Regulation of cyclic AMP phosphodiesterase from *Mucor rouxii* by phosphorylation and proteolysis

Interrelationship of the activatable and insensitive forms of the enzyme

Néstor KERNER, Silvia MORENO and Susana PASSERON*

Programa de Regulación Hormonal y Metabólica, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

(Received 7 November 1983/Accepted 21 December 1983)

DEAE-cellulose chromatography of mycelial extracts of *Mucor rouxii* grown to mid-exponential phase resolves two types of low- K_m cyclic AMP phosphodiesterase (EC 3.1.4.17; PDE): PDE I, highly activatable (4–6-fold) by phosphorylation or proteolysis, and PDE II, unresponsive to activation. The enzymic profile of PDE activity obtained from germlings shows only PDE I activity, whereas PDE activity from mycelia grown to stationary phase is eluted from the DEAE-cellulose column at the position of PDE II, and like PDE II is unresponsive to activation. Endogenous proteolysis or controlled trypsin treatment transforms PDE I into PDE II. The insensitive forms of PDE exhibit a slightly smaller sedimentation coefficient than the activatable forms, as judged by sucrose-gradient centrifugation. The basal activity of the highly activatable form of PDE is elevated almost to the value in the presence of trypsin on storage at 4°C in the absence of proteinase inhibitors. Benzamidine, leupeptin, antipain or EGTA prevents the activation produced by storage. PDE I remains strongly activatable by phosphorylation and proteolysis after resolution by polyacrylamide-gel electrophoresis.

In most tissues multiple forms of cyclic nucleotide phosphodiesterases (EC 3.1.4.17) coexist (Beavo *et al.*, 1982), including those which have low affinity for cyclic AMP and are activated by calmodulin, those with high affinity for cyclic AMP and unaffected by calmodulin, and those capable of hydrolysing both cyclic AMP and cyclic GMP. It is reasonable to assume that these enzymes are probably structurally unrelated, since they do not exhibit immunological cross-reactivity (Beavo *et al.*, 1982).

On the other hand, it has been demonstrated that the multiple forms of calmodulin-independent high-affinity cyclic AMP phosphodiesterases co-existing in rat skeletal myoblasts are derived from only one primary form, which can be converted into the others by aggregation or proteolysis (Narindrasorasak *et al.*, 1982).

Previous work from our laboratory has demonstrated the participation of cyclic AMP in the morphogenesis of *Mucor rouxii* (Paveto *et al.*, 1975;

Cantore *et al.*, 1983). In studying the underlying biochemical mechanism of cyclic AMP action we have characterized PDE which regulates, together with adenylate cyclase, the concentration of the nucleotide *in vivo* (Cantore *et al.*, 1983).

We have previously reported (Galvagno *et al.*, 1979; Moreno *et al.*, 1982) that PDE from *M. rouxii* is of the calmodulin-insensitive low- K_m type, highly specific for cyclic AMP. It was also found that *Mucor* PDE can be reversibly activated 1.5–3-fold by treatment with MgATP, cyclic AMP and homologous cyclic AMP-dependent protein kinase or catalytic subunit from bovine heart. We also demonstrated that the enzyme can be activated to the same extent by treatment with trypsin, and that activation by phosphorylation and proteolysis is not additive.

In the present paper we report that, when PDE activity was analysed from the point of view of its regulatory properties, such as the degree of activation by phosphorylation and proteolysis, the existence of a heterogeneous population of the enzyme became evident. In view of this fact, information is required on the possible structural relationship among the different forms before the

Abbreviation used: PDE, cyclic AMP phosphodiesterase.

* To whom reprint requests should be addressed.

molecular details of the control of PDE by phosphorylation and proteolysis are further investigated. We present evidence which favours the view that *Mucor* has one primary form of PDE highly activatable by phosphorylation and proteolysis, from which the less-sensitive forms are derived by proteolysis *in vivo* or *in vitro*.

Experimental

Unless otherwise indicated, experimental details were as described in our previous papers (Moreno *et al.*, 1977; Moreno & Passeron, 1980; Moreno *et al.*, 1982).

