Characterization of a rat liver cyclic GMP-activated phosphodiesterase by chromatography on hexyl-agarose

Inhibition of phosphodiesterase activity by hexyl-agarose

Dominique COUCHIE, Christophe ERNEUX and Jacques E. DUMONT
*Institute of Interdisciplinary Research, School of Medicine, Free University of Brussels, 2 rue Evers,
B-1000 Brussels, Belgium*

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Chromatography on hexyl-agarose resolved a partially purified cyclic GMP-activated phosphodiesterase from rat liver into two peaks of activity: the first was eluted with 0.5 M-KCl and was cyclic AMP-specific. The second was tightly bound to hexyl-agarose and was not eluted with KCl (0–2.0 M), which enhanced the hydrophobic interactions of this form with the matrix. It was eluted with 0.5 M-Tris, hydrolysed cyclic AMP and cyclic GMP and was specifically activated by cyclic GMP. The cyclic GMP-activated phosphodiesterase was immobilized on hexyl-agarose. Enzyme activity, quantitatively bound to hexyl-agarose, was not released from the hydrophobic matrix in the presence of cyclic AMP or cyclic GMP, under our assay conditions. The immobilized form of the enzyme retained catalytic activity, was inhibited by 0.1 mM-cyclic AMP and was activated by micromolar concentrations of cyclic GMP to a lesser extent (7-fold) than the control, i.e. the enzyme mixed with unsubstituted agarose (15-fold). When the enzyme was immobilized, inhibition of cyclic AMP phosphodiesterase activity was only observed in the presence of cyclic GMP (at 3 μ M); in its absence, activity remained unchanged. The kinetic behaviour of the immobilized enzyme is consistent with the hypothesis of a binding site distinct from the hydrolytic and activating sites.

In mammalian tissues, at least three types of phosphodiesterases have been demonstrated: a Ca^{2+} -activator (or calmodulin)-dependent form hydrolysing both cyclic AMP and cyclic GMP, a cyclic AMP-specific phosphodiesterase and a third form hydrolysing cyclic AMP and cyclic GMP, characterized by cyclic GMP activation of cyclic AMP hydrolysis (Russell *et al.*, 1973; Wells & Hardman, 1977). These enzymes are not equally distributed among the many tissues so far studied, and in horse thyroid we have shown by ultracentrifugation on sucrose gradients that they were not interconvertible forms (Erneux *et al.*, 1980).

It has been suggested that the cyclic GMP-activated phosphodiesterase prepared from rat liver behaves as a hydrophobic protein (Moss *et al.*, 1977). Therefore we wanted to immobilize this enzyme by a non-covalent method on a hydrophobic matrix. Such experiments aimed to determine whether immobilization of the enzyme would affect its catalytic hydrolytic activity and/or the cyclic GMP-activating effect.

Materials and methods

Materials

Cyclic [8- ^3H]AMP (sp. radioactivity 30 Ci/mmol) and cyclic [8- ^3H]GMP (sp. radioactivity 21 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and purified on Dowex-50 cation-exchange resin columns (Hardman & Sutherland, 1969). Unlabelled cyclic nucleotides were from Boehringer. 5'-Nucleotidase (*Crotalus atrox* venom) and phenylmethanesulphonyl fluoride were obtained from Sigma. QAE-Sephadex [quaternary diethyl-(2-hydroxypropyl)aminoethyl-Sephadex] A-25 and Sephacryl S-200 were from Pharmacia. DEAE-cellulose DEAE-52 was purchased from Whatman. Shaltiel hydrophobic chromatography kit and hexyl-agarose were obtained from Miles-Yeda. The hexyl-agarose columns used in this work consisted of hexylamine groups coupled to agarose, and probably separated enzymes on the basis of both hydrophobicity and ionic character (Hjerten, 1973). Fatty-acid-poor

bovine serum albumin was from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). PM 10 membranes were from Amicon.

Preparation of phosphodiesterases

Partially purified cyclic GMP-activated phosphodiesterase was prepared as follows. Fresh liver (40 g) from male Sprague-Dawley rats was homogenized (1 g of tissue/5 ml of buffer medium) with a motor-driven Teflon/glass homogenizer in the following buffer medium: 20 mM-Tris/HCl, pH 7.4, 2 mM-MgCl₂, 7 mM-2-mercaptoethanol, 0.25 M-sucrose, and 1.5 ml of a freshly prepared solution (50 mg/ml in dimethyl sulphoxide) of phenylmethanesulphonyl fluoride/litre. This preparation was filtered through a Teflon sieve and centrifuged at first at 2400 g for 20 min, then at 40000 g for 60 min. The final supernatant was fractionated by DEAE-cellulose chromatography (2.5 cm × 47 cm) with an exponential salt gradient (total vol. 1000 ml) of 0–0.5 M-(NH₄)₂SO₄, as previously described (Wells *et al.*, 1975; Erneux *et al.*, 1980). The enzyme (peak II) was eluted with 35% yield, immediately concentrated by precipitation with 60%-satd. (NH₄)₂SO₄ and dialysed overnight against 20 mM-Tris/HCl (pH 7.4)/2 mM-MgSO₄/100 mM-KCl/1 mM-EGTA/7 mM-2-mercaptoethanol in 20% (v/v) glycerol. Further purification was achieved by gel filtration on Sephacryl S-200 (column 1.5 cm × 90 cm) equilibrated and eluted with the same buffer used for dialysis (Erneux *et al.*, 1980). Active fractions (peak II') were pooled, dialysed against 40 mM-Tris/HCl (pH 7.4)/7 mM-2-mercaptoethanol in 20% glycerol, and stored in the presence of 4 mg of bovine serum albumin/ml at -80°C.

