Chemical Modification and Site-Directed Mutagenesis of the Single Cysteine in Motif 3 of Class II *Escherichia coli* Prolyl-tRNA Synthetase†

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ABSTRACT: Class II prolyl-tRNA synthetase (ProRS) from *Escherichia coli* contains all three of the conserved consensus motifs characteristic of class II aminoacyl-tRNA synthetases. In this study, chemical modification and site-directed mutagenesis of the single cysteine located at position 443 in motif 3 of *Escherichia coli* ProRS is carried out. We show that chemical modification of C443 blocks the ability of the enzyme to form the activated aminoacyl-adenylate, a prerequisite for $tRNA^{Pro}$ aminoacylation. Nearly complete protection from inactivation is achieved by preincubating the enzyme with ATP or ATP and proline, but not proline alone or $tRNA^{Pro}$. Mutagenesis of C443 to amino acids Ala, Gly, and Ser resulted in significant decreases (16−225-fold) in k_{cat}/K_M^{Pro} as measured by the ATP−PP_i exchange reaction. The Ala and Gly mutations have a relatively small effect (4−7-fold) on the overall aminoacylation reaction, while the activity of the C443S mutant in this same assay is substantially reduced (80-fold). A sequence comparison of the motif 3 region of class II synthetases shows that C443 aligns with residues that have been implicated in amino acid binding specificity. The results of our study suggest that while the thiol located at position 443 of *Escherichia coli* ProRS is not essential for catalysis, this residue is likely to be in a buried region that forms the prolyl-adenylate substrate binding pocket.

Aminoacyl-tRNA synthetases are an ancient family of enzymes and are key components in the process by which the genetic code is translated into protein sequences (Moras, 1992, 1993). In most living cells, there are at least 20 synthetases, 1 for each amino acid. These enzymes catalyze a two-step reaction that results in the esterification of a specific amino acid to either the 2′- or 3′-hydroxyl group on the ribose of the terminal adenosine of their cognate tRNAs (Freist, 1989). The first step involves the recognition of the amino acid and its condensation with ATP to form an enzyme-bound "activated" aminoacyl-adenylate intermediate (aa-AMP)[1] with release of pyrophosphate (PP_i) (eq 1) (Warshel et al., 1994). The second step involves the displacement of AMP by attack of the ribosyl hydroxyl of tRNA on the carbonyl carbon of the amino acid (eq 2).

$$E + aa + ATP \Leftrightarrow [E\cdot(aa\text{-}AMP)] + PP_i \quad (1)$$

$$[E\cdot(aa\text{-}AMP)] + tRNA \rightarrow aa\text{-}tRNA + AMP + E \quad (2)$$

Although all synthetases catalyze a similar chemical reaction, there is little homology with respect to their primary sequence (Eriani et al., 1990). In fact, this family of enzymes is characterized by its structural diversity (Schimmel, 1987). On the other hand, tRNA molecules show few distinguishing features (Saks et al., 1994). Each tRNA is thought to fold into a similar L-shaped tertiary structure (Kim et al., 1974a,b; Robertus et al., 1974). The "catalytically active" 3′-terminal CCA is the same in all tRNAs. Furthermore, a single amino acid may be attached to as many as six tRNA isoforms by the same synthetase.

All 20 aminoacyl-tRNA synthetases from *Escherichia coli*, and many more from other prokaryotic and eukaryotic sources, have been sequenced. These enzymes have been divided into 2 classes of 10 each based on computer-assisted sequence alignments (Eriani et al., 1990). The 10 class I synthetases are grouped according to the presence of the "HIGH" and "KMSKS" consensus sequences. They are further characterized by the attachment of the amino acid to the 2′-hydroxyl group on the tRNA 3′-terminal ribose, and exist predominantly as monomeric structures in solution (Moras, 1993). Several class I synthetases have been cocrystallized with substrate molecules (Brick et al., 1989; Rould et al., 1989, 1991; Brunie et al., 1990). The 10 class II synthetases, with 1 exception, aminoacylate the 3′-hydroxyl group and tend to form dimers and tetramers in solution (Cusack et al., 1991; Moras, 1992). Class II synthetases are characterized by three sequence regions, designated as motifs 1, 2, and 3 (Eriani et al., 1990; Cusack, 1993; Davis et al., 1994). The cocrystal structures of *Thermus thermophilus* SerRS with two seryl-adenylate analogs (Belrhali et al.,

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[1] Abbreviations: aa-AMP, aminoacyl-adenylate; AlaRS, alanyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; BSA, bovine serum albumin; βME, 2-mercaptoethanol; DTT, dithiothreitol; GlyRS, glycyl-tRNA synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HisRS, histidyl-tRNA synthetase; IAEDANS, 5-[[2-[(2-iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; IM, iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LysRS, lysyl-tRNA synthetase; PP_i, pyrophosphate; ProRS, prolyl-tRNA synthetase; SerRS, seryl-tRNA synthetase; TNB, 5-mercapto-2-nitrobenzoate.

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1994), yeast aspartyl-tRNA synthetase (AspRS) with the aspartyl-adenylate (Cavarelli et al., 1994), *E. coli* lysyl-tRNA synthetase (LysRS) with lysine (Onesti et al., 1995), and *E. coli* histidyl-tRNA synthetase (HisRS) with histidyl-adenylate (Arnez et al., 1995) show that amino acids in motifs 2 and 3 make specific contacts with the bound substrates. The crystal structures also show that these enzymes possess a very similar catalytic domain composed of a seven-stranded antiparallel β-sheet (Cusack et al., 1990; Ruff et al., 1991). A division of the class II synthetases into two subclasses, IIa and IIb, has been proposed (Cusack et al., 1991). Based on sequence alignments and secondary structural predictions, SerRS, prolyl-tRNA synthetase (ProRS), and threonyl-tRNA synthetases are closely related. These three enzymes, together with the HisRS and alanyl-tRNA synthetases (AlaRS), make up subclass IIa.

