Inactivation of Arginyl-tRNA Protein Transferase by a Bifunctional Arsenoxide: Identification of Residues Proximal to the Arsenoxide Site†

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ABSTRACT: Aminoacyl-tRNA protein transferases catalyze (posttranslational) aminoacylation of specific protein N-termini, using aminoacyl-tRNA as substrate. This modification targets the protein for ATP-dependent degradation; in eukaryotes, degradation occurs in the ubiquitin-mediated pathway. The eukaryotic transferase, which catalyzes Arg transfer to N-terminal Glu or Asp residues, is potently inhibited by phenylarsenoxides. The gene encoding Arg-tRNA protein transferase from the yeast *Saccharomyces cerevisiae* was subcloned and overexpressed in *Escherichia coli* to provide large amounts of homogeneous protein for a molecular analysis of this inhibition. The bifunctional reagent *para*-[(bromoacetyl)amino]-phenylarsenoxide is a potent and irreversible inactivator of the yeast transferase; the arsenoxide moiety of the reagent directs binding to the enzyme, while the alkyl halide moiety alkylates a residue(s) proximal to the arsenoxide site. One mole of ^{14}C-labeled reagent was covalently incorporated during inactivation, with the side chain of Cys-315 representing the major site of alkylation. Mutation of Cys-315 to Ala yielded a fully active enzyme which was still subject to stoichiometric, irreversible inactivation by the bifunctional arsenoxide. With the C315A-enzyme, the major fraction of the ^{14}C-labeled bifunctional reagent was associated with the side chain(s) of one or more of a stretch of Glu residues (Glu 339−341). These results show that phenylarsenoxides inhibit Arg-tRNA protein transferase by binding to a site that is either itself essential, or regulates an essential site. Inhibition appears to occur through a steric blockade mechanism.

Aminoacyl-tRNA protein transferases catalyze the transfer of specific amino acids from charged tRNA to N-termini of suitable proteins or peptides. In the resulting products, the amino acid is linked by a normal peptide bond to the substrate's α-amino group (Kaji, 1968; Soffer, 1970). Although aminoacyl-tRNA protein transferases were first characterized more than 20 years ago, only recently has a physiological function been described for them, involving intracellular proteolysis (below). The transferases remain poorly understood at the level of structure and mechanism.

The half-life of a protein can be related to the identity of its N-terminal residue through one of two hierarchical N-end rules (Bachmair et al., 1986; Tobias et al., 1991). In each rule, N-terminal residues are classified as stabilizing or destabilizing, with the latter residues falling into several subgroups (Varshavsky, 1992). In eukaryotes, amino acids with basic (Arg, Lys) or bulky hydrophobic side chains are primary destabilizing residues (Bachmair et al., 1986). Asp and Glu are secondary destabilizing residues because they direct N-terminal arginylation by Arg-tRNA protein transferase (Soffer, 1970; Bachmair et al., 1986; Ferber & Ciechanover, 1987). Asn and Gln are tertiary destabilizing residues; they are deamidated to expose the secondary destabilizing residues Asp and Glu (Gonda et al., 1989). A different N-end rule pertains in *Escherichia coli*, where Leu/Phe-tRNA transferase conjugates Leu or Phe (primary destabilizing residues) to N-terminal Arg or Lys (secondary destabilizing residues) (Liebowitz & Soffer, 1970; Tobias et al., 1991; Shrader et al., 1993). The difference in the identities of primary and secondary destabilizing residues in bacteria *versus* eukaryotes is consistent with the distribution of aminoacyl-tRNA transferases: Arg-tRNA transferase is confined to eukaryotes, while Leu/Phe-tRNA transferase is present in Gram-negative bacteria, but is absent from eukaryotes (Deutsch, 1984; Soffer & Savage, 1974).

The specific role of Arg-tRNA protein transferase in eukaryotic proteolysis is to make its targets susceptible to ubiquitin conjugation. In eukaryotic cells, a specific ligase (E3) recognizes proteins bearing primary destabilizing N-terminal residues and conjugates ubiquitin to the side chain of an internal Lys residue of the target [Reiss et al., 1988; Gonda et al., 1989; reviewed in Hershko and Ciechanover (1992)]. Proteins with acidic N-terminal residues are recognized by this E3 only following arginylation by Arg-tRNA transferase (Gonda et al., 1989; Elias & Ciechanover, 1990). Following ubiquitination, the target is degraded by the ATP-dependent 26S proteasome (Hershko & Ciechanover, 1992). N-end rule proteolysis in bacteria is mediated by the ATP-dependent Clp protease, but the mechanistic function of N-terminal aminoacylation in bacteria is unknown (Tobias et al., 1991). In both eukaryotes and bacteria, N-end rule components seem to mediate selective rather than bulk proteolysis (Bartel et al., 1990; Geuskens et al., 1992).

Trivalent arsenoxides are considered to bind specifically to vicinal thiol groups in proteins (Whitaker, 1947; Stevenson et al., 1978). We showed previously that several enzymes within the mammalian N-end rule proteolytic pathway are

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sensitive to trivalent arsenoxides (Klemperer & Pickart, 1989; Berleth et al., 1992a). Among these enzymes is Arg-tRNA transferase: mammalian and yeast transferases are both irreversibly inactivated by a bifunctional phenylarsenoxide bearing a *para*-alkyl halide substituent (Berleth et al., 1992b). This latter finding suggests that vicinal thiol groups and a reactive proximal nucleophile are conserved elements of transferase structure and/or mechanism. In this report we use radiolabeled bifunctional reagent and purified recombinant enzyme to identify residues that are proximal to the arsenoxide binding site of Arg-tRNA protein transferase from *Saccharomyces cerevisiae*.

EXPERIMENTAL PROCEDURES

Materials. The following were purchased from the indicated sources: L-[2,3-^{3}H]Arg (53 Ci/mmol; Dupont-NEN); crude *E. coli* tRNA (Sigma); crude mixture of *E. coli* aminoacyl-tRNA synthetases (Sigma); PAsO[1] (Aldrich). BrAcAn, NPAsO, and BrAcNPAsO were synthesized by modifications of published procedures as described (Berleth et al., 1992a). RcmBSA was synthesized as described (Evans & Wilkinson, 1985). The vector pTH4+, containing the yeast Arg-tRNA transferase gene (Balzi et al., 1990), was provided by A. Varshavsky (Cal Tech).

