Affinity Chromatography and Inhibition of Chorismate Mutase–Prephenate Dehydrogenase by Derivatives of Phenylalanine and Tyrosine

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Several derivatives of phenylalanine and tyrosine were prepared and tested for inhibition of chorismate mutase-prephenate dehydrogenase (EC 1.3.1.12) from *Escherichia coli* K12 (strain JP 232). The best inhibitors were *N*-toluene-*p*-sulphonyl-L-phenylalanine, *N*-benzenesulphonyl-L-phenylalanine and *N*-benzyloxycarbonyl-L-phenylalanine. Consequently two compounds, *N*-toluene-*p*-sulphonyl-L-*p*-aminophenylalanine and *N*-*p*-aminobenzenesulphonyl-L-phenylalanine, were synthesized for coupling to CNBr-activated Sepharose-4B. The *N*-toluene-*p*-sulphonyl-L-*p*-aminophenylalanine–Sepharose-4B conjugate was shown to bind the enzyme very strongly at pH 7.5. The enzyme was not eluted by various eluents, including 1 M-NaCl, but could be quantitatively recovered by washing with buffer of pH 9. Elution was more effective in the presence of 10 mM-1-adamantanecetic acid, a competitive inhibitor of the enzyme. This affinity-chromatography procedure results in a high degree of purification of the enzyme and can be used to prepare the enzyme in a one-step procedure from the bacterial crude extract. Such a procedure may therefore prove useful in studying this enzyme in a state that closely resembles that *in vivo*.

Chorismate mutase–prephenate dehydrogenase (EC 1.3.1.12) is a key enzyme in bacterial biosynthesis of tyrosine (Pittard & Gibson, 1970). It has the interesting property of catalysing two consecutive reactions in this pathway, and evidence is available to suggest that the active sites at which the reactions occur are close to, or contiguous with, one another (Koch *et al.*, 1972). Prephenate need not dissociate from the enzyme during reaction, and hence it is likely that when it is formed in the mutase reaction it then swings into a position that is more conducive to the subsequent dehydrogenase reaction. With this in mind, it thus seemed possible that double-ring compounds, containing structural features resembling to some extent both chorismate and prephenate, might be capable of binding simultaneously to both regions of such an active site, and hence prove to be stronger inhibitors of the enzyme than are compounds that are analogues of just one or other of the two substrates. The present work was undertaken in the hope of finding such ‘double-headed’ inhibitors that might prove to be useful, not only in studies of the catalytic and other properties of the enzyme, but also as conjugates for purification of the enzyme by affinity chromatography. Although a purification procedure has been published (Koch *et al.*, 1971) for the enzyme isolated from *Escherichia coli* K12 (strain JP232), it is time-consuming and has not yielded pure enzyme in our hands. Further, there is

considerable evidence (Reed & Cox, 1970) that this enzyme exists in several species as a multienzyme complex. Part of the aim of the present work was therefore to seek a method of purification, based on affinity chromatography, that is quick and gentle, in the hope of avoiding such steps as $(\text{NH}_4)_2\text{SO}_4$ precipitation, which is known to disrupt the structure of at least one well-characterized multienzyme complex, pyruvate dehydrogenase (Wold, 1971; Reed & Cox, 1970).

Experimental

Materials

p-Nitro-L-phenylalanine ($\text{R} = \text{NO}_2$, $\text{R}' = \text{H}$) was prepared by the method of Bergel & Stock (1954). *N*-Toluene-*p*-sulphonyl-L-phenylalanine ($\text{R} = \text{H}$, $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-CH}_3$) and *N*-toluene-*p*-sulphonyl-L-tyrosine ($\text{R} = \text{OH}$, $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-CH}_3$) were prepared by the method of Fischer & Lipschitz (1915). *N*-Toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine ($\text{R} =$

