Site-directed mutagenesis and kinetic studies have been employed to identify amino acid residues involved in aspartate binding and transition state stabilization during the formation of β-aspartyl-AMP in the reaction mechanism of *Escherichia coli* asparagine synthetase B (AS-B). Three conserved amino acids in the segment defined by residues 317–330 appear particularly crucial for enzymatic activity. For example, when Arg-325 is replaced by alanine or lysine, the resulting mutant enzymes possess no detectable asparagine synthetase activity. The catalytic activity of the R325A AS-B mutant can, however, be restored to about 1/6 of that of wild-type AS-B by the addition of guanidinium HCl (GdmHCl). Detailed kinetic analysis of the rescued activity suggests that Arg-325 is involved in stabilization of a pentacovalent intermediate leading to the formation β-aspartyl-AMP. This rescue experiment is the second example in which the function of a critical arginine residue that has been substituted by mutagenesis is restored by GdmHCl. Mutation of Thr-322 and Thr-332 also produces enzymes with altered kinetic properties, suggesting that these threonines are involved in aspartate binding and/or stabilization of intermediates en route to β-aspartyl-AMP. These experiments are the first to identify residues outside of the N-terminal glutamine amide transfer domain that have any functional role in asparagine synthesis.

A great deal of interest in asparagine metabolism has resulted from the finding that certain leukemias can be treated by the administration of l-asparaginase (see review, Ref. 1). Experiments suggest that the effectiveness of this protocol is dependent upon decreasing the circulating amount of asparagine (2). Although administration of l-asparaginase is accepted as an essential component of modern therapy, it is fraught with serious side effects and plagued by the appearance of resistant leukemias. An alternative, or adjunct, approach to the use of l-asparaginase might be to lower circulating asparagine by inhibiting asparagine synthetase (AS), the enzyme responsible for its production. Of several hundred compounds that have been evaluated as AS inhibitors, however, none have exhibited sufficient potency and specificity to warrant clinical consideration (3). This failure can be partly explained by the lack of detailed mechanistic information on AS.

Two classes of enzymes catalyzing asparagine synthesis have been described that possess no sequence similarity and may consequently have arisen by convergent evolution. Ammonia-dependent asparagine synthetases in prokaryotes such as *Klebsiella aerogenes* and *Escherichia coli* (4–6) can employ only ammonia as a nitrogen source (Reaction 1). 6–17141

\[
\text{L-Asp} + \text{NH}_3 + \text{ATP} \rightarrow \text{L-Asn} + \text{AMP} + \text{PP}, \quad (\text{Reaction 1})
\]

\[
\text{L-Asp} + \text{l-GLn} + \text{ATP} \rightarrow \text{L-Asn} + \text{l-Glu} + \text{AMP} + \text{PP}, \quad (\text{Reaction 2})
\]

\[
\text{L-Gln} \rightarrow \text{l-Glu} + \text{NH}_3 \quad (\text{Reaction 3})
\]

The second group of asparagine synthetases, on the other hand, is present in both prokaryotes and eukaryotes and employs glutamine as the predominant source of nitrogen in obtaining asparagine from aspartate and ATP (Reaction 2), although ammonia can be employed as an alternative to glutamine (7–9). In addition, this class of synthetases acts as glutaminases in the absence of aspartate (Reaction 3).

*E. coli* contains two unlinked genes coding for asparagine synthetases (7). Asparagine synthetase A (AS-A), the product of the 990-bp *asnA* gene for which the complete nucleotide sequence is known, has been isolated and exhibits strictly ammonia-dependent activity (5, 6, 10). The nucleotide sequence for the 1662-bp *asnB* gene, encoding asparagine synthetase B (AS-B), has also been cloned and sequenced (11). Based on its primary amino acid sequence, AS-B is a purF (class II) amidotransferase, possessing an N-terminal cysteine residue that is essential for glutamine-dependent activity (12).

Our recent work describing a number of site-specific AS-B mutants has identified specific amino acids in the N-terminal glutamine amide transfer (GAT) domain that are critical to glutamine-dependent nitrogen transfer (13, 14). Detailed kinetic analysis of wild-type enzyme and these mutants using alternate substrates and heavy atom isotope effects has also yielded new insights into the mechanistic role of the AS-B GAT domain (15, 16). Identification of key residues in the N-terminal region of AS-B was aided by the availability of sequences for the glutamine-utilizing domains of other purF amidotransferases. On the other hand, both BLAST and FASTP analyses indicate that the primary structure of the C-terminal synthetase domain in asparagine synthetases is unique, with the exception of a small region, termed the P-loop-like motif, that is present in a number of ATP-dependent enzymes that release sulfonylbenzoyladenosine; MetRS, methionyl tRNA aminoacyl synthetase; bp, base pair(s); GAT, glutamine amide transfer; I, intermediate.

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‡ The abbreviations used are: AS, asparagine synthetase; AS-B *E. coli* asparagine synthetase B; GdmHCl, guanidinium hydrochloride; PCR, polymerase chain reaction; CSA, cysteinesulfonic acid; FSBA, 5′-fluoro-
AMP and PP, as reaction products (17). The involvement of this motif in ATP utilization during asparagine synthesis, however, has yet to be validated using site-directed AS mutants.

While raising interesting evolutionary questions, the observation that sequence similarities between AS-A and AS-B do not exist (11) complicates the identification of critical catalytic residues in the C-terminal AS-B synthetase domain. Multiple sequence alignments for glutamine-dependent asparagine synthetases show 152 conserved residues and 92 positions in which conservative replacements are present. Of the seven regions possessing four, or more, consecutive conserved residues, two of these are in the GAT domain and appear to mediate only nitrogen transfer and glutamine hydrolysis. In this paper, we present the results of random, and site-specific, mutagenesis experiments on two of the five regions defined by residues 317–330 and 484–500, respectively (Fig. 1). While the latter region does not appear to possess a functional role in catalysis and/or substrate binding, we report evidence supporting the hypothesis that Arg-325, Thr-322, and Thr-323 mediate only nitrogen transfer and glutamine hydrolysis. In the following description, we refer to these residues as the Thr-321–Thr-323 module.

