Superproduction and Rapid Purification of Escherichia coli Aspartate Transcarbamylase and Its Catalytic Subunit under Extreme Derepression of the Pyrimidine Pathway*

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A strain of Escherichia coli has been constructed which greatly overproduces the enzyme aspartate transcarbamylase. This strain has a deletion in the pyrB region of the chromosome and also carries a leaky mutation in pyrF. Although this strain is a pyrimidine auxotroph, it will grow very slowly without pyrimidines if a plasmid containing the pyrB gene is introduced into it. Derepression occurs when this strain exhausts its uracil supply during exponential growth. Under extreme derepression, aspartate transcarbamylase can account for as much as 60% of the total cellular protein. This host strain/plasmid system can be utilized for the rapid purification of wild-type aspartate transcarbamylase or plasmid-born mutant versions of the enzyme. This system is particularly well-suited for analysis of the latter since the control of overproduction resides exclusively on the bacterial chromosome. Therefore, any plasmid bearing the pyrB operon can be made to overproduce aspartate transcarbamylase in this host strain. Based on this system, a rapid purification procedure has been developed for E. coli aspartate transcarbamylase. The purification scheme involves an ammonium sulfate fractionation followed by a single precipitation of the enzyme at its isoelectric point. In a similar fashion, this strain can also be employed to produce exclusively the catalytic subunit of the enzyme if the plasmid only carries the pyrB gene. This system may be adapted to overproduce other proteins as well by using this host strain and the strong pyrB promoter linked to another gene.

Because of recent advances in recombinant DNA technology, such as site-directed mutagenesis (Zoller and Smith, 1982), it is now relatively easy to isolate mutant versions of enzymes with single amino acid substitutions. These techniques are allowing enzymologists to use these mutant enzymes as powerful tools to investigate the mechanism of catalysis and allosterism (Dalbadie-McFarland et al., 1982; Winter et al., 1982). In order to characterize these mutant enzymes, it would be of great value to be able to rapidly purify them. As part of our analysis of mutant versions of Escherichia coli aspartate transcarbamylase, we have developed a new E. coli strain/plasmid system which will superproduce aspartate transcarbamylase. As a direct consequence of the high levels of enzyme in this system, a new purification procedure has been devised which is simple and extremely rapid.

E. coli aspartate transcarbamylase catalyzes the committed step in pyrimidine biosynthesis: the reaction of aspartate and carbamyl phosphate to form carbamyl aspartate and phosphate. This enzyme contributes to the control of the rate of the pyrimidine pathway by a combination of genetic, metabolic, and allosteric means. Beckwith et al. (1962) found that aspartate transcarbamylase can be derepressed 100- to 400-fold. Although a p-independent attenuation mechanism has been proposed (Root et al., 1982; Navre and Schachman, 1983; Turnbough et al., 1983) to account for this genetic control, by comparison to other related systems it is unlikely that attenuation alone can account for this level of regulation (Bertrand and Yanofsky, 1976; Jackson and Yanofsky, 1973). Metabolically, aspartate transcarbamylase is inhibited by CTP, the end-product of the pyrimidine pathway (Gerhart and Schachman, 1965), and is activated by ATP, the product of the parallel purine pathway (Gerhart and Schachman, 1965; Yates and Pardee, 1956). Finally, aspartate transcarbamylase exhibits homotropic cooperativity with both of its substrates, carbamyl phosphate and aspartate (Gerhart and Pardee, 1962; Bethell et al., 1968).

