Aspartate Transcarbamylase. Amino-Terminal Analyses and Peptide Maps of the Subunits*

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ABSTRACT: The amino-terminal residues of the subunits of *Escherichia coli* aspartate transcarbamylase have been determined by the cyanate method. The regulatory subunit has 0.93 mole of threonine/2.7 × 10^4 g and the catalytic subunit 1.92 moles of alanine/1.0 × 10^5 g. Two-dimensional maps show the approximate number of tryptic peptides to be expected from a single chain in the case of the regulatory subunit and from a dimer of two identical chains in the case of the catalytic subunit. The present evidence strongly suggests that there are four each of the two different kinds of peptide chains in the native enzyme (molecular weight 3.1 × 10^5). Taken together with the data of Changeux *et al.* (Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1967), *in* Regulation of Nucleic Acid and Protein Biosynthesis, Koningsberger, V. V., and Bosch, L., Ed., New York, N. Y., Elsevier, p 344) our results indicate that each of the binding sites for succinate (a competitive inhibitor of aspartatate) and cytidine triphosphate resides on a separate chain.

The aspartate transcarbamylase of *Escherichia coli* can be dissociated into catalytic subunits, which are capable of binding the substrates and stimulating the synthesis of carbamyl aspartate, and regulatory subunits, devoid of catalytic activity but fully able to bind the allosteric effector cytidine triphosphate (Gerhart and Schachman, 1965). The native enzyme, molecular weight 3.1 × 10^5, has been shown recently to have 3.9 sites for succinate (a competitive inhibitor of aspartate) and 4.0 sites for cytidine triphosphate; it is composed of two catalytic subunits, molecular weight 1.0 × 10^5, each of which can bind 1.8 molecules of succinate, and four regulatory subunits, molecular weight 2.7 × 10^4, each with a single site for cytidine triphosphate (Changeux *et al.*, 1967). Since the quaternary structure of allosteric enzymes has been postulated to play a central role in the detailed mechanism of regulation by feedback inhibition (Monod *et al.*, 1965), it seemed important to confirm by chemical means what is suggested by the data on binding sites and molecular weights; that is, that native aspartate transcarbamylase contains four each of two different kinds of polypeptide chains. The quantitative data reported in this communication are in full accord with such a model.

Materials and Methods

Enzyme. Aspartate transcarbamylase was prepared and dissociated into subunits according to the methods of Gerhart and Holoubek (1967). The derepressed mutant of *E. coli* described by those authors was provided through the kindness of Dr. Gerhart. The separated subunits gave amino acid analyses identical with those reported by Changeux and Gerhart (1967), within experimental error. The catalytic subunit and native enzyme had specific activities equivalent to or slightly greater than those of the pure preparations described by Gerhart and Holoubek (1967). The regulatory subunit was devoid of activity.

Determination of Enzymatic Activity. Samples containing from 0.02 to 0.4 μg of pure enzyme (or equivalent activity) were added to 1.0 ml of a solution containing 1 × 10^{-2} M aspartate, 5 × 10^{-3} M dilithium carbamyl phosphate, and 5 × 10^{-2} M Tris-acetate buffer (pH 8.0). After 10 min at 28°, the reaction was stopped by adding 1.0 ml of 0.5 M acetic acid. Carbamyl aspartate was determined according to Hunninghake and Grisolia (1966).

Amino-Terminal Analyses. The cyanate method of Stark and Smyth (1963) was used, including several minor alterations in procedure described recently by Stark (1967). Precautions were observed to avoid the destruction of tryptophan and separate analyses were performed to test for the presence of amino-terminal cystine or cysteine. Carbamylations were carried out in 8 M urea and carbamyl proteins were desalted by dialysis. A stock solution containing 11.4 mg of carbamyl regulatory subunit or 33 mg of carbamyl catalytic subunit was prepared and the concentrations were determined accurately by amino acid analysis. The same analyses gave the ratios of lysine and homocitrulline expected of proteins in which carbamylation of the ε-amino groups is complete (Stark and Smyth, 1963), providing assurance that the carbamylation of the α-amino groups was also complete. One-fifth of each stock solution was used for each determination.

