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# Amino acid sequence of the catalytic subunit of aspartate transcarbamoylase from *Escherichia coli*

(tryptic peptides/proteinase C peptides/cyanogen bromide fragments/Edman degradation/HPLC)

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**ABSTRACT** **We propose a primary structure for the catalytic subunit of aspartate transcarbamoylase (aspartate carbamoyltransferase; carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli* based on amino acid sequences of fragments obtained by cyanogen bromide cleavage, by tryptic digestion of the succinylated polypeptide, and by chymotryptic and proteinase C digestion of the intact catalytic chain. The protein contains 310 amino acids and has a calculated molecular weight of 33,944. The negatively and positively charged residues are distributed uniformly, and there is no indication of charge clustering in the linear sequence.**

Aspartyl transcarbamoylase (aspartate carbamoyltransferase; carbamoylphosphate:L-aspartate carbamoyltransferase, LC 2.1.3.2) from *Escherichia coli* is the enzyme that catalyzes the rate-controlling step in the biosynthesis of pyrimidines. The holoenzyme is composed of equimolar quantities of two different polypeptide chains, and the intact enzyme exhibits allosteric properties with both homo- and heterotropic interactions (for reviews see refs. 1–4). In the native enzyme, the catalytic and the regulatory chains, designated c and r chains, respectively, are arranged in a highly ordered structure, which dissociates reversibly to give trimers of the catalytic chain ($c_3$) and dimers ($r_2$) of the regulatory chains (5). The dissociated subunits retain their functional activities; the catalytic subunit ($c_3$) converts aspartate to carbamoyl aspartate, and the regulatory subunit ($r_2$) binds ATP and CTP, modulating the enzymatic activity in a reciprocal fashion (6). The native enzyme has a pseudodyad axis of symmetry and can be written as $2(c_3)\cdot 3(r_2)$, a formula consistent both with the observed molecular weight (7) and results of x-ray diffraction analysis (8). Although extensive work has been done on the mechanism of action, comparatively few studies have attempted to correlate structure and function (9–13). Up to this time, such studies have been hampered by the lack of sequence data on the c chain. The amino acid sequence of the r chain has been established (7), and a tertiary structure of the regulatory subunits has been proposed at the level of 3-Å resolution (14, 15). The primary structure of the c chain, reported here, is crucial for detailed interpretation of x-ray diffraction data, and its availability should speed the formulation of a three-dimensional model for the native enzyme at atomic resolution. In addition, it should now be possible to interpret more fully the results obtained after chemical modification of the intact enzyme in terms of structure–function relationships.

## MATERIALS AND METHODS

Aspartate transcarbamoylase was isolated by using the procedures of Gerhart and Holoubek (16) from a partially diploid, uracil-requiring mutant of *E. coli*, which was developed for enhanced aspartate transcarbamoylase production by these authors. The c chain, prepared as described (16), gave a single Coomassie blue-staining band and was judged to have a minimum purity of 97%.

The purified c chain was dissolved in 8 M guanidine hydrochloride, buffered with 0.1 M Tris·HCl (pH 8.0), and allowed to stand at 55°C in the presence of 20 mM dithiothreitol for 30 min. A 2-fold molar excess of iodo[$^{14}$C]acetamide was added, and the alkylation was allowed to proceed for 15 min at 37°C. The reagent and guanidine hydrochloride were removed by dialysis, and the carboxamidomethylated protein was lyophilized. Cyanogen bromide cleavage of [$^{14}$C]carboxamidomethylated c chain was carried out as described (17). The resulting fragments were separated, first by gel filtration on Sephadex G-100 in 50% acetic acid and then by HPLC on a Waters C-18 μBondapak column with gradient elution in which the first solvent was 0.1% trifluoroacetic acid and the second solvent was acetonitrile/0.1% trifluoroacetic acid, 60:40 (vol/vol).

Tryptic digestion was carried out on [$^{14}$C]carboxamidomethylated c chain and on [$^{14}$C]succinylated c chain under the following conditions: protein concentration, 3 mg/ml in 0.05 M $NH_4HCO_3$ (pH 8.5); trypsin-to-protein ratio, 1:100 by weight. The digestion was carried out at 37°C for 6 hr. After lyophilization, peptides from the digested, succinylated c chain were separated first by chromatography on DEAE-cellulose (see Fig. 3), then by gel filtration on Sephadex G-50, and finally, when required, by preparative paper electrophoresis.

