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Mapping the Structural Domains of *E. coli* Carbamoyl Phosphate Synthetase Using Limited Proteolysis

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Abstract—The structural and functional domains of *Escherichia coli* carbamoyl phosphate synthetase (CPS) have been identified by limited proteolysis. Incubation of CPS with several proteases, including trypsin, chymotrypsin, subtilisin and endoproteinase Asp-N, under native conditions, causes a time-dependent loss of enzymatic activity and the generation of a common fragmentation pattern. Amino-terminal sequencing studies demonstrated that the initial cleavage event by trypsin occurred at the carboxy-terminal end of the large subunit. The ultimate fragments produced in most of the proteolysis studies, 35- and 45-kDa peptides, were derived from areas corresponding to the putative ATP binding regions. Substrate protection studies showed that the addition of ligands did not affect the final fragmentation pattern of the protein. However, ornithine and UMP were found to significantly reduce the rate of inactivation by inhibition of proteolytic cleavage. MgATP and IMP provided modest protection whereas bicarbonate and glutamine showed no overall effect on proteolysis. Limited proteolysis by endoproteinase Asp-N resulted in the production of a fragment (or multiple fragments) which contained enzymatic activity but had lost all regulation by the allosteric ligands, UMP and ornithine. The small subunit has been shown to be protected from proteolysis by the large subunit. Proteolysis of the isolated small subunit resulted in the generation of a stable 31-kDa species which contained 10% of the original glutaminase activity. These studies demonstrate that a portion of the C-terminal end of the large subunit can be excised without entirely destroying the ability of CPS to catalyze the formation of carbamoyl phosphate. Therefore, the C-terminal end of CPS is required for allosteric control but not for catalysis of the enzymatic reaction.

Introduction

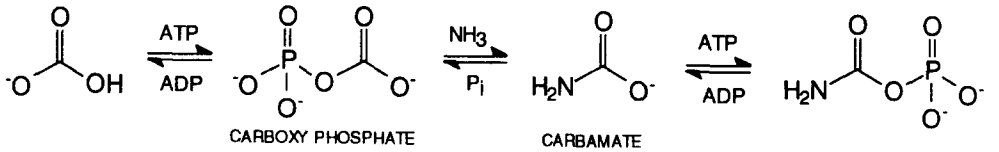
Carbamoyl phosphate synthetase (CPS) catalyzes the formation of the key intermediate in the biosynthesis of arginine and pyrimidine nucleotides. The enzyme from *Escherichia coli* catalyzes the formation of carbamoyl phosphate as shown in Equation 1. The chemical mechanism:

$$2MgATP + HCO_3^- + Gln \rightarrow 2MgADP + Glu + P_i + \text{carbamoyl phosphate} \quad (1)$$

has been shown to occur via the formation of two reactive intermediates, carboxyphosphate and carbamate, as illustrated in Scheme 1.[1-3] The heterodimeric enzyme is composed of a large subunit of molecular weight 118K and a small subunit of molecular weight 41K.[4,5]

The N- and C-terminal halves of the large subunit are homologous. The high degree of sequence identity (39%) between these two halves of the large subunit suggests that each domain may contain a separate binding site for one of the two molecules of ATP used in the overall reaction. This proposal has been supported by the kinetic properties of site-directed mutants,[6] inactivation studies with 5'-*p*-fluorosulfonylbenzoyl adenosine (FSBA),[7] inhibitory properties for a series of diadenosine-5'-polyphosphates[8] and steady state kinetic studies.[3]

Limited proteolysis investigations of CPS from a variety of sources have provided structural and functional information about the enzyme. Proteolysis of rat liver CPS by trypsin and elastase was shown to be modulated by the presence of the substrate ATP and the allosteric effector *N*-acetylglutamate.[9] The loss of enzymatic activity paralleled the loss of the intact 160-kDa monomeric enzyme when the initial cleavage occurred within the carboxy-terminal region in the presence of *N*-acetylglutamate. However, in the presence of ATP the initial cleavage site occurred at the N-terminal end of the protein and an active 120-kDa fragment was formed. The 37-kDa fragment derived from the N-terminal end was homologous to the small subunit of the *E. coli* enzyme.



Scheme 1.

