Effect of Cysteine Residues on the Activity of Arginyl-tRNA Synthetase from *Escherichia coli*[†]

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Received February 18, 1999; Revised Manuscript Received June 9, 1999

ABSTRACT: Arginyl-tRNA synthetase (ArgRS) from *Escherichia coli* (*E. coli*) contains four cysteine residues. In this study, the role of cysteine residues in the enzyme has been investigated by chemical modification and site-directed mutagenesis. Titration of sulfhydryl groups in ArgRS by 5, 5′-dithiobis(2-nitro benzoic acid) (DTNB) suggested that a disulfide bond was not formed in the enzyme and that, in the native condition, two DTNB-sensitive cysteine residues were located on the surface of ArgRS, while the other two were buried inside. Chemical modification of the native enzyme by iodoacetamide (IAA) affected only one DTNB-sensitive cysteine residue and resulted in 50% loss of enzyme activity, while modification by *N*-ethylmeimide (NEM) affected two DTNB-sensitive residues and caused a complete loss of activity. These results, when combined with substrate protection experiments, suggested that at least the two cysteine residues located on the surface of the molecule were directly involved in substrates binding and catalysis. However, changing Cys to Ala only resulted in slight loss of enzymatic activity and substrate binding, suggesting that these four cysteine residues in *E. coli* ArgRS were not essential to the enzymatic activity. Moreover, modifications of the mutant enzymes indicated that the two DTNB- and NEM-sensitive residues were Cys^{320} and Cys^{537} and the IAA-sensitive was Cys^{320}. Our study suggested that inactivation of *E. coli* ArgRS by sulfhydryl reagents is a result of steric hindrance in the enzyme.

Aminoacyl-tRNA synthetases (aaRSs) are a heterogeneous family of enzymes that catalyze the ligation of amino acids to the 3′-hydroxyl end of tRNA molecules (*1*). All 20 aaRSs, each for one regular amino acid, catalyze a two-step reaction that involves three substrates: ATP, amino acid and tRNA (*2*). The first step produces a stable enzyme-bound intermediate, called aminoacyl-adenylate (aa-AMP), from ATP and amino acid, while the second step involves the displacement of AMP by a nucleophilic attack of the ribosyl hydroxyl group of tRNA to the carbonyl carbon of the amino acid. AaRSs can be divided into two classes, with 10 members in each, based on computer-assisted sequence alignments: class I aaRSs are grouped according to the presence of the “HIGH” and “KMSKS” consensus sequence, while class II are characterized by three homologous motifs, designated as motifs 1, 2, and 3 (*3*).

Arginyl-tRNA synthetase (ArgRS)[EC 6. 1. 1.19][1] belongs to class I aaRSs with the exception of no KMSKS sequence, which is considered to be involved in the amino acid activation step in this class of aaRSs (*3*−*6*). This enzyme is also unique in this class for its another feature: like glutaminyl-tRNA synthetase (GlnRS) and glutamyl-tRNA synthetase (GluRS), ArgRS requires the presence of the cognate tRNA for the ATP−PPi exchange reaction, while the other 17 aaRSs can catalyze the first-step reaction without their cognate tRNAs (*7*, *8*). Because of these unique features, ArgRS from *Escherichia coli*, a single-peptide enzyme consisting of 577 amino acid residues with a molecular mass of 64.8 kDa, has been the focus of study in our laboratory for many years (*9*).

It has long been thought that a cysteine residue is crucial to the activities of many enzymes, and sulfhydryl reagents were usually used to detect the role of cysteine residues. For example, *N*-ethylmaleimide (NEM), a rather specific sulfhydryl reagent, was chosen to investigate the effect of thiol groups in rat liver microsomal glucose-6-phosphatase (Glc-6-Pase) on its activity. It was found that three thiol groups are important for the activity of Glc-6-Pase (*10*). A series of chemical modification experiments showed that many aaRSs could be inactivated by at least one sulfhydryl reagent, suggesting that cysteine residues might possess an important role in the catalysis of this enzyme family (*11*).

