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ENZYME-ENZYME INTERACTION AND THE BIOSYNTHESIS OF AROMATIC AMINO ACIDS IN *ESCHERICHIA COLI*

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Summary

The technique of affinity chromatography has been used to demonstrate that enzymes involved in the biosynthesis of tyrosine and phenylalanine in *Escherichia coli* undergo reversible interactions. Thus it has been shown that the aromatic amino acid aminotransferase (aromatic-amino-acid: 2-oxoglutarate amino-transferase, EC 2.6.1.57) reacts specifically with chorismate mutase-prephenate dehydrogenase (chorismate pyruvate mutase, EC 5.4.99.5 and prephenate: NAD^+ oxidoreductase (decarboxylating), EC 1.3.1.12) in the absence of reactants and with chorismate mutase-prephenatedehydratase (prephenate hydro-lyase (decarboxylating), EC 4.2.1.51) in the presence of phyenylpyruvate. Tyrosine causes dissociation of the aminotransferase: mutase-dehydrogenase complex while dissociation of the aminotransferase-mutase-dehydratase complex occurs on omission of phenylpyruvate. Only the active form of chorismate mutase-prephenate dehydrogenase participates in complex formation.

Introduction

In *Escherichia coli* the biosynthesis of phenylalanine and tyrosine from chorismate occurs by the reactions:

Chorismate → prephenate → phenylpyruvate → phenylalanine
Chorismate → prephenate → 4-hydroxyphenyl-pyruvate → tyrosine

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Two different bifunctional enzymes are responsible for the first two steps along each pathway. Thus, chorismate mutase-prephenate dehydratase (chorismate pyruvate mutase, EC 5.4.99.5, and prephenate hydro-lyase (decarboxylating), EC 4.2.1.51) [1] and chorismate mutase-prephenate dehydrogenase (chorismate pyruvate mutase, EC 5.4.99.5, and prephenate: NAD^+ oxidoreductase (decarboxylating), EC 1.3.1.12) [2] catalyse the conversion of chorismate to phenylpyruvate and 4-hydroxyphenylpyruvate, respectively. The final step of both pathways is catalysed by a single aromatic amino acid aminotransferase (aromatic-amino-acid: 2-oxoglutarate aminotransferase, EC 2.6.1.57) [3]. Because of the occurrence in microorganisms of multi-enzyme complexes, it was of interest to determine if such complexes could be formed as a result of the interaction of either of the two bifunctional enzymes with the aminotransferase. The occurrence of protein-protein interactions has been explored by using the technique of affinity chromatography which has been utilized previously for enzyme purification and for determining the order of substrate additions to an enzyme [4] as well as for quantitative studies on enzyme-substrate formation [5]. The present communication reports the reversible interaction of the aromatic amino acid aminotransferase with chorismate mutase-prephenate dehydrogenase in the absence of reactants and with chorismate mutase-prephenate dehydratase in the presence of phenylpyruvate.

Materials and Methods

Enzymes. The aromatic amino acid aminotransferase and the aspartate aminotransferase were prepared, as described previously [3], from *E. coli* strains DG34 and DG27, respectively. The activity of both aminotransferases was assayed by the method of Diamondstone [6]. The apoenzyme of the aromatic amino acid aminotransferase was prepared by treating a solution of the holoenzyme with ammonium sulphate (47 g/100 ml), dissolving the precipitate in 50 mM Tris-HCl buffer (pH 8.0) and dialyzing the solution against the same buffer [3]. The apoenzyme had a specific activity of 28 U/mg of protein when tested in the presence of 2.5 mM phenylalanine, 2.5 mM 2-oxoglutarate and 80 μM pyridoxal phosphate. Chorismate mutase-prephenate dehydrogenase was a gift from Dr. T.E. Heyde while chorismate mutase-prephenate dehydratase was a gift from Dr. R.G. Duggleby. The bifunctional enzymes were isolated from extracts of *E. coli* and assayed by determining their chorismate mutase activity as described by Koch et al. [7]. Protein was estimated by the method of Bradford [8].