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TABLE I: Amino-Terminal Analyses of the Regulatory Subunit.

	Amino-Terminal Residues (moles/27,000 g)								
	Carbamylated Enzyme				Blank			Sample − Blank	
Amino Acid	1[a]	2	3	Best Value	1	2	Best Value	Uncor	Cor[b]
Lysine	0.02	0.05	0.03	0.03	0.03	0.03	0.03	0	0
Histidine	0.48[c]	0.09	0.08	0.09	0.07	0.12	0.09	0	0
Arginine	0.28[c]	0.08	0.14	0.11	0.12	0.10	0.11	0	0
Aspartic acid	0.29	0.26	0.19	0.25	0.04	0.11	0.08	0.17	0.18
Threonine	0.32	0.35	0.24	0.30	0.01	0.02	0.02	0.28	0.93
Serine	0.04	0.07	0.06	0.06	0.03	0.07	0.05	0.01	0.05
Glutamic acid		0.18	0.08	0.13	0.12	0.13	0.13	0	0
Proline	0	0	0	0	0	0	0	0	0
Glycine	0.39	0.27	0.29	0.32	0.10	0.14	0.12	(0.20)[e]	0
Alanine	0.03	0.05	0.06	0.05	0.02	0.04	0.03	0.02	0.02
Valine	0.06[c]	0	0	0	0	0	0	0	0
Methionine	0	0.02[c]	0	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0	0	0
Leucine	0	0	0	0	0	0	0	0	0
Tyrosine	0	0	0	0	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0	0	0	0
Cysteic acid				0[d]				0	0
Tryptophan	0	0	0		0	0	0	0	0

[a] Determined after performic acid oxidation of the hydantoin fraction A, before alkaline hydrolysis. Analysis of another portion of the same fraction without oxidation gave essentially the same result. [b] The correction factors of Stark and Smyth (1963) were used. [c] Not included in calculating the best value. [d] A separate determination for amino-terminal cystine and cysteine was performed according to the procedure of Stark (1967). [e] Glycine is formed during the alkaline hydrolysis of threoninehydantoin (Stark and Smyth, 1963).

Amino Acid Analyses. Hydrolyses were carried out with 6 M HCl or 0.2 M NaOH in evacuated, sealed tubes at 110°. Amino acid analyses were performed with a Spinco Model 120 B, modified for greater sensitivity with a long-path flow cell and range card (see Fruchter and Crestfield, 1965) so that 5–10 mμmoles of a single amino acid could be estimated accurately. Buffer flow rates were 100 ml/hr and Spinco AA 15 and PA 35 resins were used for 58- and 10-cm columns, respectively.

Peptide Maps. Tryptic digests of the subunits were prepared according to Rodbell and Fredrickson (1959), except that the reaction was carried out for 6 hr at 25°. The resulting peptides were desalted, then separated in two dimensions on paper, according to Helinski and Yanofsky (1962). Electrophoresis in pyridine–acetic acid–water (1:10:289) was followed by ascending chromatography in pyridine–butanol–acetic acid–water (24.4:37.8:7.6:30.2) (Weigert and Garen, 1965). The latter solvent system improves the resolution of highly charged peptides. Peptides containing tyrosine or arginine were detected according to Acher and Crocker (1952) or Helinski and Yanofsky (1962).

Results and Discussion

Amino-Terminal Analyses. The results are shown in Tables I and II for regulatory and catalytic subunits, respectively. In addition to the amino-terminal threonine of the regulatory subunit, small amounts of aspartic acid and glycine remain when the blank values are subtracted from those for the carbamyl protein. The glycine is expected; it is a hydrolysis product of threoninehydantoin and provides confirmation that the end group is indeed threonine (Stark and Smyth, 1963).

Gerhart and Holoubek (1967) have pointed out that the native enzyme, prepared according to their method, contains 2–5% of a slowly sedimenting component. We have noted small amounts of a contaminant in our own preparation by polyacrylamide gel electrophoresis. The amino-terminal aspartic acid found in the analysis of the regulatory subunit might correspond to the terminus of this low molecular weight contaminant if it were not separated from this subunit during purification. Since aspartic acid and methionine sulfoxide are not completely resolved under the conditions used for amino acid analysis, identification as aspartic

TABLE II: Amino-Terminal Analyses of the Catalytic Subunit.