Recent studies also revealed that some aaRSs from *E. coli*, such as methionyl-tRNA synthetase (MetRS) (*12*), isoleucyl-tRNA synthetase (IleRS) (*13*), alanyl-tRNA synthetase (AlaRS) (*14*), GluRS (*15*), and so-forth, are zinc-binding proteins and possess “zinc-finger”-like motif sequence in which cysteine residues are vital to the enzymatic activity. Moreover, in AlaRS, the results of site-directed mutagenesis showed that not only those cysteine residues in the“zinc-finger”-motif-like sequence, but also those near the C-terminal are essential for its activity (*16*). However, in some enzymes, such as glycyl-tRNA synthetase (GlyRS) (*17*) and

[†] This work was supported by Grant No. 39730120 from the National Natural Science Foundation of China and Grant No. KJ951-B1-610 from Chinese Academy of Sciences.

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[1] Abbreviations: ArgRS, arginyl-tRNA synthetase; aaRS, aminoacyl-tRNA synthetase; DTNB, 5,5′-dithiobis(2-nitro benzoic acid); IAA, iodoacetamide; NEM, *N*-ethylmeimide; SH, sulfhydryl.

10.1021/bi990392q CCC: $18.00 © 1999 American Chemical Society
Published on Web 08/05/1999

Proyl-tRNA synthetase (ProRS) (*18*) from *E. coli*, aspartyl-tRNA synthetase (AspRS) from *Saccharomyces cerevisiae* (*S. cerevisiae*) (*19*), creatine kinase (*20*), NAD-malic enzyme (*21*), and firefly luciferase (*22*), the sulfhydryl groups that were shown to be essential for enzymatic activity by chemical modification are not directly involved in catalysis. Therefore, the functional role of cysteine residues in enzymes should be defined with more caution.

As deduced from the gene of *E. coli* ArgRS, four cysteine residues are located at position 24, 320, 520, and 537 (*4*). The latter two in the C-terminal domain might constitute a putative "zinc-finger"-like motif sequence of $^{517}HX_2CX_{15}HC^{537}$. To probe the role of cysteine residues in *E. coli* ArgRS, we performed chemical modification and site-directed mutagenesis. In this paper, the effects of covalent attachment of sulfhydryl reagents and replacement of Cys by Ala on the enzymatic activity are reported.

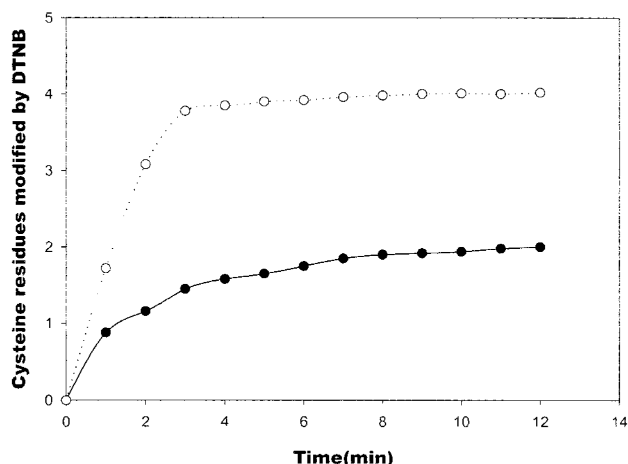

FIGURE 1: Titration of arginyl-tRNA synthetase with 5, 5′-dithiobis (2-nitrobenzoate). (●) in 100mM phosphate buffer (pH7.27) containing 5μM enzyme, 2mM DTNB; (○) in the above solution plus 0.2% SDS. Titration result was followed by absorbance at 412 nm, as described in the Materials and Methods.

MATERIALS AND METHODS

Materials. DTNB, NEM, and β-mercaptoethanol were purchased from Shanghai Dong Feng Biochemical Technology Company, China; IAA from Fluka Biochemika, Switzerland; Site-directed mutagenesis and sequencing primers were synthesized by a DNA synthesizer in Shanghai Institute of Biochemistry. Total tRNA containing $tRNA^{Arg}{}_2$ was isolated from an *E. coli* strain overproducing $tRNA^{Arg}{}_2$ (*23*). The $tRNA^{Arg}{}_2$ was purifed from total tRNA to more than 90% homogeneity through a DEAE-sepharose CL-6B and a BD-cellulose column (*23*).

