Evidence for an Arginine Residue at the Substrate Binding Site of *Escherichia coli* Adenylosuccinate Synthetase as Studied by Chemical Modification and Site-directed Mutagenesis*

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Chemical modification of adenylosuccinate synthetase from *Escherichia coli* with phenylglyoxal resulted in an inhibition of enzyme activity with a second-order rate constant of 13.6 M⁻¹ min⁻¹. The substrates, GTP or IMP, partially protected the enzyme against inactivation by the chemical modification. The other substrate, aspartate, had no such effect even at a high concentration. In the presence of both IMP and GTP during the modification, nearly complete protection of the enzyme against inactivation was observed. Stoichiometry studies with [7-'³⁵C]phenylglyoxal showed that only 1 reactive arginine residue was modified by the chemical reagent and that this arginine residue could be shielded by GTP and IMP. Sequence analysis of tryptic peptides indicated that Arg¹⁴⁷ is the site of phenylglyoxal chemical modification. This arginine has been changed to leucine by site-directed mutagenesis. The mutant enzyme (R147L) showed increased Michaelis constants for IMP and GTP relative to the wild-type system, whereas the *Kₘ* for aspartate exhibited a modest decrease as compared with the native enzyme. In addition, *kₐ₈* of the R147L mutant decreased by a factor of 1.3 × 10⁴. On the bases of these observations, it is suggested that Arg¹⁴⁷ is critical for enzyme catalysis.

Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming) (EC 6.3.4.4)) catalyzes the reaction of the first committed step during *de novo* purine biosynthesis from IMP to AMP and plays an important role in the metabolism of all living systems (1).

In spite of its position in cellular metabolism, relatively little was known regarding the mechanism of the adenylosuccinate synthetase reaction and its molecular mode of regulation until very recently. The reason for this dearth of information is clear from a cursory investigation of the literature, which shows that extremely small quantities of the synthetase exist in most cells (1). In addition, the turnover number of the enzyme is extremely small (2). It was not until recently that the *pur* A gene of *Escherichia coli*, which encodes adenylosuccinate synthetase, was cloned into the temperature-inducible high-copy-number plasmid vector (pMOB45) (3), and the overproduced adenylosuccinate synthetase, which can be purified to homogeneity in amounts sufficient for studying the enzyme structure and mechanism, became possible. So far, three mechanisms for the enzyme reaction have been proposed, and all of them are based on the random Ter Ter kinetic sequential mechanism (1). The first mechanism regards the reaction as a one-step "concerted" reaction, with all the substrates participating simultaneously (4). In the second mechanism proposed by Lieberman (5) and Fromm (6), IMP is considered to be phosphorylated at the hydroxyl group at the C-6 position by GTP, after which the 6-phosphoryl-IMP intermediate undergoes nucleophilic attack by the amino nitrogen of aspartate. The third mechanism suggests that an intermediate is formed in which aspartate attacks the C-6 of IMP, after which the intermediate is phosphorylated by GTP and converted into products (7). Recent experiments, involving the protocol of isotope exchange at chemical equilibrium, demonstrated that the mechanism is, in fact, steady-state random with aspartate and adenylosuccinate being the inner substrate-product pair (8). Positional isotope scrambling studies suggest that the chemical mechanism of the adenylosuccinate synthetase reaction involves the intermediate 6-phosphoryl IMP (9).

The amino acid sequence of the enzyme has been recently deduced from the nucleotide sequence (10). There are 4 cysteine residues in the enzyme, and none of them are involved in enzyme activity under physiological conditions (11). On the other hand, our recent chemical modification and site-directed mutagenesis studies on *E. coli* adenylosuccinate synthetase suggest that one of the 22 lysine residues (Lys¹⁴⁶) is critical for enzyme activity and probably is involved in the binding of GTP (12).

