Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3249–3253, June 1980
Biochemistry

Isolation and preliminary characterization of single amino acid substitution mutants of aspartate carbamoyltransferase

(allosteric enzyme/2-aminopurine mutagenesis/*pyrB* gene/nonsense suppression)

EVAN R. KANTROWITZ, JEFFERSON FOOTE, HAROLD W. REED, AND LESLIE A. VENSEL

Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167

Communicated by William N. Lipscomb, March 13, 1980

ABSTRACT **In order to isolate functional *Escherichia coli* aspartate carbamoyltransferase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) with single amino acid replacements, a series of *pyrB* nonsense mutants has been isolated. These nonsense mutants were induced by 2-aminopurine mutagenesis and selected by a combination of antibiotic treatments, direct enzyme assays, and suppressibility tests. Suppression of the *pyrB* nonsense mutation with various suppressors, which insert different amino acids, has resulted in the formation of a series of mutant aspartate carbamoyltransferases, each differing in one amino acid from the wild-type enzyme. After partial purification, kinetic studies revealed that some of the mutant enzymes had altered homotropic and heterotropic interactions. The mutants that had a tyrosine insert showed the most pronounced changes, followed by those with a serine insert. The mutants having a glutamine insert, however, were indistinguishable from the wild-type enzyme, supporting the conclusion that, because of the specificity of the mutagen, the glutamine insert had regenerated the wild-type enzyme.**

In the study of the interrelationship between the structure and function of a protein, the most instrumental development has been the advances in x-ray crystallography which permitted the determination of protein structures to atomic, or near atomic, resolution. However, even the information obtained from x-ray crystallographic analysis is not sufficient to fully understand how a particular protein or enzyme functions. For example, in order to propose a molecular mechanism for hemoglobin's allosteric interactions (1) it was necessary to combine the x-ray structures of the oxy- and deoxy- forms of the protein with information obtained from mutant hemoglobin molecules containing single amino acid replacements (2). For hemoglobin the isolation of these naturally occurring single amino acid substitution mutants was straightforward. However, in order to determine similar structure–function relationships for other proteins and enzymes a simple method must be available for the production of these single amino acid substitution mutants.

Of particular interest are mutants in which the function of an enzyme has been modified but not obliterated. Because these mutants still retain enzymatic activity, their isolation from bacterial sources has been difficult or impossible by standard bacteriological techniques. However, Miller and his associates have been able to produce a series of *Escherichia coli lac* repressor proteins with specific amino acid substitutions at selected points by using a combination of nonsense mutations and nonsense suppressors (3, 4). We have now used this method to produce mutant enzymes that retain enzymatic activity but that contain different amino acids substituted at a single location in the enzyme's primary structure.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

With the availability of the x-ray structure of *E. coli* aspartate carbamoyltransferase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) (5), the production and characterization of single amino acid substitution mutants is a logical next step for promoting the understanding of this important enzyme. This allosteric enzyme catalyzes the committed step in pyrimidine biosynthesis: the reaction between carbamoylphosphate and L-aspartate to form carbamoylaspartate and phosphate (6, 7). The 300,000-dalton aspartate carbamoyltransferase dodecamer is made up of two 100,000-dalton catalytic subunits, each composed of three identical catalytic chains, and three 33,000-dalton regulatory subunits, each composed of two regulatory chains (8–10). The saturation curves for both substrates are sigmoidal (11, 12), a manifestation of cooperative interactions between the enzyme's subunits. This enzyme is controlled via feedback inhibition by CTP and is allosterically activated by ATP, the product of the parallel purine biosynthesis pathway (11, 13). The regulatory dimer binds the heterotropic effectors but possesses no enzymatic activity, whereas the catalytic subunit has three active sites but is insensitive to the allosteric effectors. These various features have led aspartate carbamoyltransferase to become one of the most studied allosteric enzymes (14, 15).

Here we report a method for the production of aspartate carbamoyltransferase molecules with single amino acid substitutions. These mutant enzymes have been obtained from *E. coli* that have had a nonsense mutation in the *pyrB* gene, the gene coding for aspartate carbamoyltransferase, suppressed by a series of nonsense suppressors. The mutant aspartate carbamoyltransferase molecules have been characterized in terms of their kinetic properties and compared to the native enzyme.