Cultures

Fresh spores of *M. rouxii* (NRRL 1894) were produced and stored as described by Haidle & Storck (1966). Aerobic cultures of the fungus were grown from a spore inoculum of about 10^6 spores/ml in a complex medium (YPG) containing 2% (w/v) glucose as the main carbon source (Bartnicki-García & Nickerson, 1962), at 28°C with continuous shaking, harvested by filtration after different periods of time, and stored frozen at -70°C until used. Germlings were obtained after 6–8 h of growth, mycelia from mid-exponential phase after 10–12 h and mycelia from stationary phase after 16–18 h.

Phosphodiesterase preparation

The $(\text{NH}_4)_2\text{SO}_4$ fraction was prepared as previously described (Moreno *et al.*, 1982), except that the buffer used throughout was 10 mM-Tris/HCl (pH 7.4)/2 mM- β -mercaptoethanol/4 mM-EDTA/5 mM-EGTA/20 mM-benzamidine (buffer I). The $(\text{NH}_4)_2\text{SO}_4$ fraction, adjusted to 20 mg of protein/ml, was desalted by filtration through a Sephadex G-25 column (bed volume 10 times the volume to be desalted; height/diameter ratio 20:1) equilibrated with buffer I and poured on to a DEAE-cellulose column (10 mg of protein/ml bed volume) equilibrated with buffer I. The column was washed with 2 bed volumes of buffer I, and the protein was eluted with 10 bed volumes of a linear gradient of 0–0.35 M-NaCl in buffer I. The flow rate was regulated at 1 bed volume/h. Fractions (0.1 bed volume) were collected and samples were assayed for PDE activity under the conditions described in the legend to Fig. 1. Fractions indicated by arrows in Fig. 1 were separately pooled, adjusted to 80% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, and the protein was collected by centrifugation. The resulting pellet was dissolved in buffer I to a protein concentration of approx. 10 mg/ml, dialysed against the same buffer and stored in batches at -20°C.

Sucrose-density-gradient centrifugation

Linear 5–20% (w/v) sucrose gradients in buffer I were prepared as previously described (Moreno *et al.*, 1982), and run in a Beckman SW 55 Ti rotor at 140000 g_{av} during 16 h at 4°C. Alkaline phosphatase (*Escherichia coli*, 6.3S), peroxidase (horseradish, 3.5S) and cytochrome *c* (horse heart, 1.7S) were used as markers and assayed as previously described (Moreno & Passeron, 1980).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed at 4°C under non-denaturing conditions, following the instructions of the Canalc Manual (1980) in gels containing 7% (w/v) acrylamide plus NN' -methylenebisacrylamide, in 7 mm $\times$ 130 mm glass tubes. The gels were pre-run for 2 h at 2 mA/gel.

Assay methods

PDE standard assay. PDE activity was assayed by the modified two-step method of Thompson *et al.* (1976) as previously described (Moreno *et al.*, 1982). The standard incubation mixture contained, in a final volume of 0.1 ml: 50 mM-Tris/HCl buffer, pH 7.4, 2 mM- β -mercaptoethanol, 50 μ g of bovine serum albumin, 10 mM-MgCl₂, 5 μ M-cyclic [8-³H]-AMP (50000–80000 c.p.m.) and the enzyme preparation. Incubations were performed at 30°C for 20 min. Reaction rates were linear with time and proportional to enzyme-protein concentration. Substrate consumption was not higher than 20%.

One unit of PDE activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 pmol of cyclic AMP/min under the standard assay conditions.

Activation of PDE by trypsin. Trypsin treatment was performed as follows: appropriate samples of the enzyme preparation were preincubated for 10 min at 4°C with 10 μ g of trypsin in a final volume of 50 μ l; proteolysis was stopped by the addition of 30 μ g of egg-white trypsin inhibitor, and PDE activity was measured in the whole sample under the standard assay conditions.

