Enzymic Synthesis of *N*-Acetyl-L-Phenylalanine in *Escherichia coli* K12

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(Received 14 April 1971)

1. Cell-free extracts of *Escherichia coli* K12 catalyse the synthesis of *N*-acetyl-L-phenylalanine from acetyl-CoA and L-phenylalanine. 2. The acetyl-CoA-L-phenylalanine α -*N*-acetyltransferase was purified 160-fold from cell-free extracts. 3. The enzyme has a pH optimum of 8 and catalyses the acetylation of L-phenylalanine. Other L-amino acids such as histidine and alanine are acetylated at slower rates. 4. A transacylase was also purified from *E. coli* extracts and its substrate specificity studied. 5. The properties of both these enzymes were compared with those of other known amino acid acetyltransferases and transacylases.

During studies on the nature of acylamino acids present in *Escherichia coli* both in the free form and in proteins, it was found that *N*-acetyl-L-phenylalanine was the principal acylamino acid (Krishna, Krishnaswamy & Rajagopal Rao, 1969a). A preliminary study revealed that cell-free extracts of *E. coli* catalysed the synthesis of *N*-acetyl-L-phenylalanine from L-phenylalanine and acetyl-CoA (Krishna, Krishnaswamy & Rajagopal Rao, 1969b). The present paper describes the purification and properties of the enzymes involved.

EXPERIMENTAL

Materials

DL-, L- and D-amino acids were products of British Drug Houses Ltd., Poole, Dorset, U.K. or E. Merck A.G., Darmstadt, Germany. Most of the acylamino acids were obtained from either Calbiochem, Los Angeles, Calif., U.S.A., or Sigma Chemical Co., St Louis, Mo., U.S.A. All reagents used for preparation of buffers etc. were of analytical-reagent grade wherever possible.

L-[U-¹⁴C]Phenylalanine, specific radioactivity 50 mCi/mmol, and [U-¹⁴C]glycine, specific radioactivity 50 mCi/mmol, were products of The Radiochemical Centre, Amersham, Bucks., U.K. Sodium [1-¹⁴C]acetate, specific radioactivity 24 mCi/mmol, and DL-[1-¹⁴C]phenylalanine, specific radioactivity 24 mCi/mmol, were products of Bhabha Atomic Research Centre, Trombay, Bombay, India.

ATP and CoA were obtained from Sigma Chemical Co. Acetyl-CoA was a product of Calbiochem. Acetyl phos-

phate (dilithium salt) was obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Renal acylase I was prepared from pig kidneys by the procedure of Birnbaum, Levintow, Kingsley & Greenstein (1952). Carbonic anhydrase, ribonuclease and trypsin were obtained from Worthington Biochemicals Corp., Freehold, N.J., U.S.A. Cytochrome c and bovine serum albumin were products of Sigma Chemical Co. Acetone-dried cells of *Streptococcus faecalis* were used as source of phenylalanine decarboxylase to determine L-phenylalanine (Udenfriend, 1957).

Calcium phosphate gel was prepared by the procedure of Tsuboi & Hudson (1954). Alumina (Alcoa-301) used for grinding bacterial cell suspensions was a product of Aluminium Company of America, Pittsburgh, Pa., U.S.A. Bacto-Tryptone, Bacto-Yeast extract and agar were products of Difco Laboratories, Detroit, Mich., U.S.A.; sometimes Tryptone made by Oxoid Ltd., London E.C.4., U.K., was used. *p*-Hydroxymercuribenzoate, iodoacetamide and *N*-ethylmaleimide were products of Sigma Chemical Co. Sephadex G-75, G-100 and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50 (X4; H⁺ form; 100-200 mesh) was a product of Dow Chemical Co., Midland, Mich., U.S.A. Dowex 50 resin was recycled several times from the Na⁺ and H⁺ forms before use.

The bacterial strain, *E. coli* K12, was obtained from Dr T. Ramakrishnan, Department of Microbiology and Pharmacology, Indian Institute of Science, Bangalore, India.

Methods

Conditions of growth of E. coli K12. *E. coli* was grown on the following medium: Bacto-Tryptone (10g), Bacto-Yeast extract (5g) and NaCl (10g), dissolved in water and made up to 1 litre after adjustment to pH 7.5 with 2M-NaOH. The medium was sterilized by autoclaving at 110°C for 15 min, cooled and used. Cultures were grown at 37°C for 18 h (stationary phase) in a fermenter with forced aeration in 17-litre batches containing a 10%

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inoculum. Cells were collected by centrifugation (Sharples centrifuge) and washed thrice with 5 vol. of 0.9% NaCl. Approx. 75–80g wet wt. of cells was obtained. These were freeze-dried and the dried cells stored in a desiccator at -15°C until used.

