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EVIDENCE FOR INVOLVEMENT OF ARGINYL RESIDUE AT THE CATALYTIC SITE OF PENICILLIN ACYLASE FROM ESCHERICHIA COLI [1]

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SUMMARY: Incubation of penicillin acylase from Escherichia coli with phenylglyoxal or 2,3-butanedione results in enzyme inactivation. Both benzylpenicillin and phenylacetate protect the enzyme against the inactivation, indicating the presence of arginine at or near the catalytic site. The reactions follow pseudofirst order kinetics and the inactivation kinetics indicate the presence of a single essential arginine moiety.

Penicillin acylase (EC 3.5.1.11) from E. coli catalyses the hydrolysis of benzylpenicillin to phenylacetic acid and 6-aminopenicillanic acid (6-APA), the latter being the key intermediate in the manufacture of semisynthetic penicillins. Extensive studies have been carried out consequently on industrial aspects such as the immobilization of the enzyme and cloning of the penicillin acylase gene (for review see Ref. 1). In contrast, only a few studies have been reported on the reaction mechanism and the active centers of this group of enzymes. Benzylpenicillin acylase from E. coli, which is the most extensively studied among this class of enzymes, has been shown to be a heterodimer ($\alpha\beta$) containing an M_r 20,500 (α) and an M_r 69,000 (β) subunit (2). In subunit structure the enzyme from E. coli resembles those from Proteus rettgeri (3), Kluyvera citrophila (4) and Arthrobacter viscosus (5).

The formation of an acyl-enzyme intermediate has been postulated from the kinetics of the enzyme catalyzed reaction (6). The serine reagent phenylmethanesulfonyl fluoride, which also structurally resembles the side chain of benzylpenicillin, inactivates the enzyme; a mole of the reagent completely inactiva-

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ting a mole of the enzyme from E. coli (7) or from P. rettgeri (3). However, the serine reagent diisopropylfluorophosphate inactivates only weakly the E. coli enzyme casting ambiguity about the presence of an essential serine residue (7). The involvement of tryptophan at the active site of enzymes from P. rettgeri (8) and from E. coli (9) has been suggested from inactivation studies.

In this communication we present evidence for the presence of an essential arginine residue in the E. coli enzyme.

MATERIALS AND METHODS

Phenylglyoxal and 2,3-butanedione were obtained from Fluka, benzylpenicillin and 6-aminopenicillanic acid (6-APA) were gifts from Hindustan Antibiotics Ltd., phenylacetic acid was obtained from Aldrich, and DEAE-Sepharose CL-6B was from Pharmacia.

Escherichia coli NCIM 2350 was obtained from the National Collection of Industrial Microorganisms, Pune, and was maintained routinely on nutrient agar slants.

Penicillin acylase. The enzyme was isolated from the extract of cells of E. coli grown at 26°C for 24 h under shake flask conditions in a medium of the following composition: yeast extract, 2 g; bactopeptone, 2 g; tryptone, 1 g; beef extract, 2 g; corn steep liquor (0.5 g/ml), 25 ml; K_2HPO_4, 3 g; KH_2PO_4, 0.3 g; NaCl, 3.5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0.2 g; phenylacetic acid, 1 g; and water to make to 1 L after adjustment of pH to 7.2. Cells were harvested by centrifugation, washed with 0.01 M potassium phosphate buffer, pH 7.5 and stored at -20°C.

Cells (60 g wet weight) were thawed, suspended in 0.05 M potassium phosphate buffer, pH 7.5, (3 ml/g packed cells), cooled in an ice bath and disrupted by sonication (20 Kc, 300 W) for a total period of 10 min. Cell debris was removed by centrifugation and all subsequent operations were carried out at 0-4°C.

The clear cell-free extract was subjected to ammonium sulfate fractionation, and the fraction obtained between 0.3 - 0.8 saturation was collected, dissolved in the minimum volume of 0.05 M potassium phosphate buffer, pH 7.5, and dialyzed overnight against 100 volumes of buffer of the same composition. The dialysate was clarified by centrifugation and applied on to a DEAE-Sepharose CL-6B column (3 x 75 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The enzyme was eluted using a linear gradient of potassium phosphate buffer, pH 7.5 (0.01 - 0.1 M). The eluant with enzyme activity was concentrated by ultrafiltration and loaded on a Sephadex G-200 column equilibrated with 0.05 M potassium phosphate buffer, pH 7.5; the fractions with penicillin acylase activity were pooled and concentrated by ultrafiltration.

