SHORT COMMUNICATION

Localization of Cholinesterase in *Pseudomonas aeruginosa* Strain K

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The inducible cholinesterase of *Pseudomonas aeruginosa* strain K (ATCC 25102) degraded propionylcholine, acetylthiocholine, acetylcholine and acetyl- β -methylcholine at a high rate and butyrylcholine and succinylcholine at very low rates. The localization of the enzyme in the periplasmic space was indicated by a similar rate of acetylcholine degradation by intact cells or their extracts, by release of cholinesterase together with alkaline phosphatase into the culture medium during cell growth in a low phosphate-containing medium, by liberation of cholinesterase and alkaline phosphatase during lysozyme-induced conversion of cells to spheroplasts and by freezing and thawing. Treatment of cells with diazo-7-amino-1,3-naphthalenedisulphonic acid, which inactivates surface-located enzymes, abolished most of the cholinesterase and 5'-nucleotidase activities.

INTRODUCTION

Cholinesterase (ChE), which is rare in bacteria, occurs in cells of *Pseudomonas fluorescens* (Goldstein & Goldstein, 1953) and in *Pseudomonas aeruginosa* (Gilboa-Garber *et al.*, 1973; Tani *et al.*, 1975*a, b*). The formation of the enzyme in these bacteria is induced by acetylcholine (Goldstein & Goldstein, 1953; Fitch, 1963; Gilboa-Garber *et al.*, 1973) or choline (Fitch, 1963) and repressed by glucose (Gilboa-Garber *et al.*, 1973). A similar decomposition rate of acetylcholine was observed whether intact cells or extracts of either *P. fluorescens* or *P. aeruginosa* were employed (Fitch, 1963; Gilboa-Garber *et al.*, 1973) indicating easy availability of the substrate for the enzyme. The latter property could be related to localization of ChE at the cell surface. Surface localization has already been reported for several degradative enzymes of *P. aeruginosa*, such as alkaline phosphatase (Cheng *et al.*, 1970*b*), ribonuclease, cyclic-2',3'-phosphodiesterase, 5'-nucleotidase (Bhatti *et al.*, 1976) and L-asparaginase (Day & Ingram, 1971).

The aim of the present study was to examine the localization of the ChE of *P. aeruginosa* K using various techniques.

METHODS

Organism. *Pseudomonas aeruginosa* strain K (ATCC 25102) was maintained in 20% (w/v) glycerol at -15°C .

Media. Grelet's medium (Grelet, 1951) without glucose but with 0.4% (w/v) yeast extract (Eagon, 1956) (GE medium), nutrient broth (Difco) (NB medium), and Cheng's medium (Cheng *et al.*, 1970*a*) without glucose [C medium; containing 0.02 M-NH₄Cl, 0.02 M-KCl, 0.12 M-Tris, 0.5% (w/v) Proteose peptone (Difco) and 1.6 mM-MgSO₄, pH 7.4] were employed for growing the bacteria. Choline chloride (0.2%, w/v) was added to these media in order to induce ChE production.

Partial purification of *P. aeruginosa* K cholinesterase. Bacteria were grown with shaking in 12 l GE medium supplied with 0.2% (w/v) choline chloride at 30°C for 18 h. They were harvested by centrifugation at 16300 g

for 10 min at 4 °C, washed three times in 0.05 M-Tris/HCl buffer pH 7.5 and resuspended in the similar buffer to give 200 mg wet wt ml⁻¹. The bacteria were disintegrated by passage through a French pressure cell at 136 MPa and, after centrifugation at 16300 g for 30 min, the supernatant was used as the crude enzyme preparation. The total activity of ChE in this preparation was 7.4 units with a specific activity of 0.009 units (mg protein)⁻¹. Partial purification of the ChE was obtained by the following procedure (Laing *et al.*, 1967; Gilboa-Garber *et al.*, 1973): removal of nucleic acids with 0.7% (w/v) streptomycin sulphate at 0 °C, precipitation of foreign proteins by 40% saturation with (NH₄)₂SO₄ and by acidification to pH 5 with 2 M-acetic acid and concentration of the ChE by 60% saturation with (NH₄)₂SO₄ at pH 7.5 following overnight dialysis against 0.1 M-Tris/HCl buffer pH 7.5 at 4 °C. The specific activity of the partially purified enzyme (67% yield) was 0.084 ChE units (mg protein)⁻¹ (9.33-fold enrichment).