While several aminoacyl-tRNA synthetases have been cocrystallized with substrates and substrate analogs, each synthetase has unique aspects, and to understand amino acid and tRNA specificity and discrimination by this important class of enzymes, it will be necessary to acquire information on all 20 recognition systems. To probe the role of class II consensus motifs in substrate binding and catalysis further, *E. coli* ProRS, a class IIa synthetase of unknown structure, has been chosen for this study. ProRS is a functional dimer of identical subunits with a molecular mass of 127 kDa (Eriani et al., 1990). ProRS is unique among the tRNA synthetases in that its substrate proline is actually an *imino* acid. Recent studies have identified important tRNA recognition elements in three different domains of $tRNA^{Pro}$, but the enzyme side chains that are involved in substrate interactions are unknown (Liu & Musier-Forsyth, 1994; McClain et al., 1994; Liu et al., 1995). Furthermore, while there has been no crystal structure reported for ProRS to date, this enzyme has a relatively high degree of sequence homology with *E. coli* SerRS (14% strict identity and 37% conservative substitutions) whose structure has been solved (Cusack et al., 1990; Eriani et al., 1990). *E. coli* ProRS contains a single cysteine in its 572 residue amino acid sequence located in motif 3. This residue, C443, is seven amino acids removed from the strictly conserved arginine of motif 3 that is shown to make contact with N_3 of the adenine ring of ATP in *T. thermophilus* SerRS and yeast AspRS (Belrhali et al., 1994; Cavarelli et al., 1994). Residues in other class II synthetases located in analogous positions to C443 of *E. coli* ProRS have been implicated in helping to confer amino acid specificity (Kast & Hennecke, 1991; Ibba et al., 1994, 1995; Shi et al., 1994; Onesti et al., 1995; Arnez et al., 1995).

To probe the role of C443 in motif 3 of *E. coli* ProRS, we performed chemical modification with sulfhydryl-specific reagents and site-directed mutagenesis. In particular, chemical modification of ProRS with iodoacetamide (IM), 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was carried out. The effects of covalent attachment of these probes on ProRS activity are reported. Inactivation and substrate protection experiments suggest that the aminoacyl-adenylate binding site is in close proximity to C443 of motif 3. Site-directed mutagenesis is carried out to more precisely define the role of this active-site residue in substrate binding and catalysis.

MATERIALS AND METHODS

Materials. *E. coli* strain SY327 carrying the plasmids pGT1-2 and pGT1-2# was a gift from Mike Syvaney. pGT1-2 contains the T7 RNA polymerase gene under the heat-inducible λP_L promoter. pGT1-2# contains the ProRS gene under a T7 polymerase promoter. Wild-type ProRS purification and preparation of unmodified $tRNA^{Pro}$ by *in vitro* transcription were performed as described previously (Liu & Musier-Forsyth, 1994). Purification of histidine-tagged ProRS was performed as described below. [^{14}C]-Iodoacetamide was purchased from Amersham, and [^{32}P]-pyrophosphate was from New England Nuclear. DTNB was purchased from Sigma, and IAEDANS was from Molecular Probes, Inc.

Determination of Protein Concentration. *E. coli* ProRS concentrations were determined by the Bradford method using the Bio-Rad protein assay kit and bovine serum albumin (BSA) as the standard (Bradford, 1976). This assay was calibrated for ProRS by quantitative amino acid analysis (Tarr, 1986) performed in the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. Comparison of the results obtained by both methods, using aliquots of the same sample, reveals that the Bradford method overestimates the ProRS protein concentration by 44%. All protein determinations were corrected by this amount.

Labeling of ProRS by IM and IAEDANS. Modification of ProRS with IM and IAEDANS was conducted at approximately 1000:1 probe to enzyme ratios unless otherwise indicated. Stock solutions (50 mM) of IM and IAEDANS were prepared immediately before use in 50 mM HEPES, pH 7.5 (buffer A), and kept in the dark. Labeling reactions were initiated by addition of probe (16 mM) to ProRS (16 μM) in buffer A at room temperature (22 °C). At desired time intervals, aliquots of the reaction mixture were quenched in equal volumes of 100 mM 2-mercaptoethanol (βME) at 4 °C and kept on ice. A control reaction, using buffer A instead of probe, was performed simultaneously to assure tolerable reaction conditions. The effect of probe modification on the catalytic activity of ProRS was determined immediately after the completion of the labeling reactions. The aminoacylation activity was determined using *in vitro* $tRNA^{Pro}$ transcripts as previously described (Liu & Musier-Forsyth, 1994). The ATP−PP_i exchange activity was determined as described below.