Arg-tRNA Transferase Expression Vector. PCR was used to amplify the putative yeast transferase coding sequence and simultaneously introduce unique *Nco*I and *Bgl*II sites at the the 5′ and 3′ ends, respectively (Balzi et al., 1990). The two oligonucleotide primers were (1) forward primer, 5′-GGGAGATCTCCATGGCCGATAGATTCGTTA-3′ and (2) reverse primer, 5′-GGGAGATCTCAGCGCGATGGTGAG-3′. The underlined sequences are complementary to the transferase gene (Balzi et al., 1990). Introduction of the 5′-restriction site caused a mutation at residue two, from Ser to Ala. As described in Results, this mutation had no effect on activity, and the transferase expressed from this gene is referred to as "wild type". The PCR reaction mixture contained (100 μL): 500 ng of each primer, 150 ng of template DNA (4 kb *Hind*III fragment of the transferase gene), 2.5 units of pfu DNA polymerase (Stratagene), 100 μM dNTPs, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM ammonium sulfate, 0.1% Triton X-100, and 10 ng of nuclease-free BSA. Amplification involved 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 4 min at 75 °C. The reaction yielded a single product of the expected size, ~1550 bp. This fragment was gel-purified and cloned into the *Sma*I site of pUC19 to give the vector pUC19-ATT. The latter vector was cut with *Nco*I and *Bgl*II, and the 1550 bp product was cloned into the expression vector pET3d (Studier et al., 1990) which had previously been digested with *Nco*I and *Bam*HI. The fidelity of the coding sequence of this vector, pET3d-ATT, was confirmed by PCR sequencing (fmol kit, Promega).

[1] Abbreviations: BSA, bovine serum albumin; BrAcAn, bromoacetyl aniline; BrAcNPAsO, *para*-[(bromoacetyl)amino]phenylarsenoxide; DTT, dithiothreitol; E, Arg aminoacyl-tRNA protein transferase; I, BrAcNPAsO; IPTG, isopropyl β-D-thiolgalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonate; NEM, *N*-ethyl maleimide; NPAsO, aminophenylarsenoxide; PAGE, polyacrylamide gel electrophoresis; PAsO, phenylarsenoxide; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; rcmBSA, reduced and carboxymethylated bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; UV, ultraviolet.

Expression of Transferase in E. coli. The plasmid pET3d-ATT was used to transform *E. coli* strain BL21(DE3)/pLysS (Moffatt & Studier, 1987). Transformants were grown in 2xYT medium with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL) at 37 °C to A_{600} = 0.6−0.8. The suspension was then cooled on ice for 15 min. IPTG, 100 μM, was added, and growth was continued at 18 °C for 36 h more. Bacteria were harvested by centrifugation (3000*g*, 20 min) and pellets frozen at −20 °C.

Transferase Purification. Frozen cells were suspended in lysis buffer (50 mM Tris-HCl (5% base), 10% glycerol, 0.2 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, and 20 μg/mL leupeptin), using 3 mL/g cells. The suspension was incubated at room temperature until the cells were lysed as judged by visual inspection. DNase I (20 μg/mL) and $MgCl_2$ (1 mM) were added, and the suspension was incubated for 20 min (RT). The suspension was centrifuged (150000*g*, 30 min) and the supernatant brought to 50% saturation with solid ammonium sulfate. The ammonium sulfate pellet was dissolved in 50 mM potassium phosphate (pH 7.4), 10% glycerol, 0.2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. After dialysis against the same buffer, the solution was loaded onto a 2.5 × 15 cm column of hydoxylapatite (Bio-Rad, ~10 mg of protein/mL of resin). The column was developed with a 300 mL linear gradient of phosphate buffer (50−600 mM, pH 7.4), in 10% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM DTT; the transferase eluted at ~400 mM. Fractions enriched in transferase protein were pooled and exchanged into a buffer of 50 mM Tris (5% base), 10% glycerol, 0.2 mM EDTA, and 0.5 mM PMSF, by ammonium sulfate precipitation and dialysis. The protein concentration was adjusted to ~1 mg/mL, and the solution was loaded onto a 1 mL FPLC Mono-Q column (Pharmacia-LKB Biotech). The transferase was eluted with a two-step linear gradient at 1 mL/min, in a buffer of 50 mM Tris-HCl (5% base), 0.2 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol, and 0.5 mM PMSF (50−125 mM KCl over 5 min, followed by 125−200 mM KCl over the next 25 min). Fractions were monitored by absorbance at 280 nm and by SDS−PAGE (Laemmli, 1970). Fractions containing the transferase were pooled and stored in small aliquots at −60 °C. C315A-transferase (below) was similarly expressed and purified.

Transferase Assay. Incorporation of [^{3}H]Arg into rcmBSA was monitored as the appearance of radioactivity insoluble in TCA (Klemperer & Pickart, 1989). [^{3}H]Arg tRNA was first generated in a precincubation containing: 50 mM Tris (24% base), 5 mM $MgCl_2$, 2 mM ATP, 10 mM phosphocreatine, 0.6 unit/mL each of creatine phosphokinase and pyrophosphatase, 1.5 mg/mL *E. coli* tRNA, 30 μCi/mL [^{3}H]-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 μL of this mixture was added to 4 μL of a second solution containing the transferase and 1 mg/mL rcmBSA (final concentration). After 5 min at 37 °C, the incubation was quenched with 8 μL of 8 M urea, followed by processing as described (Klemperer & Pickart, 1989). All assays were quenched during the linear phase of the reaction. Data were corrected by subtracting a blank obtained by omitting the transferase. Electrophoresis and fluorography of assay incubations showed that >95% of protein-associated radioactivity migrated at the position of rcmBSA.

Table 1: Stoichiometric Transferase Inactivation by [^{14}C]BrAcNPAsO[a]

enzyme	pmol E	[^{14}C]BrAcNPAsO incorporated		stoichiometry (mol I/mol E)
		10^{-3} × cpm	pmol	
wild type	1035	11.6	1115	1.10
wild type	1035	9.4	895	0.87
C315A	690	5.7	545	0.79
C315A	604	6.4	610	1.0

[a] [^{14}C]BrAcNPAsO (55 μM, 10.5 cpm/pmol) was incubated with the indicated amount of enzyme for 10 min (pH 7.5, 37 °C; total volume, 35 μL). Appropriate controls showed that the enzyme was completely and irreversibly inactivated in each case. Details are given in Experimental Procedures.

PCR Mutagenesis. Cys-315 was mutated to Ala by a two-step PCR method (Landt et al., 1990). The first PCR reaction was carried out as described above, using the same reverse primer and a forward primer encoding the desired mutation (5′-ACTGGATGGCTCACAGTAAATATA-3′). The ~670 bp PCR product was gel-purified and used as the reverse primer in the second PCR reaction (~100 ng/mL); the forward primer was the same as for amplification of the wild-type gene (above; 20 ng/mL). This second PCR reaction was heated to 95 °C for 5 min before addition of pfu polymerase and 30 cycles of 1 min at 95 °C, 2 min at 60 °C, and 4 min at 75 °C. The ~1550 bp product was purified and cloned into pET3d (above). The presence of the desired mutation was verified by DNA sequencing (as above).