Purification of ArgRS. ArgRS was purified from the overproduction strain, *E. coli* TG_1 transformant containing recombinant plasmid pUC18-*argS*, via a two-step chromatography on DEAE-Sephacel by the method described previously (*24*).

Determinations of SH groups in ArgRS. SH groups in ArgRS were determined using DTNB according to Riddles et al. (*25*). The reaction was performed at 16 °C in 100mM potassium phosphate buffer (pH 7.27) containing 5μM ArgRS and 2mM DTNB. Absorbency change at 412 nm was recorded, and total SH content was calculated assuming a molar absorption coefficient (ϵ_m) of 14 150 under native condition and 13 700 in the presence of 0.2% SDS for the reagent (*25*), respectively. The concentration of ArgRS was determined by absorbency at 280 nm with ϵ_m of 76 500.

Chemical Modifications of Cysteine Residues in ArgRS. The cysteine residues in ArgRS were modified by IAA or NEM according to Riordan and Valle (*26*). The modification was performed at 16 °C in 0.5 mL of 20mM phosphate buffer (pH7.27) containing 16.5μM ArgRS, 40% glycerol, and either 60mM IAA or 0.4mM NEM. The reaction was terminated by addition of 100mM β-mercaptoethanol after various time intervals to assays for determining both the remained activity and the number of SH groups.

Assays of Aminoacylation Activity. The aminoacylation activity of ArgRS was determined by the method from S. X. Lin et al. (*9*). The assays were performed at 37 °C in a reaction mixture of 25μL containing 50mM Tris-HCl, pH 7.4, 8mM $MgCl_2$, 4mM ATP, 80mM KCl, 0.5mM dithiothreitol (DTT), 0.1mM EDTA, and 20.5 mg/ml total tRNA (corresponding to a $tRNA^{Arg}{}_2$ content of 534μM) and 0.1mM [^{3}H]arginine (25μCi/μmol). One unit was defined as the amount of enzyme that charges 1 nmol arginine to $tRNA^{Arg}$ in 1 min under the standard condition. The specific activity was defined as the units of enzyme per milligram protein. The K_m values for three substrates were determined under similar condition, except for different concentrations of the corresponding substrates.

Site-Directed Mutagenesis. Mutagenesis was carried out as described by Kunkel (*27*). The primers (25−35 bases long) for site-directed mutagenesis were complementary to the single stranded *argS*, except for the changes of codons TGC and TGT for Cys to codons GGC and GGT for Ala, respectively. Single-site mutations of Cys→Ala were made at each of the four Cys residues, Cys^{24}, Cys^{320}, Cys^{520}, and Cys^{537}, while double-site mutants were created in the same way with an additional primer corresponding to the DNA sequence at another position. The mutated *argS* as identified by dideoxy sequencing was recombined into pUC18. *E. coli* TG1 was transformed with the recombinant plasmid for overexpression.

RESULTS

Titration of Cysteine Residues in ArgRS with DTNB. The cysteine residues in ArgRS were titrated by DTNB either in the presence or absence of denaturing agent (Figure 1). Reaction of DTNB with *E. coli* ArgRS causes a time-dependent labeling of TNB on the enzyme. In the presence of 0.2% SDS, all four SH could be titrated by DTNB. However, in the native state only two of the four SH in ArgRS were DTNB-sensitive. Additional assays in the presence of arginine or ATP showed that arginine and ATP did not affect the number of titrated SH group, while in the presence of $tRNA^{Arg}$, the number of titrated SH group became three.

