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CYCLIC 3',5'-AMP PHOSPHODIESTERASE OF *SACCHAROMYCES CARLSBERGENSIS*

INHIBITION BY ADENOSINE 5'-TRIPHOSPHATE, INORGANIC PYROPHOSPHATE AND INORGANIC POLYPHOSPHATE

G. A. G. SPEZIALI AND R. VAN WIJK
Van 't Hoff Laboratory, The State University, Utrecht (The Netherlands)

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SUMMARY

1. Cyclic 3',5'-AMP phosphodiesterase activity can be demonstrated in *Saccharomyces carlsbergensis* by measuring the 5'-AMP formation from cyclic 3',5'-AMP.

2. Enzymic activity was optimal at pH 8.5 and shows a 2-fold stimulation in the presence of 4 mM Mn^{2+}; Mg^{2+}, Ca^{2+} and EDTA hardly show any effect.

3. ATP, inorganic polyphosphate and pyrophosphate are effective inhibitors of the phosphodiesterase. The nature of these inhibitions was found to be of the mixed type.

4. The possible physiological significance of this inhibition is discussed.

INTRODUCTION

SUTHERLAND AND RALL[1] have described an enzyme that hydrolyzes cyclic 3',5'-AMP to 5'-AMP. The enzyme has been found widely distributed in animal tissues[2,3], slime molds[4] and bacteria[5]. Since cyclic 3',5'-AMP occupies an important position in cellular metabolism, and phosphodiesterase may play a major role in regulating its level, a great deal of interest in this enzyme has developed. Until now, no data are known on the presence and the role of cyclic 3',5'-AMP phosphodiesterase in yeast cells. Recently[6] it could be demonstrated that in *S. carlsbergensis* the level of cyclic 3',5'-AMP varies with the physiological state of the cells. In order to study the regulation of the level of cyclic 3',5'-AMP the phosphodiesterase in this yeast was characterized. This report deals with the results of this study.

MATERIALS AND METHODS

Chemicals

Cyclic 3',5'-AMP, lactate dehydrogenase, myokinase and pyruvate kinase were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. ATP was purchased from P-L Biochemicals Inc., Milwaukee, Wisc., U.S.A. Phosphoenolpyruvate and pyrophosphate (tetrasodium salt) were obtained from Sigma Chemical Company,

St. Louis, Miss., U.S.A. NADH and tetraphosphoric acid were purchased from Koch-Light Laboratories, Colnbrook, England, and disodium hydrogen orthophosphate from British Drug Houses Ltd., Poole, England.

Yeast strain

S. carlsbergensis strain no 74, N.C.Y.C., England, was used. Yeast culturing was performed as described previously[7]. The cells were harvested during the logarithmic phase of growth.

Preparation of yeast extract

After centrifugation the yeast cells were washed twice with distilled water and resuspended in 0.04 M Tris HCl buffer (pH 8.5) containing $4 \cdot 10^{-4}$ M $MgSO_4$ to a final concentration of 50 mg yeast dry wt. per ml. The cells were broken in the cold by shaking during 1 min with glass beads (diameter 0.45–0.50 mm) in an apparatus according to MERCKENSCHLAGER *et al.*[8]. After centrifugation for 10 min at 0° and 2000 × *g*, the supernatant was removed carefully from unbroken cells and glass beads and was centrifuged at 0° for 30 min at 45 000 × *g*. The resulting supernatant was dialysed overnight against 500 vol. of the same Tris HCl Mg^{2+} buffer. Mg^{2+} was omitted when the effect of bivalent cations was tested. The dialyzed extract (containing approx. 10 mg protein per ml) can be stored at −20° with no appreciable loss of enzymatic activity for several weeks.