Chemical modification is a powerful technique for identifying residues necessary for enzyme activity. The well characterized arginine residue modification reagent, phenylglyoxal, has been widely used in experiments to identify functional arginine residues (13–15). Because all of the substrates of *E. coli* adenylosuccinate synthetase, GTP, IMP, and aspartate, are negatively charged under physiological conditions, it is reasonable to suggest that the positively charged amino acid residues such as lysine and arginine are possible functional groups in the binding of these ligands. Our recent experimental results have shown that a lysyl residue at position 140 is important for the binding of GTP (12). In addition, it has been reported that the guanidium side chain on the arginine residues is especially well suited as a phosphoryl anion recognition site for nucleotide substrate binding (16–18). The chemical modification of *E. coli* adenylosuccinate synthetase with phenylglyoxal was carried out on the basis of these considerations.

In this paper, we report the results from chemical modifi-
cation and site-directed mutagenesis experiments that show that an arginine residue (Arg411) is also essential for enzymatic activity.

EXPERIMENTAL PROCEDURES

Materials—IMP, GTP, L-aspartate, and trypsin were obtained from Sigma. Trichloroacetic acid was purchased from Fisher. Phenylglyoxal was from Aldrich, and [7-14C]phenylglyoxal and E. coli strain TG-1 were purchased from Amersham Corp. E. coli strain DT13 was purchased from Stratagene, and purA- strains H1238 (thr25, tonA49, argF58, relAl, spoT1, purA54, argI61) were obtained from Dr. B. Bachmann (Genetic Center, Yale University).

Preparation and Assay of Adenylosuccinate Synthetase—Adenylosuccinate synthetase was purified to homogeneity from E. coli as described previously (3). The purified enzyme was then dialyzed against 50 mM KP3, (potassium phosphate) buffer (pH 7.0) containing 1 mM EDTA. The concentration of pure adenylosuccinate synthetase in solution was determined spectrophotometrically by using the Bio-Rad protein assay method (19), and the concentration refers to the enzyme monomer. Adenylosuccinate synthetase activity was determined as described previously (1, 3). Activity is expressed in units (micromoles of adenylosuccinate formed per min).

Modification of Adenylosuccinate Synthetase with Phenylglyoxal and Substrate Protection Experiments—Purified adenylosuccinate synthetase (13.3 μM) was incubated with varying concentrations of phenylglyoxal at room temperature in the dark in 50 mM triethylenimine (pH 8.0). The stock solution of phenylglyoxal was prepared in 100% ethanol. The final concentration of ethanol in the incubation solution was less than 1%, and control experiments indicated that, under these conditions, the enzyme was quite stable. The reaction was initiated by adding phenylglyoxal. Ten-μl aliquots were withdrawn at various intervals, and the enzyme activities were then determined. For kinetic studies, the activity of the modified enzyme was expressed as the log of the percent activity remaining, and the data were analyzed by using the ENSFITTER computer program (20).

In the substrate protection experiments, purified adenylosuccinate synthetase (1.3 μM) was incubated with phenylglyoxal (5 mM) in the presence of different concentrations of substrates in a volume of 1 ml. The reaction was carried out in 50 mM triethylenimine (pH 8.0) at room temperature in the dark. At each time interval, 10 μl sample was withdrawn, and the enzyme activity was determined. A control experiment was carried out under identical conditions except that the same amount of ethanol was added to the incubation solution instead of phenylglyoxal.

3H]Phenylglyoxal Incorporation—Adenylosuccinate synthetase (431 μg, 4.5 μM) was incubated with 6.2 mM phenylglyoxal in 50 mM triethylenimine (pH 8.0) in a volume of 2 ml. The incubation was carried out at room temperature in the dark. At each time interval, a 150-μl sample was removed, the reaction was stopped by 10% (w/v) cold trichloroacetic acid, and a 5-μl sample was withdrawn to check the enzyme activity. At the end of the experiment, all aliquots were carefully filtered through 2-cm diameter GF/C filter paper discs (Whatman) to remove excess reagent (21, 22). The retained protein was then washed 15 times with 10 ml 10% (w/v) of cold trichloroacetic acid and finally with 2 x 30 ml water. The discs were dried, and the radioactivities were determined in a Tm-Analytic Liquid Scintillation Counter. Controls were carried out by spotting the same amount of [3H]phenylglyoxal on the discs that were used in the modification experiments. The discs were then washed with cold trichloroacetic acid and water, and the radioactivities were assayed as already described.

