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Assembly of the Aspartate Transcarbamoylase Holoenzyme from Transcriptionally Independent Catalytic and Regulatory Cistrons

KAREN F. FOLTERMANN, MARK S. SHANLEY,† AND JAMES R. WILD*

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843

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The cistrons encoding the regulatory and catalytic polypeptides of aspartate transcarbamoylase (EC 2.1.3.2) from *Eschericha coli* K-12 have been cloned separately on plasmids from different incompatability groups. The catalytic cistron (*pyrB*) was carried by pACYC184 and expressed from its own promoter, whereas the regulatory cistron was expressed from the *lac po* of pBH20. The catalytic polypeptide chains assembled into enzymatically active trimers (c_3) in vivo when expressed in the absence of regulatory subunits. Similarly, the regulatory polypeptide chains assembled into regulatory dimers (r_2) in vivo in the absence of catalytic subunits. When cellular extracts containing regulatory dimers and catalytic trimers synthesized in separate cells were combined in vitro, partial spontaneous holoenzyme assembly occurred. When *pyrB* and *pyrI* were expressed from transcriptionally independent cistrons in the same cell, all detectable catalytic polypeptides were incorporated into the functional aspartate transcarbamoylase holoenzyme, $2(c_3):3(r_2)$. Thus, it is clear that the in vivo assembly of ATCase holoenzyme is a direct, spontaneous process involving the association of preformed regulatory subunits (r_2) and catalytic subunits (c_3). This procedure provides a general method for the construction of hybrid aspartate transcarbamoylase in vivo and may be applicable to other oligomeric enzymes constructed from different polypeptides.

Aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carbamoyltransferase; EC 2.1.3.2 [ATCase]) catalyzes the first step unique to de novo pyrimidine biosynthesis in *Escherichia coli*. The ATCase holoenzyme is a dodecamer of 310,000 g/mol and is composed of six identical catalytic and six identical regulatory polypeptide chains (34, 36). Three of the catalytic chains associate to form an enzymatically functional catalytic trimer (c_3) and two of the regulatory chains comprise the stable, zinc-dependent regulatory dimer (r_2). These subunits are associated in a $2(c_3):3(r_2)$ holoenzyme through an assembly process characterized by negative enthalpy and heat capacity changes with a positive entropy change in vitro under approximate physiological conditions (24). The holoenzyme possesses a variety of intricate protein-protein interactions (for example, each catalytic chain appears to be in contact with three other catalytic chains and two regulatory chains) (17). The heterotropic allosteric effects and the homotropic substrate characteristics of the enzyme are determined by these interactions (9, 18, 30, 38). It is possible to reversibly dissociate the holoenzyme into constituent subunits by exposure to mercurials such as *p*-chloromercuribenzoate (PCMB) or neohydrin (16, 37). The catalytic trimer maintains catalytic activity but possesses no regulatory properties (16). The regulatory dimer binds nucleotide effectors, CTP and ATP, with stoichiometries consistent with those of the holoenzyme (16, 31), but it has no catalytic activity. These subunits can be physically separated and then associated in vitro in the presence of zinc acetate and dithiothreitol to reform the holoenzyme (15). The reconstructed holoenzyme is identical to the native enzyme in allosteric, physical, and catalytic characteristics (16, 31, 38).

In *E. coli*, two adjacent cistrons encode the two polypeptide chains of ATCase and are expressed in the order: promoter, leader, catalytic polypeptide, regulatory polypeptide. The *pyrB* cistron codes for the catalytic polypeptide, and *pyrI* codes for the regulatory polypeptide (19, 29). The two cistrons are organized as a bicistronic operon and possess a single control region characterized by a potential attenuator (19, 26, 28, 29, 33) which is responsive to endogenous pools of UTP (26, 32, 33). Both cistrons and their controlling region have been cloned as a 2,800-base-pair (bp) fragment into pBR322, and the complete nucleotide sequence has been determined (19; unpublished data for *pyrI*, T. A. Hoover and J. R. Wild). The cloned *pyrBI* operon synthesizes a 310,000-molecular-weight holoenzyme subject to normal repression control upon growth in uracil (19, 29).

The assembly of the holoenzyme from nascent polypeptides occurs in a series of discrete stages in vitro. First, the randomly coiled, nascent catalytic polypeptides fold into the proper conformation for assembly (8); then these competent monomers aggregate into stable, catalytically active trimers. Such trimers are much more stable thermodynamically than the dimers which contain only a single, interchain bonding domain (c:c domain) (7). It is presumed that the active sites are defined at the heterologous binding domains in the crevice between each pair of catalytic polypeptides (c1:c2, c2:c3, c1:c3) (phosphate binding to the active site involves residues from adjacent catalytic chains) (18). Furthermore, it has been assumed that the holoenzyme is formed by the spontaneous assembly of intact catalytic trimers and regulatory dimers through a series of discrete intermediates, e.g., $c_3:r_2 \rightarrow c_3:2(r_2) \rightarrow c_3:3(r_2) \rightarrow 2(c_3):3(r_2)$ (4, 5, 11). Nonetheless, alternate methods of in vivo assembly involving the initial association of nascent regulatory and catalytic monomers have not been excluded experimentally.