Modifications of ArgRS with IAA and NEM. The effects of IAA and NEM on the enzyme are shown in Figure 2, A and B, respectively. The chemical modification of the SH groups by these two reagents resulted in a time-dependent loss of enzymatic activity. IAA modified only one of the four cysteine residues and the IAA-labeled enzyme showed about 50% of the activity of the control. Two of the four cysteine residues in ArgRS were modified by NEM. As a

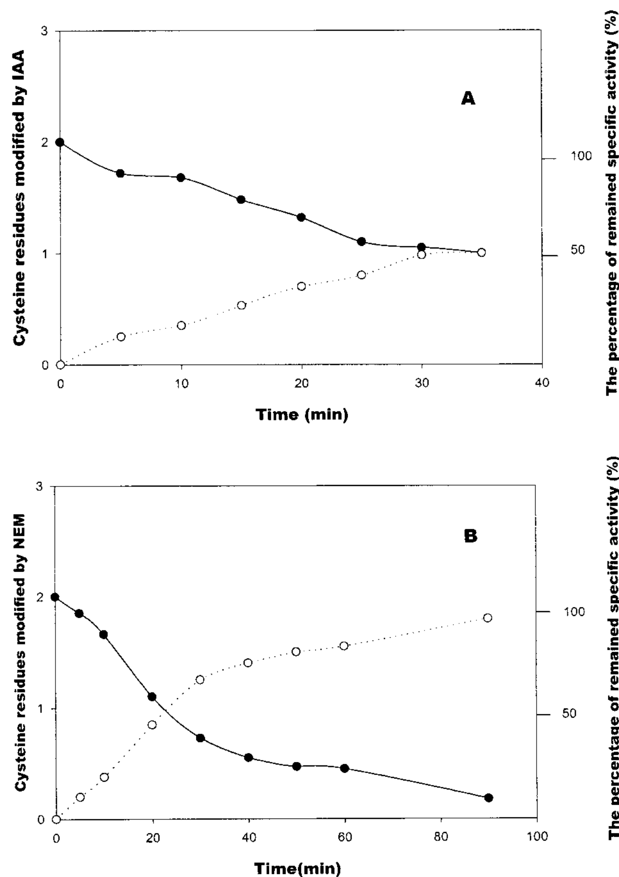

FIGURE 2: The reaction of sulfhydryl reagents with arginyl-tRNA synthetase. (A) IAA. (B) NEM. At regular intervals a 50μL aliquot of the reaction mixture was removed from the cuvette and 100mM β-mercaptoethanol added to terminate the reaction. A 5μL sample diluted 3,000−5,000-folds was used to assay the aminoacylation activity expressed as the percentage of that of a control sample containing no SH reagents (●); the rest sample was applied onto a Sephadex G-100 column (1 × 10 cm) to remove small molecules, and the protein peak was pooled and titrated by DTNB to assay the SH groups modified by SH reagents (○).

