Involvement of Arginine 143 in Nucleotide Substrate Binding at the Active Site of Adenylosuccinate Synthetase from *Escherichia coli*†

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ABSTRACT: Adenylosuccinate synthetase from *Escherichia coli* is inactivated in a biphasic reaction by guanosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate (GMPSBDB) at pH 7.1 and 25 °C. Reaction of the enzyme with [8-^{3}H]GMPSBDB results in the incorporation of 2 mol of the reagent/mol of subunit; in the presence of active site ligands the incorporation is reduced to 1 mol of reagent/mol of subunit. GMPSBDB reacts with Cys-291 in the initial rapid reaction which is accompanied by loss of 50% of the enzymatic activity; this reaction is not affected by the presence of active site ligands. In the slower reaction, GMPSBDB inactivates the enzyme by reacting with Arg-143. The inactivation kinetics of the slower phase are consistent with the formation of an enzyme−GMPSBDB complex having a K_d of 42 μM. Active site nucleotides, either adenylosuccinate or IMP + GTP, prevent both slower phase inactivation and labeling of Arg-143. Replacement of Arg-143 with a Leu by site-directed mutagenesis does not change the catalytic constant or the K_m for aspartate but does significantly impair nucleotide binding: the Michaelis constants for IMP and GTP increase by 60-fold and 10-fold, respectively, in the R143L mutant. The crystal structure of the *E. coli* enzyme [Poland, B. W., Silva, M. M., Serra, M. A., Cho, Y., Kim, K. H., Harris, E. M. S., & Honzatko, R. B. (1993) *J. Biol. Chem. 268,* 25334−25342] shows that Arg-143 from one subunit projects into the putative active site of the other subunit. These results indicate that both subunits of dimeric adenylosuccinate synthetase contribute to each active site and that Arg-143 plays an important role in nucleotide binding.

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4], a key enzyme in the conversion of IMP to AMP, couples the hydrolysis of GTP to the formation of adenylosuccinate (SAMP)[1] from IMP and aspartate:

$$\text{IMP} + \text{GTP} + \text{L-aspartate} \overset{[Mg^{2+}]}{\rightleftharpoons} \text{adenylosuccinate} + \text{GDP} + P_i$$

The *Escherichia coli* enzyme is a homodimer, each subunit consisting of 431 amino acids and exhibiting a molecular weight of 47 300 (Bass *et al*., 1987; Poland *et al*., 1993). Steady-state kinetic studies have demonstrated that the *E. coli* enzyme follows a rapid equilibrium random mechanism (Rudolph & Fromm, 1969), but isotope exchange experiments point to a preferred pathway in which aspartate binds after the addition of nucleotide substrates (Cooper *et al*., 1986). The currently favored chemical mechanism involves nucleophilic attack by the 6-hydroxyl group of IMP on the γ-phosphoryl group of GTP, forming a 6-phosphoryl-IMP intermediate which is subsequently attacked by the amino group of aspartate to form adenylosuccinate (Webb *et al*., 1984; Bass *et al*., 1984).

The crystal structure of adenylosuccinate synthetase from *E. coli* in the absence of substrates has been determined to a resolution of 2.8 Å (Poland *et al*., 1993). Chemical modification and site-directed mutagenesis studies have indicated the importance of Asp-333, Lys-140, Arg-147, and a glycine-rich N-terminal sequence for the proper functioning of the enzyme (Kang *et al*., 1994; Dong & Fromm, 1990; Dong *et al*., 1991; Liu *et al*., 1992). Site-directed mutagenesis has also implicated Arg-303, Arg-304, and Arg-305 in the binding of aspartate (Wang *et al*., 1995).

This paper reports the inactivation of *E. coli* adenylosuccinate synthetase by guanosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate (GMPSBDB), a new nucleotide affinity label (Vollmer *et al*., 1994). Our results indicate that GMPSBDB binds at the enzyme active site and modifies Arg-143, a residue that plays a key role in nucleotide substrate binding. A preliminary version of this work has been presented (Moe *et al*., 1996).

EXPERIMENTAL PROCEDURES

Materials. L-Aspartic acid, BSA, GTP, IMP, guanosine, HEPES, PIPES, sodium borohydride, and NEM were purchased from Sigma. Radioactive [8-^{3}H]guanosine was

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[1] Abbreviations: BSA, bovine serum albumin; GMPS, guanosine 5′-*O*-thiophosphate; GMPSBDB, guanosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography, NEM, *N*-ethylmaleimide; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PROXYL, 2,2,5,5-tetramethyl-1-pyrrolidinyloxy; PTH, phenylthiohydantoin; SAMP, adenylosuccinate.

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purchased from Moravek Biochemicals. A site-directed mutagenesis kit was obtained from Amersham. Restriction enzymes were purchased from Promega. *E. coli* strain XL-1 blue was obtained from Stratagene, and *E. coli* strain *purA*$^-$ H1238 was a gift from Dr. B. Bachman (Genetic Center, Yale University). The 1,4-dibromobutanedione was purchased from Aldrich and was recrystallized from petroleum ether before use. Staphylococcal protease from *Staphylococcal aureus*, strain V8, was obtained from ICN Pharmaceuticals.

Wild-type and mutant adenylosuccinate synthetases were purified using phenyl-Sepharose CL-4B, Cibracron blue 3GA, and DEAE-TSK HPLC columns, as described elsewhere (Kang & Fromm, 1994; Bass *et al*., 1987). Enzyme purity was checked by SDS−PAGE (Laemmli, 1970). Adenylosuccinate synthetase activity was determined at 25 °C by measuring the increase in absorbance at 280 nm that accompanies the conversion of IMP to adenylosuccinate ($\Delta\epsilon_{280} = 1.17 \times 10^4\ M^{-1}\ cm^{-1}$) (Rudolph & Fromm, 1969). Prior to inactivation and incorporation experiments in this paper, the enzyme was dialyzed versus two changes of 40 mM PIPES buffer, pH 7.0, containing 100 μM EDTA.

The protein concentration was initially determined by a method involving the difference in protein absorbance at 224 and 233 nm ($\Delta A_{224-233nm}$), using BSA as a standard (Groves *et al*., 1968). The $\Delta A_{224-233nm}$ method was then used to determine the extinction coefficient for adenylosuccinate synthetase at 280 nm: $E^{0.1\%} = 1.05$. The protein concentrations of all dialyzed stock enzyme solutions were routinely determined from A_{280nm} measurements using a molecular mass of 47 300 Da for each identical subunit (Bass *et al*., 1987). The dialyzed enzyme was stored in aliquots at −80 °C. After modification by GMPSBDB, the protein concentration was determined by the Bio-Rad protein assay method (Bradford, 1976) using stock-dialyzed adenylosuccinate synthetase as a standard.

Site-Directed Mutagenesis. Recombinant DNA manipulation was performed using standard procedures (Sambrook *et al*., 1989). The sequencing primer used was CTGCTGCTGTCTGAAGCA, and the R143L mutagenic oligonucleotide was CAGACCGCGAAGTGCTACTTTATC, the underlined letter indicating the mismatch. The oligonucleotide primers were synthesized on a Bioresearch 8570EX automated DNA synthesizer at the DNA facility at Iowa State University. Mutagenesis was carried out according to the protocol provided by the Amersham Co. in which a plasmid containing a 1.8 kb *Bam*HI−*Hin*dIII fragment from PMS204 ligated into the phagemid PUC118 was used to make a single-stranded DNA template for mutagenesis. A phosphorylated mutagenesis primer was annealed to the template. The subsequent synthesis of double-stranded closed circular phagemid containing the mutation was carried out using Klenow DNA polymerase and T4 DNA ligase, incorporating dCTPγS into the newly synthesized DNA instead of CTP. Restriction enzyme *Nci*I and exonuclease III were used to nick and digest the nonmutant strand. The final repolymerization reaction generated the phagemid containing the desired mutation. The mutations were confirmed by DNA sequencing using the chain termination method (Sanger *et al*., 1977). The newly formed pMS204-R143L was selected and transformed into XL-1 blue cells. The plasmid isolated from that strain was used to transform *E. coli* strain *purA*$^-$ H1238, which was the strain used for protein purification.