E. coli aspartate transcarbamylase is composed of twelve polypeptides of two types. The larger 33,000 molecular weight chain is the product of the pyrB gene, while the smaller 17,000 molecular weight chain is the product of the pyrI gene. Three of the larger or catalytic chains spontaneously combine to form a catalytic trimer and two smaller or regulatory chains combine to form a regulatory dimer (Burns and Schachman, 1982a; Burns and Schachman, 1982b). These subunits then spontaneously assemble to form the holoenzyme which is composed of two catalytic subunits and three regulatory subunits (Rosenbusch and Weber, 1971; Foltermann et al., 1984). The pyrB and pyrI genes are contiguous on the E. coli chromosome at 96 min on the genetic map (Bachmann, 1983; Pauza et al., 1982). The regulatory subunits which are necessary for the allosteric interactions of the enzyme are not required for catalytic activity since all the enzymatic activity resides on the catalytic subunit. Strains which have the pyrI gene deleted are not pyrimidine auxotrophs since they produce functional catalytic trimers (Foltermann et al., 1984).

Gerhart and Holoubek (1967) were able to purify large amounts of aspartate transcarbamylase from an E. coli strain which contained a leaky mutation in the pyrF gene. When this strain was grown in medium containing limited amounts of pyrimidines, derepression occurred when the pyrimidine supply was exhausted. Using this strain, approximately 30 mg

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of crude enzyme was obtained per liter of culture. These authors were able to obtain approximately 15 mg of pure enzyme/liter of culture by a seven-step purification procedure employing one column chromatography step.

The purification of mutant versions of aspartate transcarbamylase has proven to be difficult (West et al., 1985), especially if activity is reduced or abolished. Here, we report the development of a new strain of _E. coli_ which overproduces aspartate transcarbamylase. Furthermore, this strain will overproduce either wild-type or a mutant aspartate transcarbamylase as long as the pyrBI operon is carried on a plasmid, since control for the overproduction of the enzyme is located on the bacterial chromosome. To insure that the overproduced enzyme is the exclusive product of the pyrB operon carried on the plasmid, _pyrB_ has been deleted from the chromosome. The yield of enzyme from this strain is approximately 10-20-fold higher than the original Gerhart and Holoubek strain (Gerhart and Holoubek, 1967). This high level of expression has made possible a new simple purification of both the wild-type and mutant versions of the enzyme without any column chromatography.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbamyl phosphate, L-aspartate, agar, ascorbate, ampicillin, potassium dihydrogen phosphate, and carbamyl L-aspartate were obtained from Sigma. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann, and casamino acids were purchased from Difco. The casamino acids were tested to verify the absence of sufficient tryptophan and uracil to support the growth of tryptophan and pyrimidine auxotrophs, respectively. Restriction endonucleases were obtained from either Bethesda Research Laboratories or New England Biolabs and used according to the supplier's recommendations. Carbamyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at -20°C (Gerhart and Pardee, 1962).

**Enzyme Activity**—The transcarbamylase activity was measured at 25°C and pH 8.3 by either a colorimetric (Pastra-Landis et al., 1981) or pH-stat method (Wu and Hammes, 1973). pH-stat assays were carried out with a Radiometer TT180 titrator and an ABU 80 auto-burette. Enzymatic assays were performed at 4.8 mM carbamyl phosphate and 30 mM aspartate.

**Bacterial Strains**—The various bacterial strains used in this work are listed in Table I. All bacteria growth was carried out at 37°C. The _E. coli_ strain U39a, obtained from J. Wild, Texas A & M University, was used to construct the overproducing strain.

**Preparation of Bacterial Extracts**—Bacteria were grown overnight to an absorbance of approximately 1.50 at 560 nm in M9 medium (Miller, 1972) supplemented with 0.5% casamino acids and the appropriate amounts of uracil. The bacterial culture was centrifuged at 10,000 x g for 10 min, resuspended in 0.01 M Tris-acetate, pH 8.5, to one-fifth the original volume, and sonicated for 3 min using a Branson sonicator (Model 200) equipped with a microtip. The disrupted cells were centrifuged for 25 min at 20,000 x _g_ and the supernatant was used directly for the pH-stat assay.

| Strain | Source |
|--------|--------|
| EK005  | J. C. Gerhart |
| EK017  | This laboratory |
| EK1102 | This work |
| EK1104 | This work |
| KL701  | K. B. Low, CGSC 4256* |
| U39a   | J. Wild |

* Cell strains are _E. coli_ K12.