Peptides obtained by proteinase C digestion of [$^{14}$C]carboxamidomethyl c chain were separated by HPLC by using the same conditions described for the cyanogen bromide fragments. Digestions of the carboxamidomethyl c chain with other enzymes were carried out under standard conditions (18), and separations were performed by gel filtration, ion-exchange chromatography, and HPLC. Dansyl–Edman degradations and carboxypeptidase A and B digestions were done in the usual way (19, 20). Automatic sequence determination of the intact c chain was carried out on a Beckman 890C sequenator using the Quadrol program (21), and the resulting phenylthiohydantoin derivatives were identified either by gas chromatography or by amino acid analysis after hydrolysis with hydroiodic acid for 18 hr at 130°C.

## RESULTS AND DISCUSSION

The primary structure proposed for the c chain of aspartate transcarbamoylase (Fig. 1) represents a compilation of sequence data collected on cyanogen bromide fragments, on tryptic peptides obtained from succinylated c chain, and on proteinase C, chymotrypsin, and *Staphylococcus aureus* protease diges-

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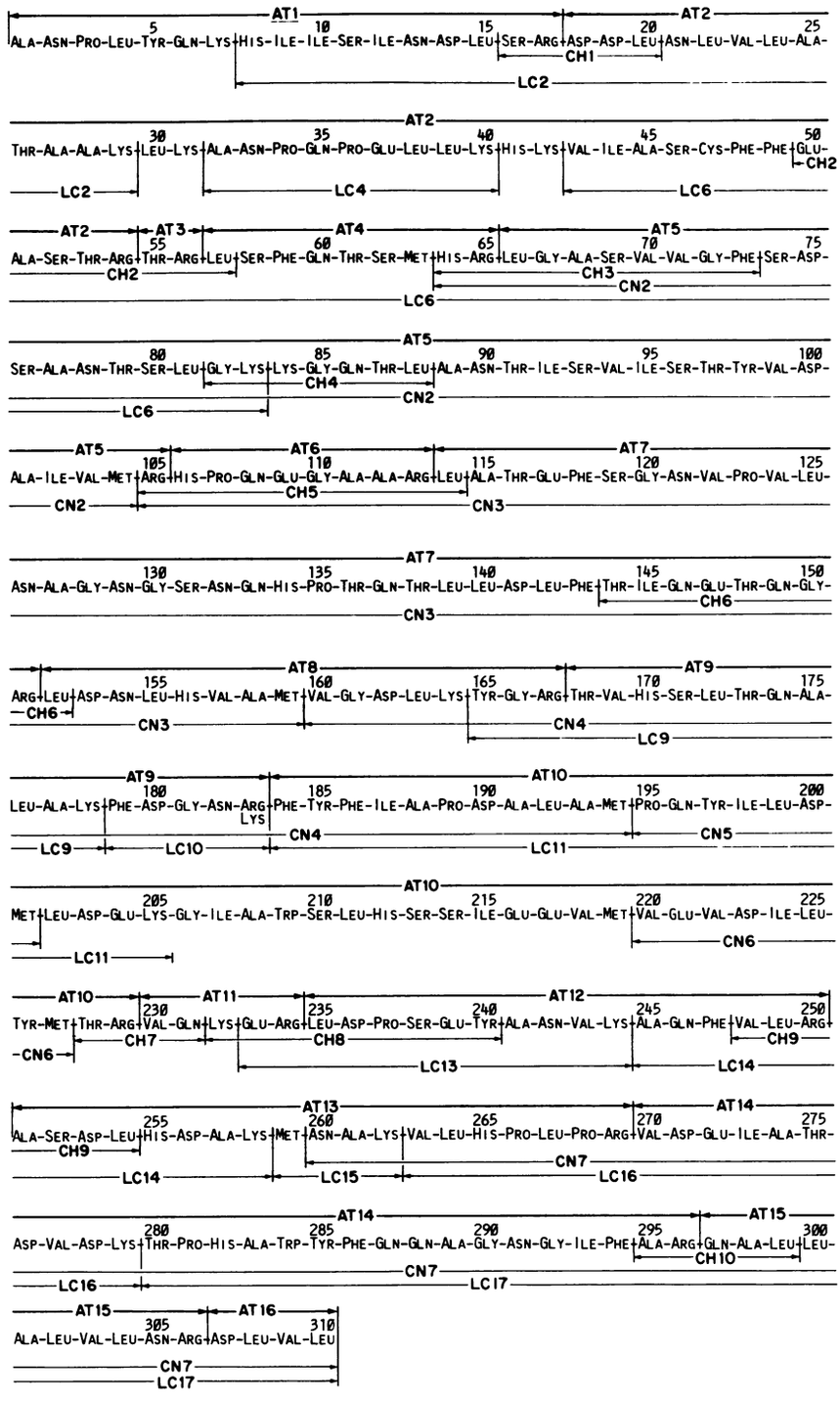