Activation of PDE by phosphorylation. The activation was directly measured during the PDE assay by the addition to the standard incubation mixture of 0.1 mM-ATP and 30 units of catalytic subunit of bovine heart protein kinase. [One unit of protein kinase activity represents the incorporation of 1 pmol of [³²P]P_i into histone/min under the standard assay conditions previously described (Moreno *et al.*, 1982).]

Protein assay

Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard.

Chemicals

Cyclic [^3H]AMP (sp. radioactivity 38 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. DEAE-cellulose (microgranular), bovine pancreatic trypsin (type III), chicken egg-white trypsin inhibitor (type III), protein kinase catalytic subunit from bovine heart, heat-stable protein kinase inhibitor from rabbit skeletal muscle, snake venom (*Ophiophagus hannah*), benzamidine, Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 'TLCK') phenylmethanesulphonyl fluoride, antipain, leupeptin, *NN'*-methylenebisacrylamide and the proteins used as markers were from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide was from Merck-Schuchardt, Darmstadt, West Germany. All other chemicals were of analytical grade.

Results

DEAE-cellulose column chromatography of PDE preparations from mycelia harvested at different growth stages

A typical profile of PDE activity after DEAE-cellulose column chromatography of the (NH₄)₂SO₄ fraction prepared from mycelium of mid-exponential stage is shown in Fig. 1(a). Extracts prepared from mycelium of this stage were routinely used previously (Galvagno *et al.*, 1979; Moreno *et al.*, 1982) to demonstrate the activation of PDE by phosphorylation and proteolysis. PDE activity was eluted as a broad peak over a wide range of salt concentration. When basal activity was compared with that assayed under phosphorylating conditions or after trypsin treatment, it was observed that the fractions eluted at lower ionic strength were strongly activatable, whereas those eluted at higher salt concentrations were insensitive to trypsin treatment or phosphorylation. For simplicity we arbitrarily designate the two fractions as PDE I and II respectively, without the implication that they represent single molecular forms. The two forms of PDE were consistently obtained, although their relative amounts varied slightly from one preparation to another.

The results in Fig. 1(a) also show that the two methods of activating the enzyme lead to similar changes in enzymic activity. This result, together with those previously reported (Moreno *et al.*, 1982), namely that activation by phosphorylation and trypsin are not additive, suggest that both mechanisms of activation involve the same region of the enzyme. Definite proof of this statement will be obtained if removal of the phosphorylation site by controlled proteolysis is demonstrated.

It is also evident that the activity eluted at lower salt concentration (PDE I) is increased about 4–6-

fold by trypsin treatment or phosphorylation, significantly greater than the activation of 1.5–3-fold previously reported (Moreno *et al.*, 1982). However, it must be borne in mind that, although we had worked with similar preparations, the activation by trypsin treatment or phosphorylation was routinely performed with the whole peak of PDE from DEAE-cellulose chromatography in which only basal activity had been tested.

It is quite conceivable that the broadness of the peak is due to the existence of a heterogeneous population of PDE, very probably arising from endogenous proteolysis occurring *in vivo* or during the enzyme preparation, despite the inclusion in buffers of proteinase inhibitors (see Table 1).

Experiments were undertaken to see whether the heterogeneous population of PDE was dependent on the stage of growth of the organism. Two different stages of growth were chosen for the analysis: one was the onset of germ-tube emergence, in which the whole population is composed of round germ cells with two or three nascent hyphae per cell; the other was the stationary phase, in which growth has almost stopped and autolysis of older hyphae has begun.

Typical profiles from both stages are shown in Figs. 1(b) and 1(c). As shown in Fig. 1(b), all of the eluted PDE activity could be activated by phosphorylation or proteolysis, and no activity that was insensitive to activation and that was eluted at a higher ionic strength could be detected. PDE preparations obtained from mycelium harvested in the stationary phase showed a very different profile on DEAE-cellulose column chromatography (Fig. 1c). The enzyme activity was eluted as a narrower peak, almost insensitive to activation by trypsin or phosphorylation, at the ionic strength corresponding to PDE II. It is possible that this insensitive form(s) arises from activatable form(s) of the enzyme by endogenous proteolysis occurring *in vivo*. It is well known that the arrival of the stationary phase in filamentous fungi is accompanied by autolysis, with the consequent release of degradative enzymes, many of them proteinases.