EXPERIMENTAL PROCEDURES

Materials. ATP, CTP, carbamoylphosphate, succinate, L-aspartate, and cycloserine were purchased from Sigma. Ultrapure, enzyme-grade ammonium sulfate and 2-aminopurine were purchased from Schwarz/Mann and Vega Biochemicals (Tucson, AZ), respectively. Ampicillin hydrate was a gift of Bristol Laboratories (Syracuse, NY). Carbamoylphosphate was purified as described (11).

Enzyme Activity. Two methods were used for measuring transferase activity. The first was a pH-stat assay (16) and the second was a colorimetric assay based on the "Method II" procedure of Prescott and Jones (17) with modifications similar to those described (18). All assays were carried out at 25°C. Standard pH-stat assays were performed at 4.8 mM carbamoylphosphate and 30 mM L-aspartate.

Bacterial Strains. The various bacterial strains used in this work are listed in Table 1. Strains were either constructed in this laboratory or obtained through the *E. coli* Genetic Stock Center, Yale University.

Table 1. Bacterial strains used

Strain*	Sex	Genetic markers†	Source‡
KL185	F⁻	*thi,pyrD,his,trp,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	K. B. Low/CGSC 4222
EK012	F⁻	"Wild-type"	CGSC 5073
EK017	F⁻	*thi,his,trp,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	P1 transduction with EK012 as donor
EK110	F⁻	*thi,pyrB110,his,trp,galK,mtl,xyl,malA,rspL*,λ^r,λ^-	This work
EK117	F⁻	*thi,pyrB117,his,trp,galK,mtl,xyl,malA,rpsL*,$\lambda^r\lambda^-$	This work
CA165	Hfr	*thi,relA,supB*	S. Brenner/CGSC 4980
CA167	Hfr	*thi,relA,supC*	S. Brenner/CGSC 5401
WH-1	F′	*his,trp,phoA,recA,rpsL*,Val^r/F′*his*$^+$,*supD*	Wilhelm & Hoffman/CGSC 4612
AB3590	F⁻	*thi,his,trp*,Δ*ilv,mtl,malA,rpsL,ton,tsx*,λ^r	D. Duggan/CGSC 3590
EK045	F⁻	*thi,his,trp*,Δ*ilv,mtl,malA,rpsL,ton,tsx*,Nal^r,λ^r	This work
EK046	F′	*thi,his,trp*,Δ*ilv,mtl,malA,rpsL,ton,tsx*,Nal^r,λ^r/F′*his*$^+$*supD*	EK045 × WH-1
EK110B	F⁻	*thi,pyrB110,supB,his,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	EK110 × CA165
EK110C	F⁻	*thi,pyrB110,supC,his,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	EK110 × CA167
EK110D	F′	*thi,pyrB110,his,trp,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-/F′*his*$^+$*supD*	EK110 × EK046
EK117B	F⁻	*thi,pyrB117,supB,his,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	EK117 × CA165
EK117C	F⁻	*thi,pyrB117,supC,his,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-	EK117 × CA167
EK117D	F′	*thi,pyrB117,his,trp,galK,mtl,xyl,malA,rpsL*,λ^r,λ^-/F′*his*$^+$*supD*	EK117 × ED046

* All strains are *E. coli* K-12.
† See ref. 19.
‡ CGSC is the *E. coli* Genetic Stock Center, Yale University.

Mutagenesis and Selection for Uracil-Requiring Mutants. EK017, a suppressor-free strain, was the parent of all uracil-requiring mutants prepared in this study. Strain EK017 was mutagenized with 2-aminopurine as follows. Dilutions of overnight cultures containing 100 cells per ml were innoculated into separate tubes containing 600 μg of 2-aminopurine per ml (20). More than 600 cultures were prepared. These were grown to saturation at 37°C in uracil-containing medium. The cultures then underwent two successive ampicillin/cycloserine selections (20), after which samples of the cultures were plated out on minimal M9 medium plates (20) containing 0.6 μg of uracil per ml. On these plates uracil-requiring colonies grew very slowly. The slowly growing colonies were tested for their uracil requirement, purified, and tested again. Only those colonies that showed no growth whatsoever on uracil-negative plates were saved. Uracil-negative colonies were grown to saturation in minimal medium plus uracil and stored at −20°C in 40% (vol/vol) glycerol. To ensure that each mutant was of independent origin, we saved only one mutant from any one original 2-aminopurine culture.