NO_2 , $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-CH}_3$) and *N*-toluene-*p*-sulphonyl-*L*-*p*-aminophenylalanine ($\text{R} = \text{NH}_2$, $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-CH}_3$) were prepared by the method of Jameson *et al.* (1973). *N*-Benzoyl-*L*-phenylalanine ($\text{R} = \text{H}$, $\text{R}' = \text{CO-C}_6\text{H}_5$) was prepared by the method of Fischer & Mouneyrat (1900). *N*-Benzyl-oxy-carbonyl-*L*-phenylalanine ($\text{R} = \text{H}$, $\text{R}' = \text{CO-O-CH}_2\text{-C}_6\text{H}_5$) was prepared by the method of Bergmann *et al.* (1934). *N*-Acetyl-*L*-phenylalanine ($\text{R} = \text{H}$, $\text{R}' = \text{CO-CH}_3$) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-(*p*-Nitrobenzenesulphonyl)-*L*-phenylalanine ($\text{R} = \text{H}$, $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-NO}_2$) was prepared from *L*-phenylalanine and *p*-nitrobenzenesulphonyl chloride by the method of Jameson *et al.* (1973) as used for the preparation of the corresponding *N*-toluene-*p*-sulphonyl derivative. After recrystallization from ethyl acetate/light petroleum (b.p. 40–60°C), it had m.p. 166–169°C (Found: C, 51.0; H, 4.1; N, 7.9%. $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_6\text{S}$ requires C, 51.42; H, 4.03; N, 7.99%). *N*-(*p*-Aminobenzenesulphonyl)-*L*-phenylalanine hydrochloride ($\text{R} = \text{H}$, $\text{R}' = \text{SO}_2\text{-C}_6\text{H}_4\text{-NH}_3^+\text{Cl}^-$) was prepared by hydrogenation of *N*-(*p*-nitrobenzenesulphonyl)-*L*-phenylalanine by the method of Jameson *et al.* (1973) as used for the preparation of *N*-toluene-*p*-sulphonyl-*L*-*p*-aminophenylalanine. After recrystallization from ethanol/diethyl ether it had m.p. 172–175°C (decomp.) (Found: C, 50.0; H, 4.9; N, 7.8%. $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_4\text{ClS}$ requires C, 50.49; H, 4.80; N, 7.85%).

CNBr-activated Sepharose-4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. 1-Adamantanecetic acid [tricyclo(3,3,1,^{3,7})decane-1-acetic acid] was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Chorismic acid was prepared by the method of Gibson (1970). Barium prephenate was kindly given by Dr. I. G. Young, John Curtin School of Medical Research, Australian National University, Canberra. Protamine sulphate and NAD^+ were obtained from Sigma, St. Louis, MO, U.S.A. Aquacide III [flake poly(ethylene glycol)] was obtained from Calbiochem, San Diego, CA, U.S.A. All other reagents and chemicals were of high purity and obtained from commercial sources.

Methods

N-(*p*-Aminobenzenesulphonyl)-*L*-phenylalanine-Sepharose-4B and *N*-toluene-*p*-sulphonyl-*L*-*p*-aminophenylalanine-Sepharose-4B conjugates. *N*-(*p*-Aminobenzenesulphonyl)-*L*-phenylalanine and *N*-toluene-*p*-sulphonyl-*L*-*p*-aminophenylalanine were coupled covalently to CNBr-activated Sepharose-4B by the procedure recommended by Pharmacia as supplied with their product. In a typical preparation, CNBr-activated Sepharose-4B (5g of dry powder) was washed and swelled with ice-cold 1M-HCl on a sintered-glass funnel. Approx. 1 litre of solution

was used, in several batches, the supernatant being sucked off between successive additions. The gel was then added to a suspension (10mm) of ligand in ice-cold 0.1M- NaHCO_3 /0.5M- NaCl [30ml, containing 10% (v/v) ethanol or dioxan] and shaken gently overnight at 4°C. The gel was filtered off and washed with more NaHCO_3 / NaCl solution (2litres) and then with 1M-HCl containing 20% (v/v) ethanol (2litres). The gel was washed in a short column with the chromatography buffer (0.05M-Tris/HCl/1mM-disodium EDTA/0.2mM-dithiothreitol, pH7.5 at 25°C). When not in use the column was washed with water or buffer containing 1mM- NaN_3 and stored at 4°C.