Evans and Balon have carried out controlled proteolysis of the ammonia-dependent CPS from Syrian hamster liver.[10] The initial cleavage of the protein at the C-terminal end resulted in the complete loss of enzymatic activity upon formation of a 145-kDa species. Further cleavage of this protein resulted in the generation of 40-, 37- and 62-kDa fragments. Although this enzyme cannot utilize glutamine as a nitrogen source, it has a vestigial glutaminase domain that is represented by the 40-kDa fragment. Limited proteolysis of the multifunctional CAD protein resulted in the formation of 18 fragments.[11] Initial proteolytic cleavage of the glutaminase and carbamoyl phosphate synthetase subdomain occurred at the carboxy-terminal end of the 156-kDa species. The resulting 142-kDa species was further cleaved to 31-, 50- and 40-kDa fragments.

In the study reported here, limited proteolysis was utilized to further investigate the structural and functional properties of the *E. coli* enzyme. Various proteases, including trypsin, chymotrypsin, endoproteinase Asp-N and subtilisin, were utilized to map the subdomain structure of this complex enzyme. The CPS from *E. coli* was cleaved at sites similar to those previously observed with this enzyme from other sources. However, for the first time, partial enzymatic activity was detected after cleavage of small fragments from the carboxy terminal end of the large subunit. The extreme C-terminal domain appears to control the allosteric regulation by UMP.

Results

Limited proteolysis by trypsin

Partial digestion of carbamoyl phosphate synthetase by trypsin was assayed by enzyme activity measurements and correlated to specific fragmentation pathways by SDS/polyacrylamide gel electrophoresis. Proteolysis by trypsin caused a reproducible, time-dependent decrease in enzymatic activity. Initial cleavage of the large subunit resulted in the generation of a large, inactive peptide with a molecular weight of approximately 100 kDa (Fig. 1). Upon further incubation, an 80-kDa fragment was produced prior to a cleavage that generated two additional fragments of approximately 45- and 35-kDa each. The 80-kDa fragment was electroeluted on to a ProBlott membrane and subsequent sequencing of the amino-terminal end of this protein identified the first 11 amino acids as MPKRTDIKSIL (Table 1). Comparison of the amino acid sequence of this peptide to the known sequence of the large subunit demonstrated that the amino-terminal

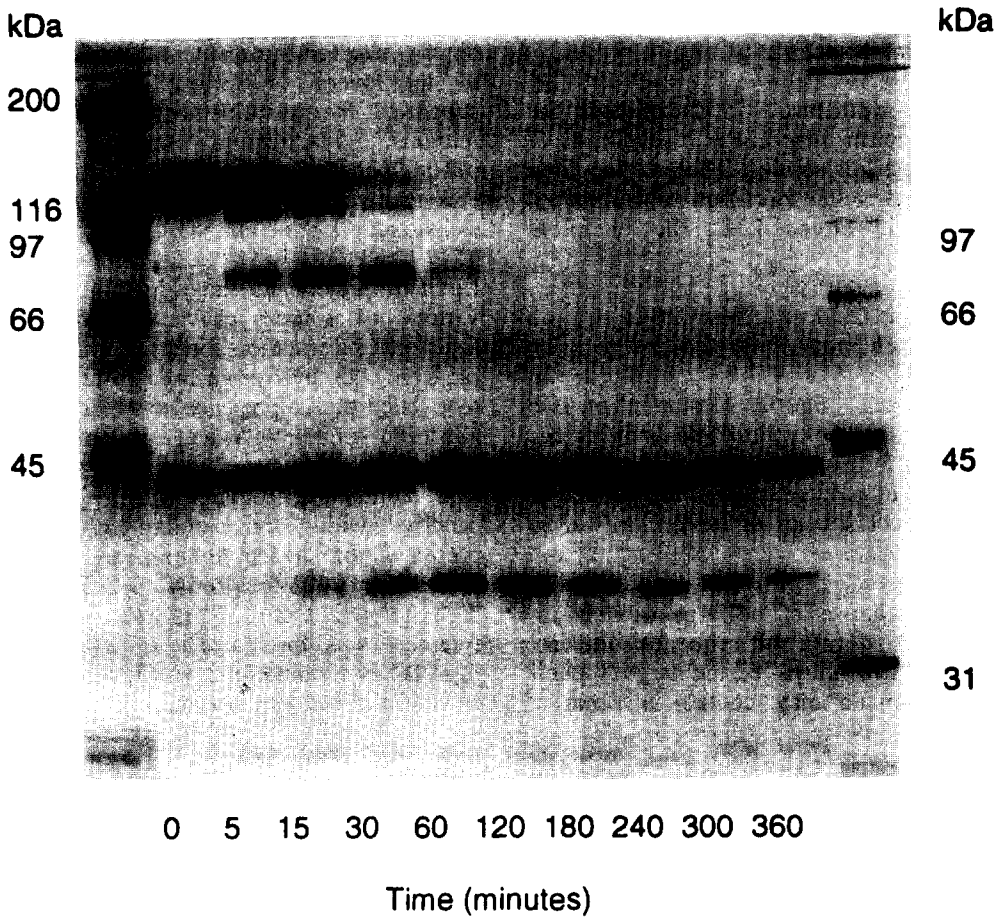


Figure 1. SDS-PAGE of carbamoyl phosphate synthetase fragments obtained after trypsin proteolysis. The digestion was performed at 25 °C in 60 mM HEPES, pH 7.6 with 8 mg CPS and 0.7 mg trypsin. Additional details are provided in the text.