**RESULTS**

**Construction of pEK2**—Plasmid pEK2, which carries the _pyrB_ operon in pUC8, was created by restricting pUC8 and pPBlh04 (Roef et al., 1982) with _PstI_ and _SalI_ followed by ligation. The ligated mixture was then transformed into competent U39a cells (Maniatis et al., 1982) followed by selection on M9 medium plates supplemented with 0.5% casamino acids and 40 µg/ml ampicillin. Rapid plasmid purification (Maniatis et al., 1982) was performed on a number of candidates. A plasmid was selected which exhibited the proper size, as judged by ascorbic gel electrophoresis as well as the appropriate restriction pattern.

**Construction of pEK1**—A substantial portion of the gene for the regulatory chain of aspartate transcarbamylase (pyrF) was deleted from pEK2 to generate pEK1 which produces only the catalytic subunit of the enzyme. This was accomplished by first restricting pEK2 into three fragments with _BglII_ and _BamHI_. The largest fragment, 4.5 kilobases, is comprised of the pUC8 vector, the entire _pyrF_ gene, and its control region, along with a fragment corresponding to approximately one-third of the _pyrF_ gene. This fragment, with complementary ends, was ligated upon itself followed by transfection into U39a. A number of colonies were selected that were Amp+ and _Ura*. The candidate plasmids from these strains were isolated and one was chosen which had the proper size and restriction pattern. This plasmid, pEK1, was then transformed into EK1104 to create a strain which would overproduce only the catalytic subunit of aspartate transcarbamylase.

**Construction of pEK2**—Plasmid pEK2 was identical to pEK2 except that the DNA sequence in the region corresponding to tryptophan 209 of the catalytic chain was converted to a tyrosine codon by site-directed mutagenesis. Details of the construction of this plasmid will be described elsewhere.

**Construction of EK1102 (ΔpyrB, trp)**—Strain U39a was subjected to methanesulfonic acid ethyl ester mutagenesis (Miller, 1972), followed by two selective ampicillin/cycloserine selections (Kantrowitz et al., 1981) in M9 medium supplemented with 0.5% casamino acids and 30 µg/ml uracil. After the selections, _Trp_ auxotrophs were detected as minute colonies on M9 medium plates supplemented with 0.5% casamino acids, 30 µg/ml uracil, and 0.8 µg/ml tryptophan. Introduction of episome F′123, which carries the entire _trpABCDE_ operon, confirmed that the _Trp_ mutation was in the region of the chromosome which could be cotransduced with _pyrF_.

**Construction of EK1104 (ΔpyrB, _pyrF* )**—In order to overproduce aspartate transcarbamylase from a plasmid carrying the _pyrBI_ gene, a strain was constructed which had both a deletion in the _pyrB_ gene and also carried the leaky _pyrF_ (_pyrF*) defect originally employed by Gerhart and Holoubek (1967). The construction of this strain was accomplished by introducing this _pyrF* _ allele into EK1102 by _F_1 transduction and selection for _Trp*. A number of _Trp*, _Ura* candidates

**TABLE I**

| Strain* | Sex | Genetic markers* | Source |
|---------|-----|------------------|--------|
| EK005   | F− | his, _pyrF* | J. C. Gerhart |
| EK017   | F− | his, _trp, galK, mtl, xyl, malA, _rpsL_ | This laboratory |
| EK1102  | F− | ara, _trp, galK, _ΔpyrB_ | This work |
| EK1104  | F− | ara, _ΔpyrB, _ΔpyrF*_, _rpsL_ | This work |
| KL701   | F− | _pyrD_, _trp, his, recA, _thr, galK, malA, xyl, _rpsL, _ΔpyrF*_, _rpsL_ | K. B. Low, CGSC 4256* |
| U39a    | F− | ara, _ΔpyrB, _ΔpyrF*, _rpsL_ | J. Wild |

* Cell strains are _E. coli_ K12.