Preparation of spheroplasts and their extracts. Bacteria were grown in C medium for 18 h with shaking at 28 °C, harvested as described above and washed three times with a solution containing 0.22 M-NaCl, 0.026 M-MgCl₂ and 0.01 M-KCl, adjusted to pH 7 with 0.01 M-NaOH (Forsberg *et al.*, 1970). 'Magnesium' spheroplasts were prepared according to Cheng *et al.* (1970b) using 10% (wet wt/v) cells with 1 mg lysozyme ml⁻¹ (Sigma) and 1 h incubation in the presence of 0.2 M-MgCl₂. Spheroplast forms appeared after 15 min treatment with 0.01 M-MgCl₂ in 0.01 M-Tris/HCl buffer pH 8.4. The supernatants of the lysozyme/0.2 M-MgCl₂ medium and that containing 0.01 M-MgCl₂, which leads to spheroplast formation, were combined for the enzymic determinations. The spheroplasts were disintegrated by 3 min treatment in an ultrasonic disintegrator (MSE, 150 W) at 0 °C.

Determination of enzymes released into the medium during growth. Bacteria were grown with shaking in 250 ml C medium in 500 ml Erlenmeyer flasks at 28 °C for 18 h (0.36 mg dry wt ml⁻¹). The growth medium was separated from the bacteria by centrifuging and the bacteria were washed three times in 0.05 M-Tris/HCl buffer pH 7.5, suspended in 5 ml of similar buffer and ultrasonically disrupted for 3 min at 0 °C. Both the medium (C medium) and the disintegrated cells suspension were examined for enzymic activities.

Release of periplasmic enzymes by repeated freezing and thawing (Furth, 1975). Bacteria were grown in C medium, harvested and washed as described in the preceding section. The packed cells were divided into 1 ml portions and kept at -20 °C. Each sample was thawed with an equal volume of 0.2 M-acetic acid/sodium acetate buffer pH 4.8 containing DNAase (10 µg ml⁻¹, Sigma), frozen in an acetone/solid CO₂ mixture and thawed in a 37 °C water bath. After freezing and thawing twice more, 2 ml 0.2 M-acetate buffer pH 4.8 was added to the treated bacteria followed by stirring for 20 min at room temperature. The treated suspension was centrifuged at 33400 g at 2 °C for 1 h. The supernatant was kept at -20 °C and the cell pellet was suspended in 4 ml 0.05 M-Tris/HCl buffer pH 7.5, ultrasonically disrupted for 3 min and kept at -20 °C. Control bacteria were prepared under the same conditions but without freezing and thawing.

Diazotization of bacteria. Diazotization was performed according to Day & Ingram (1971) and Day *et al.* (1975) using diazo-7-amino-1,3-naphthalenedisulphonic acid (NDS). The bacteria were grown overnight at 28 °C in GE medium, sedimented by centrifugation, washed three times in 0.05 M-Tris/HCl buffer pH 7.5 and suspended at 5% (wet wt/v) in similar buffer. NDS reagent (1 ml) was added to 9 ml bacterial suspension and the mixture was incubated for 1 h at room temperature. The treated bacteria were washed three times in 0.05 M-Tris/HCl buffer pH 7.5 and resuspended to 10% (wet wt/v) in similar buffer. For determination of the enzymic activities, the diazotized cell suspension was subjected to ultrasonic treatment for 3 min.

Enzyme activities.