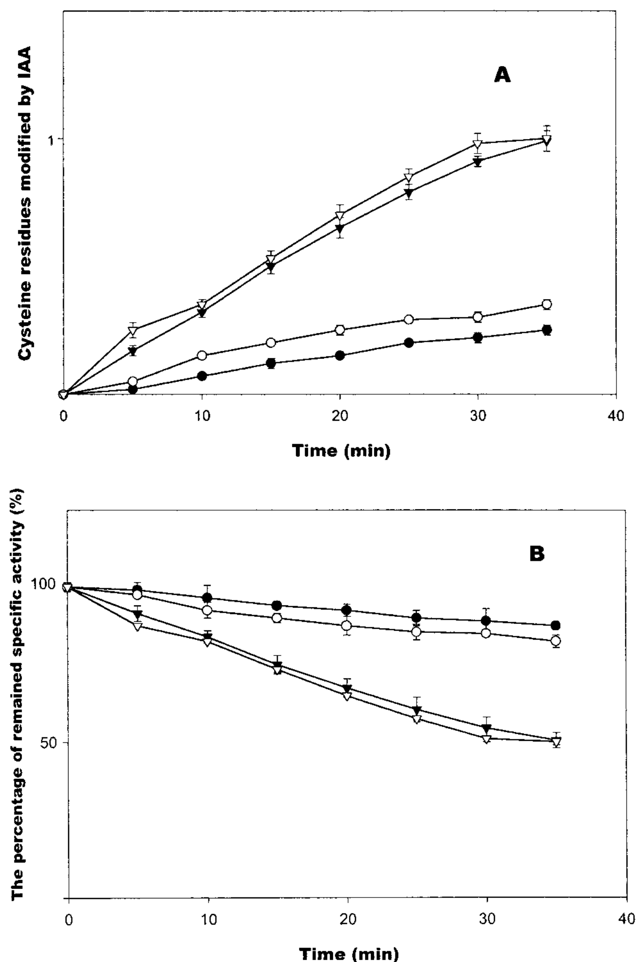

FIGURE 3: The effect of IAA on arginyl-tRNA synthetase in the presence or absence of substrates. (A) The SH groups. (B) The specific activity of ArgRS. The reactions were carried out in the presence of $tRNA^{Arg}$ (●), ATP (○), arginine (▼) or absence of substrate (▽). Experimental details similar to those given in Figure 2.

result, more than 90% of enzyme activity was lost. The IAA-sensitive Cys residue was shown to be one of the two DTNB-sensitive ones and the two NEM-sensitive Cys residues to be identical to the two DTNB-sensitive ones by DTNB titration.

Substrate protection experiments showed that ATP and $tRNA^{Arg}$ but not arginine (Figure 3, A and B) protected modification of the enzyme with IAA and NEM.

Site-Directed Mutagenesis and Expression of Mutant Genes. To determine more precisely the functional role of each Cys in substrate binding and catalysis, we prepared mutants: C24A, C320A, C520A, C537A, C24−320A, C520−537A, and C320−537A. The seven mutant proteins were successfully overproduced and purified.

Determinations of SH Groups in Mutant Enzymes. The purified mutant proteins of ArgRS were also titrated by DTNB either in the native state or in the presence of denaturing agent. In 0.2% SDS, three SH groups in single-site mutant enzymes and two in double-site mutant enzymes were determined by DTNB, as shown in Table 1. In the native state, only one cysteine residue could be detected in ArgRSC320A and ArgRSC537A and none in ArgRSC320−537A, suggesting that the two DTNB-sensitive cysteine residues must be Cys^{320} and Cys^{537}, while Cys^{24} and Cys^{520} were

Table 1: Sulfhydryl Content of Arginyl-tRNA Synthetase and Its Mutants[a]

enzyme	sulfhydryl content (SH/mole) native condition	with 0.2% SDS
ArgRS	2.10 ± 0.10	4.06 ± 0.20
ArgRSC24A	2.05 ± 0.05	3.05 ± 0.15
ArgRSC320A	1.04 ± 0.15	3.04 ± 0.06
ArgRSC520A	2.06 ± 0.14	3.06 ± 0.04
ArgRSC537A	1.00 ± 0.05	3.05 ± 0.03
ArgRSC24−320A	1.05 ± 0.06	2.04 ± 0.05
ArgRSC520−537A	1.02 ± 0.09	2.05 ± 0.09
ArgRSC320−537A	0.10 ± 0.05	2.03 ± 0.07

[a] Samples containing 5μM of purified enzyme, 2mM DTNB in 100mM phosphate buffer (pH7.27). Absorbency at 412 nm was followed until no further increase was observed. All values are the average of three experiments given as means ± S.E.M. See the experimental section for further details.

DTNB-insensitive. Additional experiments verified that in the presence of $tRNA^{Arg}$, Cys^{520} became DTNB-sensitive.

Chemical Modifications of the Mutant Enzymes. After the modifications of ArgRS and five of its mutants (ArgRSC24A, ArgRSC320A, ArgRSC520A, ArgRSC537A, and ArgRSC320−537A) with IAA for 60 min, the remaining SH groups and activities were determined (Table 2). Like the native enzyme, ArgRSC24A, ArgRSC520A, and