Enzyme assay. Penicillin acylase activity was assayed essentially as described by Balasingham et al. (10) by measuring the amount of 6-APA produced at 40°C using 4% benzylpenicillin as substrate in 0.1 M potassium phosphate buffer, pH 7.8 and 6-APA produced was measured with p-dimethylaminobenzaldehyde according to the procedure of Bomstein and Evans (11). One unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions.

Protein assay. Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as standard.

Gel electrophoresis. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was carried out in 7.5% gels using Tris-glycine buffer, pH 8.8 as described by Laemmlli (13).

Treatment with arginine modifying reagents. Penicillin acylase was incubated at 25°C with either phenylglyoxal (2.5-20 mM) in 0.05 M potassium phosphate buffer, pH 8.0) or 2,3-butanedione (10 - 40 mM) in 0.05 M sodium borate buffer, pH 8.0. The reaction system was shielded from light when 2,3-butanedione was used as the arginine modifying reagent (14). Enzyme incubated with buffers in absence of the modifying reagents served as controls. Aliquots were withdrawn at different time intervals for assay of enzymatic activity.

Protection of the enzyme against inactivation by the arginine reagents was tested with benzylpenicillin-K salt and phenylacetate. The compounds were tested at 50 mM final concentration and pH 8.0 and were added immediately before the addition of phenylglyoxal (20 mM final concentration) or 2,3-butanedione (40 mM final concentration).

RESULTS

Enzyme purification: The purified enzyme had a specific activity of 27 U/mg protein. The preparation was homogenous on the basis of SDS-polyacrylamide gel electrophoresis, only two polypeptide bands of $M_r \sim 20,000$ and $M_r \sim 70,000$ being observed on staining.

Inactivation by Enzyme Modifying Reagents: E. coli penicillin acylase was rapidly inactivated on incubation at pH 8.0 with phenylglyoxal or 2,3-butanedione, the former being a more potent inactivator (Fig. 1). The rates of inactivation followed pseudo first-order kinetics in both cases, plots of the logarithm residual activity versus time of contact with the reagents being linear upto the test period of 60 min when the residual activity in presence of 20 mM phenylglyoxal was less than 5% initial enzyme activity, the corresponding value in presence of 40 mM 2,3-butanedione being 26%.

