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INCORPORATION OF AMINO ACID ANALOGS DURING THE BIOSYNTHESIS OF *ESCHERICHIA COLI* ASPARTATE TRANSCARBAMYLASE

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Summary

Amino acid-requiring mutants capable of producing derepressed levels of aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) were obtained and used for the incorporation in this enzyme of eight different amino acid analogs. These amino acid replacements enabled the biosynthesis of a series of modified aspartate transcarbamylases altered in their catalytic or regulatory properties. The enzyme in which phenylalanine was replaced by 2-fluorophenylalanine was purified to homogeneity and appeared to have the same specific activity as normal asparate transcarbamylase but lacking both homotropic and heterotropic interactions.

Introduction

Aspartate transcarbamylase (carbamoylphosphate:L-asparate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli*, the first enzyme of the pyrimidine pathway, shows both homotropic and heterotropic regulatory interactions and is extensively studied as a model system of metabolic regulation. This enzyme is made up of two trimeric catalytic subunits and three dimeric regulatory subunits, the association of which requires the presence of six atoms of zinc per molecule of native enzyme [1—4]. The catalytic and regulatory chains are different polypeptides [5] and their amino acid sequences are almost entirely known (Refs. 6, 7 and W. Konigsberg, personal communication). The

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three-dimensional structure of this protein has been determined with a resolution of about 3 Å [8].

During the biosynthesis of *E. coli* aspartate transcarbamylase, the synthesis of the two kinds of polypeptide is ordered and correlated, suggesting that the genes coding for the two types of chain are part of the same operon, the gene coding for the catalytic chain being proximal to the operator and the gene coding for the regulatory chain being distal to it [9,10]. This interpretation is confirmed by the fact that these two genes are cotransducible (Feller, A., Pierard, A. and Glansdorff, N., unpublished data).

A considerable amount of information on the conformational changes involved in the complex regulation of aspartate transcarbamylase activity is available and has been reviewed [12—14] but the precise mechanisms of these regulatory processes are still unknown. Numerous informative results were obtained using aspartate transcarbamylases which were altered by chemical reagents [15—22] or modified under the influence of 2-thiouracil [4,23—25]. A possible way of obtaining some specifically modified enzymes is the use of amino acid analogs which can be incorporated in vivo into the protein during its biosynthesis. This method provided interesting results in the case of β-galactosidase [26], alkaline phosphatase [27—32], T-even bacteriophage coat protein [33—36], *Pseudomonas aeruginosa* flagella [37] and *Aspergillus nidulans* phosphatase [38]. Furthermore the incorporation of amino acid analogs might lead to the biosynthesis of catalytic and regulatory subunits unable to associate.

In the present work, mutants of *E. coli* were isolated associating auxotrophy for certain amino acids to the mutation allowing for a high yield of aspartate transcarbamylase production. These mutants were used to assay the incorporation in aspartate transcarbamylase of eight different amino acid analogs (2-, 3- and 4-fluorophenylalanines, 1, 2, 4-triazoalanine, 2-methylhistidine, azatryptophan, canavanine, ethionine) leading to the biosynthesis of a series of aspartate transcarbamylases modified in several ways in their catalytic and regulatory properties. One of these modified enzymes was purified to homogeneity.

Materials and Methods

Chemicals. Leucine, histidine, cytidine triphosphate, azatryptophan and canavanine were purchased from Sigma; aspartate, carbamylphosphate and 2-, 3- and 4-fluorophenylalanines from Fluka; uracil from Serlabo; ethionine from Schuchardt; methylhistidine from Cyclo; triazolalanine from Fox; [^{14}C]aspartate from Service de Biochimie, C.E.N.-Saclay (Ref. CB51 in the C.E.A. catalog); other chemicals from Prolabo or Merck.

Bacterial strains. The mutants used in this study were constructed in order to bear the pyrF bradytrophy ensuring a high production of aspartate transcarbamylase and the mutations resulting in requirement for different amino acids. Thus all the *pyrF* mutants are leaky for this mutation.

M2: (F'*pyrB*$^+$/*his, pyrF*). This strain auxotrophic for histidine is a gift from Dr. J. Gerhart, University of Berkeley, U.S.A. it was used for the incorporation of triazolalanine and methylhistidine.