Segregation of *pyrB* Mutants. Each uracil-requiring mutant was tested to determine if functional aspartate carbamoyltransferase was present. Five-milliliter cultures of the various mutants were grown in medium containing 12 μg of uracil per ml. Under these conditions derepression of the pyrimidine pathway occurred after about 12 hr. The cultures were allowed to grow for a full 24 hr before they were sonicated, centrifuged to remove cell debris, and assayed for aspartate carbamoyltransferase activity. Cultures that showed no detectable aspartate carbamoyltransferase activity were considered *pyrB* mutants.

Isolation of *pyrB* Nonsense Strains and Suppression of Nonsense Codon. *pyrB* nonsense strains were selected and nonsense suppressed strains were created by Hfr crosses with various nonsense suppressor strains or by episome transfers (see Table 1).

Growth of Cells and Partial Purification of Aspartate Carbamoyltransferase. *E. coli* strain EK017 and nonsense suppressed versions of EK017 were grown in M9 medium (20) supplemented with thiamine, tryptophan, and 1.5% Casamino acids. Cultures were grown to a density of about 8×10^8 cells per ml at 37°C with aeration in 0.5- to 6-liter batches. Cells were pelleted by centrifugation at 10,000 × *g* for 15 min and the pellet was then suspended in 0.1 M Tris acetate buffer (pH 9.2) and lysed by sonication. Debris was removed by centrifugation at 10,000 × *g* for 15 min. Aspartate carbamoyltransferase was partially purified by the combination of a heat step and ammonium sulfate fractionation (21). The partially purified enzyme was exhaustively dialyzed into 0.1 M Tris acetate/1 mM 2-mercaptoethanol, pH 8.3. No loss of activity was observed over the course of 1 month.

RESULTS AND DISCUSSION

Aspartate carbamoyltransferase with a single amino acid substitution was produced by suppression of a nonsense codon in the *E. coli pyrB* gene. The *pyrB* nonsense strains were produced by mutagenesis of the *E. coli* K-12 strain EK017 by use of 2-aminopurine. This mutagen was selected because of its specificity in causing only A·T ⇌ G·C base transitions (22), which will result only in the generation of a nonsense codon from either a tryptophan or a glutamine codon. The parent strain, EK017, was derived from the suppressor-free strain KL185 by phage P1 generalized transduction by using a lysate of wild-type *E. coli* K-12.

After mutagenesis with 2-aminopurine, uracil-requiring pyrimidine pathway mutants were selected by two successive ampicillin/cycloserine treatments, followed by direct aspartate carbamoyltransferase assays to segregate the *pyrB* mutants. *pyrB* nonsense mutants were detected by replica plate mating (23) with CA167 or replica plating onto a lawn of phage φ80 carrying *supF* (or both). The actual construction of the suppressed *pyrB* nonsense strains was accomplished by appropriate matings or Hfr crosses in liquid culture (see Table 1).

These mutant aspartate carbamoyltransferase enzymes were initially characterized by comparison of their kinetic behavior to that of the wild-type enzyme. Aspartate carbamoyltransferase prepared in parallel from the parent strain EK017 was therefore used as control for evaluation of kinetic data obtained for the mutant enzymes. With the suppression of the nonsense codon in the *pyrB* gene, the newly formed strains no longer required pyrimidines for growth. Under these circumstances, the normal procedure (21) for production of large quantities of enzyme by a derepression mechanism was impossible. We therefore chose to perform initial characterizations of the mutants with partially purified enzyme.

Comparisons between mutant and wild-type enzyme kinetics

were made by aspartate saturation curves in the presence and absence of a fixed concentration of the allosteric activator ATP or the allosteric inhibitor CTP. Additional comparisons included determination of the maximal activation or inhibition of the allosteric effectors by means of effector concentration dependence data. With the partially purified enzymes, their respective specific activities could not be compared. These comparisons must await the purification of milligram quantities of these mutant aspartate carbamoyltransferases.

In order to verify that the partially purified enzyme preparations exhibited the same kinetic behavior as the pure enzyme, aspartate saturation curves were determined in the presence and absence of the allosteric effectors for the partially purified enzyme isolated from EK017, the wild-type parent of all suppressed *pyrB* nonsense strains. Fig. 1 shows the sigmoidal aspartate saturation curve of partially purified EK017 aspartate carbamoyltransferase along with the saturation curves in the presence of the allosteric activator ATP and the allosteric inhibitor CTP. As expected, ATP shifts the saturation curve to lower K_m whereas CTP shifts the saturation curve to higher K_m. These curves are almost identical to curves obtained for the purified enzyme.

