not bind in an obligatory fashion to sulfhydryl groups derived either from Cys residues or from a prosthetic group.

Chemically Vicinal Thiols Do Not Mediate ^{125}I -PAsO Binding to the Transferase in 8 M Urea. Even though Cys residues of the transferase apparently did not mediate arsenoxide binding under native conditions (above), it was possible that a more conventional mechanism would apply in arsenoxide binding to previously denatured enzyme. Studies on disulfide bond formation in small Cys-Ala_n-Cys peptides in a denaturing solvent showed that both Cys-Cys and Cys-Ala₂-Cys form disulfide bonds; the disulfide in Cys-Ala₂-Cys was the most stable among all tested (Zhang & Snyder, 1989). Thus, in the denatured transferase, both "chemically vicinal" thiol pairs of the transferase (Cys-20-Cys-23 and Cys-94-Cys-95) would appear to have potential to bind phenylarsenoxide. A stoichiometry of 2:1 would be expected if both putative sites were occupied.

This issue was addressed using the SDS-PAGE assay, which permits binding to be monitored in the absence of activity. The results showed that the transferase bound ^{125}I -PAsO in 8 M urea, with only slightly reduced affinity relative to native conditions (Figure 2, panel B vs A). This is consistent with our finding that the enzyme binds to PAsO-Sepharose in 8 M urea (not shown). These results suggest

Table 4: Binding of ^{125}I -PAsO to Mutant Enzymes in 8 M Urea^a

name	mutation(s)	activity (% wt)	bound ^{125}I -PAsO	
			cpm	mol I/mol E
Mut-1	C315A	100	12850	0.71
Mut-2	C23A	~1	14386	0.80
Mut-3	C94S, C95A	~1	12907	0.72

^a Mutant enzymes (2.3 μM) were incubated with 7.5 μM ^{125}I -PAsO (600 cpm/pmol) in 8 M urea, 20 mM MOPS (pH 7.2), and 0.1 mM βME at 37 °C (13 μL volume). After 10 min, the incubation was quenched with SDS-PAGE sample buffer (lacking βME) and loaded onto a 10% SDS gel. The gel was stained with Coomassie blue, and the transferase band was excised and counted.

either that the arsenoxide-binding residues are in close apposition in the primary sequence or that the transferase is not fully denatured in 8 M urea. While the conformational state of the transferase in 8 M urea has not been assessed, the enzyme is known to be completely inactivated by 4 M urea (Li & Pickart, 1995). The binding stoichiometry observed in urea was ~1:1 (Figure 2B). Failure to observe a 2:1 stoichiometry could indicate that the complex involving Cys-20-Cys-23 was more stable than that involving Cys-94-Cys-95 (or vice versa). However, mutational disruption of either of pair (C23A; C94S, C95A) failed to eliminate ^{125}I -PAsO binding in urea (Table 4). Therefore, as seen under native conditions (above), the E·I complex formed under denaturing conditions is unlikely to involve vicinal thiols. It is likely, but not proven, that binding occurs at the same site in denatured and native enzyme.

Relation to Previous Results. We showed previously that the transferase is inactivated by NEM with $k_2 = 4500 \text{ M}^{-1} \text{ min}^{-1}$, indicating the presence of one or more essential thiols (Li & Pickart, 1995). The mutagenesis data revealed that several Cys residues are indeed important for activity (Table 1). If NEM and phenylarsenoxides interacted with a common essential thiol, there might be protection against irreversible inactivation by NEM when the enzyme was complexed to phenylarsenoxide. Such behavior is seen with lecithin-cholesterol acyl transferase (Jauhainen et al., 1988) and with ubiquitin conjugating enzyme E2-230K.² However, a saturating concentration of PAsO did not alter the rate of inactivation by NEM (Li & Pickart, 1995), even though PAsO did not dissociate from the enzyme on the time scale of this experiment (above). The simplest of the several possible explanations for this lack of protection, that the ligands of bound PAsO are not thiols, is fully consistent with the mutagenesis data presented above. In preliminary studies, we failed to obtain any evidence that bound PAsO protected specific thiols against reaction with ^{14}C -NEM.³ This result is also consistent with the conclusion that thiols do not mediate PAsO binding.

DISCUSSION

Site-Specific Mutagenesis: Implications for Mechanism. We used a simple PCR procedure to mutagenize each of the 15 Cys residues of the transferase to Ala (13 Cys) or Ser (2 Cys). Three or four Cys residues (Cys-20, Cys-23, Cys-94, and/or Cys-95), all fairly close to the N-terminus, proved to be important for activity. Interestingly, the corresponding

² E. Berleth and C. Pickart, manuscript in preparation.