DAT01: (*his, pyrF*) is the corresponding F$^-$ strain [9].

RCB6: (*metA*, *argH*, *his*, *rif*) was a gift from Dr. C. Babinet, Institut Pasteur, Paris.

DAT08: (*metA*, *argH*, *his*, *pyrF*, *rif*, *strA*) was obtained from DAT01 using a stock of P1 sv phage grown on RCB6. It was used for the incorporation of canavanine and ethionine.

VO30: (*metA*, *argH*, *his*, *pyrF*, *rif*, *trpE*10220, *strA*) was prepared from DAT08 as previously described [10]. It was used for the incorporation of azatryptophan.

DAT37: (*aroB*, *his*, *arg*, *pyrF*) was used for the incorporation of 2-, 3- and 4-fluorophenylalanines.

P1 sv phage: was a gift from Dr. M. Hofnung, Institut Pasteur, Paris.

Cell growth and aspartate transcarbamylase biosynthesis in the presence of amino acid analogs. The different *E. coli* mutants were grown in the mineral medium previously described [9] in the presence of 50 μg/ml of the required amino acids and 30 μg/ml of uracil, a concentration which assures the repression of aspartate transcarbamylase biosynthesis. The bacteria were spun down, washed and resuspended in the grown medium supplemented with the amino acid analog to be incorporated (100 μg/ml) but deprived of the corresponding amino acid and uracil. Cells were harvested, resuspended in the same volume of 10 mM potassium phosphate buffer (pH 7)/1 mM β-mercaptoethanol and disrupted by sonication under the conditions previously described [9].

Determination of the aspartate transcarbamylase activity and its sensitivity to the effector cytidine triphosphate. The aspartate transcarbamylase activity was measured as previously described [9]. The specific activity of the purified enzymes is expressed as units per mg of protein and was calculated taking into account the fact that the serum albumin standard gives a 20% overestimate of aspartate transcarbamylase content [24]. 1 unit is defined as that amount of enzyme which catalyses the formation of 1 μmol of carbamylphosphate per h under the standard conditions used. The specific activity of the crude enzyme preparations is expressed as activity (in units) per mg total protein. The influence of the effector cytidine triphosphate is determined as previously described [14,23].

Ratio of activities at pH 7 and pH 8.4. The ratio of the aspartate transcarbamylase activity at pH 7 and 8.4, the value of which reflects the existence or absence of homotropic cooperative interactions between the catalytic sites for aspartate binding [23], was determined as previously described in the presence of 5 mM aspartate.

Amino acid analysis. The amino acid analysis and the protein amino acid content determinations were performed using a Technicon 3A1 autoanalyzer in the Service de Biochimie, C.E.N.-Saclay.

Dissociation and reassociation of aspartate transcarbamylase subunits. The dissociation of aspartate transcarbamylase into catalytic and regulatory subunits and the reassociation of subunits for the preparation of hybrid aspartate transcarbamylase molecules were performed as already described [4].

Results

Biosynthesis of aspartate transcarbamylase in the presence of the different amino acid analogs

The different mutants (10 ml cultures) were grown in the presence of 30 μg/ml of uracil and 50 μg/ml of the required amino acids until an absorbance of 0.4 at 546 nm (about $2 \cdot 10^8$ bacteria per ml) was reached. The cells were then spun down, washed twice with 10 ml of non-supplemented mineral medium and resuspended in the same volume of growth medium deprived of uracil but supplemented either with the required amino acids (50 μg/ml) or one of the corresponding analogs (100 μg/ml). They were then incubated for 18 h at 37°C under these conditions of derepression for the biosynthesis of aspartate transcarbamylase. After centrifugation, the cells were resuspended in 10 ml of potassium phosphate 10 mM pH 7/1 mM β-mercaptoethanol and disrupted by sonication as indiated in Materials and Methods. During each of these experiments a parallel culture was treated the same way but in the presence of the corresponding amino acid (50 μg/ml). After elimination of the cell debris the extracts obtained were analysed by electrophoresis on polyacrylamide gels. In all cases except when canavanine was used they showed a strongly coloured band which has been previously identified as aspartate transcarbamylase [9]. No free catalytic or regulatory subunit could be detected in any case.