Cholinesterase (EC 3.1.1.7) was determined according to Ellman *et al.* (1961) in a reaction mixture containing, in 1 ml: 0.1 M-Tris/HCl buffer pH 7.5, 0.3 M-NaCl, 0.05 M-MgCl₂, enzyme sample and 1 µmol acetylthiocholine (Sigma). One unit of ChE was defined as the amount of enzyme which decomposed 1 µmol acetylthiocholine min⁻¹ at 30 °C and pH 7.5. Decomposition of other choline esters was followed manometrically (Pollock, 1952). Each substrate was tested at a concentration of 3.5 µmol ml⁻¹ in the presence of 10 µmol NaCl ml⁻¹.

3-Phosphoglycerate kinase (EC 2.7.2.3) was determined according to McFadden & Schuster (1972). One unit of 3-phosphoglycerate kinase was defined as the amount of enzyme which produced 1 µmol 1,3-diphosphoglyceric acid from 3-phosphoglyceric acid min⁻¹ at 30 °C and pH 7.4.

Alkaline phosphatase (EC 3.1.3.1) activity was measured by the method of Bessey *et al.* (1946). One unit of alkaline phosphatase was defined as the amount of enzyme which produced 1 µmol *p*-nitrophenol from *p*-nitrophenyl phosphate min⁻¹ at 37 °C and pH 10.5.

ATPase (EC 3.6.1.3) was measured by the method of Thacker & Eagon (1969). One unit of ATPase was defined as the amount of enzyme which liberated 1 µmol inorganic phosphate from ATP min⁻¹ at 37 °C and pH 9.

5'-Nucleotidase (EC 3.1.3.5) was measured according to the method of Neu & Heppel (1965) with some variations. The reaction mixture contained, in 0.9 ml: 5'-AMP (13 µmol), CaCl₂ (3.3 µmol), CoCl₂ (0.3 µmol) and sodium acetate buffer pH 5.8 (33.3 µmol). The reaction was stopped after 20 min at 37 °C by adding 0.9 ml 20% (w/v) trichloroacetic acid and the inorganic phosphate was assayed using the method of Chen

Fig. 1. Relative rates of hydrolysis of various choline esters by *P. aeruginosa* K cholinesterase. Ester hydrolyses were examined manometrically (Pollock, 1952) at 30 °C. The reaction mixture contained, in 3 ml: 10.5 μ mol choline ester, 30 μ mol NaCl, 21.5 μ mol NaHCO₃, 2.5 mg gelatin and the partially purified cholinesterase preparation [4.32 mg protein, 0.084 units (mg protein)⁻¹]. Cholinesterase activity on acetylcholine was taken as 100%. ACh, Acetylcholine; AtCh, acetylthiocholine; PrCh, propionylcholine; ButCh, butyrylcholine; SucCh, succinylcholine; AβmCh, acetyl-β-methylcholine.

et al. (1956). One unit of 5'-nucleotidase was defined as the amount of enzyme which liberated 1 μ mol inorganic phosphate from 5'-AMP min⁻¹ at 37 °C and pH 5.8.

Protein concentrations were determined by the method of Lowry.

RESULTS

Decomposition of various choline esters. The partially purified ChE was examined for its activity against several esterified choline derivatives (Fig. 1). The rate of propionylcholine degradation was the highest. Acetylcholine above 4 mM inhibited ChE activity. A similar rate of acetylcholine degradation (0.044 μ mol min⁻¹) was found whether intact cells or extracts of *P. aeruginosa* K were used.

Release of ChE and other enzymes into the culture medium. Bacteria grown in a low phosphate C medium at 28 °C for 18 h released 38.5 % of the total ChE activity into the medium. Under the same conditions 39.7 % of the total alkaline phosphatase activity was liberated, while no release of 3-phosphoglycerate kinase and ATPase was observed.