Synthesis of Radiolabeled Bifunctional Arsenoxide. This reagent was synthesized as described previously (Adamson et al., 1984) from NPAsO and bromo[1-^{14}C]acetic acid (40 mCi/mmol; Dupont-NEN), except that the ratio of labeled to unlabeled bromoacetic acid was increased (to 1:2.9) to increase the specific radioactivity of the product. The product was purified on a C18 reverse-phase HPLC column, using a linear gradient of acetonitrile developed in water (0−100% in 35 min; 1 mL/min). Absorbance was monitored at 259 nm; fractions at 17−18 min containing a peak of radioactivity and UV absorbance (corresponding to the elution position of unlabeled BrAcNPAsO) were pooled and dried down, and the residue was suspended in methanol. The concentration of [^{14}C]BrAcNPAsO was determined by UV absorbance (Berleth et al., 1992a). Its specific radioactivity was 10.5 cpm/pmol.

Preparative Labeling of Transferase with Bifunctional Arsenoxide. Purified wild-type or mutant transferase was incubated with [^{14}C]BrAcNPAsO (37 °C, 10 min; 35 μL) as follows: 50 mM Tris (5% base), 0.28 M KCl, 10% glycerol, 55 μM [^{14}C]BrAcNPAsO (10.5 cpm/pmol), and transferase protein as indicated in Table 1. The labeled protein was separated from unincorporated radioactivity by precipitation with 15% TCA. The pellet was washed twice with 150 μL of cold acetone, and then dissolved in 50 μL of 0.4 M ammonium bicarbonate and 8 M urea. An aliquot was counted and the remainder digested with trypsin (below).

Isolation of Labeled Peptides. About 1 nmol of [^{14}C]-BrAcNPAsO-labeled wild-type transferase was digested with 2 μg of trypsin in 2 M urea and 0.1 M ammonium bicarbonate (37 °C, 12 h; 200 μL). A 100 μL aliquot of the digestion was loaded onto a C18 (80 Å) reverse-phase HPLC column. The column was developed at 1 mL/min with a linear gradient of acetonitrile in 0.1% TFA (0−10% over 10 min; 10−50% over the next 70 min). Fractions of 1 mL were collected; 100 μL aliquots were counted (here and below, absorbance was monitored at 215 nm). Fractions corresponding to the major radioactive peak were dried down. This procedure was repeated with the second 100 μL aliquot of the trypsin digest. The combined residues of the peak fractions were dissolved in 100 μL of buffer containing 2 M urea and 0.1 M ammonium bicarbonate and digested for 12 h (37 °C) with 53 ng of endoprotease Asp-N (sequencing grade, Boehringer-Mannheim). The Asp-N digest was loaded onto the C18 column and eluted with a linear gradient of acetonitrile in 0.1% TFA, at 1 mL/min (0−40% over 80 min). Fractions (1 mL) were collected, and the peak radioactive fraction was dried down. This material was sequenced (Results). Labeled C315A mutant transferase (labeling as above) was similarly digested with trypsin. The digest was loaded onto a C8 reverse-phase column and eluted at 1 mL/min with a linear acetonitrile gradient developed in 0.1% TFA (0−20% over 10 min; 20−50% over the next 30 min; 50−100% over the last 10 min). Fractions corresponding to the major radioactive peak were dried down and further purified using a C-18 (300 Å) column developed with a linear gradient of acetonitrile in 0.1% TFA, at 1 mL/min (0−40% over 80 min). Recovery of radioactivity and UV absorbance during HPLC was typically ~50%.

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1   MSDRF VIWAP SMHNE PAAKC GYCHG NKGGN MDQLF ALDSW AHRYM NKMDV

51  VKIEN CTIGS FVEHM DVATY DRMCN MGFRR SGKFL YKVDP LRNCC RLYTI

101 RTAPQ ELNMT KELKK CISRF ASRIT SEDYC PAAVA SSDFV GKIVN AEMNS

151 KTFYT RFEPA LYSEE KYHLF VKYQE KVHQD YNNSP KSFKR FLCDT PFGPE

201 AVLGT QESWE QLNNW QRMKP GEKLK MMGPV HECYY YEGKL IRITV SDILP

251 SGISS VYFIW DPDYS KWSLG KLSAL RDLAI IQRTN LQYYY LGYYI EDCPK

301 MNYKA NYGAE VLDVC HSKYI PLKPI QDMIS RGKLF VIGEE ETKVT KELYL

351 VDSET GRGEG FPTDN VVKYK NIAEE IYGVG GCAFK SANES ALELK ELYGI

401 PIEEE DLDTI YHLKE HNGHA PNGIP NVVPG LLPLW ELLDI MQSGK ITDLE

451 GRLFL FEIET EGIRP LINFY SEPPN VKKRI CDVIR LFGFE TCMKA VILYS

501 EQM
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FIGURE 1: Sequence of transferase from *S. cerevisiae*. The protein used in this work had an Ala at position two (Results). Peptides modified by BrAcNPAsO are underlined (see text).

Protein Sequencing. Matrix-assisted laser desorption time-of-flight mass spectrometric analyses were carried out at the Harvard Microchemistry Facility (Chicz et al., 1993). Protein sequence analysis was done at the same facility, using an ABI 477A sequencer coupled to an online PTH-amino acid analyzer.