Enzyme preparations. The enzyme sample used for the inhibition studies was prepared by the method of Kock *et al.* (1971) and stored in 0.1M-Tris/HCl/1mM-EDTA/0.2mM-dithiothreitol (pH7.5)/30% (v/v) glycerol. For assays the enzyme was diluted directly in the assay buffer. This enzyme sample had a specific activity of 0.9 $\mu\text{mol/min}$ per mg of protein and showed several bands on electrophoresis. It was devoid of chorismate mutase-prephenate dehydratase (EC 4.2.1.51) activity. All other enzyme preparations were made by the procedure described herein.

Bacteria. *Escherichia coli* JP 232 was kindly supplied by Professor J. Pittard, Dept. of Microbiology, University of Melbourne, Parkville, Vic., Australia, and grown at 37°C in a glucose/mineral-salts medium supplemented with thiamin (Koch *et al.*, 1971). Cells were collected by centrifugation (8300g, 0°C, 20min). The yield was approx. 7.5g wet wt. of cells/litre of culture. Cells were harvested in a Sorvall RC2-B centrifuge and used immediately for enzyme preparation. Cells were disrupted in an Aminco French Pressure Cell at 138 MPa (20000lb/in²).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel disc electrophoresis was used with Tris/glycine buffer as in the method of Davis (1964), but with the following modifications. The sample and stacking gels were eliminated, and the sample (0.02 ml) containing enzyme in 30% (v/v) glycerol/buffer was applied directly to the top of the resolving gel. Gels were run at room temperature (25°C) and 2mA per tube. Protein bands were detected by staining with Coomassie Blue (Weber & Osborn, 1969). Enzyme activity on gels was detected by the method of Koch *et al.* (1970).

Chromatography buffers. All experiments described were done in 0.05M-Tris/1mM-disodium EDTA/0.2mM-dithiothreitol adjusted either to pH7.5, 8.3 or 9.0 with HCl at room temperature, unless otherwise indicated.

Enzyme assays. The inhibition studies were performed by measuring the rate of the overall reaction from chorismate to 4-hydroxyphenylpyruvate (Koch

et al., 1972) in 0.1 M-Tris/HCl/1 mM-disodium EDTA, pH 8.1. Reaction was commenced by adding the enzyme to the preincubated cuvette at 30°C. The final concentration of chorismate was 0.11 mM, that of NAD⁺ was 0.2 mM and that of inhibitor was as indicated. The reaction was followed at 340 nm in a Varian Techtron (model 635) recording spectrophotometer. This assay showed a slight lag in the product-time curve, by virtue of the coupling of the two consecutive reactions, but became linear within approx. 60–90 s. None of the inhibitors significantly changed the lag period.

In assays for mutase activity specifically, the decrease in A_{274} was recorded directly (Koch *et al.*, 1972), or, alternatively, the rate of formation of prephenate was measured by conversion into phenylpyruvate (Koch *et al.*, 1970). The latter method was used for assaying fractions eluted during column chromatography.

Chorismate mutase-prephenate dehydratase (EC 4.2.1.51) activity was assayed by the method of Cotton & Gibson (1965) or by the method of Koch *et al.* (1970) for chorismate mutase, but was modified by terminating the reaction directly with 1 M-NaOH rather than with 1 M-HCl. In this procedure any prephenate present is not converted into phenylpyruvate and hence an increase in A_{320} during the assay represents prephenate dehydratase activity.

Protein determination. Protein concentrations for specific-activity determinations were determined by the method of Hartree (1972), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard. In column elutions A_{280} was used as a measure of total protein concentration, assuming that a solution of 1 mg/ml gives an A_{280} of 1.0 in 1 cm-path-length cuvettes.

