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ENZYMATIC HYDROLYSIS OF ADENOSINE 3',5'-CYCLIC PHOSPHOROTHIOATE

FRITZ ECKSTEIN AND HANS-PETER BÄR

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen (Germany) and Institute for Biomedical Research, Education and Research Foundation, American Medical Association, Chicago, Ill. (U.S.A.)

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SUMMARY

3',5'-AMPS (adenosine 3',5'-cyclic phosphorothioate) does not serve as a substrate or inhibitor of 3',5'-cyclic nucleotide phosphodiesterase from rat and rabbit brain and from beef heart. Other enzymes, presumably phosphodiesterases, present in the preparations hydrolyze 3',5'-AMPS with simultaneous displacement of sulfur. Snake venom phosphodiesterases from *Crotalus adamanteus* and *Crotalus terr. terr.* hydrolyze 3',5'-AMPS to 5'-AMP, with adenosine 5'-phosphorothioate (AMPS) as an intermediate.

INTRODUCTION

Adenosine 3',5'-cyclic phosphate (3',5'-AMP) is a common intracellular mediator of the action of several hormones[1,2]. Derivatives and analogs of 3',5'-AMP are, therefore, of interest because of their possible pharmacological action in replacing or interfering with 3',5'-AMP in biological systems[1,3]. Since nucleoside 5'-phosphorothioates have been found to be resistant to phosphatases[4] and dinucleoside phosphorothioate to some diesterases[5], adenosine 3',5'-cyclic phosphorothioate (3',5'-AMPS) was synthesized[6] in the hope that it might also be resistant to the action of specific 3',5'-cyclic nucleotide phosphodiesterases which interfere with kinetic and pharmacological studies of reactions involving 3',5'-AMP. When 3',5'-AMPS was tested as a stimulator of lipolysis in adipose cells from rat epididymal fat tissues (T. BRAUN, unpublished observation, 1968), it was found that it possessed lipolytic activity similar to 3',5'-AMP (the intracellular mediator of lipolytic hormones (ref. 7 and T. BRAUN, unpublished observations, 1968), but a strong smell of H_2S was noted during the experiment, indicating loss of sulfur during the incubation. Thus, a series of studies was conducted to investigate the enzymatic stability of 3',5'-AMPS and to establish the nature and mechanism of action of enzymes involved in its hydrolysis.

Abbreviations: AMPS, adenosine 5'-phosphorothioate; 3',5'-AMPS, adenosine 3',5'-cyclic phosphorothioate; 3',5'-AMP, adenosine 3',5'-cyclic phosphate.

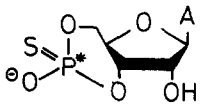

Fig. 1. Adenosine 3',5'-cyclic phosphorothioate. A = adenine. Mixture of stereoisomers of unknown ratio.

MATERIALS AND METHODS

The following enzymes were purchased from Sigma: Adenosine deaminase (EC 3.5.4.4), bacterial alkaline phosphatase (EC 3.1.3.1), snake venom from *Crotalus atrox* and *Crotalus adamanteus*, 5'-nucleotidase (EC 3.1.3.5) and phosphodiesterase (EC 3.1.4.1) both from *Crotalus adamanteus*; from Boehringer: Adenosine deaminase (EC 3.5.4.4), acid phosphatase (EC 3.1.3.2), intestinal alkaline phosphatase (EC 3.1.3.1), and snake venom phosphodiesterase (EC 3.1.4.1) from *Crotalus terr. terr.*; from Worthington: Spleen phosphodiesterase (EC 3.1.4.1). 3',5'-Cyclic nucleotide phosphodiesterase from beef heart was a generous gift from Dr. G. Weimann, Boehringer, Mannheim (Tutzing, Germany). 3',5'-Cyclic nucleotide phosphodiesterase from rat and rabbit brain was prepared as described[8,9]. These brain 3',5'-cyclic nucleotide phosphodiesterase preparations were not pure, containing a number of additional nucleotide and nucleoside catabolising enzymes. For example, based on the 3',5'-cyclic nucleotide phosphodiesterase content (100%), the following activities were noted with a rat-brain 3',5'-cyclic nucleotide phosphodiesterase preparation, when assayed without the addition of Mg^{2+} at pH 7.5 by the deamination method (see below) at substrate concentrations of 16 μM: Adenosine deaminase (62%), AMP-hydrolyzing activity (5'-nucleotidase and phosphatase) (136%).