Labeling ProRS with DTNB. DTNB modification was accomplished using a 25:1 probe to ProRS ratio in 100 mM Tris-HCl, pH 8.0 (buffer B). A 2 mM DTNB stock solution was prepared immediately prior to use in 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, and kept in the dark. G-50 Sephadex (Pharmacia) spin columns were equilibrated with buffer B, such that the packed resin volume was 1.5 mL, and kept at 4 °C until use. Two identical samples of enzyme (150 μL at 21 μM) were prepared in buffer B (label and control) and allowed to come to room temperature (10 min at 22 °C). Prior to probe addition, 50 μL aliquots of each solution were eluted through the spin columns and reserved for the initial activity determination. The labeling reaction was initiated by addition of DTNB to a final concentration of 400 μM. For the control reaction, an equal amount of buffer B was substituted for the DTNB. After 60 min, the reaction mixture was applied to a spin column to remove unreacted DTNB. Reactivation of ProRS was accomplished by reacting 100

μL of the DTNB-labeled enzyme solution with 5 mM dithiothreitol (DTT) for 1 h at 4 °C. The release of 5-mercapto-2-nitrobenzoate (TNB) was monitored at 412 nm until the signal remained constant (20 min at 22 °C). The free DTNB in this sample was then removed from ProRS using spin columns as described above. All of the samples were stored at 4 °C pending activity analysis.

Determinations of the DTNB labeling stoichiometry and ATP−PP_i exchange activity were performed on the same day as the labeling experiment. The amount of DTNB attached to ProRS was determined by denaturing the enzyme in an equal volume of 6 N guanidine hydrochloride with 10 mM DTT. The amount of released TNB was quantitated by absorbance spectroscopy ($\epsilon_{412} = 14$ mM^{-1}) (Riddles et al., 1983). The effects of DTNB modification and release on the activity of ProRS were determined in duplicate by the ATP−PP_i exchange assay as described below.

Determination of Labeling Stoichiometry Using [^{14}C]-IM. ProRS was labeled with [^{14}C]-IM (60 mCi/mmol) as described above for IM. The specific activity (cpm per picomole of IM) of the reaction mixture was determined by counting 2 μL aliquots injected directly into scintillation fluor. Two control reactions, one that did not include ProRS and the other without [^{14}C]-IM, were performed simultaneously. At desired time points, excess reagent was removed by gel filtration on G-50 Sephadex spin columns (1.5 mL packed column volume). Aliquots (10 μL) were counted to determine the amount of ^{14}C in the column eluant. The control reaction without ProRS showed that 99% of the ^{14}C was removed by the spin columns. The ProRS concentration in the column eluant was also determined for each sample. The labeling stoichiometry was calculated from the ratio of the [^{14}C]-IM to protein concentrations. The activity of the [^{14}C]-IM-labeled ProRS samples was determined by the ATP−PP_i exchange assay as described below.

Substrate Protection Studies. Substrate protection studies were performed by preincubation of a mixture containing 0.17 μM ProRS, 0.25 mg/mL BSA, and 50 mM HEPES, pH 7.5, in the presence of varying amounts of proline, ATP, ATP + proline, or $tRNA^{Pro}$ for 15 min at 22 °C prior to addition of 15 mM IM or IAEDANS. IM and IAEDANS stock solutions were prepared immediately before use as described above and kept from light. Two control reactions were run simultaneously. One control reaction did not include substrate, and the other contained neither probe nor substrate. Aliquots were removed at the desired time intervals, quenched in equal volumes of 100 mM βME, 4 °C, and assayed for ATP−PP_i exchange activity as described below.

Activity Assays. Aminoacylation assays were performed as previously described (Jasin et al., 1985; Liu & Musier-Forsyth, 1994). The steady-state kinetic parameters for ATP and proline were determined using the ATP−PP_i exchange assay as previously described (Calendar & Berg, 1966; Hill & Schimmel, 1989; Heacock et al., 1996). The rate constants were determined using substrate concentrations ranging from ~5-fold higher to ~5-fold lower than the deduced K_M values and a 10-fold excess over K_M for the nonvaried substrate. ATP was varied from 0.05 to 1.0 mM with a constant 10 mM proline. Proline was varied from 0.05 to 6.0 mM with 2.4 mM ATP included. The kinetic constants were derived from a Lineweaver−Burk plot. For the substrate protection experiments using ATP and/or proline, the concentration of the substrates in the assay mixture was reduced to account for their presence in the enzyme solutions.

Plasmids, Site-Directed Mutagenesis, and Purification of Mutant Proteins. To allow purification of overexpressed mutant proteins from wild-type endogenous *E. coli* ProRS, the gene encoding the wild-type protein was first cloned into the *Bam*HI/*Hin*dIII sites of plasmid pQE-30 (Qiagen). This allowed overexpression of a ProRS variant that terminated in 6 histidine residues, and had a total of 18 additional amino acids at the N-terminus of the enzyme. A mutation at the start codon of ProRS was also introduced to replace the Met with a Ser (M1S). The plasmid containing the histidine-tagged version of ProRS was designated pCS-M1S.

Site-directed mutagenesis was accomplished by the Kunkel method (Kunkel, 1985). The entire gene (in the case of the M1S mutation) or a 790 nucleotide long fragment of the gene (in the case of the C443X mutations) was cloned into a pBluescript II KS+ phagemid vector to allow preparation of single-stranded DNA. To generate the C443X mutations, a mixture of deoxynucleotide primers randomized at the C443 codon was used. Following all mutagenesis procedures, the entire gene or the 790 nucleotide long fragment of the gene was sequenced to confirm the presence of only the desired mutation.

