A Cysteine in the C-Terminal Region of Alanyl-tRNA Synthetase Is Important for Aminoacylation Activity†

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ABSTRACT: Alanyl-tRNA synthetase (AlaRS) from *Escherichia coli* is a multimeric enzyme that catalyzes the esterification of alanine to $tRNA^{Ala}$ in the ATP-dependent aminoacylation reaction. The functional binding of all three substrates follows Michaelis–Menten kinetics. The role of cysteines in this enzyme has been evaluated *via* modification of these residues with *p*-(hydroxymercuri)phenylsulfonic acid, monobromobimane, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The former two reagents induce nearly complete inactivation of AlaRS aminoacylation activity and the release of all tightly bound zinc. In the case of mild DTNB treatment, only two of the six cysteines in AlaRS are modified, with release of all zinc and partial loss of aminoacylation activity. These experiments indicate the importance of one or more cysteines, other than those thought to be coordinated with zinc, in the aminoacylation reaction. Substitution of each of the cysteine residues outside the zinc-binding motif with serine does not disrupt zinc binding. However, the cysteine most removed in primary sequence from the active site (Cys665) is identified as important in the aminoacylation step. Mutation of Cys665 to serine induces a 120-fold decrease in the catalytic efficiency of this enzyme, primarily through a k_{cat} effect, and introduces sigmoidal kinetics (n_H = 1.8) with respect to the RNA substrate. The results demonstrate that a simple manipulation in the C-terminal region can introduce positive cooperativity in this otherwise noncooperative enzyme.

Many large proteins contain compact, locally folded regions of tertiary structure organized in a modular fashion along the sequence of the polypeptide. Such an organization of functional domains exists in the family of aminoacyl-tRNA synthetases (aaRSs)[1] [reviewed in Delarue and Moras (1993) and Landro and Schimmel (1993b)]. The first aaRS to be characterized in this manner was AlaRS from *Escherichia coli*, which was shown by systematic gene deletions to contain separate but contiguous alanyladenylate synthesis, tRNA binding, and oligomerization domains (Jasin et al., 1983). Integration exists between these domains, as demonstrated by the ability of a piece of the C-terminal domain to activate the catalytic site when coupled through subunit interactions (Jasin et al., 1984). Furthermore, single glycine to aspartate mutations in this portion adversely affect aminoacylation activity (Jasin et al., 1985). The mechanism and functional role of this integration remain unclear. We report here the construction of a cysteine to serine mutation in the C-terminal domain that also causes diminution in aminoacylation activity while introducing positive cooperativity with respect to the cognate tRNA ($tRNA^{Ala}$). Positive cooperativity with respect to the RNA substrate has not, to our knowledge, been observed previously in the aaRS family.

Aminoacyl-tRNA synthetases catalyze the esterification of amino acids to their cognate tRNAs during the course of protein synthesis [reviewed in Carter (1993)]. This reaction proceeds in two steps through the formation of an aminoacyl-adenylate intermediate (Freist, 1989). It has long been known that one or both steps of the reaction may be inhibited by mercurial agents such as *p*-mercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), suggesting that cysteine residues play a crucial role in this family of enzymes (Stern et al., 1966; Cassio, 1968; Iaccarino & Berg, 1969; Kuo & Deluca, 1969; Ostrem & Berg, 1974; Murayama et al., 1975; Remy & Ebel, 1976). However, the advent of site-directed mutagenesis led to results showing that cysteines presumed essential may not be required for catalysis. Specifically, substitution of presumed essential cysteines by alanine in *E. coli* GlyRS yields active protein (Profy & Schimmel, 1986), and none of the three cysteines in *Saccharomyces cerevisiae* cytoplasmic AspRS are essential for its activity (Kern et al., 1990).

Rather than participating directly in catalysis, cysteine residues may serve other functions in aaRSs. For example, the zinc-binding ability of several aaRSs is apparently due to the presence of cysteine-containing metal-binding motifs (Coleman, 1992), first identified in these enzymes by Berg (1986). In *E. coli* MetRS the zinc atom is coordinated with a cysteine box sequence (**Cys**-Xaa_2-**Cys**-Xaa_9-**Cys**-Xaa_2-**Cys**) within the connective polypeptide 1 that is inserted between two halves of the nucleotide-binding fold (Landro & Schimmel, 1993a; Xu et al., 1993; Fourmy et al., 1993). The *E. coli* AlaRS putative metal-binding domain is a cysteine–histidine box (**Cys**178-Xaa_2-**Cys**181-Xaa_6-**His**188-Xaa_2-**His**191; Miller et al., 1991) located in the N-terminal portion of the protein that also contains the ATP-binding domain (Jasin et al., 1983). Several additional aaRSs have been proposed or shown to contain similar metal-binding motifs (Berg, 1986; Miller et al., 1991; Nureki et al., 1991, 1993).

