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The inhibition of chloramphenicol-O-acetyltransferase by adenine nucleotides

Chloramphenicol acetylation catalyzed by chloramphenicol-O-acetyltransferase in the presence of acetyl-CoA, which contributes to the resistance of Enterobacteria and Staphylococci to the drug[1–6], was reported by us to be inhibited by ATP[7]. The inhibition, investigated in subcellular preparation of *Escherichia coli* B, was found to be of a noncompetitive type in relation to chloramphenicol. The relation of the enzyme inhibition by various nucleotides to acetyl-CoA, the acetyl donor for the acetylation of chloramphenicol, was the aim of the present study.

The cell-free extracts of *E. coli* B carrying R factor were prepared by a modification of the previously described method[8] which included suspension of the cells in 25% (wet weight/v) concentration in 0.01 M Tris–HCl buffer (pH 7.8), disruption of the cells by sonic vibrations (using a Raytheon 10KC 250W sonic oscillator for 5 min) and centrifugation at 30 900 × *g* for 30 min at 2° (refrigerated centrifuge, Sorvall RC2-B). The supernatant obtained was partially purified by passing it through a Sephadex G-50-80 (Sigma) column (1.1 × 19 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 7.8) before use[3] in order to prevent the inactivation of the enzyme by dilution. Chloramphenicol acetylation was followed by determination of the acetyl-CoA disappearance using a spectrophotometric 5,5′-dithiobis-2-nitrobenzoic acid assay[5].

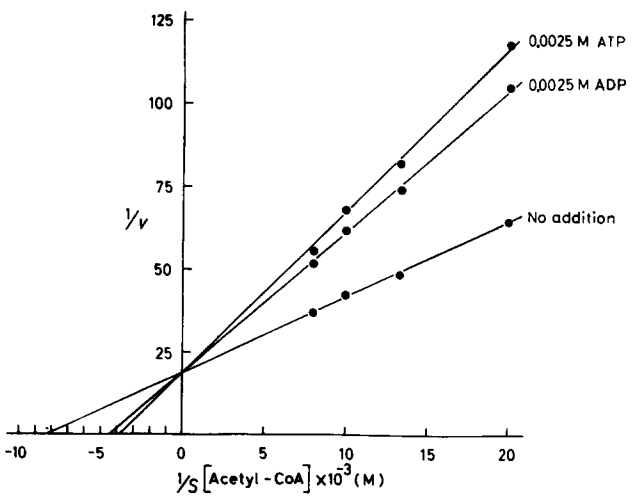

Fig. 1. Effect of ATP and ADP on chloramphenicol acetylation in the presence of varying concentrations of acetyl-CoA. The reaction mixture[5] contained the following in a volume of 1 ml: 1 μmole 5,5′-dithiobis-2-nitrobenzoic acid, 100 μmoles Tris–HCl buffer (pH 7.8), 0.1 μmole chloramphenicol, 0.050–0.125 μmole S-acetyl-CoA (Sigma), ATP and ADP at the concentration noted in the figure and 8.6 μg protein of a partially purified enzyme preparation of *E. coli* B carrying R factor. Acetyl-CoA disappearance was measured by following the absorption at 412 nm at 37° during 5 min using a Zeiss spectrophotometer PMQ II. The initial velocity (v) is expressed in μmoles of chloramphenicol acetylated per min per ml.

The effects of the adenine nucleotides on the kinetics of the enzyme activity, in the presence of varying concentrations of acetyl-CoA, indicate that ATP and ADP inhibit chloramphenicol-O-acetyltransferase by competition with acetyl-CoA (Fig. 1). The K_i values were found to be $2.25 \cdot 10^{-3}$ M for ATP and $3.02 \cdot 10^{-3}$ M for ADP. The K_m for acetyl-CoA was found to be $1.2 \cdot 10^{-4}$ M. Other related compounds, including 5'AMP, adenosine and pyrophosphate, in 2.5 mM concentration, were not inhibitory. It seems therefore that a combination of adenosine with at least two phosphate groups is a prerequisite for inhibition of the enzyme.