The studies reported in this paper demonstrated that the assembly of catalytic trimers and regulatory dimers proceeds without the participation of heterologous subunits from transcriptionally independent cistrons in vivo. Subsequent assembly of the holoenzyme appeared to occur spontaneous-

* Corresponding author.
† Present address: Department of Biology, Yale University, New Haven, CT 06511.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Markers[a]	Genotype	Reference
E. coli TB2		*pyrB pyrI argI*	29
E. coli HB101		*pro leu thi hsdR endA recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*	3
E. coli HB101-4442		HB101 + *pyrB*	This study
Plasmid pBH20	Ap^r Tc^r	pBR322 + 203 bp of the *lac* operon	21
Plasmid pACYC184	Cm^r Tc^r	Derived from P15A	12
Plasmid pPBh105	*pyrBI*	2.8 kbp of *E. coli* in pBR322	29
Plasmid pPBc201	Tc^r *pyrB*	5.3 kbp of *E. coli* in pBR322	29
Plasmid pPBr101	Ap^r *pyrI*	Fig. 3	This study
Plasmid pPBc301	Cm^r *pyrB*	Fig. 2	This study

[a] Genetic markers used for selection after transformation.

ly in vivo. In vitro, the formation of holoenzyme is extremely rapid and occurs in seconds at concentrations equivalent to those approximating one molecule per cell in vivo (4). Unlike many other oligomeric enzymes, ATCase has little tendency to dissociate into subunits at concentrations as low as 10^{-10} M at neutral pH (20). These studies characterized the formation of catalytic trimers and regulatory dimers in separate cells and the formation of holoenzyme within the same cell from transcriptionally independent cistrons carried on separate plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in these studies are listed in Table 1. Bacterial strains were grown on tryptone-yeast extract or M56 media as described earlier (19, 29). Restriction endonucleases and DNA ligase were obtained from Bethesda Research Laboratories or Boehringer Mannheim Corp. and used according to the recommendations of the supplier. Ligation mixtures were incubated overnight at 4°C. T4 DNA polymerase was obtained from BRL, and blunt-ended ligation was performed by the adapted methods of Maniatis et al. (23). Frozen competent cells (14) were thawed in an ice-water bath, exposed to recombinant plasmids, and incubated at 45°C for 3 min. L broth (1 ml) was added, and the culture was grown for 2 h at 30°C. Selective platings were performed on tryptone-yeast extract plates containing the appropriate antibiotics in the following concentrations: ampicillin, 40 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 25 μg/ml. Plasmid inserts were identified with the rapid plasmid isolation procedure of Barnes (2), and restriction digests were analyzed by gel electrophoresis as described previously (29).

Enzyme isolation and assay. Bacterial cultures for assay were grown at 37°C in M56 medium supplemented with 0.2% glucose, 50 μg of leucine, 50 μg of proline, and 2 μg of thiamin per ml. The pPBr101 (plasmid, pyrimidine B operon, regulatory, producing number 101)-containing strains did not produce catalytic polypeptide and required uracil (50 μg/ml) for growth. Cultures were harvested during exponential growth (100 Klett units; green filter no. 54), washed in 40 mM potassium phosphate buffer (pH 7.5) plus 10 mM EDTA, and frozen. Cell extracts were prepared by sonication as described earlier (35). Enzymatic assays for ATCase and estimation of the molecular weight of the holoenzyme or its subunits by using ascending Sephadex G-200 chromatography have been previously described (35). The assay was performed at 28°C in the presence of 5 mM aspartate and 4 mM carbamoyl phosphate. The concentration of nucleotide effectors, ATP and CTP, was 2 mM. Protein concentration was estimated by the method of Lowry et al. (22).