Cell-free extracts. Freeze-dried cells were ground with alumina (1 part of cells to 2 parts of alumina; McIlwain, 1948) with a small amount of 0.05M-TME* buffer for 15 min. The pasty mass was diluted with 40 vol. of the same buffer and centrifuged at 10000g for 30 min at $0-5^{\circ}\text{C}$ to remove cell debris. The supernatant was the cell-free extract and was used for purification of the enzymes.

Paper chromatography. Acetylaminic acids were chromatographed on Whatman no. 1 paper with (i) propan-2-ol-aq. NH_3 soln.-water (8:1:1, by vol.) and (ii) butan-1-ol-acetic acid-water (20:1:4, by vol.) as solvents (Zenk & Schmitt, 1968).

Isolation of acylamino acids. Acylamino acids were separated from amino acids by chromatography on a column of Dowex 50 (H^+ form) and elution with water. The effluent containing the acylamino acids was concentrated under reduced pressure at $40-50^{\circ}\text{C}$.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. During calcium phosphate-gel treatment and Sephadex-gel filtration, protein was determined by the procedure of Warburg & Christian (1941).

Polyacrylamide-gel electrophoresis. The apparatus and experimental conditions used were similar to those described by Davis (1964). Polyacrylamide-gel concentration was 7.5% and tris-glycine buffer, pH 8.3 (I.044), containing 1 mM-mercaptoethanol was used.

Ultracentrifugal studies. Sedimentation-velocity determinations were made at 59780 rev./min on a 1% soln. of the protein (α -N-acetyltransferase) in 0.05M-TME buffer at 4°C . A Spinco model E ultracentrifuge fitted with phase-plate schlieren optics was used. From the photographs, taken at intervals of 16 min, $s_{20,w}$ values were calculated by the standard procedure (Schachman, 1959). Molecular-weight determinations were made by the sedimentation-equilibrium method of Archibald, as described by Schachman (1959). The concentration factor was determined in a synthetic-boundary cell. A partial specific volume of 0.76 was assumed for calculating the molecular weight.

Molecular weight by gel filtration. The elution volumes of proteins of known molecular weight and the α -N-acetyltransferase were individually determined on Sephadex G-75 and G-100 (Andrews, 1964). The molecular weight of the α -N-acetyltransferase was then determined by the method described by Determann (1968).

Determination of thiols. The thiol group of CoA was determined by the procedure of Grunert & Phillips (1951). Acetyl-CoA was determined by the procedure of Lipmann & Tuttle (1945).

Determination of radioactivity. Radioactivity was determined in samples of 0.1–1 ml (1000–5000 c.p.m.), which were transferred to glass scintillation vials; 10 ml of the scintillation fluid (Bray, 1960) was added and radioactivity counted in a Packard Tri-Carb scintillation

spectrometer of 75% efficiency for ^{14}C . Radioactivity was also determined on samples plated on aluminium or stainless-steel planchets, which were counted in a windowless gas-flow counter (Tracerlab model SC-16 or Nuclear-Chicago model D-47) of about 40% efficiency for ^{14}C .

Enzyme assays

Assay of acetate-CoA ligase. Reactions catalysed:

The reaction mixture for the assay of acetate-CoA ligase (EC 6.2.1.1) contained, in a final volume of 1 ml: tris-HCl buffer, pH 8.3, 100 μmol ; sodium acetate, 20 μmol ; ATP, 10 μmol ; MgCl_2 , 5 μmol ; CoA, 100 μg ; GSH, cysteine or mercaptoethanol, 5 μmol ; neutralized hydroxylamine, 200 μmol ; enzyme, 100–200 μg . After incubation at 37°C for 15 min, 4 ml of FeCl_3 -trichloroacetic acid reagent was added and the amount of ferric acetohydroxamate complex formed was determined at 540 nm (Lipmann & Tuttle, 1945). One unit of enzyme activity is defined as the amount of the enzyme required for the formation of 1 μmol of acetohydroxamate under these conditions. Specific activity is defined as the number of units/mg of protein. An acetohydroxamate standard was prepared by the procedure of Stadtman (1957).