	Amino-Terminal Residues (moles/100,000 g)									
	Carbamylated Enzyme					Blank			Sample − Blank	
Amino Acid	1[a]	2	3	4	Best Value	1	2	Best Value	Uncor	Cor[b]
Lysine	0.04	0.03			0.03	0.06		0.06	0	0
Histidine	0.61	0.09	0.33	0.19	0.20	0.24	0.25	0.25	0	0
Arginine	0.68	0.14	0.31	0.24	0.23	0.21	0.19	0.20	0.03	0.03
Aspartic acid	0.40	0.06	0.07	0.18	0.10	0.34[e]	0.09	0.09	0.01	0.01
Threonine	0.12	0.03	0.04	0.05	0.04	0	0.01	0.01	0.03	0.09
Serine	0.37	0.06	0.06	0.11	0.08	0.03	0.04	0.04	0.04	0.20
Glutamic acid	0.76	0.20	0.24	0.27	0.24	0.13	0.24	0.18	0.06	0.09
Proline	0	0	0	0	0	0	0	0	0	0
Glycine	0.61	0.12	0.18	0.36	0.22	0.15	0.11	0.13	0.09	0.09
Alanine	2.57	0.86[c]	1.92	1.96	1.94	0.05	0.07	0.06	1.88	1.92
Valine	0.05	0	0	0	0	0	0	0	0	0
Methionine	0.01	0	0	0	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0	0	0	0
Leucine	0.04	0	0.02	0.02	0.02	0	0	0	0.02	0.02
Tyrosine	0	0	0	0	0	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0	0	0	0	0
Cysteic acid					0[d]				0	0
Tryptophan	0	0			0	0		0	0	0

[a] This analysis is obviously contaminated and has not been used in calculating best values. It is included in the table because it does show approximately two residues of amino-terminal alanine. [b] The correction factors of Stark and Smyth (1963) were used. [c] The value for amino-terminal alanine was unaccountably low in this analysis and has not been used in further calculations. [d] A separate determination for amino-terminal cystine and cysteine was performed according to the procedure of Stark (1967). [e] Not included in calculating the best value.

acid was confirmed by oxidizing with performic acid a portion of the hydantoin fraction A in determination 3. The amount of aspartic acid remained essentially unchanged and only atr ace of methioninesulfone, which *is* completely resolved from aspartic acid, was observed in the oxidized sample.

A small amount of serine is seen in addition to the amino-terminal alanine of the catalytic subunit (Table II). The corrected value of about 0.1 residue/peptide chain has been derived from an uncorrected value of about 0.02 residue/chain. Such a small difference between sample and blank is probably not significant. The low values of threonine and glycine may reflect contamination of the catalytic subunits by a trace of regulatory subunit or native enzyme.

Peptide Maps. Figure 1A–B shows the peptide maps for the regulatory and catalytic subunits, respectively. The total number of peptides that contain tyrosine, lysine, and arginine is given in Table III and is compared with the number of each of these residues found in the complete amino acid analysis of the separate subunits (Changeux and Gerhart, 1968).

Since the catalytic subunit of molecular weight 1.0×10^5 contains 21 residues of lysine and 22 of arginine, the tryptic digest should contain 86 different peptides if the two chains terminated by alanine are different but only 43 if the chains are identical; 32 different peptides were found. In the case of the regulatory subunit, the sum of the number of residues of lysine and arginine is 29 for a molecular weight of 2.7×10^4 and 24 different peptides were found. The number of tryptic peptides observed is usually lower than the maximum number calculated from the amino acid analysis (see, for example, the results of Helinski and Yanofsky (1962) and Baldwin and Berg (1966)). This is to be expected for many reasons. For example, the tryptic digestion may not be complete, some peptides may be ninhydrin negative, some peptides may cochromatograph or remain at the origin during mapping, and some residues of lysine and arginine probably will occur in sequences that are not sensitive to trypsin, such as Lys · Pro.