Immunological procedures. ATCase was purified by the method of Gerhart and Holoubek (15), and isolated subunits were prepared by dissociation with PCMB or neohydrin (15, 16, 37). Young, white New Zealand rabbits (2 to 3 kg) were injected with 2 ml of an emulsion of Freund incomplete adjuvant and 100 μg of holoenzyme, catalytic subunit, or regulatory subunit. The rabbits were subsequently injected after 14, 27, 42, and 63 days at several sites across the nuchal region. Blood was drawn from the animals by ear bleeding them every four to six weeks, and serum was prepared by letting the freshly drawn blood sit undisturbed at room temperature for 1 h and removing the clot by centrifugation. The serum was stored at −20°C, and the antisera were used without further purification. The three classes of antiserum exhibited unique patterns of reactivity to purified holoenzyme and the separated subunits. Anti-holoenzyme was cross-reactive with holoenzyme, regulatory dimer, and catalytic trimers by the Ouchterlony immunoprecipitin assay (27). Anti-c_3 produced precipitin bands with holoenzyme and catalytic trimer and the anti-r_2 sera reacted poorly with regulatory dimer. Rocket immunoelectrophoresis was used to identify the location of regulatory subunits after molecular weight separation of Sephadex G-200 chromatography (1).

RESULTS

Construction of plasmid carrying *pyrI*$^+$ cistron (pPBr101). Fragments containing *pyrI* were generated by digesting pPBh105 DNA first with *Sal*I and secondarily with *Bst*EII. *Bst*EII cuts into the catalytic cistron thus destroying catalytic activity of ATCase (Fig. 1). Plasmid pBH20 DNA was restricted with *Hin*dIII. Both restricted DNAs were made blunt-ended by using T4 DNA polymerase, mixed together, blunt-end ligated by T4 DNA ligase (Fig. 2), and transformed into a $\Delta pyrB^-I^-$ background. Selective platings were performed on ampicillin plates. Colonies were picked and screened for the following characteristics: Ap^r Tc^s Ura^-. A total of 30 colonies were screened for the expected plasmid size (6.5 kilobase pairs [kbp]) of pBH20 containing the regulatory cistron. Of the 30 colonies containing plasmids of approximately this size, 7 were evaluated for regulatory gene product by using antibody techniques described above (1, 27). Three of the seven clones seemed to produce regulatory gene products. An Ap^r Tc^s *pyrB*$^-$ *pyrI*$^+$ clone was selected and designated pPBr101.

Construction of plasmid carrying *pyrB*$^+$ cistron. Fragments containing *pyrB* were generated by restricting pPBc201 with *Bgl*II followed by a second restriction with *Pst*I. This produced a fragment which contained a functional *pyrB* catalytic gene and a nonfunctional *pyrI*, owing to deletion of ca. two-thirds of the *pyrI* region (Fig. 1). Plasmid pACYC184