RESULTS

Transferase Expression and Purification. The yeast transferase gene in the vector pTH4+ (Balzi et al., 1990) was modified by PCR to produce convenient restriction sites for cloning into the *E. coli* vector pET3d, which directs IPTG-inducible expression (Studier et al., 1990; Figure 1). Introduction of the 5′-*Nco*I site caused a Ser to Ala change at residue two. Based on assays of crude lysates of *E. coli* harboring either pTH4+ or pET3d-ATT, this change did not affect enzymatic activity (not shown). For reasons of convenience, we will refer to this S2A-transferase as "wild

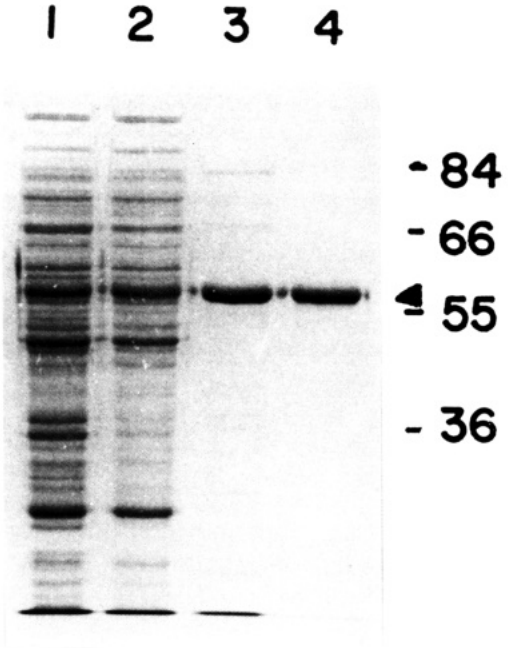

FIGURE 2: Transferase protein purification. Samples of material at different purification stages were run on a 10% SDS gel, followed by Commassie blue staining. (Lane 1) 150000*g* supernatant (~30 μg); (lane 2) 0−50% ammonium sulfate cut (~20 μg); (lane 3) hydroxylapatite peak (~5 μg); (lane 4) Mono Q peak (~4 μg). The arrowhead denotes transferase (58 kDa).

type". Under standard conditions of growth at 37 °C and induction with 400 μM IPTG, all of the recombinant transferase partitioned to inclusion bodies. However, induction at lower temperature and IPTG concentration (18 °C and 100 μM, respectively) resulted in expression of ~60% of the protein in a soluble form. This soluble protein had the expected subunit molecular mass, ~58 kDa, and was purified to homogeneity (Figure 2). Usually 5 to 10 mg of electrophoretically homogeneous protein was recovered from 20 g of cells.

The standard transferase assay involves incubation of the enzyme with presynthesized [^{3}H]Arg-tRNA and an acceptor protein, rcmBSA (N-terminal Asp). In pilot studies we found that the yeast transferase did not discriminate markedly between yeast and *E. coli* Arg-tRNA. Therefore we routinely used *E. coli* tRNA and an *E. coli* aminoacyl-tRNA synthetase mixture for assays. The concentration of rcmBSA in the standard assay, 1 mg/mL, is not fully saturating (not shown), indicating that the K_m of the yeast enzyme for this substrate is higher than that of the mammalian enzyme (K_m ~25 μg/mL for the mammalian enzyme; Berleth et al., 1992b). The rate of Arg transfer catalyzed by the purified yeast enzyme under standard conditions (1 mg/mL rcmBSA) yields a turnover number of ~4 min^{-1}. This may be compared to a value of 8−10 min^{-1} for the rabbit liver enzyme (Soffer, 1970).

Transferase Inhibition by Phenylarsenoxides. Previous qualitative assays carried out in crude lysates of recombinant *E. coli* harboring pTH4+ indicated that the yeast enzyme, like its mammalian counterpart, was arsenoxide-sensitive (Berleth et al., 1992b). Purification of the yeast enzyme permitted a quantitative analysis of this inhibition. As shown by the *open circles* in Figure 3A, the transferase (1.5 μM) was inhibited by PAsO with $K_{0.5}$ ~ 1.2 μM. In this experiment, the transferase was preincubated with the indicated concentration of PAsO for 5 min at 37 °C, further

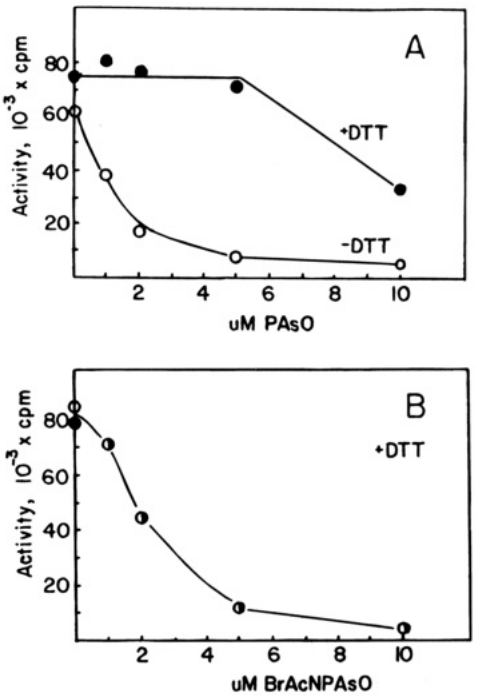

FIGURE 3: Inhibition by phenylarsenoxides. (A) Reversible inhibition by monofunctional arsenoxide (PAsO). Purified wild-type enzyme, 1.5 μM, was preincubated with the indicated concentration of PAsO for 5 min at 37 °C (50 mM MOPS, pH 7.2), diluted 4-fold into a 10-min incubation on ice without (open circles) or with (filled circles) 4 mM DTT, and then diluted 4-fold into a standard assay at 37 °C (Experimental Procedures). (B) Irreversible inactivation by bifunctional arsenoxide (BrAcNPAsO). As described above, wild-type enzyme, 1.5 μM, was preincubated with the indicated concentration of unlabeled (open circles) or ^{14}C-labeled (filled circles) BrAcNPAsO, further incubated with 4 mM DTT for 10 min at 0 °C, and diluted into the assay (dilution factors as above). The lines in each panel have no theoretical basis.

incubated on ice with or without DTT, and then diluted into a standard assay. Appropriate controls showed that the maximum inhibition achievable at a given PAsO concentration was obtained within 5 min at 37 °C. The persistence of inhibition despite the large dilution (16-fold overall) requires that bound PAsO dissociates extremely slowly; consistent with this idea, a PAsO−transferase complex, with a stoichiometry of ~1 mol of arsenoxide/mol of E, is readily isolated by several methods.[2] The concentration dependence of inhibition was intermediate between linear and hyperbolic, probably reflecting very tight binding of PAsO to the relatively high concentration of enzyme (1.5 μM). Under these conditions, the observed value of $K_{0.5}$ is likely to underestimate the true affinity (Cogan et al., 1976), and data obtained at a lower enzyme concentration indeed gave a proportionally lower value for $K_{0.5}$ (not shown). NPAsO inhibited similarly to PAsO ($K_{0.5}$ ~ 2.5 μM at 1.5 μM enzyme; not shown). Thus arsenoxide binding is relatively insensitive to *para* substitution of the phenyl ring.