FIG. 1. Amino acid sequence of the c chain of aspartate transcarbamoylase. AT, peptides obtained after tryptic digestion of succinylated c chain; CH, peptides isolated after chymotryptic digestion of succinylated c chain; LC, peptides isolated after proteinase C digestion of carboxamidomethylated c chain; CN, peptides obtained after cyanogen bromide cleavage of the c chain.

tion fragments of carboxamidomethyl c chain. In addition, automatic sequence determination of the c chain prior to proteolytic or chemical cleavage gave sequence information for the first 39 residues of the polypeptide. Overlap peptides were obtained from chymotryptic and proteinase C digests and from cyanogen bromide cleavage, which allowed the tryptic peptides of the succinylated c chain to be ordered unambiguously as shown in Fig. 1.

The strategy used in determining the sequence is summarized in Fig. 2. The decision to use trypsin for cleavage of succinylated c chain was based on finding 13 arginine residues in the c chain, which we anticipated would yield a small enough number of peptides to permit their isolation in pure form and in high yield. All of the tryptic peptides expected from the succinylated c chains were isolated in sufficient quantity to be used as the main source for obtaining the primary structure of the catalytic polypeptide. We concluded that all of the residues in the c chain are accounted for because (*i*) the sum of the compositions of the tryptic peptides (Table 1) agrees with the previously determined amino acid composition of the intact c chain (22, 23), (*ii*) the overlap peptides span large enough regions on both sides of the arginine residues to provide assurance that no tryptic peptides have been missed, (*iii*) all of the peptides, which were isolated from a number of different digests of the c chain, are consistent with the sequence shown in Fig. 1, and (*iv*) the primary structure (with the exception of amide assignments)

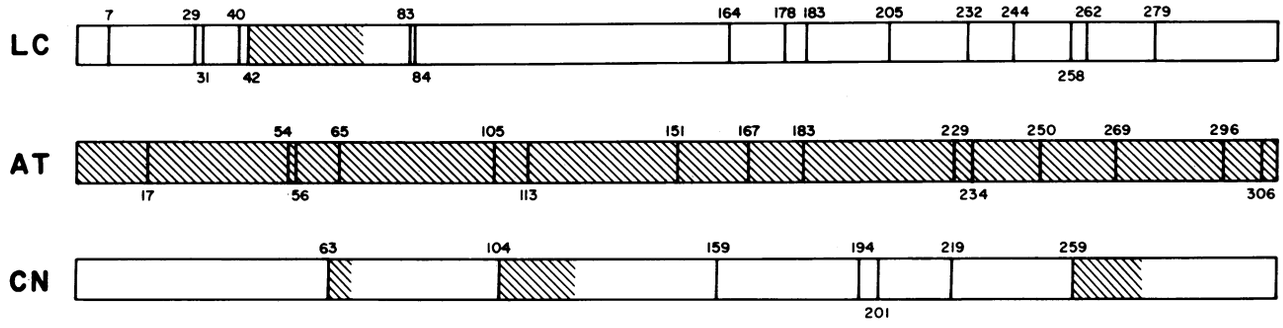


FIG. 2. Summary of the distribution and location of fragments generated by proteinase C on the c chain (LC), trypsin on succinylated c chain (AT), and cyanogen bromide cleavage of the c chain (CN). The vertical lines denote cleavage sites, and the numbers indicate the positions of the cleavage sites in the polypeptide chain. The hatched areas indicate regions where the sequence of the fragments was determined by Edman degradation and carboxypeptidase digestion.

agrees with the recently determined nucleotide sequence of the *pyrB* gene (24).