Results

Inhibition studies

The enzyme that was prepared for testing the potential inhibitors exhibited sigmoidal kinetics in its mutase reaction. This may have been a result of it not being pure, but nevertheless presented a problem for the inhibition kinetics in that precise inhibition constants could not be defined.

The value of the chorismate concentration at which the enzymic rate was half the maximum velocity ($V/2$) was determined as 0.11 mM ($s_{0.5}$). The effect of each inhibitor was then determined in assays using this chorismate concentration, by measuring half the inhibitor concentration that gave 50% inhibition ($I_{0.5}$). These values are given in Table 1. In the analogous situation of an enzyme displaying Michaelis-Menten kinetics and being competitively inhibited such a procedure would yield the precise inhibition constant K_i .

Table 1. *Inhibition of chorismate mutase-prephenate dehydrogenase by derivatives of phenylalanine*

$I_{0.5}$ values were determined at a chorismate concentration of 0.11 mM ($s_{0.5}$) as described in the text.

Compound	$I_{0.5}$ (mM)
<i>N</i> -Toluene- <i>p</i> -sulphonyl-L-phenylalanine	0.69
<i>N</i> -Benzyloxycarbonyl-L-phenylalanine	0.71
<i>N</i> -Toluene- <i>p</i> -sulphonyl-L- <i>p</i> -aminophenylalanine	1.1
<i>N</i> -Benzoyl-L-phenylalanine*	>2
<i>N</i> -Acetyl-L-phenylalanine*	>2

* Exact values could not be obtained owing to insolubility at the relevant concentration.

Affinity chromatography

The two columns originally tested consisted of the *N* - toluene - *p* - sulphonyl - L - *p* - aminophenyl - alanine-Sephacrose-4B and *N*-(*p*-aminobenzene-sulphonyl) - L - phenylalanine-Sephacrose - 4B conjugates. A sample of the enzyme used for the above inhibition studies was applied to each column (18 cm × 1 cm) in pH 7.5 buffer at 25°C and the columns were washed with the same buffer. The former column gave complete retention of the enzyme after exhaustive washing, and it (or one prepared similarly) was used for all subsequent experiments; the latter column only gave partial retention and was not used further. A variety of eluents were used in an attempt to wash the enzyme from the column. The following were unsuccessful: L-tyrosine (0.2 and 2 mM), NaCl (0.3 and 1 M), 1-adamantaneacetic acid [shown by Andrews & Smith (1975) to be a strong competitive inhibitor of the enzyme; 0.2 and 10 mM] (all in pH 7.5 buffer), pH 8.3 buffer, and 0.04 M-potassium phosphate buffer (pH 6); the enzyme was not eluted at pH 7.5 (pH refers to 25°C when made up) in the temperature range 4–25°C. However, with pH 9 buffer the enzyme was eluted quantitatively from the column, although in a relatively large elution volume. In subsequent experiments it was found that the inclusion of 10 mM-1-adamantaneacetic acid in the pH 9 buffer decreased the elution volume by one-third.

Having found conditions whereby the relatively pure enzyme preparation could be bound to and then eluted from the columns, we experimented with procedures for using the columns to isolate the enzyme from bacterial cells. The following procedure has been used as a routine, all steps being performed at 0–4°C except as indicated.

Bacterial cells (from 3 litres of culture) were suspended in pH 7.5 buffer (4 ml/g wet wt. of cells) and disrupted in the French Pressure Cell (138 MPa). Cell debris was removed by centrifugation (20 200g, 0°C, 20 min), and to the supernatant was added 2% (w/v) protamine sulphate (1 vol./4 vol. of cell extract)

Fig. 1. *Affinity chromatography of chorismate mutase-prephenate dehydrogenase*

The elution profile has been measured with respect to both A_{280} (●) and enzyme activity. Total chorismate mutase activity (○) and chorismate mutase-prephenate dehydrogenase activity (△) are both shown. Dehydrogenase activity can be estimated by difference. The fraction volume was 5 ml.