For concentrations of PAsO below 5 μM, inhibition was fully reversed by excess DTT (filled circles, Figure 3A), but not by a monothiol reagent such as β-mercaptoethanol (not shown). At concentrations of PAsO above 10 μM, inhibition was only partially reversed by DTT (Figure 3A). This may reflect an additional, lower-affinity binding mode, leading to irreversible enzyme aggregation, which becomes signifi-

[2] J. Li and C. Pickart, manuscript in preparation.

cant as the concentration of PAsO strongly exceeds that of enzyme. Alternatively, the DTT−arsenoxide complex may bind in an inhibitory fashion to a lower-affinity site. Selective reversal of inhibition by dithiol reagents is expected if arsenoxides inhibit by binding to vicinal thiols, leading to the formation of a dithioarsenite ring structure (Whittaker, 1947; Webb, 1966; Zahler & Cleveland, 1968; Stevenson et al., 1978; eq 1).

$$E(SH)_2 + R\text{-}As{=}O \underset{k_{off}}{\rightleftharpoons} E\begin{matrix}S\\ \diagup\ \diagdown\\ S\end{matrix}As\text{-}R + H_2O \qquad (1)$$

However, reversal by DTT does not *prove* such a structure for the inhibited enzyme. According to eq 1, DTT acts by binding free arsenoxide and displacing the equilibrium toward uncomplexed enzyme; such an effect of DTT will operate independent of the structure of the inhibited enzyme. Equation 1 predicts that appearance of activity following DTT addition will be rate-limited by arsenoxide dissociation (k_{off}). In the case of the transferase, there is complete reactivation within 10 min of DTT addition (legend, Figure 3), but dissociation of arsenoxide does not occur detectably on a time scale of hours (for example, during gel filtration chromatography).[2] Thus regardless of the structure of the enzyme−arsenoxide complex, the role of DTT in promoting reactivation must be a more active one—perhaps involving ligand exchange—than outlined in eq 1.

The transferase was rapidly inactivated by the thiol-specific alkylating agent NEM. At pH 7.5 and 37 °C, inactivation by 200 μM NEM followed pseudo-first-order kinetics with a half-time of 0.9 min. The pseudo-first-order rate constant was directly proportional to [NEM], yielding a second-order rate constant of 4500 M^{-1} min^{-1}. This high sensitivity to NEM strongly suggests that the transferase has one or more essential thiol groups. To address whether an essential thiol mediates arsenoxide binding, we determined whether PAsO protected against inactivation by NEM. This seemed a reasonable approach, given the slow rate of PAsO dissociation (above). However, preincubation of the transferase (0.34 μM) with PAsO (5 μM) neither prevented irreversible inactivation by NEM (200 μM), nor altered the NEM inactivation rate (not shown).

The failure of bound PAsO to protect against NEM, and the large number of Cys residues of the enzyme (Figure 1), suggested that a selective labeling approach to identification of the Cys residues (presumably) mediating arsenoxide binding would be difficult. However, a bifunctional arsenoxide (BrAcNPAsO) provided a simple method for identifying enzymic residues proximal to the arsenoxide binding site. This reagent has an activated alkyl halide moiety *para* to the arsenoxide moiety (Adamson & Stevenson, 1981). BrAcNPAsO inhibited similarly to the monofunctional reagent PAsO, exhibiting $K_{0.5} \sim 2$ μM at 1.5 μM enzyme (compare open circles, Figure 3 panels B and A). Inactivation by the bifunctional reagent was not reversed by DTT (DTT was present at 4 mM in all the assays shown in Figure 3B). Therefore inactivation by BrAcNPAsO appears to be due to irreversible alkylation of a residue that is itself essential, or resides within an essential site. This site is proximal to the arsenoxide binding site, because BrAcAn, an otherwise identical reagent that lacks the arsenoxide moiety, has no effect on enzyme activity [data not shown; see also Berleth et al. (1992b)]. Thus a specific interaction between the enzyme and the arsenoxide moiety brings the bromacetamido moiety into the essential site.

Identification of Proximal Nucleophile(s). [^{14}C]BrAcNPAsO inhibited the transferase identically to unlabeled BrAcNPAsO (Figure 3B, filled versus open circles). The existence of a specific interaction between the transferase and the bifunctional arsenoxide is shown by the finding that 1 mol of labeled reagent is covalently incorporated during inactivation (average 0.98 mol of I/mol of E, Table 1). As expected if BrAcNPAsO specifically alkylates a residue within the arsenoxide site, identical labeling was seen if treatment with [^{14}C]BrAcNPAsO was carried out in the presence of a 10-fold excess of unlabled BrAcAn (data not shown). Following digestion of the labeled enzyme with trypsin, most (~75%) of the radioactivity was associated with a single peptide (Figure 4A). Digestion of this labeled peptide with endoprotease Asp-N, which cleaves on the N-terminal side of Asp, shifted the labeled species to an earlier elution position (Figure 4B). This indicated the presence of an Asp residue in the original tryptic peptide and provided homogeneous material for protein chemical analysis.

The Asp-N-digested labeled peptide (Figure 4B) was composed of residues 313−318 (Table 2, sample 1). Although residue 1 could not be identified due to contamination with free amino acids, use of Asp-N to generate the peptide confirmed the presence of the N-terminal Asp residue predicted by the protein sequence (Figure 1). The absence of a signal at position 315 (Cys) was expected, since the protein was not reduced and carboxymethylated prior to digestion. However, if a specific residue other than Cys-315 was the predominant site of alkylation by BrAcNPAsO, a strongly reduced yield of the PTH derivative would be expected at this position. The failure to observe such a "blank" suggested that Cys-315 was a major site of alkylation. To confirm this inference, a portion of the product of each cleavage cycle was collected, prior to resolution of the PTH derivatives, during analysis of an independently generated sample. The results showed that two-thirds of the radioactivity was associated with Cys-315 and the remainder with His-316 (Table 2, sample 2).

Matrix-assisted laser desorption time-of-flight mass spectrometric analyses were done on samples 1 and 2. In each case, a peak of 945 ± 1 daltons was detected (data not shown). These results are consistent with the sequence shown in Table 1, assuming that the Cys-linked arsenoxide moiety underwent oxidation (to an arsenate) during the extensive manipulations conducted on the samples. In this case, the mass of the modified peptide should be 946 daltons.

Cys-315 was mutated to Ala by a PCR method (Experimental Procedures). The specific activity of the purified C315A protein was identical to that of the wild-type enzyme (not shown). Since it is highly unlikely that Ala can substitute for Cys in any catalytic function, inactivation of wild-type transferase following alkylation of Cys-315 (Figure 3B) must be due to steric blockade of an essential site.