Tryptic Peptide Mapping—Adenylosuccinate synthetase (4.31 mg) was incubated with 39 mM phenylglyoxal in the absence or presence of substrates (8.9 mM GTP and 19.2 mM IMP) in a total volume of 3 ml. The reactions were carried out for 60 min at room temperature in 50 mM triethylenimine (pH 8.0). The concentration of the modified enzyme was checked, and the reaction was stopped by adding 7 ml of 10% cold trichloroacetic acid to the reaction mixture. The precipitated enzyme was washed three times with 5% cold trichloroacetic acid and finally with 95% ethanol. The denatured precipitate was dissolved with 250 μl of 0.1 M ammonium bicarbonate and 1 mM ethyl chloromethyl ketone-treated trypsin from bovine pancreas at 37°C for 6 h in 2.3 ml of 0.1 M ammonium bicarbonate and 1 mM CaCl2. The reaction was stopped by acidifying the digest with 0.1% trifluoroacetic acid. After centrifugation, the clear supernatant fluids were taken and prepared for C-18 reverse-phase HPLC separations.

HPLC Separation of Tryptic Peptides and Amino Acid Sequence Analysis—Initial resolution of tryptic peptides was performed by applying the tryptic digest to a 300 x 3.9-mm μBondapak C-18 column. Partial characterization of HPLC components was achieved by using HPLC with a 5% (v/v) acetonitrile gradient in a mobile phase containing 5% trifluoroacetic acid. Peptides were eluted with 0.1% trifluoroacetic acid as the mobile phase (buffer A) and 0.1% trifluoroacetic acid in 85% acetonitrile as the organic modifier (buffer B). The gradient used was: 0 min, 100% buffer A; 0% buffer B; 30 min, 50% buffer A; 50% buffer B; 40 min, 90% buffer B; 50 min, 0% buffer A; 100% buffer B; 60 min, 100% buffer A, 0% buffer B. The flow rate was 1 ml/min. The peptides were monitored for absorbance at 220 nm, and the radioactivities in individual fractions were determined by liquid scintillation counting. Fractions containing the radioactive peptides were pooled and rechromatographed on C-6 reverse-phase column. All of the HPLC analyses were performed using a Waters model 510 liquid chromatography system equipped with a Lambda Max model 481 LC spectrophotometer and a Spectra Physics 4270 integrator. The complete amino acid sequences of the peptides were determined by automatic Edman degradation by using the 470A Sequencer at the Protein Structure Facility, University of Iowa, under the direction of Dr. Alan Bergold.

Site-directed Mutagenesis—A 1.9-kilobase pair HindIII-BamHI fragment of pMS204 (3) containing a part of the adenylosuccinate synthetase gene was ligated into the HindIII-BamHI site of pUC118. The ligation mixture was used to transform competent TG-1 cells. Colonies were isolated from Luria broth plates containing ampicillin, and the plasmid was digested with BamHI and HindIII. A plasmid of the proper size and orientation was used for DNA sequencing and site-directed mutagenesis.

The following oligonucleotides were prepared: (a) mutagenic oligonucleotide, 5'CCTCGCAACAA GCGAGCCGCC (this had a C-A base pair change that substituted Arg411 in the wild-type adenylosuccinate synthetase with a Leu); (b) sequencing oligonucleotide, sGGAA GTTGTGATATT (this oligonucleotide primes 47 nucleotides upstream from the first nucleotide in the mutagenic oligonucleotide).