Materials and Methods

Restriction and modifying enzymes were purchased from Promega (Madison, WI), Life Technologies, Inc., or New England Biolabs (Bever- ly, MA). Deoxyadenosine 5′-O-(3′-thiotriphosphate) triethylammonium salt (Sε, εomer, 1000 dnmol) was purchased from Amersham Corp. All other reagents were the highest possible quality. Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer by the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Polymerase chain reactions (PCR) were performed on an Erichrom (San Diego, CA) thermocycler using the GeneAmp DNA Amplification Reagent Kit with AmpliTaq from Perkin-Elmer. Thirty-five cycles consisting of denaturation at 94°C for 1 min, annealing at 54°C for 1 min during megaprimer reactions or 52°C for 1 min during megaproduct reactions, and extension at 72°C for 1 min were followed by a 10-min completion cycle at 72°C. Megaprimers were purified by polyethylene glycol precipitation (0.6 volumes of 20% polyethylene glycol 8000 in 2.5 mM NaCl) were added to the PCR reaction, incubated at 37°C for 10 min, centrifuged at 10,000 rpm for 10 min, and was washed 2 times with 80% EtOH or agarose gel electrophoresis. After gel electrophoresis, the PCR product was extracted using a Gene Clean II kit from Bio 101, Inc. (Vista, CA). Double-stranded DNA sequencing was performed using the U. S. Biochemical Corp. Sequenase 2.0 Sequencing Kit. Preparation of the template for sequencing was performed by the alkaline lysis method.

Bacterial Strains and Plasmids—All strains were derivatives of E. coli K-12: BL21DE3PlyS (F′,ompT, rbi, mbl) generously supplied by Studier and Moffatt (19), while NM522 (sup E, thi lac-proAB), hsd5, (r–m–)/λpro AB proA, lac I Z M15) and plasmid pBluescript were obtained from Stratagene (La Jolla, CA). Plasmid pETB was prepared as described previously (12), and the hosts were transformed according to the procedure of Hanahan (20).

Sequence Alignments—The amino acid sequences of known glutamine-dependent asparagine synthetases were aligned using a simplification of the progressive alignment method of Feng and Doolittle (21), as implemented in the program PILEUP in the GCG Sequence Analysis Software Package (22).

Construction of Mutants—All directed random mutants were constructed using the PCR megaprimer strategy (23) using template pETB, and oligonucleotide primer pairs SS123 and SS124 or SS125 and SS126 (Table I). Primer SS123 and SS124 contained degenerate oligonucleotide sequence alignments for several glutamine-dependent asparagine synthetases were aligned using a simplification of the progressive alignment method of Feng and Doolittle (21), as implemented in the program PILEUP in the GCG Sequence Analysis Software Package (22).

Sequence alignments using a simplification of the progressive alignment method of Feng and Doolittle (21), as implemented in the program PILEUP in the GCG Sequence Analysis Software Package (22). The identity of the mutated insert was verified by sequencing.

Site-directed mutants with single amino acid changes were obtained as follows. A mutant in which Thr-322 was replaced by serine (T322S), created the unique SacI site, was generated using mutagenic primers SS196 and SS145 (Table I) as a template to construct a cloning cassette. The template was chosen because a unique SacI site, 3′ of the mutagenic region, was created during the original mutagenesis. Megaprimers were utilized with primers SS196 and SS145 (Table I) creating a unique KpnI site 5′ of the mutagenic area. The megaproduction utilized the megaprimers and SS197 (Table I) containing a 126-bp cassette between the KpnI and SacI sites. pETB-KS was then used as the template to construct site-directed random mutants of Thr-323 and Arg-325. SS198 and SS145, containing the KpnI site and the correct sequence for Thr-322, along with SS197 (Thr-323) or SS195 (Arg-325) were used to generate a 126-bp product which was cut by KpnI and cloned into pETB-KS digested with the same enzymes. Additional site-specific mutations were generated utilizing pETB-KS as a template and SS224 and SS351-SS363 and SS370 (Table I) to produce T322Y, T322A, T322V, T323Y, T323F, T323A, R325S, R325K, R325V, D320A, Y319A, Y318A, E317A, and E317Q. The 534-bp product was digested with SalI and KpnI and cloned into template T322S, thus creating a new template pETB-KS, containing a 126-bp cassette between the KpnI and SalI sites. pETB-KS was then used as the template to construct single random mutants of Thr-323 and Arg-325. SS184, containing the KpnI site and the correct sequence for Thr-322, along with SS197 (Thr-323) or SS195 (Arg-325) were used to generate a 126-bp product which was cut by SalI and KpnI and cloned into pETB-KS digested with the same enzymes.

Additional site-specific mutations were generated utilizing pETB-KS as a template and SS224 and SS351-SS363 and SS370 (Table I) to produce T322Y, T322A, T322V, T323Y, T323F, T323A, R325S, R325K, R325V, D320A, Y319A, Y318A, E317A, and E317Q. The 534-bp product was digested with SalI and KpnI and cloned into pETB-KS digested with the same enzymes. The identity of mutated inserts generated by PCR and the accompanying cloning sites was confirmed by sequencing before protein expression.

Screening of Directed Random AS-B Mutants—Several preliminary assays were performed to determine which AS-B mutants warranted further investigation. First, the solubility of the mutant protein was evaluated by SDS-polyacrylamide gel electrophoresis. Second, the soluble cellular fraction containing crude overexpressed AS-B or AS-B mutant enzymes were assayed by measuring the conversion of aspartate to asparagine as monitored by high performance liquid chromatography amino acid analysis on an Applied Biosystems 420A derivitizer and 130A separation system. Standard assay conditions were as follows: 100 mM NaH2OAc or 10 mM glutamine, 10 mM ATP, 10 mM L-aspartate, 17 mM Mg(OAc)2, and 50 mM Tris-HCl, pH 7.5. Reactions were initiated with 10 μM of the substrate. All reactions were performed in duplicate. Procedures for the purification and expression of pETB and mutant enzymes have been described elsewhere (13). Protein concentrations were determined with an assay kit supplied by Bio-Rad using γ-globulin to construct a standard curve.