Histidine-tagged "wild-type" ProRS and C443X mutant proteins were maintained in the K-12-derived *E. coli* strain M15 [pREP4] (Qiagen), overexpressed, and purified as follows. Two liters of LB media supplemented with 0.1 mg/mL ampicillin and 0.025 mg/mL kanamycin was inoculated with an overnight culture of the strain containing the expression plasmid and grown at 37 °C to an OD_{600} of about 0.5. The cells were induced by addition of 1 mM isopropyl thiogalactoside, and grown for an additional 4 h. All subsequent steps were carried out at 4 °C. Cells (~10 g wet weight) were harvested by centrifugation and stored overnight as frozen pellets at 80 °C. Cells were resuspended in 100 mL of sonication buffer [100 mM K_2HPO_4 (pH 7.8), 0.3 M NaCl, 2 mM β-mercaptoethanol, 0.16 mg/mL benzamidine, 4 μg/mL leupeptin, 4 μg/mL pepstatin A, and a 0.1%/volume of a saturated 2-propanol solution of phenylmethanesulfonyl fluoride]. Following sonication, cells were centrifuged for 45 min at 15000*g*. Protamine sulfate (0.5 mg/mL) was added to the supernatant and stirred slowly for 10 min. The precipitated nucleic acids were removed by centrifugation for 20 min at 15000*g*. The supernatant was loaded directly onto a 3 mL column packed with Ni^{2+}−nitrilotriacetic acid modified resin (Ni−NTA, Qiagen) equilibrated with 25 mL of sonication buffer. Fifty milliliters of wash buffer [100 mM K_2HPO_4 (pH 6.0), 0.3 M NaCl, 2 mM β-mercaptoethanol, and 10% glycerol] was applied to the column followed by 10 mL of wash buffer containing 0.2 M imidazole. The desired protein was eluted with 5 mL aliquots of 0.5 M imidazole in wash buffer. Peak fractions were detected by SDS−polyacrylamide gel electrophoresis, pooled, and concentrated using Centricon 30 (Amicon) concentrators. Protein was judged to be >95% pure by SDS−polyacrylamide gel electrophoresis and stored at −20 °C in 25 mM HEPES (pH 7.5) and 40% glycerol.

RESULTS

Figure 1 shows the effect of covalent modification of ProRS with the sulfhydryl-specific probes IM and IAEDANS.

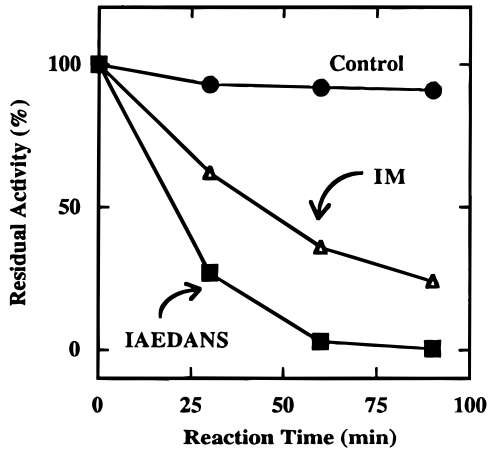

FIGURE 1: Time course of inactivation of ProRS ATP−PP_i exchange activity upon reaction with IM and IAEDANS. 17 μM ProRS was reacted with either 15 mM IM (△), 15 mM IAEDANS (■), or without probe (control, ●) in 50 mM HEPES, pH 7.5 at 22 °C. Aliquots were removed at the indicated time points, and the activity was determined by the ATP−PP_i exchange assay.

Table 1: Covalent Modification of ProRS by [^{14}C]-IM[a]

reaction time (min)	[^{14}C]-IM incorporation (%)	residual activity (%)[b]	unlabeled control activity (%)[b]
0	0	100	100
45	54	54	96
90	102	25	91

[a] Labeling experiments were carried out as described under Materials and Methods. Results are the averages of two trials, and standard errors are less than ±10%. [b] Activity was determined using the ATP−PP_i exchange assay (Calendar & Berg, 1966; Hill & Schimmel, 1989).

Reaction of a 1000-fold excess of these two reagents with ProRS causes a time-dependent inactivation of the ATP−PP_i exchange activity (Figure 1). The ability of the enzyme to form the aminoacyl-adenylate is, therefore, blocked upon reaction with these sulfhydryl-specific probes. A control reaction was conducted simultaneously in the absence of either probe, and ProRS maintains >90% activity under the labeling reaction conditions (Figure 1). As expected, a similar decrease in the $tRNA^{Pro}$ aminoacylation activity was also observed upon reaction with these probes (data not shown). In a separate experiment, the inactivation study using IM was repeated and allowed to proceed to longer times. The same rate of inactivation was observed as shown in Figure 1, but activity was completely abolished by 150 min (data not shown). The stoichiometry of the ProRS labeling reaction was determined using [^{14}C]-IM. As shown in Table 1, the amount of inactivation over time is approximately equal to the amount of [^{14}C]-IM incorporated. When 1:1 labeling is achieved, 75% inactivation is observed. The control reaction maintained >90% of its activity over the same period.

To characterize the inactivation of ProRS by IM and IAEDANS further, labeling reactions were conducted in the presence of saturating amounts of substrates. The steady-state kinetic parameters were first determined for ATP and proline using the ATP−PP_i exchange assay (Hill & Schimmel, 1989). The K_M values for ATP and proline were found to be 0.12 (±0.05) mM and 0.30 (±0.1) mM, respectively. The k_{cat} value was 81 (±40) s^{-1}. Labeling studies in the presence of ATP and proline were then conducted. Preincubation of 0.17 μM ProRS with 10 mM ATP or 10 mM ATP + 10 mM proline affords >90% protection from inactivation by 15 mM IM (Figure 2). In contrast, the rate of inactivation was only decreased by about 10% in the presence of 10 mM proline alone (Figure 2). The substrate protection experiments were repeated using IAEDANS instead of IM, and the same protection trends were observed even up to 25 mM of each substrate (data not shown). Preincubation of ProRS with $tRNA^{Pro}$ showed no evidence of protection from either IM or IAEDANS inactivation (data not shown).