Site of Alkylation in Mutant Protein. C315A-transferase was inhibited by PAsO in a DTT-reversible fashion; the properties of inhibition were essentially identical to those of the wild-type enzyme (not shown). Surprisingly, C315A-transferase remained susceptible to irreversible inactivation by BrAcNPAsO and was labeled by [^{14}C]BrAcNPAsO with

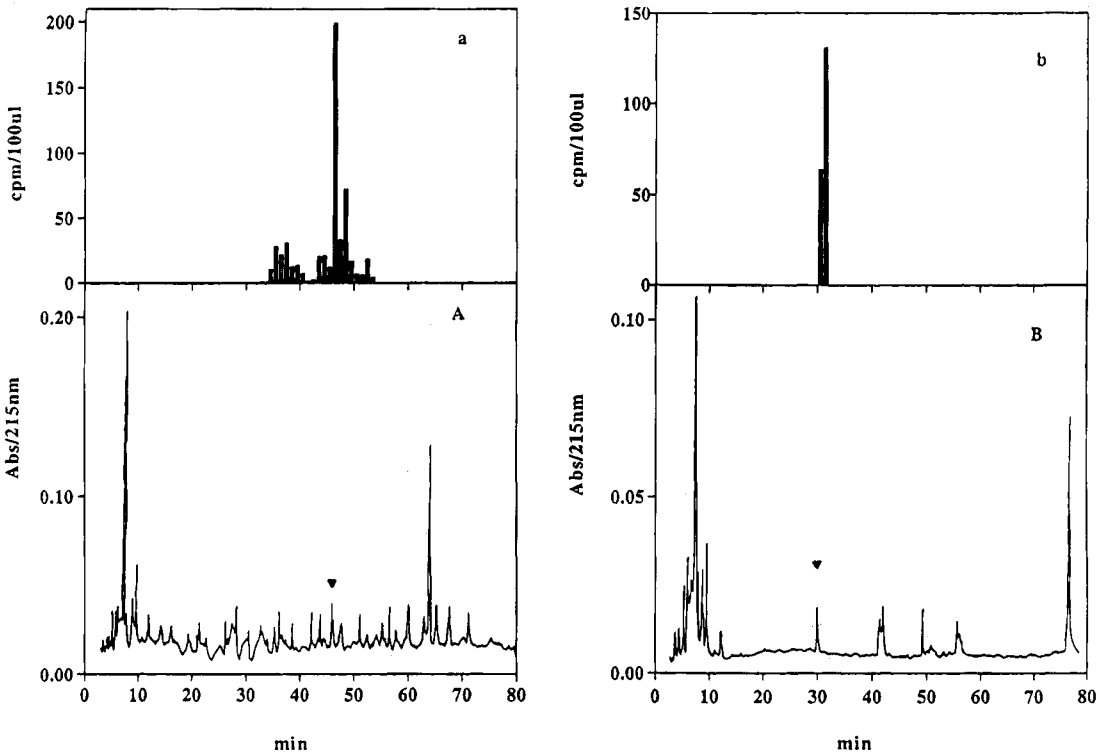

FIGURE 4: Purification of labeled peptide from reaction of wild-type enzyme with [^{14}C]BrAcNPAsO. The data shown constitute the purification of sample 1 in Table 2. (A) First HPLC column. Tryptic digest derived from 500 pmol of labeled enzyme (5000 cpm) was loaded onto the column. (Top) Profile of radioactivity; (bottom) profile of UV absorbance. Several pilot experiments indicated that only fractions 35 to 55 contained significant radioactivity. Here and elsewhere, counts were corrected by subtracting a blank consisting of counts obtained in fraction 1. (B) Second HPLC column. Fraction 46 (arrowhead, panel A), and the corresponding fraction from a second run, were pooled, digested with Asp-N, and analyzed by HPLC as described in Experimental Procedures (top, radioactivity; bottom, UV).

Table 2: Identification of Proximal Nucleophiles in Wild-Type and Mutant Transferases

sample 1 (wt)			sample 2 (wt)			sample 3 (C315A)		
exp[a]	obsd[b]	pmol	obsd	pmol	counts[c]	exp	obsd	pmol
Asp-313	—[d]	—	Asp	—[d]	—	Leu-334	Leu	23
Val	Val	35	Val	30	0	Phe	Phe	20
Cys	—[e]	—	—[e]	—	540	Val	Val	17
His	His	7	His	5	225	Ile	Ile	19
Ser	Ser	14	Ser	8	0	Gly	Gly	15
Lys	Lys	12	Lys	7	0	Glu	Glu	10
						Glu	Glu	12
						Glu	Glu	11
						Thr	Thr	6.5
						Lys	Lys	3.1

[a] Residue expected, based on transferase protein sequence (Figure 1). [b] Residue observed in protein sequencing. [c] An equal portion of the product of each sequencing cycle, collected prior to resolution of the PTH derivative, was counted for 15 min. Radioactivity in each fraction has been corrected for the (observed) sequencing lag. Counts in the sample from cycle 1, which were identical to those in a vial lacking any sample, were subtracted to correct the counts in subsequent cycles. [d] In samples 1 and 2, several residues (Ser, Thr, Gly) were detected in cycle 1, apparently due to contaminating free amino acids. In sample 2, Asp was predominant among these. The transferase sequence (Figure 1) also identifies this residue as Asp-313. [e] Unknown peak seen in PTH-amino acid chromatogram (presumed PTH derivative of BrAcNPAsO-akylated Cys).

unit stoichiometry (average 0.9 mol of I/mol of E, Table 1). The simplest explanation of this latter result is that elimination of Cys-315, the primary reaction site in the wild-type protein, permitted alkylation of a different residue(s) that is also proximal to the arsenoxide site.

Two major labeled peptides were detected in the C315A-transferase tryptic digest (Figure 5A). The predominant one, representing two-thirds of the radioactivity, eluted in fractions 22 and 23 (Figure 5A, top) and was purified by a somewhat different regimen than for the peptide derived from wild-type enzyme (Figure 5B; Experimental Procedures). Sequence analysis showed that this mutant-derived peptide was distinct from that obtained with the wild type, spanning residues 334−343 instead of 313−318 (Table 2, sample 3). This conclusion was further supported by the following observations (data not shown): (1) digestion of the labeled, mutant-derived peptide with Asp-N did not shift its HPLC elution position, indicating that Asp was absent; (2) digestion with Glu-C led to the appearance of two new HPLC peaks, indicating that one or more Glu residues was present. As shown in Table 2, the mutant peptide lacked Asp and contained three Glu residues (Table 2, sample 3).