Assay of acetyl-CoA-L-phenylalanine α -N-acetyltransferase. Reaction catalysed:

The reaction mixture for the assay of the α -N-acetyltransferase contained all the components described for the assay of acetate-CoA ligase except hydroxylamine. Instead of hydroxylamine, L-phenylalanine, 10 μmol , containing either DL-[1- ^{14}C]phenylalanine (8×10^4 c.p.m.) or L-[U- ^{14}C]phenylalanine (4×10^4 c.p.m.), and 10–100 μg of the acetyltransferase were added. The final volume was 1 ml. After incubation at 37°C for 15 min, the reaction was terminated by immersion of the assay tubes in boiling water for 3 min and the tubes were later centrifuged for 10 min. The entire supernatant was applied to a Dowex 50 (H^+ form) column (1 cm $\times$ 4 cm), eluted with water and the effluent was made up to 25 ml. The amount of radioactivity was determined in appropriate fractions. Individual omissions of the acetyl-CoA-generating system or of L-phenylalanine, and boiled enzyme controls, were used as blanks. In the first few steps of enzyme fractionation there was no necessity to add a separate source of acetate-CoA ligase as a considerable excess was present in the acetyltransferase itself. However, this point was checked by a separate acetate-CoA ligase assay on these fractions. At the later stages of enzyme fractionation, when separation of acetate-CoA ligase and α -N-acetyltransferase was apparent, an excess of purified acetate-CoA ligase, which was essentially free of α -N-acetyltransferase, was added to the assay mixture.

The amount of enzyme required to catalyse formation of 1 μmol of N-acetylphenylalanine under the assay conditions is defined as one unit of activity. Specific activity

* Abbreviation: TME buffer, tris-HCl buffer, pH 8.3, containing 1 mM-2-mercaptoethanol.

Table 1. *Requirements for the enzymic synthesis of N-acetyl-L-phenylalanine*

Standard assay conditions for acetate-CoA ligase and α -N-acetyltransferase as described under 'Methods' were used with the addition of 5 μ mol of NaF to inhibit adenosine triphosphatase activity. Dialysed cell-free extract (2 mg of protein) was used as the source of enzyme*.

System	Acetohydroxamate formed (μ mol)	N-Acetyl-L-phenylalanine formed (μ mol)
Complete	3.20	1.0
– Acetate	0.09	0.1
– ATP	0.10	0.08
– CoA	0.20	0.10
– Mg ²⁺	0.10	0.15
With boiled enzyme	0	0.05

* Cell-free extract (10 ml; 50 mg of protein) was dialysed against 500 ml of 0.05 M-TME buffer for 4 h at 0–5°C with a change of buffer at 2 h.

is defined as units/mg of protein. Alternatively, [1-¹⁴C]-acetate was used instead of radioactive phenylalanine and the acylamino acid fractions from the Dowex 50 (H⁺ form) columns were repeatedly evaporated to dryness with addition of water to remove the volatile [¹⁴C]acetic acid and the residue was later counted for radioactivity.

Reversibility of α -N-acetyltransferase reaction. The reaction mixture consisted of the following components, in a total volume of 1 ml: TME buffer, 50 μ mol; L-phenylalanine, 20 μ mol; N-acetyl-DL-[1-¹⁴C]phenylalanine, 6 $\times$ 10⁴ c.p.m.; MgCl₂, 10 μ mol; CoA, 100 μ g; α -N-acetyltransferase, 100 μ g. After incubation at 37°C for 15 min, the reaction was stopped by heat denaturation, the mixture was centrifuged and the supernatant was applied to a Dowex 50 (H⁺ form) column (1 cm $\times$ 5 cm). The column was washed with water (total volume 25 ml) and amino acids were eluted with aq. 2 M-NH₃ soln. (total vol. 25 ml). The eluates were concentrated *in vacuo* at 40°C and residues dissolved in 4 ml of water. Samples were taken for determination of radioactivity.

Assay of transacylase. Reaction catalysed:

The reaction mixture for the assay of transacylase contained the following components, in a final volume of 1 ml: tris-HCl buffer, pH 8.3, 100 μ mol; N-acetyl-L-amino acid, 10 μ mol (or N-acetyl-DL-amino acid, 20 μ mol); DL-[1-¹⁴C]-phenylalanine, 8 $\times$ 10⁴ c.p.m. (or L-[U-¹⁴C]phenylalanine, 4 $\times$ 10⁴ c.p.m.); transacylase (corresponding to 400–500 μ g of protein). After incubation at 37°C for 15 min, the mixture was processed as described in the assay of the α -N-acetyltransferase.