Table 1. Amino-terminal sequence and approximate molecular mass of fragments obtained by limited proteolysis of carbamoyl phosphate synthetase by trypsin

kDa	Amino-terminal sequence	Subunit involved
80	^{1}MPKRTDIKSIL	large subunit
35	^{374}LTTQMSKSVG	large subunit
33	^{374}LTTQMSKSVG	large subunit
31	^{374}LTTQMSKSVG	large subunit
31	^{94}NTED	small subunit

end of the large subunit was intact and thus the initial tryptic cleavage sites were located at the carboxy-terminal end of the large subunit. Amino-terminal sequence analysis of the 35-kDa peptide indicated that this peptide starts with the sequence LTTQMSKSVG. Comparison with the amino acid sequence of the wild type protein showed that the 35-kDa peptide begins with Leu-374 of the native enzyme. This observation demonstrates that this peptide was derived from thecarboxy-terminal half of the 80-kDa species. Attempts to obtain the amino-terminal sequence of the 45-kDa peptide were unsuccessful but it is likely that this peptide was generated from the amino-terminal end of the 80-kDa species. The 35-kDa peptide was further cleaved into smaller peptides (33- and 31-kDa peptides) that were shown to have the same amino-terminal end that begins with Leu-374 of the native enzyme. These results are summarized in Table 1.

Substrate protection studies were conducted using 5 mM MgATP, 5 mM ornithine, 50 μM UMP, 0.5 mM IMP, 5 mM glutamine and 40 mM bicarbonate. The best protection against cleavage was provided by ornithine and UMP. Moderate protection was provided by MgATP and IMP while bicarbonate and glutamine had no effect on the rate of cleavage of the large subunit (data not shown). The fragmentation patterns of the digestion reactions carried out in the presence of substrates and allosteric ligands were similar to those observed in the absence of these materials. The effect of added substrates on the rate of inactivation of carbamoyl phosphate synthetase are presented in Figure 2. These studies indicate that the decrease in the rate of loss of catalytic activity of the enzyme is directly attributed to a slower rate of cleavage by the protease.

The small subunit was cleaved by trypsin at a significantly slower rate than the large subunit. Cleavage of the isolated small subunit resulted in the generation of a 31-kDa peptide (data not shown). Sequence analysis demonstrated that the N-terminal amino acid sequence was NTED (Table 1). These results demonstrated that the amino-terminal end of the 31-kDa peptide starts with residue Asp-94 of the small subunit. Kinetic studies showed that 20% of the original glutaminase activity was obtained after the intact small subunit had completely disappeared from the gel.

Limited proteolysis by chymotrypsin

Proteolysis of the native enzyme by chymotrypsin resulted in fragments of similar sizes to those obtained with trypsin (data not shown). The initial cleavage site resulted in the generation of an inactive 100-kDa fragment. Further cleavage produced an 80-kDa peptide and then a fragment of approximately 70-kDa. Subsequent cleavages generated 45- and 25-kDa fragments. The 45- and 25-kDa peptides resulted from the cleavage of either the 70- or 80-kDa fragment. The small subunit appeared to be more susceptible to proteolysis relative to comparable digestion reactions with trypsin. The small subunit, however, was cleaved only after substantial digestion of the large subunit had occurred.