Table 2. *Summary of affinity chromatography of chorismate mutase-prephenate dehydrogenase*

The Table shows the total activity and total protein obtained during the chromatography of the enzyme, together with the corresponding specific activity, enrichment and yield. The last-mentioned parameters have been calculated with respect to the protamine sulphate extract. In the calculation of the activity of the enzyme in the presence of adamantaneacetic acid ('combined fractions 46-49') the inhibitory effect of this compound has been estimated and allowed for from its known apparent inhibition constant of 0.12 mM (P. R. Andrews, E. N. Cain, E. Rizzardo & G. D. Smith, unpublished work).

Stage of preparation	Total activity (μmol/min)	Total protein (mg)	Sp. activity (μmol/min per mg)	Enrichment (fold)	Yield (%)
Initial crude extract	63.0	1352	0.047	—	—
Protamine sulphate extract	31.7	922	0.034	1	100
Combined fractions 46-49	11.6	6.6	1.76	52	37
Fractions 46-49 after concentration and dialysis	4.6	6.6	0.70	21	15

to remove nucleic acids. After 10 min the precipitate was removed by centrifugation (20200g, 0°C, 20 min) and the supernatant applied to the column (18 cm × 1 cm) that had been previously equilibrated with pH 7.5 buffer at 0-4°C. The column was then washed with pH 7.5 buffer (50-60 ml) to remove most of the components of the crude extract remaining on the column. It was then washed with 1 M-NaCl in pH 7.5 buffer (80-90 ml) and again with pH 7.5 buffer (30-40 ml). The column was then equilibrated at room temperature for 10-15 min, and enzyme eluted with 10 mM-1-adamantaneacetic acid in pH 9 buffer. It was important that this final elution should be done at room temperature; virtually no elution was obtained at 0-4°C. An elution profile corresponding to the above procedure is shown in Fig. 1, in which both chorismate mutase-prephenate dehydrogenase and

chorismate mutase-prephenate dehydratase were eluted together. Other enzymes of the aromatic pathway were not assayed. Clearly there was not coincidence of the activity and protein (as measured by A_{280}), and this was always observed. In the preparation described by Fig. 1 fractions 46-49 were pooled, concentrated by placing in a dialysis sac and leaving in Aquacide, and then dialysed against pH 7.5 buffer/30% glycerol. The enzyme was stored at -20°C in this form. Table 2 summarizes the preparation with respect to yield and enrichment. The procedure could be completed in a single day.

Considerable loss of activity occurred on protamine sulphate treatment. Experiments in which this treatment was omitted were unsatisfactory, as direct application of the viscous crude extract to the column resulted in an extremely slow flow rate. Hence yield

and enrichment have been calculated relative to the protamine sulphate extract, rather than to the initial extract, as values calculated in this way are more representative of the chromatography procedure. Of concern is the loss of activity that occurred during concentration and dialysis against glycerol buffers. However, no better method of storing the enzyme was found, and the loss of activity may in fact represent reversible changes in the structure of this complex enzyme. The non-coincidence of the protein and activity peaks in Fig. 1 may also indicate that the enzyme can exist in two different oligomeric forms with different activity (D. J. Llewellyn & G. D. Smith, unpublished work). The final preparation exhibited 12–15 bands on polyacrylamide-gel electrophoresis.

In this procedure considerable enrichment of the enzyme was obtained from crude extracts in a single step. Such a preparation should prove useful for studies of the structure and function of the enzyme as a possible multienzyme complex.

In some preparations $(\text{NH}_4)_2\text{SO}_4$ fractionation of the protamine sulphate extract was included. It was found that most enzymic activity could be precipitated between 20.5 and 34.9 g/100 ml of extract. The precipitate was collected by centrifugation and dissolved in and dialysed overnight against pH 7.5 buffer before application as a small volume to the chromatography column. The inclusion of this step did not appear to enhance the yield or enrichment of the enzyme.