The insertion of a single tyrosine residue into a position previously occupied by a tryptophan or glutamine residue results in drastic changes in the enzyme's kinetic properties. For example, when the *pyrB* nonsense codon of strain EK117 is suppressed with *supC* (tyrosine insert), a functionally active aspartate carbamoyltransferase is formed. Kinetic data for this mutant enzyme (EK117C) are shown in Fig. 2. The enzyme's homotropic interactions were markedly reduced and the K_m shifted from 12 to 20 mM; in addition, both the activation by ATP and the inhibition by CTP were substantially reduced at the same concentrations of effectors as used for the control (see Fig. 1).

Enzyme isolated from strain EK110C again shows the effect that a single tyrosine insertion can have on the kinetic properties of aspartate carbamoyltransferase (Fig. 3). If the nonsense codon in EK110 is suppressed with *supB* (glutamine insert) or *supD* (serine insert), the mutant enzymes are much less altered kinetically (Fig. 3). In the primary sequence of the catalytic chain of aspartate carbamoyltransferase there are 15 glutamine residues and 2 tryptophan residues; hence a nonsense mutation has an 88% chance of arising from a glutamine codon. We would expect that suppression of a *pyrB* nonsense mutant with a glutamine suppressor should have a high probability of restoring the enzyme to its wild-type form. Enzyme from strain EK110B, which has a *pyrB* nonsense codon suppressed with *supB*, exhibits kinetic properties identical to wild-type enzyme (see Fig. 4). As seen in Fig. 3, when this same nonsense codon is suppressed with *supD* there is a shift in the partially purified enzyme's K_m, although the extent of homotropic cooperativity is about the same as for the wild-type enzyme. Likewise, when the *pyrB* nonsense codon of strain EK117 is suppressed with *supB*, the kinetic properties are restored to that observed for the wild-type enzyme (Fig. 4). Suppression of EK117 with *supD* shifts the aspartate saturation curve only slightly, to a higher K_m (Fig. 5). The aspartate saturation curves for mutant enzymes EK110C and EK110D are significantly different from those of EK117C and EK117D, respectively (compare Figs. 3

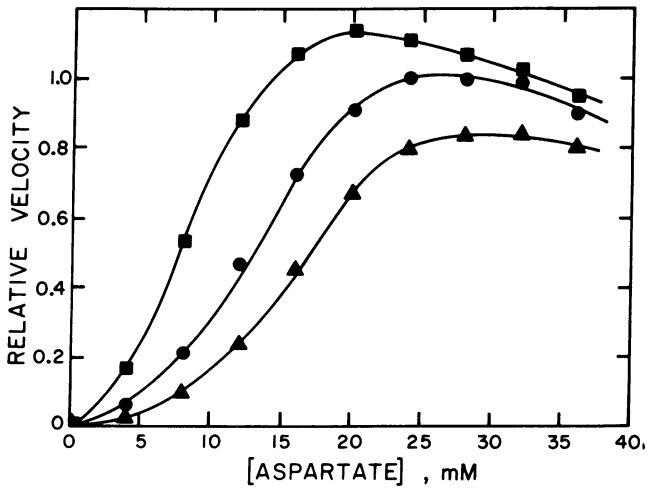

FIG. 1. Aspartate saturation curves of aspartate carbamoyltransferase partially purified from strain EK017. The enzymatic activity was determined by the colorimetric assay in 0.1 M Tris acetate buffer (pH 8.3) in the presence of 4.8 mM carbamoylphosphate. Relative velocities are normalized to the highest measured velocity obtained without effectors. ●, EK017 aspartate carbamoyltransferase alone; ■, with 4 mM ATP; ▲, with 0.3 mM CTP.