Table 2: Effect of Sulfhydryl Reagents on Arginyl-tRNA Synthetase and Its Mutants[a]

sulfhydryl reagent	enzyme	remained sulfhydryl (SH/mole)	remained activity (%)
IAA	ArgRS	1.05 ± 0.05	50.0 ± 3.0
	ArgRSC24A	0.80 ± 0.15	46.8 ± 3.0
	ArgRSC320A	0.88 ± 0.13	95.1 ± 3.3
	ArgRSC520A	1.00 ± 0.05	47.7 ± 3.8
	ArgRSC537A	0.20 ± 0.00	56.0 ± 5.0
	ArgRSC320−537A	0.10 ± 0.03	93.8 ± 1.1
NEM	ArgRS	0.05 ± 0.05	6.0 ± 1.0
	ArgRSC24A	0.20 ± 0.15	10.5 ± 1.5
	ArgRSC320A	0.20 ± 0.04	35.0 ± 2.0
	ArgRSC520A	0.06 ± 0.03	2.1 ± 1.6
	ArgRSC537A	0.10 ± 0.10	25.5 ± 3.0
	ArgRSC320−537A	0.02 ± 0.00	96.2 ± 1.2

[a] 16.5μM ArgRS or its mutants were incubated with 60mM IAA in 20mM phosphate buffer (pH7.27) for 60 min or with 0.4mM NEM for 120 min. The remaining SH contents were determined by DTNB titration. The activities of the modified enzyme were determined under standard conditions (see the experimental section) and presented as % of control. All values are the average of three independent determinations given as mean ± S.E.M.

ArgRSC537A retained about 50% of their original activities, while ArgRSC320A and ArgRSC320−537A remained fully active. The results showed that the particular IAA-sensitive residue must be Cys^{320}.

After ArgRS and five of its mutants were completely modified with 0.4 mM NEM for 2 h, the remaining SH groups and activities were determined, as shown in Table 2. Like the native enzyme, ArgRSC24A and ArgRSC520A were almost completely inactivated by NEM. However, ArgRSC320A and ArgRSC537A still retained some activities and the activity of ArgRSC320−537A remained unchanged. These results showed that Cys^{320} and Cys^{537}, as were identified to be sensitive to DTNB, were also sensitive to NEM.

Aminoacylation Activities and Steady-State Kinetics of ArgRS and Its Mutants. Aminoacylation activities and steady-state kinetics of ArgRS and its mutants were presented in Table 3. Mutation at Cys^{320} resulted in the biggest loss of enzyme activity among single-site mutants. On the other hand, mutation at Cys^{24} reduced the activity by 20%. Mutations at these two sites also slightly decreased the bindings of ATP and arginine (the values of K_m increased from 20 to 60%) but did not affect the binding of $tRNA^{Arg}$. Single-site mutations at Cys^{520} and Cys^{537}, which might be the elements of "zinc-finger"-like motif in the C-terminal domain, did not significantly affect the enzymatic activity. However, the mutations significantly affected binding of ATP (K_{mATP} increased more than 100%). It affected binding of the other two substrates as well. ArgRSC24−320A maintained about 50% of original enzyme activity. ArgRSC520−537A retained only about of 30% activity, although its two corresponding single-site mutants displayed similar activity to the native enzyme. ArgRSC320−537A still had about 28% activity although modification of Cys^{320} and Cys^{537} by NEM caused a total loss of activity. The values of K_m for ATP and arginine of these three double-site mutants were similar, which were about 2-folds as those of native enzyme. The values of $K_{mtRNA}{}^{Arg}$ of the latter two double-site mutants increased slightly, while $K_{mtRNA}{}^{Arg}$ of ArgRSC24−320A did not change. In general, replacement of Cys by Ala mainly

Table 3: Aminoacylation Activity and Kinetic Constants for the Aminoacylation Reaction by Arginyl-tRNA Synthetase and Its Mutants[a]

enzyme	specific activity (U/mg)	relative activity (%)	k_{cat} (s^{-1})	$K_{m.Arg}$ (μM)	$K_{m.ATP}$ (mM)	$K_{m.tRNA}{}^{Arg}$ (μM)
ArgRS	14 000	100.0	28.0	12.0	0.9	2.5
ArgRSC24A	10 480	75.0	21.4	16.0	1.2	2.4
ArgRSC320A	9 000	64.2	19.2	20.0	1.1	2.6
ArgRSC520A	13 200	94.3	22.5	17.0	2.0	2.7
ArgRSC537A	13 500	96.0	25.0	22.0	2.0	2.8
ArgRSC24−320A	7 500	53.6	14.6	23.0	2.5	2.6
ArgRSC520−537A	4 100	29.3	8.3	26.0	2.1	3.3
ArgRSC320−537A	4 030	28.8	8.4	23.7	2.0	3.4