Release of ChE and other enzymes into the medium during the conversion of bacteria to spheroplasts. Conversion of bacteria to spheroplasts (Cheng *et al.*, 1970b) led to a considerable release of ChE and alkaline phosphatase into the medium (30 % and 80 % of their total activities, respectively). Under identical conditions release of ATPase and 3-phosphoglycerate kinase was considerably lower (18 % and 5 % of their total activities, respectively).

Effect of repeated freezing and thawing on the release of ChE and other enzymes. Repeated freezing and thawing of bacteria (Furth, 1975) led to a considerable release of ChE and

alkaline phosphatase into the medium (73% and 47% of their total activities, respectively), whilst 3-phosphoglycerate kinase and ATPase were not released.

Effect of diazotization on various enzyme activities. Treatment of bacteria with NDS reagent at room temperature for 1 h led to considerable inactivation of ChE, ATPase and 5'-nucleotidase activities (60%, 90.2% and 88.4% inactivation, respectively), while 3-phosphoglycerate kinase activity was unaffected.

DISCUSSION

The ChE of *P. aeruginosa* strain K resembles that of *P. fluorescens* (Laing *et al.*, 1967) and *P. aeruginosa* strain A-16 (Tani *et al.*, 1975*a, b*) in its activity on several choline esters (except for a somewhat higher activity on propionylcholine) and in its inhibition by an excess of acetylcholine. The latter property and the ability of the bacterial ChEs to decompose acetyl- β -methylcholine indicate their similarity to brain (Myers, 1953) and erythrocyte (Myers, 1952) acetylcholinesterases as opposed to human blood plasma pseudo-cholinesterase (Myers, 1952, 1953). Another similarity between the true acetylcholinesterase of the erythrocyte and the *Pseudomonas* enzyme was the similar activity that was found in intact cells and extracts for acetylcholine decomposition (Fitch, 1963; Gilboa-Garber *et al.*, 1973). This observation led us to examine the possibility that the bacterial enzyme was located on the cell surface. By comparison with the behaviour, under different experimental conditions, of enzymes known to be within the periplasmic space, such as alkaline phosphatase and 5'-nucleotidase (Cheng *et al.*, 1970*b*; Bhatti *et al.*, 1976), of ATPase, which is a plasma membrane enzyme (Thacker & Eagon, 1969), and of 3-phosphoglycerate kinase, which is a cytoplasmic enzyme, we concluded that ChE had a similar location to that of alkaline phosphatase and 5'-nucleotidase. This conclusion is based on the following experimental results. During growth in a low phosphate-containing medium, which leads to a release of periplasmic enzymes such as the alkaline phosphatase of *P. aeruginosa* (Cheng *et al.*, 1970*b*), the ChE was released with alkaline phosphatase, but ATPase and 3-phosphoglycerate kinase were not. By conversion of cells to spheroplasts, a process in which the alkaline phosphatase of *Escherichia coli* is released from the cells (Malamy & Horecker, 1964; Heppel, 1967; Brockman & Heppel, 1968), the *P. aeruginosa* K ChE and alkaline phosphatase were released to the surrounding medium; there was no such release of ATPase and 3-phosphoglycerate kinase activities. Repeated freezing and thawing of *P. aeruginosa*, which liberates periplasmic β -lactamase (Furth, 1975), also led to a significant release of ChE and alkaline phosphatase activities, but not of 3-phosphoglycerate kinase.

Differentiation between the *Pseudomonas* enzymes based on their localization was also obtained using NDS reagent (Pardee & Watanabe, 1968), which is known to react with and inactivate periplasmic enzymes. Internal enzymes are protected against NDS action by the cytoplasmic membrane (Day & Ingram, 1971; Day *et al.*, 1975). In *P. aeruginosa* K, NDS led to a considerably inactivated ChE, as well as 5'-nucleotidase and ATPase, without affecting 3-phosphoglycerate kinase activity.

In conclusion, the ChE of *Pseudomonas aeruginosa* behaves like known periplasmic enzymes, indicating its periplasmic localization.

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