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase (for specific aaRSs, aa is replaced by the corresponding three-letter amino acid code); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)phenylsulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Thiolyte MB, monobromobimane.

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The discovery of cysteine-rich zinc-binding motifs in aaRSs has led to an interest in reevaluating the chemical modification of cysteines in these enzymes. A recent study by Nureki et al. (1993) demonstrated the ability of *p*-(hydroxymercuri)-phenylsulfonic acid (PMPS) to liberate zinc from the cysteine-rich zinc-binding motifs of *Thermus thermophilus* IleRS and MetRS and from *E. coli* IleRS, PheRS, and ThrRS. With the exception of PheRS, these enzymes exhibited loss of aminoacylation activity upon cysteine modification. The implication that at least one cysteine is coordinated with zinc in AlaRS led to the present investigation of the effects of various cysteine modification reagents on this enzyme. The modification data identified two classes of cysteines that affect aminoacylation activity, one of which is not related to zinc coordination. Subsequent mutagenesis of cysteine residues outside the cysteine–histidine box identified Cys665, in the C-terminal domain, to be crucial to the aminoacylation reaction. The role of Cys665 is considered in the context of a C-terminal domain that participates in the maintenance of a more active enzyme form.

EXPERIMENTAL PROCEDURES

Materials. Purified *E. coli* $tRNA^{Ala}$ was purchased from Subriden RNA, the concentration of which was determined from its capacity to be charged with [^{14}C]alanine (Francklyn et al., 1992). Crude *E. coli* tRNA was from Boehringer Mannheim. Phagemid pTZ18U and helper phage M13K07 were from Bio-Rad. The reagents PMPS, DTNB, and 4-(2-pyridylazo)resorcinol (PAR) were from Sigma, and monobromobimane (Thiolyte MB) was from Calbiochem-Behring.

Mutagenesis. Mutagenesis of *alaS* was conducted in pSF351 as described by Filley and Hill (1993). All four cysteine codons outside the cysteine–histidine box coding region were mutated to serine codons. The mutations were incorporated separately into wild-type *alaS* with complementary oligodeoxyribonucleotides (National Biosciences) containing one or two base mismatches. Subsequent DNA sequencing with Sequenase (United States Biochemicals) confirmed the presence of the serine codons and the fidelity of the adjacent bases in *alaS*. Production of the mutant proteins *in vivo* was confirmed by Western blot analysis using antibody raised against *E. coli* AlaRS (HTI Bio-Products, Inc.).

Protein Purification. Wild-type and mutant AlaRS were purified from an *alaS* deletion strain (W3110 *alaS*Δ2; Jasin & Schimmel, 1984; gift of P. Schimmel) by modification of the procedures of Putney et al. (1981) and Regan (1986). Typically, 1-L overnight cultures were employed. Following centrifugation, the cell pellets were washed in 0.8% NaCl, resuspended in 10 mL of cold extraction buffer [0.1 M NaCl, 50 mM potassium phosphate, pH 7.5, 50 mM 2-mercaptoethanol (2-ME), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1.0 μg mL^{-1} leupeptin, 15 μg mL^{-1} soybean trypsin inhibitor, 1.0 μg mL^{-1} pepstatin, 0.1 mM sodium metabisulfite, and 0.05 mM benzamidine], and subjected to sonication. The resulting crude extracts were clarified by centrifugation at 60000*g* at 4 °C for 1 h. The supernatants were then treated with ammonium sulfate; proteins that precipitated between 35 and 50% of saturated solution were recovered and dissolved in 10 mL of 25 mM Tris-HCl, pH 7.5, and 1 mM 2-ME containing the protease inhibitors mentioned above. The resulting protein solutions were filtered through 0.45-μm Autovial syringeless filters (Genex) and stored overnight in 50% glycerol at −20 °C.

In preparation for FPLC the crude protein solutions were diluted to 5% glycerol and then loaded onto a Q Sepharose Fast Flow (Pharmacia) anion-exchange column (2.6 × 16 cm) preequilibrated at 4 °C in column buffer (25 mM Tris-HCl, pH 7.5, 1.0 mM 2-ME, 0.2 mM PMSF, and 0.1 M NaCl). Proteins were eluted with a 560-mL NaCl gradient (0.10–0.18 M over 160 mL; 0.18–0.22 M over 240 mL; 0.22–0.40 M over 160 mL) at a flow rate of 8 mL min^{-1}. Fractions (20 mL) were collected and assayed for protein with alanine-dependent aminoacylation activity, which eluted at 0.2 M NaCl. Fractions containing activity were pooled, concentrated by precipitation in a 50% saturated solution of ammonium sulfate, resuspended in 5 mL of 10 mM sodium phosphate, pH 7.5, and clarified for 2 min in a microcentrifuge. Supernatants were stored overnight in 50% glycerol at −20 °C. The protein composition of these pools was shown by SDS–polyacrylamide gel electrophoresis (PAGE; Laemmli, 1970) to be >50% AlaRS.