Determination of cyclic 3′,5′-AMP phosphodiesterase activity

Cyclic 3′, 5′-AMP phosphodiesterase activity was determined by a modification of the method of CHEUNG[9]. The enzyme preparation was incubated at 37° for 40 min. The reaction mixture contained in a final vol. of 1 ml: 40 μmoles Tris HCl buffer pH 8.5, 2 μmoles Na_2HPO_4, 0.4 μmoles $MgSO_4$, 2 μmoles cyclic 3′,5′-AMP and 0.3 mg protein. After 10 min of preincubation the reaction was started by the addition of the substrate cyclic 3′,5′-AMP. The reaction was stopped after 40 min by addition of 0.1 ml of 3 M $HClO_4$. After centrifugation the supernatant was neutralized with $KHCO_3$ crystals. Insoluble $KClO_4$ was then removed by low speed centrifugation and the supernatant was used for the assay of 5′-AMP. Determination of the amount of 5′-AMP was performed by the use of the coupled reaction of pyruvate kinase, lactate dehydrogenase and myokinase[10]. For this determination 5 units of each enzyme was present in a final volume of 3 ml. The myokinase was added last, when there was no more change in the absorbance at 340 nm, using a spectrophotometer Beckmann DK 2. Protein was assayed according to the method of LOWRY *et al.*[11] using bovine serum albumin as a standard. Cyclic 3′,5′-AMP phosphodiesterase specific activity is expressed as nmoles AMP formed per min per mg protein.

RESULTS

The use of phosphate during incubation

The assay methods commonly used to measure cyclic 3′,5′-AMP specific phosphodiesterase activity are based on the assumption that the enzyme activity is proportional to the amount of 5′-AMP present in the incubation mixture after incubation with cyclic 3′,5′-AMP. By using this method it was observed that the

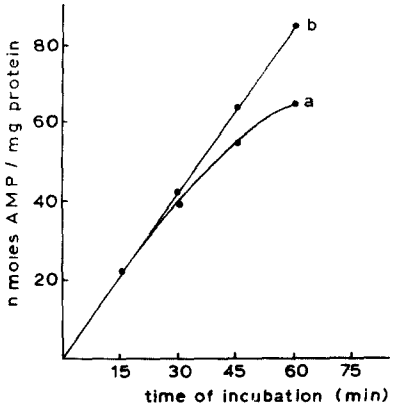
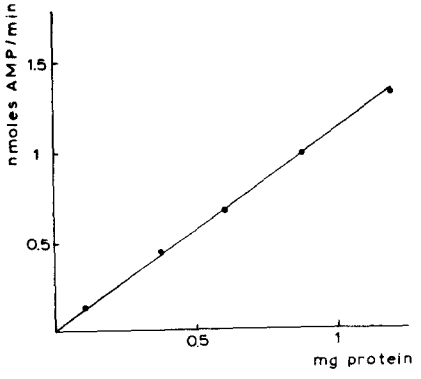


Fig. 1. Effect of Na_2HPO_4 on the apparent cyclic 3′, 5′-AMP phosphodiesterase activity. The tubes contained per ml: 2 μmoles cyclic 3′, 5′-AMP, 40 μmoles Tris–HCl buffer (pH 8.5), 0.4 μmoles $MgSO_4$ and 1 mg protein. a. amount of 5′-AMP measured during incubation in the absence of Na_2HPO_4. b. *idem* in the presence of 2 μmoles Na_2HPO_4.

formation of 5′-AMP during incubation of the extract with 2 mM cyclic 3′,5′-AMP shows no linear rate (Fig. 1). However in the presence of 2 mM Na_2HPO_4 the amount of 5′-AMP formed from cyclic 3′,5′-AMP maintained a linear rate up to 60 min (Fig. 1, Curve b).

The effect of inorganic phosphate can be explained by the occurrence in crude preparations of other enzymes that catalyze the hydrolysis of the reaction product 5′-AMP[12]. In separate experiments the presence of a 5′-AMP splitting activity in preparations of *S. carlsbergensis* was measured by the incubation of a crude extract with 5′-AMP and determining the decrease in the amount of 5′-AMP by the use of the coupled reaction of pyruvate kinase, lactate dehydrogenase and myokinase[10]. It was found that the 5′-AMP conversion is completely inhibited by the addition of 2 mM Na_2HPO_4.



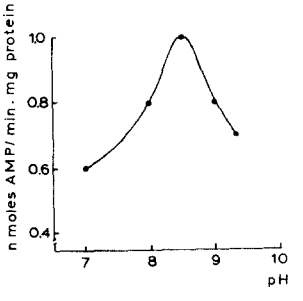


Fig. 2. Activity of cyclic 3′, 5′-AMP phosphodiesterase as a function of the protein concentration. Other conditions were as discussed in the text.