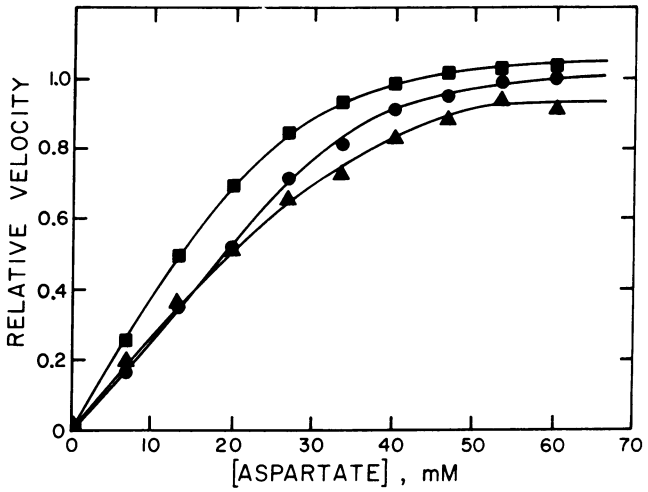

FIG. 2. Aspartate saturation curves of aspartate carbamoyltransferase partially purified from strain EK117C. This strain was obtained by suppression of the *pyrB117* nonsense mutation with *supC*, which inserts tyrosine. Velocities were determined and reported as for Fig. 1. ●, EK117C aspartate carbamoyltransferase alone; ■, with 4 mM ATP; ▲, with 0.3 mM CTP.

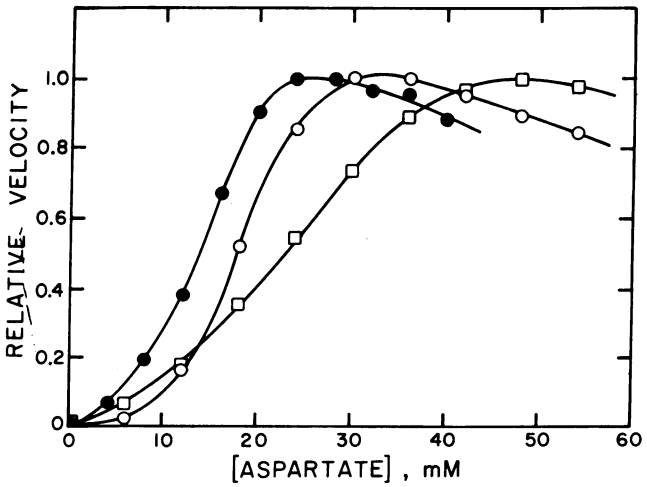

FIG. 3. Aspartate saturation curves of aspartate carbamoyltransferase partially purified from different suppressed versions of EK110. Enzyme purified from EK110B (●), EK110C (□), and EK110D (○) have the *pyrB110* nonsense mutation suppressed with *supB*, *supC*, and *supD*, respectively. Velocities were determined as for Fig. 1 and normalized to the highest value for each curve.

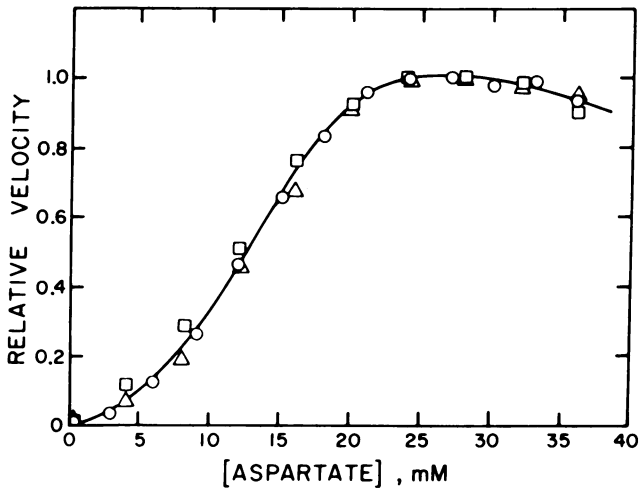

FIG. 4. Aspartate saturation curve of partially purified aspartate carbamoyltransferase. Enzyme was purified from the wild-type parent EK017 (○), EK110B (△), and EK117B (□). Strains EK110B and EK117B were formed by suppression of the respective nonsense mutations with *supB*. Velocities were determined as for Fig. 1 and normalized to the highest value for each curve.

and 5), furnishing evidence that the site of the original *pyrB* nonsense mutation is different in strains EK110 and EK117.

As a second method for characterizing the single amino acid substitution mutants, we examined their response to the allosteric activator ATP and the allosteric inhibitor CTP (Fig. 6). The maximal activation of enzymes partially purified from strains EK017 and EK110B were identical, along with the concentration dependence of the activation effect. This agrees with our previous results which suggest that aspartate carbamoyltransferase of EK110B is indistinguishable from that of EK017 by virtue of the fact that *pyrB110* nonsense codon suppressed with *supB* would most likely regenerate the wild-type enzyme. On the other hand, when the *pyrB* nonsense codon of EK110 is suppressed with either *supC* or *supD*, the maximal activation by ATP changes and, at least for EK110D, there is an alteration in the concentration dependence of the activation. The extent of inhibition observed with CTP is also affected by the nature of the inserted amino acid (data not shown).