The different degree of activatability of PDE depending on the stage of growth of the organism, presented here, points to the potential importance of the regulation of PDE by phosphorylation *in vivo*.

Activation of PDE I by endogenous proteolysis

Activation of PDE from several sources by endogenous proteolytic activity has been reported (Epstein *et al.*, 1978; Tucker *et al.*, 1981). The possibility that the activatable form of *Mucor* PDE (PDE I) could be stimulated by endogenous proteinases was investigated.

Fig. 1. Elution profiles of PDE activity from DEAE-cellulose chromatography of extracts from mycelia harvested in different growth stages

(a) $(\text{NH}_4)_2\text{SO}_4$ fraction (175 mg) from mycelium harvested in mid-exponential stage; (b) $(\text{NH}_4)_2\text{SO}_4$ fraction (50 mg) of germlings; (c) $(\text{NH}_4)_2\text{SO}_4$ fraction (167 mg) from mycelium harvested in stationary phase. Chromatography was performed as described in the Experimental section, on $1.4\text{ cm} \times 14.5\text{ cm}$ columns in (a) and (c) and on $1\text{ cm} \times 6\text{ cm}$ columns in (b). Appropriate samples were tested for PDE activity under basal conditions (●), after trypsin treatment (○) and under phosphorylating conditions (□), and for protein (A_{595} ;). —, Conductivity measured throughout the gradient.

Table 1. *Effect of trypsin on PDE activity after storage in the presence of proteinase inhibitors*

PDE I (500 units) was rapidly filtered through Sephadex G-25 as described in the text. A 2 μ l sample of the effluent was immediately tested for PDE activity with or without prior trypsin treatment. The values obtained, 10.1 and 3.0 units respectively, were taken as controls. The remaining eluate was divided in 20 μ l batches and stored at 4°C for 24h with the indicated additions. After the storage period, 2 μ l samples were withdrawn and assayed for PDE activity with or without trypsin treatment as described in the Experimental section. Under these conditions, proteinase inhibitors were diluted 40-fold during the preincubation with trypsin. All assays were done in triplicate. The divergence between the average and the individual values in no case exceeded $\pm 10\%$.

Additions	PDE activity (units)		Activation (%)
	Basal	+ Trypsin	
None	8.7	10.4	119
Tos-Lys-CH ₂ Cl (0.1 mM)	5.3	10.7	201
Phenylmethane- sulphonyl fluoride (0.4 mM)	4.5	10.5	471
Benzamidine (20 mM)	1.9	9.6	505
EGTA (5 mM)	2.1	10.4	495
Antipain (3 μ g/ml)	2.0	10.1	505
Leupeptin (20 μ g/ml)	1.8	9.8	544

Accordingly, a sample of the PDE I fraction was freed of the proteinase inhibitors added to buffer I (EGTA and benzamidine) by rapid filtration through a Sephadex G-25 column equilibrated with 10 mM-Tris/HCl (pH 7.4)/2 mM- β -mercaptoethanol/1.5 mM-EDTA. Immediately after filtration, a sample of the effluent was tested for PDE activity, with or without trypsin treatment. These values were taken as controls (see Table 1). The remaining eluate was divided into several batches and stored at 4°C for 24h in the absence or in the presence of the proteinase inhibitors indicated in Table 1. After the storage period, PDE activity was assayed with or without prior trypsin treatment. The results obtained are shown in Table 1. The basal activity of the enzyme stored in the absence of inhibitors is elevated almost to that of trypsin-treated enzyme, yielding an activation of 1.2-fold. In some preparations, the fold activation decreased the limiting value of 1 after 24h at 4°C.