³ J. Li and C. Pickart, unpublished experiments involving cleavage of radiolabeled enzyme with CNBr or trypsin.

mutant enzymes (C20S, C23A, and C94S,C95A) all exhibited low but detectable activity, ~1% relative to wild type enzyme (Table 2). The importance of these Cys residues for catalytic activity is unlikely to derive from their involvement in disulfide bond(s), since the transferase is fully active in the presence of 5 mM DTT (Li & Pickart, 1995) and moreover is a cytosolic enzyme. Nor is any of these Cys residues likely to function as an obligatory nucleophilic element (i.e., site of formation of an arginyl thiol ester), since repeated efforts have failed to reveal any credible arginyl-enzyme intermediate in the transferase reaction.⁴ Moreover, if any of these Cys residues functioned as nucleophilic elements, the mutations would probably have decreased activity by more than 100-fold [e.g., Carter and Wells (1988)]. It is unlikely that these mutant enzymes utilize a different mechanism than the wild type enzyme, both *a priori* and because the residual activity remains arsenoxide-sensitive (Table 3). Further work will be necessary to understand the functions of these Cys residues.

Mechanistic Basis of Arsenoxide Inhibition. Our previous results suggested that irreversible inactivation by the bifunctional reagent BrAcNPAsO was due to steric blockade of a functionally important site by the bulky phenyl group. Thus, mutagenesis of the primary alkylation site, Cys-315 (to Ala), had no effect on enzyme activity, indicating that the alkylated residue did not serve a catalytic function (Li & Pickart, 1995). Steric blockade of an essential site may similarly underlie reversible inhibition by the monofunctional reagent PAsO and its *para*-iodo derivative. The mechanistic function of this site remains unclear. Phenylarsenoxides do not appear to inhibit by competing with substrates, since high concentrations of rcMBSA and tRNA did not diminish the binding of ¹²⁵I-PAsO (not shown).

Mechanism of Arsenoxide Binding. ¹²⁵I-PAsO and BrAcNPAsO bound to the transferase at the same site, with ~1:1 stoichiometry in each case (Figures 3 and 5; Li & Pickart, 1995). These two results are most simply explained if there is a single phenylarsenoxide binding site on the transferase. Surprisingly, no Cys residue proved to be essential for phenylarsenoxide (or BrAcNPAsO) binding (Tables 2 and 3). Our mutagenesis was rationalized on the assumption that arsenoxides bind to vicinal thiols (see the introduction). We thus began with the expectation that single mutation of *each* of the two vicinal Cys residues would eliminate binding. The failure of *any* Cys mutation to affect binding is thus a very strong negative result. However, given the unexpected nature of the conclusion that thiols do not mediate binding, several potential caveats need to be considered.

One way in which our results could be misleading would be if there were two negatively interacting arsenoxide binding sites. In this case, the 1:1 stoichiometry could result from mutually exclusive binding, and removal of one site by mutagenesis might allow the other to be occupied. If this rather complex model is correct, then each of the two sites must have a proximal nucleophile, since in no case did Cys mutagenesis abolish irreversible reaction of the bifunctional reagent BrAcNPAsO (Table 3). However, labeling by BrAcNPAsO defined a single locus (Li & Pickart, 1995).

A second potential complication derives from the nature of two of the mutations. Small-molecule studies in the gas

phase indicate that a hydroxyl and a sulfhydryl have some potential to form a ring structure with phenylarsenoxide (Hannestad & Sorbo, 1980). Such complexes must be very weak in solution, since modest concentrations of mercaptoethanol do not relieve inhibition of the transferase or other arsenoxide-inhibited enzymes. However, these considerations raise the possibility that mutating Cys to Ser might not abolish binding. In our studies, Cys-20 and Cys-94 were mutated to Ser (Table 2). Thus if these two residues constituted the vicinal Cys pair, the C20S and C94S,C95A mutant enzymes could have given misleading binding results. However, it seems unlikely that changing the arsenoxide ligand from a thiol to a hydroxyl would not change binding affinity. Thus, we tentatively exclude this model on the basis of the finding that the ¹²⁵I-PAsO titration curves of the C20S and C94S,C95A mutant enzymes were similar to that of the wild type enzyme (not shown).

A final possibility is that there is redundancy in the abilities of Cys residues of the transferase to bind arsenoxide. In a complex situation where several Cys residues are spatially vicinal and any two of them can bind arsenoxide, single-Cys mutations might fail to reveal an essential role for Cys residues in binding. This possibility cannot be rigorously excluded, but is unlikely. Thus, several multiple mutants retained full binding capacity (Table 2). Furthermore, the high specificity with which BrAcNPAsO interacts with the transferase (Li & Pickart, 1995) tends to argue against much flexibility in the transferase-arsenoxide interaction.

Our unexpected results challenge the traditional view of the mechanism of enzyme inhibition by arsenoxides. While there is no question that vicinal thiols interact strongly with arsenoxides, we note that there is actually little direct evidence to support the proposal that vicinal thiols are the only, or even the predominant, site of arsenoxide binding to proteins. The thermodynamic stability of the cyclic dithiol-arsenite complex provides the basis for reversal of enzymic inhibition by chemical dithiols (see the introduction). But relief of inhibition is in fact a mechanistically neutral observation: as long as binding to the enzyme is reversible, a sufficient concentration of dithiol will relieve inhibition by thermodynamic competition, independent of how the arsenoxide binds to the enzyme. Nonetheless, for many arsenoxide-interacting proteins, reversal of the interaction by DTT is the sole basis for postulating the existence of vicinal thiols. Further characterization of arsenoxide binding to the transferase should provide insight into the spectrum of binding modes available to arsenoxides.

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⁴ J. Li and C. Pickart, unpublished experiments.

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