Preparation of affinity matrices. Pyridoxamine phosphate-Sepharose was prepared by the procedure of Miller et al. [9] using the ethylenediamine spacer arm. The amount of bound pyridoxamine was estimated fluorimetrically as 5 μmol/ml of wet gel. Phenylalanine-Sepharose was prepared by coupling phenylalanine (0.1 M) to cyanogen bromide-activated Sepharose 4B at pH 9. The substituted gel contained 5 μmol of phenylalanine/ml of wet gel.

Chromatography. Columns (0.9 X 2.4 cm; 1.5 ml) were run at 4°C at a flow rate of 10 ml/h and fractions (0.7 ml) were collected automatically. Pyridoxamine phosphate-Sepharose columns were equilibrated and loaded in a Tris buffer mixture, containing 50 mM Tris, 1 mM sodium citrate and 2 mM

mercaptoethanol, which had been adjusted to pH 8.0 with HCl. The aromatic amino acid aminotransferase was eluted with a cacodylate buffer mixture containing 20 mM sodium cacodylate (pH 6.8), 0.1 M NaCl, 2 mM mercaptoethanol and 0.2 mM pyridoxal phosphate.

Phenylalanine-Sepharose columns were equilibrated and loaded in 20 mM sodium phosphate (pH 6.2) containing 0.2 M NaCl, and 2 mM mercaptoethanol. Chorismate mutase-prephenate dehydratase was eluted with 50 mM Tris-HCl buffer (pH 8.1) containing 2 mM mercaptoethanol.

Results

To determine if there were any interaction between the aromatic amino acid aminotransferase and chorismate mutase-prephenate dehydrogenase or chorismate mutase-prephenate dehydratase, one of the enzymes was adsorbed specifically onto an affinity column and a study made of the retention or retardation of one of the other enzymes on its passage through the column. The initial studies involved use of a pyridoxamine phosphate-Sepharose column to which the aromatic amino acid aminotransferase could be adsorbed and from which it could be eluted with the cacodylate buffer mixture [3]. To ensure that any interaction between the aminotransferase and one of the bifunctional enzymes was specific, a series of preliminary tests was performed with each substituted Sepharose column.

Control tests with pyridoxamine phosphate-Sepharose columns

Controls tests showed that the binding of the aminotransferase to, and its elution from, a pyridoxamine phosphate-Sepharose column was unaffected by the addition to the buffer of phenylalanine, tyrosine or phenylpyruvate. Further, it was observed that bovine serum albumin and cytochrome *c* chromatographed in the same way on the substituted column loaded with aminotransferase as they did on an unloaded, substituted column or an unsubstituted Sepharose column. Chorismate mutase-prephenate dehydrogenase was retarded on the pyridoxamine phosphate-Sepharose column by one column volume, as compared with bovine serum albumin, while no such retardation was observed on a column of unsubstituted Sepharose. Since chorismate mutase-prephenate dehydrogenase is inhibited by pyridoxal phosphate, the retardation probably results from the interaction of the enzyme with bound pyridoxamine phosphate.

Interaction of chorismate mutase-prephenate dehydrogenase with aromatic amino acid aminotransferase

A column of pyridoxamine phosphate-Sepharose was saturated with aromatic amino acid aminotransferase (7 U, 0.25 mg) after which chorismate mutase-prephenate dehydrogenase (10 U, 0.57 mg) was applied to the column. On washing with the Tris buffer mixture (pH 8.0) excess chorismate mutase-prephenate dehydrogenase and inactive protein (1.25 U, 0.43 mg) was eluted from the column with the expected retardation of one column volume (see above). Neither the chorismate mutase-prephenate dehydrogenase (8.75 U) nor the aminotransferase (7 U) was eluted by those buffers that normally elute

these enzymes (cf. Material and Methods) and no protein could be detected in the washes. However, when sodium phosphate (0.2 M, pH 4.0) was applied to the column, virtually all the protein (0.35 mg) and 4.5 U of aminotransferase activity were eluted from the column. The failure to detect any mutase or dehydrogenase activity is due to the fact that chorismate mutase-prephenate dehydrogenase undergoes irreversible inactivation under the conditions used for elution of the column.