[a] The activity of purified ArgRS and its mutants were measured by aminoacylation of tRNA under standard conditions (see the Experimental Section) and are presented as specific activity and as % of the activity of native enzyme. Parameters were determined from standard assays assuming simple Michaelis−Menten kinetics. The values of K_{cat} were extrapolated from experiments performed at ATP and arginine concentrations of 4mM and 100μM respectively, with varying $tRNA^{Arg}$ concentration. The values of K_m for arginine ($K_{m.Arg}$) or for ATP ($K_{m.ATP}$) were determined using total *E. coli* tRNA (contained 50% $tRNA^{Arg}$) (*23*), and arginine concentrations ranged from 0.78 to 100μM or ATP concentrations ranged from 31μM to 4mM. The K_m values for tRNA ($K_{m.tRNA}$) were obtained using *E. coli* $tRNA^{Arg}$ concentrations ranged from 0.2 to 50μM.

affected the binding of ATP and arginine, with exception of mutation at Cys537, which appeared to slightly affect the binding of $tRNA^{Arg}$ instead.

The K_{cat} values of all the mutants were decreased with those of the three double-site mutants to a greater extent, as compared with that of ArgRS. In the single-site mutants, Cys^{24}→Ala and Cys^{320}→Ala might affect the active site indirectly and play an indirect role in enzymatic catalysis, although they did not obviously affect the binding of the three substrates to the enzyme. However, the single change of Cys^{520} or Cys^{537} to Ala in the putative "zinc-finger"-like motif did not affect the activity of ArgRS. Interestingly, when both cys520 and cys537 were mutated, the K_{cat} of the double-site mutant decreased about 70%. The simultaneously mutation of the two cysteine residues located on the surface of the molecule, i.e. Cys^{320} and Cys^{537}, also reduced the K_{cat} by 70%.

DISCUSSION

E. coli ArgRS, which contains four cysteine residues at position 24, 320, 520, and 537, was chosen as a model for the study on the role of cysteine residues in the aaRSs (*4*). On the basis of the crystal structure of ArgRS from *Saccharomyces cerevisiae*, the four Cys in *E. coli* ArgRS are located within three α-helices: Cys^{24} in H_3 of the additional domain-1, Cys^{320} in H_{13} of the catalytic domain, and Cys^{520} and Cys^{537} in H_{22} of the additional domain-2 (*28*). Titration by DTNB showed that four Cys could be labeled in the denaturing condition and only two were sensitive in the native state (Figure 1), suggesting that no disulfide bonds were formed in the molecule and that two Cys were located on the surface of the molecule. According to the crystal structure of ArgRS, three Cys-containing α-helix are distributed in different domains and the two Cys residues in the C-terminal domain are located at opposite ends of H_{22} (*28*). Therefore, it is not likely to form disulfide bond between cysteine residues.

To probe the functional significance of Cys residues in substrate binding and catalytic activity, chemical modification by sulfhydryl site-specific reagents IAA and NEM and substrate protection experiments were performed. The results suggested that the two Cys residues located on the surface of the enzyme molecule might be essential for catalytic activity and likely to be involved in the binding of the two substrates $tRNA^{Arg}$ and ATP (Figure 2 and Figure 3).

To determine if the inactivation of ArgRS with these two reagents was a result of steric hindrance by the bulky chemical group attached near the active site, we constructed seven mutants with alterations of Cys→Ala at either single-site or double-site. The activities and steady-state kinetic constants of these mutants showed that not only the DTNB- and NEM-sensitive Cys^{320} and Cys^{537} but also Cys^{24} and Cys^{520} were not crucial for the enzymatic activity, and that they only affect the catalytic activity indirectly (Table 3). Moreover, the fluorescent spectrum of the native enzyme and its mutants also showed that replacement of Cys by Ala did not cause detectable conformational change of the enzyme molecule (data not shown). Therefore, it was the steric interference introduced by NEM and IAA that prevented the proper positioning of substrates for catalysis and resulted in a decreased reaction velocity. Although chemical modification is a simple and effective method to probe the role of particular residues in enzymatic activity, it is often limited by the introduction of steric hindrance and the presence of unspecific modification. Wrong conclusions may be drawn if chemical modification is depended on solely (*17*−*22*).