Kinetic Characterization of Wild-type AS-B and AS-B Mutants—Affinity constants (Km(app)) for AS-B substrates were determined by incubating purified wild-type or mutant AS-B in reaction mixtures (total volume 160 μl) in which all but one of the substrates were saturating, i.e., at approximately 10 times their Km(app) value, unless otherwise noted. The highest aspartate concentration used in any of the assays was 100 mM. Enzyme activity assays were performed until the initial velocities were reached. Determination of secondary and tertiary structures of proteins was performed to determine the effect of substrate concentration for both wild-type and mutant enzymes. The initial velocity of each reaction was determined spectropho-to.
Aspartate Activation by Asparagine Synthetase

The codes for oligonucleotides synthesis are as follows: a, 91% A, 3% T, 3% G, 3% C; c, 91% C, 3% T, 3% G, 3% A; g, 91% G, 3% T, 3% C, 3% A; *, 25% A, 25% T, 25% G, 25% C.

### Table I

**Oligonucleotides used in construction of site-directed mutants of AS-B**

| Mutation       | Oligo number | Oligonucleotide sequence                      |
|----------------|--------------|---------------------------------------------|
| N terminus     | SS85         | 5’ A GCT TCC CAT ATG TGT TCA ATT TTT GGC GTA TTC GAT 3’ |
| C terminus     | SS82         | 5’ CGC TTT GTT GGC ACG CGC GCA GTC 3’           |
| kpn site       | SS148        | 5’ AAC CAT CGT GCT ACC GTC GTG CAT CAC 3’        |
| Random 1       | SS123        | 5’ gas acT Tat GaT gTg acc acT att cgc gG TACa ccg aTg TAT TTA 3’ |
| Random 2       | SS124        | 5’ gcc TCG TCC Tac aac acc GCA GGT CAC 3’         |
| R325X          | SS195        | 5’ CAT CGG TGT CGA CGC *** AAT AGT GAT 3’         |
| T322Y          | SS196        | 5’ CAT CGG TGT CGA CGC AAT AGT ATA CAC 3’         |
| T323X          | SS197        | 5’ CAT CGG TGT CGA CGC AAT *** GAT 3’             |
| T322V          | SS351        | 5’ CAT CGG TGT CGA CGC AAT AGT GAC CAC 3’         |
| T322S          | SS352        | 5’ CAT CGG TGT CGA CGC AAT AGT GAT GCA CAC 3’     |
| R325A          | SS353        | 5’ CAT CGG TGT CGA CGC AAT GAT AGT GGC CAC 3’    |
| R325K          | SS354        | 5’ CAT CGG TGT CGA CGC AAT AAC GAT GGC CAC 3’    |
| V321A          | SS355        | 5’ CAT CGG TGT CGA CGC AAT AGT AGT GAT GGC CAC 3’ |
| D320A          | SS356        | 5’ CAT CGG TGT CGA CGC AAT AGT GAT GGC CAC 3’    |
| Y319A          | SS360        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| T323V          | SS357        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| T323S          | SS358        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| T322A          | SS359        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| R325D          | SS361        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| Y319F          | SS362        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| E317A          | SS363        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |
| E317Q          | SS370        | 5’ CAT CGG TGT CGA CGC AAT GAT GAT GGC CAC 3’    |

### Table II

**Activity of the AS-B mutants obtained using directed random mutagenesis**

| Mutant              | AS activity (+/-) | Mutant              | AS activity (+/-) |
|---------------------|-------------------|---------------------|-------------------|
| Mutants with changes in the region defined by residues 317–330 |
| E317V               | +                 | E317V: P329A         | -                 |
| Y219F               | +                 | Y219F: Y232I         | -                 |
| E317V               | +                 | E317V: R322P         | -                 |
| T322P               | -                 | T322P: M330I         | -                 |
| T322M               | -                 | T322M: T323N         | -                 |
| T322S               | +                 | T322S: T322S; T323P  | -                 |
| T328S               | +                 | T328S: D320A:T323A   | -                 |
| Mutants with changes in the region defined by residues 484–500 |
| F485L               | +                 | F485L: T489K         | +                 |
| F485C               | +                 | F485C: E500K         | +                 |
| F486R               | +                 | F486R: E500K         | +                 |
| F487C               | +                 | F487C: E500K         | +                 |
| F489S               | +                 | F489S: E500K         | +                 |
| T491S               | +                 | T491S: E500K         | +                 |
| E494Q               | +                 | E494Q: E500K         | +                 |
| F485L.S492C         | +                 | F485L.S492C          | +                 |
| F486L.N488D         | +                 | F486L.N488D          | +                 |

RESULTS

**Preliminary Functional Evaluation of C-terminal Segments 317–330 and 484–500.**—An initial evaluation of the functional importance of amino acid residues located in regions 317–330 and 484–500 was carried out using “directed random mutagenesis.” Oligonucleotides SS123 and SS124 (Table I), having a calculated average of two mismatches per oligonucleotide, were used to create the two sets of mutations. Twelve independent clones containing mutations in region 317–330 and 17 independent clones for region 484–500 were evaluated (Table II). Each of these clones was characterized by sequence determination of the inserts and measurement of glutaminase and asparagine synthetase activities in extracts of the expressed protein. Although random mutagenesis of region 484–500 gave eight double mutants, two triple mutants, and seven single mutants, representing a variety of conservative replacements (T489S, S492T, and F485L) and changes in local charge and conformational modifications (P486L:T489K, R484D:E500K), all of the associated proteins retained their ability to catalyze asparagine synthesis (Table II). We therefore conclude that residues 484–500 are not involved in direct catalysis and/or substrate binding.