UTP was found to have a lower inhibitory power than ATP, while the inhibitory effects of GTP and CTP were found to be very weak (Table I). The inhibitory effect of

TABLE I

THE EFFECT OF NUCLEOTIDES ON CHLORAMPHENICOL ACETYLATION CATALYZED BY CELL-FREE EXTRACT OF *E. coli* B CARRYING R FACTOR

The reaction mixture and conditions were those described in the legend for Fig. 1, except the acetyl-CoA concentration which was 0.1 μmole.

Nucleotide	*Concentration (mM)*	*Enzymatic activity (units/ml)**	*Inhibition (%)*
None	—	0.0047	0.0
ATP	5.0	0.0017	62.5
UTP	5.0	0.0028	40.5
GTP	5.0	0.0042	11.0
CTP	5.0	0.0042	11.0

* One unit is defined as the amount of enzyme sufficient to acetylate 1 μmole of chloramphenicol per min (ref. 5).

ATP (5 mM) was neither reversed by preincubation of the ATP with Mg^{2+} (10 mM) nor by addition of these ions to the reaction mixture. Mg^{2+} is known to reverse some regulatory enzyme inhibitions by ATP[9].

The competition of ATP and ADP with acetyl-CoA for chloramphenicol-O-acetyltransferase indicates the possibility that acetyl-CoA is attached to the enzyme through its adenine nucleotide moiety. A recognition of the adenine nucleotide moiety of CoA was already ascribed to the phosphotransacetylases of *E. coli* B and *Clostridium kluyveri* which are inhibited by adenine nucleotides[10]. An additional example of inhibition of an enzyme by ATP, competing with the adenine nucleotide containing coenzyme and not with the substrate, is that of the NAD-linked pea malate dehydrogenase[11].

The conclusions can be summarized as follows: (1) ATP and ADP inhibit chloramphenicol-O-acetyltransferase of *E. coli* B by competing with acetyl-CoA and not with chloramphenicol. (2) This competitive inhibition may be regarded as an indication that the adenine nucleotide of acetyl-CoA is the moiety recognized by the enzyme. (3) The inhibitory power of the adenine nucleotides is dependent on the number of phosphate groups in the molecule. (4) Pyrophosphate, adenosine and 5'AMP, at a similar concentration, have no effect on chloramphenicol acetylation. (5) UTP is a weaker inhibitor of chloramphenicol-O-acetyltransferase than ATP, while CTP

and GTP are the weakest inhibitors. (6) Mg^{2+} does not abolish the inhibition of chloramphenicol acetylation exerted by ATP.

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The preparation of trypsin chemically attached to nylon tubes

Although enzymes have been insolubilised by a wide variety of methods[1], the resulting products have so far only been used in the form of particles, both for continuously stirred reactors[2] and packed beds[3], in the form of porous sheets[4] and in the form of membranes[5]. The present work describes two new methods for the insolubilisation of an enzyme by its attachment to nylon, together with the novel use of a water-insoluble enzyme in the form of a tube.

The inside surface of a nylon tube (4.0 m long, 0.1 cm internal diameter) made of "Type 6" nylon (John Tullis, Alloa, Scotland) was partially hydrolysed by perfusion through the tube with 3.0 M HCl for 30 min at 30° at a flow rate of 2 ml/min, after which the hydrolysis was arrested by washing through the tube with water. At this stage the presence of liberated amine groups was confirmed by the red coloration produced on the inside surface of the tube, when a small portion of the latter was treated with a 0.1% (w/v) solution of 2,4,6-trinitrobenzenesulphonate in saturated sodium tetraborate. So as not to interfere in subsequent steps in the process, the amine groups were destroyed by perfusing the tube for 2 min with an ice-cold solution of 1% (w/v) $NaNO_2$ in 0.5 M HCl and then warming the tube to 40° and continuing the perfusion for a further 20 min. The destruction of the primary amine groups was confirmed by the inability of a small sample of the inner surface of the tube to form a red-coloured trinitrophenyl derivative with 2,4,6-trinitrobenzenesulphonate.

The tube was then cut into two equal lengths and to one portion benzidine was coupled to the carboxyl groups by perfusion through the tube at 10° with a