The amount of enzyme required to catalyse formation of 1 μ mol of the N-acetyl-L-[¹⁴C]phenylalanine under the conditions of assay is defined as one unit of enzyme activity; specific activity is defined as units/mg of protein. Omission of the acetyl amino acid from the complete system and complete system incubated with boiled enzyme formed controls for the assay.

Assay of transpeptidase. Reaction catalysed:

The reaction mixture for assay of transpeptidase contained the following components, in a final volume of 1 ml: TME buffer, pH 8.3, 100 μ mol; L-phenylalanyl-L-tyrosine, 20 μ mol; L-alanine, 20 μ mol; enzyme, 400–500 μ g. After incubation for 15 min at 37°C, the reaction was stopped by addition of 3 ml of ethanol and the product centrifuged. Samples (50–100 μ l) of the supernatant were chromatographed and the new amino acid (phenylalanine in this case) was determined by the procedure of Giri, Radhakrishnan & Vaidyanathan (1952). Large amounts were spotted on to Whatman 3 MM paper to detect traces of reaction.

RESULTS

Cell-free extracts catalysed the synthesis of N-acetyl-L-phenylalanine (Table 1). Omission of CoA, ATP or Mg²⁺ from the assay system had marked influence both on the formation of acetohydroxamate and N-acetyl-L-phenylalanine, indicating that two distinct reactions, (A) and (B), were probably involved. On fractionation of the cell-free extract (Table 2) it was found that two different protein fractions were needed to catalyse reactions (A) and (B).

The enzymes acetate-CoA ligase and α -N-acetyltransferase, catalysing reactions (A) and (B), have been purified and their properties studied.

Purification of acetate-CoA ligase, α -N-acetyltransferase and transacylase

Details of the steps involved are given below. Results obtained in a typical fractionation are given in Table 2.

Table 2. *Purification of acetate-CoA ligase, α -N-acetyltransferase and transacylase from freeze-dried cells*

The starting material was 10 g of freeze-dried cells.

Step no.	Stage of purification	Vol. (ml)	Protein (mg/ml)	Acetate-CoA ligase			α -N-Acetyltransferase			Transacylase		
				Specific activity	Total units	Recovery (%)	Specific activity	Total units	Recovery (%)	Specific activity	Total units	Recovery (%)
1	Cell-free extract	360	5	1.55	2790	100	0.45	810	100	0.15	280	100
2	(NH ₄) ₂ SO ₄ fractionation: (a) 40-60% saturation (fraction 1) (b) 60-90% saturation (fraction 2)	22 60	19 13.6	4.00 0.50	1672 450	60 16	0.26 0.73	110 600	13.5 74	— 0.25	— 225	— 80
3	Further purification of acetate-CoA ligase: (a) Cs ₃ (PO ₄) ₂ -gel treatment of fraction 1, gel supernatant (b) Sephadex G-100 gel filtration	18 5	14.0 1.5	5.50 110	1400 840	50 30	0.10 —	26 —	3.2 —	— —	— —	— —
4	Separation of acetyltransferase and transacylase by Cs ₃ (PO ₄) ₂ -gel treatment of fraction 2 (gel supernatant)	25	9.0	—	—	—	—	—	—	0.60	135	48
5	Further purification of acetyltransferase: (a) Gel eluate (b) pH 5 treatment and (NH ₄) ₂ SO ₄ precipitation (c) Sephadex G-100 gel filtration (d) CM-Sephadex C-50 chromatography	47 30 5 3	12.3 4 1.5 1.0	— — — —	— No activity — —	— — — —	1.10 3.00 36.00 75.00	630 360 270 225	77 45 33 25	— — — —	— — — —	— — — —
6	Further purification of transacylase; Sephadex G-75 gel filtration of gel supernatant from step 4	3	2.3	—	—	—	—	—	—	8.50	58	27

Preparation of cell-free extracts. Freeze-dried cells (10g) were extracted as described under 'Methods' by using 400ml of 0.05M-TME buffer. The enzyme fractionation was carried out at 0–5°C.