In contrast, of the six mutant enzymes associated with single mutations in region 317–330, three were unable to synthesize asparagine, and of the six mutants containing multiple alterations in their sequence, none had detectable AS activity (Table II). All of these mutants were soluble and retained the potential ability to catalyze glutamine hydrolysis (data not shown), suggesting that these conformational changes were not responsible for the significant loss of synthetase activity. Three AS-B single mutants retaining synthetase activity (Y319F, T322S, and T328S) were then purified according to standard procedures (13), and kinetic constants for their glutaminase (Table III) and synthetase activity were measured.
Aspartate Activation by Asparagine Synthetase

Kinetic constants for the glutaminase activity of wild-type AS-B and AS-B mutants

All initial rates were determined measuring glutamate production in a coupled assay.

| Mutant     | Glutamine + 5 mM ATP | Glutamine only |
|------------|----------------------|----------------|
|            | $K_m$ ($\mu$M) | $k_{cat}$ ($\mu$M s$^{-1}$) | $k_{cat}/K_m$ | $K_m$ ($\mu$M) | $k_{cat}$ ($\mu$M s$^{-1}$) | $k_{cat}/K_m$ |
| wt AS      | 1.29 ± 0.07         | 1.38 ± 0.02    | 993            | 1.94 ± 0.18     | 0.80 ± 0.03    | 412             |
| E317A      | 6.12 ± 0.54         | 0.59 ± 0.01    | 96             | 8.44 ± 0.48     | 0.64 ± 0.01    | 76              |
| E317Q      | 2.60 ± 0.07         | 0.18 ± 0.004   | 69             | 4.01 ± 0.35     | 0.18 ± 0.004   | 45              |
| T318A      | 1.04 ± 0.08         | 1.30 ± 0.03    | 1250           | 1.45 ± 0.17     | 1.23 ± 0.05    | 848             |
| Y319A      | 1.15 ± 0.10         | 1.24 ± 0.03    | 1078           | 1.23 ± 0.21     | 0.38 ± 0.02    | 309             |
| T323F      | 1.03 ± 0.09         | 1.0 ± 0.02     | 968            | 1.90 ± 0.10     | 0.67 ± 0.02    | 556             |
| D328A      | 1.98 ± 0.18         | 1.49 ± 0.05    | 788            | 3.14 ± 0.33     | 1.35 ± 0.06    | 430             |
| V321A      | 1.11 ± 0.06         | 1.16 ± 0.02    | 1045           | 1.51 ± 0.27     | 1.06 ± 0.06    | 702             |
| T322A      | 1.34 ± 0.08         | 1.49 ± 0.03    | 1112           | 1.39 ± 0.19     | 0.97 ± 0.04    | 698             |
| T322S      | 1.23 ± 0.08         | 1.60 ± 0.03    | 1301           | 1.42 ± 0.16     | 1.12 ± 0.04    | 789             |
| T322V      | 1.21 ± 0.07         | 1.72 ± 0.03    | 1421           | 1.78 ± 0.22     | 1.34 ± 0.06    | 753             |
| T322Y      | 0.90 ± 0.07         | 0.57 ± 0.01    | 633            | 1.11 ± 0.10     | 0.44 ± 0.01    | 396             |
| T323A      | 0.98 ± 0.05         | 0.86 ± 0.01    | 878            | 1.38 ± 0.08     | 0.77 ± 0.01    | 558             |
| T323I      | 0.71 ± 0.04         | 0.74 ± 0.01    | 1042           | 0.95 ± 0.07     | 0.54 ± 0.01    | 568             |
| T323L      | 0.94 ± 0.09         | 0.79 ± 0.02    | 840            | 1.57 ± 0.20     | 0.80 ± 0.03    | 510             |
| T323S      | 0.84 ± 0.11         | 0.74 ± 0.03    | 881            | 1.64 ± 0.16     | 0.69 ± 0.02    | 610             |
| T323V      | 1.14 ± 0.10         | 1.11 ± 0.03    | 974            | 1.97 ± 0.19     | 0.94 ± 0.04    | 686             |
| R325A      | 1.59 ± 0.25         | 0.77 ± 0.04    | 484            | 2.02 ± 0.16     | 0.84 ± 0.02    | 333             |
| R325K      | 0.91 ± 0.05         | 0.71 ± 0.01    | 780            | 1.43 ± 0.14     | 0.70 ± 0.02    | 490             |
| T322S      | 0.92 ± 0.04         | 0.71 ± 0.01    | 772            | 2.18 ± 0.26     | 0.49 ± 0.02    | 225             |

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Aspartate Activation by Asparagine Synthetase

**Table IV**  
Kinetic constants for the glutamine-dependent synthetase activity of wild-type AS-B and the AS-B mutants

| Mutant       | Glutamate | Aspartate | ATP |
|--------------|-----------|-----------|-----|
|              | $k_{in}$  | $k_{cat}$ | $k_{cat}/K_m$ |
|              | $s^{-1}$  | $s^{-1}$  | $s^{-1}$ |
|              | $mM$      | $mM$      | $mM$ |
| wt AS-B      | 0.89 ± 0.07 | 1.01 ± 0.05 | 74.4 ± 1.9 |
| E317A        | 3.47 ± 0.42 | 0.55 ± 0.02 | 1.5 ± 0.05 |
| E317Q        | 4.67 ± 0.50 | 0.15 ± 0.01 | 1.7 ± 0.05 |
| T318A        | 0.25 ± 0.02 | 0.25 ± 0.003 | 757.60 ± 0.54 |
| T319A        | 0.04 ± 0.07 | 0.04 ± 0.03 | 12.09 ± 0.08 |
| T320A        | 0.67 ± 0.09 | 0.25 ± 0.01 | 375.20 ± 0.18 |
| V321A        | 0.57 ± 0.03 | 0.74 ± 0.02 | 1298 ± 3.2 |
| T322A        | 0.43 ± 0.08 | 0.14 ± 0.01 | 330 ± 2.1 |
| T322S        | 0.19 ± 0.03 | 0.13 ± 0.01 | 684 ± 1.2 |
| T322V        | 0.18 ± 0.03 | 0.045 ± 0.002 | 575 ± 2.8 |
| T323A        | 0.09 ± 0.07 | 0.022 ± 0.001 | 128 ± 0.54 |
| T323I        | 0.50 ± 0.02 | 0.240 ± 0.004 | 480 ± 15.6 |
| T323L        | 0.41 ± 0.03 | 0.25 ± 0.01 | 3511 ± 20.1 |
| T323S        | 0.30 ± 0.02 | 0.47 ± 0.01 | 566 ± 3.50 |
| D320A        | 0.50 ± 0.02 | 0.240 ± 0.004 | 480 ± 15.6 |
| Y319A        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319F        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319H        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319I        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319K        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319L        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319T        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |
| Y319Q        | 0.12 ± 0.002 | 14 | 73.8 ± 19.6 |

* Kinetic constants for this mutant were determined using an end point assay.