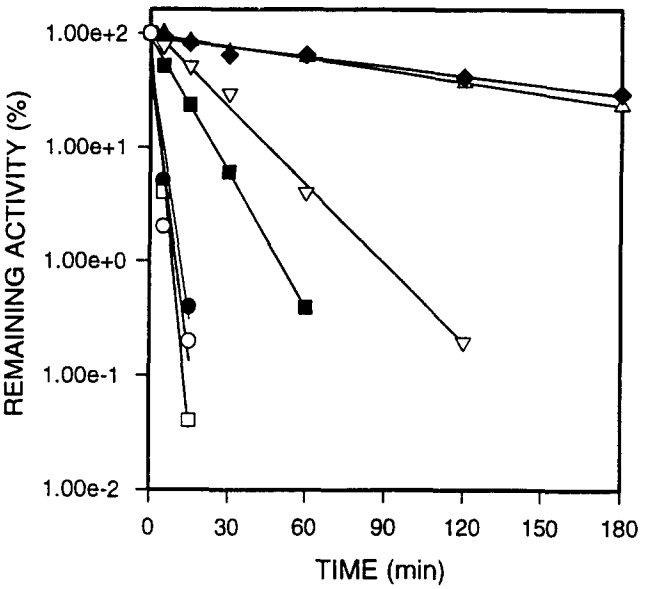


Figure 2. Inactivation of the glutamine-dependent ATPase activity of carbamoyl phosphate synthetase by trypsin cleavage. Substrate protection studies were carried out in the presence of (●) no additions, (■) 5 mM MgATP, (Δ) 5 mM ornithine, (◆) 50 μM UMP, (▽) 0.5 mM IMP, (❑) 5 mM glutamine and (○) 40 mM bicarbonate.

Limited proteolysis by subtilisin

Proteolysis in the presence of a 1:10000 ratio (w/w) of subtilisin to CPS resulted in cleavage of the native enzyme (Fig. 3). Initial cleavage of the large subunit resulted in the generation of several inactive large fragments of molecular weights 98–115 kDa. A predominant peptide of about 80 kDa was made simultaneous with the formation of 70- and 65-kDa fragments. Two peptides with molecular weights of approximately 45 kDa were generated at the end of the time course as shown in Figure 3. When more subtilisin was used in the digestion reaction mixture (1:20 ratio;

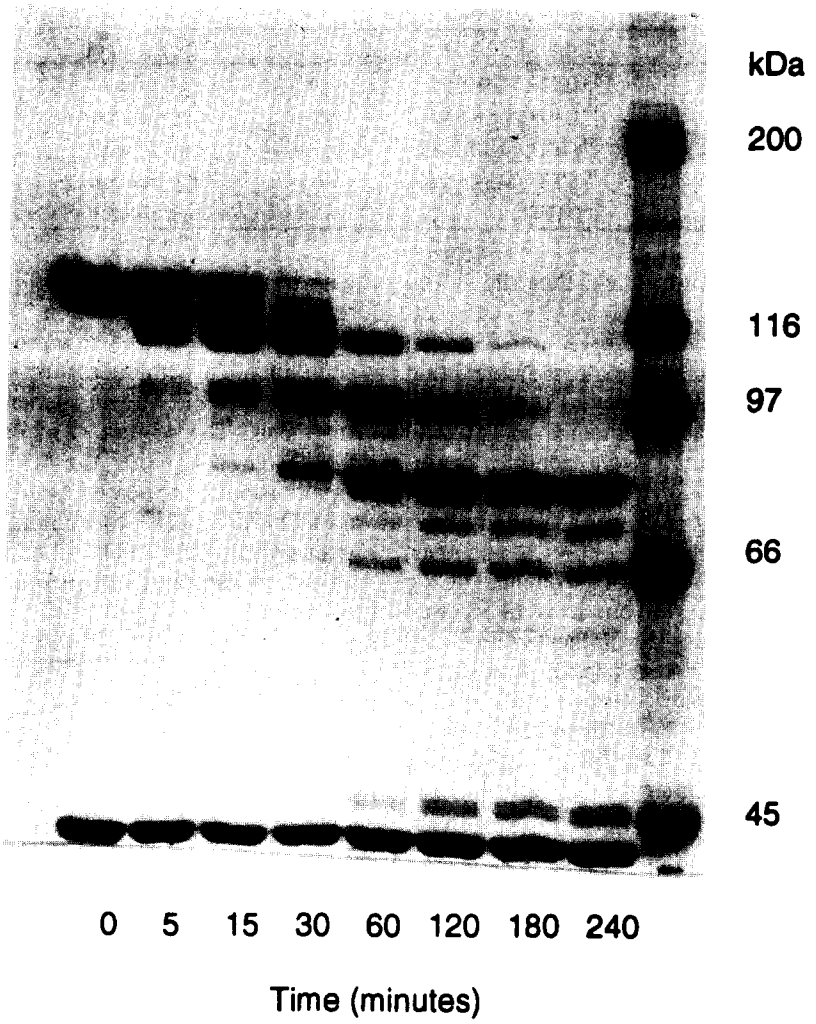


Figure 3. Limited proteolysis of carbamoyl phosphate synthetase by subtilisin. Digestion reactions were inhibited by adding PMSF to give a final concentration of 10 mM. The CPS (2 mg) was incubated at 25 °C with 0.1 μg of subtilisin and aliquots were analyzed on an 8% gel.

protease to substrate), the initial fragments observed from the large subunit were 80-, 70- and 60-kDa peptides (data not shown). Subsequent cleavage generated 35-, 33- and 25-kDa fragments which were stable towards further proteolysis.

