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CYCLIC AMP PHOSPHODIESTERASES OF *FUNARIA HYGROMETRICA*

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Key Word Index—*Funaria hygrometrica*; Bryidae; moss; protonema; cyclic AMP phosphodiesterase; high-affinity; calcium dependence.

Abstract—High affinity cAMP phosphodiesterase (PDE) activity has been purified (*ca* 474-fold) from the chloronema cells of the moss *Funaria hygrometrica*. The activity in the 15 000 *g* supernatant was precipitated at 40–80% saturation with $(NH_4)_2SO_4$ and separated by DEAE-cellulose chromatography into two peaks of activity A and B with pH optima at pH 5.5 and 7.5, respectively. When rechromatographed on DEAE-cellulose, the PDE B activity redistributed into two peaks (A′ and B′). The recovery of PDE activity in the latter peaks suggests a conversion of alkaline PDE into acid PDE. The phosphodiesterase activity B was further resolved by affinity chromatography on a cAMP–agarose column into PDE I and PDE II. The latter was free from nucleotidases, was Ca^{2+}-dependent and showed only a high-affinity component towards cAMP as the substrate, the K_m being 11.8 ± 4.8 μM.

INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDE) have been studied in a number of higher plant tissues [1–6]. Most of these are acid phosphodiesterases, which possess properties considerably different from animal [7] and bacterial [8] phosphodiesterases. The PDE from algae [9] on the other hand are similar to those from animal and bacterial sources.

We have previously demonstrated the presence of two forms of cyclic nucleotide phosphodiesterases in the chloronema cells from the protonema of the moss *Funaria hygrometrica* [10]. These were separated by affinity chromatography on a cAMP–agarose column. The activity present in the buffer wash (PDE I), exhibits an acidic pH optimum, is insensitive to methylxanthines and imidazole, and copurifies with nucleotidases. Thus it resembles the PDE from higher plants. The other activity, PDE II, binds to cAMP–agarose and is eluted by 100 μM cAMP. This enzyme fraction has properties similar to animal phosphodiesterases as it is free from nucleotidases and produces only 5′-AMP, the maximum activity being at pH 7.5–8.0. Further, it is inhibited by methylxanthines, and stimulated by imidazole [10].

The phosphodiesterase activity from *Funaria* exhibited a single pH optimum in the acidic range (pH 5.0–5.5) in the crude extracts [11]. Therefore in our previous studies [10], the PDE activity was monitored at pH 5.4 during purification. Consequently there was a selective purification of the acid PDE. Though the alkaline PDE survived this purification procedure, its yield was low. The present studies were undertaken (a) to find out the relationship between the acid and alkaline phosphodiesterases and (b) to purify the alkaline phosphodiesterase for further characterization.

RESULTS

Purification

The details of the purification of the cAMP phosphodiesterases are given in Table 1. Two major peaks of

Table 1. Purification of cAMP PDE from chloronema cells

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Crude extract	760	1583	0.48	1.2	100
15 000 *g* supernatant	460	1179	0.39	1.0	61
40–80% Satd $(NH_4)_2SO_4$	220	310	0.71	1.8	29
DEAE-Cellulose					
Peak A	187	11	17.0	43.6	25
Peak B	136	98	1.39	3.6	18
cAMP–agarose					
Peak I	66	65	1.01	2.6	8.7
Peak II	26.6	0.14	185	474.4	3.5

PDE activity were obtained after chromatography on DEAE-cellulose; peak A in the buffer wash and peak B eluting at 0.45 M NaCl. Sixty eight units of activity B were precipitated by $(NH_4)_2SO_4$ (80%), dissolved in 50 mM Tris–acetate, pH 7.5, dialysed and loaded on a 5 ml cAMP–agarose column. PDE I and II were eluted by buffer and 100 μM cAMP respectively. A 474-fold purification was observed for PDE II, the recovery being only 3.5%. For PDE II, 132-fold purification was achieved by affinity chromatography alone. PDE I, however, was purified only 2.6-fold. With the exception of PDE II, all the other PDE activities contained appreciable levels of nucleotidases and the major product of cAMP degradation was adenosine. PDE II produced only 5'-AMP upon hydrolysis of cAMP and showed a pH optimum of 7.5–8.0.