**Table V**  
Kinetic constants for the ammonia-dependent synthetase activity of wild-type AS-B, AS-B mutants

| Mutant       | Glutamate | Aspartate | ATP |
|--------------|-----------|-----------|-----|
|              | $k_{in}$  | $k_{cat}$ | $k_{cat}/K_m$ |
|              | $s^{-1}$  | $s^{-1}$  | $s^{-1}$ |
|              | $mM$      | $mM$      | $mM$ |
| wt AS-B      | 12.0 ± 0.8 | 0.69 ± 0.02 | 58 |
| E317A        | 4.5 ± 0.43 | 0.69 ± 0.02 | 153 |
| E317Q        | 8.5 ± 0.43 | 0.69 ± 0.02 | 153 |
| T318A        | 10.17 ± 1.05 | 0.26 ± 0.01 | 26 |
| Y319A        | 17.4 ± 1.4 | 0.56 ± 0.02 | 32 |
| Y319F        | 8.14 ± 1.18 | 0.72 ± 0.04 | 88 |
| D320A        | 16.6 ± 1.6 | 0.20 ± 0.01 | 12 |
| V321A        | 22.6 ± 1.9 | 0.85 ± 0.04 | 38 |
| T322A        | 13.4 ± 2.2 | 0.080 ± 0.006 | 6 |
| T322S        | 11.1 ± 1.8 | 0.16 ± 0.01 | 14 |
| T322V        | 4.6 ± 0.07 | 0.040 ± 0.001 | 8.9 |
| Y323A        | 2.60 ± 0.05 | 0.16 ± 0.001 | 27 |
| Y323I        | 23.3 ± 1.3 | 0.36 ± 0.008 | 15.4 |
| Y323L        | 12.05 ± 1.70 | 0.21 ± 0.008 | 17 |
| Y323S        | 9.13 ± 0.86 | 0.56 ± 0.02 | 81 |
| Y323V        | 12.08 ± 0.69 | 0.38 ± 0.01 | 31 |
| Y323S        | 9.4 ± 1.3 | 0.47 ± 0.02 | 50 |

using this assay were unchanged from our experiments using the standard assay, within experimental error (data not shown).

**Kinetic Characterization and Chemical Rescue of Arg-325 AS-B Mutants**—Using random mutagenesis methods, Arg-325 was changed to lysine, histidine, isoleucine, leucine, threonine, and glutamine to yield a series of AS-B mutants that exhibited no detectable synthetase activity, but glutaminase activity was unaltered. The catalytic role of this residue was then probed using chemical rescue experiments involving the R325A, and the conservatively altered R325K AS-B mutants, both of which possessed unaltered glutaminase activity (in the presence and absence of ATP) when compared with wild-type AS-B (Table III). In the presence of saturating substrates, methyamine, urea, thiourea, GdmHCl, methylguanidine, or tetramethylguanidine were added to wild-type AS-B, R325A, and R325K at varying concentrations (0.5–50 mM). The addition of 50 mM GdmHCl to the R325A AS-B mutant restored synthetase activity (150 mM s⁻¹/mg), to a level of about 15% that of wild-type AS-B. Chemical rescue saturated at approximately 50 mM GdmHCl, whether the aspartate concentration was 50 or 200 mM (Fig. 2), with no further activation at concentrations up to 150 mM GdmHCl (data not shown). Plots of initial velocity versus either glutamine or ATP concentration in 50 mM GdmHCl showed normal Michaelis-Menten behavior, and the $K_m$ (app) values for ATP and glutamine were similar to those of wild-type AS-B. In contrast, aspartate did not show saturation kinetics, the plot of initial velocity versus aspartate concentration remaining linear up to concentrations of 200 mM regardless of the concentration of GdmHCl in the reaction mixture (5–50 mM) (Fig. 3). Addition of methylguanidine also restored the enzymatic activity of the R325A AS-B mutant, albeit at a much lower rate. To establish whether restoration of R325A activity was a specific effect, we examined the behavior of the AS-B mutant R49A, in which Arg-49 is replaced by alanine. Arg-49 is known to be involved in glutamine recognition and binding, and mutation of this GAT-domain residue severely diminishes glutamine-dependent synthetase activity. No stimulation of the R49A AS-B synthetase activity was detected at any concentration of GdmHCl (data not shown). Kinetic constants were

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3. S. K. Boehlein, unpublished observations.
then determined for the glutamine-dependent synthetase reaction catalyzed by R325A in the presence of 50 mM GdmHCl (Table VII). No concentration of any of these exogenous agents affected glutamine-dependent synthetase activity in either wild-type AS-B or the R325K AS-B mutant.

Alanine Scanning Mutagenesis of Other Conserved Residues in AS-B Residues 317–330—To investigate the roles of other conserved amino acids in the region defined by residues 317–330, single-site AS-B mutants were expressed in which these residues were systematically replaced by alanine. After mutagenesis, sequencing, and enzyme purification, kinetic constants for the resulting AS-B mutants were determined (Tables III–V). Replacement of Glu-317 by alanine resulted in a large change in the kinetic parameters associated with the glutaminase activity of this AS-B mutant. $K_m^{\text{app}}$ for glutamine, in the absence of ATP, was increased almost 5-fold, and ATP stimulation of hydrolysis activity was lost in the E317A AS-B mutant.