Limited proteolysis by endoproteinase Asp-N

This protease generated a large fragment (or large fragments) which still contained enzymatic activity. The early stages of the digestion process are presented in Figure 4. Approximately 10% of the original activity was retained after the large subunit was no longer visible on the gel. The inactivation of the partial reactions catalyzed by carbamoyl phosphate synthetase are shown in Figure 5. The effects of added allosteric ligands on the glutamine-dependent ATPase activity of the digested protein were determined and the results are shown in Table 2. Insignificant changes in the Michaelis constant of MgATP were observed in the presence of each individual effector.

Table 2. Effect of allosteric effectors on the glutamine-dependent ATPase activity of endoproteinase Asp-N-cleaved carbamoyl phosphate synthetase[a]

	Ligand							
	NONE		10 mM Orn		100 μM UMP		1 mM IMP	
Enzyme	V_{max} units mg^{-1}	K_{ATP} mM	V_{max} units mg^{-1}	K_{ATP} mM	V_{max} units mg^{-1}	K_{ATP} mM	V_{max} units mg^{-1}	K_{ATP} mM
WT	3.9 ± 0.2	0.52 ± 0.06	3.5 ± 0.4	0.05 ± 0.01	1.9 ± 0.1	1.1 ± 0.2	3.3 ± 0.03	0.39 ± 0.03
Digestion mixture	0.42 ± 0.03	3.9 ± 0.6	0.34 ±0.01	2.8 ± 0.1	0.55 ± 0.04	4.4 ± 0.5	0.40 ± 0.02	3.0 ± 0.3

[a]Assays were performed at 25 °C in the presence of 10 mM glutamine, variable ATP, 40 mM bicarbonate, 20 mM Mg^{2+} and pH 7.6.

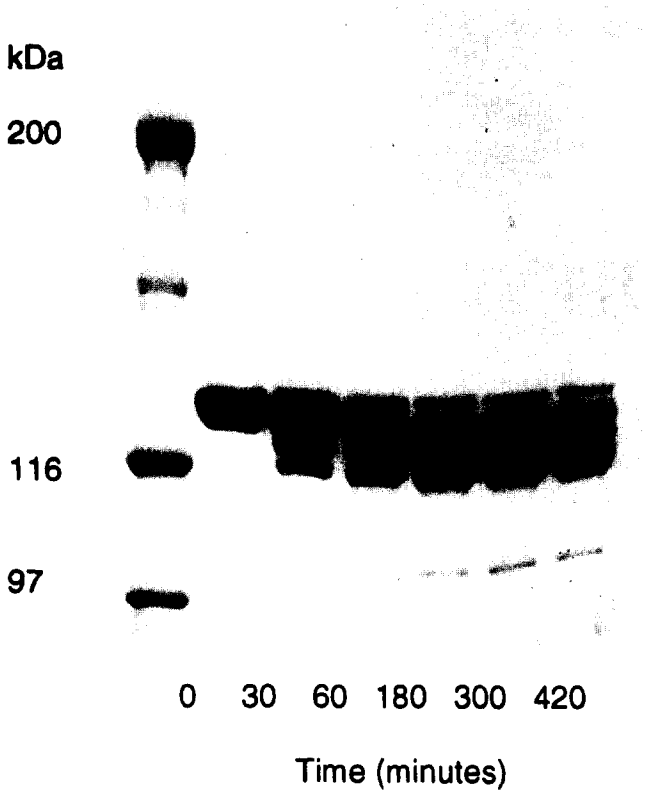


Figure 4. Endoproteinase Asp-N cleavage of the large subunit of carbamoyl phosphate synthetase. The CPS (2.7 mg) was incubated with 1 μg of the protease at 37 °C in 60 mM HEPES, pH 7.6. Timed aliquots were inhibited by adding EDTA to give a final concentration of 10 mM. The samples were analyzed by SDS-PAGE on a 5% gel.