Two procedures were used for measurement of 3',5'-AMPS hydrolysis. (a) Deamination: Inclusion of bacterial alkaline phosphatase and adenosine deaminase in the assay lead to rapid quantitative formation of inosine through the following reaction sequence: 3',5'-AMPS → AMP → adenosine → inosine. The last step is accompanied by a decrease in absorption at 265 mμ. Unless otherwise noted, reactions were carried out in 1-cm cells, containing 1.5 ml of 0.05 M Tris–HCl buffer (pH 7.5), 0.1 μg adenosine deaminase, 20 μg of bacterial alkaline phosphatase, and 3',5'-AMPS and other additions as indicated. The decrease in absorbance at 265 mμ was recorded with a Gilford or a Cary 14 spectrophotometer at 25°; $\Delta\varepsilon = -8200\ cm^2 \cdot M^{-1}$ at pH 7.5. The hydrolysis of 3',5'-AMP could be measured in the same way. AMP hydrolysis was determined after omission of phosphatase and adenosine deaminase activity after omission of both phosphatase and deaminase. (b) Trapping of SH^- with Ellman's reagent: The release of SH^- was measured by reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) (ELLMAN's[10] reagent) and by recording the resulting increase in absorbance at 412 mμ; $\Delta\varepsilon = +13600\ cm^2 \cdot M^{-1}$ at pH 7.5. This procedure was also used for measurement of AMPS hydrolysis. In both methods, initial rates were determined from the slopes of spectrophotometer recordings and are given in arbitrary units.

Descending paper chromatography

Schleicher and Schüll (2043b washed) or Whatman No. 1 paper was used; Solvent A: ethanol–1 M ammonium acetate (7:3, v/v); Solvent B: isopropanol–conc. ammonia–water (7:1:2, by vol.); Solvent C: saturated $(NH_4)_2SO_4$–1 M sodium acetate–isopropanol (80:18:2, by vol.).

TABLE I

ASSAY OF VARIOUS SUBSTRATES WITH 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE PREPARATIONS AND SNAKE VENOMS

Assay method and derivatives from standard incubations are indicated in the first column in parenthesis.

Enzyme (method)	*Substrate*	*Substrate concn. (M)*	*Rates*
Rat-brain 3',5'-cyclic nucleotide phosphodiesterase (deamination; $7 \cdot 10^{-3}$ M $MgCl_2$)	3',5'-AMP	$0.16 \cdot 10^{-4}$	9
	3',5'-AMPS	$0.16 \cdot 10^{-4}$	7.5
	Both		17
Rabbit-brain 3',5'-cyclic nucleotide phosphodiesterase (deamination; $7 \cdot 10^{-3}$ M $MgCl_2$)	3',5'-AMP	$0.16 \cdot 10^{-4}$	6
	3',5'-AMPS	$0.16 \cdot 10^{-4}$	4.5
	Both	$0.16 \cdot 10^{-4}$	10.5
Beef-heart 3',5'-cyclic nucleotide phosphodiesterase (deamination)	3',5'-AMP	$0.05 \cdot 10^{-4}$	10
	3',5'-AMPS	$0.39 \cdot 10^{-4}$	3
	Both		14
C. adamanteus phosphodiesterase (absorbance increase at 405 μm and Ellman's reagent; pH 9)	*p*-Nitrophenyl-TMP	$0.33 \cdot 10^{-3}$	7
	3',5'-AMPS	$0.16 \cdot 10^{-3}$	11
	Both		10
	3',5'-AMPS	$0.16 \cdot 10^{-3}$	17
	AMPS	$0.16 \cdot 10^{-4}$	8
	Both		16
C. terr. terr. phosphodiesterase (Ellman's reagent)	3',5'-AMPS	$0.16 \cdot 10^{-4}$	23
	AMPS	$0.10 \cdot 10^{-5}$	15
	Both		29

RESULTS

3',5'-Cyclic nucleotide phosphodiesterase from beef heart

Two batches of beef-heart 3',5'-cyclic nucleotide phosphodiesterase were obtained. Incubation of 3',5'-AMPS with one batch yielded 5'-AMP, as shown by paper chromatography in System A, in a very slow reaction. To distinguish this material from AMPS which has a similar R_F value, it was degraded by *Escherichia coli* alkaline phosphatase to adenosine. AMPS is resistant to phosphatases. The relative rates of hydrolysis of 3',5'-AMP and 3',5'-AMPS were 14 and 1, respectively, measured at concentrations of $0.16 \cdot 10^{-4}$ M with the deamination method. No mutual inhibition was observed when the two compounds were incubated together at enzyme saturating concentrations of 3',5'-AMPS (Table I). AMPS was not hydrolyzed by this 3',5'-cyclic nucleotide phosphodiesterase preparation. A second batch of beef-heart 3',5'-cyclic nucleotide phosphodiesterase did not hydrolyze 3',5'-AMPS at all. This latter preparation apparently was purer and did not contain any contaminating 3',5'-AMPS hydrolyzing activity. 3',5'-AMPS did not inhibit the rate of 3',5'-AMP hydrolysis.

