Studies of the 2':3'-Cyclic Nucleotide Phosphodiesterase of *Haemophilus influenzae*

By BRUCE M. ANDERSON,* DAVID W. KAHN AND
CONSTANCE D. ANDERSON

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University,
Blacksburg, Virginia 24061, USA

(Received 17 January 1985; revised 14 March 1985)

The 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase of *Haemophilus influenzae* was purified from a periplasmic preparation by affinity chromatographic techniques. The enzyme-catalysed hydrolysis of 2':3'-cyclic AMP to adenosine without accumulation of the intermediate substrate 3'-AMP was demonstrated by high performance liquid chromatography. Competitive inhibition of the enzyme by a variety of nucleosides and mononucleotides indicated the presence of either purine or pyrimidine bases to be essential for selective interactions with the enzyme, and confirmed the need for a 3'-position phosphate for the functioning of mononucleotides as substrates for the enzyme. The enzyme had a molecular weight of 79000, was stable at low temperatures and was thermally denatured at temperatures above 50 °C.

INTRODUCTION

Enzymes catalysing the sequential hydrolysis of 2':3'-cyclic nucleotides to nucleosides through a 3'-mononucleotide intermediate have been demonstrated in several micro-organisms (Anraku, 1964*a, b*; Kohn & Reis, 1963; Neu, 1968; Kier *et al.*, 1977; Weppelman *et al.*, 1977). Rodden & Scoeca (1972) presented convincing evidence that the cyclic nucleotide phosphodiesterase:3'-nucleotidase of *Haemophilus influenzae* is a periplasmic enzyme. The purified enzyme was demonstrated to catalyse the hydrolysis of several 2':3'-cyclic nucleotides, 3'-mononucleotides and bis(*p*-nitrophenyl) phosphate, and to be inhibited by 2'- and 5'-mononucleotides. In current studies of the role of a periplasmic nucleotide pyrophosphatase in the NAD metabolism of *H. influenzae* (Kahn & Anderson, 1983), it became important to elucidate further the interactions of mono- and dinucleotides with the cyclic phosphodiesterase, since the latter enzyme tended to co-purify partially with the nucleotide pyrophosphatase. In addition, further studies of substrate specificity, selective inhibition, and related properties of the phosphodiesterase could aid in further understanding the physiological role of this enzyme.

METHODS

Chemicals. NAD, NADP, cyclic-NADP, ADP-ribose, ATP, ADP, 3':5'-cyclic AMP, 2':3'-cyclic AMP, 2'-AMP, 3'-AMP, ribose-5-phosphate, bis(*p*-nitrophenyl) phosphate, *p*-nitrophenyl phenylphosphonate, yeast alcohol dehydrogenase, thymidine *p*-nitrophenyl phosphate, protein molecular weight standards, Sephacryl S-200, and all purine and pyrimidine nucleosides and 5'-mononucleotides were from Sigma. Matrex green A and matrex blue A gels were from Amicon. Phosphocellulose was from Whatman. Reverse phase resin, RSIL C18HL (5 µm) for high pressure liquid chromatography was from Alltech.

Enzyme extraction. *H. influenzae* cells grown in brain heart infusion medium were harvested at late exponential phase, and periplasmic enzymes were released by a modification of the procedure described by Malamy & Horecker (1964). The resulting periplasmic preparation was dialysed, and proteins of interest were precipitated at 45–65% saturation of ammonium sulphate. The protein solution was fractionated on a phosphocellulose column equilibrated with 50 mM-Tris/HCl buffer, pH 8.5. The 2':3'-cyclic nucleotide phosphodiesterase activity measured spectrophotometrically at 405 nm with bis(*p*-nitrophenyl) phosphate as substrate was eluted from the

column (1.5×30 cm) with a linear gradient of 0.2–1 M-KCl. Fractions containing enzyme activity were dialysed in 50 mM-Tris/HCl, pH 8.5, and applied to a column (1.2×8 cm) containing Amicon matrix green A gel. Enzyme was eluted with a linear gradient of 0–500 mM-KCl in 50 mM-Tris/HCl, pH 8.5. The enzyme fraction was dialysed against 50 mM-Tris/HCl, pH 8.0, and applied to a column (1.2×8 cm) containing Amicon matrix blue A gel. The 2':3'-cyclic nucleotide phosphodiesterase was eluted from this final column with a linear gradient of 0–1 M-KCl in 50 mM-Tris/HCl, pH 8.0. The specific activity of the resulting enzyme preparation was $0.4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