Fig. 3. Effect of pH on cyclic 3′, 5′-AMP phosphodiesterase activity. 40 mM Tris–HCl was used as a buffer, containing 0.4 mM $MgSO_4$.

TABLE I

EFFECT OF BIVALENT CATIONS AND EDTA ON THE ACTIVITY OF CYCLIC 3',5'-AMP PHOSPHODIESTERASE

Enzymic activity in this experiment was determined by measuring the initial rate of 5'-AMP formation in the absence of 2 mM Na_2HPO_4. The activity in the absence of Mg^{2+} ions was taken as 100%. When EDTA was tested the incubation mixture was preincubated for 40 min in the absence of the substrate cyclic 3',5'-AMP.

Addition	*Concentration (mM)*	*Activity (%)*
None	–	100
$MgSO_4$	0.4	116
	4	116
	10	108
$MgCl_2$	4	115
$MnCl_2$	1	158
	1.5	196
	4	310
	10	250
$CaCl_2$	1	92
EDTA	1	100

Effect of protein concentration and pH

When various amounts of protein were incubated for 45 min the amount of 5'-AMP formed appeared to be directly proportional to the protein concentration in the reaction mixture up to 1.25 mg per ml (Fig. 2).

The dependence on the pH of the incubation medium was determined in the range of pH 7 to 9.3 using Tris–HCl buffer. As is shown in Fig. 3 the cyclic 3',5'-AMP phosphodiesterase shows a maximal activity in this pH range at approximately 8.5.

Effect of divalent cations and EDTA

Most of the cyclic 3',5'-AMP phosphodiesterases described in the literature require Mg^{2+} or Mn^{2+} to express a full activity[13,14]. In Table I the data obtained for Mg^{2+}, Mn^{2+}, Ca^{2+} and EDTA were expressed as a percentage of the blank activity, *i.e.* the activity measured in a Tris–HCl buffer containing no additional amounts of bivalent cations or EDTA.

It can be seen in Table I that the activity is not strongly dependent on the presence of Mg^{2+}: 0.4 mM is already enough to cause full activity which is only 16% higher than the activity obtained in the complete absence of Mg^{2+}. In the presence of Mn^{2+} the yeast cyclic 3',5'-AMP phosphodiesterase shows a significant higher activity: a stimulation of approx. 200% could be obtained at a concentration of 4 mM. On the other hand Ca^{2+} was found to exhibit a slight inhibitory effect, while no effect at all was observed in the presence of 2 mM EDTA.

Michaelis–Menten constant and the n of Hill

The K_m value of cyclic 3',5'-AMP phosphodiesterase was determined in an experiment illustrated in Fig. 4.

From these data a K_m could be calculated of 0.22 mM. From the same experimental data, a Hill plot (Fig. 5) was computed by plotting $\log (v/v_{max} - v)$ as a

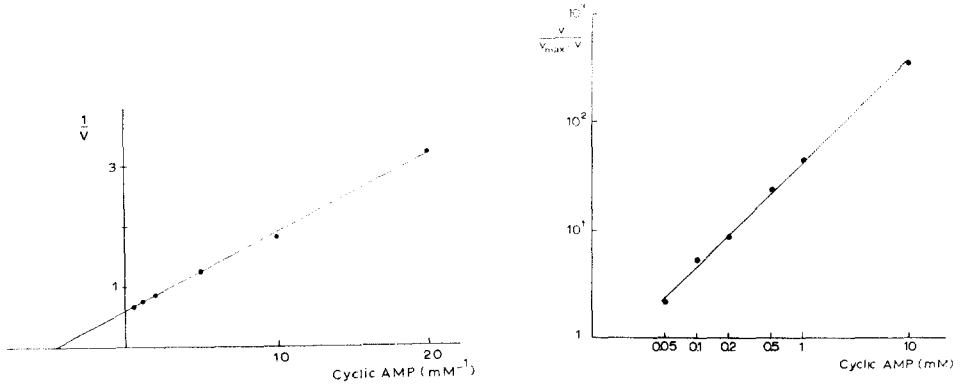


Fig. 4. Lineweaver–Burk plot of cyclic 3′, 5′-AMP phosphodiesterase activity with cyclic 3′, 5′-AMP as substrate. K_m value is 0.22 mM. v, initial rate of the formation of 5′-AMP (nmoles formed/min per mg protein).