3',5'-Cyclic nucleotide phosphodiesterase from brain

When 3',5'-AMPS was incubated with either rat- or rabbit-brain 3',5'-cyclic nucleotide phosphodiesterase, inosine formation could be noted, as evidenced by a characteristic change in the ultraviolet absorption spectrum from the adenosine to the inosine system as well as by paper chromatography of the product in Solvent A and B. With 3',5'-AMP as the substrate, inosine was produced, too. However, 3',5'-AMP and 3',5'-AMPS hydrolyzing activities did not appear identical since 3',5'-AMP

TABLE II

$(NH_4)_2SO_4$ FRACTIONATION OF RAT-BRAIN 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

Assays were conducted with the deamination method, substrates were $0.16 \cdot 10^{-4}$ M; $MgCl_2$, $7 \cdot 10^{-3}$ M.

$(NH_4)_2SO_4$ fraction (saturation at 4°)	*Relative rates of hydrolysis with*		
	3',5'-AMP	*3',5'-AMPS*	*AMPS*
0	100	175	225
0–33%	100	220	470
33–50%	100	240	470
50–80%	100	84	470

and 3',5'-AMPS were not mutually inhibitory; *i.e.* when assayed together, their rates of hydrolysis were additive (Table I). An attempt to fractionate rat-brain 3',5'-cyclic nucleotide phosphodiesterase with $(NH_4)_2SO_4$ resulted in a partial separation of 3',5'-AMPS hydrolyzing activity from 3',5'-cyclic nucleotide phosphodiesterase, nucleotidase and phosphatase as shown in Table II.

Phosphodiesterase from snake venom

(a) Phosphodiesterase from *C. adamanteus* hydrolyzed 3',5'-AMPS as well as 5'-AMPS to 5'-AMP as shown by paper chromatography in System C and by anion-exchange chromatography on polyethyleneimine-cellulose thin layers[11] with 0.3 M LiCl for development. At enzyme saturation levels of substrates, the combined rates of hydrolysis of 3',5'-AMPS and 5'-AMPS as well as 3',5'-AMPS and *p*-nitrophenyl-TMP were not additive (Table I). Stepwise heat denaturation of phosphodiesterase at 80° for 30 and 60 sec, respectively, affected the relative rates of 3',5'-AMPS and *p*-nitrophenyl-TMP hydrolysis in a parallel way. Furthermore, the relative rates of hydrolysis of AMPS and 3',5'-AMPS with crude venom, 5'-nucleotidase and phosphodiesterase were parallel (Table III). All these observations are compatible with the suggestion that the same enzymatic activity is involved in the hydrolysis of all three compounds.

The K_m values were estimated from double reciprocal plots of rate data and found to be $2.6 \cdot 10^{-5}$ M for 3',5'-AMPS and $1.3 \cdot 10^{-6}$ M for 5'-AMPS at pH 7.5, as determined for the release of SH^- with Ellman's reagent. The pH optimum was above pH 9–9.5 for both compounds.

TABLE III

HYDROLYSIS OF 3',5'-AMPS AND AMPS BY SNAKE VENOM ENZYME FRACTIONS FROM *Crotalus adamanteus*

Assayed with Ellman's reagent at pH 7.5, substrates at $0.16 \cdot 10^{-3}$ M.