High pressure liquid chromatography. This was done on an RSIL C18HL reverse phase column at 50°C with a flow rate of 1.5 ml min^{-1} . The eluting buffer was 50 mM-potassium phosphate, pH 5.5, containing 4 mM-tetrapropylammonium hydroxide and 5% (v/v) methanol. Separations were done on a Spectra-Physics SP8000 HPLC equipped with a SP770 UV spectrophotometric detector. Nucleotides were assayed spectrophotometrically at 260 nm. Yeast alcohol dehydrogenase assays were done in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-ethanol and 250 μg enzyme in a total volume of 1 ml. Reduction of NAD was monitored at 340 nm. Spectrophotometric measurements were made on a Beckman Acta M6 recording spectrophotometer. Protein concentrations were determined by the Coomassie blue method (microprotein assay; Bradford, 1976) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The 2':3'-cyclic nucleotide phosphodiesterase of *H. influenzae* was demonstrated by Rodden & Scocca (1972) to be a periplasmic enzyme which could be solubilized from washed cells by treatment with lysozyme in Tris/HCl buffer (pH 7.6) containing 3 mM-EDTA and 30% (w/v) sucrose. In the present study, enzyme solubilized in this fashion was purified through ammonium sulphate precipitation followed by successive chromatographic steps using phosphocellulose, Amicon matrix gel blue A and Amicon matrix gel green A. At this stage of purification the enzyme had a specific activity of $0.4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, which compared favourably with the enzyme purified previously through a completely different purification procedure (Rodden & Scocca, 1972). Both purification procedures resulted in a rather low yield of purified enzyme (3–6%), which might be attributable to the assay used to monitor the purification procedure. The continuous spectrophotometric assay with bis(*p*-nitrophenyl) phosphate as substrate underestimated the yield of purified enzyme, since more than one enzyme capable of using this substrate was present in the initial periplasmic preparation. For example, in inhibitor analyses described in detail below, adenosine was demonstrated to inhibit the purified cyclic nucleotide phosphodiesterase in an effective and substrate-competitive fashion. At a concentration of adenosine sufficient to inhibit completely the purified enzyme, only 26% of the bis(*p*-nitrophenyl) phosphate hydrolase activity of the initial periplasmic preparation was inhibited, suggesting that only one-fourth of this activity could be attributed to the functioning of the cyclic nucleotide phosphodiesterase.

In previous studies of the cyclic nucleotide phosphodiesterase (Rodden & Scocca, 1972), 2':3'-cyclic GMP, 2':3'-cyclic CMP, 2':3'-cyclic UMP, 3'-AMP, 3'-GMP, and 3'-CMP were shown to be substrates for the enzyme. In the present study, the substrate specificity was defined further by investigating the activity of the enzyme towards a variety of mono- and dinucleotides. 2':3'-Cyclic AMP was demonstrated to be a substrate by HPLC analysis. Since 3'-AMP is also a substrate for the enzyme, hydrolysis of 2':3'-cyclic AMP should proceed to adenosine and inorganic phosphate as final products. Adenosine, 3'-AMP, and 2':3'-cyclic AMP can be separated and assayed by HPLC. 2':3'-Cyclic AMP (200 μM) was incubated at 25°C in 0.1 M-Tris/HCl buffer, pH 8.0, with 20 μg purified 2':3'-cyclic phosphodiesterase. At timed intervals, 0.1 ml samples of the reaction mixture were removed, diluted with eluting buffer and assayed by HPLC. Over a 60 min reaction period, the 2':3'-cyclic AMP was converted to adenosine without any observed accumulation of the intermediate 3'-AMP. Under the same conditions, 200 μM -3'-AMP was completely hydrolysed to adenosine in less than 20 min. These observations are consistent with a rate-limiting hydrolysis of the cyclic phosphate to 3'-AMP followed by a rapid hydrolysis of 3'-AMP to adenosine. The observed rapid hydrolysis of 3'-AMP does not however rule out an alternative, concerted mechanism for the hydrolysis of the 2':3'-cyclic AMP on the enzyme. After a 75 min incubation of 3':5'-cyclic AMP, 2'-AMP and 5'-AMP with the same

Fig. 1

Fig. 2

Fig. 1. Competitive inhibition of *H. influenzae* 2':3'-cyclic nucleotide phosphodiesterase by 5'-GMP. Initial velocities were measured at 25 °C in 0.1 M-Tris/HCl buffer, pH 8.0. The concentration of the substrate, bis(*p*-nitrophenyl) phosphate was varied from 10 to 50 μM .