The specific aspartate transcarbamylase activity of the different extracts was measured, as well as the ratio of their activities at pH 7 and 8.4, since it has been shown previously that the value of this ratio reflects the existence or absence of homotropic cooperative interaction between the catalytic sites for aspartate binding [23]. Finally, the sensitivity to the feedback inhibitor cytidine triphosphate of the aspartate transcarbamylase activity present in these extracts was determined. The results obtained are presented in Table I in comparison with the corresponding properties of purified aspartate transcarbamylase and its isolated catalytic subunits.

Azatryptophan and ethionine. The incorporation of azatryptophan and ethionine instead of tryptophan and methionine, respectively, leads to the biosynthesis of a normally active aspartate transcarbamylase though the specific activity of the extracts derived from the cells derepressed in the presence of the analogs is significantly lower. The pH 7: 8.4 activity ratio and the sensitivity to CTP show that these modified enzymes exhibit both homotropic and heterotropic interactions. Thus it seems that the replacement of tryptophan or methionine by these two analogs does not alter significantly the catalytic and regulatory properties of the enzyme.

1,2,4-Triazolalanine and 2-methylhistidine. In spite of the fact that the incorporation of 1,2,4-triazolalanine and 2-methylhistidine leads to the presence of a strongly coloured band of aspartate transcarbamylase the specific activity of the corresponding extracts is very low, indicating that the incorporation of these two changes results in the biosynthesis of an inactive enzyme. A similar observation has been made in the case of alkaline phosphatase [28,30]. This result provides additional indication that histidine residue is involved in the mechanism of action of the catalytic sites of aspartate transcarbamylase [39]. The small activity observed shows normal pH 7 : 8.4 activity ratio and

TABLE I

CHARACTERISTICS OF THE ASPARTATE TRANSCARBAMYLASE (ATCase) ACTIVITIES PRESENT IN THE EXTRACTS OF AMINO ACID REQUIRING MUTANTS OF *E. COLI* DEREPRESSED IN THE PRESENCE OF AMINO ACID ANALOGS

The different amino acid requiring mutants were derepressed for the biosynthesis of asparatate transcarbamylases under the condition described in the text. The specific activity and the pH 7 : 8.4 activity ratio were determined as reported in Materials and Methods. The feedback inhibition by cytidine triphosphate was measured as indicated in Materials and Methods in the presence of 5 mM aspartate and 1 mM CTP. The percentage of inhibition by CTP is expressed as follows:

$$\% = \frac{(A_0 - A_n) \times 100}{A_0}$$

where A_0 is the catalytic activity in absence of CTP, and A_n the catalytic activity in the presence of this nucleotide

Amino acid or analog	ATCase activity per mg total protein (u/mg)	pH 7 : 8.4 activity ratio	% inhibition by CTP (1 mM)
Tryptophan	360	3.0	44
Azatryptophan	128	4.8	55
Arginine	480	3.1	48
Canavanine	0		
Phenylalanine	600	3.8	52
2-Fluorophenyl-alanine	320	0.67	6
3-Flurophenyl-alanine	225	0.68	6
4-Fluorophenyl-alanine	110	0.56	8
Methionine	750	4	61
Ethionine	332	2.9	75
Histidine	880	3.0	51
Triazolalanine	45	1.6	54
Methylhistidine	59	3	59
Purified ATCase	16 000	2.4	55
Purified catalytic subunits	24 000	0.9	8

sensitivity to cytidine triphosphate, suggesting that this light residual activity is due to the biosynthesis of a small amount of catalytic chains containing histidine in the catalytic site; this possibility will be discussed further.