Chorismate mutase-prephenate dehydrogenase could be eluted almost quantitatively from a pyridoxamine phosphate-Sepharose column loaded with aromatic amino acid aminotransferase by the inclusion in the buffer of tyrosine which is a product of the aminotransferase reaction and an allosteric inhibitor of the mutase and dehydrogenase reaction (Fig. 1). Thus development of a column containing aminotransferase and chorismate mutase-prephenate dehydrogenase with the Tris buffer mixture containing 1 mM tyrosine, leads to the elution of chorismate mutase-prephenate dehydrogenase in a sharp peak. This treatment does not elute the aminotransferase, but the latter enzyme can be eluted by the cacodylate buffer mixture which contains 0.2 mM pyridoxal phosphate.

Interaction of chorismate mutase-prephenate dehydratase with aromatic amino acid aminotransferase

Chorismate mutase-prephenate dehydratase, like the aminotransferase and

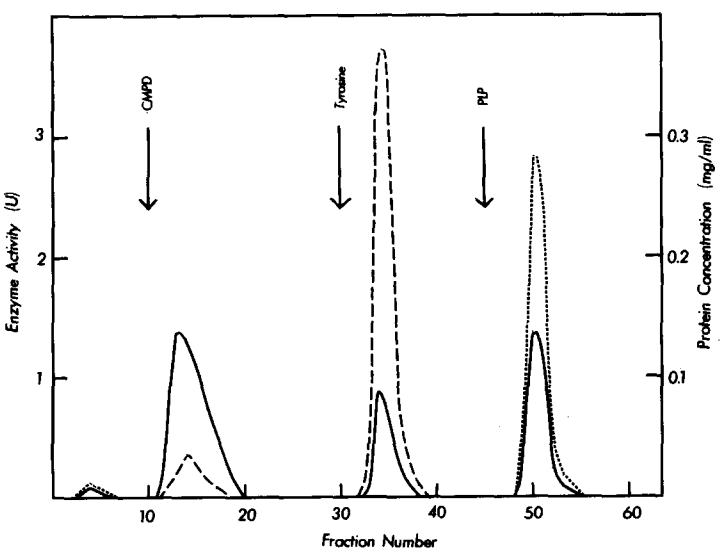

Fig. 1. Interaction of chorismate mutase-prephenate dehydrogenase with the aromatic amino acid aminotransferase bound to a column of pyridoxamine phosphate. Aminotransferase (7 U, 0.25 mg) was applied to the column (1.5 ml) at pH 8.0 in the Tris-HCl buffer mixture described in Materials and Methods and washed with the same buffer to remove the excess enzyme. Chorismate mutase-prephenate dehydrogenase (CMPD; 10 U, 0.57 mg) was then applied and after extensive washing to remove excess mutase-dehydrogenase (1.25 U) and inert protein (0.43 mg), tyrosine (1 mM) was added to the Tris-HCl to elute this enzyme (7.8 U, 0.14 mg). The aminotransferase (6.9 U, 0.23 mg) was eluted with the cacodylate buffer mixture described in Materials and Methods which contained 0.2 mM pyridoxal phosphate. The various additions are indicated by arrows. ———, protein concentration; - - - - - -, chorismate mutase activity; · · · · · ·, aminotransferase activity.

unlike chorismate mutase-prephenate dehydrogenase, is retained on a column of pyridoxamine phosphate-Sepharose in the presence of the Tris buffer mixture (pH 8.0). However, whereas the aminotransferase is eluted from the column with the cacodylate buffer mixture containing pyridoxal phosphate, chorismate mutase-prephenate dehydratase is not eluted. Further, it was found that chorismate mutase-prephenate dehydratase could be adsorbed onto a column which was apparently saturated with the aminotransferase and that the addition of phenylpyruvate (2 mM) to the cacodylate buffer mixture caused elution of both enzymes. Such a result is difficult to interpret and an alternative approach, involving the use of phenylalanine-Sepharose, was adopted to demonstrate enzyme-enzyme interaction.

Control tests with phenylalanine-Sepharose columns

Chorismate mutase-prephenate dehydratase is bound to phenylalanine-Sepharose at high salt and low pH (0.2 M NaCl, pH 6.1) and eluted at low salt and higher pH [1]. The aromatic amino acid aminotransferase is not bound to phenylalanine-Sepharose under either of these conditions or in the presence of 2 mM phenylpyruvate. Further, this enzyme does not bind to a column of phenylalanine-Sepharose which is saturated with chorismate mutase-prephenate dehydratase (6 U, 0.1 mg) and does not affect the subsequent elution of chorismate mutase-prephenate dehydratase.