Fig. 5. Hill plot computed from the data presented in Fig. 4. v, nmoles 5′-AMP formed/min per mg protein.

function of log(s). This should give a straight line with a slope n[15,16]. The n is the number of substrate molecules cooperatively reacting with each molecule of enzyme; from the data illustrated in Fig. 5, n appears to be approx. 1.

Inhibition by ATP, inorganic pyrophosphate (PP_i) and polyphosphate

ATP, PP_i and polyphosphate appear to inhibit the cyclic 3′,5′-AMP phosphodiesterase activity of *S. carlsbergensis*. Figs. 6, 7 and 8 indicate the nature of the inhibition by ATP, PP_i and inorganic polyphosphate respectively.

In Fig. 6 a K_m of 0.22 mM with no ATP increases to 0.60 mM at 1 mM ATP The fact that the intercepts do not coincide on the ordinate indicates that this is not a simple competitive inhibition but a mixed type[17].

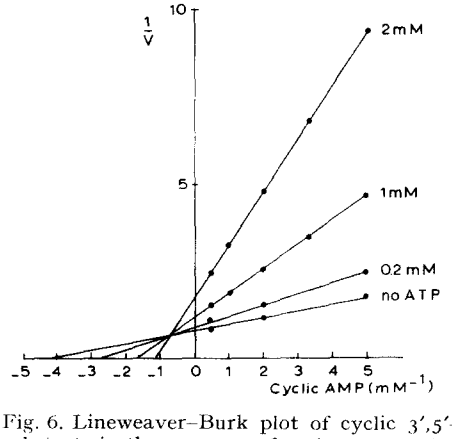


Fig. 6. Lineweaver–Burk plot of cyclic 3′,5′-AMP phosphodiesterase with cyclic 3′,5′-AMP as substrate in the presence of various amounts of ATP.

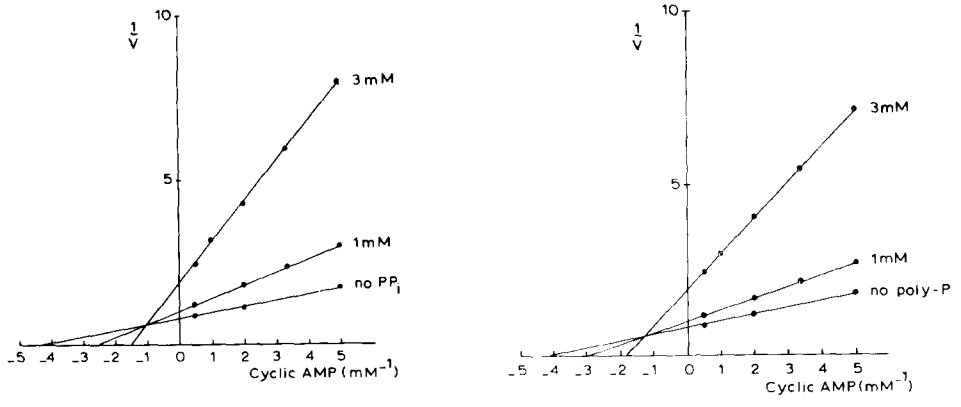


Fig. 7. Lineweaver Burk plot of cyclic 3',5'-AMP phosphodiesterase with cyclic 3',5'-AMP as substrate in the presence of various amounts of inorganic pyrophosphate (PP_i).

Fig. 8. Lineweaver-Burk plot of cyclic 3',5'-AMP phosphodiesterase with cyclic 3',5'-AMP as substrate in the presence of various amounts of inorganic polyphosphate (poly-P).

Figs. 7 and 8 illustrate that the inhibition by PP_i and inorganic polyphosphate exhibit characteristics similar to those of ATP in Fig. 6. Again the K_m increases from 0.22 mM with no inhibitor to 0.37 mM in the presence of 1 mM PP_i (Fig. 7) and 0.32 mM in the presence of 1 mM inorganic polyphosphate (Fig. 8) respectively. In all three figures (Figs. 6, 7 and 8) v_{max} decreases as a function of the inhibitor concentration.