Sanger sequencing was performed by using the mutagenic oligonucleotide primers (23). Site-directed mutagenesis was accomplished by the method of Nakamaye and Eckstein (24). The presence of the mutation was confirmed by DNA sequencing. The HindIII-BamHI fragments encoding the Leu mutation were cloned back into pMS204, and the resulting plasmids were designated pR147L. TG-1 cells were transformed by using this mutant construct. Colonies were selected from Luria broth plates containing chloramphenicol (25 μg/ml). Plasmids were prepared by the method of Biraboin and Doly (25) from a few of the colonies obtained after transformation, and a colony containing a plasmid with the correct orientation and size (as confirmed by digestion with EcoRI) was used as a source for each of the mutant forms of the enzyme. The selected plasmid was used to transform E. coli strain XL1-Blue. The plasmids isolated from this strain were then used to transform E. coli purA strain H1238. The mutant forms of adenylosuccinate synthetase were purified by using protocols identical to those reported for the wild-type enzyme (3) except for the phenyl-Sepharose chromatography step at which the enzyme was eluted by washing the column with buffer instead of 50 mM KP3 (pH 7.0).

RESULTS

Inactivation of Adenylosuccinate Synthetase with Phenylglyoxal—Incubation of adenylosuccinate synthetase from E. coli with varying concentrations of phenylglyoxal results in a time-dependent loss of enzyme activity (Fig. 1). When the enzyme (1.3 μM) was incubated with 5 mM of phenylglyoxal at 25°C for 15 min, only 25% of the enzyme activity remained, suggesting that an arginine residue may be important for adenylosuccinate synthetase activity. The inactivation of the enzyme with excess phenylglyoxal follows pseudo-first-order kinetics (Fig. 1A). A replot of the apparent first-order rate constants (K') versus phenylglyoxal concentrations is linear (Fig. 1B), indicating that the reaction order with respect to the concentration of phenylglyoxal obeys second-order kinetics.

1The abbreviations used are: HPLC, high-performance liquid chromatography.
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-2.80
-3.20

B !0-1
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00

CPhenylglyoxal (mM)

FIG. 1. Inactivation of E. coli adenylosuccinate synthetase by varying concentrations of phenylglyoxal. A, the enzyme (1.3 μM) was treated with phenylglyoxal at the following concentrations: 0.1 mM (×), 0.5 mM (▼), 1 mM (○), 3 mM (■), 5 mM (+) and 7.5 mM (△). All incubations and assays were performed in 0.2 M triethylamine buffer (pH 8.0) at 25 °C in the dark. For the details, see "Experimental Procedures." B, dependence of apparent first-order rate constant on phenylglyoxal concentrations. The observed rate constant for the reaction was calculated to be 13.6 M⁻¹ min⁻¹. This kinetic result shows that a significant covalent phenylglyoxal-enzyme complex is formed during the inactivation process.

Substrate Protection against Phenylglyoxal Inactivation—Substrate protection experiments were carried out to determine whether this inactivation is due to a direct modification of an essential amino acid residue at the enzyme's active site or due to the modification of an amino acid residue remote from the active site, which results in an inactive enzyme form. Fig. 2 shows the chemical modification results in the presence of different substrates in the absence of substrates during the modification, the enzyme was inactivated in about 30 min. Similar results were found when one of the substrates, aspartate, was present during the modification. On the other hand, GTP or IMP significantly protected the enzyme against phenylglyoxal modification (Fig. 2A). In addition, in the presence of both GTP and IMP, the protection is almost complete (Fig. 2B). These results strongly suggest that the arginine residue(s) that can be modified by phenylglyoxal is (are) in some way involved in substrate binding or catalysis.

Stoichiometry of [7-¹⁴C]Phenylglyoxal Binding to Adenylosuccinate Synthetase—To establish the stoichiometry of phenylglyoxal binding to E. coli Adenylosuccinate synthetase, ¹⁴C-labeled phenylglyoxal was employed in the modification experiments. Studies have shown that phenylglyoxal can irreversibly bind to arginine residues of proteins with a stoichiometry of two to one (26). As can be seen in Table 1, in the absence of substrates, about 2 mol of [7-¹⁴C]phenylglyoxal can be incorporated per mol of enzyme subunit, indicating that 1 arginine residue was modified. The loss of enzyme activity correlated with the incorporation of phenylglyoxal, indicating that this arginine residue is critical for enzyme activity (Fig. 3). On the other hand, in the presence of substrates IMP and GTP during the modification, no significant incorporation of phenylglyoxal into the enzyme could be detected; however, a 13% loss of activity was observed in the
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TABLE I