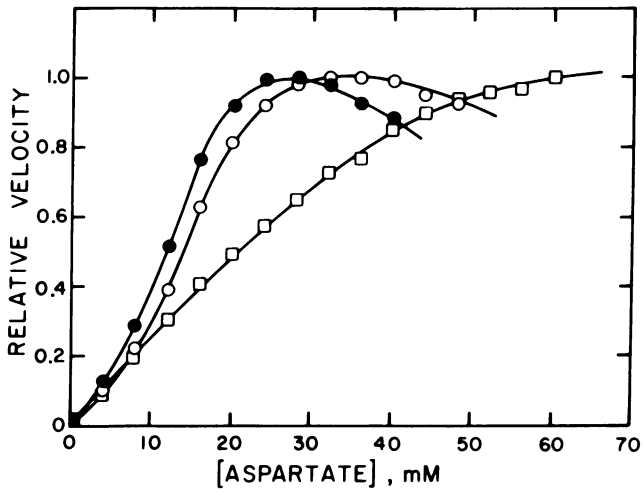

FIG. 5. Aspartate saturation curves of aspartate carbamoyltransferase partially purified from different suppressed versions of EK117. Enzymes purified from EK117B (●), EK117C (□), and EK117D (○) have the *pyrB117* nonsense mutation suppressed with *supB*, *supC*, and *supD*, respectively. Velocities were determined as for Fig. 1 and normalized to the highest value for each curve.

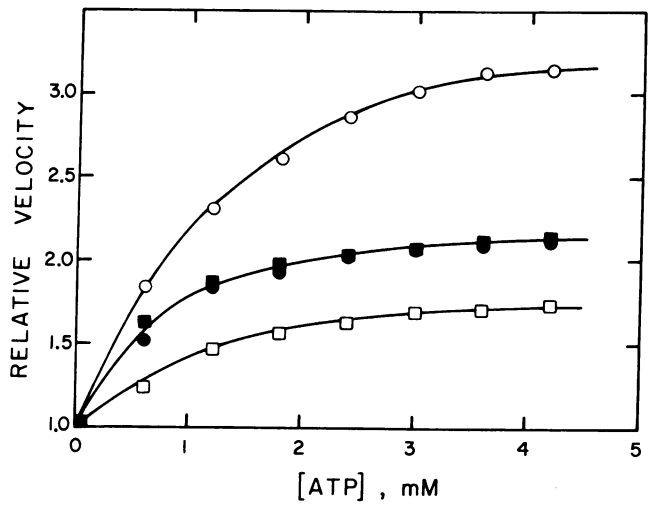

FIG. 6. Activation of aspartate carbamoyltransferase by ATP. Represented is the enzyme from EK017 and different suppressed versions of EK110. Enzyme was partially purified from EK017 (■), EK110B (●), EK110C (□), and EK110D (○). The enzymatic activity was determined by the colorimetric assay in 0.1 M Tris acetate buffer (pH 8.3) in the presence of 4.8 mM carbamoylphosphate and 15 mM L-aspartate. Velocities are reported relative to velocity observed with no ATP present.

Any *pyrB* mutants isolated by this procedure must necessarily be in the enzyme's catalytic chain because the catalytic chain of aspartate carbamoyltransferase is transcribed before the regulatory chain (24). If the mutation occurred in the regulatory chain, then active catalytic trimers would still be produced, resulting in a strain that was not a uracil auxotroph and therefore would not be selected for by our procedure.

With the availability of the high-resolution x-ray structure of aspartate carbamoyltransferase (5), the explanation of why a particular enzyme modification affects a particular enzyme function can now be approached in a meaningful and quantitative way. The genetic modification method that we have outlined here has a number of important advantages over other methods of protein modification. First, the amino acid replacement is at only one unique site on the catalytic chain of the enzyme; second, because of the specificity of the suppressors used, the inserted amino acid is known; third, because of the mutagen used the site of the substitution can be only at glutamine or at tryptophan sites; fourth, the inserted amino acid can be varied to some degree; and fifth, the mutants produced are enzymatically active, which aids in their characterization. One disadvantage to this approach, however, is that the isolation of the original nonsense mutants is difficult, especially in a gene such as *pyrB* for which there is no direct method for mutant selection.