Analytical Methods. Radioactivity was determined using a Packard Tricarb Model 300 liquid scintillation counter. Circular dichroism spectra for wild-type and mutant adenylosuccinate synthetase were acquired on a JASCO J700 spectropolarimeter equipped with a data processor. Samples (150 μg/mL) were placed in a 1 mm cuvette, and data points were collected in 0.10 nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed using instrumental software. The data were analyzed by JASCO analysis or PSIPLOT. Peptide molecular weights were determined using a Fisons Autospec Q mass spectrometer equipped with an electrospray attachment. Molecular modeling of adenylosuccinate synthetase was carried out on a Silicon Graphics Indigo II workstation, using the programs Sybyl and Biopolymer from Tripos, Inc. The crystal coordinates for *E. coli* adenylosuccinate synthetase were kindly supplied by Dr. R. B. Honzatko of Iowa State University. Energy minimizations for modified residues utilized the "minimize subset" command of the software, which varied the orientation of the modified residue within the rigid local protein environment until a lowest energy position of the modified residue was attained.

Enzyme Assays Used in Inactivation and Incorporation Experiments. The standard assay solution had a volume of 900 μL and contained 10 mM $MgCl_2$, 5 mM aspartate, 300 μM IMP, and 150 μM GTP in a 20 mM HEPES buffer at pH 7.7. The reaction was initiated by addition of 1−10 μg of enzyme and was carried out at 25 °C using a Perkin-Elmer Model 553 UV/visible spectrophotometer with an expanded (0−0.1) absorbance scale to follow the change in absorbance at 280 nm.

Kinetic Study of Wild-Type and Mutant Adenylosuccinate Synthetase. The assay solution contained 20 mM HEPES, pH 7.7, and 5 mM $MgCl_2$ at 25 °C. To determine the K_m for IMP, the concentrations of GTP and aspartate were fixed at 300 μM and 5 mM, respectively. To determine the K_m for GTP, aspartate was held at 5 mM and IMP was fixed at either 450 μM for the wild-type enzyme or 12 mM for the R143L mutant. To determine the K_m for aspartate, GTP was fixed at 300 μM and IMP was fixed at either 450 μM for the wild-type enzyme or 12 mM for the R143L mutant. In order to reduce the background absorbance from high IMP concentrations, the reaction was followed at 290 nm (instead of 280 nm) using 2 mm quartz cuvettes.

Synthesis of [8-^{3}H]GMPS and [8-^{3}H]GMPSBDB. [8-^{3}H]-GMPS was synthesized from 1.5 mmol of guanosine and 2.5 mCi of [8-^{3}H]guanosine using a previously described method for the synthesis of GMPS (Ozturk *et al*., 1992). [8-^{3}H]GMPSBDB was prepared via coupling with 1,4-dibromobutanedione as previously described (Vollmer *et al*., 1994). The final [8-^{3}H]GMPSBDB product had a specific radioactivity of 1.75 cpm/pmol.

Inactivation of Adenylosuccinate Synthetase by GMPSBDB. The enzyme (1−4 μM subunit) was incubated in the presence of 14−787 μM GMPSBDB in 25 mM PIPES buffer at pH 7.1 at 25 °C. At indicated times, aliquots (10−30 μL) were withdrawn from the reaction mixtures and added to 900 μL of the standard assay mixture at 25 °C to determine the enzyme activity remaining. An enzyme control without GMPSBDB was incubated and assayed under the same conditions. Residual activity is expressed as E/E_0, the ratio of the activity of the inactivated enzyme to that of the control.

To determine the effect of active site ligands on the inactivation of adenylosuccinate synthetase by GMPSBDB, the enzyme was preincubated with ligands for 10 min at 25 °C in 25 mM PIPES, pH 7.1, after which 350 μM GMPSBDB was added to initiate the inactivation reaction. Aliquots were removed as a function of time and assayed for enzyme activity.

Incorporation of [8-^{3}H]GMPSBDB into Adenylosuccinate Synthetase. Incubation solutions were prepared to contain enzyme (2.9−23 μM) and [8-^{3}H]GMPSBDB (340−400 μM) in a 0.025 M PIPES buffer at pH 7.1. After incubation at 25 °C for a specified length of time, the reactions were stopped by adding aliquots of 0.30 M $NaBH_4$ to give a final $NaBH_4$ concentration of 10 mM (test experiments showed that reduction of GMPSBDB by 10 mM $NaBH_4$ would completely stop inactivation of the enzyme by GMPSBDB). The borohydride-treated incubation mixture was dialyzed at 5 °C versus four changes of 20 mM PIPES buffer, pH 7.0, containing 50 μM EDTA. After dialysis, the amount of [8-^{3}H]GMPSBDB incorporated was determined by liquid scintillation counting, and the protein concentration was determined by the Bio-Rad protein assay method. As a control, to evaluate the extent of removal of noncovalently bound reagent, [8-^{3}H]GMPSBDB was reduced by $NaBH_4$ before it was added to adenylosuccinate synthetase in the incubation solution. To prepare labeled enzyme for digestion by *S. aureus* protease, a 20 mM sodium acetate buffer, pH 4.0, replaced the 20 mM PIPES buffer in the dialysis step following $NaBH_4$ reduction.

To determine the effect of active site ligands on [8-^{3}H]-GMPSBDB incorporation, the enzyme was preincubated at 25 °C for 10 min in the pH 7.0 PIPES buffer with specific concentrations of the active site ligands, after which [8-^{3}H]-GMPSBDB (340−400 μM) was added to the incubation mixtures. The reactions were stopped by the addition of $NaBH_4$, and the modified enzyme was dialyzed against pH 7.0 PIPES buffer as described above.

In one experiment, NEM was used to block unreacted sulfhydryl groups immediately after the incorporation was stopped by $NaBH_4$. In this experiment, sufficient solid urea was dissolved in the stopped incubation solution to yield a final urea concentration of 8 M, and an aliquot of 50 mM NEM was immediately added to bring the final NEM concentration to 10 mM. After the reaction was allowed to proceed for 10 min at 25 °C, the mixture was dialyzed against pH 4.0 ammonium acetate buffer.

Proteolytic Digestion of Adenylosuccinate Synthetase Modified by [8-^{3}H]GMPSBDB. Following dialysis of labeled adenylosuccinate synthetase in 20 mM ammonium acetate buffer, pH 4.0, digestion of the suspended enzyme by the protease from *S. aureus*, strain V8, was conducted at 37 °C for 5 h with a total of 8−15% (w/w) protease as compared with adenylosuccinate synthetase. The final concentration of the *S. aureus* protease in the digestion mixtures ranged from 1 to 6 μM. Enzyme digests were centrifuged at 14 000 rpm for 4 min to remove insoluble protein, and the resulting supernatants were filtered through a 0.45 μm Millipore membrane filter to prepare them for HPLC.

Fractionation of [8-^{3}H]GMPSBDB-Labeled Peptides by High-Performance Liquid Chromatography. Radioactively labeled peptides were separated on a Varian Model 5000 HPLC system equipped with a Vydac C_{18} column (0.46 × 25 cm). Separations were carried out at an elution rate of 1 mL/min in 0.1% aqueous trifluoroacetic acid (solvent A), from 0 to 10 min, and then by a linear gradient from solvent A to 30% solvent B (0.075% trifluoroacetic acid in acetonitrile) between 10 and 70 min, followed by a linear gradient from 30% solvent B to 80% solvent B between 70 and 320 min. Fractions of 1.0 mL were collected, the effluents were monitored at 220 nm, and 100 μL aliquots from the eluted fractions were assayed for radioactivity by liquid scintillation counting.