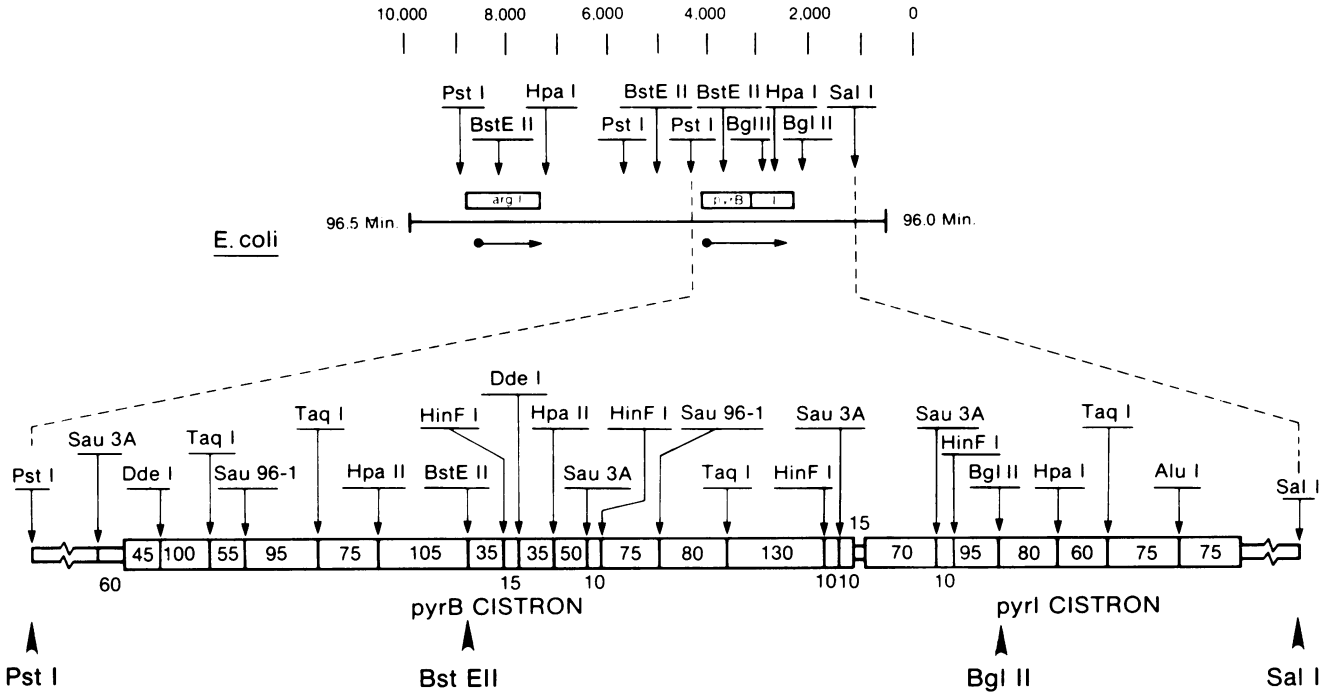

FIG. 1. Endonuclease restriction map of the *pyrBI-argI* region (96.5 min) of *E. coli* K-12 (29). Overlapping restriction endonuclease patterns were used to define the positional relationship of the *pyrBI* operon and *argI*. These gene loci mapped ca. 4,000 bp apart. *pyrB* encoded the catalytic polypeptide of ATCase; *pyrI* encoded the regulatory polypeptide; and *argI* encoded the catalytic polypeptide of ornithine carbamoyltransferase. Transcription occurred counterclockwise relative to the normal orientation of the *E. coli* genetic map for both of the gene systems. The *Pst*I-*Bgl*II fragment expressed only the catalytic polypeptide of ATCase upon subcloning, and the *Bst*EII-*Sal*I fragment placed in an appropriate expression vector expressed only the regulatory polypeptide of ATCase.

was restricted with *Bam*HI, and all fragments were made blunt-ended with T4 DNA polymerase. The DNAs were mixed together as previously described, blunt-end ligated (Fig. 3), and transformed into a $pyrB^-$ background. Selective platings were performed on chloramphenicol medium. Appropriate colonies were screened for Cm^r Tc^s Pyr^+ characteristics and checked for plasmid size. A $pyrB^+$ Cm^r Tc^s clone containing a plasmid of ca. 5.4 kbp was selected and designated pPBc301.

Construction of strain containing both plasmids. Initially, each plasmid was transformed into *E. coli* u39a which was $pyrB^-I^-$ ara^- Δ(*lac pro*). The strain had been made $pyrB^-I^-$ by the insertion and excision of Mu d1(*lac* Ap^r) (10). However, it was noted that when plasmid pPBr101 was placed in this background, a large number of $pyrB^+$ colonies appeared. Plasmid DNAs from pPBr101 and pPBc301 were reisolated by using a cleared lysate technique followed by phenol extraction (no CsCl purification was done) (29). This DNA was then transformed into strain HB101-4442 (derived from insertion and subsequent excision of Mu *cts* which is $\Delta pyrB^-I^-$ and $recA^-$ (Table 1). Little or no reversion occurs in this background. To construct a cell containing both plasmids, HB101-4442 cells containing pPBc301 DNA (carried by pACYC184) were made competent, and plasmid pPBr101 DNA (carried by a pBR322-derived plasmid) was transformed into them. Colonies were selected onto plates containing chloramphenicol and ampicillin. These colonies were screened for the following characteristics: Cm^r Ap^r Tc^s Pyr^+. Clones were further screened for presence of a 6.5-kbp plasmid and a 5.4-kbp plasmid.

Evaluation of holoenzyme assembly. Effector responses and molecular weight species were estimated for ATCase assayed from extracts prepared from cultures which harbored the $pyrI^+$ plasmid, the $pyrB^+$ plasmid, or both plasmids in the same cell. After the cells were resuspended in the appropriate amount of buffer, the sample was divided in half. One cell extract was prepared by sonication in the presence of 0.2 mg of PCMB per ml, whereas the other was sonicated in the absence of PCMB. Control extracts (treated and evaluated in the same manner) were included to demonstrate that PCMB used at this concentration would not cause appreciable dissociation of holoenzyme nor would this concentration allow association to occur between free subunits. Thus, the holoenzyme was not being formed or dissociated in cell extracts during or after sonication. The first control extract, designated "pPBh105 (intact *pyrBI*)" in Tables 2 and 3, was prepared from a culture which harbored a plasmid (pPBh105) carrying the entire *pyrBI* operon and demonstrated that holoenzyme was not being dissociated in the presence of PCMB. Effector response of intact *pyrBI* extracts in the presence and absence of PCMB (Table 2) was that expected of *E. coli* holoenzyme. Molecular weight determination of intact *pyrBI* extracts in the presence and absence of PCMB (Table 3) showed that ATCase was recovered only as holoenzyme (molecular weight, 310,000). Thus, PCMB at this concentration did not cause dissociation of holoenzyme. The second control, designated "pPBr101 + pPBc301 (mix)" in Tables 2 and 3, was prepared by growing two separate cultures (one containing a plasmid carrying *pyrI* only and another carrying *pyrB* only) which were mixed together before sonication in the presence and absence of PCMB. The effector response of the mixed sample showed that in the absence of PCMB, partial association was apparent since some effector response did occur (CTP inhibition) (Table 2). Both ATCase holoenzyme and catalytic trimer were observed by molecular weight determinations (Table 3) of the mixed sample. This indicated that some reassociation occurred during or after sonication in the absence of PCMB.