Ammonium sulphate fractionation. The cell-free extract was treated with solid ammonium sulphate (0.24g/ml), which was added gradually with continuous stirring to obtain about 40% saturation. After 15min, the suspension was centrifuged at 15000g for 15min. The precipitate was discarded. The supernatant obtained was similarly treated with additional ammonium sulphate (0.13g/ml) to give about 60% saturation and the suspension was again centrifuged. The precipitate obtained was dissolved in about 20ml of 0.02M-TME buffer and dialysed against 1 litre of the same buffer for 12h with a change of buffer at 6h (fraction 1). The supernatant remaining after 60% saturation was raised to about 90% saturation by addition of more ammonium sulphate (0.23g/ml) as described above. The precipitate was dissolved in 60ml of 0.02M-TME buffer and dialysed against 50 vol. of the same buffer as described above (fraction 2).

Further purification of acetate-CoA ligase

Calcium phosphate-gel treatment and Sephadex-gel filtration. Fraction 1 was diluted with buffer to contain 10mg of protein/ml and stirred with calcium phosphate gel for 15min [protein/dry wt. of gel (w/w) ratio of 3]. It was then centrifuged and the gel discarded. The gel supernatant was applied to a column (1.8cm × 50cm; void volume 43ml) of Sephadex G-100 in two portions (the second portion was added after complete elution of protein from the first addition) and eluted with 0.05M-TME buffer containing 0.05M-potassium chloride. Fractions (5ml) were collected. The acetate-CoA ligase activity was present in fractions 16, 17 and 18 and was clearly separated from the principal protein peak. The combined fractions (specific activity 110) were used as a source of acetate-CoA ligase in all experiments.

Separation of acetyltransferase and transacylase by calcium phosphate-gel treatment. Fraction 2 from the ammonium sulphate fraction was also diluted with buffer and treated with calcium phosphate gel as described above. The α -N-acetyltransferase was adsorbed on to the gel, whereas the transacylase remained in the supernatant.

Further purification of acetyltransferase

Elution from calcium phosphate gel. The α -N-acetyltransferase was eluted from the gel by stirring with 25ml of 10% ammonium sulphate solution (pH 8) for 20min and centrifuging. The gel pellet was again suspended in another 25ml of 10%

ammonium sulphate and treated similarly. The combined eluates (47ml) contained predominantly the α -N-acetyltransferase activity.

pH 5 treatment. The pH of the combined ammonium sulphate eluates was adjusted to 5 by dropwise addition of ice-cold 2M-acetic acid with continuous stirring and centrifuged at 10000g for 10min. The precipitate was discarded and the pH of the supernatant adjusted to 8 by addition of 0.1M-sodium hydroxide.

Ammonium sulphate precipitation. Ammonium sulphate (0.66g/ml) was added to the above supernatant to give about 80% saturation and the suspension was centrifuged after 15min. The precipitate was dissolved in about 12ml of 0.02M-TME buffer.

Gel filtration. The solution from the previous step was applied to a column (2cm × 49cm; void volume 52ml) of Sephadex G-100. 0.02M-TME buffer containing 0.05M-potassium chloride was used as eluent and fractions (5ml) were collected. Most of the enzyme was present in fractions 20, 21 and 22.

CM-Sephadex chromatography. The combined fractions from the above step were applied to a column (1.4cm × 20cm) of CM-Sephadex C-50 and eluted with 0.02M-TME buffer containing potassium chloride (linear gradient 0.05–0.15M-potassium chloride). Fractions (3ml) were collected. Most of the activity was recovered in fractions 17–20 (Fig. 1). The α -N-acetyltransferase was purified nearly 160-fold overall (specific activity 75) by this procedure.

Further purification of transacylase

Gel filtration. The supernatant from the calcium phosphate-gel treatment of fraction 2 was applied to a column (1.5cm × 55cm; void volume 36ml) of

Fig. 1. Chromatography of α -N-acetyltransferase on CM-Sephadex C-50. Experimental details are given in the text. ○, Total protein in fraction; △, sp. activity of enzyme.

Sephadex G-75 in two equal portions and eluted with 0.02M-TME buffer. Fractions (4ml) were collected (Fig. 2). Enzyme activity was recovered in fractions 21, 22 and 23.

Properties of α -N-acetyltransferase

Purity. The enzyme sedimented as a single component ($s_{20,w}$ 1.8S) in the ultracentrifuge (Fig. 3). Molecular weight as determined by the Archibald method was 24000. It showed one major and one minor component by polyacrylamide-gel electrophoresis. The enzyme was eluted as a single component on gel filtration on Sephadex G-75 or G-100 and molecular-weight determination by the methods of Andrews (1964) and Determann (1968) gave a value of 18000. The purified enzyme is free of transacylase activity.