The most obvious nucleophilic residue in this peptide, Lys-343, was unmodified, based on successful tryptic cleavage. This left one or more of the Glu residues (339−341), or Thr-342, as candidates for site(s) of modification. Glu modification would be most consistent with the lack of a "blank" in the sequence, since the ester adduct formed from Glu should be lost *via* hydrolysis during the multiple exposures to alkali that occur during peptide coupling to the solid support and repetitive Edman degradations. Consistent with the presence of an ester adduct, the bulk of the radioactivity associated with the C315A protein was labile to treatment with 0.1 N NaOH for 30 min (Figure 6, lanes 1 versus 2). We conclude that one or more of the Glu residues (339−341) was the primary site of alkylation in the C315A protein.

As shown in Figure 6, lanes 3 versus 4, a fraction of the label associated with the wild-type enzyme was also lost during alkali treatment, suggesting that a fraction of the wild-

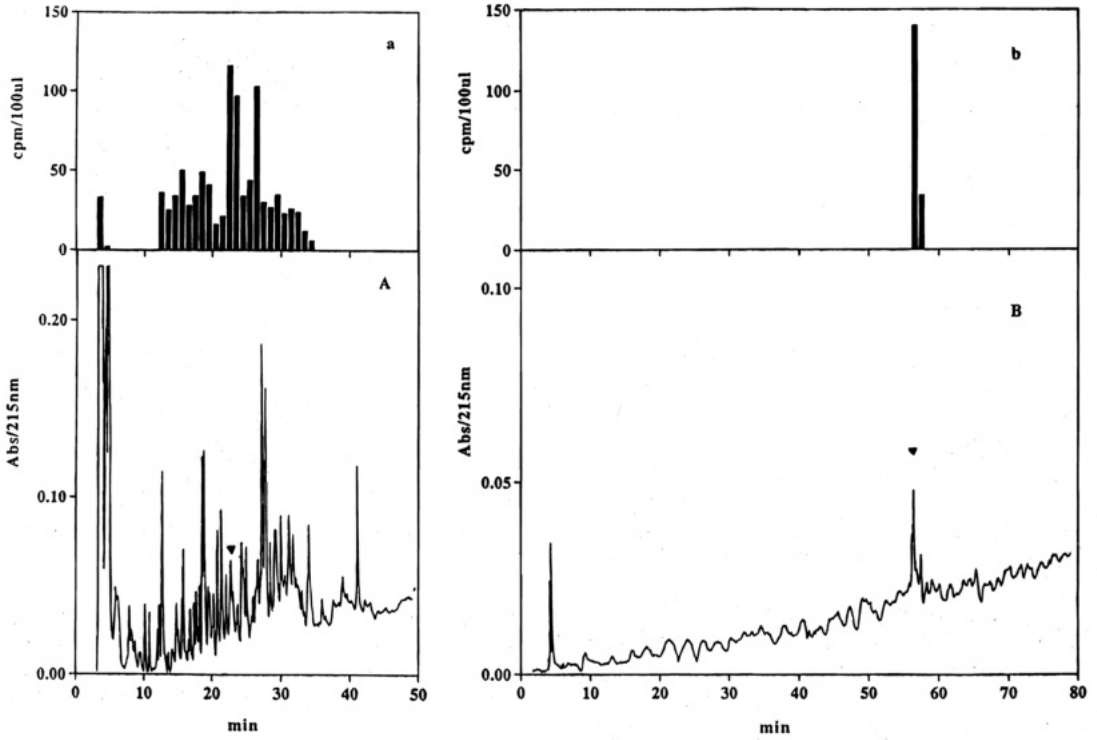

FIGURE 5: Purification of labeled peptide derived from reaction of C315A protein with [^{14}C]BrAcNPAsO. Detailed methods are given in Experimental Procedures. The tryptic digest derived from 1 nmol of labeled enzyme (10 000 cpm) was loaded. (A) First HPLC column. (Top) profile of radioactivity; bottom, UV absorbance profile. (B) Second HPLC column. The peptide eluting at the arrowhead in panel A (fractions 22 and 23) was rerun, using a different gradient (top, radioactivity; bottom, UV).

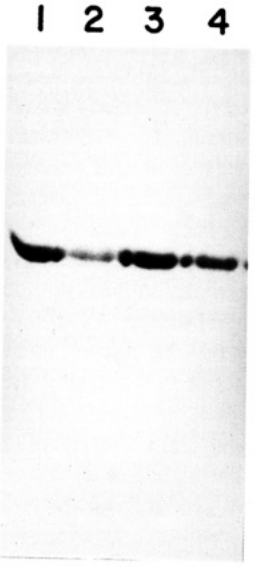

FIGURE 6: Alkaline sensitivity of labeled adducts of wild-type and C315A enzymes (fluorograph). To generate the labeled adduct, wild-type or C315A enzyme (6 μM) was incubated with 10 μM [^{14}C]BrAcNPAsO at 37 °C, in 50 mM MOPS (pH 7.2), 50 mM KCl, and 1 mM β-mercaptoethanol (total volume, 24 μL). After 10 min, the inactivation reaction was quenched with 1 mM DTT and sampled for activity. Each labeled enzyme was precipitated with TCA, solublized in 8 M urea, and then incubated at RT for 30 min, with or without addition of 0.1 N NaOH (see Experimental Procedures). The samples were neutralized as required and then electrophoresed on a 10% SDS gel. The gel was impregnated with Autofluor (National Diagnostics), dried, and fluorographed. (Lane 1) C315A enzyme, no NaOH; (lane 2) C315A enzyme, plus NaOH; (lane 3) wild-type enzyme, no NaOH; (lane 4) wild-type enzyme, plus NaOH.

type enzyme was modified at a carboxylate side chain(s). If so, this could not have occurred to a very significant degree, since the corresponding peptide should have been recovered during the first HPLC run, based on results with the mutant. As a thiol is a much stronger nucleophile than a carboxylate, Cys alkylation is expected to predominate if there is an opportunity for competition. Another possible explanation for the partial alkaline lability of the wild-type adduct is occurrence of slow, irreversible protein aggregation in alkali. We have observed alkali-induced aggregation in other experiments.[3] In any case, the substantially greater alkaline lability of the mutant protein adduct confirms that the major fraction of the mutant protein is modified at a site different from the wild type and with the chemical properties expected for an ester. The His side chain is also more nucleophilic the Glu side chain. The question arises as to whether the secondary labeled peptide detected with the mutant protein (fraction 26 in Figure 6A) represented the product of reaction at His-316. In a single experiment, the elution position of the radioactivity associated with this peptide was not altered following digestion with Asp-N. This contrasts with the behavior expected if His-316 was the secondary labeling site (above). Further studies with this secondary labeled peptide were prevented by its low yield.