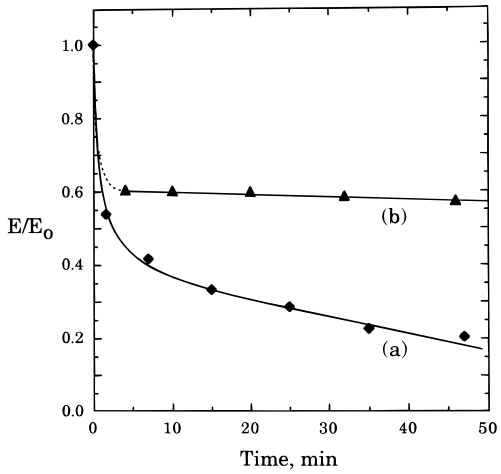

FIGURE 1: Effect of GMPSBDB on activity of adenylosuccinate synthetase. The enzyme (1.1 μM subunit) was incubated with (a) 340 μM GMPSBDB or (b) 340 μM GMPSBDB + 55 μM SAMP at 25 °C in 25 mM PIPES buffer, pH 7.1. At the indicated times, aliquots were withdrawn and assayed as described under Experimental Procedures. The solid line in (a) represents a least-squares fit to eq 1 in the text. The solid line shown in (b) represents a linear least-squares fit to the data. The dashed portion of the line in (b) is drawn arbitrarily.

Sequence Analysis of Isolated Peptides. HPLC fractions to be sequenced were lyophilized to dryness and redissolved in a mixture of 35 μL of 0.1% trifluoroacetic acid and 35 μL of 0.075% trifluoroacetic acid in acetonitrile. Sequence analyses were performed on an Applied Biosystems gas-phase protein (peptide) sequencer, Model 470, equipped with an on-line phenylthiohydantion analyze, Model 120, and computer, Model 900A. Typically, 20−1000 pmol of peptide was used for each analysis.

RESULTS

Inactivation of Adenylosuccinate Synthetase by GMPSBDB. GMPSBDB was tested as an inactivator of adenylosuccinate synthetase by incubating 340 μM GMPSBDB with the enzyme in PIPES buffer at pH 7.1 and 25 °C and measuring the residual enzyme activity (E/E_0) as a function of reaction time. Activity loss was observed to follow a biphasic, first-order exponential decay, as shown in Figure 1, line a. The experimental data in line a, which consist of a rapid initial inactivation to *ca*. 50% residual activity followed by a slower first-order inactivation, were fit to the following equation for a biphasic exponential inactivation process (DeCamp & Colman, 1989):

$$\frac{E}{E_0} = (1 - F)e^{-k_{fast}t} + (F)e^{-k_{slow}t} \qquad (1)$$

In this equation, F represents the fraction of residual enzyme activity after complete reaction in the fast phase; k_{fast} and k_{slow} represent the pseudo-first-order rate constants for the

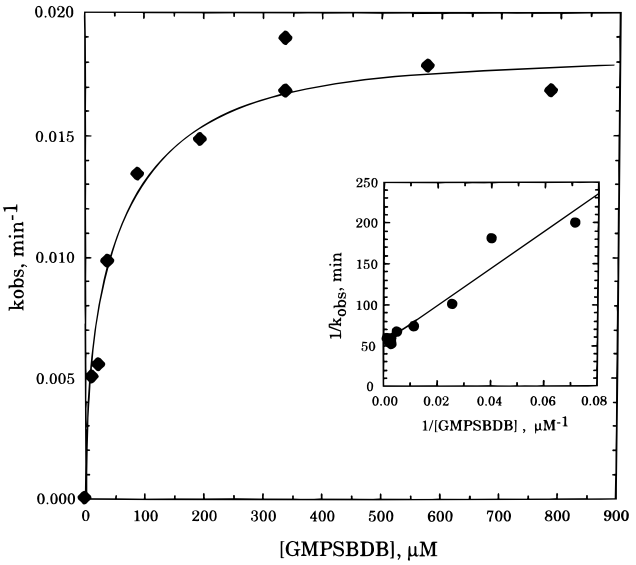

FIGURE 2: Dependence of the pseudo-first-order rate constant (k_{obs}) for slow-phase inactivation of adenylosuccinate synthetase on the concentration of GMPSBDB. Adenylosuccinate synthetase (1−3 μM subunit) was incubated with GMPSBDB (14−787 μM) in 25 mM PIPES buffer, pH 7.1. At the indicated times, aliquots were withdrawn and assayed as described under Experimental Procedures. Inset: Double-reciprocal plot used to evaluate k_{max} and $K_{GMPSBDB}$ through a linear least-squares fit (solid line) of the data to eq 2 in the text.

fast and slow phases. The data in Figure 1 afford an excellent fit to the slow phase, yielding a pseudo-first-order rate constant (k_{obs}) of 0.0196 min^{-1}, but only an approximation of the fast phase (k_{obs} = 1.2 min^{-1}) which is based primarily on one to two data points. The value for F in Figure 1 is 0.46.

Adenylosuccinate synthetase was incubated with 14−787 μM GMPSBDB, and rate constants were calculated from least-squares fits of the experimental data to eq 1 over the first 50 min. [The multiple correlation statistic, R^2, was determined for all least-squares fits to eq 1, and the values were found to range between 0.97 and 1.00, indicating excellent fits for all inactivation experiments (Draper & Smith, 1966).] The 50 min time period selected for the determination of the rate constants was chosen to be short relative to the rate of hydrolysis of GMPSBDB [$t_{1/2}$ = 120 min under these conditions (Vollmer *et al*., 1994)]. For the eight GMPSBDB concentrations employed, the average value for the F parameter in eq 1 was 0.51, with a standard deviation of ±0.04. A plot of k_{obs} for the slow phase versus [GMPSBDB], shown in Figure 2, reveals saturation behavior. This result can be interpreted as indicating reversible binding of the nucleotide analog to the enzyme prior to irreversible inactivation. The double-reciprocal form of the equation relating k_{obs} to [GMPSBDB] for a reversible binding model is given by the equation (Huang & Colman, 1984):

$$\frac{1}{k_{obs}} = \frac{1}{k_{max}} + \left[\frac{K_{GMPSDBD}}{k_{max}}\right]\frac{1}{[GMPSDBD]} \qquad (2)$$

In this equation, $K_{GMPSBDB}$ is the apparent dissociation constant for the enzyme−GMPSBDB complex and k_{max} is the maximal rate constant for inactivation. The inset in Figure 2 shows a linear plot of $1/k_{obs}$ versus 1/[GMPSBDB]. A least-squares fit to the data yields a dissociation constant of 42 μM for GMPSBDB and a k_{max} of 0.0185 min^{-1}.

Table 1: Effect of Active Site Ligands on the Slow Rate Constant for Inactivation of Adenylosuccinate Synthetase by 340 μM GMPSBDB[a]

ligands added to incubation mixture	k_{+L}/k_{-L}
none	1.00
10 mM $MgCl_2$	1.08
10 mM $MgCl_2$ + 5.0 mM aspartate	1.00
10 mM $MgCl_2$ + 500 μM GTP	0.90
10 mM $MgCl_2$ + 5.0 mM IMP	0.37
110 μM SAMP	0
10 mM $MgCl_2$ + 150 μM GTP + 300 μM IMP	0

[a] Reactions were conducted in 25 mM PIPES, pH 7.1, at 25 °C as described in Experimental Procedures. The value, k_{+L}/k_{-L}, is the ratio of the rate constant for inactivation in the presence of an active site ligand to that in its absence.

Analysis of the fast phase of kinetic inactivation was imprecise due to the limited number of data points we were able to collect in the brief initial time interval of inactivation. Although there was considerable scatter in the data, the best-fit k_{fast} values clearly increased as [GMPSBDB] increased, showing no evidence of saturation (data not shown).