Conclusions

Taken together, the molecular weights (Gerhart and Schachman, 1965), number of binding sites (Changeux *et al.*, 1967), amino-terminal analyses, and peptide maps indicate that each binding site for succinate or cytidine triphosphate resides on a single peptide chain of the appropriate subunit. Since precautions were

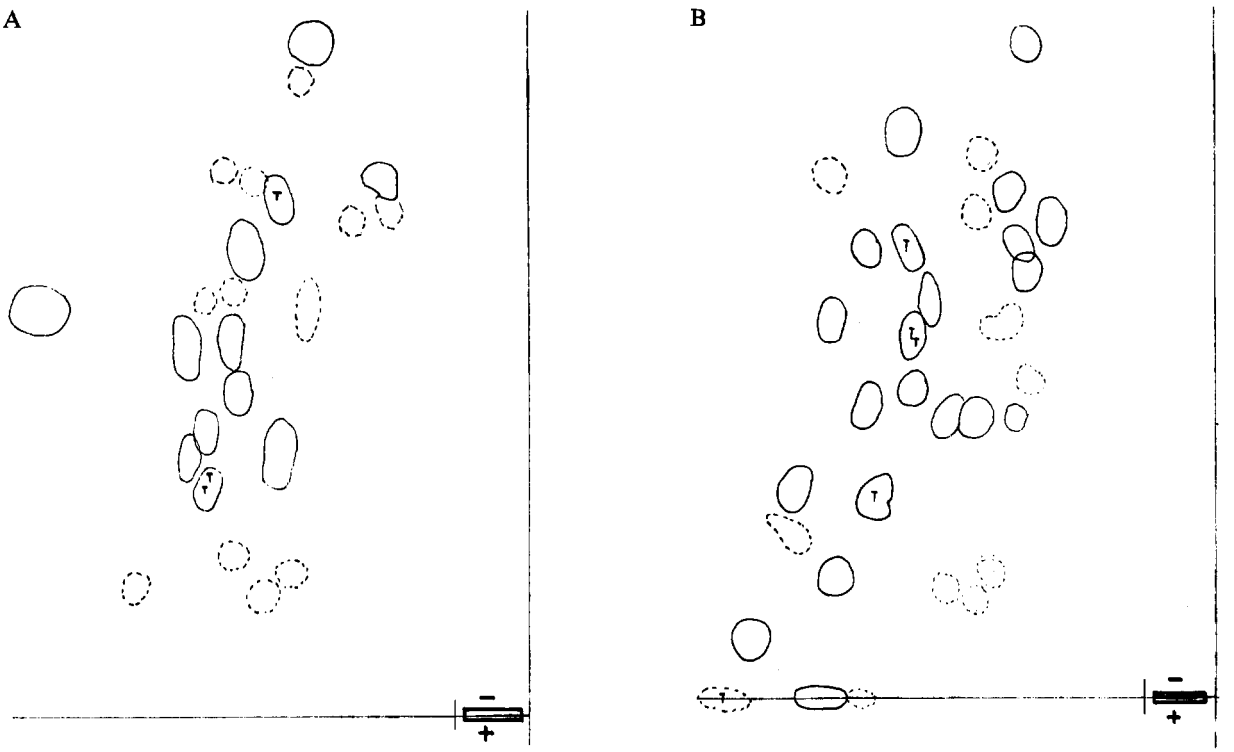

FIGURE 1: Maps of the tryptic peptides from the subunits of *E. coli* aspartate transcarbamylase. See the text for experimental details. Solid lines: strongly ninhydrin positive; dotted lines: weakly ninhydrin positive; T: positive for tyrosine; TT: strongly positive for tyrosine. (A) Regulatory subunit: 1 mg of protein was used in the digestion. (B) Catalytic subunit: 2 mg of protein was used in the digestion.

TABLE III: Comparison of the Number of Tryptic Peptides Containing Lysine, Arginine, and Tyrosine with the Number of Each of These Residues in Each Subunit.