Interconversion of phosphodiesterase activities

The pH optima for A and B activities were 5.5 and 7.5 respectively. Upon rechromatography on DEAE-cellulose, the B activity redistributed itself into two peaks, A' and B', which were eluted with buffer and 0.35 M NaCl respectively. The PDE activities of peaks A, B, A' and B' at pH 5.5 and 7.5 are shown in Table 2. The buffer-eluted peaks (A and A') showed higher activity at pH 5.5 while the NaCl eluted peaks (B and B') exhibited higher activity at pH 7.5. It is noteworthy that the rechromatography of peak B activity on DEAE-cellulose resulted in an increased yield of acid PDE activity. When 71 units of acid PDE activity (peak B) were rechromatographed, 102 units of acid PDE activity were recovered in peaks A' and B'. At the same time the activity at pH 7.5 decreased from 136 units (peak B) to 97 units (peaks A' and B'). Thus the increase in acid PDE activity of 31 units was accompanied by a decrease of 39 units in the alkaline PDE activity.

Properties of PDE II

Kinetic studies of PDE II showed a linear double reciprocal plot for cAMP hydrolysis at substrate concentrations ranging from 5 μM to 5 mM. The K_m and V_{max} values obtained were 11.8 ± 4.8 μM and 208 ± 17 units/mg protein.

Table 2. Phosphodiesterase activity of the PDE peaks* obtained by fractionation on a DEAE-cellulose column

Phosphodiesterase peak	Activity (units) pH 5.5	pH 7.5
A	288	187
B	71	136
A'	78	61
B'	24	36

*PDE peaks A and B were obtained in the buffer wash and 0.45 M NaCl eluate respectively, after chromatography on DEAE-cellulose. Upon rechromatography on DEAE-cellulose, peak B activity redistributed itself into A' and B', once again eluting in the buffer wash and 0.35 M NaCl respectively.

Table 3. Effect of EDTA and divalent metal ions on PDE II activity

Compound added	Concentration (mM)	Activity (% control)*
None (control)		100
EDTA	1.0	22.5 ± 1.7(4)
1.0 mM EDTA + Ca^{2+}†	1.0	67.5 ± 3.4(4)
	5.0	107.0 ± 2.8(4)
1.0 mM EDTA + Mg^{2+}†	1.0	32.0 ± 2.5(4)
	5.0	41.5 ± 3.7(4)
1.0 mM EDTA + Zn^{2+}‡	1.0	29.0 ± 2.7(4)
	5.0	16.5 ± 1.7(4)

*The activity in the control was 1.26 ± 0.1 (4) units/ml.
†Chloride salt.
‡Acetate salt.

PDE activity was inhibited by 80% upon the addition of 1 mM EDTA (Table 3). The activity was restored completely by the addition of 5 mM Ca^{2+}, whereas Mg^{2+} and Zn^{2+} showed only a marginal effect (Table 3). Monovalent ions had no effect on PDE II.

DISCUSSION

The study of cyclic nucleotide phosphodiesterase in *Funaria* as well as other organisms has been complicated due to the multiplicity of enzyme forms. We have earlier reported the presence of multiple forms of PDE with respect to pH optima and kinetic behaviour [10]. The results presented in this paper show that the partially purified PDE (peak B), with activities of 71 and 136 units at pH 5.5 and 7.5 respectively, redistributes itself into two fractions with a total activity of 102 and 97 units at pH 5.5 and 7.5. This result is strongly indicative of an interconversion of the alkaline PDE into the acid PDE. Interconversions of one molecular form of phosphodiesterase to another have been reported in *Dictyostelium* [12, 13] and human platelets [14]; and have been implicated in a number of mammalian tissues [15, 16]. In *Dictyostelium* the high K_m enzyme is formed due to the interaction of the low K_m enzyme with an inhibitor protein [17]. It has also been suggested by Orlow *et al.* [12] that several MW forms of phosphodiesterase in *Dictyostelium* are due to binding of some uncharacterized acidic material to the same catalytic form.