In the case of the T318A AS-B mutant, the kinetic parameters were consistent with altered aspartate binding. Thus, a 10–12-fold increase in $K_m^{\text{app}}$ for aspartate was seen when either glutamine or ammonia were used as the nitrogen source (see Tables IV and V). The $k_{cat}$ was decreased approximately 3-fold, and the specificity constant $k_{cat}/K_m^{\text{app}}$ was decreased approximately 30–40-fold. The kinetic constants obtained when varying glutamine, ATP, or ammonia are very similar to that of wild-type AS-B. For T318A, $K_m^{\text{app}}$ for aspartate (6.6 mM) and the apparent CSA $K_I$ (90 mM) gave a $K_I^{\text{(CSA)}}/K_m^{\text{app}}$ (Asp) of 13.6.

Replacement of Tyr-319 by either alanine or phenylalanine caused only minor alterations in the kinetic parameters for both the glutamine-dependent and ammonia-dependent AS reactions (Tables IV and V). The $K_I$ (CSA)/$K_m^{\text{app}}$ (Asp) ratio was about 8, indicating that both aspartate and CSA bind similarly to these mutants and also that aspartate binding was not altered by the mutation.

Substitution of alanine for Asp-320 gave the D320A AS-B mutant for which $k_{cat}$ was decreased from 4- to 6-fold in both glutamine- and ammonia-dependent asparagine synthetase reactions. The associated specificity constant ($k_{cat}/K_m^{\text{app}}$) was lowered 14–22-fold since $K_m^{\text{app}}$ for aspartate was increased 4-fold. In contrast to the modest increase in this kinetic parameter, the $K_I$ for CSA became greater than 150 mM (Table VI).

Mutation of Val-321 to alanine caused a 4-fold increase in $K_m^{\text{app}}$ for aspartate in the synthetase activity using any nitrogen source. In addition, there was a 4-fold increase in the $K_I$ for CSA, consistent with the hypothesis that the change in $K_m^{\text{app}}$ of aspartate reflects perturbed aspartate binding (Table VI).

**DISCUSSION**

Although the detailed mechanism of nitrogen transfer from glutamine remains to be defined for class II amidotransferases, especially in the light of recent kinetic isotope effect measurements (15), ATP-dependent aspartate activation has been dem-

**TABLE VI**

|                  | $K_m^{\text{app}}$ | $K_I^{\text{(CSA)}}/K_m^{\text{app}}$ (Asp) |
|------------------|--------------------|---------------------------------------------|
| wt AS-B          | 5.4                | 7.9                                         |
| T318A            | 90                 | 13.6                                        |
| Y319A            | 8.4                | 7.9                                         |
| Y319F            | 6.7                | 8.2                                         |
| D320A            | 159.3              | 61.3                                        |
| V321A            | 21.7               | 7                                           |
| T322A            | 41.5               | 19.8                                        |
| T322S            | 15.6               | 13                                          |
| T323S            | 64                 | 19                                          |
| T328S            | 5.9                | 10.7                                        |

* CSA inhibition was determined at approximately twice the aspartate concentration for each mutant.

* Refers to the aspartate value for glutamine-dependent asparagine synthesis.

**Fig. 2.** Kinetic behavior of the R325A AS-B mutant at various fixed concentrations of GdmHCl. Aspartate concentrations were varied from 50 to 200 mM, at the fixed GdmHCl concentrations indicated on the figure. In all experiments, the concentrations of glutamine, ATP, and Mg$^{2+}$, in 100 mM Tris-HCl, pH 8, were maintained at 10, 2.5, and 8 mM, respectively. Each initial velocity represents the average value determined from two parallel experiments.

**Fig. 3.** Chemical rescue of the R325A AS-B mutant by GdmHCl at various fixed concentrations of aspartate. GdmHCl concentrations were varied from 0 to 50 mM, at the fixed aspartate concentrations indicated on the figure. In all experiments, the concentrations of glutamine, ATP, and Mg$^{2+}$, in 100 mM Tris-HCl, pH 8, were maintained at 10, 2.5, and 8 mM, respectively. Each initial velocity represents the average value determined from two parallel experiments.
Aspartate Activation by Asparagine Synthetase

No saturation of activity was observed for aspartate at concentrations of up to 200 mM.

| Glutamine | ATP |
|-----------|-----|
| $K_{m}$  | $k_{cat}$ | $k_{cat}/K_{m}$ | $K_{m}$ | $k_{cat}$ | $k_{cat}/K_{m}$ |
| mM  | s$^{-1}$ | s$^{-1}$ | mM  | s$^{-1}$ | s$^{-1}$ |
| wt AS-B$^a$ | 0.69 ± 0.07 | 1.01 ± 0.05 | 1463 | 0.15 ± 0.01 | 1.10 ± 0.03 | 6111 |
| R325A | 0.58 ± 0.66 | 0.16 ± 0.006 | 282 | 0.39 ± 0.05 | 0.225 ± 0.007 | 577 |

$^a$ Included from Table IV for ease of comparison.

| Glutamine | ATP |
|-----------|-----|
| $K_{m}$  | $k_{cat}$ | $k_{cat}/K_{m}$ | $K_{m}$ | $k_{cat}$ | $k_{cat}/K_{m}$ |
| mM  | s$^{-1}$ | s$^{-1}$ | mM  | s$^{-1}$ | s$^{-1}$ |
| wt AS-B$^a$ | 0.69 ± 0.07 | 1.01 ± 0.05 | 1463 | 0.15 ± 0.01 | 1.10 ± 0.03 | 6111 |
| R325A | 0.58 ± 0.66 | 0.16 ± 0.006 | 282 | 0.39 ± 0.05 | 0.225 ± 0.007 | 577 |

$^a$ Included from Table IV for ease of comparison.

onstrated to proceed via the formation of β-aspartyl-AMP for a number of asparagine synthetases (Fig. 4) (5, 18). Thus, the β-carboxylate anion of aspartate reacts with the α-phosphorus of ATP generating a pentacovalent intermediate (I). Release of pyrophosphate then gives the desired intermediate that can undergo attack by nucleophilic nitrogen to give asparagine and AMP (Fig. 4). In forming (I), however, a number of chemical problems must be overcome by the synthetase domain of the enzyme. First, the resonance stabilized carboxylate, which is ordinarily a poor nucleophile, must be activated. Second, the α-phosphorus must be made more electrophilic, and the transition state leading to the formation of the pentacovalent intermediate must be stabilized.