DISCUSSION

Overexpression and purification of the yeast Arg-tRNA protein transferase permitted the current studies on transferase structure−function. The purified enzyme is active when Arg-tRNA is generated by *E. coli* Arg-tRNA synthetase (Results) and fractionates as an apparent monomer during native gel permeation chromatography.[3] Mammalian Arg-tRNA transferase exists predominantly in a heterooligomeric complex with Arg-tRNA synthetase (Ciechanover et al., 1988). This complex is dissociated only with great difficulty

[3] J. Li and C. Pickart, unpublished experiments.

(Ciechanover et al., 1988), suggesting that Arg-tRNA may normally be "channeled" from synthetase to transferase, even though the mammalian enzyme can utilize free, exogenous Arg-tRNA (e.g., Berleth et al., 1992b). Whether there is a stable transferase−synthetase complex in yeast cells is unknown. However, our data suggest that any such complex is not obligatory for transferase catalysis, since it is unlikely that a stable complex is formed with the heterologous *E. coli* synthetase.

An initial concern about expressing Arg-tRNA transferase in *E. coli* was the potential for destabilization of endogenous proteins bearing N-terminal Glu or Asp residues. In turn this could lead to deleterious effects on growth or selection against high expression of the recombinant transferase. Destabilization would arise as a result of the *E. coli* N-end rule: endogenous proteins with N-terminal Glu or Asp residues would be arginylated by the recombinant transferase (which utilizes *E. coli* Arg-tRNA), and then conjugated to Leu or Phe by the *E. coli* transferase, which recognizes proteins bearing N-terminal Arg residues (Liebowitz & Soffer, 1970; Tobias et al., 1991). Leu and Phe are primary destabilizing residues in *E. coli*; their presence can lead to degradation by the Clp protease (Tobias et al., 1991). In the event, however, BL21(DE3)/pLysS, the *E. coli* strain used for Arg-tRNA transferase expression, was found to be deficient in endogenous Leu/Phe-tRNA transferase activity ($\leq$2% of the activity detected in strain TB1),[3] eliminating the possibility of such complications. The basis for the low Leu/Phe-tRNA transferase activity in BL21(DE3)/pLysS is not known.

PAsO is an extremely potent, stoichiometric inhibitor of the yeast transferase (Figure 3; Table 1; Results). Phenylarsenoxides are also potent inhibitors of the mammalian transferase ($K_{0.5} = 8\ \mu$M for NPAsO with the rabbit enzyme; Berleth et al., 1992b). Inhibition by phenylarsenoxides is generally considered to be diagnostic for the presence of the vicinal thiol motif, since no other high-affinity arsenoxide binding mode is known for proteins. Although the properties of transferase inhibition are consistent with vicinal thiols mediating arsenoxide binding (Results), this mechanism remains speculative pending identification of the proposed ligands. The 15 Cys residues of the transferase include two pairs that are relatively close in the primary sequence (Cys-20/23 and Cys-94/95; Figure 1). The side chains of these pairs have some potential to bind arsenoxides (Brown et al., 1989; Hoffman & Lane, 1992). However, vicinal thiols may also derive from linearly distant Cys residues that are brought together by folding (Jauhiainen et al., 1988). Identifying the enzymic ligands of the bound arsenic is the objective of ongoing studies, as is identifying the specific step in transferase catalysis that is inhibited by arsenoxides. The current studies showed that the transferase has one or more essential thiols (cf. high sensitivity to NEM). However, the results failed to show that these essential thiol(s) mediate arsenoxide binding.

BrAcNPAsO was originally described as an active-site-directed inactivator of pyruvate dehydrogenase complex. In this case, the arsenoxide moiety of this bifunctional reagent binds to the vicinal thiols of the lipoamide prosthetic group of lipoamide acetyltransferase (Stevenson et al., 1978), while the bromacetamido moiety alkylates His-444 of lipoamide dehydrogenase (Adamson & Stevenson, 1981; Adamson et al., 1984; Holmes & Stevenson, 1986).

Identification of the proximal nucleophile of mammalian Arg-tRNA transferase was prevented by the extreme difficulty of mammalian transferase purification (Berleth et al., 1992b). Such studies became possible with the availability of large amounts of homogeneous yeast transferase. A highly specific interaction between enzyme and bifunctional arsenoxide is indicated by high affinity (Figure 3B), the finding of a single predominant alkylated peptide in wild type and C315A enzymes (Figures 4 and 5), and the unit alkylation stoichiometry seen with both proteins (Table 1). In the wild-type enzyme, this reagent targets primarily Cys-315. Inactivation is apparently due to steric blockade, since mutation of Cys-315 to Ala has no effect on transferase catalytic properties.

Perhaps the strongest evidence for the specificity of interaction of this reagent, however, is that it primarily targets one or more residues within a sequence of three contiguous Glu residues in the C315A mutant protein. Thus the bound bifunctional reagent must be positioned in a highly advantageous way for alkylation of the weakly nucleophilic Glu side chain. Presumably this latter reaction occurs less significantly in the wild type due to competition from the more nucleophilic thiol of Cys-315. The identical specific activities of the mutant and wild-type proteins, and their quantitatively identical properties with respect to arsenoxide inhibition, make it very unlikely that the C315A mutation caused a qualitative change in the site of arsenoxide binding. However, subtle changes in the site, for example, leading to decreased nucleophilicity of His-316 in the mutant protein, are not excluded.

Given the potent inhibition of yeast and mammalian transferases by phenylarsenoxides, the arsenoxide binding site either is an essential site or regulates an essential site. Our finding that Cys-315 and residues 339−341 are proximal to the arsenoxide binding site provides the first structure−function information for any aminoacyl-tRNA transferase. The quantitative conservation of phenylarsenoxide inhibition between yeast and mammalian enzymes (Berleth et al., 1992b) indicates some likelihood that the residues identified in the present study will be conserved in transferases from higher eukaryotes, for which primary structures are not yet available.

We did not evaluate the catalytic essentiality of the Glu triplet (residues 339−341) because it is not known whether the bifunctional arsenoxide targets one or all of these residues. In principle, the Glu side chain could act as a general base catalyst or, less likely, as a nucleophile. It is also possible that a cluster of Glu residues could function in binding of the positively charged Arg substrate, either at the level of Arg-tRNA or perhaps in a covalent Arg−enzyme intermediate. However, pK_a values of one or more residues in the Glu triplet are likely to be perturbed upward, such that ionization of all of them is unlikely. In any case, based on the results obtained with the wild type enzyme, alkylation of one or more of these Glu residues is very likely to inactivate by a steric blockade mechanism even if the alkylated residue(s) is nonessential.

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