The detailed evidence for the structures of the tryptic peptides will be documented elsewhere, but the approach involved either direct automated sequence determination or fragmentation of the larger peptides with enzymes such as chymotrypsin, thermolysin, and *Staphylococcus aureus* protease prior to sequence assay. After separation by ion-exchange chromatography or paper electrophoresis, the sequences of the smaller peptides were determined by dansyl–Edman degradation and carboxypeptidase A and B digestion. Glutamine and asparagine assignments were made on the basis of the electrophoretic mobility of their respective peptides on paper or cellulose thin-layer plates (25). Because of the ever present possibility of deamidation either during the preparation of the protein or during the isolation of the peptides, the amide assignments have been considered to be tentative. They should be compared and corrected to correspond with the structure derived from the sequence of the *pyrB* structural gene (24).

The initial fractionation of the tryptic peptides was carried out by chromatography on DEAE-cellulose (DE-52), which provided a partial separation (Fig. 3). Further fractionation was achieved by gel filtration on Sephadex G-50, and, in some cases, by semipreparative electrophoresis on paper or cellulose thin-layer plates at pH 8.6 and pH 5.6. The amino acid composition, elution position from DE-52, yield, and location in the polypeptide chain are given in Table 1. In a separate experiment, a proteinase C digest of the c chain was separated by HPLC as shown in Fig. 4. Because proteinase C cleaves at lysine but not at arginine residues, a number of overlap peptides were obtained, which are depicted in Fig. 1. As with the tryptic peptides, the relevant data on the proteinase C peptides are listed in Table 2.

On the basis of the proposed structure, a molecular weight of 33,944 can be calculated, which is in good agreement with the value estimated by the mobility of the c chain in $NaDodSO_4$

Table 1. Amino acid composition of tryptic peptides (AT) from the c chain of succinylated ATCase

| | Composition, by pool (Fig. 3) and peptide | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 8 AT1 | 9 AT2 | 1 AT3 | 2 AT4 | 9 AT5 | 3 AT6 | 6 AT7 | 6 AT8 | 7 AT9 | 10 AT10* | 2 AT11 | 4 AT12 | 5 AT13 | 9 AT14* | 3 AT15 | 3 AT16 |
| Lysine | 0.9 | 4.1 | | | 2.2 | | | 1.1 | 1.5 | 0.9 | 1.0 | 1.1 | 2.0 | 1.1 | | |
| Histidine | 0.9 | 0.8 | | 1.0 | | 0.9 | 1.1 | 1.1 | 0.9 | 1.1 | | | 1.8 | 1.2 | | |
| Arginine | 1.1 | 1.0 | 1.1 | 1.0 | 0.9 | 1.0 | 1.0 | 0.9 | 0.6 | 1.2 | 1.0 | 0.9 | 0.9 | 0.8 | 1.0 | |
| CM cysteine | | 1.1 | | | | | | | | | | | | | | |
| Aspartic acid | 3.1 | 3.8 | | | 3.8 | | 4.8 | 2.9 | 1.9 | 3.8 | | 1.8 | 2.7 | 3.9 | 1.1 | 1.0 |
| Threonine | | 2 | 0.9 | 0.9 | 4.0 | | 4.8 | | 2.1 | 0.9 | | | | 2.1 | | |
| Serine | 2.1 | 2.1 | | 2.1 | 6.2 | | 2.1 | | 1.1 | 3.4 | | 1.1 | 1.1 | | | |
| Glutamic acid | 0.9 | 2.9 | | 0.9 | 0.8 | 1.8 | 6.2 | | 0.8 | 3.7 | 2.1 | 1.9 | | 2.9 | 1.0 | |
| Proline | 1.2 | 2.1 | | | | 1.1 | 2.1 | | | 2.2 | | 1.1 | 2.1 | 1.1 | | |
| Glycine | 0.4 | | | | 3.8 | 0.9 | 4.2 | 2.1 | 1.1 | 1.4 | | | | 2.2 | | |
| Alanine | 1.0 | 6.3 | | | 4.1 | 2.0 | 2.0 | 1.2 | 2.0 | 3.8 | | 2.0 | 3.0 | 4.0 | 2.0 | |
| Valine | | 1.8 | | | 4.7 | | 1.8 | 1.7 | 0.9 | 2.7 | 0.9 | 1.8 | 1.1 | 1.8 | 0.8 | 0.9 |
| Methionine | | | | 1.2 | 1.1 | | | 0.9 | | 3.6 | | | 0.9 | | | |
| Isoleucine | 2.8 | 0.7 | | | 2.8 | | 0.7 | | | 4.6 | | | | 1.7 | | |
| Leucine | 2.1 | 6.2 | | 1.0 | 2.8 | | 5.2 | 3.0 | 2.0 | 5.6 | | 2.1 | 3.0 | | 4.1 | 2.0 |
| Tyrosine | 1.2 | | | | 0.7 | | | 0.7 | 0.8 | 2.5 | | 0.7 | | 0.9 | | |
| Phenylalanine | | 2.0 | | 0.9 | 0.9 | | 2.1 | | | 1.7 | | 0.9 | | 2.1 | | |
| Total residues | 17 | 37 | 2 | 9 | 40 | 8 | 38 | 16 | 16 | 46 | 5 | 16 | 19 | 27 | 10 | 4 |
| % yield | 35 | 55 | 90 | 85 | 60 | 80 | 45 | 70 | 10 | 10 | 90 | 75 | 70 | 60 | 50 | 85 |
| Location | (1) | (18) | (55) | (57) | (66) | (106) | (114) | (152) | (168) | (184) | (230) | (235) | (241) | (270) | (297) | (307) |

