Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 779-785

# CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE PHOSPHODIESTERASE FROM MUCOR ROUXII: REGULATION OF ENZYME ACTIVITY BY PHOSPHORYLATION AND DEPHOSPHORYLATION

M. A. Galvagno, S. Moreno, M. L. Cantore and S. Passeron

Departamento de Química Biológica and Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina

Received June 8,1979

SUMMARY: Crude preparations of cyclic adenosine 3',5'-monophosphate phosphodiesterase were activated 1.5 to 2 fold by incubation with ATP, $Mg^{2+}$ and cyclic AMP in a reaction which was both, time and temperature dependent. Cyclic AMP phosphodiesterase remained in an activated state upon filtration of the enzymatic preparation through Sephadex G-25 and ion-exchange chromatography. Activation of the enzyme in the presence of [$\gamma$ $^{32}$P]ATP resulted in a significant amount of [$^{32}$P] protein-bound radioactivity. Reversible deactivation of cyclic AMP phosphodiesterase was enhanced by $Mg^{2+}$ and was accompanied by the release of [$^{32}$P]protein bound radioactivity. The evidence is consistent with a mechanism for controlling cyclic AMP phosphodiesterase through phosphorylation-dephosphorylation sequence.

A number of key regulatory enzymes in eucaryotic organisms are known to be controlled by phosphorylation-dephosphorylation mechanism and there is increasing evidence that these interconversion reactions underlie the short term regulation of metabolism by external physiological stimuli (1).

Recent studies in our laboratory (2, 3) have shown that cyclic AMP plays an important role in the morphogenesis of the dimorphic fungus Mucor rouxii. We have also reported that the action of cyclic AMP could be mediated through the activation of a cyclic AMP dependent protein kinase (4).

Cyclic adenosine 3',5'-monophosphate phosphodiesterase (PDE), the enzyme that hydrolyzes cyclic AMP, was also partially characterized from extracts of the fungus. The soluble enzyme exists in only one molecular form and has a Km for cyclic AMP of 1 $\mu$M, well within the range of the intracellular level of the nu-

Abbreviation: PDE, cyclic AMP phosphodiesterase.

0006-291X/79/150779-07$01.00/0

cleotide. The partially purified enzyme was neither activated nor inhibited by ATP up to concentrations of 0.5 mM (3). However the observation that ATP activates PDE in crude preparations of the fungus, prompted a further investigation of the action of ATP.

In the present communication we show a concentration and time dependent activation of PDE by enzymatic phosphorylating conditions. We also show a reversible deactivation of the enzyme in a $Mg^{2+}$ stimulated reaction. These observations suggest that PDE activity could be regulated by a phosphorylation-dephosphorylation sequence.

It has been reported that cyclic AMP and ATP can increase the activity of the activator-deficient high Km PDE from rat brain in a $Ca^{2+}$ dependent fashion, but a different mechanism was proposed for the action of cyclic AMP in that system (5).

## MATERIALS AND METHODS

[$\gamma$-$^{32}$P] ATP (specific activity 4000 cpm/pmol) was prepared according to Chang et al. (6). [$^{3}$H] Cyclic AMP (specific activity 42.3 Ci/mmol) was obtained from New England Nuclear.

Mucor rouxii (NRRL 1894) was used throughout this work. Frozen mycelial powder obtained as previously described (4) was homogenized with 2 vol. of 10 mM Tris-HCl buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM mercaptoethanol (buffer A); the homogenate was centrifuged at 8,000 x g for 20 min and the supernatant was spun down at 100,000 x g for 60 min. The clear supernatant was brought to 55% saturation with saturated neutralized ammonium sulfate solution. Protein was allowed to precipitate for $\frac{1}{2}$h and was then collected at 12,000 x g for 20 min. The pellet was dissolved in a minimum volume of buffer A, clarified and desalted through a Sephadex G-25 column equilibrated with buffer A and the void volume, designated SA fraction, was used as enzyme source. All of the above steps were carried out at 4ºC.