2, 3 or 4-fluorophenylalanines. In order to detect any influence of the position of the fluorine atom on the aromatic ring of phenylalanine the *ortho-* (2-) *meta-* (3-) or *para-* (4-) fluorophenylalanines were used. The results presented in Table I show that the aspartate transcarbamylase specific activity of the bacterial extracts decreases as a function of the location of the fluorine atom in the order 2-, 3-, 4-. In all three cases the aspartate transcarbamyl activity is optimum at pH 7: 8.4 ratio which is characteristic of the lack of homotropic cooperative interactions. In addition this activity appears to be insensitive to the presence of the feedback-inhibitor cytidine triphosphate. These results indicated that under these conditions either free catalytic subunits unable to associate to the regulatory subunits or a desensitized aspartate trans-

carbamylase were synthesized. In order to test these two possibilities the extracts of bacteria derepressed for the biosynthesis of aspartate transcarbamylase in the presence of 2-, 3- or 4-fluorophenylalanines were analysed by zonal centrifugation in sucrose gradients in comparison with purified aspartate transcarbamylase and its isolated catalytic subunits. The results presented in Fig. 1 show that in the presence of the three fluorophenylalanines a complete enzyme of normal molecular weight is synthesized, which lacks homotropic cooperative interactions and which is not sensitive to the feedback inhibitor cytidine triphosphate.

Properties of the purified 2-fluorophenylalanine-aspartate transcarbamylase

Purification. Since the procedure employed for the purification of aspartate transcarbamylase makes use of a thermal denaturation step [40], the thermosensitivity of the enzymes synthesized in the presence of the three fluorinated derivatives of phenylalanine was investigated. Fig. 2 shows that 2-fluorophenyl-

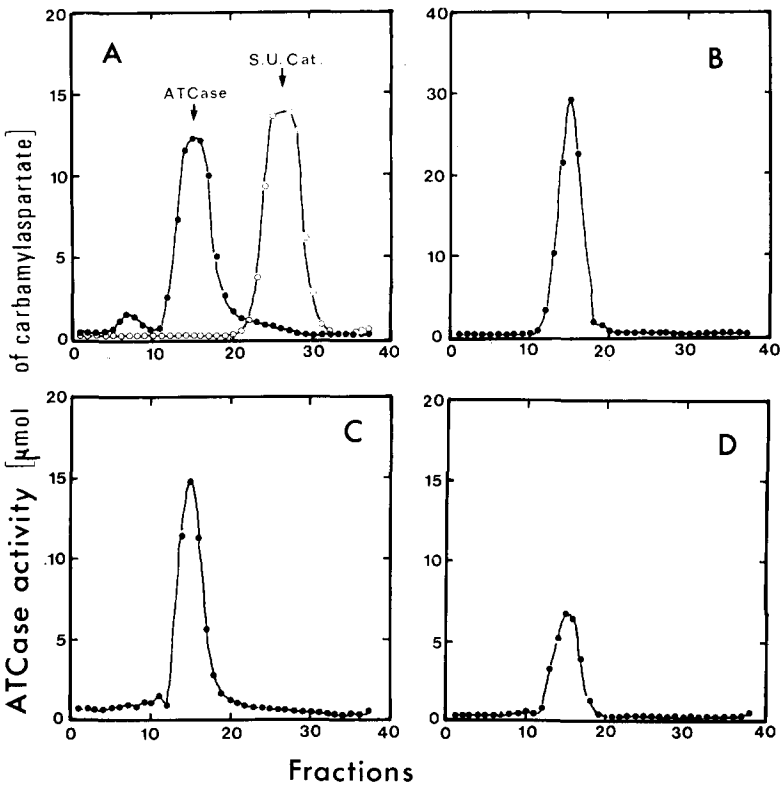

Fig. 1. Analysis by centrifugation in sucrose gradients of the extracts from bacteria derepressed for the biosynthesis of aspartate transcarbamylase (ATCase) in the presence of 2-, 3- or 4-fluorophenylalanine. The extracts from bacteria derepressed for the biosynthesis of aspartate transcarbamylase in the presence of the three fluorinated derivatives of phenylalanine were analysed as indicated in Materials and Methods by centrifugation in 5—20% saccharose gradient for 11 h at 36 000 rev./min in a SW39 Beckman rotor. 10 drop fractions were collected and 100 µl of each fraction were used for the determination of aspartate transcarbamylase activity. A, mixture of native aspartate transcarbamylase and free catalytic subunits (5 µg each). B, extract of bacteria derepressed in the presence of 2-fluorophenylalanine (40 µg of protein). C, extract of bacteria derepressed in the presence of 3-fluorophenylalanine (30 µg of protein). D, extract of bacteria derepressed in the presence of 4-fluorophenylalanine (30 µg of protein).