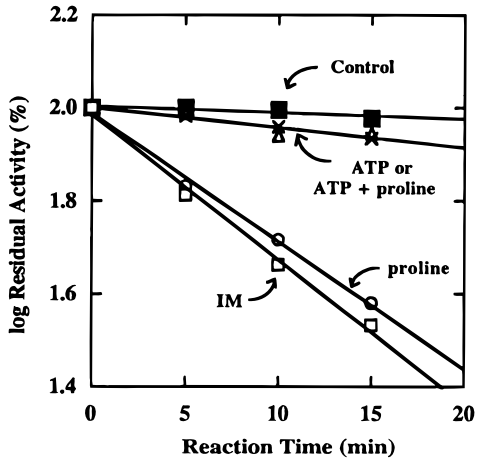

FIGURE 2: Time course of inactivation of ProRS ATP−PP_i exchange activity upon reaction with IM in the presence and absence of substrates. 0.17 μM ProRS was reacted with 15 mM IM at 22 °C in 50 mM HEPES, pH 7.5, 0.25 mg/mL bovine serum albumin, following preincubation of the enzyme with the indicated substrates [no substrates (□), 10 mM proline (○), 10 mM ATP (×), 10 mM ATP + 10 mM proline (△)]. The control reaction (■) contained neither IM nor substrates.

Table 2: Reversible ProRS Inactivation by DTNB[a]

reaction time (min)	DTNB incorporation[b]	residual activity (%)[c]	unlabeled control activity (%)[c]
0	0	100	100
60	94	5	98
DTT treatment[d]	29	65	89

[a] All experiments were carried out as described under Materials and Methods. Results are the averages of two trials, and the standard error is less than ±5%. [b] DTNB incorporation was determined spectrophotometrically (ϵ_{412} = 14 mM^{-1}) (Riddles et al., 1983) after addition of 5 mM DTT under denaturing conditions. [c] Activity was determined using the ATP−PP_i exchange assay (Calendar & Berg, 1966; Hill & Schimmel, 1989). [d] DTT (5 mM) was added under native conditions to release covalently bound TNB.

The labeling experiments were repeated using 17 μM ProRS and 0.4 mM DTNB, a reversible sulfhydryl-specific probe (Bodwell et al., 1984; Ploux et al., 1995). After 60 min of labeling, about 95% of the ATP−PP_i exchange activity was lost relative to the zero time point (Table 2). Approximately 94% of the ProRS in the sample was labeled by DTNB. This was determined by treating the DTNB-labeled ProRS sample with DTT under denaturing conditions and measuring the absorbance at 412 nm caused by release of the bound mercaptan (Bodwell et al., 1984; Ploux et al., 1995). The nonlabeled control reaction lost only 2% of its activity over the same time period (Table 2). Addition of 5 mM DTT to the labeled ProRS sample under native conditions resulted in significant restoration of the ATP−PP_i exchange activity (68% compared to the 60-min time point) (Table 2). Once again, denaturing conditions were used to

Table 3: Kinetic Constants for *E. coli* ProRS Mutants in the ATP−PP_i Exchange Reaction[a]

enzyme	K_M^{Pro} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M^{Pro} (s^{-1} mM^{-1})	relative k_{cat}/K_M^{Pro}
wild-type	0.3 ± 0.1	81 ± 40	270	1.0
C443A	1.7 ± 1.3	28 ± 22	17	0.063
C443G	2.4 ± 0.9	15 ± 2	6.3	0.023
C443S	3.1 ± 1.8	3.7 ± 1.6	1.2	0.004

[a] ATP−PP_i exchange assays were carried out as described under Materials and Methods. Results are averages of two or three trials.

Table 4: Aminoacylation Activity of *E. coli* ProRS Mutants[a]

enzyme	relative k_{cat}/K_M	*x*-fold decrease
wild-type	1	
C443A	0.15	7
C443G	0.26	4
C443S	0.013	80

[a] For technical reasons, aminoacylation assays were carried out using subsaturating amounts (20 μM) of proline and 1−12 μM $tRNA^{Pro}$. Accurate individual kinetic parameters could, therefore, not be determined, and only relative k_{cat}/K_M values are reported. Results are averages of 2−4 trials.

quantitate the amount of DTNB remaining covalently attached to this reactivated sample. This result indicated that the sample was still 29% labeled (Table 2). Therefore, the amount of reactivation (68%) corresponds well with the amount of DTNB released (69%). The nonlabeled control sample retained 89% of its activity after undergoing the same treatment (Table 2).

To determine the functional role of the Cys at position 443, we prepared four site-directed changes: C443A, C443G, C443S, and C443W. To facilitate purification, these changes were made in the context of a histidine-tagged ProRS. The histidine-tag did not affect the kinetic parameters of wild-type ProRS. In the course of this work, we determined the sequence of the entire *E. coli* ProRS gene. We note that these experiments revealed three differences (I26M, V27L, and Q205S) between our wild-type (both non-His-tagged and His-tagged) clone and the *E. coli* ProRS sequence published by Eriani et al. (1990). The ATP−PP_i exchange reaction was used to monitor adenylate formation. Substitution of the smaller residues Ala, Gly, and Ser at position 443 increased the K_M for proline by 6−10-fold (Table 3). Of these three changes, the Ser mutation had the largest effect on the k_{cat} of the ATP−PP_i exchange reaction, decreasing k_{cat} by 22-fold (Table 3). Upon Trp substitution at position 443, we were unable to detect an overexpressed protein on an SDS gel following our standard overexpression protocol. We confirmed that the promoter region of the expression plasmid and the entire coding region of the C443W mutant gene were intact by sequencing, and that the only mutation was the desired change at position 443. Attempts to obtain stably folded C443W protein by varying the growth conditions of the cells were also unsuccessful.