For further purification, pools were applied to a Sephacryl S-300 (Pharmacia) column (2.5 × 116 cm) equilibrated in 10 mM sodium phosphate, pH 7.5, 1.0 mM 2-ME, and 0.2 mM PMSF at 4 °C. Proteins were eluted at a flow rate of 3.0 mL min^{-1} in 3-mL fractions, and absorbance (280 nm) peaks were checked for protein purity by SDS–PAGE. Fractions were pooled appropriately, concentrated to 10–20 mg mL^{-1} with YM30 membranes (Amicon; 30 000 MW cutoff) in stirred cells, and stored at −20 °C. At this point the synthetases were judged to be >98% pure. The concentration of purified AlaRS was determined by A_{280} measurements (ϵ = 0.83 L g^{-1} cm^{-1}; unpublished work).

Cysteine Modification. Cysteine residues in AlaRS were modified with PMPS (Boyer, 1954), DTNB (Ellman, 1959), and Thiolyte MB (Dailey, 1984; Yagi & Hatefi, 1984). Prior to treatment with these reagents, the protein solutions were dialyzed against 50 mM HEPES, pH 7.5, and 20 mM KCl in order to ensure no interference by 2-ME. When PMPS was employed, mercaptide bond formation was monitored at 250 nm. In the case of DTNB, the extent of cysteine modification was determined by monitoring the formation of the reaction product, 2-nitro-5-mercaptobenzoic acid, at 412 nm (ϵ = 13 600 M^{-1} cm^{-1}). Thiolyte MB is a labeling reagent that reacts extremely rapidly with thiol groups, under physiological conditions, to yield highly fluorescent products. The thiol adducts formed upon reaction with AlaRS were monitored by fluorescence spectroscopy with the excitation and emission wavelengths set at 375 and 475 nm, respectively.

Zinc Stoichiometry. Zinc content was determined by monitoring formation of the zinc/PAR_2 complex (A_{500}; ϵ = 66 000 M^{-1} cm^{-1}) following modification of cysteine residues in AlaRS (Hunt et al., 1985; Shang et al., 1989; Han et al., 1990) or by atomic absorption analysis. The latter was conducted at 214 nm using an Instrumentation Laboratory 251 spectrometer. Standard curves were generated using solutions containing 0.02–1.0 μg mL^{-1} zinc, and the AlaRS concentration was typically between 5 and 10 μM.

Analytical Molecular Sieve Chromatography. The molecular weights of wild-type AlaRS and mutant Cys665Ser were determined by analytical gel filtration according to the *High Molecular Weight Gel Filtration Calibration Kit Instruction Manual* (Pharmacia). A Sephacryl S-300 HR (Pharmacia) gel filtration column (1.6 × 83 cm), with a flow rate of 1.0 mL min^{-1}, was employed to resolve protein standards and AlaRS. Values for K_{av} were calculated from elution and void volumes and then plotted against the log of the molecular weights of the standard proteins. The molecular weights of the synthetases were estimated from these standard curves.

Table 1: Characterization of Mutant Alanyl-tRNA Synthetases

AlaRS	zinc content[a] (mol of Zn/mol of subunit)	DTNB-sens. cysteines[b]	$K_{m,ATP}$[c] (μM)	$K_{m,Ala}$[c] (μM)	k_{cat}[c] (s^{-1})	$[S_{0.5}]_{tRNA^{Ala}}$[d,e] (μM)	k_{cat}[d] (s^{-1})	$k_{cat}/[S_{0.5}]_{tRNA^{Ala}}$[d] ($s^{-1}$ μM^{-1})	n_H[d,f]
wild type	0.79; 0.89	1.9	83	340	84	1.1	1.1	1.0	1.0
Cys76Ser	0.95; 1.2	1.8	180	330	120	1.1	0.98	0.89	1.3
Cys290Ser	0.61; 0.95	1.4	210	500	110	1.3	0.96	0.74	1.1
Cys412Ser	0.61; 0.81	0.88	120	470	79	0.62	0.92	1.53	1.0
Cys665Ser	0.89; 0.82	1.6	95	230	89	2.4	0.017	0.0071	1.8

[a] Zinc concentrations were determined either by monitoring formation of the zinc/PAR_2 complex upon modification with DTNB (first number in column) or by atomic absorption analysis (second number in column; average values from two determinations are given). Protein concentrations were determined by use of the Bio-Rad protein assay. [b] Average values are given from two experiments using 250 μM DTNB at 25 °C. [c] *Via* the ATP–PP_i exchange assay. [d] *Via* the spectrophotometric aminoacylation assay. [e] Note that $[S_{0.5}] = K_m$ when $n_H = 1$. [f] Hill coefficient with respect to $tRNA^{Ala}$.