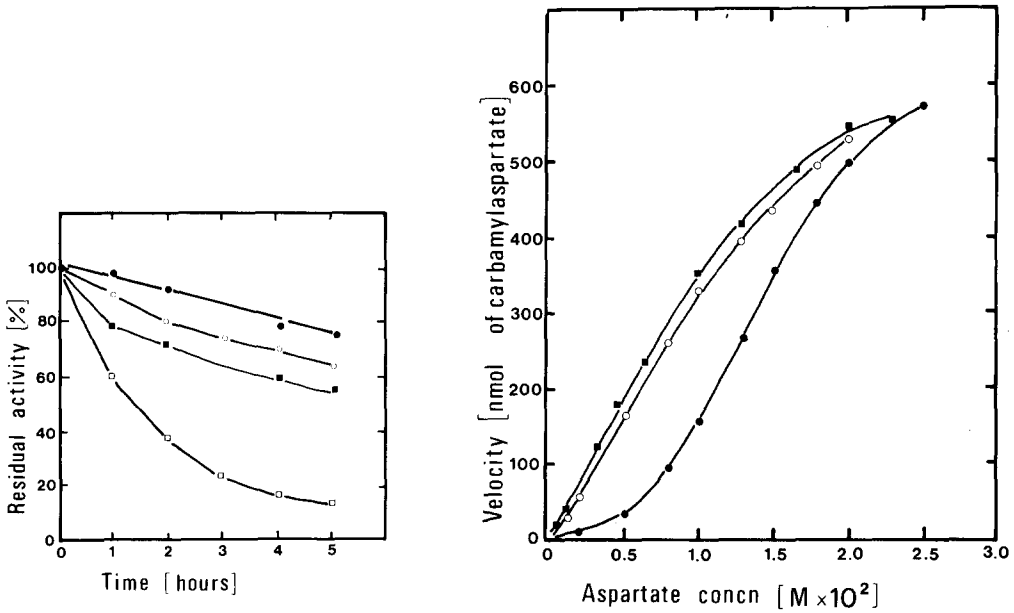

Fig. 2. Thermodenaturation of aspartate transcarbamylase synthesized in the presence of 2-, 3- or 4-fluorophenylalanine. 600 µl samples of extracts, coming from bacteria derepressed for the biosynthesis of aspartate transcarbamylase in the presence of the three fluorinated derivatives of phenylalanine, were incubated at 60°C at the same time as a sample of purified aspartate transcarbamylase (4.5 µg in 600 µl of the same buffer). At different time intervals 20-µl samples were taken and assayed for aspartate transcarbamylase activity as indicated in Materials and Methods. ●———●, aspartate transcarbamylase; ○———○, 2-fluorophenylalanine-aspartate transcarbamylase; ■———■, 3-fluorophenylalanine-aspartate transcarbamylase, □———□, 4-fluorophenylalanine-aspartate trancarbamylase.

Fig. 3. Saturation curve for aspartate of purified 2-fluorophenylalanine-aspartate transcarbamylase. 0.25 µg of aspartate transcarbamylase (●———●), 0.13 µg of catalytic subunits (■———■) and 0.30 µg of 2-fluorophenylalanine-aspartate transcarbamylase (○———○) were incubated as indicated in Materials and Methods but in the presence of increasing amounts of [^{14}C]-aspartate (from 0.6 to 25 mM). Activity is expressed as nmol of carbamyl aspartate formed during 10 min incubation.

alanine-aspartate transcarbamylase and 3-fluorophenylalanine-aspartate transcarbamylase are slightly less resistant to thermodenaturation than aspartate transcarbamylase and that 4-fluorophenylanaline-aspartate transcarbamylase is considerably more heat-labile. Consequently, 2-fluorophenylalanine aspartate carbamylase was purified as indicated in Materials and Methods except that the thermodenaturation step was performed at 55°C as previously reported in the case of 2-Thiouracil-aspartate transcarbamylase [23].