Fig. 2. Thermal denaturation of *H. influenzae* 2':3'-cyclic nucleotide phosphodiesterase. Enzyme (20 $\mu\text{g ml}^{-1}$) was incubated in 0.1 M-Tris/HCl buffer, pH 8.0, at the four temperatures indicated.

amount of enzyme, HPLC showed that these compounds were not substrates. Although 2':3'-cyclic AMP was a very effective substrate, the dinucleotide derivative, 2':3'-cyclic NADP was totally ineffective, since incubation of 2':3'-cyclic NADP with enzyme under the same conditions produced no NAD, as determined by a yeast alcohol dehydrogenase assay. Further hydrolysis of any NAD produced was ruled out by the absence of hydrolysis of added NAD under the same conditions.

In the earlier studies of the cyclic nucleotide phosphodiesterase (Rodden & Scocca, 1972) the K_m for bis(*p*-nitrophenyl) phosphate hydrolysis at pH 5.6 was reported to be 50 μM . The K_m for this substrate in Tris/HCl buffer, pH 8.0, was determined spectrophotometrically in the present study to be 22 μM . Under the same conditions both *p*-nitrophenyl phenylphosphonate and thymidine *p*-nitrophenyl phosphate were substrates, with K_m values of 33 μM and 167 μM respectively. Since neither of the latter two substrates provided any kinetic advantage, the hydrolysis of bis(*p*-nitrophenyl) phosphate was used for further studies of selective inhibition of the enzyme.

The inhibition of the 2':3'-cyclic phosphodiesterase by a variety of nucleotides and nucleosides was studied at 25 °C in 0.1 M-Tris/HCl buffer, pH 8.0, with bis(*p*-nitrophenyl) phosphate as substrate at concentrations from 10–50 μM . Competitive inhibition of the enzyme by 5'-GMP is shown in Fig. 1. From these data, an inhibitor dissociation constant (K_i) of 2.9 μM was determined. The inhibition was reversible upon dilution and no time-dependent loss of enzyme activity was observed by incubation with 5'-GMP. This experimental approach was used to investigate selective inhibition by a variety of purine and pyrimidine derivatives. Where inhibition occurred, the inhibition was observed to be competitive with respect to substrate; the K_i values obtained are listed in Table 1. The effective inhibition of the enzyme by both purine and pyrimidine nucleosides and mononucleotides was not greatly influenced by the nature of the nitrogen bases or the presence of phosphate in these derivatives. However, in the absence of a nitrogen base, as observed with ribose 5-phosphate, no inhibition was obtained. With the exception of ADP-ribose, all adenosine derivatives studies inhibited in the concentration range of 2.4–6.5 μM , regardless of degree of phosphorylation or the presence of the 3':5'-cyclic phosphate. The small variation observed in the binding of purine and pyrimidine nucleosides is consistent with the earlier observations (Rodden & Scocca, 1972) that the 2':3'-cyclic phosphates of both purine and pyrimidine nucleosides are substrates for the enzyme.

Table 1. *Competitive inhibition of 2':3'-cyclic nucleotide phosphodiesterase*

Inhibitor	K_i (μM)
5'-Mononucleotides	
5'-AMP	6.5
5'-GMP	2.9
5'-CMP	8.7
5'-UMP	11.0
Adenosine derivatives	
5'-AMP	6.5
5'-ADP	2.7
5'-ATP	2.8
ADP-ribose	13.6
3':5' c-AMP	5.6
2'-AMP	2.4
Ribose 5-phosphate	—*
Nucleosides	
Adenosine	3.9
Guanosine	5.3
Cytidine	5.3
Uridine	3.0

* No inhibition at 1 mM.

The molecular weight of the native, purified 2':3'-cyclic nucleotide phosphodiesterase was determined by gel filtration chromatography on Sephacryl S-200. The column (1.6 × 79 cm) was equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-KCl, and eluted with a flow rate of 15.4 ml h⁻¹. The column was calibrated with the molecular weight standards yeast alcohol dehydrogenase, bovine serum albumin, horse liver alcohol dehydrogenase, ovalbumin and catalase. A molecular weight of 79000 for the cyclic nucleotide phosphodiesterase was obtained.