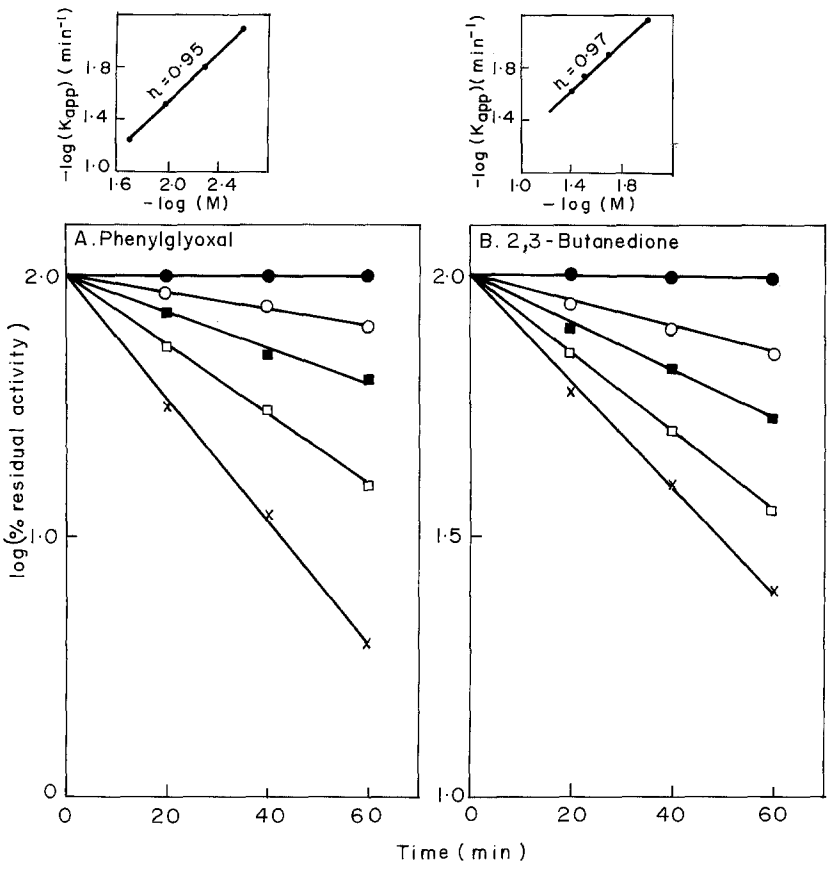

Fig. 1. Inactivation of penicillin acylase from E. coli by α-carbonyl reagents.

The enzyme (60 μg/ml) was incubated at 25°C in 50 mM potassium phosphate buffer (pH 8.0) and varying concentrations of phenylglyoxal or in 50 mM sodium borate buffer (pH 8.0) and varying concentrations of 2,3-butanedione

(A) Phenylglyoxal 0 mM (-●-), 2.5 mM (-○-), 5 mM (-■-), 10 mM (-□-) and 20 mM (-X-).

(B) 2,3-butanedione 0 mM (-●-), 10 mM (-○-), 20 mM (-■-), 30 mM (-□-), and 40 mM (-X-).

At the indicated periods of time aliquots were withdrawn for assay of enzyme activity.

Insets: Determination of the order of the reaction with respect to phenylglyoxal (A) and 2,3-butanedione (B).

The reaction order (n) with respect to the arginine reagent was determined from the plot of the logarithm of the apparent first order rate constant, K_{app} versus the logarithm of the

Table 1. Protection of E. coli penicillin acylase against inactivation by arginine specific reagents

Treatment[a]	Enzyme activity[b] (% initial activity)
None	100
Phenylglyoxal (20 mM)	4
Benzylpenicillin (50 mM) + phenylglyoxal (20 mM)	95
Phenylacetate (50 mM) + phenylglyoxal (20 mM)	60
2,3-Butanedione (40 mM)	26
Benzylpenicillin (50 mM) + 2,3-butanedione (40 mM)	98
Phenylacetate (50 mM) + 2,3-butanedione (40 mM)	92

[a]Test compounds when used were added immediately before the α-carbonyl reagent. Enzyme (60 µg/ml) was treated at 25°C as indicated in Table and samples were withdrawn periodically for assay of enzyme activity.

[b]Values obtained after 60 min.

reagent concentration and the value of n = 0.95 for phenylglyoxal and of n = 0.97 for 2,3-butanedione indicate that loss of enzyme activity results from the reaction of one arginine per mole penicillin acylase.

Protection from inactivation by arginine reagents: Table 1 summarizes the data on protection against inactivation of the penicillin acylase by phenylglyoxal and 2,3-butanedione in the presence of benzylpenicillin or of phenylacetate.

Benzylpenicillin (50 mM) protected the enzyme almost fully against inactivation by both 20 mM phenylglyoxal and 40 mM 2,3-butanedione, 95% and 98% respectively of initial activity being retained at the end of the test period of 60 min, compared to 4% and 26%, respectively in the absence of substrate. The corresponding values for protection by phenylacetate are 60% against inactivation of the enzyme by phenylglyoxal and 92% against inactivation by 2,3-butanedione.

DISCUSSION

Penicillin acylase from E. coli has been shown in the present study to be inactivated by arginine specific reagents such

as phenylglyoxal and 2,3-butanedione. The kinetics of inactivation indicate that the reaction of 1 mole reagent per mole enzyme is required for the inactivation. The presence of the substrate, benzylpenicillin or of the product, phenylacetate, which functions as a weak competitive inhibitor (10) protects the enzyme against inactivation by phenylglyoxal and 2,3-butanedione indicating, that the arginine residue is at or near the active site of the enzyme. The involvement of an essential arginine residue has not been reported hitherto in this group of enzymes

The presence of an essential arginine residue has been reported extensively in enzymes that act on anionic substrates or those that require anionic cofactors (15). The present study would add benzylpenicillin acylase from E. coli to this list of enzymes that depend on an arginyl residue to act upon a negatively charged substrate molecule.

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