See Bachmann, 1983.

CGSC is the _E. coli_ Genetic Stock Center, Yale University.
were then tested for the presence of pyrF* by transformation of competent cells with pEK2, a pUC8-based plasmid carrying the entire pyrBI operon. Colonies that exhibited Amp' were selected. Transformants with a pyrF* allele would grow normally on M9 medium plates supplemented with casamino acids (Trp-free) and ampicillin, while transformants with a pyrF* gene yielded extremely slow-growing colonies. The ratio of pyrF* to pyrF+ colonies was in agreement with the 44% cotransduction frequency found between pyrF and the nearby Trp operon (Bachmann, 1983; Wu, 1966).

**Expression of Aspartate Transcarbamylase**—The level of expression of aspartate transcarbamylase can be elevated greatly by derepression of the pathway (Gerhart and Holoubek, 1967). The original strain constructed for the overproduction of the enzyme utilized a leaky mutation in the pyrF gene along with a second copy of the pyrBI gene carried on an episome (Gerhart and Holoubek, 1967). For comparative purposes, we used strain EK005 which carries the same pyrF* allele but is not diploid for pyrBI. As shown in Table II, when strain EK005 is grown in the presence of 12 µg/ml uracil, there is a 28-fold increase in the level of aspartate transcarbamylase over a comparable strain which has no defects in the pyrimidine pathway (EK017). Under these conditions, the uracil is exhausted during late exponential growth phase, at which time the pathway undergoes derepression. If a plasmid which carries the pyrF* gene is inserted into a strain with no other pyrimidine pathway defects (U39a/pEK2), the level of the enzyme increases by a factor of 8. This increase is presumably due to the existence of multiple copies of the gene within the cell. The pyrB gene has been deleted from the chromosome in strain U39a; therefore, the aspartate transcarbamylase produced will be the exclusive gene-product of the pyrB gene carried on the plasmid.

By introducing the pyrF* allele into strain U39a, overproduction of aspartate transcarbamylase can occur under conditions of limited uracil. EK1104, a pyrimidine auxotroph, has the pyrF* allele in a U39a background. The introduction of pEK2 into this strain eliminates the pyrimidine requirement but this strain grows poorly without added pyrimidines due to the pyrF* defect. When this strain (EK1104/pEK2) is grown with limiting 12 µg/ml uracil, drastic overproduction of aspartate transcarbamylase occurs. The derepression causes a 35-fold increase in the level of aspartate transcarbamylase compared with U39a/pEK2. Under these conditions, approximately 60% of the cell protein is aspartate transcarbamylase (see Fig. 1).

**Expression of the Aspartate Transcarbamylase Catalytic Subunit**—Strain EK1104/pEK17 will produce only the catalytic subunit of aspartate transcarbamylase from the intact pyrB gene. As shown in Table II, the specific activity observed for EK1104/pEK17 is twice that observed for EK1104/pEK2. Gel electrophoresis, under non-denaturing conditions (Ornstein, 1964; Davis, 1964), of cell extracts from this strain shows no detectable holoenzyme.

**Effect of Uracil Concentration on Aspartate Transcarbamylase Levels**—The levels of aspartate transcarbamylase in these strains are variable depending on the uracil concentration. Fig. 2 compares the production of aspartate transcarbamylase from strains U39a and EK1104, both containing pEK2. When no uracil is present in the medium, EK1104/pEK2 produces almost no detectable aspartate transcarbamylase because this strain barely grows without uracil. Under these same conditions, U39a/pEK2 produces approximately 5 times as much enzyme as a "wild-type" strain. Derepression of the pathway occurs in these strains during exponential growth, if the initial uracil concentration is below approximately 20 µg/ml. The fraction of aspartate transcarbamylase to total cell protein increases dramatically at levels of uracil below 20 µg/ml. As seen in Fig. 2, if the concentration of uracil is increased above 20 µg/ml, the fraction of aspartate