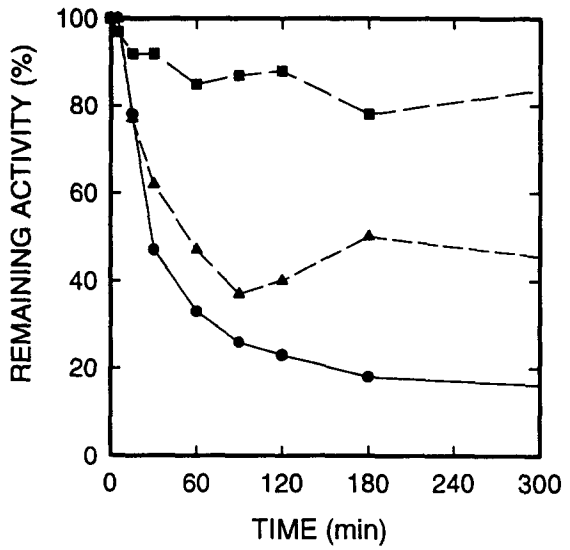


Figure 5. Inactivation profile for carbamoyl phosphate synthetase by endoproteinase Asp-N. Enzymatic activity was monitored for the (●) glutamine-dependent ATPase reaction, the (■) bicarbonate-dependent ATPase reaction and the (▲) ATP synthesis reaction.

Discussion

Limited proteolysis has been utilized here as a technique to probe the domain structure of *E. coli* CPS. Cleavage of short peptides from the C-terminal end of the large subunit results in the generation of several large fragments of approximately 100 kDa in size. Further cleavage of this peptide with all of the enzymes tested subsequently produces a smaller 80-kDa fragment. Positive identification of an N-terminal sequence in the 80-kDa fragment that corresponds exactly with the intact large subunit demonstrates that the initial cleavage sites are located at the carboxy-terminal end of the large subunit. The 80-kDa fragment is then further cleaved into 45- and 35-kDa pieces when trypsin is used to proteolyze CPS. Sequence analysis has shown that the amino-terminal half of the 35-kDa species starts at Leu-374. This observation indicates that the 45-kDa fragment is derived from the amino-terminal half of the 80-kDa peptide while the 35-kDa peptide derives from the C-terminal half. The 80-, 45- and 35-kDa fragments appear in experiments with all of the proteases tested, suggesting that protease labile sites exist between the various structural domains of the large subunit of CPS. A summary of the fragmentation pattern obtained with trypsin is presented in Figure 6.

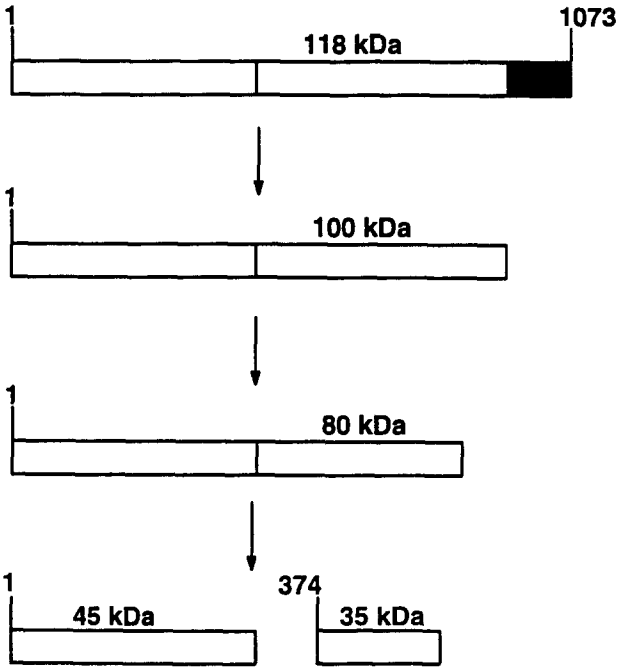


Figure 6. Summary of the trypsin cleavage sites of the large subunit of *E. coli* carbamoyl phosphate synthetase.

The pattern of protease cleavage sites in the large subunit of CPS from *E. coli* resembles those previously observed with CPS from other sources. Independent and folded structural domains corresponding to the small subunit have been obtained in the limited proteolysis studies of other CPS enzymes.[9-11] Moreover, the generation of 45- and 35-kDa fragments in this study are derived from the same general regions as the 37- and 62-kDa fragments from the hamster liver CPS, and the 50- and 40-kDa fragments from the CAD CPS. The regions from which these fragments originate correspond roughly to the putative ATP bindings sites within the large subunit.[6-8] It is therefore likely that these fragments are independently folded structural domains involved in the binding of ATP.