Effect of Active Site Ligands on the Inactivation of Adenylosuccinate Synthetase by GMPSBDB. The ability of various active site ligands to protect against inactivation by 350 μM GMPSBDB was evaluated in 25 mM PIPES, pH 7.1, at 25 °C. Concentrations of ligands were selected to be high relative to their enzyme−ligand dissociation constants. For example, Figure 1, line b, shows the effect of 55 μM SAMP on the inactivation of adenylosuccinate synthetase by GMPSBDB. The presence of SAMP has virtually no effect on the rate of inactivation during the fast phase but strikingly decreases the slower phase. The 55 μM SAMP concentration is 30 times higher than the reported 1.8 μM dissociation constant for the SAMP−enzyme complex (Soans & Fromm, 1991). For Figure 1, the rate constant estimated for GMPSBDB inactivation in the presence of 55 μM SAMP is 0.00155 min^{-1}, which corresponds to a 92% reduction in the inactivation rate (0.0196 min^{-1}) obtained in the absence of SAMP.

A systematic survey of the effects of active site ligands on the slow phase of inactivation of adenylosuccinate synthetase by GMPSBDB is shown in Table 1. The effect of a protecting ligand is expressed quantitatively as k_{+L}/k_{-L}, the ratio of the slower phase rate constant for inactivation in the presence of an active site ligand to that in its absence. The k_{+L}/k_{-L} values indicate that the greatest protection is afforded by the reaction product, SAMP, and by the combination of $MgCl_2$, GTP, and IMP. The combination of IMP and $MgCl_2$ also shows significant protection, while the combination of $MgCl_2$ and GTP provides a weak, but reproducible protective effect. Neither $MgCl_2$ by itself nor in combination with aspartate shows any protection against GMPSBDB inactivation.

A separate determination of the concentration dependence of IMP in protecting against GMPSBDB is shown in Figure 3, which plots k_{+L}/k_{-L} versus [IMP]. Figure 3 shows that increasing the IMP concentration decreases k_{+L}/k_{-L} to a limiting value of 0.37. The data in Figure 3 were analyzed by assuming a model in which IMP exerts a competitive, but only partial, protection against GMPSBDB inactivation; a slow, residual rate of inactivation by GMPSBDB still occurs in the presence of saturating IMP. The following

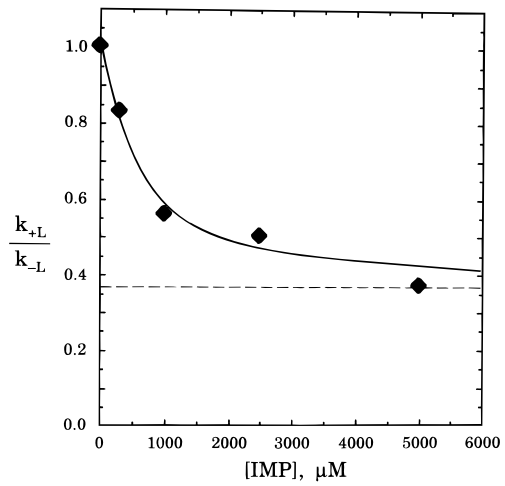

FIGURE 3: Effect of IMP concentration on the rate constant for inactivation of adenylosuccinate synthetase by GMPSBDB. GMPSBDB (350 μM) was added to enzyme which had been preincubated with 10 mM $MgCl_2$ and IMP (0−5 mM) in 25 mM PIPES, pH 7.1, at 25 °C. Aliquots were withdrawn as a function of time and assayed as described under Experimental Procedures. The solid line shown represents a least-squares fit to eq 3 in the text, and the dashed line represents the residual rate of inactivation that is not affected by IMP.

equation, based on the model of partial protection, was used to fit the data in Figure 3:

$$\frac{k_{+L}}{k_{-L}} = R + \frac{(1-R)\left[1+\dfrac{K_{GMPSBDB}}{[GMPSBDB]}\right]}{\left[1+\dfrac{K_{GMPSDBD}}{[GMPSBDB]}\left(1+\dfrac{[IMP]}{K_{IMP}}\right)\right]} \quad (3)$$

In this equation, R is the k_{+L}/k_{-L} ratio for the residual rate of inactivation by GMPSBDB in the presence of saturating IMP and K_{IMP} is the dissociation constant for IMP. Using $K_{GMPSBDB}$ of 42 μM as determined earlier, R is determined to be 0.37 and K_{IMP} is calculated to be 59 μM, in good agreement with an IMP dissociation constant of 39 μM determined by direct binding measurements (Soans & Fromm, 1991).

Incorporation of [8-^{3}H]GMPSBDB by Adenylosuccinate Synthetase. The amount of nucleotide analog incorporated into adenylosuccinate synthetase was determined by incubating the enzyme with 350 μM [8-^{3}H]GMPSBDB as a function of time in 25 mM PIPES, pH 7.1, at 25 °C. At each of the times indicated in Table 2, incorporation was stopped by the addition of $NaBH_4$, and the modified enzyme was dialyzed exhaustively to remove unreacted reagent. Incorporation was determined both in the absence of ligands (designated "unprotected samples") and in the presence of protecting ligands (termed "protected samples"). Also shown in Table 2 are residual activities (E/E_0 values) associated with protected and unprotected samples at each incubation time.

The data in Table 2 indicate that 1 mol of GMPSBDB was incorporated per mole of adenylosuccinate synthetase subunit within the first 4 min of reaction time (when the enzyme had lost one-third of its original activity) and that, in the protected samples, no further incorporation occurred during the next 2 h. In unprotected samples, however, incorporation continued at a slower rate, approaching a value of 2 mol of GMPSBDB incorporated per mole of enzyme subunit when the enzyme was 90% inactivated. A measure of the incorporation of GMPSBDB into the slower reacting site is provided by the difference in incorporation between the protected and unprotected samples, shown in the last column in Table 2. The data indicate a direct correspondence between GMPSBDB incorporation and residual enzyme activity for the slower unprotected inactivation reaction.

Isolation of Peptides after Proteolysis of [8-^{3}H]GMPSBDB-Modified Adenylosuccinate Synthetase. An HPLC chromatogram for the separation of peptides from an *S. aureus* protease digest of [8-^{3}H]GMPSBDB-modified adenylosuccinate synthetase is shown in Figure 4. In this experiment, enzyme (23 μM) was incubated with GMPSBDB (350 μM) for 2 h in the absence of protecting ligands and exhibited an 11% residual activity. (Repetition of this experiment several times gave reproducible HPLC patterns.) Major radioactive peaks are indicated in the chromatogram by reference numbers at the top of the figure.

Figure 5 compares the distribution of radioactive peaks from digests of enzyme modified for 2 h in the absence of protecting ligands (panel A), modified for 2 h in the presence of 10 mM $MgCl_2$, 300 μM GTP, and 600 μM IMP (panel B), and modified for 4 min in the absence of ligands (panel C). Samples shown in panels A, B, and C exhibited 11%, 83%, and 60% residual activity at the end of the period of incubation with GMPSBDB. A comparison of the radioactive peaks in panels A and B shows that the presence of GTP and IMP dramatically reduces the levels of radioactive incorporation in peaks 2a and 2b, while having little effect on the incorporation in peaks 1a−1d.

A comparison of the radioactive peaks in panels A and C shows that a short incubation time nearly eliminates incorporation in peaks 2a and 2b while still allowing incorporation in peaks 1a−1d. These comparisons suggest that incorporation in peaks 1a−1d results from the fast-reacting kinetic phase of the GMPSBDB modification reaction (see Figure 1), a reaction that is not affected by active site ligands. Incorporation in peaks 2a and 2b, in contrast, results from the slowly reacting kinetic phase of the GMPSBDB modification reaction, a reaction that is prevented by certain active site ligands (see Figure 1 and Table 1).