The three mutant proteins that were successfully overexpressed and purified were also tested for overall aminoacylation activity. Due to technical limitations, the aminoacylation experiments were performed at subsaturating proline concentrations. Therefore, the k_{cat} values for the aminoacylation reactions are underestimated, and comparisons between the wild-type enzyme and the three mutants, which have different apparent K_M values for proline (Table 3), may not be valid. Nevertheless, under the experimental conditions employed, the effect on the overall aminoacylation reaction is much greater in the case of the Ser mutation (80-fold decrease in k_{cat}/K_M) than for the C443G and C443A mutations, which resulted in only 4- and 7-fold decreases in k_{cat}/K_M, respectively (Table 4).

DISCUSSION

E. coli ProRS contains a single cysteine at position 443 that is located in the motif 3 consensus sequence common to all class II synthetases (Eriani et al., 1990). To investigate the functional significance of this active site residue in ProRS−substrate interactions, the sulfhydryl-specific fluorescent probe IAEDANS was initially chosen for attachment to C443 (Ferguson & Yang, 1986; First et al., 1989). Chemical modification of ProRS with this reporter molecule was found to cause a time-dependent inactivation of the ATP−PP_i exchange activity (Figure 1). To determine if the inactivation was a result of steric interference by the bulky naphthalene moiety attached near the active site, IM was used to label ProRS. A similar inactivation of the ATP−PP_i exchange activity was observed (Figure 1). Interestingly, the rate of inactivation by IAEDANS is significantly faster than inactivation by the same concentration of IM, despite the fact that IM is much smaller than IAEDANS and would be expected to have greater access to solvent-exposed residues in the active site. The increased reactivity of IAEDANS over IM may be a result of structural factors that attract the probe to the ATP binding site of ProRS (see below). Labeling experiments were also conducted in the presence of substrates. Addition of 10 mM ATP or 10 mM ATP + 10 mM proline to the labeling reaction mixtures resulted in significant protection from inactivation of ProRS by all of the probes tested (Figure 2 and data not shown). Proline alone does not afford significant protection, even at concentrations 20 times the K_M. These results strongly suggest that C443 of *E. coli* ProRS is involved in forming the binding pocket for the activated prolyl-adenylate.

The labeling stoichiometry was determined using [^{14}C]-IM (Table 1). The extent of inactivation approximately parallels the level of probe incorporation. The amount of probe incorporated into ProRS is slightly higher than the amount of inactivation at both time points (≈25%). This may be due to reaction with other amino acids. To confirm that inactivation was a result of modification of only the single cysteine residue at position 443, covalent attachment of DTNB to ProRS was carried out. Similar to IM and IAEDANS, DTNB inactivates the aminoacyl-adenylate-forming activity of ProRS in a time-dependent manner. The level of inactivation correlates closely with the level of DTNB incorporation into ProRS as determined by spectrophotometric analysis (Table 2) (Riddles et al., 1983). Unlike the stable thioether bond formed when iodoacetamides react with cysteine residues, DTNB attaches through formation of a disulfide bond. Release of TNB from modified cysteines can, therefore, be specifically accomplished by addition of reducing agents such as DTT. Addition of DTT to DTNB-labeled ProRS under native conditions removed about 70% of the attached probe, with a concomitant recovery of aminoacyl-adenylate activity (Table 2). The correlation of recovered activity with the amount of TNB released clearly

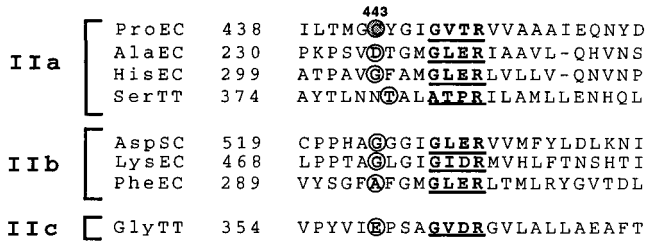

FIGURE 3: Motif 3 region of the class II aminoacyl-tRNA synthetases according to published sequence alignments (Belrhali et al., 1994; Logan et al., 1995). Synthetases are grouped according to subclass (IIa, IIb, or IIc). The GLER tetrapeptide motif is underlined. The single cysteine (C443) in *E. coli* ProRS is indicated by a shaded circle. Open circles surround residues that are discussed in the text. EC refers to *E. coli*; TT, *T. thermophilus*; SC, *S. cerevisiae*.

demonstrates that covalent modification of C443 is directly responsible for the observed inactivation.