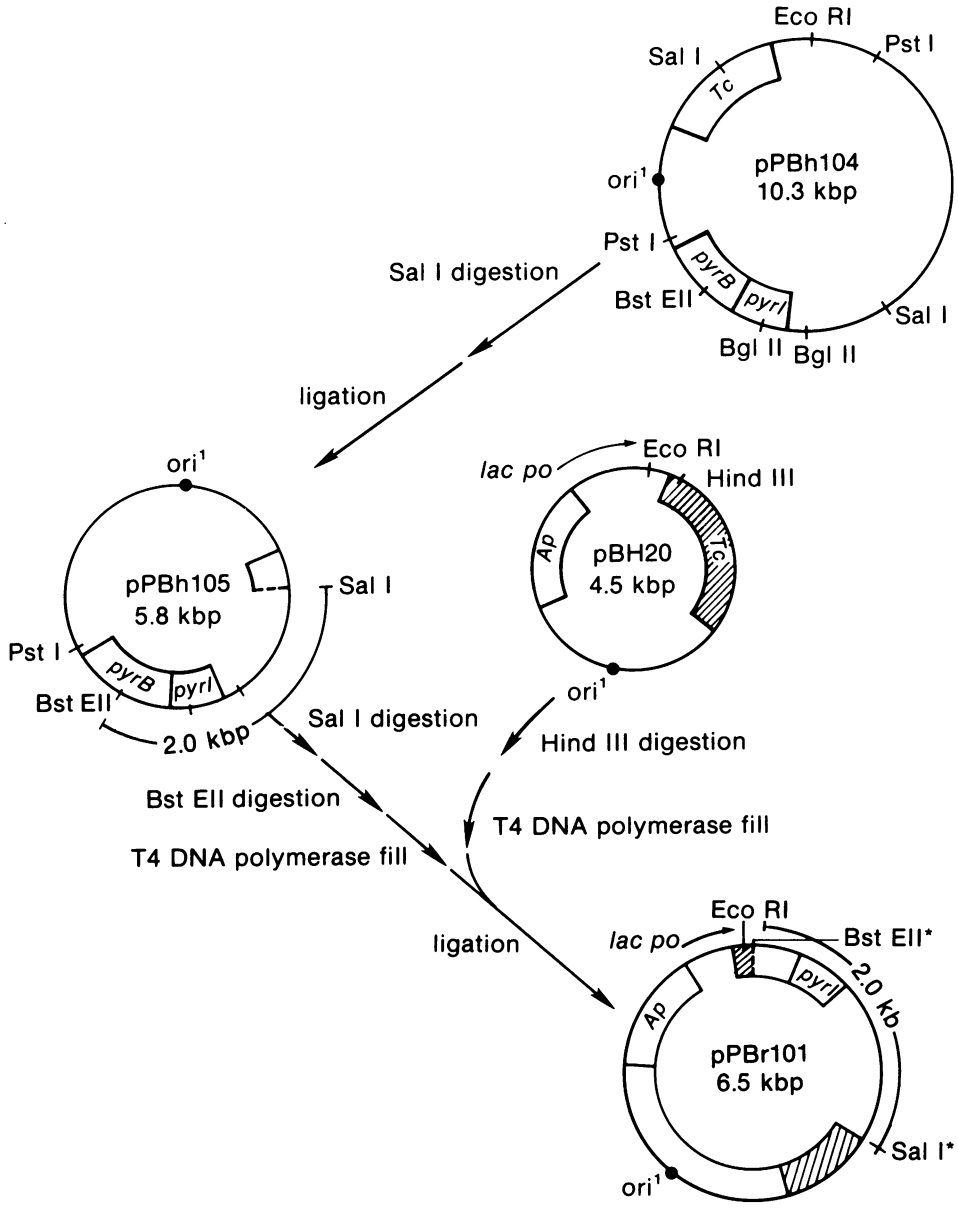

FIG. 2. Schematic diagram demonstrating the genealogy of pPBr101 used in the current study. A 2.0-kbp fragment defined by *Bst*EII-*Sal*I was transferred into pBH20. The resulting plasmid, pPBr101, was Ap^r Tc^s $pyrB^-$ $pyrI^+$ and replicated from an origin derived from pBR322 (29). This origin is designated ori^1. Restriction endonuclease sites subjected to fill reactions and blunt-ended ligation are indicated by asterisked mnemonics (e.g., *Bst*EII*).