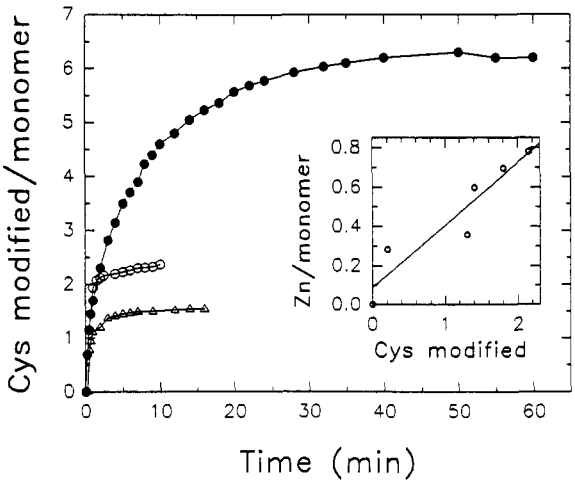

FIGURE 1: Time course of DTNB modification of cysteine residues in AlaRS. Cysteine modification on AlaRS was monitored at 410 nm *via* formation of 2-nitro-5-mercaptobenzoic acid. Reactions of 100 μM DTNB with 2.5 μM AlaRS were performed at pH 7.5 in the dark at room temperature with the following additions: none (○), 2% SDS (●), or 5 mg mL^{-1} crude *E. coli* tRNA (△). The relationship between zinc release and DTNB modification of cysteine residues in AlaRS is shown in the inset. In this case, the number of cysteines modified on AlaRS was determined following the addition of 1–25 μM DTNB. Zinc release induced by DTNB was monitored at 500 nm *via* formation of the zinc/PAR_2 complex. The line shown was generated *via* first-order regression of these data.

Catalytic Activity. The alanyladenylate synthesis activity of AlaRS was assayed at 37 °C by the adenylate burst assay (Fersht et al., 1975) or the ATP–PP_i exchange assay (Calendar & Berg, 1966) as adapted by Hill and Schimmel (1989). The overall aminoacylation of $tRNA^{Ala}$ was monitored routinely at 37 °C using a spectrophotometric assay (Wu & Hill, 1993), with the exception that the radioisotope assay (Schreier & Schimmel, 1972; Jasin et al., 1985) was employed during the enzyme purification procedure. Kinetic data were fit *via* SigmaPlot software to the modified Hill equation $v = V_{max}[S]^{n_H}/([S_{0.5}]^{n_H} + [S]^{n_H})$, where v, V_{max}, and S have the usual meanings, and n_H is the Hill coefficient (Hill, 1910; Wedding et al., 1990; note that this equation reduces to the Michaelis–Menten equation and $[S_{0.5}] = K_m$ when $n_H = 1$).

UV-Difference Spectra. Difference spectra were recorded over the wavelength range 190–300 nm using a Lambda 2 Perkin-Elmer spectrophotometer and analyzed with the PECSS (Perkin-Elmer) software package. Protein concentrations were approximately 0.2 mg mL^{-1} in 50 mM HEPES, pH 7.4, and 20 mM KCl. The temperature in the sample chamber was maintained with a circulating water bath.

RESULTS

Cysteine Modification. Alanyl-tRNA synthetase from *E. coli* is a tetramer of identical 875 amino acid subunits, each containing six cysteine residues (Putney et al., 1981). Mild treatment of this enzyme with DTNB, in the presence of 2% SDS, results in the modification of all cysteines (Figure 1). However, only two cysteines are rapidly modified in the absence of the denaturing agent. These are referred to as "DTNB-sensitive" cysteines to distinguish them from the other four. Transfer RNA protects one of the DTNB-sensitive cysteines from modification. Simultaneous monitoring of zinc released to PAR indicates that all of the enzyme-bound metal [based on 0.8 mol of zinc/mol of monomer; Table 1 and Miller et al. (1991)] is liberated upon modification of these two DTNB-sensitive residues (Figure 1, inset).

Having established that two cysteines in AlaRS could be modified differentially, it was of interest to consider the effects of the mild DTNB treatment on aminoacylation activity. Titration of AlaRS with DTNB produced a curve with half-maximal inactivation at approximately 3 μM DTNB (Figure 2a). Of particular note is the incomplete inactivation; approximately 55% activity remains after treatment with up to 90 μM DTNB. Addition of 4 mM dithiothreitol (DTT) to these reaction mixtures causes reactivation to initial levels, indicating that the DTNB-induced inactivation is due to modification of free thiols on AlaRS. The data in Figure 2b confirm that DTNB modification of the two DTNB-sensitive cysteines leads to an approximate 50% loss in activity.