PDE Assay. PDE activity was assayed by a modification of the two step method of Thompson and Appleman (7). The standard incubation mixture, in a final volume of 0.1 ml, contained 10 $\mu$M cyclic AMP (50,000-70,000 cpm of $^{3}$H), 50 mM Tris-HCl buffer, pH 7.4, 10 mM $MnCl_2$, 2 mM mercaptoethanol and 25-50 $\mu$g of the SA fraction. The incubation was carried out for 20 min at 30ºC. Each assay was run in triplicate.

Phosphorylation Reaction. Unless otherwise stated, the incorporation of phosphate into endogenous proteins from [$\gamma$-$^{32}$P] ATP was performed as previous-

ly described (4). The assay mixture contained: 10 mM $MgCl_2$, 0.2 mM [$\gamma$-$^{32}$P]ATP (specific activity 1300 cpm/pmol), 2 mM mercaptoethanol, 50 mM Tris-HCl buffer, pH 7.4, 10 $\mu$M cyclic AMP and protein as indicated in a final volume of 0.1 ml. All reactions were carried out at 30ºC. [$^{32}$P] Protein-bound radioactivity was rendered acid soluble after hydrolysis in 1 N KOH for 1 h at 37ºC indicating that $^{32}$P was bound to protein.

Activation of PDE. The standard incubation mixture was similar to the one described for the phosphorylation reaction except that unlabeled ATP was used. The mixture was incubated at 30ºC and the reaction was started by the addition of the SA fraction. Aliquots were removed and assayed for PDE activity as described above. These assays gave linear time courses indicating that activation did not continue during the assay.

Protein was assayed by the method of Lowry et al. (8) with bovine serum albumin as standard. Radioactivity was determined by liquid scintillation spectrometry.

## RESULTS AND DISCUSSION

Incubation of the SA fraction from M. rouxii extracts with ATP, $Mg^{2+}$ and cyclic AMP caused a nearly 2-fold increase in PDE activity (Table I). Some further experiments were carried out to identify the components necessary for activation of the enzyme. As can be seen, no activation was observed in the absence of either ATP, $Mg^{2+}$ or cyclic AMP. When ATP was replaced by the imido analog of ATP (the terminal phosphate of which cannot be utilized for protein phosphorylation) no activation was observed.

The time course of activation of PDE by ATP was examined. At 30ºC, the activation occurred very rapidly and was maximal within 2 min after adding the enzymatic preparation. As shown in Fig. 1, incubation of the SA fraction with ATP, $Mg^{2+}$ and cyclic AMP at 21ºC resulted in a time dependent activation of PDE activity and incorporation of [$^{32}$P] protein-bound radioactivity from [$\gamma$-$^{32}$P] ATP. The activation was maximal after 5 min of addition of the SA fraction.

The extent of enzyme activation was dependent on ATP concentration. Half-maximal activation of PDE was attained at 0.04 mM ATP and maximal activation at about 0.2 mM (data not shown).

TABLE I: Requirements for the Activation of PDE

| Preincubation conditions | PDE activity (pmol [$^3$H]cyclic AMP hydrolyzed/20 min) | % Maximal activity |
|---|---|---|
| Complete mixture | 72 | 100 |
| -ATP, -$Mg^{2+}$, - cyclic AMP | 39 | 54.1 |
| -ATP | 38 | 52.7 |
| -cyclic AMP | 44.9 | 62.3 |
| -$Mg^{2+}$ (+ 10 mM EDTA) | 39.4 | 54.7 |
| -ATP (+ 0.2 mM $\beta,\gamma$-imido ATP) | 37.8 | 52.5 |

Aliquots of the SA fraction containing each 360 µg of protein were preincubated during 6 min at 30ºC in a final volume of 0.1 ml (see Materials and Methods). Preincubations were stopped with 20 mM EDTA and 10 µl aliquots of each preincubation mixture were assayed for PDE activity.