ATCase, aspartate transcarbamoylase. Values are expressed as residues per mol. The peptides were obtained from the pooled fractions after DEAE-cellulose column chromatography (Fig. 3) and repurified as described. The peptides, designated AT, are both numbered and listed in the order that they appear in the polypeptide chain. The percentage yield is based on the amount of the peptide (estimated by amino acid analysis of a suitable aliquot) to have been present in the fractions from the DE-52 column before repurification. The location of each peptide is indicated by the position of the $NH_2$-terminal residue of each tryptic peptide in the protein. Peptide impurities of 0.3 residues or less were omitted for simplicity in reading the table. The peptides with an asterisk contain one residue of tryptophan as determined spectrophotometrically.

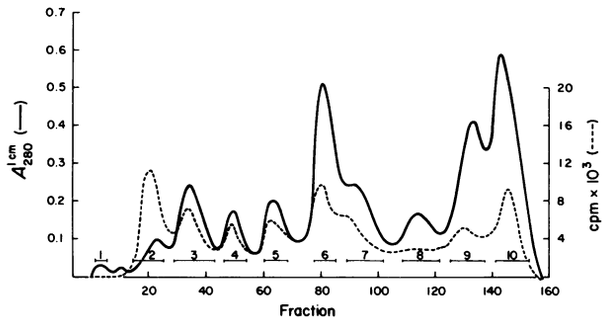


FIG. 3. Elution pattern of a tryptic digest of [$^{14}$C]-succinylated c chain from a 30 × 1.5 cm column of DEAE-cellulose (DE-52). The peptides were eluted with a gradient of increasing chloride ion concentration in a buffer of 0.1 M Tris·HCl maintained at pH 8.2. The chloride ion concentration was varied from 0.1 M to 1.0 M. Fractions with absorbance at 280 nM and radioactivity were collected according to the location of the peaks, pooled, lyophilized, and desalted by gel filtration on Sephadex G-50 prior to subsequent fractionation.

gel electrophoresis and with other physicochemical estimates of the molecular weight of the catalytic polypeptide (26).

Prior to this study, sequences of small sections of the c chain had been determined in order to locate amino acid substitutions from mutant (27, 28) or chemically modified aspartate transcarbamoylases (9–13). The $NH_2$-terminal sequence of aspartate transcarbamoylase was compared with the corresponding region in ornithine transcarbamoylase from *E. coli*, and extensive homology was observed (29). The results presented here are in agreement with the partial sequences reported previously by us and by others (27–29) and with the primary structure derived from the nucleotide sequence of the *pyrB* gene (24).

Only one cysteine residue is present in the c chain, and it was found that this residue could be carboxymethylated fully only in strong denaturing solvents such as 8 M guanidine hydrochloride at 60°C, thus suggesting that the cysteine residue is buried in a highly stable, folded domain.