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**Table II**

| Strain | Plasmid | Relevant genotype | [Uracil] | Specific activity* |
|--------|---------|------------------|---------|------------------|
|        |         |                  | µg/ml   |                  |
| U39a   | pEKO5   | ΔpyrBI           | 12      | 0                |
| EK104  | pEKO5   | ΔpyrBI           | 12      | 0                |
| EK017  | pEKO5   | ΔpyrBI           | 12      | 34               |
| EK005  | pEKO5   | ΔpyrBI*, pyrF*   | 12      | 600              |
| U39a   | pEKO2   | (pyrBI)         | 12      | 260              |
| EK104  | pEKO2   | (pyrBI)         | 12      | 9100             |
| U39a   | pEKO17  | (pyrB*)         | 12      | 450              |
| EK104  | pEKO17  | (pyrB*)         | 12      | 18200            |

*Specific activity is reported in units of µmol of carbamyl aspartate formed/h/mg of protein.

**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples during the three stages of the purification of wild-type aspartate transcarbamylase. The two major bands in each gel correspond to the 33,000 molecular weight catalytic and 17,000 molecular weight regulatory chains of aspartate transcarbamylase. Lane A is a sample of the supernatant after the sonication step; lane B is a sample after the 65% ammonium sulfate fractionation; and lane C is a sample of the final material after the pH 5.9 isoelectric precipitation. In each case, approximately 800 units of activity (µmol of carbamyl aspartate formed/h) were loaded onto the gel.
Superproduction of E. coli Aspartate Transcarbamylase

![Graph showing the effect of uracil concentration on levels of aspartate transcarbamylase produced.]

**FIG. 2.** Effect of uracil concentration on levels of aspartate transcarbamylase produced. The levels of aspartate transcarbamylase were measured in strains U39a and EK1104 both of which carried the pyrBI gene on pEK2. All cultures were inoculated at exactly the same density and grown for 20-22 h at 37 °C. The cell extract was prepared as described (see "Experimental Procedures") followed by pH-stat assays to determine aspartate transcarbamylase activity. Specific activity is reported in units of mmol of carbamyl aspartate/h/mg.

**FIG. 1**

**DISCUSSION**

With the development of *in vitro* mutagenesis techniques, multiple forms of a particular protein or enzyme with single amino acid substitutions can now be constructed readily (Zoller and Smith, 1982). Although the actual genetic manipulations often can be accomplished in a short period of time, the purification and biochemical characterization of these mutant proteins and enzymes is often very time consuming.

To analyze the catalytic and regulatory properties of aspartate transcarbamylase, we have begun to generate a series of single amino acid substitution mutants. In order to purify some of these mutants a rather complex purification scheme is often required (West *et al.*, 1985). By constructing a special strain of *E. coli* which overproduces this enzyme in large quantities, the purification is vastly simplified.

The strain EK1104, which carries a deletion in the pyrB region of the chromosome as well as a leaky pyrF allele, is ideal for the production of large quantities of aspartate transcarbamylase. Furthermore, the only way that this strain can produce aspartate transcarbamylase activity is if it carries a plasmid encoding the pyrB gene. Of particular significance is that this strain will overproduce aspartate transcarbamylase from any plasmid which carries the pyrBI gene, mutant or wild-type. Since the control of pyrBI expression is solely on the bacterial chromosome, no genetic manipulations of strain EK1104 have to be performed to cause overproduction of the aspartate transcarbamylase holoenzyme; this strain can be transformed with a plasmid which carries the pyrBI operon and then grown under limited uracil conditions. This system is therefore ideal for purifying mutant enzymes produced by *in vitro* mutagenesis techniques in which the mutation is introduced directly into the pyrB or pyrI genes carried on a plasmid.