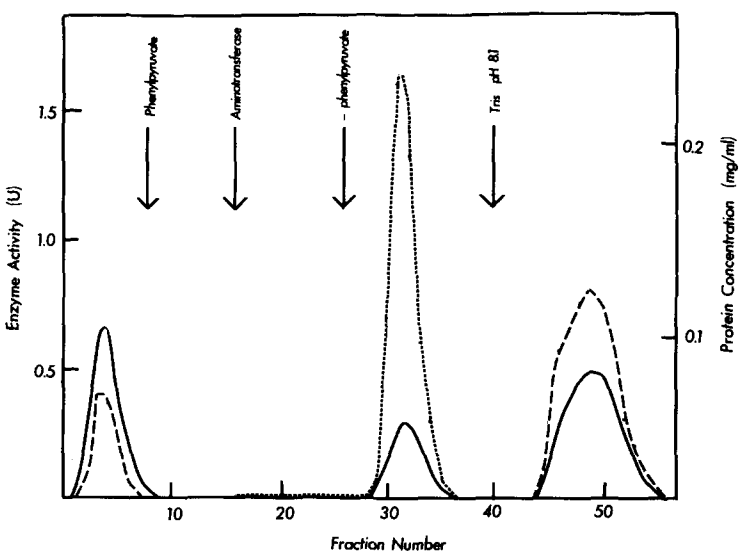

Fig. 2. Interaction of the aromatic amino acid aminotransferase with chorismate mutase-prephenate dehydratase bound to a column of phenylalanine-Sepharose. Chorismate mutase-prephenate dehydratase (7 U, 1.2 mg) was applied to the column (1.5 ml) at pH 6.2 in the presence of the sodium phosphate/NaCl buffer described under Materials and Methods. The same buffer containing 2 mM phenylpyruvate was used to wash the column, for the application of the aminotransferase and for further washing of the column. The aminotransferase was eluted by removal of phenylpyruvate from the buffer. Chorismate mutase-prephenate dehydratase was eluted at pH 8.1 with the Tris-HCl buffer described in Materials and Methods. The various additions are indicated by arrows. ———, protein concentration; · · · · · ·, aminotransferase activity; - - - - - -, chorismate mutase activity.

Interaction of aromatic amino acid aminotransferase with chorismate mutase-prephenate dehydratase in presence of phenylpyruvate

In the presence of 2 mM phenylpyruvate, aminotransferase (up to a maximum of 3.1 U, 0.11 mg) was bound to a column of phenylalanine-Sepharose to which 6 U of chorismate mutase-prephenate dehydratase was adsorbed (Fig. 2). Removal of the phenylpyruvate from the buffer resulted in elution of the aminotransferase while the chorismate mutase-prephenate dehydratase could then be eluted by lowering the salt concentration and increasing the pH of the buffer to pH 8.1. The latter enzyme did not elute sharply as in the case when the aminotransferase had not been applied (cf. Ref. 10).

Specificity of the enzyme-enzyme interactions

As an additional check on the specificity of the interaction between the aromatic amino acid aminotransferase and the two bifunctional enzymes from *E. coli*, control tests were performed with aspartate aminotransferase which had been isolated in pure form from the same source [3]. This enzyme has properties in common with the aromatic amino acid aminotransferase including the ability to utilize phenylpyruvate and 4-hydroxyphenylpyruvate as substrates. Aspartate aminotransferase was bound to a column of pyridoxamine phosphate-Sepharose from which it could be eluted with sodium phosphate (50 mM, pH 6.8) but it was not retained on a column of phenylalanine-Sepharose. The control tests provided no evidence for the specific interaction of aspartate aminotransferase with either of the bifunctional enzymes.

When the aromatic amino acid aminotransferase was coupled directly to cyanogen bromide-activated Sepharose at pH 9, the bound enzyme (0.14 mg of protein/ml of wet gel) possessed negligible activity. The matrix also failed to retain either of the bifunctional enzymes under conditions where they interact with non-covalently bound aromatic amino acid aminotransferase.