EGTA, antipain, leupeptin and benzamidine were the best inhibitors of the increase in basal activity occurring on storage. Tos-Lys-CH₂Cl and phenylmethanesulphonyl fluoride only partially prevented this process.

The activity of the trypsin-treated enzyme after the storage period was similar under all conditions

and attained the same activity as the trypsin-treated control, indicating that no significant denaturation of the enzyme had occurred on storage. The slight decrease in fold activation observed in the control probably arises by endogenous proteolysis occurring during the enzyme assay.

Since experiments shown in Fig. 1 suggest that PDE I is the form susceptible to activation by phosphorylation and proteolysis, it is possible that this is the precursor form. On the other hand, results in Table 1 indicate that *Mucor* PDE can be activated by endogenous proteinases present in the preparation. Accordingly, we attempted to convert PDE I into PDE II by endogenous proteolysis. Fig. 2 shows that, when PDE I was stored in the absence of proteinase inhibitors, which resulted in 5-fold activation, and was rechromatographed on DEAE-cellulose, a form similar to PDE II was predominant, with trace amounts of PDE I, very probably owing to incomplete activation by proteolysis. Similar results were obtained when PDE I was previously subjected to trypsin treatment and then chromatographed on DEAE-cellulose (results not shown).

These results resemble those reported by Narindrasorasak *et al.* (1982). They found that rat myoblasts contain multiple forms of low- K_m phosphodiesterases and demonstrated that only one is susceptible to regulation by phosphorylation and proteolysis, the other forms being derived from that precursor.

Analysis by sucrose-density-gradient centrifugation of the activatable and insensitive forms of PDE

The different forms of PDE were subjected to centrifugation on linear 5–20% (w/v) sucrose gradients (results not shown). PDE I and PDE from germlings sediment with an apparent sedimentation coefficient of $4.3 \pm 0.2S$. PDE II, PDE obtained from mycelium from stationary phase, and trypsin-treated or endogenously proteolysed PDE I had a sedimentation coefficient of $3.3 \pm 0.2S$ (means $\pm$ S.D.; $n = 4$). From these results it can be concluded that, no matter where the enzyme insensitive to activation by phosphorylation or proteolysis was obtained from, it exhibited a smaller sedimentation coefficient than did the activatable enzyme.

These results reinforce the idea that the loss of responsiveness is a consequence of proteolytic cleavage of the regulatory domain of the enzyme molecule.

Analysis of PDE activities by polyacrylamide-gel electrophoresis

Non-denaturing polyacrylamide-gel electrophoresis of activatable (PDE I) and insensitive

Fig. 2. Elution profile of PDE activity from DEAE-cellulose chromatography of endogenously proteolysed PDE I. PDE I (4mg) obtained from the DEAE-cellulose column of Fig. 1(a) was dialysed for 24h at 4°C against 10mM-Tris/HCl (pH 7.4)/2mM- β -mercaptoethanol/1.5mM-EDTA and then chromatographed on a 0.8cm $\times$ 3cm column. Appropriate samples were tested for PDE activity under basal conditions (●), after trypsin treatment (○) and under phosphorylating conditions (□). —, Conductivity measured throughout the gradient.

(PDE II) forms of the enzyme yielded symmetrical peaks of enzyme activities, with R_F values of 0.60 and 0.69 respectively. As shown in Fig. 3(a), PDE I remained strongly activatable by phosphorylation or by trypsin treatment after electrophoresis, whereas PDE II (Fig. 3b) remained insensitive to both activation processes.

Discussion

The results presented in this paper support the idea that the presence of the form of PDE highly activatable by proteolysis or phosphorylation depends on the stage of growth of the organism, this being the only form present in germlings. Evidence is also presented that the insensitive form of the enzyme is derived by endogenous proteolysis or trypsin treatment of the activatable one. This insensitive form is the only one observed in mycelium from stationary phase. The profiles of trypsin-treated or endogenously proteolysed PDE I and of PDE from stationary-phase mycelium on DEAE-cellulose and on sucrose gradients are similar. These results could indicate that the sites of proteolysis are on the same region of the enzyme.