To further understand the role of C443, site-directed mutagenesis was performed. Eliminating the side chain at position 443 (C443G) increases the K_M^{Pro} about 8-fold, and has a slightly smaller effect (5-fold decrease) on the k_{cat} of the ATP−PP_i exchange reaction (Table 3). The effect of the Gly mutation on the overall aminoacylation reaction was determined to be relatively minor (∼4-fold) under the experimental conditions employed (Table 4). Replacing the thiol of Cys by hydrogen (C443A) increases the K_M^{Pro} (6-fold) and results in a 3-fold decrease in the k_{cat} for adenylate formation (Table 3). The Ala and Gly substitutions show that the thiol of the cysteine residue is not catalytically essential, since a significant amount of activity is maintained in each case (Tables 3 and 4). The chemical modification studies, on the other hand, clearly showed that modification of the thiol results in inactivation (Figure 1). Furthermore, the substitution of a hydroxyl group for the thiol (C443S) has a large effect on both steps of the reaction, resulting in a 225-fold decrease in the k_{cat}/K_M for adenylate formation (Table 3) and an 80-fold decrease in the overall aminoacylation reaction (Table 4). ProRS appears to be sensitive to the change in polarity and/or differences in hydrogen bonding properties caused by the substitution of a Ser residue at position 443. Ser residues are much more hydrophilic than Cys residues and are typically solvent-exposed. In contrast, Cys (even as a free SH form) is generally buried (Richardson & Richardson, 1989). Therefore, the greater effect of the C443S substitution is consistent with the conclusion that C443 is buried in the substrate binding pocket. Placement of a bulky tryptophan residue at this site did not allow production of a stable protein. The instability of the C443W variant suggests that a large residue cannot fit into the buried site normally occupied by C443.

Figure 3 shows a sequence alignment in the motif 3 region of *E. coli* ProRS (Eriani et al., 1990) along with seven additional class II synthetases (Belrhali et al., 1994; Logan et al., 1995). The characteristic GLER tetrapeptide (underlined) contains the arginine that is absolutely conserved in motif 3 of all class II synthetases. In the *T. thermophilus* SerRS cocrystal structures with substrate analogs, this conserved arginine (R386) makes an indirect contact with N_3 of adenine in the aminoacyl-adenylate through a water molecule (Belrhali et al., 1994). The position of C443 in *E. coli* ProRS is indicated by the shaded circle. Open circles are also placed around residues in the other synthetases shown in this figure that align with C443 of *E. coli* ProRS and/or have been implicated in formation of the aminoacyl-adenylate binding site. For example, threonine 380, which is six residues away from R386 of *T. thermophilus* SerRS (Figure 3), forms a hydrogen bond with the side chain of the serine substrate. In the cocrystal structure of yeast AspRS with the aspartyl-adenylate, the backbone carbonyl of G524 is shown to interact with the hydrogen bonding network that stabilizes the bound amino acid (Cavarelli et al., 1994). Glycine 524 aligns with C443 of *E. coli* ProRS (Figure 3). In *E. coli* AlaRS, mutation of D235, which also aligns with C443 of ProRS, has been shown to cause significant loss of aminoacylation activity (Shi et al., 1994). Furthermore, while the amino acid activation rate was reduced only 7-fold, the rate of transfer of the enzyme-bound adenylate was reduced 15-fold in the D235A mutant. In this study, the researchers concluded that D235 of *E. coli* AlaRS is needed for formation of the pocket that binds the 3′-end of the tRNA and/or for catalysis. In *E. coli* HisRS, the residue in the analogous position to C443 of *E. coli* ProRS is G304 (Figure 3). In the *E. coli* HisRS cocrystal structure with histidyl-adenylate, G304 lies under the imidazole ring of the bound adenylate (Arnez et al., 1995). Moreover, based on the structure, a side chain at this position would be predicted to interfere with histidyl-adenylate binding (C. S. Francklyn, personal communication). Site-directed mutagenesis studies of the analogous residue in *E. coli* phenylalanyl-tRNA synthetase (A294) showed that it is involved in defining the size of the amino acid binding pocket (Kast & Hennecke, 1991; Ibba et al., 1994). Specifically, the A294G mutation causes a change of substrate specificity to para-halogenated phenylalanine analogs. Recently, *E. coli* LysRS has been cocrystallized with its amino acid substrate (Onesti et al., 1995). Glycine 473 of LysRS aligns with C443 of ProRS (Figure 3), and its backbone carbonyl is shown to form an important part of the hydrogen bonding network around the ϵ-amine group of the lysine substrate. Based on the crystal structure of *T. thermophilus* glycyl-tRNA synthetase (GlyRS) and molecular modeling, E359, the residue that aligns with C443, is predicted to be part of a rigid wall of side chains that help prevent larger amino acids from entering the GlyRS aminoacyl-adenylate binding pocket (Logan et al., 1995). In each of these seven class II synthetases, therefore, the residue that aligns with C443 of *E. coli* ProRS provides a significant and distinct contribution to the formation of the aminoacyl-adenylate binding pocket. The chemical modification, substrate protection, and site-directed mutagenesis studies reported here provide experimental evidence that C443 in motif 3 of *E. coli* ProRS is likely to be a hydrogen bond donor in a buried region that forms the prolyl-adenylate binding pocket.

While C443 appears to be involved in the formation of the substrate binding pocket in *E. coli* ProRS, a Cys residue is not strictly conserved at this position in ProRS sequences from different species. The residues Ser and Thr are also found at the analogous position of other known prokaryotic synthetases specific for proline (based on unpublished sequence alignments by K. Shiba, personal communication). Species-specific differences in substrate interactions by this class of enzymes are of great interest and may be exploited for drug design (Quinn et al., 1995; Heacock et al., 1996). Interestingly, the location and primary sequence of motif 3 in all known eukaryotic ProRS species are significantly

different from those of the prokaryotic enzymes (Cerini et al., 1991; Fett & Knippers, 1991; K. Shiba, personal communication). The putative motif 3 of eukaryotic ProRS species contains a conserved serine residue that is six residues removed from a strictly conserved arginine. Whether this serine residue plays the same role as C443 of *E. coli* ProRS (which is seven residues removed for the strictly conserved arginine of motif 3) remains to be seen. Studies to investigate the functional role of this residue and other species-specific differences in the primary structure of human ProRS are currently underway.