Discussion

The technique of affinity chromatography has permitted the study of enzyme-enzyme interaction with the use of relatively small amounts of material. Further, the technique has allowed ready determination of the effect of reactants on the formation and dissociation of enzyme-enzyme complexes. Control tests indicate that the observed interactions are specific. However, such interactions may differ from those which might exist in aqueous solution because of the possible participation of the affinity matrix (Sepharose) in complex formation. The intracellular interaction of enzymes may also involve membranes or organelles with the formation of solid state assemblies.

The results (Fig. 1) indicate that there is specific interaction between the aromatic amino acid aminotransferase and chorismate mutase-prephenate dehydrogenase from *E. coli* and that it is only the active form of the latter enzyme which participates in complex formation. The chorismate mutase-prephenate dehydrogenase used in these studies had been isolated as a homogeneous preparation with a specific activity of 35, but on storage over several months, the specific acitivity had fallen to 18. The denatured enzyme

in the sample applied to the pyridoxamine phosphate-Sepharose column saturated with the aminotransferase was not retained and calculation showed that the retained enzyme should have a specific activity of 62. This value agrees well with that of 56 obtained for the enzyme eluted by the addition of tyrosine to the Tris buffer. The chromatographic procedure described in Fig. 1 provides a means of isolating active chorismate mutase-prephenate dehydrogenase from denatured enzyme.

Recent findings (Heyde, E. and Llewellyn, D., unpublished data) have indicated that the apoenzyme of the aromatic amino acid aminotransferase neither undergoes interaction with chorismate mutase-prephenate dehydrogenase, as judged by the results of active enzyme centrifugation studies, nor has any kinetic effects on the mutase and dehydrogenase activities of the enzyme. It may well be that attachment of pyridoxal phosphate, or pyridoxamine phosphate, to the aminotransferase is essential for complex formation. Experiments could not be performed in the presence of pyridoxal phosphate as this compound acts as a strong inhibitor of the activities of chorismate mutase-prephenate dehydrogenase.

It is attractive to consider that tyrosine brings about dissociation of the aminotransferase: chorismate mutase-prephenate dehydrogenase complex because of conformation changes induced by its reaction at the allosteric site of the bifunctional enzyme. However, tyrosine also functions as a substrate of the aminotransferase reaction [3] and hence no definitive conclusion can be reached about the mode of action of tyrosine. But it should be noted that tyrosine does not bring about elution of the aminotransferase from the pyridoxamine phosphate-Sepharose column.

In contrast to the direct interaction that occurs between the aromatic amino acid aminotransferase and chorismate mutase-prephenate dehydrogenase, the formation of a specific complex between the aminotransferase and chorismate mutase-prephenate dehydratase requires the presence of phenylpyruvate. Since the latter compound is a substrate for the aminotransferase [3] as well as a product of the dehydratase reaction there could be several reasons as to why phenylpyruvate is required for complex formation. In this connection it should be mentioned that substrates could not be used in tests of complex formation because both the mutase and dehydratase reactions require only a single substrate and each reaction is essentially irreversible. The addition of either chorismate or prephenate would result virtually in their quantitative conversion to phenylpyruvate.

The metabolic significance of the formation of multi-enzyme complexes with the enzymes involved in the biosynthesis of phenylalanine and tyrosine is yet to be determined. The enhancement of each catalytic activity through complex formation (cf. Refs. 11, 12) would be in accord with the findings that the allosteric inhibitor, tyrosine, causes dissociation of the chorismate mutase-prephenate dehydrogenase:aminotransferase complex while the intermediate metabolite, phenylpyruvate facilitates the formation of a chorismate mutase-prephenate dehydratase:aminotransferase complex.

As all three enzymes used in the present study have molecular weights in the range of 80—90 000 [1—3], a comparison of the amounts of each pair retained on a column gives an indication of the stoichiometries of the interactions. Such

a comparison suggests that the stoichiometry is not greater than 2 : 1 for the aminotransferase mutase-dehydrogenase complex and is 1 : 1 for the aminotransferase: mutase-dehydratase complex.

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