2-Aminoethylarsonic acid as an analogue of ethanolamine phosphate

Endowment of ethanolamine-phosphate cytidyltransferase with CTP pyrophosphatase activity

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2-Aminoethylarsonic acid was tested for its ability to act as a substrate for ethanolamine-phosphate cytidyltransferase as a cytidyl acceptor in place of ethanolamine phosphate. The expected product, like all mixed anhydrides of arsonic acids, should hydrolyse spontaneously with regeneration of the substrate analogue and CMP formation; such CMP production was observed. The limiting velocity with aminoethylarsonic acid is about 90% that with ethanolamine phosphate, and the Michaelis constant is below 20 mM.

INTRODUCTION

Aminoethyl phosphate (*O*-phosphoethanolamine, ethanolamine phosphate, $\text{HO}_3\text{POCH}_2\text{CH}_2\text{NH}_3^+$) is incorporated into phospholipids via CDP-ethanolamine, which is synthesized by the transfer of a cytidyl group from CTP on to the phospho group of the aminoethyl phosphate. Its analogue, aminoethylphosphonic acid, $\text{HO}_3\text{PCH}_2\text{CH}_2\text{NH}_3^+$, is incorporated into the phosphonolipids of some lower organisms by a similar route of cytidyl transfer to form a CDP derivative [Liang & Rosenberg (1966); see also reviews by Florin-Christensen *et al.* (1986) and Kittredge & Roberts (1969)]. Further, the mammalian ethanolamine-phosphate cytidyltransferase accepts the phosphonate as a substrate to catalyse this reaction (Tamari *et al.*, 1973). This led us to think that it might also accept the corresponding arsonate, 2-aminoethylarsonic acid, $\text{HO}_3\text{AsCH}_2\text{CH}_2\text{NH}_3^+$.

The interest in this would be that the transferase would be endowed by the analogue with a CTP pyrophosphatase activity, since esters and anhydrides of arsonic acids hydrolyse spontaneously in water (see Scheme 1). They do so because the arsenic atom, being larger than phosphorus, passes more easily to the penta-co-ordinate transition state. This hydrolysis is the basis of well-known effects of arsenate on several enzyme systems [Gresser (1981) for ATP synthase; Warburg & Christian (1939) for glyceraldehyde-phosphate dehydrogenase; Braunstein (1931) for the general background]. It is also the basis of the finding that arsonic acids in which the arsonomethyl group, $\text{H}_2\text{O}_3\text{AsCH}_2-$, replaces the phosphono-oxy group, $\text{H}_2\text{O}_3\text{PO}-$, transform several reactions in which they participate. Such an analogue of AMP acts as a substrate for adenylate kinase and therefore endows this enzyme with ATPase activity (Adams *et al.*, 1984), since the analogue of ADP is rapidly hydrolysed; similarly such an analogue ($\text{H}_2\text{O}_3\text{AsCH}_2\text{PO}_3\text{H}_2$) of diphosphate acts as a substrate for RNA polymerase, and therefore gives this enzyme artificial exonuclease activity (Rozovskaya *et al.*, 1984). The present work shows that 2-aminoethylarsonic acid

similarly endows ethanolamine-phosphate cytidyltransferase (EC 2.7.7.14) with a CTP pyrophosphatase activity (Scheme 1).

MATERIALS AND METHODS

2-Aminoethylarsonic acid

This was prepared as described in the preceding paper by Geoghegan & Dixon (1989).

Ethanolamine-phosphate cytidyltransferase (EC 2.7.7.14)

The enzyme was prepared from rat liver by the method of Sundler (1975), involving homogenization, collection of the material precipitated by 25–50% saturation with $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose. On chromatography in Sundler's (1975) Tris/HCl buffer, pH 7.6, containing dithiothreitol, and a gradient from 0 to 0.3 M-NaCl, the active fractions were found to be those of 0.2–0.3 M-NaCl, although Sundler (1975) had found the enzyme to be eluted at slightly lower salt concentrations. The active fractions (30 ml from 15 g of rat liver) were pooled and stored at 0 °C.

Separation of cytidine nucleotides

Samples were analysed for CMP and CDP-ethanolamine by chromatography on a column (1 cm × 10 cm) of the strongly basic resin Dowex 1 (X8; 200–400 mesh; formate form) equilibrated and developed with a solution of 40 mM-formic acid/20 mM-triethylamine, pH 3.7, run at 0.9 ml/min. The effluent was monitored at 270 nm. In this system 2 ml samples of 0.25 mM-nucleotide were applied; CDP-ethanolamine ran at 2.8 column volumes, CMP at 8 column volumes, and CTP did not emerge by 12 column volumes.