No previous studies have clearly identified the amino acids involved in mediating aspartate binding and/or activation in asparagine synthesis. In an effort to identify catalytically important residues in the synthetase domain of AS-B, two regions that were highly conserved in all known asparagine synthetases were chosen for alteration using random mutagenesis methods (Fig. 1). Of these two regions, numerous single and double mutants in residues 484–500 showed insignificant effects on the specific activity of the enzyme, indicating that this region was not directly involved in either substrate binding or catalysis. In sharp contrast, preliminary mutagenesis experiments revealed that even conservative changes to several residues in region 317–330 gave mutants possessing no detectable synthetase activity without affecting glutaminase activity or the effect of ATP on the glutaminase activity.

The specific functional roles of conserved amino acids in this region were therefore investigated using a combination of alanine scanning and site-directed mutagenesis. Before being subject to detailed kinetic analysis, each AS-B mutant had to meet several criteria demonstrating its overall structural integrity. First, mutant enzymes had to be soluble. Furthermore, in the absence of a crystal structure for AS-B, we utilized the unique characteristics of the two separable reactions catalyzed by all asparagine synthetases; the synthetase activity of site-specific mutants was fully characterized only if the mutant AS-B exhibited essentially unaltered kinetic parameters for glutaminase activity, in the presence and absence of ATP, relative to wild-type enzyme. If these criteria were met, then we assumed that changes in activity due to site-specific replacements arose from local effects. We note that, of all the AS-B mutants constructed in this study, only E317A and E317Q exhibited diminished glutaminase activity, suggesting that mutation of this residue had caused large structural changes in the enzyme. It is also possible that this residue may function in inter-domain communication, linking the synthetase and the GAT domain through hydrogen bonding or salt-bridge interactions. Consequently, its mutation resulted in perturbation of both the glutaminase and synthetase activities. Validation of this hypothesis, however, awaits detailed structural characterization of AS-B.

After extensive kinetic analysis of a large number of site-directed AS-B mutants, it was clear that several mutations resulted in considerable increases in $K_{m(app)}$ for aspartate in both glutamine- and ammonia-dependent asparagine synthesis. To verify that these increases in the Michaelis constant reflected, at least in part, a decreased ability of the enzyme to bind aspartate, we examined whether CSA binding to each AS-B mutant was similarly reduced. In previous studies, we have shown that CSA is a competitive inhibitor with respect to only aspartate and no other AS-B substrate (26). With the one notable exception of the D320A AS-B mutant, we observed a good correlation between increases in $K_{m(app)}$ for aspartate and the loss of the ability of CSA to inhibit asparagine synthesis (Table VI). This behavior supports the idea that mutations raising $K_{m(app)}$ for aspartate reflect an involvement in aspartate recognition and binding by the cognate residue in the wild-type enzyme. In experiments employing conformationally constrained amino acids, we have demonstrated that in the bound conformation of aspartate, all of the ionizable groups are located on one face of the substrate (26). It is therefore possible that the side chains of Thr-318 and Val-321 are positioned to interact with the hydrophobic face of the bound aspartate. In the case of the D320A AS-B mutant, we observed that while $K_{m(app)}$ for aspartate was only moderately increased, the apparent $K_{i}$ for CSA was greater than 150 mM (Table VI), giving a $K_{i}$ (CSA)/$K_{m(app)}$ (Asp) ratio of approximately 61. Hence, the D320A AS-B mutant appears, in contrast to wild-type AS-B, to be able to distinguish structural differences in the carboxylate and sulfonate groups of aspartate and CSA, respectively.

Having tentatively identified residues participating primarily in aspartate binding (Thr-318 and Val-321), we next sought those that had a catalytic function in the mechanism of β-aspartyl-AMP formation. The observations that site-specific mutations of Thr-322 mostly affected the turnover number of the synthetase activity, while changes to Thr-323 caused both a decrease in $k_{cat}$ and an increase in $K_{m(app)}$ for aspartate, were therefore noteworthy. Our results suggest that Thr-323 is most likely involved in aspartate binding, given that changes in both kinetic parameters for aspartate appeared to reflect the size and shape of the amino acid replacing threonine in any given AS-B mutant, whereas Thr-322 is important in catalysis.

Having tentatively identified residues participating primarily in aspartate binding (Thr-318 and Val-321), we next sought those that had a catalytic function in the mechanism of β-aspartyl-AMP formation. The observations that site-specific mutations of Thr-322 mostly affected the turnover number of the synthetase activity, while changes to Thr-323 caused both a decrease in $k_{cat}$ and an increase in $K_{m(app)}$ for aspartate, were therefore noteworthy. Our results suggest that Thr-323 is most likely involved in aspartate binding, given that changes in both kinetic parameters for aspartate appeared to reflect the size and shape of the amino acid replacing threonine in any given AS-B mutant, whereas Thr-322 is important in catalysis. Hence, no substitution for Thr-322 significantly affected $K_{m(app)}$ for aspartate with the exception of tyrosine. The alterations to both kinetic parameters by the latter, however, were most likely the result of structural perturbations due to the size of the tyrosine side chain. Furthermore, mutation of Thr-322 also caused an increased affinity for ATP in the T322A, T322S, and T322V AS-B mutants relative to wild-type enzyme, as reflected by the $K_{m(app)}$ values for ATP (Tables IV and V). That $K_{i}$ for ATP was decreased in these mutants was confirmed by determining the ability of ATP to protect against FSBA inactivation of these three AS-B mutants (see "Results"). Energy released on ATP binding to wild-type AS-B may therefore result in a conformational change facilitating either aspartate binding or the formation of β-aspartyl-AMP. Although our data suggest that some structural conservation of the Thr-322 side chain is required for aspartate binding, the $K_{i}$ for aspar-
tate was only increased 10-fold for the T322V AS-B mutant. We
determined that the ATPase and synthetase activities of the
mutant enzymes were not uncoupled since the amounts of
pyrophosphate and asparagine formed were equal in independ-
ent assays.