There does not appear to be any marked clustering of positively or negatively charged amino acid residues, nor are there long stretches of uninterrupted hydrophobic residues. Two features of the sequence however are worth noting. First, five of the nine histidine residues, all in the $NH_2$-terminal half of the molecule, occur adjacent to arginine or lysine. Second, position 183 can be occupied by either arginine or lysine. This was shown by the following results: (*i*) We isolated a tryptic peptide (AT9) spanning residues 168–183 (which has COOH-terminal arginine); (*ii*) we also obtained and partially determined the sequence of a tryptic peptide spanning residues 168–229, which had ε-succinyllysine at position 183 and clearly had not undergone tryptic cleavage at that position; and (*iii*) we isolated peptides LC9, LC10, and LC11 from a proteinase C digest of carboxamidomethylated c chain and obtained a low yield of a peptide LC10–LC11, which contained one arginine (position 183) and one lysine residue (position 205). All of these data are consistent with dual occupancy at position 183. The simplest explanation for this finding is that the stock of *E. coli* cells used for large-scale production of the enzyme was a mixture of two genotypes where the structural genes for the c chains differed by a single nucleotide.

Because of recent revisions that we have made in the amino acid sequence, residues that previously were identified by other workers as being near the active site can now be assigned to their correct position in the polypeptide chain. These revisions were the result of a reinvestigation, which was prompted by a discrepancy between our previously proposed structure and the nucleotide sequence of the *pyrB* gene, which was sent to us by J. Wild and co-workers in advance of publication. Specifically, we reisolated AT-10, a large succinylated tryptic peptide, cleaved it with CNBr, and isolated an eight-residue segment spanning residues 220–227, a section that we had previously missed. We subjected this peptide to Edman degradation and found that its primary structure was the same as the one predicted from the DNA sequence (24). With this finding, the primary structure of the c chain is now congruent with the nucleotide sequence of the *pyrB* gene (24). The residues that previously were identified by chemical modification include cysteine-47 (9), lysine-84 (11), and tyrosine-165 and -240 (13).

X-ray crystallographic studies on aspartate transcarbamoylase, which have been carried out in W. N. Lipscomb's laboratory, have provided electron density maps at a resolution of

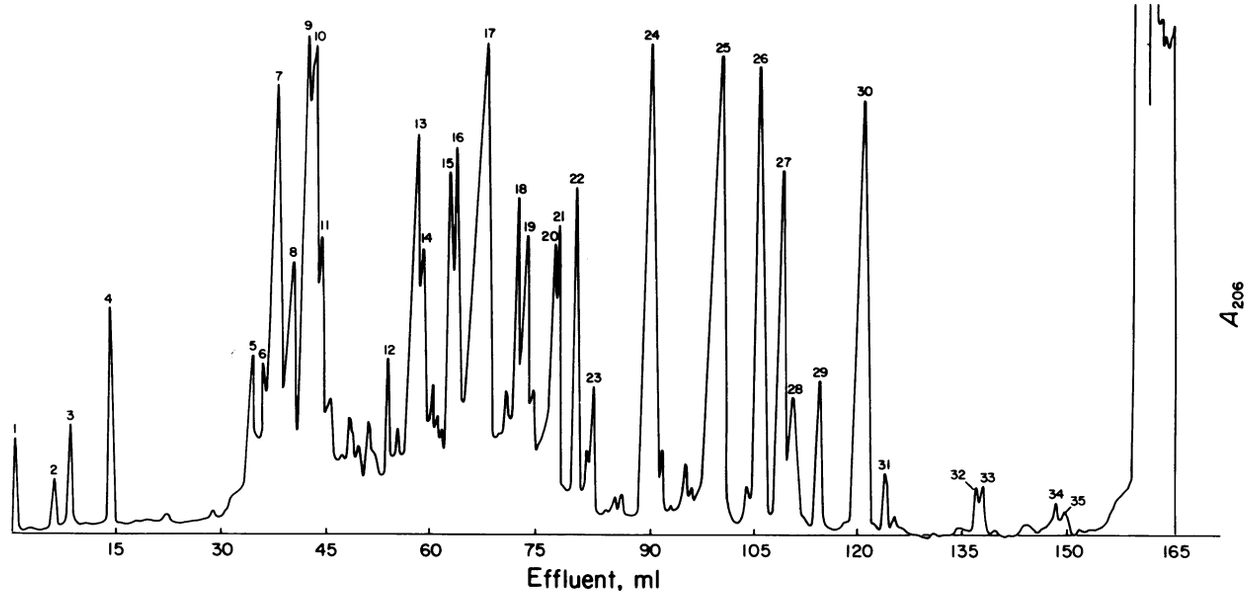


FIG. 4. HPLC of a proteinase C digest of [$^{14}$C]carboxamidomethylated c chain. The sample (50 nmol) was applied to a Waters $C_{18}$ μ-Bondapak column in 0.1% trifluoroacetic acid. The column was developed with a gradient ranging from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid. Peak fractions were collected, pooled, and lyophilized prior to hydrolysis or Edman degradation.