In the presence of PCMB, the mixed sample had no appreciable effector response (Table 2), and molecular weight determination showed that only catalytic trimer was present (Table 3). Nonspecific inhibition of the catalytic trimer by CTP and ATP has been reported in earlier studies (31), and values of 85 to 100% activity are expected. Thus, PCMB at this concentration (0.2 mg/ml) prevented in vitro association. The extract prepared from a culture containing both *pyrB* and *pyrI* on different plasmids in the same cell behaved exactly as the intact *pyrBI* extract with normal ATP and CTP responses (Table 2) and only intact holoenzyme was recovered in the presence and absence of PCMB (Table 3). No independent catalytic trimers were detected in extracts from cells containing both $pyrB^+$ and $pyrI^+$ on separate plasmids. It is apparent that excess r_2 is produced from the *lac* promoter in pPBr101 since all c_3 is accommodated in holoenzyme.

Although the molecular weights of catalytically active aggregates (i.e., holoenzyme and catalytic trimers) were readily determined after separation by Sephadex G-200 chromatography (Table 3), it was more difficult to monitor the catalytically inactive species (i.e., regulatory dimers, monomers, and catalytic monomers). Therefore, it was necessary to utilize immunological analyses to assess the production of regulatory polypeptides in the absence of catalytic subunits or to determine the presence of inactive catalytic species. When the regulatory polypeptide was expressed singularly from pPBr101, all detectable cross-reacting material from cell extracts had a molecular weight of 35,000 g/mol, corresponding to regulatory dimer (data not