Enzyme assay

The ethanolamine-phosphate cytidyltransferase was assayed by incubating 0.5 ml samples of enzyme (representing the enzyme from 0.25 g of liver) at 37 °C with 0.5 ml of a solution containing 4 mM-CTP, 2 mM-

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Normal reaction

Reaction modified by arsenical analogue

Scheme 1. Endowment of ethanolamine-phosphate cytidylyltransferase with the ability to hydrolyse CTP to CMP and diphosphate by the addition of 2-aminoethylarsonate

If the natural substrate, which may be 2-aminoethylphosphonate, $\text{OPO}_2^- \text{--CH}_2\text{CH}_2\text{NH}_3^+$, or, more commonly, *O*-phosphoethanolamine (ethanolamine phosphate, 2-aminoethyl phosphate), $\text{OPO}_2^- \text{--OCH}_2\text{CH}_2\text{NH}_3^+$, is replaced by 2-aminoethylarsonate, $\text{OAsO}_2^- \text{--CH}_2\text{CH}_2\text{NH}_3^+$, the instability of anhydrides of arsonic acids leads to the cycle shown.

ethanolamine phosphate, 20 mM-MgCl₂, 10 mM-dithiothreitol and 40 mM-Tris that had been adjusted with conc. HCl to pH 7.8. Incubations were for 0.5, 1.0, 1.5 and 2.0 h. The reaction was stopped by adding 1 ml of the much more acid chromatographic buffer (40 mM-formic acid/20 mM-triethylamine). It was then heated at 100 °C for 20 min, and the precipitate was removed by centrifugation. A 1 ml sample of the supernatant was then subjected to chromatography, as above, and the amount of CDP-ethanolamine produced was assessed from the area of the peak determined by its absorbance at 280 nm. When aminoethylarsonic acid was used in place of ethanolamine phosphate, the area of the peak of CMP was similarly assessed. The rate was measured from the slope of the progress curves, which remain constant for 2 h.

RESULTS

The active fraction of enzyme was incubated with 0.5 mM-ethanolamine phosphate and 1 mM-CTP. A rate of production of CDP-ethanolamine of 6.8×10^{-4} mM/min was observed. Since the Michaelis constant of this enzyme is 65 μM for ethanolamine phosphate (Sundler, 1975), the limiting velocity was about 7.7×10^{-4} mM/min.

When 2-aminoethylarsonic acid (20 mM and 50 mM) was used, CMP was produced at rates of 5.85×10^{-4} mM/min and 6.38×10^{-4} mM/min respectively. This corresponds to a limiting velocity of about 6.8×10^{-4} mM/min and a Michaelis constant of well under 20 mM (about 3 mM).

DISCUSSION

Catalysed reaction

Far the simplest interpretation of the production of CMP that we have demonstrated is that the 2-aminoethylarsonate is a substrate for the cytidylyltransferase (Scheme 1). This analogue is isosteric with aminoethylphosphonate, shown by Tamari *et al.* (1973) to be a substrate, so substrate action is not surprising. The presumed product must be unstable, so spontaneous hydrolysis completes the cycle shown in the lower half of Scheme 1. We have not, however, actually demonstrated that the mixed anhydride is an intermediate. Such an intermediate anhydride was trapped by Gresser (1981) for the mixed anhydride of ADP with arsenate. Similarly Teipel & Koshland (1970) obtained kinetic evidence for the finite lifetime of the 3-phosphoglyceroyl arsonate formed by glyceraldehyde-3-phosphate dehydrogenase

when it oxidizes glyceraldehyde 3-phosphate in the presence of arsenate.

Chromatographic properties

The basic resin might have been expected to retard CDP-ethanolamine more than CMP, since it possesses $-\text{PO}_2^-\text{OPO}_2^-\text{CH}_2\text{CH}_2\text{NH}_3^+$ in place of $-\text{PO}_3\text{H}^-$, and the extra negative charge should have more effect than an extra positive charge, since it will be closer to the exchanging groups of the resin. The fact that the reverse was found may be explained by the ability of the $-\text{PO}_3\text{H}^-$ group of the CMP to dissociate to $-\text{PO}_3^{2-}$, which may be the predominant form bound even at a pH where $-\text{PO}_3\text{H}^-$ predominates in solution, and by the hydrophilic properties of the diphosphate grouping.

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