Modification of the enzyme by sulfhydryl reagents showed Cys^{320} and Cys^{537} might be located on the surface of the molecule. From the structure of yeast ArgRS, both of the Cys^{320}-contained H_{13} and the Cys^{537}-contained H_{22} appear to be located outside (*28*). Substrate protection experiment showed that in the presence of $tRNA^{Arg}$, Cys^{520} also became DTNB-sensitive, indicating that a conformation change of ArgRS occurred upon binding of $tRNA^{Arg}$ and implying that Cys^{520} might be located near the binding site of $tRNA^{Arg}$. In fact, the model of Cavarelli et al. showed that Cys^{520}-containing H_{22} is actually involved in the protein-tRNA interface (*28*).

Site-directed mutagenesis also provided a tool to further investigate the role of each Cys in the function of the enzyme. N-terminus is considered to be vital to the tRNA binding of ArgRS. According to the model of Cavarelli et al., the tRNA binding domain is at the N-terminus of ArgRS (*28*). However, the value of K_m $tRNA^{Arg}$ for ArgRSC24A was not changed, suggesting that Cys→Ala at this position did not affect binding of $tRNA^{Arg}$. Cys^{320} is the only Cys that located in active site. However, mutations of this residue did not change the catalysis dramatically. These results suggest that this sulfhydryl group is not essential for the enzymatic activity and may only play an indirect role. An alignment of 15 ArgRS sequences from different sources also shows that Cys^{24} and Cys^{320} are not conserved.

While Cys^{520} and Cys^{537} appear to be involved in the formation of a "zinc-finger"-like motif sequence, substrate binding ability and catalytic activity of ArgRSC520A and ArgRSC537A were changed less than 2-folds. However, in some aaRSs containing Zn^{2+}-binding motif, mutations of Cys in the motif were reported to reduce the catalytic efficiency by more than 1000-folds (*12*−*16*). Therefore, the sequence from residue 517 to 537, $H-X_2-C-X_{15}-H-C$, may not be a "zinc-finger" motif in *E. coli* ArgRS. Nureki et al. also indicated that no Zn^{2+} ion was detected in the enzyme (*29*). Although these two Cys did not form Zn^{2+}-binding motif, the kinetic constants of the two single-site mutants and one double-site mutant showed that the binding of three substrates were affected. For ATP and arginine, the values of K_m increased about 2-fold, and to the mutants containing mutation at both positions, binding of $tRNA^{Arg}$ was also affected (Table 3). According to the model of Cavarelli et al., helices H_{21}, H_{22} and H_{23} interact underneath the floor of the active site with HIGH-containing helix H_6 (the 'HIGH' signature motif in ArgRS is involved in binding of both ATP and arginine); and the helices $H_{17}-H_{18}$ and H_{22} are crucial to tRNA recognition (*28*). An alignment of 15 ArgRS sequences from different sources indicates that Cys^{520} and Cys^{537} are semi-conserved but not present in *S. cerevisae* ArgRS.

Cysteine residues are involved in the catalysis of many enzymes (*10*). Recently, some studies still indicated that the cysteine residues in some aaRSs were involved in the binding of substrate molecules (*12*−*16*, *29*). In our study, chemical modification, substrate protection, and site-directed mutagenesis provided experimental evidence that the cysteine residues in *E. coli* ArgRS affected substrate binding indirectly. More importantly, they were not essential for the catalysis of the enzyme. Therefore, in addition to GlyRS and ProRS from *E. coli* and AspRS from *S. cerevisiae*, *E. coli* ArgRS is another aaRS in which cysteine residues are actually not crucial for its catalytic activity.

ACKNOWLEDGMENT

We thank Dr. Doris M. Benbrook at the University of Oklahoma Health Science Center for her careful reading of this manuscript and Dr. Cai-Zhong Jiang at Mendel Biotechnology, Inc. for his review of the manuscript.

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BI990392Q