In contrast to DTNB, Thiolyte MB (Figure 2a) and PMPS (data not shown) induce near complete inactivation of AlaRS aminoacylation activity, consistent with the results of PMPS treatment of several other aaRSs (Nureki et al., 1993). Titration of nondenatured AlaRS with PMPS produces an increasing A_{250} that reaches a plateau at approximately six PMPS equivalents, indicating that modification of all six sulfhydryl groups occurs. Figure 3 shows that all zinc is released upon PMPS treatment.

Characterization of Mutant Proteins. The chemical modification results imply that the aminoacylation activity of AlaRS is influenced by two classes of cysteines, one of which contains cysteines other than those required for zinc binding. At least one of the cysteines in the cysteine–histidine box is thought to function in a zinc-binding capacity (Miller et al., 1991), but little is known about the roles of the four cysteines outside this motif. In order to assess the functional importance of these cysteines, we generated four mutant AlaRSs, each containing serine at one of the wild-type cysteine positions (Cys67Ser, Cys290Ser, Cys412Ser, and Cys665Ser). These mutants were purified and subjected to zinc analysis as shown in Table 1. Each of the mutant enzymes contains a molar ratio of zinc similar to that of wild-type AlaRS, demonstrating that cysteine residues outside the cysteine–histidine box are not required for zinc binding. Subsequent DTNB treatment indicated that approximately 2 DTNB-sensitive cysteines remain in all except the Cys412Ser mutant, in which the number is reduced to 1 (Table 1). Cysteine 412, outside the

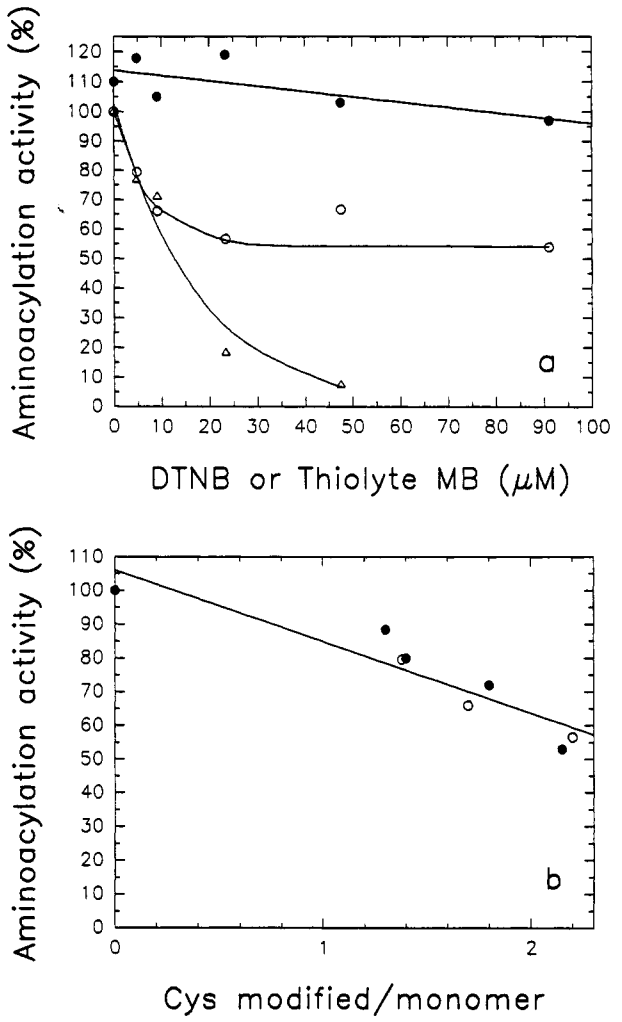

FIGURE 2: Inactivation of AlaRS by DTNB and Thiolyte MB. The enzyme was preincubated for 5 min in various concentrations of modification reagent and then assayed for aminoacylation activity *via* the spectrophotometric assay. Data are plotted to show (a) the DTNB-induced inactivation (○) and the recovery of this activity upon addition of 4 mM DTT (●) to the assay cuvette, the Thiolyte MB-induced inactivation (△), and (b) the relationship between AlaRS activity and DTNB modification of the two DTNB-sensitive cysteine residues [data from two experiments are shown (○, ●)]. The number of cysteines modified on AlaRS was determined as in Figure 1. The inactivation data in panel a were fit to double-(○) and single-exponential decay equations (△), and the straight lines in panels a and b were generated *via* first-order regression of the respective data.