2-Fluorophenylalanine content of the purified protein. It was important to determine to what extent phenylalanine was replaced by its fluorinated analog in the modified enzyme. Prior to determining the amino acid composition of the purified protein it was verified that 2-fluorophenylalanine resists the acid hydrolysis of the protein by 6 N HCl. During the subsequent amino acid analysis 2-fluorophenylalanine was eluted at the same position as tyrosine. This determination showed that under the conditions used only 60% of the phenylalanine residues of aspartate transcarbamylase were replaced by 2-fluorophenylalanine. Obviously these replacements must occur randomly along the polypeptide chains.

TABLE II

COMPARATIVE PROPERTIES OF 2-FLUOROPHENYLALANINE-ASPARTATE TRANSCARBAMYLASE, ASPARTATE TRANSCARBAMYLASE (ATCase) AND CATALYTIC SUBUNITS

2—Fluorophe-nylalanine-aspartate transcarbomylase, was purified as reported in the text. Aspartate transcarbomylase and catalytic subunit were purified as reported in Materials and Methods. The properties of these three enzymatic species were determined as indicated in Table I.

	Specific activity (μ/mg)	pH 7 : 8.4 activity ratio	% inhibition by CTP (1 mM)
2-fluorophe-nylalanine-ATCase	17 000	0.9	5
Aspartate trans-carbomylase	16 000	2.4	55
Catalytic subunits	24 000	0.9	8

Catalytic and regulatory properties of the purified 2-fluorophenylalanine-aspartate transcarbamylase. The properties of the purified 2-fluorophenylalanine-aspartate transcarbamylase are shown in Table II. Its specific activity was 17 000 u/mg, similar to the specific activity of normal aspartate transcarbamylase. The catalytic activity of this modified enzyme appears to be insensitive to the feedback-inhibitor cytidine triphosphate. The pH 7: 8.4 activity ratio is identical to that of free catalytic subunits. In order to verify that 2-fluorophenylalanine-aspartate transcarbamylase does not show homotropic

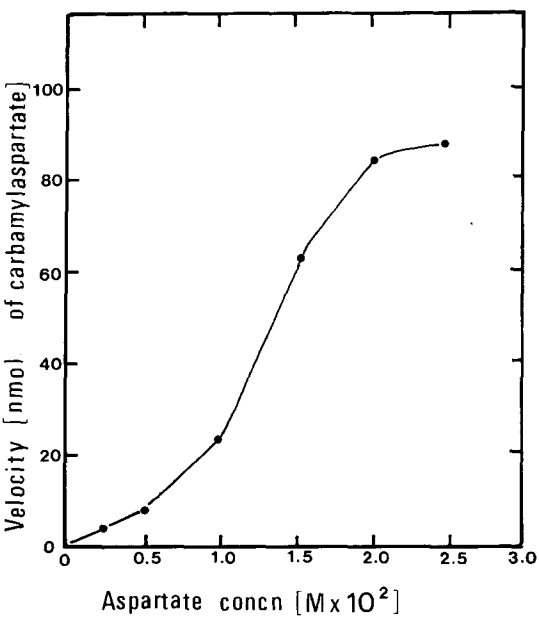

Fig. 4. Saturation curve for aspartate of the hybrid enzyme made up from 2-fluorophenylalanine-catalytic subunits and normal regulatory subunits. The hybrid aspartate transcarbamylase made up from 2-fluorophenylalanine-catalytic subunits and normal regulatory subunits was incubated as indicated in Materials and Methods but in the presence of increasing amounts of [^{14}C]-aspartate. Activity is expressed as indicated in Fig. 3.

TABLE III

EFFECT OF CTP ON THE HYBRID ASPARTATE TRANSCARBAMYLASE (ATCase) MADE UP OF 2-FLUOROPHENYLALANINE-CATALYTIC (CAT) SUBUNITS AND NORMAL REGULATORY (REG) SUBUNITS

2-fluorophenylalanine-catalytic subunits and normal regulatory subunits were reassociated as indicated in Materials and Methods and the sensitivity of the different enzymes species to feedback inhibition by CTP was determined as indicated in Table I

Enzymatic species	% inhibition by 1 mM CTP
Catalytic subunits	9
2-fluorophenylalanine-ATCase	6
2-fluorophenylalanine-Cat normal-Reg hybrid	50

cooperative interactions, its saturation curve for aspartate was determined at the same time as aspartate transcarbamylase and free catalytic subunits as controls. The results obtained are presented in Fig. 3 where it can be seen that the saturation curve of the modified enzyme is hyperbolic as is that of the free catalytic subunits.