Characterization of Modified Peptides. Samples from all major radioactive HPLC peaks were sequenced using an automated gas-phase sequenator. Results for peak 2a are shown in Figure 6, which plots the yield of the PTH-amino acid derivative versus the Edman degradation cycle number. For the HPLC run analyzed in Figure 6, peak 2a contained a single peptide (Asp-139 to Glu-155) for which the level of Arg-143 was significantly depleted. A fit of the data in Figure 6 to an exponential function (solid line) was used to estimate an expected yield of 51 pmol of Arg-143 in cycle 5. The 8.1 pmol of Arg-143 obtained indicates an 84% reduction in the amount of free Arg expected at position 143. In contrast, Arg-144 (cycle 6) and Arg-147 (cycle 9) are present at expected levels in this peptide. These results indicate that Arg-143 of this peptide has been chemically modified and that the PTH derivative of this modified Arg is not observed in the normal HPLC detection system of the sequenator.

By extrapolating the line for the exponential fit in Figure 6 back to cycle 1, we estimate that 90−95 pmol of peptide was applied to the sequenator, giving a 1:1 stoichiometry

Table 2: Incorporation of [8-^{3}H]GMPSBDB by Adenylosuccinate Synthetase[a]

incubation time (min)	protected samples[b] E/E_0	protected samples[b] mol of GMPSBDB/ mol of subunit	unprotected samples E/E_0	unprotected samples mol of GMPSBDB/ mol of subunit	Δ mol of GMPSBDB/ mol of subunit
4	0.74	0.96	0.66	1.22	0.26
22	0.71	0.99	0.33	1.45	0.45
45	0.72	1.18	0.19	1.86	0.68
120	0.56	1.06	0.09	1.81	0.75
130	0.56	0.89	0.10	1.73	0.84

[a] Incubation solutions contained 2.9−3.3 μM enzyme and 350 μM [8-^{3}H]GMPSBDB in 25 mM PIPES buffer, pH 7.1, at 25 °C. Protocol for determining incorporation is given in Experimental Procedures. [b] Protection was afforded by pre- and coincubation of the enzyme with 10 mM $MgCl_2$, 150 μM GTP, and 300 μM IMP in 25 mM PIPES buffer, pH 7.1. In the case of the 120 min incubation only, protection was afforded by 110 μM SAMP.

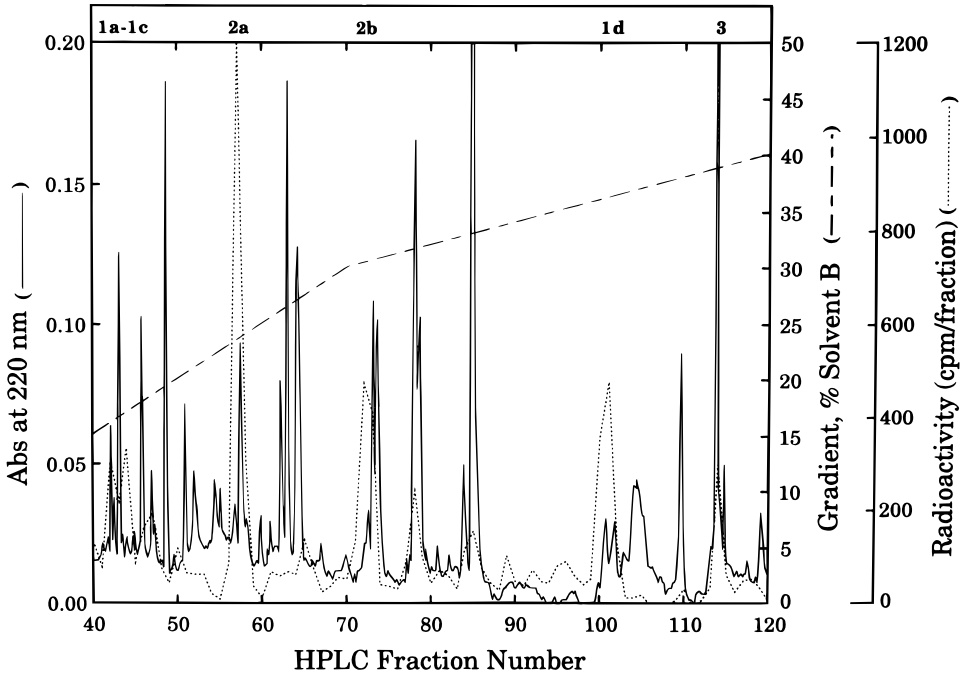

FIGURE 4: Separation of the V8 protease digest of [8-^{3}H]GMPSBDB-labeled adenylosuccinate synthetase by reverse-phase HPLC. Peptides were separated by gradient elution on a C_{18} column at a flow rate of 1 mL/min. The gradient, shown as the dashed line in the plot, was produced by mixing solvent A (0.1% aqueous trifluoroacetic acid) with solvent B (0.075% trifluoroacetic acid in acetonitrile). A 100 μL aliquot of each fraction was assayed for radioactivity. The solid line represents A_{220nm}, and the dotted line is the net radioactivity per fraction. The numbers above the plot are reference labels for the major radioactive peaks.

with the 90 pmol (160 cpm) of [8-^{3}H]GMPSBDB applied. Scintillation counting of the application filter and the sequenator fractions from the experiment in Figure 6 indicated that the radioactivity did not elute with any PTH derivative but remained bound to the filter. Similar observations have been made for other polar nucleotidyl peptides (Vollmer *et al*., 1994). HPLC peak 2b was found by gas-phase sequencing to contain a peptide starting with Ala-102 which was not completely cleaved by *S. aureus* protease and was likely to contain Arg-143; however, the peptide was too long to verify by complete sequencing.

The Arg−GMPSBDB adduct results from bond formation between the two free guanidino nitrogens of Arg-143 and the two carbonyl carbons of the diketone moiety of GMPSBDB to form a cyclic vicinal dihydroxy adduct analogous to those shown in the papers of Wrzeszczynski and Colman (1994) and Pathy and Smith (1975). The reactive terminal methylene carbon of the affinity label, which carries the bromide leaving group, is therefore not directly involved in the arginine modification reaction. The terminal bromide group is, however, readily displaced by water in a nucleophilic displacement reaction (Vollmer *et al*., 1994) which has a half-life of 120 min. We therefore expect extensive release of the bromide ion and replacement by −OH during the lengthy procedures for the purification of modified peptides.

We used electrospray mass spectrometry to provide additional evidence for the chemical modification of the peptide in peak 2a by GMPSBDB. Peptides having masses of 1973 amu (*ca*. 77% of total) and 2453 amu (*ca*. 23%) were detected. The mass of 1973 amu is identical to the predicted mass of the unmodified form of the peptide (Asp-139 to Glu-155), while the mass of 2453 amu corresponds to the predicted mass of the GMPSBDB-modified peptide in which the bromide group of GMPSBDB had been replaced by an −OH group. Thus, although the GMPSBDB−Arg adduct was not entirely stable to electrospray mass spectrometry, sufficient adduct survived to directly demonstrate chemical modification of the peptide.

Sequence determinations for peaks 1a, 1b, and 1d, the results of which are shown in Table 3, implicate Cys-291 as the residue that is modified in the fast kinetic phase of the

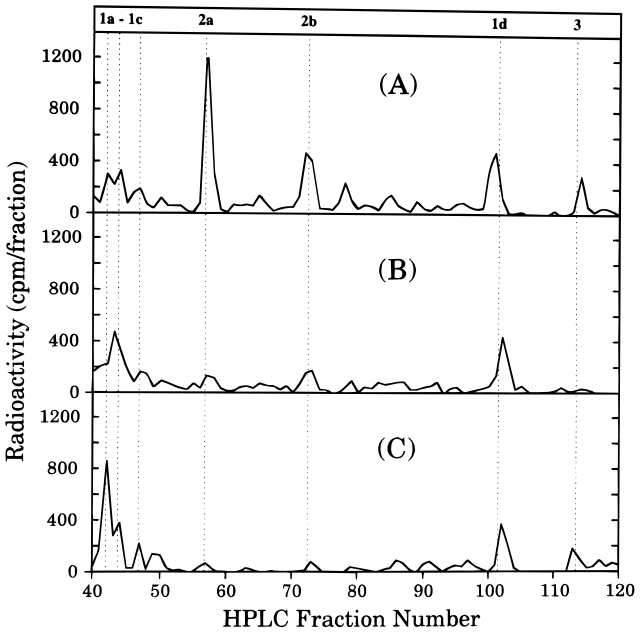

FIGURE 5: Separation of radioactive V8-digested peptides of [8-^{3}H]-GMPSBDB-labeled adenylosuccinate synthetase by reverse-phase HPLC. The peptides were separated by gradient elution as described in Experimental Procedures. Normalized cpm data (solid lines in each panel) are plotted to account for differing dilutions during dialysis and differing levels of solubilization during digestion.[2] The sample in (A) was incubated with 350 μM GMPSBDB for 2 h in the absence of ligands and exhibited 11% residual activity. The sample in (B) was incubated with 350 μM GMPSBDB for 2 h in the presence of 10 mM $MgCl_2$, 300 μM GTP, and 600 μM IMP and exhibited 83% residual activity. The sample in (C) was incubated in the absence of ligands with 350 μM GMPSBDB for 4 min and exhibited 60% residual activity. The recoveries of radioactivity from the HPLC columns were typically on the order of 50%.