Activity of cyclic 3',5'-AMP phosphodiesterase activity under variable conditions of catabolite repression

Growth of *S. carlsbergensis* cells in media containing different carbon sources was found to lead to different cellular amounts of cyclic 3',5'-AMP. In a previous paper[6] the intracellular concentration was found to be 0.04 μM in cells which were grown in a synthetic medium with 2% glucose as the sole source of carbon, while it was 0.25 μM when 2% galactose was the carbon source.

The measurement of the cyclic 3',5'-AMP phosphodiesterase under these two conditions reveals that the activity of this enzyme is the same, namely 0.9 nmoles AMP formed per min per mg protein.

DISCUSSION

For the measurement of the cyclic 3',5'-AMP phosphodiesterase we used the method of CHEUNG[9] which measures activity as a function of the increase in 5'-AMP. In yeast extracts this appears only applicable if there is no further degradation of 5'-AMP. This condition can be approximated when the rate of 5'-AMP formation is measured in the presence of 2 mM orthophosphate.

For the cyclic 3',5'-AMP phosphodiesterase of *S. carlsbergensis* a number of basic characteristics were determined: the optimal pH was found to be 8.5, a K_m was found of 0.22 mM and the activity appeared to be stimulated in the presence of Mn^{2+} up to 4 mM. We studied the cyclic 3',5'-AMP phosphodiesterase in order to

explain the different amounts of cyclic 3′,5′-AMP present in cells which were grown either on 2% glucose or on 2% galactose[6]. Principally this difference can be caused by differences in the rate of synthesis of cyclic 3′,5′-AMP or by differences in the rate of breakdown of cyclic 3′,5′-AMP. Results of the present study show that the same amount of cyclic 3′,5′-AMP phosphodiesterase is present in extracts which contain different cyclic 3′,5′-AMP concentrations. However, it was observed that the activity of this enzyme can be inhibited by ATP, PP_i and inorganic polyphosphate. The strong inhibition by ATP at concentrations of approximately physiological levels[18] suggests that phosphodiesterase activity *in vivo* might be regulated by this inhibition as well as by that by polyphosphate, a compound that is also present in high amounts in yeast[19]. Thus, phosphodiesterase in its turn could constitute a useful *in vivo* control mechanism of the intracellular amount of cyclic 3′,5′-AMP.

REFERENCES

1 E. W. SUTHERLAND AND T. W. RALL, *J. Biol. Chem.*, 232 (1958) 1077.
2 R. W. BUTCHER AND E. W. SUTHERLAND, *J. Biol. Chem.*, 237 (1962) 1244.
3 G. L. DRUMMOND AND S. PERROTT-YEE, *J. Biol. Chem.*, 236 (1961) 1126.
4 Y. Y. CHANG, *Science*, 160 (1968) 57.
5 H. BRANA AND F. CHYTIL, *Folia Microbiologica*, 11 (1965) 43.
6 R. VAN WIJK AND T. M. KONIJN, *FEBS Letters*, 13 (1971) 184.
7 R. VAN WIJK, *Proc. Kon. Ned. Akad. Wetenschap.*, C71 (1968) 60.
8 M. MERCKENSCHLAGER, K. SCHLOSSMANN AND W. KURZ, *Biochem. Z.*, 329 (1957) 392.
9 W. Y. CHEUNG, *Biochem. Biophys. Res. Commun.*, 23 (1966) 214.
10 H. ADAMS, in H. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1963, p. 57.
11 O. H. LOWRY, N. T. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
12 D. G. THERRIAULT AND V. G. WINTERS, *Life Sciences*, 9 (1970) 1053.
13 W. Y. CHEUNG, *Biochemistry*, 6 (1967) 1079.
14 T. DOUSA AND I. RYCHLIK, *Biochim. Biophys. Acta*, 204 (1970) 10.
15 J. C. CHANGEUX, *Cold Spring Harb. Symp. Quant. Biol.*, 28 (1963) 487.
16 D. E. ATKINSON, J. A. HATHAWAY AND E. C. SMITH, *J. Biol. Chem.*, 240 (1965) 2682.
17 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, London, 1964, p. 324.
18 R. VAN WIJK, K. W. VAN DE POLL AND G. A. G. SPEZIALI, *Proc. Kon. Ned. Akad. Wetenschap.*, B 73 (1970) 357.
19 K. LOHMANN AND P. LANGEN, *Biochem. Z.*, 328 (1956) 1.