Table 2. Amino acid composition of peptides (LC) from a proteinase C digest of the c chain of ATCase

| | Composition, by peak (Fig. 4) and peptide | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 17 LC2 | 26 LC4 | 20 LC6 | 30 LC9 | 5 LC10 | 22 LC11 | 25 LC13 | 24 LC14 | 6 LC15 | 27 LC16 | 9 LC17* |
| Lysine | 0.9 | 0.8 | 1.2 | 1.2 | 0.8 | 1.1 | 1.1 | 0.9 | 1.0 | 1.2 | |
| Histidine | 0.9 | | 0.9 | 0.9 | | | | 1.1 | | 0.8 | 0.9 |
| Arginine | 1.1 | | 2.8 | 0.9 | | | 0.9 | 1.0 | | 0.9 | 2.1 |
| CM cysteine | | | 0.9 | | | | | | | | |
| Aspartic acid | 5.2 | 1.0 | 1.9 | | 2.2 | 2.9 | 2.1 | 2.1 | 1.1 | 3.1 | 2.9 |
| Threonine | 0.9 | | 3.8 | 1.8 | | | | | | 0.9 | 0.9 |
| Serine | 2.1 | | 7.7 | 0.9 | | | 1.1 | 1.1 | | | 0.4 |
| Glutamic acid | | 2.1 | 2.1 | 1.0 | | 2.0 | 2.0 | 1.0 | | 1.0 | 3.1 |
| Proline | | 1.8 | | | | 2.2 | 0.8 | | | 2.1 | 1.1 |
| Glycine | 0.4 | | 3.1 | 1.1 | 1.1 | | | | | | 2.2 |
| Alanine | 3.0 | 1.1 | 4.2 | 2.0 | | 2.9 | 1.0 | 3.1 | 1.0 | 1.0 | 5.3 |
| Valine | 0.8 | | 2.7 | 0.9 | | | 0.8 | 0.9 | | 2.9 | 1.7 |
| Methionine | | | 0.9 | | | 1.7 | | | 0.9 | | |
| Isoleucine | 2.7 | | 0.9 | | | 1.8 | | | | 0.8 | 0.7 |
| Leucine | 4.2 | 2.0 | 3.0 | 2.0 | | 3.0 | 1.0 | 2.0 | | 2.0 | 5.7 |
| Tyrosine | | | | 0.9 | | 1.8 | 0.9 | | | | 0.9 |
| Phenylalanine | | | 3.7 | | 1.2 | 1.9 | | 0.9 | | | 1.9 |
| Total residues | 22 | 9 | 41 | 14 | 5 | 22 | 12 | 14 | 4 | 17 | 31 |
| % yield | 40 | 55 | 40 | 50 | 15 | 10 | 65 | 60 | 70 | 15 | 40 |
| Location | (8) | (32) | (43) | (165) | (179) | (184) | (233) | (245) | (259) | (263) | (280) |

ATCase, aspartate transcarbamoylase. Values are expressed as residues per mol. The peptides, designated LC, correspond to the peaks obtained from the HPLC separation of the digest (Fig. 4) and are numbered according to their position in the polypeptide. The percentage yield and the location of each peptide were determined and designated as in Table 1. The peptide with an asterisk contains one residue of tryptophan as determined spectrophotometrically.

about 3 Å. The x-ray work, which has been performed on the native enzyme both in the presence and absence of ligands and inhibitors (30), shows that although the fit between the crystallographic data and the chemical sequence of the r chains is in perfect agreement (9, 10), the electron-density maps and the chemically determined sequence of the c chain have not been matched completely as yet. Nevertheless, it is still possible to place certain regions of the amino acid sequence within the domains described by Honzatko *et al.* (31). This has been done in fact by Hoover *et al.* in the accompanying paper (24). The information obtained from the structural studies still needs to be correlated with the extensive enzymatic and physicochemical work that has been completed; taken together, the results should provide the basis for a coherent model for the mechanism of action of this important, multimeric, regulatory enzyme.

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