GMPSBDB inactivation reaction. The sequence for peak 1d was determined from an experiment in which free cysteine residues of urea-denatured enzyme had been blocked by reaction with NEM immediately following the reaction with GMPSBDB (see Experimental Procedures). The PTH derivative of NEM-modified Cys elutes on the sequencer as a characteristic double peak between the PTH derivatives of Pro and Met (Smyth & Colman, 1991). The sequence of the peptide from peak 1d, shown in Table 3, shows the complete absence of the expected NEM adduct of Cys-291 and strongly argues that Cys-291 is the site of GMPSBDB incorporation. NEM adducts of two of the remaining cysteines in the enzyme, Cys-103 and Cys-344, were identified in other fractions of the same HPLC run (data not shown), indicating that NEM modification of the enzyme had occurred. Note that the peak 1d peptide begins with an N-terminal Thr-286 and continues through two potential cleavage points, Glu-288 and Glu-296. From the known sequence of the enzyme, the *minimum* size of this peptide is 55 amino acids (Thr-286 to Glu-340).

Analysis of peak 1a, the major peak in the 4 min unprotected incubation (see Figure 5), yielded an octapeptide

[2] The normalization in Figure 5 was based on incorporation data (Table 2) which showed that all modification reactions gave an incorporation of 1 mol of reagent/mol of enzyme subunit at the fast reacting site. After identification of peaks 1a−1d as the fast-reacting, unprotected sites, counts per minute were normalized on the basis of the total radioactivity in peaks 1a−1d.

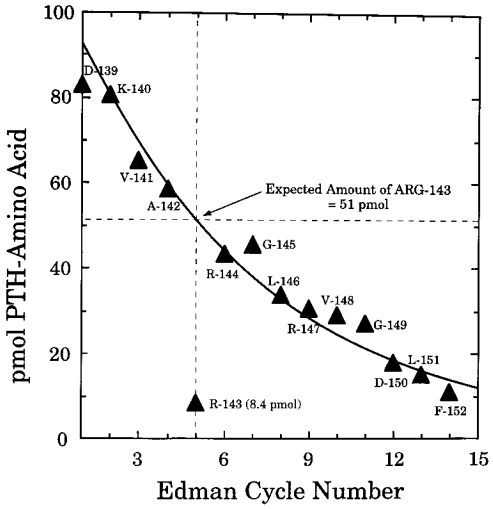

FIGURE 6: Edman yield plot for sequence determination of labeled peptide from HPLC peak 2a. In this plot, the pmol of the phenylthiohydantoin-amino acid derivative released during each cycle of the Edman degradation is plotted versus the cycle number.

Table 3: Summary of Sequence of Peptides Containing Cys-291[a]

	HPLC peak 1a		HPLC peak 1b		HPLC peak 1d	
cycle no.	amino acid	pmol	amino acid	pmol	amino acid	pmol
1	Phe-289	726	Thr-286	263	Thr-286	32
2	Leu-290	712	Gly-287	323	Gly-287	52
3	**Cys-291**		Glu-288	306	Glu-288	34
4	Lys-292	710	Phe-289	320	Phe-289	27
5	Gln-293	551	Leu-290	350	Leu-290	28
6	Gly-294	335	**Cys-291**		**Cys-291**	**0**
7	Asn-295	375	Lys-292	352	Lys-292	16
8	Glu-296	295	Gln-293	265	Gln-293	21
9	(end)		Gly-294	174	Gly-294	20
10			Asn-295	176	Asn-295	18
11			Glu-296	94	Glu-296	16
12			(end)		Phe-297	14
13−17					(continues)	

[a] In the case of HPLC peak 1d, NEM was used to derivatize all free cysteine residues in the modified enzyme as described in Experimental Procedures. For peptides **1a** and **1b**, free cysteine residues were not derivatized and could not be determined by the sequencing methods used. These are representative sequences. The amounts of amino acids shown here and in Figure 6 do not reflect the relative amounts of the radioactive peaks, since they were taken from different runs and different aliquots of the peak fractions were used for sequence determinations.

which begins at Phe-289 and includes the Cys-291 residue. Cys-291-containing peptides were also found in HPLC peak 1b (Table 3) and peak 1c (data not shown). HPLC peak 3 (see Figure 5), present in small, but variable amounts in different preparations, was found to be a complex mixture of incompletely digested peptides likely to contain both Arg-143 and Cys-291 residues (data not shown).

In addition to the samples shown in Figure 5, HPLC separations were also carried out for experiments in which the reaction of the enzyme with GMPSBDB was conducted in the presence of IMP/$MgCl_2$, GTP/$MgCl_2$, and SAMP (chromatograms not shown). To compare the different HPLC runs, the total radioactivity incorporated into the Arg-143-containing peptides (peaks 2a and 2b) was summed and compared in Table 4, as a ratio, with the total radioactivity from the Cys-291-containing peaks (peaks 1a−1d). The Arg-143/Cys-291 ratio, shown in the last column of Table

Table 4: Comparison of Radioactivity Incorporated into HPLC Peaks Containing Peptides Labeled at Cys-291 and Arg-143

modified enzyme sample	fraction of inactivation during slow phase	Arg-143/ Cys-291 ratio[a]
unprotected, 2 h incubation	0.87	0.97
protected, 600 μM GTP, 10 mM $MgCl_2$, 2 h incubation	0.63	0.63
protected, 5 mM IMP, 10 mM $MgCl_2$, 2 h incubation	0.23	0.33
protected, 600 μM IMP, 300 μM GTP, 10 mM $MgCl_2$, 2 h incubation	0.00	0.23
protected, 110 μM SAMP, 0 mM $MgCl_2$, 2 h incubation	0.00	0.17
unprotected, 4 min incubation	0.00	0.08

[a] This ratio is defined as the ratio of total radioactivity found in peaks 2a and 2b to the total radioactivity found in peaks 1a−1d (see Figure 4).

Table 5: Kinetic Parameters of Wild-Type and Mutant Adenylosuccinate Synthetases from *E. coli*[a]

protein	k_{cat} (s^{-1})	K_m^{GTP} (μM)	K_m^{IMP} (mM)	K_m^{ASP} (mM)
wild type	1.00 ± 0.05	26.2 ± 2.3	0.03 ± 0.00	0.23 ± 0.04
R143L mutant	1.04 ± 0.01	269 ± 17	1.73 ± 0.14	0.34 ± 0.06

[a] Enzyme assays were carried out at 25 °C in 20 mM HEPES buffer, pH 7.7, containing 5 mM $MgCl_2$, as described in Experimental Procedures.

4, shows equal incorporation of tritium from [8-^{3}H]GMPSBDB into Cys-291 and Arg-143 for a 2 h unprotected incubation. The Arg-143/Cys-291 ratio further demonstrates a low level of protection against incorporation at the Arg-143 site by GTP, with increasing levels of protection by IMP, by a combination of GTP and IMP, and by SAMP. Very low incorporation into Arg-143-containing peptides is observed in the 4 min unprotected incubation.