REFERENCES

Arnez, J. G., Harris, D. C., Mitschler, A., Rees, B., Francklyn, C. S., & Moras, D. (1995) *EMBO J. 14*, 4143−4155.

Belrhali, H., Yaremchuk, A., Tukalo, M., Larsen, K., Berthet-Colominas, C., Leberman, R., Beijer, B., Sproat, B., Als-Nielsen, J., Grübel, G., Legrand, J.-F., Lehmann, M., & Cusack, S. (1994) *Science 263*, 1432−1436.

Bodwell, J. E., Holbrook, N. J., & Munck, A. (1984) *Biochemistry 23*, 1392−1398.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Brick, P., Bhat, T. N., & Blow, D. M. (1989) *J. Mol. Biol. 208*, 83−98.

Brunie, S., Zelwer, C., & Risler, J.-L. (1990) *J. Mol. Biol. 216*, 411−424.

Calendar, R., & Berg, P. (1966) *Biochemistry 5*, 1690−1695.

Cavarelli, J., Eriani, G., Rees, B., Ruff, M., Boeglin, M., Mitschler, A., Martin, F., Gangloff, J., Thierry, J.-C., & Moras, D. (1994) *EMBO J. 13*, 327−337.

Cerini, C., Kerjan, P., Astier, M., Gratecos, D., Mirande, M., & Semeriva, M. (1991) *EMBO J. 10*, 4267−4277.

Cusack, S. (1993) *Biochimie 75*, 1077−1081.

Cusack, S., Berthet-Colominas, C., Härtlein, M., Nassar, N., & Leberman, R. (1990) *Nature 347*, 249−265.

Cusack, S., Härtlein, M., & Leberman, R. (1991) *Nucleic Acids Res. 19*, 3489−3498.

Davis, M. W., Buechter, D. D., & Schimmel, P. (1994) *Biochemistry 33*, 9904−9911.

Eriani, G., Delarue, M., Poch, O., Gangloff, J., & Moras, D. (1990) *Nature 347*, 203−206.

Ferguson, B. Q., & Yang, D. C. H. (1986) *Biochemistry 25*, 2743−2748.

Fett, R., & Knippers, R. (1991) *J. Biol. Chem. 226*, 1448−1455.

First, E. A., Johnson, D. A., & Taylor, S. S. (1989) *Biochemistry 28*, 3606−3613.

Freist, W. (1989) *Biochemistry 28*, 6787−6795.

Heacock, D., Forsyth, C. J., Shiba, K., & Musier-Forsyth, K. (1996) *Bioorg. Chem. 24*, 273−289.

Hill, K., & Schimmel, P. (1989) *Biochemistry 28*, 2577−2586.

Ibba, M., Kast, P., & Henneke, H. (1994) *Biochemistry 33*, 7107−7112.

Ibba, M., Johnson, C. M., Hennecke, H., & Fersht, A. R. (1995) *FEBS Lett. 358*, 293−296.

Jasin, M., Regan, L., & Schimmel, P. (1985) *J. Biol. Chem. 260*, 2226−2230.

Kast, P., & Hennecke, H. (1991) *J. Mol. Biol. 222*, 99−124.

Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C., & Rich, A. (1974a) *Science 185*, 435−440.

Kim, S. H., Sussman, J. L., Suddath, F. L., Quigley, G. J., McPherson, A., Wang, A. H., Seeman, N. C., & Rich, A. (1974b) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4970−4974.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Liu, H., & Musier-Forsyth, K. (1994) *Biochemistry 33*, 12708−12714.

Liu, H., Peterson, R., Kessler, J., & Musier-Forsyth, K. (1995) *Nucleic Acids Res. 23*, 165−169.

Logan, D. T., Mazauric, M.-H., Kern, D., & Moras, D. (1995) *EMBO J. 14*, 4156−4167.

McClain, W. H., Schneider, J., & Gabriel, K. (1994) *Nucleic Acids Res. 22*, 522−529.

Moras, D. (1992) *Trends Biochem. Sci. 17*, 159−164.

Moras, D. (1993) *Biochimie 75*, 651−657.

Onesti, S., Miller, A. D., & Brick, P. (1995) *Structure 3*, 163−176.

Ploux, O., Lei, Y., & Liu, H. (1995) *Biochemistry 34*, 4159−4168.

Quinn, C. L., Tao, N., & Schimmel, P. (1995) *Biochemistry 34*, 12489−12495.

Richardson, J. S., & Richardson, D. C. (1989) *in Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G. D., Ed.) pp 1−98, Plenum Press, New York.

Riddles, P. W., Blakely, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49−60.

Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C., & Klug, A. (1974) *Nature 250*, 546−551.

Rould, M. A., Perona, J. J., Söll, D., & Steitz, T. A. (1989) *Science 246*, 1135−1142.

Rould, M. A., Perona, J. J., Söll, D., & Steitz, T. A. (1991) *Nature 352*, 213−218.

Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J. C., & Moras, D. (1991) *Science 252*, 1682−1689.

Saks, M. E., Sampson, J. R., & Abelson, J. N. (1994) *Science 263*, 191−197.

Schimmel, P. (1987) *Annu. Rev. Biochem. 56*, 125−158.

Shi, J.-P., Musier-Forsyth, K., & Schimmel, P. (1994) *Biochemistry 33*, 5312−5318.

Tarr, G. E. (1986) in *Methods of Protein Microcharacterization* (Shively, J. E., Ed.) pp 155−194, Humana Press, Clifton, NJ.

Warshel, A., Schweins, T., & Fothergill, M. (1994) *J. Am. Chem. Soc. 116*, 8437−8442.

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