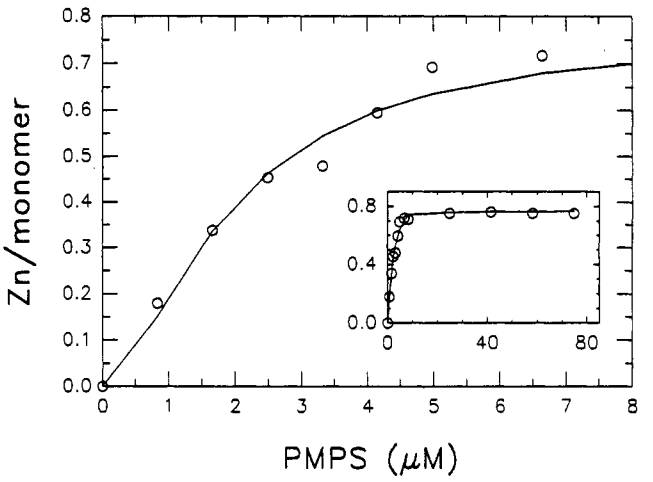

FIGURE 3: Zinc release from native AlaRS upon treatment with PMPS. Zinc release from AlaRS was monitored as in Figure 1. The experimental data were best fit (solid line, SigmaPlot Software) to a modified Hill equation (Hill, 1910) as described by Wedding et al. (1990) to yield a Hill coefficient of 1.7, a maximum of 0.77 zinc ion released per monomer, and half-maximal zinc release at 1.9 μM PMPS. The inset plot shows the complete titration, and the large plot shows the initial portion of the curve in detail.

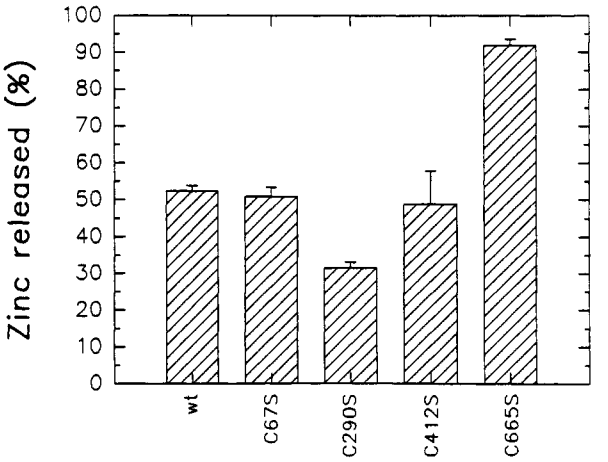

FIGURE 4: Comparison of zinc release from wild-type and mutant AlaRSs in the absence of thiol modification reagents. Zinc release was monitored as in Figure 1 in the presence of 0.5 mM PAR at 25 °C in 50 mM HEPES, pH 7.5. Average values from two experiments are given.

cysteine–histidine box, is thus apparently one of the DTNB-sensitive cysteines.

Although all of the cysteine to serine mutant proteins bind approximately equimolar quantities of zinc, the Cys665Ser substitution causes change with respect to the extraction of this metal by the chelator PAR in the absence of thiol modification reagents. The data in Figure 4 show that approximately twice as much zinc is released to this reagent by AlaRS(Cys665Ser) than by the other proteins tested. It is concluded that this substitution in the C-terminal region of AlaRS induces a structural change that makes the metal, which is thought to be coordinated primarily by residues in the N-terminal region, more accessible to PAR. In a similar experiment, wild-type and mutant Cys665Ser enzymes were preincubated for 20 min with 1.2 mg mL^{-1} crude *E. coli* tRNA. Subsequent addition of PAR and monitoring at 500 nm revealed no zinc released to this chelator by the wild-type enzyme, whereas 89% of the zinc on the mutant was released. Thus tRNA is able to protect the zinc from extraction by this chelator in the wild-type enzyme but not in the Cys665Ser mutant.

Further evidence for a structural change was obtained upon comparing the thermal stability of wild-type AlaRS with that of the Cys665Ser mutant (Figure 5). When the temperature dependence of the UV-difference spectrum for each protein was monitored, the inflection point of the denaturation curve was found to be 14 °C lower for the mutant than for wild-type AlaRS. However, this single mutation does not disrupt functional substrate binding at 37 °C (see below).

Knowing that point mutations near the oligomerization domain can disrupt the tetrameric structure of AlaRS (Jasin et al., 1985), we analyzed the Cys665Ser mutant on an analytical S-300 gel filtration column. The protein molecular mass was calculated to be 370 000 Da, confirming its retention of tetrameric structure.