| Substrate | Remaining activity | No. of PG moles incorporated/subunit | Arginine residues labeled<sup>a</sup> |
|-----------|-------------------|------------------------------------|--------------------------------------|
| None      | 5.0 ± 0.2         | 1.720 ± 0.060                      | 0.850 ± 0.030                        |
| Aspartate | 5.2 ± 2.0         | 2.020 ± 0.200                      | 1.010 ± 0.100                        |
| GTP       | 58.1 ± 14.6       | 0.200 ± 0.160                      | 0.100 ± 0.080                        |
| IMP       | 44.6 ± 11.5       | 0.130 ± 0.070                      | 0.065 ± 0.035                        |
| GTP + IMP | 86.6 ± 6.6        | 0.150 ± 0.090                      | 0.075 ± 0.045                        |

<sup>a</sup> Calculated on the basis of 2 phenylglyoxal residues/arginine residue modified (28).

Fig. 3. Relation between E. coli adenylosuccinate synthetase inactivation and [7-14C]phenylglyoxal incorporation. Incorporation of [7-14C]phenylglyoxal into adenylosuccinate synthetase (+) was carried out as described under "Experimental Procedures." The activity of the modified enzyme was measured in the same experiment (Δ).

The activity of the modified enzyme was measured in the same experiment (Δ).

Presence of GTP + IMP. This small activity loss is not thought to be significant (see Fig. 3).

Comparison of Tryptic Peptide Maps of Phenylglyoxal-inactivated and Substrate-protected Adenylosuccinate Synthetase—

A comparison of the tryptic peptide maps of phenylglyoxal-inactivated and substrate-protected adenylosuccinate synthetase by reverse-phase HPLC allows one to identify the peptide containing the phenylglyoxal-modified arginine residue. As can be seen in Fig. 4, a radioactive peak appeared at about 9 min when the phenylglyoxal-modified adenylosuccinate synthetase tryptic peptides were injected into a C-18 reverse-phase column. This radioactive peak could not be observed in the substrate-protected adenylosuccinate synthetase tryptic peptides. This radioactive fraction was further purified by using a C-8 reverse-phase column. The amino acid sequence of the purified peptide was then determined. Sequence analysis showed that the amino acid sequence of this peptide spans from residues 145 to 147 (Gly-Leu-Arg), in which Arg showed a very low yield of phenylthiohydantoin derivative amino acid. Therefore, the arginine at position 147 was assumed to be the essential residue modified by phenylglyoxal.

Preparation and Properties of the (R147L) Mutant—To further identify the role of Arg<sup>147</sup> in the enzyme catalytic mechanism, site-directed mutagenesis experiments were carried out. The mutant enzyme was expressed in E. coli strain H1238 which carries a mutation in the gene coding for adenylosuccinate synthetase. Because H1238 expresses a DNA restriction/modification system, the mutant overproducing plasmid pMS204 isolated from TG-1 could not transform the strain directly. To solve this problem, we first transformed the plasmid into a restriction minus/modification plus E. coli strain, XL1-Blue. The modified plasmids were then isolated and used to transform E. coli purA<sup>−</sup> strain H1238.

The mutant enzyme (R147L) showed a different chromatographic behavior compared with the wild-type enzyme. As can be seen in Fig. 5, the wild-type enzyme can be eluted from the phenyl-Sepharose affinity column when the linear gradient is about 80 mM of KP buffer (pH 7.0). On the other hand, the mutant enzyme binds tightly to the column under conditions.
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TABLE II
Kinetic parameters of wild-type (WT) and mutant adenylosuccinate synthetase from E. coli.

| Protein | WT | R147L | WT/R147L |
|---------|----|-------|----------|
| kcat (s⁻¹) | 1.56 × 10⁴ | 1.17 | 1.30 × 10⁴ |
| Km,IMP (µM) | 22.1 | 133 | 0.18 |
| kcat/Km,IMP (µM) | 24.3 | 118 | 0.21 |
| kcat/Km,IMP (s⁻¹ M⁻¹) | 191 | 115 | 1.66 |