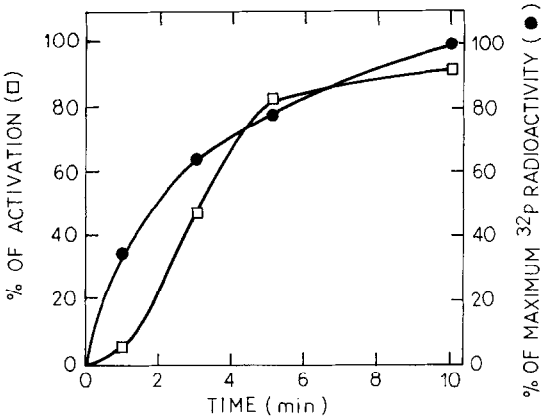


Fig. 1. Time course of protein phosphorylation by endogenous protein kinases and PDE activation.
Two samples (720 µg) of SA fraction were preincubated at 21ºC in 0.2 ml of the complete activation mixture as described in Materials and Methods. One of the preincubation mixtures contained [$\gamma$-$^{32}$P] ATP (1300 cpm/pmol). At the indicated times 10 µl aliquots from each incubation were withdrawn, 20 mM EDTA was added and the samples were assayed for PDE activity ( □ ) and [$^{32}$P] protein-bound radioactivity ( ● ). Basal PDE activity was 39 pmol of cyclic AMP hydrolyzed/20 min/10 µl and maximum [$^{32}$P] protein-bound radioactivity was 18,000 cpm/10 µl.

The above results strongly suggest that PDE can be activated by a phosphorylation reaction catalyzed by endogenous cyclic AMP dependent protein kinase.

Attempts were made to reverse the activation of PDE by incubation under conditions that might be expected to promote dephosphorylation. Deactivation of pre-

TABLE II: Effect of $Mg^{2+}$ on Deactivation of PDE and [$^{32}$P]Protein Bound Radioactivity Release

| | PDE | | [$^{32}$P] Protein bound radioactivity | |
|---|---|---|---|---|
| | pmol cyclic AMP hydrolyzed/ mg/20 min | % initial activity | pmol $^{32}$P bound/mg | % initial value |
| Initial value | 1387 | 100 | 302 | 100 |
| After 20 min incubation with | | | | |
| no addition | 1026 | 74 | 211 | 70 |
| 5 mM $Mg^{2+}$ | 568 | 41 | 91 | 30 |
| 5 mM $Mg^{2+}$ followed by 6 min activation | 1109 | 80 | 272 | 90 |

Two aliquots of 720 μg of the SA fraction were activated for 6 min as described in Materials and Methods. One of the incubation mixtures contained [$\gamma$-$^{32}$P]ATP (specific activity 1300 cpm/pmol). The activated preparations were filtered through Sephadex G-25 columns to remove ATP, $Mg^{2+}$ and cyclic AMP. Sephadex G-25 treated activated preparations were assayed for initial PDE activity and [$^{32}$P] protein-bound radioactivity, and then incubated for 20 min at 30ºC in buffer A with the indicated additions. After incubation in the presence of 5 mM $Mg^{2+}$ for 20 min, ATP (0.2 mM) and cyclic AMP (10 μM) were added and the incubation was continued for additional 6 min. One of the samples received [$\gamma$-$^{32}$P]ATP.

viously activated PDE by endogenous enzymes in the SA fraction was studied after gel filtration to remove $Mg^{2+}$, ATP and cyclic AMP. As shown in Table II both, enzymatic activity and $^{32}$P protein-bound radioactivity decreased markedly (by 59% and 70%, respectively) during a 20 min incubation in the presence of 5 mM $Mg^{2+}$.

That the deactivation was truly reversible (and not inactivation by denaturation) is shown by 80% restoration of initial activity, when after 20 min incubation with $Mg^{2+}$ the preparation was incubated for 6 min with ATP and cyclic AMP. The lack of full restoration of initial activity in the subsequent incubation with ATP and cyclic AMP could be due to denaturation rather than dephosphorylation.