Replacement of Arg-143 by Leu-143 by Site-Directed Mutagenesis. Replacement of the arginine with a leucine at position 143 of adenylosuccinate synthetase to create the R143L mutant was carried out by site-directed mutagenesis using the methods described in Experimental Procedures. Circular dichroism spectra of the wild-type enzyme and the R143L mutant were essentially indistinguishable, indicating no major differences in secondary structure (data not shown). Michaelis constants and catalytic constants determined for the wild-type adenylosuccinate synthetase and the R143L mutant form of the enzyme are given in Table 5. The mutant enzyme shows a 60-fold increase in the K_m for IMP and 10-fold increase in the K_m for GTP, indicating weaker interactions of both nucleotides with the enzyme. In contrast, neither the catalytic constant for the reaction nor the K_m for aspartate is significantly affected by the mutagenic replacement.

DISCUSSION

Results from kinetic inactivation and radioactive incorporation experiments demonstrate that GMPSBDB reacts with *E. coli* adenylosuccinate synthetase in two phases: an initial fast kinetic phase that results in the incorporation of 1 mol of reagent/mol of enzyme subunit and approximately 50% loss of activity and a slower second phase in which the remaining activity is lost through incorporation of an additional 1 mol of GMPSBDB/mol of subunit. The residues Cys-291 and Arg-143 have been identified as the amino acid side chains that react with GMPSBDB.

Labeling of Cys-291 is complete within 4 min, indicating that Cys-291 is the residue that reacts with GMPSBDB in the initial fast kinetic phase of inactivation. Active site ligands are unable to prevent reaction at Cys-291, suggesting that Cys-291 is not at the active site of the enzyme. NMR studies of the *E. coli* enzyme in which Cys-291 had been modified with a PROXYL spin label have indicated that Cys-291 is not close (*i.e.*, >9.8 Å) to the guanine ring of GTP bound at the active site (Dong *et al*., 1990). A representation of the crystal structure of the enzyme dimer (Figure 7) indicates that Cys-291 (shown in yellow) is located at an external surface of each subunit, where it would be expected to react readily as a solvent-accessible nucleophile. Modification of Cys-291 with NEM results in 83% residual enzymatic activity (Dong *et al*., 1990). We might expect, therefore, that modification of Cys-291 by the larger GMPSBDB could cause an even greater activity loss, possibly by a secondary conformational change following nucleotide binding. We have determined that the residual activity following modification of Cys-291 by GMPSBDB in the absence of active site ligands is 50%; in the presence of SAMP or the GTP/IMP combination, modification of Cys-291 by GMPSBDB, while still occurring at a rapid rate, results in a higher residual activity of 67 ± 8% (based on 11 determinations). Thus, active site ligands may be able to shield the enzyme from some effects of a secondary conformational change.

Reaction of GMPSBDB with Arg-143 is responsible for the second slower kinetic phase of inactivation. This reaction exhibits saturation kinetics with respect to GMPSBDB concentration. A strong correlation exists between the extent of incorporation of reagent into Arg-143 and the extent of kinetic inactivation in the slower phase. The rate constant for the slow-phase inactivation is markedly decreased by certain active site ligands. These results argue that GMPSBDB does function in the second slower phase as a true affinity label for adenylosuccinate synthetase, binding competitively as a substrate analog at the active site of the enzyme and covalently labeling Arg-143.

From kinetic inactivation and protection studies, it is evident that either SAMP or the combination of IMP and GTP can afford complete protection against the inactivation by GMPSBDB. GTP alone, however, exerts only a very weak protective effect. IMP is most effective at concentrations ≥5 mM, but its maximal protective effect is only partial, providing a 63% reduction in the rate of inactivation by GMPSBDB. The extent of protection by active site ligands against kinetic inactivation closely parallels the corresponding protection against the labeling of Arg-143 by [8-^{3}H]GMPSBDB.

The protection data may be explained by assuming that GMPSBDB can bind at either the IMP or GTP sites but that the reaction with Arg-143 occurs more rapidly when GMPSBDB is bound to the IMP site. Protection by saturating IMP is only partial because IMP does not protect against GMPSBDB binding at the GTP site, where the inactivation reaction occurs at a reduced rate. SAMP, which differs structurally from IMP at the 6 position of the purine ring (the 6-hydroxyl moiety of IMP is replaced by a succinyl group covalently attached to a 6-amino group),

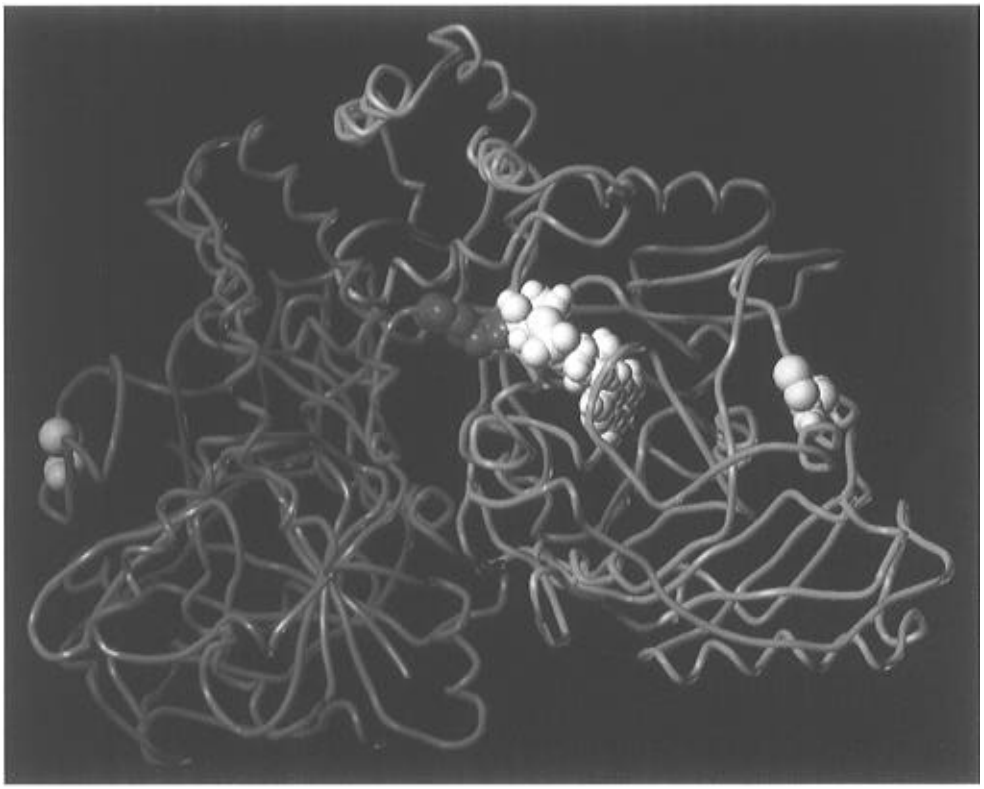

FIGURE 7: Predicted orientation of GMPSBDB-modified Arg-143 in adenylosuccinate synthetase. The molecular modeling program Sybyl was used create a GMPSBDB-modified Arg-143 residue on one subunit of adenylosuccinate synthetase. An energy minimization subroutine was then used to predict the positioning of the modified arginine side chain as described in Experimental Procedures. Polypeptide backbones are displayed in tubular form. The only amino acid side chains displayed are the Cys-291 residues on each subunit, shown in yellow, and a single modified Arg-143 residue in which the arginine itself is colored red and the affinity label is white.