The results that we report here conclusively show that it is feasible to produce single amino acid substitution mutants of aspartate carbamoyltransferase and, furthermore, that it is possible to insert a number of different amino acids into the same location in the enzyme's primary structure. For both nonsense mutants reported here the substitution of tyrosine has a much more drastic effect on the kinetic properties of the partially purified enzymes than does the insertion of serine. However, even more interesting is that the enzyme's homotropic and heterotropic interactions are exceedingly sensitive to changes in even a single amino acid. The complete analysis of these mutant enzymes and the correlation of the resulting data to the enzyme's three-dimensional structure should provide information vital to an understanding of the allosteric interactions of aspartate carbamoyltransferase. Only by means of

such molecular level perturbations of the enzyme's structure can the allosteric phenomenon of aspartate carbamoyltransferase be understood.

We thank Dr. B. Bachmann of the *E. coli* Genetic Stock Center for her helpful discussions and for supplying many of the *E. coli* strains needed for this work. We also express gratitude to R. Brent for supplying the P1 phage, to D. Freifelder for the $\phi 80$ phage, and to K. Abatzis, R. Ferraro, D. Browinski, and T. Connolly for isolating the hundreds of uracil-requiring mutants. Finally, we acknowledge support of the National Institutes of Health through Grant GM-26237.

1. Perutz, M. F. (1970) *Nature (London)* **228,** 726–739.
2. Perutz, M. F. & Lehmann, H. (1968) *Nature (London)* **219,** 902–909.
3. Kolata, G. B. (1976) *Science* **191,** 373.
4. Miller, J. H., Coulondre, C., Schmeissner, U., Schmitz, A., Hofer, M. & Galas, M. in *Nonsense Mutations and tRNA Suppressors,* eds. Celis, J. E. & Smith, J. D. (Academic, New York), pp. 127–132.
5. Monaco, H. L., Crawford, J. C. & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5276–5280.
6. Jones, M. E., Spector, L. & Lipmann, F. (1955) *J. Am. Chem. Soc.* **77,** 819–820.
7. Reichard, P. & Hanshoff, G. (1956) *Acta Chem. Scand.* **10,** 548–566.
8. Weber, K. (1968) *Nature (London)* **218,** 1116–1119.
9. Wiley, D. C. & Lipscomb, W. N. (1968) *Nature (London)* **218,** 1119–1121.
10. Meighen, E. A., Pigiet, V. & Schachman, H. K. (1970) *Proc. Natl. Acad. Sci. USA* **65,** 234–241.
11. Gerhart, J. C. & Pardee, A. B. (1962) *J. Biol. Chem.* **237,** 891–896.
12. Bethell, M. R., Smith, K. E., White, J. S. & Jones, M. E. (1968) *Proc. Natl. Acad. Sci. USA* **60,** 1442–1449.
13. Yates, R. A. & Pardee, A. B. (1956) *J. Biol. Chem.* **221,** 757–770.
14. Gerhart, J. C. (1970) *Curr. Top. Cell. Regul.* **2,** 275–325.
15. Jacobson, G. R. & Stark, G. R. (1973) in *The Enzymes,* ed. Boyer, P. D. (Academic, New York), Vol. 9, pp. 225–308.
16. Kantrowitz, E. R. & Lipscomb, W. N. (1976) *J. Biol. Chem.* **251,** 2688–2695.
17. Prescott, L. M. & Jones, M. E. (1969) *Anal. Biochem.* **32,** 408–419.
18. Pastra-Landis, S. C., Evans, D. R. & Lipscomb, W. N. (1978) *J. Biol. Chem.* **253,** 4624–4630.
19. Bachmann, B., Low, K. B. & Taylor, A. L. (1976) *Bacteriol. Rev.* **40,** 116–167.
20. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
21. Gerhart, J. C. & Holoubek, H. (1967) *J. Biol. Chem.* **242,** 2886–2892.
22. Drake, J. W. (1970) *The Molecular Basis of Mutation,* (Holden-Day, San Francisco), pp. 161–176.
23. Low, K. B. (1973) *J. Bacteriol.* **113,** 798–812.
24. Perbal, B. & Hervé, G. (1972) *J. Mol. Biol.* **70,** 511–529.