The table also shows reincorporation of [$^{32}$P] protein-bound radioactivity into the previous inactivated preparation. The results are compatible with reversible

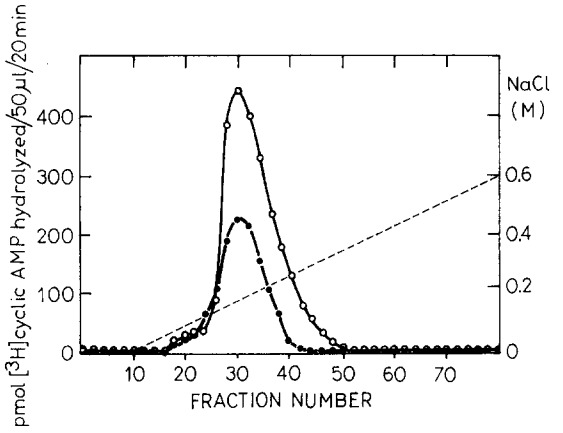


Fig. 2. DEAE-cellulose column chromatography of activated and control preparations.
Two aliquots of the SA fraction containing 18 mg of protein were incubated for 6 min at 30ºC under complete phosphorylating conditions ( ○ ) or without ATP ( ● ) in a final volume of 8 ml. They were diluted twice with buffer A and loaded into DEAE-cellulose columns (0.9 x 8 cm, 5 ml of resin) and the protein was eluted with a linear gradient of 0-0.6 M NaCl made in buffer A (total volume, 50 ml) in 0.5 ml fractions. PDE activity was assayed in 50 µl aliquots of each fraction.

deactivation by endogenous phosphatase(s). In a control experiment the SA fraction was not activated and the subsequent incubation with or without $Mg^{2+}$ resulted in a small loss of activity (10 to 20%).

PDE which had been incubated under phosphorylating conditions remained in an activated state after passing the enzyme preparation through a Sephadex G-25 column and could not be further activated upon incubation with ATP, $Mg^{2+}$ and cyclic AMP. Moreover, the activated and control enzyme showed identical elution pattern in DEAE-cellulose column chromatography except that the total activity of the activated preparation was twice that of the control (Fig. 2). The recovery of enzymatic activities of both was over 80%.

The data reported in this paper indicate that PDE activity from M. rouxii can be controlled by a phosphorylation-dephosphorylation reaction sequence. They represent the first evidence that such mechanism is involved in the regulation of the enzyme. The ATP-dependent activation of PDE activity appears to involve enzyma-

tic modification of some protein of the enzymatic system, as indicated by the dependence of the activation on time, temperature and ATP concentration. The question of whether it is the enzyme itself that is phosphorylated or some other protein which interacts with it, is still under study.

Acknowledgements. This work has been supported in part by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Secretaría de Ciencia y Tecnología (Argentina). S. M. is a Post-graduate Fellow and S. P. a Career Investigator of CONICET. The authors wish to express their gratitude to Dr. C. E. Cardini for his constant and enthusiastic support.

REFERENCES

1. Nimmo, H. G. and Cohen, P. (1977) Adv. Cycl. Nucl. Res. 8, 145-166.
2. Paveto, C., Epstein, A. and Passeron, S. (1975) Arch. Biochem. Biophys. 169, 449-457.
3. Cantore, M. L., Galvagno, M. A. and Passeron, S. (1979) submitted for publication.
4. Moreno, S., Paveto, C. and Passeron, S. (1977) Arch. Biochem. Biophys. 180, 225-231.
5. Gnegy, M. E., Costa, E. and Uzunov, P. (1976) Proc. Nat. Acad. Sci. U. S. A. 73, 352-355.
6. Chang, K., Marcus, N. and Cuatrecasas, P. (1974) J. Biol. Chem. 249, 6854-6865.
7. Thompson, W. J. and Appleman, M. (1971) Biochemistry 10, 311-316.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.