Subunit	Residue	Residues/ Molecule, from Amino Acid Anal.[a]	No. of Peptides Containing Each Type of Residue
Regulatory, mol wt 2.7×10^4	Lysine + arginine	29	24[b]
	Arginine	12	9[c]
	Lysine	17	15
	Tyrosine	5	2
Catalytic, mol wt 1.0×10^5	Lysine + arginine	86	32[b]
	Arginine	44	14[c]
	Lysine	42	18
	Tyrosine	22	4

[a] Calculated from the data of Changeux and Gerhart (1967). [b] Sum of the spots in Figures 1A,B. [c] Determined in a separate experiment in which the peptides were stained for arginine (Sakaguchi reaction) rather than with ninhydrin.

taken to protect tryptophan during the formation of hydantoins and since no cysteic acid was found in the separate procedure used to detect amino-terminal cystine or cysteine, we can say with confidence that the catalytic subunit has no terminal amino group other than that of alanine and that the regulatory subunit has no terminal amino group other than that of threonine. The possibility remains that each subunit

may contain additional peptide chains terminated by an acylamino group or by a residue of pyrrolidonecarboxylic acid. These would have to be bound covalently to the chains with free amino termini by disulfide bridges or very firmly by noncovalent interactions. We consider these possibilities unlikely.

The catalytic subunit of molecular weight 1.0×10^5 appears to be a dimer of identical chains, each with molecular weight 5.0×10^4, a single amino-terminal alanine and a single binding site for succinate. In this regard, it is pertinent to note that the catalytic subunit dissociates into smaller units in denaturing solvents (Changeux *et al.*, 1967).

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References

Acher, R., and Crocker, C. (1952), *Biochim. Biophys. Acta 9*, 704.

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.

Changeux, J.-P., and Gerhart, J. C. (1968), *Biochemistry* (in press).

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1967), *in* Regulation of Nucleic Acid and Protein Biosynthesis, Koningsberger, V. V., and Bosch, L., Ed., New York, N. Y., Elsevier, p 344.

Fruchter, R. G., and Crestfield, A. M. (1965), *J. Biol. Chem. 240*, 3875.

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Helinski, D. R., and Yanofsky, C. (1962), *Biochim. Biophys. Acta 63*, 10.

Hunninghake, D., and Grisolia, S. (1966), *Anal. Biochem. 16*, 200.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Rodbell, M., and Fredrickson, D. S. (1959), *J. Biol. Chem. 234*, 562.

Stark, G. R. (1967), *Methods Enzymol. 11*, 125.

Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.

Weigert, M. G., and Garen, A. (1965), *J. Mol. Biol. 12*, 448.

Crystal Spectra of Some Ferric Hemoproteins*

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ABSTRACT: Polarized crystal spectra of the low-spin compounds ferricytochrome *c* and ferrimyoglobin cyanide are reported. In both spectra a weak band at approximately 8000 cm^{-1} was found to be polarized in the heme plane. In cytochrome *c* there is a further band at 10,700 cm^{-1} which indicates that the heme complex contains a few per cent of the high-spin form. Measurement of the spectra of metmyoglobin and ferricytochrome *c* crystals which had been bathed in D_2O confirm our previous assignment of the 6500 cm^{-1} band as a water overtone. The relationship between the band intensities in the crystal and solution is discussed. The lower intensity of the near-infrared absorption in crystalline myoglobin derivatives compared with that found in solution spectra may result from splitting of the degenerate excited state.

It is generally considered that the absorption spectra of heme complexes can be used as a guide to the spin state of the central metal atom (Williams, 1956). In a previous paper (Day *et al.*, 1967) we used this argument in a comparison between the solution and crystal spectra of a series of myoglobin derivatives in high- or mixed-spin states. We have now extended our measurements to myoglobin cyanide and ferricytochrome *c*, which are believed to be of the low-spin type from magnetic susceptibility measurements. Single crystals of the latter compound have already been studied from 28,000 to 13,000 cm^{-1} by Eaton and Hochstrasser (1967), but no measurements were reported in the near-infrared region. Crystal spectra in

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