We still do not have enough evidence to decide whether a profile such as that depicted in Fig. 1(a) is the result of the coexistence of two defined forms of PDE, activatable and insensitive, or whether it represents a heterogeneous population of the enzyme with variable degrees of activatability.

The results of polyacrylamide-gel electrophoresis strongly support the hypothesis that the activation of PDE I by phosphorylation and proteolysis results from the modification of the enzyme molecule itself. To investigate further the molecular details of the control of PDE by phosphorylation and proteolysis and the interrelationship of the two mechanisms of activation, a highly purified preparation of the activatable PDE must be available. To achieve this goal, it will be necessary to find an alternative to the harsh homogenization procedure used at present to fragment large quantities of mycelia. Despite the results presented in Table 1, we could not reproducibly eliminate progressive endogenous proteolysis in further steps of purification of PDE, even with the inclusion of EGTA and antipain in buffers. Therefore we are continuously modifying our purification protocol to try to minimize proteolytic action, in an effort to prepare a stable activatable enzyme.

Fig. 3. Polyacrylamide-gel electrophoresis of PDE I and PDE II

The gels were loaded with 100 μ l of PDE I (6 mg of protein/ml) (a) and 100 μ l of PDE II (5 mg of protein/ml) (b) and run as described in the Experimental section. After electrophoresis, the gels were cut into 2 mm slices. PDE activity was assayed in quarters of slices under basal conditions ($\bullet$), after trypsin treatment ($\circ$) or under phosphorylating conditions ($\square$), in a final volume of 150 μ l for 60 min at 30°C. After this period, the second step of the assay was performed on 100 μ l samples. Other details were as described in the Experimental section.

The fold activation by phosphorylation and trypsin treatment is taken as an invaluable assay in pursuing such studies.

We are grateful to Dr. M. A. Galvagno for helpful criticism of the manuscript. This work has been supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Subsecretaría de Estado de Ciencia y Tecnología and Comisión Nacional de Energía Atómica. N. K. is a fellow of CONICET; S. M. and S. P. are Career Investigators of the same Institution.

References

- Bartnicki-García, S. & Nickerson, W. J. (1962) *J. Bacteriol.* **84**, 829–840
- Beavo, J. A., Hansen, R. S., Harrison, S. A., Hurwitz, R. L., Martins, T. J. & Mumby, M. C. (1982) *Mol. Cell. Endocrinol.* **28**, 387–410
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cantore, M. L., Galvagno, M. A. & Passeron, S. (1983) *Cell Biol. Int. Rep.* **7**, 947–955
- Epstein, P., Pledger, W. J., Gardner, E., Stancel, G., Thompson, W. J. & Strada, S. (1978) *Biochim. Biophys. Acta* **527**, 442–445
- Galvagno, M. A., Moreno, S., Cantore, M. L. & Passeron, S. (1979) *Biochem. Biophys. Res. Commun.* **89**, 779–785
- Haide, C. W. & Storck, R. (1966) *J. Bacteriol.* **92**, 1236–1244
- Moreno, S. & Passeron, S. (1980) *Arch. Biochem. Biophys.* **199**, 321–330
- Moreno, S., Paveto, C. & Passeron, S. (1977) *Arch. Biochem. Biophys.* **180**, 225–231
- Moreno, S., Galvagno, M. A. & Passeron, S. (1982) *Arch. Biochem. Biophys.* **214**, 573–580
- Narindrasorasak, S., Tan, L. U., Seth, P. K. & Sanwal, B. D. (1982) *J. Biol. Chem.* **257**, 4618–4626
- Paveto, C., Epstein, A. & Passeron, S. (1975) *Arch. Biochem. Biophys.* **169**, 449–457
- Thompson, W. J., Ross, C., Pledger, W. J., Strada, S., Banner, R. & Hersh, E. (1976) *J. Biol. Chem.* **251**, 4922–4929
- Tucker, M., Robinson, J. B., Jr. & Stellwagen, E. (1981) *J. Biol. Chem.* **256**, 9051–9058