Discussion

Considerable doubt exists as to whether many of the chromatographic procedures described in the literature as 'affinity chromatography' are any more than simple ion-exchange chromatography (O'Carra *et al.*, 1974), and hence care must be taken in describing a new chromatographic procedure as such. O'Carra *et al.* (1974) showed that in many published examples of affinity chromatography there has been a failure to distinguish between biospecific and non-biospecific adsorption and that in many cases the non-specific adsorption is so large that it tends to mask any specific adsorption that might also be present. In a number of instances this can be attributed to the unavoidable ion-exchange effects of either the ligand attached to the column material or to the 'spacer arm' that is used. The most common reason for non-specific adsorption, however, appears to be due to the use of hydrophobic spacer arms, e.g. the hexamethylene grouping, which tends to bind many enzymes with hydrophobic or aromatic substrates. We believe that the procedure described in this paper can reasonably be assumed to be an affinity method for the following reasons. First, the column does not have a spacer arm between the Sepharose and the inhibitor to which it is attached and hence would not bind proteins

as a result of the hydrophobic nature of such a spacer arm. The possibility of an ion-exchange effect operating with this particular column cannot be eliminated, since an essential part of the substrate and inhibitors of this enzyme is the presence of at least one carboxyl group. However, the second reason for claiming an affinity effect is the failure of 1 M-NaCl to elute chorismate mutase-prephenate dehydrogenase activity from the column. Thirdly, elution at pH 9 is considerably enhanced by the presence of the competitive inhibitor, 1-adamantaneacetic acid. That the enzyme is eluted at pH 9, when it is not eluted at pH 7.5–8.3, is indicative of a binding role of an ionizing group in the active site of the enzyme with a pK value greater than 8.3. This group may be lysine, for which there is considerable evidence (B. Davidson & J. Morrison, personal communications). A group with such a high pK would be expected to be present, since bond formation between the enzyme and the carboxyl groups of chorismate, prephenate and transition state is likely to be involved in the catalytic function of this enzyme (Andrews *et al.*, 1973). Similar arguments presumably also apply to whatever other enzymes may be retained on the column; because of the aromatic nature of the affinity group on the column it is possible that other enzymes of the aromatic pathway are retained.

In this particular column, the ligand is not attached to the column material by a spacer arm, and, although the enzyme does obviously bind to the ligand-Sepharose conjugate, it may not in fact be the optimum structure. Cuatrecasas (1972) has shown that a suitable spacer arm improves the effective binding capacity of a column, and this may well be an important factor for our particular enzyme, which has a high molecular weight, and hence steric factors involving approach to the ligand on the column may be important.

The premise on which this work was based, i.e. that chorismate and prephenate occupy adjacent positions within a single active site of the enzyme, is of course neither proved nor disproved by the present work. For such proof, based on inhibition studies, a large number of inhibitors in which structural features were systematically varied would have to be studied. The main evidence for a single active site for both the mutase and the dehydrogenase reactions is that of Koch *et al.* (1972), who showed that prephenate concentrations in overall assays are low, suggesting that prephenate, when formed, does not necessarily leave the enzyme before being converted into 4-hydroxyphenylpyruvate. Whether or not there is any physical translocation of prephenate at all, from one reaction to the next, is not known. Nevertheless, our observations are consistent with such a model, and the observation, for example, that *N*-acetyl-L-phenylalanine is a much weaker inhibitor than diaromatic compounds containing L-phenylalanine

indicates that both rings are involved in binding to the active site.

The purpose of this work was to develop a fast method of extraction of the enzyme and one which would avoid potential damage to the physical structure of the enzyme as it exists in the cell, perhaps associated with other enzymes of the aromatic amino acid biosynthetic pathway. The simplicity and specificity of the described method encourage one to hope that both of these aims are achieved. In conjunction with purification steps described previously by Koch *et al.* (1971) the affinity chromatography may facilitate the rapid preparation of pure enzyme.

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