The predominant cleavage pattern observed in the limited proteolysis experiments of the *E. coli* CPS enzyme is not altered in the presence of substrates or allosteric ligands. Ornithine, MgATP, UMP and IMP kinetically stabilize the enzyme against proteolysis by trypsin but the same sized peptides are eventually produced. Glutamine and HCO_3^- do not provide any protection against proteolysis. The substantial decrease in the rate of inactivation induced by the various proteases is directly correlated with a decrease in the rate of degradation of the intact large subunit. There is previous literature evidence to suggest that UMP, IMP and possibly ornithine bind to the extreme carboxy-terminal region of the large subunit.[12-14] The direct binding of these allosteric effectors to the domain that contains the most labile proteolytic sites must substantially alter the conformation of these regions within the large subunit to the point where these exposed sites are no longer available to the proteases.

The small subunit is quite resistant to cleavage by the various proteases until the large subunit is nearly fully digested. This result demonstrates that the large subunit protects the smaller subunit from cleavage during the initial stages of incubation with the proteolytic enzymes. The hydrolysis of the isolated small subunit occurs at a rate that is significantly faster than the proteolytic cleavage of the intact heterodimer. A reasonably stable 31-kDa fragment is generated and sequence analysis indicates that the amino-terminal end of this peptide starts with Asp-94. Approximately 10% of the original glutaminase activity is retained with this fragment supporting previous suggestions that the carboxy-terminal half of the small amidotransferase subunit (382 residues) contains all of the catalytic machinery required for the hydrolysis of glutamine.[15-17]

Endoproteinase Asp-N has been shown to generate a fragment of the large subunit (or a group of fragments) of the *E. coli* CPS that still contains substantial enzymatic activity. This is the first time that a truncated version of the large subunit has exhibited some catalytic activity. The changes in the kinetic constants, V_{max} and K_m, in the presence of the allosteric ligands IMP, UMP and ornithine, were measured in an attempt to determine whether these compounds could still exert an effect on the kinetic parameters of the enzyme. However, essentially all of the allosteric control is lost after incubation of the native enzyme with the endoproteinase Asp-N enzyme (see Table 2). These studies demonstrate that a portion of the C-terminal end of the large subunit can be excised without destroying the ability of CPS to synthesize carbamoyl phosphate. However, this region of the large subunit must contain the binding sites for the binding of the allosteric ligands. This domain can be trimmed to create a modified protein that is no longer under allosteric control. Experiments are in progress to genetically create truncated mutants of defined length and site-directed changes at conserved residues in order to more closely identify the specific allosteric binding sites of CPS.

The studies carried out here have identified structural and functional domains of CPS which are generated by limited proteolysis. The carboxy-terminal end has been found to be quite susceptible to proteolytic cleavage. Initial cleavage at this end of the large subunit generates inactive fragments. The fragments obtained from proteolysis by trypsin, 35- and 45-kDa species, are derived from regions of the large subunit which are believed to bind MgATP. However, as an exception, proteolysis catalyzed by endoproteinase Asp-N has generated an active peptide (or active peptides) of approximately 115 kDa that is devoid of the allosteric control features of the native enzyme.

Experimental

Materials

Pyruvate kinase, lactate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, trypsin, ornithine transcarbamylase and other assay chemicals were obtained from Sigma. The proteases, chymotrypsin, subtilisin and endoproteinase Asp-N were purchased from Boehringer Mannheim. Wild type carbamoyl phosphate synthetase was purified from *E. coli* XL1-Blue cells carrying the plasmid pDP412 as previously described.[18] The purified protein was stored at −70 °C until required.

Determination of enzymatic activity

An indirect method of determining carbamoyl phosphate synthesis was used. This procedure involved measuring the formation of MgADP using a pyruvate kinase/lactate dehydrogenase (PK/LDH) coupling system. Each 2.5 mL reaction mixture contained 50 mM HEPES, pH 7.5, 1 mM phosphoenol pyruvate (PEP), 100 mM KCl, 0.2 mM NADH, 10 mM ornithine, 20 mM $MgCl_2$, 2 mM ATP, 10 mM glutamine, 40 mM $NaHCO_3$, 20 units PK and 30 units LDH. After the addition of 10–40 μg of CPS, the reaction was monitored spectrophotometrically at 340 nm. For determination of the bicarbonate-dependent ATPase reaction, the formation of MgADP was measured using the PK/LDH coupled system with the omission of glutamine from the reaction mixture.

For the determination of MgATP synthesis from carbamoyl phosphate and MgADP, the reaction was followed using a hexokinase/glucose-6-phosphate dehydrogenase (HK/G-6-PDH) coupling system. Each 2.0 mL reaction mixture contained 50 mM glycylglycine, pH 7.6, 3 mM ADP, 0.75 mM NAD, 15 mM magnesium acetate, 1.0 mM glucose, 7.6 mM carbamoyl phosphate, 100 mM KCl, 20 units HK and 10 units G-6-PDH. After the addition of 20–40 μg of CPS, the reaction was monitored spectrophotometrically at 340 nm.