Enzyme	*Relative rates corrected for protein*	
	3',5'-AMPS	*AMPS*
Crude venom	9.40	10.5
5'-Nucleotidase	1.16	1.4
Phosphodiesterase	100	100

(b) Phosphodiesterase from *C. terr. terr.* hydrolyzed 3',5'-AMPS as well as AMPS with formation of 5'-AMP as could be shown by paper chromatography in System A and transformation of the products to adenosine by alkaline phosphatase. Rate data were obtained with 3',5'-AMPS and AMPS as substrates, and kinetic parameters were determined graphically from double reciprocal plots. At pH 7.4 the K_m for 3',5'-AMPS was found to be $1.2 \cdot 10^{-5}$ M as determined by the deamination method and $3 \cdot 10^{-5}$ M with Ellman's reagent, which is in close agreement. The K_m for AMPS under the same conditions was $2 \cdot 10^{-6}$ M as measured with Ellman's reagent. Both compounds have similar v_{max} values of $1 \cdot 10^{-2}$ and $0.7 \cdot 10^{-2}$ (AMPS)moles $\cdot$ min^{-1} $\cdot$ mg^{-1}, which is expected if the same enzyme hydrolyzes both substrates and if AMPS is a rapidly hydrolyzed intermediate of 3',5'-AMPS hydrolysis. Further evidence in support of this assumption is the fact that AMPS and 3',5'-AMPS were mutually inhibitory (Table I). The hydrolysis of *p*-nitrophenyl-TMP by this diesterase was also inhibited by 3',5'-AMPS at pH 8.7. The rate of *p*-nitrophenol release from *p*-nitrophenyl-TMP $0.3 \cdot 10^{-3}$ M was decreased by 60% in the presence of 3',5'-AMPS ($0.2 \cdot 10^{-3}$ M).

Other enzymes

Bacterial alkaline phosphatase, acid phosphomonoesterase and spleen phosphodiesterase did not attack 3',5'-AMPS.

DISCUSSION

A certain number of nucleotide analogs in which the phosphate is replaced by a thiophosphate group have been synthesized in recent years[4,5,12-14]. All of these interact with the appropriate enzyme either as substrates or as inhibitors. It was, therefore, to be expected by analogy that 3',5'-AMPS could either be a substrate or an inhibitor of 3',5'-cyclic nucleotide phosphodiesterase.

Although the above studies were carried out with enzymes of varying degree of purity, it must be concluded that 3',5'-AMPS is neither a substrate nor an inhibitor of 3',5'-cyclic nucleotide phosphodiesterase from beef heart and rat as well as rabbit brain. This complete lack of interaction indicates an unusually high selectivity of the enzyme for the unmodified phosphate group. This is in contrast to a wide tolerance towards variations in the base group, since the 3',5'-cyclic phosphates of guanosine and uridine are hydrolyzed as well[9]. The enzymatic hydrolysis of 3',5'-AMPS by various enzyme preparations from mammalian tissues and snake venom, reflects an interesting property of the involved phosphorylytic enzymes, presumably phosphodiesterases. Our experiments with mammalian enzymes show that both the opening of the diester bond and the loss of sulfur in the form of H_2S are catalyzed by the same enzymatic activity because neither 3',5'-AMP nor AMPS appear as free intermediates in the reaction. These compounds are either unaffected by the enzyme preparations, as in the case of highly pure beef-heart 3',5'-cyclic nucleotide phosphodiesterase, or when added to the assay, do not reduce the rate of 3',5'-AMP hydrolysis.

The results obtained with the phosphodiesterases from *C. adamanteus* and *C. terr. terr.* are different, however. These enzymes hydrolyze AMPS as well as 3',5'-AMPS, while 3',5'-AMP is unaffected. For these two enzymes, AMPS seems to be an intermediate in the hydrolysis of 3',5'-AMPS as indicated by the mutual inhibition. One must conclude, therefore, that the opening of the diester bond precedes the loss of

sulfur. It is tempting to assume that in the case of the mammalian enzyme preparations the same sequence of events takes place, but AMPS is not released as a free intermediate. In this case, the following could constitute the simplest sequence (E = enzyme), in accordance with our results:

$$3',5'\text{-AMPS} + E \rightleftharpoons [3',5'\text{-AMPS} - E] \xrightleftharpoons{+\,H_2O} [\text{AMPS} - E] \xrightleftharpoons{+\,H_2O} \text{AMP} + E$$

$$[\text{AMPS} - E] \xrightarrow{-\,H_2S} \text{AMPS} + E$$

C. adamanteus and *C. terr. terr.* only

The final step in the above sequence appears to be very selective, since 3',5'-AMP does not react or interfere competitively.

Clearly, purified enzyme preparations are required in order to answer certain questions about the detailed steps of hydrolysis of diester bond and displacement of sulfur. Moreover, it would be desirable for extended studies to employ pure isomers of 3',5'-AMPS, the separation of which is in progress.

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