Effect of pH on enzyme activity. The pH optimum of the reaction as found by using different buffers was about 8.

Product of the reaction. Assay mixtures from several experiments were combined and used to identify the acylamino acid formed. The acyl-amino acid was identified as *N*-acetyl-L-phenylalanine by direct paper chromatography (Zenk & Schmitt, 1968) and radioautography. Further it was hydrolysed both enzymically (renal acylase I) and by acid. The volatile acid formed was steam-distilled and identified as acetate via acetohydroxamate (Bergman & Segal, 1956). Phenylalanine was identified by paper chromatography as well as by reaction with a L-phenylalanine specific decarboxylase (Udenfriend, 1957).

Fig. 2. Gel filtration of transacylase on Sephadex G-75. Experimental details are given in the text. Fractions were collected after the void volume. ○, Total protein in fraction; Δ, sp. activity of enzyme.

Substrate specificity. A number of L-amino acids and D-amino acids, as well as β -alanine, γ -aminobutyric acid, 3,4-dihydroxyphenylalanine, *o*- and *p*-aminobenzoic acid, glucosamine and imidazole were tested in addition to the compounds mentioned in Table 3. The enzyme catalyses maximally the acetylation of L-phenylalanine, but L-histidine and L-alanine were also acetylated at lower rates. The rates with L-tyrosine and L-phenylalanyl-L-tyrosine are too low to be of any significance. The enzyme is specific for the L-isomer only, as D-amino acids, amino sugars, amines and miscellaneous amino compounds mentioned above are not acetylated.

Determination of Michaelis constants. The effect of various concentrations of L-phenylalanine and acetyl-CoA on the enzyme reaction was studied and the results were analysed by the method of Lineweaver & Burk (1934). It was found that the K_m values for L-phenylalanine and acetyl-CoA were 3.3 and 0.13mM respectively.

Stoichiometry of the reaction. The reaction was carried out under standard assay conditions. The

Fig. 3. Sedimentation-velocity analysis of α -N-acetyltransferase at 4°C at 59780 rev./min in a Spinco model 1E ultracentrifuge. Bar angle 55°. Upper pattern: protein concn. 1.1% in 0.05M-TME buffer; lower pattern: protein concn. 0.8% in 0.05M-tris-HCl buffer, pH 8.3, without mercaptoethanol. The photograph was taken 97 min after attainment of maximum speed. Sedimentation is from left to right.

Table 3. *Substrate specificity of α -N-acetyltransferase*

Standard conditions of assay of the enzyme were used with 50 μ g of acetate-CoA ligase and 50 μ g of highly purified α -N-acetyltransferase. L-Tyrosine was used at a concentration of 3 mM because of its low solubility; other substrates were used at 10 mM.

Substrate	Acetyl amino acid formed (μ mol)	Relative rates
L-Phenylalanine	3.0	100
L-Phenylalanyl-L-tyrosine	0.25	8
L-Tyrosine	0.06	2
L-Histidine	1.80	60
L-Alanine	0.87	29

initial and final concentrations of the reactants and products were determined by procedures described under 'Methods'. The results are given in Table 4. It is apparent from the results that there is a fairly good proportionality between the amounts of reactants used up and products formed.

Effect of thiol-blocking agents. *p*-Hydroxymercuribenzoate, *N*-ethylmaleimide and iodoacetamide were tested at various concentrations. The enzyme (50 μ g) was preincubated with these compounds in tris-hydrochloride buffer, pH 8.3 (100 μ mol without mercaptoethanol), for 5 min and then other components of the assay mixture were added. Acetyl-CoA was used instead of the acetyl-CoA-generating system because the test compounds inhibited the acetate-CoA ligase (Table 5). It is evident from the results that the acetyltransferase is a thiol enzyme, as it is inhibited by low concentrations (0.08 mM) of thiol-blocking agents.

Reversibility of the reaction. The α -N-acetyltransferase catalysed only the forward reaction leading to the synthesis of *N*-acetyl-L-phenylalanine. No reversibility could be detected by using labelled substrates.

Transacylase

The presence of a transacylase in crude extracts was revealed by the exchange reaction between *N*-acetyl-L-phenylalanine and L-[14 C]phenylalanine with the formation of *N*-acetyl-L-[14 C]phenylalanine (Table 6).