The level of expression of aspartate transcarbamylase in this overproducing strain is exceptional. Under conditions of maximal derepression, aspartate transcarbamylase accounts for more than 60% of the total cell protein. Although this value seems exceedingly high, it is consistent with the enzyme levels expected in a strain which contains a multicopy plasmid under pyrimidine derepression. Strain EK005, which has a single copy of the pyrB gene on the chromosome, can also be

**TABLE IV**

Purification of mutant aspartate transcarbamylase from EK1104/pEK20

| Step                        | Total protein | Activitya | Specific activityb |
|-----------------------------|--------------|-----------|-------------------|
| Cell extract                | 198.7 mg     | 1.58      | 7950              |
| After 65% Ammonium sulfate  | 122.7 mg     | 1.14      | 9290              |
| After isoelectric Precipitation | 36.74 mg | 0.55      | 15000             |

a Activity is reported in units of mol of carbamyl aspartate formed/h.

b Specific activity is reported in units of μmol of carbamyl aspartate formed/h/mg of protein.

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**TABLE III**

Purification of aspartate transcarbamylase from EK1104/pEK2

| Step                        | Total protein | Activity | Specific activity |
|-----------------------------|--------------|----------|------------------|
| Cell extract                | 223.0 mg     | 2.13     | 9550             |
| After 65% Ammonium sulfate  | 149.5 mg     | 1.88     | 11200            |
| After isoelectric Precipitation | 65.6 mg | 0.94     | 14300            |

a Activity is reported in units of mol of carbamyl aspartate formed/h.

b Specific activity is reported in units of μmol of carbamyl aspartate formed/h/mg of protein.

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Purification of mutant aspartate transcarbamylase from EK1104/pEK20

| Step                        | Total protein | Activity | Specific activity |
|-----------------------------|--------------|----------|------------------|
| Cell extract                | 198.7 mg     | 1.58     | 7950             |
| After 65% Ammonium sulfate  | 122.7 mg     | 1.14     | 9290             |
| After isoelectric Precipitation | 36.74 mg | 0.55     | 15000            |

a Activity is reported in units of mol of carbamyl aspartate formed/h.

b Specific activity is reported in units of μmol of carbamyl aspartate formed/h/mg of protein.
Superproduction of *E. coli* Aspartate Transcarbamylase

derepressed in a similar fashion, although this strain exhibits 15-fold lower enzyme levels than EKI104/pEK2. The introduction of the plasmid alone accounts for a 15-fold increase in enzyme levels.

*E. coli* strains that have the *pyrI* gene deleted produce only active catalytic trimers (Foltermann et al., 1984); therefore, if a plasmid containing only the *pyrB* gene is introduced into EKI104, this strain should overproduce the catalytic subunit of the enzyme. To test this hypothesis, plasmid pEK17 was constructed. Approximately two-thirds of the proteins are concentrated by ammonium sulfate precipitation. The aspartate transcarbamylase is then purified by a single precipitation at its isoelectric point. Using this procedure, we have been able to obtain gram quantities of pure enzyme as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific activity and kinetic properties of the enzyme produced in this fashion are identical to that found for enzyme produced by the original procedure (Gerhart and Holoubek, 1967).

The overproducing strain that was constructed in this work was designed for use with both wild-type aspartate transcarbamylase as well as mutant versions of the enzyme in which a mutant *pyrB1* operon is carried on a plasmid. Using plasmid pEK20 which carries such a mutation, purification of a mutant version of aspartate transcarbamylase was accomplished (see Table IV). Although the purification of this particular mutant was carried out under exactly the same protocol as for the wild-type, mutant enzymes which have amino acid substitutions that affect the enzyme’s isoelectric point will require variations to this procedure. However, because of the very high expression of the mutant enzyme in the overproducing host strain, it is unlikely that significant problems would be encountered in the purification.

High expression induced by derepression of EKI104 may be utilized for production of other proteins if their structural genes were linked downstream from the *pyrB* control region. Therefore, this system is not restricted to the production of large quantities of aspartate transcarbamylase.

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