Kinetic data were fitted to equation 2 using the computer programs obtained from Savanna Shell

Software. In this equation, v is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis constant, and [S] is the substrate concentration. In some cases, variable concentrations of ATP and ADP were utilized in order to determine the effect of allosteric effectors on the binding of the nucleotides. Saturating concentrations of ornithine (10 mM), UMP (100 μM) and IMP (1 mM) were added separately to the assay mixtures to determine their effects on the Michaelis constant of the nucleotides.

$$v = (V_{max}\,[S]) / (K_m + [S]) \qquad (2)$$

Limited Proteolysis of CPS

Trypsin. Purified CPS was incubated at 25 °C with trypsin at ratios of approximately 1:10 (w/w; protease:substrate) in 60 mM HEPES, pH 7.6. At specified intervals, aliquots were removed from the digestion reaction and were immediately inhibited by the addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 10 mM. The samples were then added to SDS-polyacrylamide electrophoresis solubilization buffer (70 mM Tris–Cl, pH 6.8, 2.5% SDS, 0.02% Bromophenol Blue and 5% β-mercaptoethanol), placed in boiling water for 5 min, and then subjected to SDS-polyacrylamide gel electrophoresis.

Chymotrypsin. CPS was incubated with chymotrypsin at 25 °C at protease:substrate ratios of approximately 1:30 (w/w) in 60 mM HEPES, pH 7.6. Aliquots were collected and inhibited by the addition of PMSF and the samples were analyzed as described above.

Subtilisin. Digestion reactions were conducted by incubating CPS with subtilisin at 25 °C in 60 mM HEPES, pH 7.6 (1:20 ratio; protease:substrate; w/w). Aliquots were quenched by the addition of 10 mM PMSF and analyzed by SDS-polyacrylamide gel electrophoresis.

Endoproteinase Asp-N. Incubation of CPS with this protease was carried out in the absence of EDTA. Protease to substrate ratios of 1:3000 (w/w) were used for the hydrolysis at 37°C. Reaction aliquots were inhibited by the addition of 10 mM EDTA.

Inactivation of the native enzyme was followed concurrently by activity assays on aliquots as a function of time. Protection studies were carried out in the presence of each of the following substrates and allosteric effectors: 5 mM ornithine, 5 mM MgATP, 50 μM UMP, 0.5 mM IMP, 5 mM glutamine and 40 mM bicarbonate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Digestion samples were prepared for SDS-PAGE analysis and either 8% or 10% gels were run according to the procedure of Laemmli.[19] The gels were stained in 0.1% Coomassie Brilliant Blue R-250 (Sigma) in 50% w/v trichloroacetic acid. Destaining was carried out overnight in 7% acetic acid. In cases where samples were required for amino-terminal end sequencing, modifications in the procedure were utilized. Pre-electrophoresis of the gel for 30 min at 50 V was carried out in the presence of 0.2 mM thioglycolate (upper buffer) to help keep the amino-terminal end from being blocked. Samples were denatured in 1% SDS and incubated at 37 °C for 1 h (or at 65 °C for 15 min). After electrophoresis, the protein was either electroblotted on to membranes, or stained as described above before excising and electroeluting.

Electroblotting of protein on to membranes

Electroblotting with ProBlott (Applied Biosystems) was performed by the method of Matsudaira.[20] After electrophoresis, electroblotting was carried out in the transfer buffer (10 mM CAPS, pH 11, 10% methanol) for 16–20 h at 25 V. The membrane was then rinsed with deionized water, saturated with 100% methanol for a few seconds and stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol:1% acetic acid for 1 min. Destaining was performed with 50% methanol and the membrane was extensively rinsed with deionized water, air-dried and stored at –20 °C.

Electroelution of protein samples

CPS proteolytic fragments were isolated from preparative gels (1.5 mm slab gels) run according to the procedure of Laemmli[19] and were electroeluted using the procedure of Hunkapiller *et al.*[21] The larger protein fragments were run on 5% gels to obtain optimal separation and smaller fragments were run on 8% or 10% gels. Electroelution was carried out using a BioRad Model 422 Electro-Eluter for 12 h at 60 V. The protein elution buffer contained 50 mM NH_4HCO_3 and 0.1% SDS.

Amino acid sequence determination

Edman degradation was carried out by the Biotechnology Support Laboratory, Texas A & M University, with an Applied Biosystems model 473A Gas Phase Protein Sequencer.

Acknowledgement

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