Table 6: Alignment of the *E. coli* Adenylosuccinate Synthetase Sequence 138−148 with Other Adenylosuccinate Synthetase Sequences[a]

source	sequence	reference
Bacillus subtilis	M-D-K-A-A-**R**-I-G-I-R-I	Mantsala & Zalkin, 1992
Dictyostelium discoideum	S-S-K-A-S-**R**-G-G-L-R-V	Wiesmuller *et al*., 1991
E. coli	E-D-K-V-A-**R**-R-G-L-R-V	Wolfe & Smith, 1988
human liver	S-S-K-A-A-**R**-S-G-L-R-M	Powell *et al*., 1992
mouse muscle	S-S-K-A-A-**R**-T-G-L-R-I	Guicherit *et al*., 1991
mouse T-lymphoma cells	S-S-K-A-A-**R**-S-G-L-R-M	Guicherit *et al*., 1994
Schizosaccharomyces pombe	S-T-K-A-T-**R**-S-G-I-R-V	Speiser *et al*., 1992
Thiobacillus ferrooxidans	E-D-K-V-R-**R**-R-A-L-R-V	Kusano *et al*., 1992

[a] The conserved arginine residue corresponding to Arg-143 in *E. coli* adenylosuccinate synthetase from different sources is shown with a bold character.

may be able to block both the IMP and GTP sites (the latter site via the succinyl residue of SAMP), completely preventing the inactivation reaction. The combination of IMP and GTP also blocks both binding sites and yields complete protection.

Further evidence for the location of Arg-143 at the active site of adenylosuccinate synthetase and its role in nucleotide binding is provided by the replacement of Arg-143 with Leu-143 by mutagenesis. Kinetic analysis of the R143L mutant indicates that the predominant effect of this replacement is a weakening of the binding of the two nucleotide substrates, IMP and GTP. The Michaelis constants for GTP and IMP, K_m^{GTP} and K_m^{IMP}, are increased by factors of 10 and 60, respectively. In contrast, values for K_m^{ASP} and k_{cat} for the R143L mutant are essentially unchanged from those of the wild-type enzyme. These results suggest that Arg-143 may be involved in the binding of both IMP and GTP to the adenylosuccinate active site but that its role is more important in IMP binding.

Table 6 provides a comparison of aligned adenylosuccinate synthetase sequences from various sources. The comparison shows that the arginine at position 143 in the *E. coli* enzyme is highly conserved, consistent with its importance in adenylosuccinate synthetase.

Crystallographic studies have shown that Arg-143 from one subunit projects into the putative active site crevice of the second subunit (Poland *et al*., 1993). A representation of the orientation of the modified Arg-143 residue in adenylsuccinate synthetase is shown in Figure 7. In this figure, molecular modeling software was used to covalently link GMPSBDB to Arg-143. An energy minimization subroutine was then used to predict the most stable positioning of the modified arginine side chain. The modeled protein in Figure 7 predicts that the labeled arginine residue from one subunit projects across the subunit interface where it fits into a crevice in the second subunit.

In summary, this work provides two independent lines of evidence for the role of Arg-143 in nucleotide binding in

adenylosuccinate synthetase from *E. coli*. Affinity labeling studies strongly suggest that GMPSBDB binds at both the IMP and GTP binding sites of the enzyme where it causes inactivation by reacting with Arg-143. IMP provides more effective protection against inactivation by GMPSBDB than does GTP, and both are required for full protection. Site-directed mutagenesis studies show that binding of IMP, and to a lesser extent GTP, is weakened when the positively charged Arg-143 is replaced by the uncharged leucine. Both lines of evidence thus indicate that Arg-143 plays a more important role in IMP binding than in GTP binding. Recent crystallographic evidence shows that Arg-143 from one subunit is ligated to the 5′-phosphate of IMP in the other subunit (R. B. Honzatko, personal communication). Interpreted in light of crystallographic data, the evidence presented here also suggests that an active site of *E. coli* adenylosuccinate synthetase is formed from amino acid side chains that are contributed by both subunits of the dimeric structure.

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REFERENCES

Bass, M. B., Fromm, H. J., & Rudolph, F. B. (1984) *J. Biol. Chem. 259*, 12330−12333.
Bass, M. B., Fromm, H. J., & Stayton, M. M. (1987) *Arch. Biochem. Biophys. 256*, 335−342.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Cooper, B. F., Fromm, H. J., & Rudolph, F. B. (1986) *Biochemistry 25*, 7323−7327.
DeCamp, D. L., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 8430−8441.
Dong, Q., & Fromm, H. J. (1990) *J. Biol. Chem. 265*, 6235−6240.
Dong, Q., Soans, C., Liu, F., & Fromm, H. J. (1990) *Arch. Biochem. Biophys. 276*, 77−84.
Dong, Q., Liu, F., Myers, A. M., & Fromm, H. J. (1991) *J. Biol. Chem. 266*, 12228−12233.
Draper, N. R., & Smith, H. (1966) *Applied Regression Analysis*, John Wiley & Sons, New York.
Groves, W. E., Davis, F. C., Jr., & Sells, B. H. (1968) *Anal. Biochem. 22*, 195−210.
Guicherit, O. M., Rudolph, F. B., Kellems, R. E., & Schleicher, M. (1991) *J. Biol. Chem. 266*, 2480−2485.
Guicherit, O. M., Cooper, B. F., Rudolph, F. B., & Kellems, R. E. (1994) *J. Biol. Chem. 269*, 4488−4496.
Huang, Y.-C., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 12481−12488.
Kang, C., & Fromm, H. J. (1994) *Arch. Biochem. Biophys. 310*, 475−480.
Kang, C., Sun, N., Honzatko, R. B., & Fromm, H. J. (1994) *J. Biol. Chem. 269*, 24046−24049.
Kusano, T., Takeshima, T., Sugawara, K., Inoue, C., Shiratori, T., Yano, T., Fukimori, Y., & Yamanaka, T. (1992) *J. Biol. Chem. 267*, 11242−11247.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Liu, F., Dong, Q., & Fromm, H. J. (1992) *J. Biol. Chem. 267*, 2388−2392.
Mantsala, P., & Zalkin, H. (1992) *J. Bacteriol. 174*, 1883−1890.
Moe, O. A., Baker-Malcom, J. F., Wang, W., Kang, C., Fromm, H. J., & Colman, R. F. (1996) *FASEB J. 10*, A1101.
Ozturk, D. H., Park, I., & Colman, R. F. (1992) *Biochemistry 31*, 10544−10555.
Pathy, L., & Smith, E. L. (1975) *J. Biol. Chem. 250*, 557−564.
Poland, B. W., Silva, M. M., Serra, M. A., Cho, Y., Kim, K. H., Harris, E. M. S., & Hanzatko, R. B. (1993) *J. Biol. Chem. 268*, 25334−25342.
Powell, S. M., Zalkin, H., & Dixon, J. E. (1992) *FEBS Lett. 303*, 4−10.
Rudolph, F. B., & Fromm, H. J. (1969) *J. Biol. Chem. 244*, 3832−3839.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Smyth, G. E., & Colman, R. F. (1991) *J. Biol. Chem. 266*, 14918−14925.
Soans, C., & Fromm, H. J. (1991) *Arch. Biochem. Biophys. 291*, 107−112.
Speiser, D. M., Ortiz, D. F., Kreppel, L., Scheel, G., McDonald, G., & Ow, D. W. (1992) *Mol. Cell. Biol. 12*, 5301−5310.
Vollmer, S. A., Walner, M. B., Tarbell, K. V., & Colman, R. F. (1994) *J. Biol. Chem. 269*, 8082−8090.
Wang, W., Honzatko, R. B., & Fromm, H. J. (1995) *FASEB J. 9*, A1435.
Webb, M. R., Cooper, B. F., Rudolph, F. B., & Reed, G. H. (1984) *J. Biol. Chem. 259*, 3044−3046.
Wiesmuller, L., Wittbrod, J., Noegel, A. A., & Schleicher, M. (1991) *J. Biol. Chem. 266*, 22582−22587.
Wolfe, S. A., & Smith, J. M. (1988) *J. Biol. Chem. 263*, 19147−19153.
Wrzeszczynski, K. O., & Colman, R. F. (1994) *Biochemistry 33*, 11544−11553.

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