The purified enzyme was stable when stored for one week at either 4 °C or -12 °C, although it was denatured at temperatures above 50 °C. Incubation of the enzyme (20 $\mu\text{g ml}^{-1}$) in 0.1 M-Tris/HCl buffer, pH 8.0, at higher temperatures resulted in first-order rates of thermal inactivation (Fig. 2). At 60 °C, the half-life for thermal denaturation was 12.2 min, giving a first-order rate constant of 0.057 min⁻¹. The competitive inhibitors adenosine and 5'-AMP at five times their respective K_i values did not protect the enzyme against thermal denaturation, although the substrate 3'-AMP (3 μM) provided 25% protection. The greater stability of the enzyme in the presence of substrate suggests an induced conformational change during substrate binding.

The substrate specificity of the *H. influenzae* 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase is similar to that of the specific phosphodiesterase of *Salmonella typhimurium* studied by Uerkvitz & Beck (1981). The physiological functioning of these periplasmic enzymes has been suggested to involve the scavenging of organic phosphates that normally would not traverse the cytoplasmic membrane. The tendency to co-purify with the periplasmic nucleotide pyrophosphatase of *H. influenzae* appears to be related simply to the affinity of the phosphodiesterase for the Amicon dye gels as observed with other enzymes exhibiting selective interactions with adenine nucleotides (Beissner & Rudolph, 1978; Farmer & Easterby, 1984; Pongsawasdi & Anderson, 1984; Yost & Anderson, 1981).

These studies were supported by NIH Biomedical Research Support Grant 2S07 RR709517 and Research Grant PCM 8206712 from the National Science Foundation.

REFERENCES

- ANRAKU, Y. (1964*a*). A new cyclic phosphodiesterase having a 3'-nucleotidase activity from *Escherichia coli* B. I. Purification and some properties of the enzyme. *Journal of Biological Chemistry* **239**, 3412–3419.
- ANRAKU, Y. (1964*b*). A new cyclic phosphodiesterase having a 3'-nucleotidase activity from *Escherichia coli* B. II. Further studies on substrate specificity and mode of action of the enzyme. *Journal of Biological Chemistry* **239**, 3420–3424.

- BEISSNER, R. S. & RUDOLPH, F. B. (1978). Interactions of Cibracon blue F3GA and related dyes with nucleotide-requiring enzymes. *Archives of Biochemistry and Biophysics* **189**, 76–80.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–253.
- FARMER, E. E. & EASTERBY, J. S. (1984). The purification of yeast glucose-6-phosphate by dye-ligand chromatography. *Analytical Biochemistry* **141**, 79–82.
- KAHN, D. W. & ANDERSON, B. M. (1983). Studies of *Haemophilus influenzae* NAD pyrophosphatase. *Biochemistry* **22**, 8a.
- KIER, L. D., WEPPELMAN, R. & AMES, B. N. (1977). Resolution and purification of three periplasmic phosphatases of *Salmonella typhimurium*. *Journal of Bacteriology* **130**, 399–410.
- KOHN, J. & REIS, J. L. (1963). Bacterial nucleotidases. *Journal of Bacteriology* **86**, 713–716.
- MALAMY, M. H. & HORECKER, B. L. (1964). Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. *Biochemistry* **3**, 1889–1897.
- NEU, H. C. (1968). The 5'-nucleotidases and cyclic phosphodiesterases (3'-nucleotidases) of the Enterobacteriaceae. *Journal of Bacteriology* **95**, 1732–1737.
- PONGSAWASDI, P. & ANDERSON, B. M. (1984). Kinetic studies of rat ovarian 20- α -hydroxysteroid dehydrogenase. *Biochimica et biophysica acta* **799**, 51–58.
- RODDEN, J. L. & SCOCCA, J. J. (1972). Purification and properties of cyclic phosphodiesterase:3'-nucleotidase, a periplasmic enzyme of *Haemophilus influenzae*. *Archives of Biochemistry and Biophysics* **153**, 837–844.
- UERKVITZ, W. & BECK, C. F. (1981). Periplasmic phosphatases in *Salmonella typhimurium* LT2. *Journal of Biological Chemistry* **256**, 382–389.
- WEPPELMAN, R., KIER, L. D. & AMES, B. N. (1977). Properties of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. *Journal of Bacteriology* **130**, 411–419.
- YOST, D. A. & ANDERSON, B. M. (1981). Purification and properties of the soluble NAD glycohydrolase from *Bungarus fasciatus* venom. *Journal of Biological Chemistry* **256**, 3647–3653.