The results of analysis of all cysteine to serine mutants in the ATP–PP_i exchange reaction are presented in Table 1. No significant changes relative to wild-type AlaRS were detected in the kinetic constants for this reaction. When assayed for

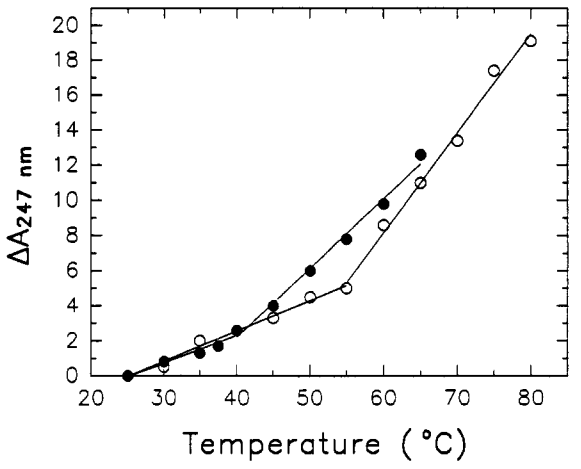

FIGURE 5: Comparison of thermal stabilities of wild-type (○) and Cys665Ser (●) forms of AlaRS. Absorption spectra of each protein were recorded at 247 nm following 5-min equilibrations at the designated temperatures. The absorbance at 25 °C was subtracted from the absorbances at the temperatures indicated. The lines shown were generated *via* first-order regression of the data points in each phase of the denaturation curve.

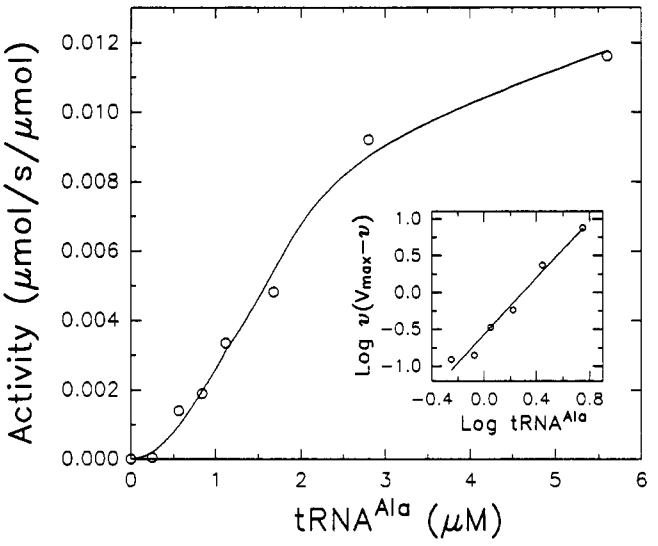

FIGURE 6: Aminoacylation activity of AlaRS(Cys665Ser) as a function of $tRNA^{Ala}$ concentration. Activity is reported as micromoles of alanyl-$tRNA^{Ala}$ formed per second per micromole of enzyme. Experimental data (○) were fit to a modified version of the Michaelis–Menton equation (solid line) as described by Wedding et al. (1990). The inset Hill plot of the same data was determined by first-order regression to have a slope of 1.8.

aminoacylation activity, three of the mutants (Cys67Ser, Cys290Ser, and Cys412Ser) produced kinetic constants that were also indistinguishable from those for the wild-type enzyme. However, the Cys665Ser mutation produces an approximate 120-fold decrease in catalytic efficiency, primarily through a k_{cat} effect. We have thus identified a cysteine residue that is not required for zinc binding yet influences the aminoacylation activity, in accord with the chemical modification data presented above. Of particular interest is the observation that the tRNA saturation curves obtained with this mutant are sigmoidal (Figure 6), rather than the hyperbolic curves obtained with wild-type AlaRS and the other three mutants. Apparent Hill coefficients calculated for each enzyme are reported in Table 1. In contrast to values near unity for wild-type AlaRS and the other mutants, the Hill coefficient for AlaRS(Cys665Ser) is increased to 1.8 (Figure 6, inset).

DISCUSSION

The functional significance of the AlaRS cysteine–histidine box was investigated initially (Miller et al., 1991) by site-directed mutagenesis of each amino acid in this region whose side chain was predicted to be a potential ligand for zinc (Vallee & Auld, 1990). Of the eight mutants constructed, only mutation of Cys178, His188, and His191 caused inability of AlaRS to complement growth of the *alaS* deletion strain (W3110 *alaS*Δ2). Following introduction into this strain maintained by fragment 461N, Western blot analysis of these three inactive enzymes indicated little or no accumulation of the full-length proteins. This was taken to be evidence that these residues are critical for enzyme stability and/or activity, perhaps through coordination with zinc. The potential for the cysteine–histidine motif to bind zinc was subsequently demonstrated by the observation that a corresponding synthetic model peptide can bind the metal (Miller et al., 1991). Our results concur that, in the context of the complete enzyme sequence, at least one free thiol in the cysteine–histidine box is required for zinc coordination. None of the four cysteines outside the cysteine–histidine box are necessary for metal binding to occur (Table 1).