In spite of using a basic pH for cell homogenization and DEAE-cellulose chromatography, the elution profiles of PDE activity and the properties of the two PDE peaks obtained upon affinity chromatography, were remarkably similar to the PDE I and PDE II reported earlier [10]. Therefore we believe that they represent the same enzyme activities and hence the same nomenclature of PDE I and II has been used in this paper.

In contrast to the non-linear kinetics observed at an acidic pH, both in the crude extract [11] and in partially purified cAMP phosphodiesterase [10] from *Funaria*, PDE II showed a linear high-affinity profile at pH 7.6, with a K_m of 11.8 ± 4.8 μM. The lack of cooperativity in the kinetic behaviour of PDE II cannot be attributed to a change in the assay pH, because both the peak B enzyme, as well as PDE I, exhibited non-linear kinetics at pH 7.6

(data not shown). More likely, such behaviour of PDE II is caused by the removal of the low affinity cAMP PDE or some other regulatory component. Linear kinetics for cAMP hydrolysis have also been observed for the high-affinity cAMP PDE purified from dog kidney [18] and rabbit skeletal muscle [19].

The K_m value of 11.8 μM is comparable to the low K_m value (8.7 μM) of the partially purified cAMP PDE [10]. As pointed out earlier [10] this range of K_m value is about one order of magnitude higher than the endogenous levels of cAMP in chloronema cells and therefore PDE II is likely to be physiologically relevant in *Funaria*. It is interesting to note that PDE II is Ca^{2+}-dependent, unlike the acid phosphodiesterase of *Funaria* which is Zn^{2+}-dependent [10]. The high affinity K_m value and the Ca^{2+} dependence substantiate the mammalian features of PDE II enzyme.

In most mammalian tissues, the Ca^{2+}-dependent regulatory protein (calmodulin) confers Ca^{2+} sensitivity to the cyclic nucleotide phosphodiesterase [20]. The activity of phosphodiesterases from higher plants such as *Phaseolus* [21] and *Spinacea* [22] is stimulated by about 30% by Ca^{2+} ions. The occurrence of calmodulin has also been reported in fungi [23], barley [23] and pea [24]. The Ca^{2+} dependence of PDE II from *Funaria* could also be caused by a calmodulin-like protein. However, the existence of such a protein in *Funaria* remains to be established.

EXPERIMENTAL

Cultures of chloronema cells. Chloronema cells from the protonema of the moss *Funaria hygrometrica* Hedw. were used for enzyme preparation. These cells were grown in axenic suspension cultures as described earlier [10].

Assay of cAMP phosphodiesterase. The reaction mixture consisted of 50 mM Tris–acetate buffer, pH 7.5, 20 μM cAMP, 0.1 μCi of [8-^{3}H] cAMP and enzyme preparation, in a total vol. of 0.25 ml. For assays at pH 5.5, the Tris–acetate buffer was replaced by 100 mM NaOAc buffer, pH 5.5. The reaction was carried out at 37° for 30 min to 2 hr and terminated by boiling for 3 min. A small aliquot of reaction products was spotted on to a silica gel coated plate along with unlabeled nucleotides as carriers and chromatographed using the solvent system 1-BuOH–MeOH–EtOAc–NH_3 (7:3:4:4). The nucleotide spots were located under UV light, scraped into Bray's scintillation fluid, and counted in a Packard Tri-Carb liquid scintillation spectrometer. One unit of enzyme activity is defined as 1.0 nmol cAMP hydrolysed/hr of incubation.

Purification of cAMP phosphodiesterase. The procedure for enzyme purification was as described previously [10], except for the following modifications. The buffer used for homogenization of cells was 50 mM Tris–acetate, pH 7.5 and the protein fraction precipitating at 40–80% saturation with $(NH_4)_2SO_4$ was used for further purification. The DEAE-cellulose column was washed with 50 mM Tris–acetate buffer, pH 7.5, followed by elution with a linear gradient of 0.10 to 1.0 M NaCl in the same buffer.

Protein estimation. Protein was determined by the method of Hartree *et al.* [25] using bovine serum albumin as the standard.

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