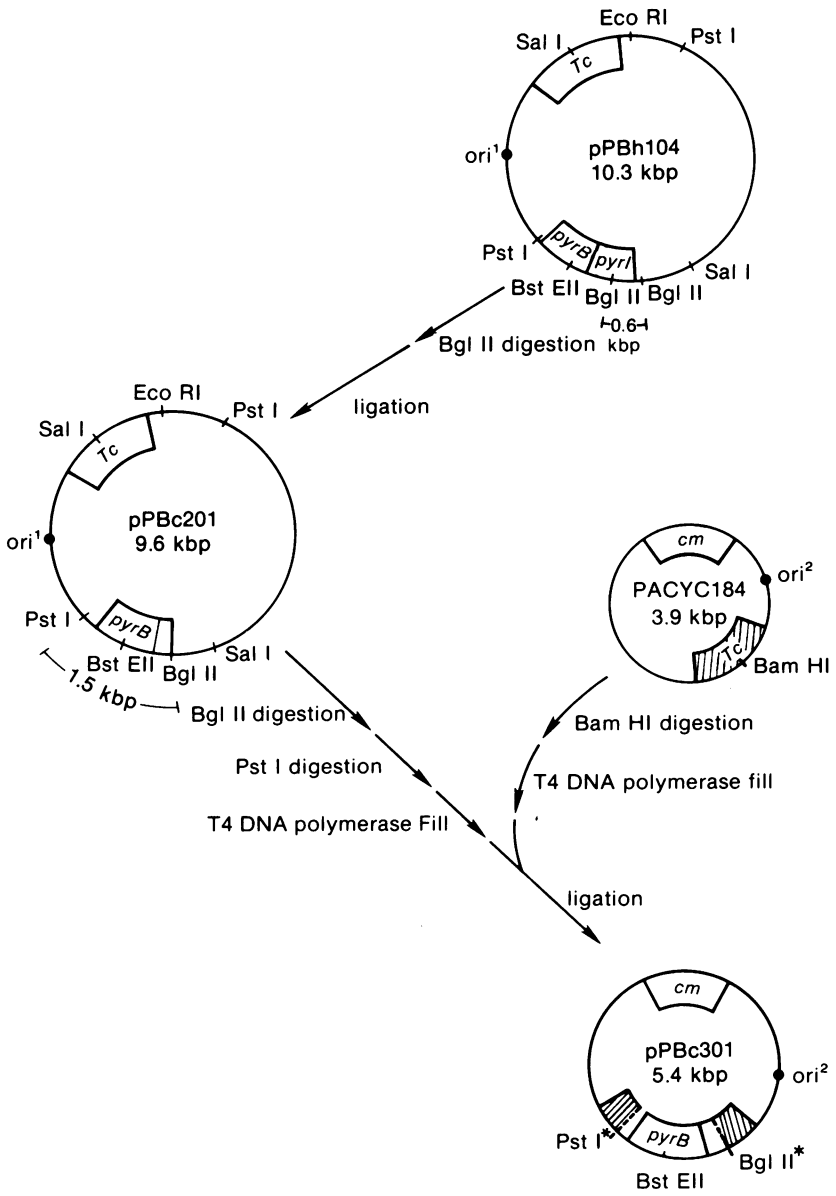

FIG. 3. Schematic diagram demonstrating the genealogy of pPBc301 used in the current study. A 0.6-kbp fragment was removed from pPHh104 by *Bgl*II-*Bgl*II deletion excision, and the 1.5-kbp fragment defined by *Pst*I-*Bgl*II was transferred into pACYC184. The resulting plasmid, pPBc301, was Cm^r Tc^s $pyrB^+$ $pyrI^-$ and replicated from an origin derived from P15A plasmid (12). This origin is designated ori^2. Restriction endonuclease sites subjected to fill reactions and blunt-ended ligation are indicated by asterisked mnemonics (e.g., *Bst*EII*).

shown). No precipitin was detected in fractions corresponding to monomer, 10,000 to 20,000 g/mol. Similarly, no precipitin corresponding to catalytic monomers was found in cell extracts expressing only catalytic polypeptides, pPBc301. All regulatory monomers appeared to be assembled as regulatory dimers and catalytic monomers assembled as catalytic trimers. It is estimated that less than 5% of the regulatory polypeptide could be present as monomers and escape detection. Furthermore, it was possible that the monomers may have different antigenic responses and thus escape detection, but the polyclonal antibodies being used have shown broad recognition characteristics (i.e., anti-holoenzyme recognizes both separate regulatory dimers and catalytic trimers from dissociated holoenzyme) (unpublished data).

DISCUSSION

Recent studies by Burns and Schachman have demonstrated that the assembly of ATCase catalytic trimers from unfolded monomers (purified catalytic trimers treated with 4.5 M urea) is consistent with a multistep assembly mechanism. The first step appears to be the rapid collapse of randomly coiled monomers into partially folded polypeptides which are catalytically inactive and cannot associate into trimers (catalytic trimers $[c_3]$ = C subunits) (8). A slow, rate-limiting conformational change converts the collapsed polypeptides into competent monomers which assemble into active trimers through a series of associative reactions (7). It was assumed that the regulatory dimers possessed some analogous assembly mechanism, but that process was not

TABLE 2. ATCase effector response in the presence and absence of PCMB

Sample description	PCMB	Sp act without effector (%)[a]	
		2 mM ATP	2 mM CTP
pPBh105 (intact *pyrBI*)	None	127	28
pPBh105 (intact *pyrBI*)	0.2 mg/ml	124	29
pPBc301	None	90[b]	97[b]
pPBr101	None	No detectable activity	No detectable activity
pPBr101 + pPBc301 (mix)[c]	None	100	69
pPBr101 + pPBc301 (mix)	0.2 mg/ml	88[b]	92[b]
pPBr101, pPBc301 (same cell)[d]	None	121	19
pPBr101, pPBc301 (same cell)	0.2 mg/ml	121	31

[a] Specific activity is presented as the percentage of activity measured without nucleotide effector for each sample.
[b] Nonspecific phosphate inhibition resulting in reduced activity is expected for the catalytic trimer (31).
[c] Each of the plasmids were expressed in different cells, and the cultures were mixed before sonication (assembly would have to occur during or after sonication).
[d] Both plasmids were carried within the same cell (assembly could occur in vivo or during or after sonication).

demonstrated experimentally. The ATCase holoenzyme is composed of two catalytic trimers, $2(c_3)$, and three regulatory dimers, $3(r_2)$, associated in a noncovalent manner such that each catalytic monomer contacts the two other monomers of that catalytic trimer and is linked with a catalytic polypeptide in the other trimer through a regulatory dimer (17, 18, 24, 34, 36). The architecture of the holoenzyme, $2(c_3):3(r_2)$ provides six c:c bonding domains within the catalytic trimers, three r:r bonding domains within the regulatory dimers and six r:c domains linking the regulatory and catalytic subunits (13). It has been suggested from X-ray diffraction studies that there may be some additional contact between the catalytic chains in separate trimers although there is a large aqueous center in the holoenzyme (17, 18, 25). The assembly of catalytic trimers and regulatory dimers into the holoenzyme architecture appears to follow a variety of alternative pathways in vitro (4, 5, 11). Because of the strong negative enthalpy of assembly (24), this association is extremely rapid and the folding of the individual monomers into competent assembly components may be the rate-limiting factor (7). It has been suggested that the virtually irreversible association of catalytic and regulatory subunits at physiological conditions is due to the combined strength of the multiple subunit interactions (6, 20).