Our mutagenesis experiments also showed that Arg-325 was
absolutely critical for both glutamine- and ammonia-dependent
asparagine formation. The catalytic role of this residue was
therefore probed by investigating chemical rescue of the R325A
and the conservatively altered R325K, AS-B mutants. Both of
these mutants met our criteria for structural integrity. Inter-
pretation of these experiments was aided by the growing liter-
ature on the rescue of catalytic activity by addition of exoge-
 nous compounds to inactive or impaired enzyme mutants (28–
34). For example, exogenous amines rescue an aspartate
aminotransferase mutant in which a critical lysine residue is
replaced by alanine (28). The only direct precedent for our
studies came from experiments in which the activity of car-
boxypeptidase A mutants lacking Arg-127 was rescued by the
addition of several guanidine derivatives (29). In these exper-
iments, the addition of the rescuing agent restored $k_{\text{cat}}$ without
affecting $K_m^{\text{(app)}}$, an observation consistent with the hypothe-
sis that the guanidine side chain of Arg-127 stabilizes the
rate-limiting transition state for peptide hydrolysis (35). For the
R325A AS-B mutant, 50 mM GdmHCl restored 15% of the
synthetase activity of the wild-type enzyme. Rescue of activity
was specific in that methylamine or other guanidine and urea
derivatives failed to restore activity to any significant level.

In another series of experiments on MetRS, Tyr-258 was re-
estudied in our experiments. Thus, Arg-325 may play two functional roles in AS-B, being in-
involved either in binding aspartate in a catalytically competent
manner or in stabilization of the transition state leading to a
pentacovalent intermediate (I). Many enzymes employ the gua-
idine group for substrate binding (37–49), and if this were the
function of Arg-325, then our failure to observe saturation of
the rescued enzyme by aspartate might result from an insuffi-
cient amount of properly oriented guanidinium ion in the active
site at concentrations that do not denature the protein. On the
other hand, there is ample precedence in the literature for
participation of critical arginine residues in stabilizing transi-
tion states involving a nucleotide (50–59). For example, syn-
thesis of aminoacylated tRNAs by aminoacyl tRNA synthetases
proceeds via an $\alpha$-aminoacyl-AMP intermediate, which is
chemically similar to $\beta$-aspartyl-AMP. The availability of x-ray
crystal data for several class I and class II tRNA synthetases
has also established functional roles for numerous residues,
including conserved arginines.

Active site residues in methionyl tRNA synthetase (MetRS)
that mediate formation of $\alpha$-methionyl-AMP are similar to
those identified in the experiments reported here. For example,
Arg-322 in MetRS, which is conserved in one subgroup of 6
class I tRNA synthetases, appears critical in the activation of
the methionine $\alpha$-carboxylate. When this residue was changed
to a glutamate, the resulting R322Q MetRS mutant exhibited
a 60,000-fold decrease in $k_{\text{cat}}/K_m^{\text{(app)}}$ for ATP-PPi exchange (53).
In addition, a 25-fold increase in the $K_m^{\text{(app)}}$ for methionine
was observed with no change in the $K_m^{\text{(app)}}$ values for either
ATP or tRNA-Met. These results for the R322Q MetRS mutant
were consistent with the hypothesis that the mutation reduced
the stability of the methionyl-AMP intermediate by elimination
of important contacts with methionine in the transition state.

In another series of experiments on MetRS, Tyr-258 was re-
placed by alanine (60). Mutation of this residue led to a 2,000-
fold decrease in $k_{\text{cat}}$ for the ATP-PPi exchange reaction but had
no significant effect on the $K_m^{\text{(app)}}$ values for ATP or methio-
nine. The rate of pyrophosphorylation for the Y258A MetRS
mutant was also decreased upon the addition of PP$i^\text{ii}$ without
affecting $K_m^{\text{(app)}}$ for PP$i^\text{ii}$. This observation was interpreted to
suggest that Tyr-258 binds the $\alpha$-phosphate of ATP in the
transition state, enhancing the rate of methionyl-AMP forma-
tion.

The effects of these changes in the active site of MetRS are
strikingly similar to those observed for the AS-B mutations
studied in our experiments. Thus, $K_m^{\text{(app)}}$ values for both glu-
tamline and ATP in asparaginyl synthesis by the rescued R325A
mutant were similar to those for wild-type AS-B, and satura-

FIG. 4. Mechanism for formation of $\beta$-aspartyl-AMP. Nucleophilic attack of the side chain carboxylate on bound aspartate on the
$\alpha$-phosphorus of ATP initially yields a pentacovalent intermediate (I). Release of pyrophosphate from (I) then yields $\beta$-aspartyl-AMP.
tion behavior for aspartate, the substrate requiring activation, was not observed under our experimental conditions. Arg-325 therefore appears to play a role in AS-B similar to that of the active site arginine in MetRS. Furthermore, the kinetic behavior of the Thr-322 AS-B mutants paralleled those observed for changes to Tyr-358 in MetRS in that $k_{\text{cat}}$ values for all substrates were unchanged compared with wild-type AS-B, whereas $k_{\text{cat}}$ was significantly decreased in both glutamine and ammonia-dependent asparagine synthesis. Hence, Thr-322 might also interact with the $\alpha$-phosphate of ATP in the transition state leading to pentacovalent intermediate (I) and, subsequently, $\beta$-aspartyl-AMP. Given the similarities in the kinetic behavior of the mutants of these two enzymes, it will be of interest to determine if there are structural relationships between asparagine synthetases and tRNA synthetases.

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Mutagenesis and Chemical Rescue Indicate Residues Involved in β-Aspartyl-AMP Formation by *Escherichia coli* Asparagine Synthetase B
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