Properties of the hybrid made up of 2-fluorophenylalanine-catalytic subunits and normal regulatory subunits. In order to determine which of the two kinds of subunit is responsible for the lack of regulatory properties, 2-fluorophenylalanine-aspartate transcarbamylase was dissociated into its catalytic and regulatory subunits as indicated in Materials and Methods and the 2-fluorophenylalanine-catalytic subunits obtained were reassociated with normal regulatory subunits. The saturation curve, for aspartate, of the hybrid enzyme obtained is reported in Fig. 4. The sigmoidal curve obtained indicates that the homotropic cooperative interactions are restored in the hybrid. The effect of cytidine triphosphate on the catalytic activity of the hybrid enzyme is shown in Table III in comparison with the same effect on aspartate transcarbamylase and its isolated catalytic subunits. It appears that the sensitivity to the feedback inhibitor is also restored in the hybrid aspartate transcarbamylase. These results indicate that it is the incorporation of 2-fluorophenylalanine in the regulatory chains which is responsible for the lack of homotropic and heterotropic interactions in 2-fluorophenylalanine-aspartate transcarbamylase.

Discussion

Azatryptophan, ethionine, 1,2,4-triazolalanine, 2-methylhistidine, *ortho-*, *meta-* and *para*-fluorophenylalanines can be incorporated into proteins upon starvation for the corresponding amino acid of auxotrophic bacteria. However, under these conditions the replacement of the normal amino acid by the analog is only partial. The results presented here show that during the biosynthesis of aspartate transcarbamylase only 60% of the phenylalanine residues are replaced by 2-fluorophenylalanine. It has been reported that under comparable conditions about 50% of the same replacement is observed in *E. coli* alkaline phosphatase [27]. In the same protein about 60% of the arginine residues could be replaced by canavanine [31] and 80% of the tryptophan residues could be replaced by azatryptophan [29]. The fact that a complete replacement cannot

be obtained must result from a small amount of proteolysis providing traces of amino acids. The proportion of replacement will then depend essentially on the relative affinity of the aminoacyl-tRNA synthetase for the amino acid and its analog. Since there is no reason to believe that the incorporation of the amino acid analog does not occur randomly along the polypeptide chain, the modified protein obtained is thus a statistically heterogeneous population of molecules. The presence of traces of normal amino acids inside the cells when the media is changed to induce derepression might also contribute to the incomplete replacement of these analogs by their analogs.

The replacement of tryptophan by azatryptophan and the replacement of methionine by ethionine does not seem to alter significantly the catalytic and regulatory properties of aspartate transcarbamylase, except that the specific aspartate transcarbamylase activity of the bacterial extracts is significantly lower. This is probably due to a decreased enzyme biosynthesis since the same phenomenon is observed in the case of 2-fluorophenylalanine in spite of the fact that purified 2-fluorophenylalanine-aspartate transcarbamylase has the same specific activity as purified aspartate transcarbamylase. On the other hand, the replacement of histidine by 2-methylhistidine or 1,2,4-triazolalanine leads to the biosynthesis of an inactive aspartate transcarbamylase, providing additional indication that histidine residue is involved in the catalytic site of this enzyme [39]. This lack of catalytic activity did not allow a direct test to ascertain whether the same replacement had an effect on the homotropic and heterotropic interactions and it will be interesting to investigate the regulatory properties of a hybrid aspartate transcarbamylase made up from normal catalytic subunits and 2-methylhistidine- or 1,2,4-triazolalanine-containing regulatory subunits.

The replacement of phenylalanine by 2-fluorophenylalanine leads to the biosynthesis of an aspartate transcarbamylase which, after complete purification, appears to have a normal specific catalytic activity but lacks both homotropic and heterotropic interactions. This property must result from a defect in catalytic and regulatory subunit interactions which can be attributed to the regulatory chains. This result confirms the idea that the three-dimensional structure of the regulatory subunits is essential for the manifestation of both homotropic and heterotropic interactions. It is interesting to note that in none of the cases tested here was the structural alteration important enough to completely hinder the subunit association.

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