The transacylase has been partially purified (Table 2) and its substrate specificity studied. With DL-[1- 14 C]phenylalanine or L-[U- 14 C]phenylalanine as the acceptor amino acid in the assay system for transacylase, various acylamino acids such as formyl, acetyl, benzoyl and phthalyl derivatives of DL-isomers of alanine, leucine, valine, methionine, cysteine and phenylalanine were tested as

Table 4. *Stoichiometry of the α -N-acetyltransferase reaction*

Standard assay conditions were used; incubation was for 20 min. The initial and final concentrations of substrates utilized and products formed were determined as described under 'Methods'.

Reactants	Concn. (μ mol)		
	Initial	Final	Change
Phenylalanine	5.0	2.56	-2.44
<i>N</i> -Acetyl-L-phenylalanine	0.15	2.20	+2.05
Acetyl-CoA	2.60	0.50	-2.10
CoA	0.50	2.48	+1.90

Table 5. *Inhibition by thiol-blocking agents*

Highly purified enzyme (50 μ g of protein) was incubated in tris-HCl buffer, pH 8.3 (100 μ mol), with the inhibitor (*p*-hydroxymercuribenzoate, *N*-ethylmaleimide or iodoacetamide) at 37°C for 5 min. Acetyl-CoA (100 μ g) was used instead of the acetate-CoA ligase system. The reaction was started by addition of L-phenylalanine.

Inhibitor	Concn. (mM)	Inhibition (%)
<i>p</i> -Hydroxymercuribenzoate	0.10	100
	0.08	90
	0.05	55
	0.01	36
<i>N</i> -Ethylmaleimide	0.10	100
	0.08	80
	0.05	50
	0.01	28
Iodoacetamide	0.50	100
	0.08	85
	0.05	60
	0.01	34

donors of acyl groups (Table 7). It is evident from the results that the transacylase catalyses the transfer of acetyl groups from several *N*-acetyl-L-amino acids to L-phenylalanine. The activity with formylamino acids was lower than that with acetyl-amino acids.

With *N*-acetyl-L-phenylalanine as donor of acetyl groups, labelled phenylalanine, leucine and histidine were used to test the specificity for the acceptor amino acid for transacylation (Table 8). The *N*-acetyl amino acids formed in the reaction, i.e. *N*-acetyl-L-[14 C]phenylalanine, *N*-acetyl-L-[14 C]leucine and *N*-acetyl-L-[14 C]histidine, were identified by paper chromatography (Zenk & Schmitt, 1968). Further, the acylamino acid was identified by acid hydrolysis and characterization of the amino acid by paper chromatography. *N*-Acetyl-D-amino acids and D-amino acids were inert as donor and acceptor respectively.

DISCUSSION

It has been shown in the present work that cell-free extracts of *E. coli* catalyse the acetyl-CoA-dependent acetylation of L-phenylalanine. The enzyme acetyl-CoA-L-phenylalanine α -N-acetyltransferase has been purified 160-fold by ammonium sulphate fractionation, calcium phosphate-gel treatment, Sephadex-gel filtration and chromatography on CM-Sephadex. The purified enzyme was monodispersed by ultracentrifugation and has an approximate molecular weight of 24 000. It showed, however, one major and a minor component by polyacrylamide-gel electrophoresis. The enzyme needs essential thiol groups for activity. Only acetyl-CoA was active as the acetyl-group donor and acetyl phosphate was inert in the standard assay system.

The purified *E. coli* K12 α -N-acetyltransferase described here differs from the acetylating system described in cell-free extracts of another strain of *E. coli* (Maas, Novelli & Lipmann, 1953), in which the acetyl donor was acetyl phosphate and acceptor was L-glutamate. In these experiments the possibility that the extracts might contain a phosphotransacetylase, which could convert acetyl phosphate into acetyl-CoA and thus use catalytic quantities of CoA present in the extracts, was not

ruled out. However, cell-free extracts of *Clostridium kluyveri* are also known to catalyse a cyanide-dependent acetylation of a few amino acids (glycine, leucine, serine, lysine, glutamic acid and aspartic acid) and the acetyl donor is acetyl phosphate (Lieberman & Barker, 1955). Acetyl phosphate provides the acetyl groups for acetylation of δ -amino group of L-ornithine in certain bacteria, frogs etc. (Grisolia & Harmon, 1962). The ϵ -amino group of L-lysine is also acetylated by acetyl phosphate in ox heart mitochondrial preparations (Paik & Kim, 1964). Acetyl-CoA-dependent acetylation of aspartic acid in rat and cat brain has been studied and the α -N-acetyltransferase purified (Knizley, 1967; Goldstein, 1959, 1969). An acetyl-

Table 6. *Transacetylase activity in E. coli K12*

The standard assay system for transacetylase described under 'Methods' was used. Dialysed cell-free extract (2.3 mg of protein) was used as the enzyme*.