TABLE III
Comparison of sequences surrounding residue 147 in E. coli and other adenylosuccinate synthetases.

| Enzyme | Residues¹ | 140 | 147 |
|--------|-----------|-----|-----|
| ECAS | G I G P | A | Y E D K V A K R G L R V G D L |
| MMAS | G I G P | T Y S S | K A A T G L R I C D L |
| DDAS | G I G C Y S S | K A A S G G L R V C D L |

¹ ECAS, E. coli adenylosuccinate synthetase (10); MMAS, mouse muscle adenylosuccinate synthetase (F. B. Rudolph, Rice University, personal communication); DDAS, D. discoideum adenylosuccinate synthetase (27).

This condition and can be eluted only by washing the column with water. The purity of the R147L mutant was checked on sodium dodecyl sulfate-polyacrylamide gel electrophoresis after the phenyl-Sepharose chromatography. It was shown that the mutant enzyme was pure and both the mutant and wild-type enzymes had the same migration distance on the gel (data not shown).

The R147L mutant enzyme has a low activity relative to the wild-type enzyme. The kinetic properties of the mutant enzyme were compared with the wild-type enzyme, and the results obtained are summarized in Table II. The Km for GTP and IMP of the mutant enzyme increased 4- to 6-fold, whereas the Km for Asp was almost the same as that of the wild-type system. A very significant decrease, by as much as 10⁴, in km for both nucleotide substrates, aspartate, provided no protection against enzyme inactivation. Almost complete protection can be observed if both nucleotide substrates are present during the modification reaction. On the other hand, the other substrate, aspartate, provided no protection against enzyme inactivation by phenylglyoxal. These observations are consistent with results from product-inhibition experiments that show that IMP and aspartate bind to the enzyme at topologically different locations (1, 2). Replacement of arginine at position 147 of the amino acid sequence with leucine by site-directed mutagenesis resulted in a significant change in Km for both nucleotide substrates, but not for aspartate (Table II).

DISCUSSION

There are 28 arginine residues in each subunit of E. coli adenylosuccinate synthetase. Our present results show, however, that only one of these arginine residues is sensitive to phenylglyoxal modification. This uniquely highly reactive residue is likely to be located in the active site of the enzyme. Replacement of Arg¹⁴⁷ with leucine causes the mutant protein to be more hydrophobic than the wild-type enzyme. The effect of this change between the mutant and native enzyme can be seen very clearly from their different binding behaviors during phenyl-Sepharose affinity chromatography (Fig. 5).

The incorporation of phenylglyoxal by Arg¹⁴⁷ results in a correlational loss of enzyme activity (Fig. 3), suggesting that Arg¹⁴⁷ may play a role in enzyme function. Alignment of the E. coli adenylosuccinate synthetase with the mouse muscle enzyme and Dictyostelium discoideum enzyme (27) shows a high homology among these three enzymes. Table III shows the sequences surrounding residue 147 in E. coli and the other adenylosuccinate synthetases. It is of interest that both Lys¹⁴⁰ and Arg¹⁴⁷ are conserved in the three different enzymes. On the other hand, none of the cysteine residues in adenylosuccinate synthetase are conserved, and they are not in any way involved in enzyme activity under physiological conditions as we found in previous experiments (11). These observations provide further evidence that Arg¹⁴⁷ is important for enzyme activity.

Additional information on the role of Arg¹⁴⁷ in enzyme function is provided by the results from substrate protection experiments. As can be seen in Fig. 2, among the three substrates of the enzyme (aspartate, IMP, and GTP), only the two nucleotide substrates showed significant protection against phenylglyoxal inactivation. Almost complete protection can be observed if both nucleotide substrates are present during the modification reaction. On the other hand, the other substrate, aspartate, provided no protection against enzyme inactivation by phenylglyoxal. These observations are consistent with results from product-inhibition experiments that show that IMP and aspartate bind to the enzyme at topologically different locations (1, 2). Replacement of arginine at position 147 of the amino acid sequence with leucine by site-directed mutagenesis resulted in a significant change in Km for both nucleotide substrates, but not for aspartate (Table II).

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