We have further explored the functional significance of the four cysteines outside the metal-binding motif and report that the one most removed (Cys665) in the primary structure from the active site is important in the aminoacylation step. Cysteine 665 is located in the C-terminal region of AlaRS, near the domain that is required for oligomerization of the native subunits. Enzymes from which the C-terminal region has been deleted are monomers that retain full alanyladenylate synthesis activity but have reduced capacity to aminoacylate $tRNA^{Ala}$ (Jasin et al., 1983). Using small RNAs corresponding mostly to the acceptor stem of $tRNA^{Ala}$ and a truncated form of AlaRS (461N), Beuchter and Schimmel (1993) demonstrated recently that the C-terminal region of AlaRS is crucial for interactions with parts of $tRNA^{Ala}$ distal to the acceptor helix. The relatively small change in the $[S_{0.5}]_{tRNA^{Ala}}$ obtained here upon mutation of Cys665 indicates that this cysteine is not one of the residues in this region important for $tRNA^{Ala}$ binding *per se*.

As with most oligomeric enzymes, AlaRS does not display allosteric behavior with respect to any of its substrates. For $tRNA^{Ala}$ in particular, both the equilibrium binding curve (at pH 5; Regan et al., 1987) and the aminoacylation activity saturation curve (at pH 7.5; Regan et al., 1988; Wu & Hill, 1993) are hyperbolic. However, we report here a site-directed point mutant (Cys665Ser) of *E. coli* AlaRS that introduces positive cooperativity as portrayed in a sigmoidal response of aminoacylation activity upon titration with $tRNA^{Ala}$. The degree of $tRNA^{Ala}$ cooperativity can be quantified using the Hill coefficient (n_H), which goes from unity (no cooperativity) for wild-type AlaRS to 1.8 for the Cys665Ser mutant (Table 1). The latter value could represent either strong cooperativity between two $tRNA^{Ala}$ sites or, more likely for this tetramer, weaker cooperativity between four $tRNA^{Ala}$ sites. Cooperative interactions are possible between the subunits in AlaRS-(Cys665Ser) because the mutation does not disrupt the quaternary structure.

Sigmoidal ligand saturation curves may also be obtained with kinetic data from multiple-substrate reactions in which there is a preferred (but not exclusive) pathway to the active ternary complex (Segel, 1975). It is possible that the sigmoidal kinetics obtained with AlaRS(Cys665Ser) result solely from a subunit-independent change in the preference of the kinetic pathway to the final ternary complex. However, the possibility

of a random kinetic mechanism is minimized in this case because the aminoacylation assays are conducted in the presence of saturating levels of the other two substrates (alanine and ATP). The precise mechanism of cooperativity remains to be established. In either case the mutant tetramer is in a lower activity conformation that is activated upon binding $tRNA^{Ala}$.

Several reports have appeared recently concerning the conferral of positive cooperativity-like behavior in multimeric enzymes, including an aaRS, through the introduction of a single mutation. In the cases of ornithine transcarbamoylase (Kuo et al., 1989), aspartate transcarbamoylase (Stebbins et al., 1989; Stebbins & Kantrowitz, 1992), and TyrRS (First & Fersht, 1993), the mutations replace residues in or near the active site. These single amino acid substitutions introduce 5–20-fold decreases in the catalytic rate constants. The sigmoidal kinetics discovered with TyrRS(Lys233Ala) are with respect to ATP in the ATP–PP_i exchange reaction; no positive cooperativity with respect to $tRNA^{Tyr}$ has been reported.

The case of AlaRS is most similar to that of glutathione reductase (Scrutton et al., 1992) in which a Gly418Trp mutation in the dimer interface introduces sigmoidal kinetics with respect to both of its substrates (glutathione and NADPH). Although the interface domain of AlaRS is not well defined in the absence of a solved tertiary structure, deletion analysis suggests that Cys665 is adjacent to a domain required for oligomerization (Gly699–Glu808; Jasin et al., 1983). Thus single mutations in both enzymes demonstrate that cooperativity can be conferred by simple manipulation in or near the subunit interface.

The 20 aaRSs have been partitioned into two classes on the basis of known and predicted structural motifs (Eriani et al., 1990; Cusack et al., 1990; Burbaum & Schimmel, 1991); both classes contain enzymes characterized by functional integration between distal domains. Examples of three such class I aaRSs are MetRS, TyrRS, and GlnRS (Kim et al., 1993; Jones et al., 1985; First & Fersht, 1993; Rogers et al., 1994). Dimeric AspRS and tetrameric AlaRS are both class II enzymes that appear to have functionally interdependent subunits (Kern et al., 1985; Eriani et al., 1993; Jasin et al., 1984, 1985). The data presented here confirm the presence of intersubunit communication in AlaRS and provide a rationale for the oligomeric structure of all class II aaRSs.

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