System	¹⁴ C-labelled acetyl amino acid formed (c.p.m.)
Complete	1400
Without N-acetyl-L-phenylalanine	210
With boiled enzyme	95

* Conditions of dialysis are described in Table 1.

Table 7. *Substrate specificity of transacetylase*

As acceptor, DL-[1-¹⁴C]phenylalanine was used and, as donor, various N-acetyl-DL-amino acids. The experimental conditions are described under 'Methods'; 450 μ g of 50-fold-purified enzyme (sp. activity 7.5) was used. Acylamino acids were used at 20 mM and incubation was for 30 min at 37°C.

Acyl donor	Acylamino acid exchanged (μ mol)
Acetyl-DL-alanine	2.0
Acetyl-D-alanine	0
Formyl-DL-alanine	0.2
Acetyl-DL-leucine	1.4
Formyl-DL-leucine	0.1
Acetyl-DL-valine	0.8
Formyl-DL-valine	0.15
Acetyl-DL-methionine	1.0
Acetyl-D-methionine	0.05
Formyl-DL-methionine	0.56
N-Acetyl-L-cysteine	1.30
Acetyl-L-phenylalanine	3.60
Acetyl-DL-phenylalanine	3.20
Acetyl-D-phenylalanine	0.04
Formyl-DL-phenylalanine	3.20
Benzoyl-DL-phenylalanine	0.50
Phthalyl-DL-phenylalanine	0.20

Table 8. *Substrate specificity of transacetylase*

As donor, N-acetyl-L-phenylalanine was used and, as acceptor, various L-amino acids. The standard assay system for transacetylase was used, but with 70 000 c.p.m. of the respective acceptor ¹⁴C-labelled amino acids.

Donor	Acceptor	10 ⁻⁴ × Radioactivity of acetyl amino acid exchanged (c.p.m.)
N-Acetyl-L-phenylalanine	L-[U- ¹⁴ C]Phenylalanine	2.5
	DL-[1- ¹⁴ C]Leucine	0.76
	DL-[1- ¹⁴ C]Phenylalanine	2.13
	L-[1- ¹⁴ C]Histidine	0.90
	Boiled enzyme control	0.17
N-Acetyl-D-phenylalanine	DL-[1- ¹⁴ C]Phenylalanine	0.15
	Boiled enzyme control	0.10

CoA-dependent α -*N*-acetyltransferase has been purified to near homogeneity from baker's yeast and is specific for a number of D-amino acids (Zenk & Schmitt, 1968).

The possibility of formation of *N*-acetyl-L-phenylalanine by pathways other than direct acylation has also been explored. During these investigations it was found that *E. coli* extracts had a transacylase that catalysed the transfer of acetyl groups from a few *N*-acetyl-L-amino acids to L-phenylalanine. *N*-Acetyl-D-phenylalanine was inactive in the transacylase system. The purified transacylase was free of α -*N*-acetyltransferase activity and did not have any transpeptidase activity, although the reaction catalysed is similar to a transpeptidation reaction.

A transacylation reaction from δ -acetyl-L-ornithine to glutamate has been demonstrated in *Micrococcus glutamicus* (Udaka & Kinoshita, 1958). *N*-Acetyl-L-glutamate is the acetyl donor in the formation of β -acetyl- α,β -diaminopropionic acid in the plant *Acacia mollisma* (Seneviratne & Fowden, 1968). By studies with [²H]acetate the possibility of transacylation in mammalian systems has been reported (Bloch & Rittenberg, 1947), but the enzyme systems involved have not been investigated so far.

The occurrence of free *N*-acetyl-L-phenylalanine in *E. coli* K12 can be explained by the presence of α -*N*-acetyltransferase but the exact mechanism by which it occurs in *E. coli* protein is still unknown (R. V. Krishna, P. R. Krishnaswamy & D. Rajagopal Rao, unpublished work).

We thank Dr O. Siddiqi and his colleagues at the Molecular Biology Division of Tata Institute of Fundamental Research, Bombay, India, for the facilities extended to us for a part of the investigation. Our thanks are due to Dr M. S. Narasinga Rao for help in the experiments with the ultracentrifuge. The present work was partially supported by Public Law 480 funds of U.S.A. Communications should be addressed to D.R.R.

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