In this study, the *pyrBI* operon of *E. coli* (19, 28, 29) has been dissected into separate genes (*pyrB* encoding the catalytic chain of ATCase and *pyrI* encoding the regulatory chain). The *pyrI* gene has been removed from the promoter-distal end of the operon and placed under control of the *lac* operator-promoter of pBH20. The *pyrB* cistron under its own promoter has been cloned into pACYC184, a plasmid derived from a different incompatability group (12). This cloning procedure makes it possible to transform a single cell with both plasmids and place *pyrB* and *pyrI* together yet under independent transcriptional control. When *pyrB* is singularly expressed in a recipient mutated in its chromosomal *pyrBI* operon by Mu *cts* transpositional mutagenesis (10) the catalytic monomers assemble into mature, catalytically active trimers. Similarly, the regulatory subunit appears to be assembled into regulatory dimers.

TABLE 3. Molecular weight of catalytically active species of ATCase produced from different plasmid constructs

Plasmid construct	PCMB[a]	Molecular weight species[b]	
		300,000	100,000
pPBh105 (intact *pyrBI*)	Without	+	−
pPBh105 (intact *pyrBI*)	With	+	−
pPBr101 + pPBc301 (mix)[c]	Without	+	+
pPBr101 + pPBc301 (mix)	With	−	+
pPBr101-pPBc301 (same cell)	Without	+	−
pPBr101-pPBc301 (same cell)	With	+	−

[a] PCMB concentration (0.2 mg/ml) prevents assembly of ATCase holoenzyme in crude extracts but does not dissociate preformed enzymes.
[b] Molecular weight species of ca. 300,000 g/mol are interpreted to be holoenzyme (determined by Sephadex G-200). Molecular weight species of ca. 100,000 g/mol are interpreted to be catalytic subunits (c_3). +, Present; −, absent.
[c] Approximately 30% of the catalytic activity was observed as holoenzyme and 70% was observed as catalytic trimers.

Once it had been determined that the catalytic chains spontaneously assemble into trimers and the regulatory chains assemble into dimers without the heterologous chains, it was necessary to demonstrate that the appropriate subunits would assemble into the holoenzyme in vivo. It had already been shown that the mixing of crude extracts of regulatory dimer and catalytic trimers would promote a partial, spontaneous formation of the holoenzyme. Therefore, it was necessary to demonstrate that the regulatory subunits (r_2) and the catalytic subunits (c_3) were assembling within the cell and not subsequent to cell disruption. It was possible to determine a concentration of PCMB (0.2 mg/ml) that would prohibit assembly of subunits into holoenzyme when r_2 from one cell extract expressing only regulatory chains ($pyrB^-$ $pyrI^+$) was mixed with cell extract containing only c_3 ($pyrB^+$ $pyrI^-$). This concentration of PCMB did not separate preformed holoenzyme since it was a 20-fold-lower concentration than that used for dissociation (16). However, this concentration did prevent in vitro assembly from the mixed extracts (Table 3). Under the conditions described here, when catalytic and regulatory polypeptides were produced in the same cell from separate plasmids, the holoenzyme was already intact when the cells were disrupted. The presence of PCMB at this lower concentration did not affect the recovery of the catalytic activity as holoenzyme (molecular weight, 310,000 g/mol).

Although it has been reported that regulatory dimers and catalytic trimers will spontaneously assemble in vitro, it was not known whether catalytic and regulatory chains could assemble into trimers and dimers in vivo in the absence of the other. These studies have demonstrated three characteristics relative to the assembly of catalytic and regulatory chains of ATCase in vivo: (i) the catalytic monomers spontaneously assemble into catalytically active trimers with a molecular weight of ca. 100,000 without the participation of the regulatory subunits; (ii) the regulatory chains assemble

into regulatory dimers with a molecular weight of ca. 35,000 without the participation of the catalytic monomers or trimers; and (iii) even if expressed from separate cistrons in *trans* positions on different plasmids, the regulatory dimers and catalytic trimers spontaneously assemble into holoenzyme in vivo before cell disruption.

It has been possible to use this technique to form hybrid ATCase enzymes from the catalytic polypeptides of *Serratia marcescens* and the regulatory dimers of *E. coli*. The hybrids thus formed demonstrated the same enzymatic characteristics as hybrids formed from purified subunits (M. S. Shanley, Ph.D. dissertation, Texas A&M University, College Station, Tex., 1983). It appears, therefore, that this approach has great facility for the formation of a variety of oligomeric hybrids in vivo.

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