Assay procedure

For phosphodiesterase assay, the standard reaction mixture contained, in 100 µl: 40 mM-Tris/HCl, pH 7.4, 5 mM-MgSO₄, 5 mM-2-mercaptoethanol, 1 mg of bovine serum albumin/ml and 0.01–100 µM tritiated cyclic nucleotide in addition to the enzyme. The reaction was carried out as previously described (Erneux *et al.*, 1980). Product accumulation was linear with time and protein concentration (Erneux *et al.*, 1977), and the enzyme concentration was always chosen so as to keep substrate conversion below 25%. Protein concentration was measured by the method of Peterson (1977), with bovine serum albumin as standard. All the results are means of duplicate or triplicate determinations.

Chromatography on hexyl-agarose

Hexyl-agarose was equilibrated in a column (0.9 cm × 12 cm) at pH 7.4 with 0.1 M-Na₂HPO₄/NaH₂PO₄/2 mM-MgCl₂/7 mM-2-mercaptoethanol/5% glycerol (flow rate 0.5 ml/min, void volume 15 ml; 5 ml fractions were collected). The enzyme

preparation, previously dialysed against the equilibration buffer, was applied and the column was washed with the equilibration buffer. The initial wash (40 ml) was followed by a linear 0–2.0 M-KCl gradient (total vol. 90 ml) at fraction 9, and at fraction 27 by 20 ml of the equilibration buffer (pH 7.4). At fraction 31, the elution was continued with 90 ml of 0.5 M-Tris/HCl, added in the equilibration buffer at pH 7.4.

Enzyme immobilization

The enzyme was immobilized on alkyl (C₆)-agarose gel. Agarose without substitution was used as a control. Enzyme was mixed with an equal volume (0.5 ml) of the appropriate gel, in the presence of the complete buffer assay medium including 3 µM unlabelled substrate: cyclic AMP if cyclic AMP phosphodiesterase was measured, and cyclic GMP for cyclic GMP phosphodiesterase activity. This suspension was left under stirring for 10 min, at 0° or 30°C. A sample (usually 20 µl) was taken to measure phosphodiesterase activity. Similar results were obtained in the absence of cyclic nucleotide during the initial 10 min preincubation.

Results

Chromatography of cyclic GMP-activated phosphodiesterase from rat liver on hexyl-agarose (Fig. 1)

The cyclic GMP-activated phosphodiesterase from rat liver was at first prepared by DEAE-cellulose chromatography. After this step, it was separated from the calmodulin-dependent phosphodiesterase, but was contaminated with a 'low-*K_m*' cyclic AMP phosphodiesterase (Erneux *et al.*, 1980). It was further fractionated by chromatography on hexyl-agarose into two forms, IIa and IIb (Fig. 1). Form IIa was eluted with low concentrations of KCl (0.5 M); it specifically hydrolysed cyclic AMP. The second peak (IIb) was tightly bound to the hydrophobic matrix: it was not eluted by KCl at concentrations up to 2.0 M. As reported by Moss *et al.* (1977), form IIb was eluted with Tris at 0.5 M. We noticed that in the presence of both Tris (0.5 M) and KCl (2.0 M) the enzyme was not detached from the hydrophobic column. Form IIb hydrolysed both cyclic nucleotides, but was more active against cyclic GMP: at 3 µM, the ratio of cyclic GMP to cyclic AMP hydrolysis was 3.5 (results not shown). Hydrolysis of cyclic AMP (3 µM) by form IIb was specifically stimulated by cyclic GMP (3 µM). The resolution of two peaks for this enzyme form (Fig. 1) was found in some but not all of a large number of different rat liver preparations. The resolution of the enzyme form IIb increased the factor of stimulation of cyclic AMP hydrolysis by cyclic GMP: before separation, this factor was only 4, but after resolution of form IIb, it became 27 (Fig. 1).

Fig. 1. Hydrophobic-interaction elution profile of the liver cyclic GMP-activated phosphodiesterase from DEAE-cellulose chromatography (peak II)

The enzyme preparation (0.79 mg of protein in 6 ml) was put on a hexyl-agarose column (0.9 cm $\times$ 12 cm) and eluted as described in the Materials and methods section. Phosphodiesterase activity was measured at 3 μ M-cyclic AMP in the presence (○) and absence (●) of 3 μ M-cyclic GMP. Activity was expressed per 10 μ l over 20 min of incubation. Results are means of duplicates and are representative of experiments with four different preparations. In the absence of cyclic GMP, recovery was 76% for form IIa and 11% for form IIb; in the presence of cyclic GMP, recovery was 11% for form IIa and 66% for form IIb.

Characterization of immobilized cyclic GMP-activated phosphodiesterase on hexyl-agarose

This study was performed with the partially purified rat liver cyclic GMP-activated enzyme (after gel filtration on Sephacryl S-200, i.e. peak II'). This enzyme (cyclic GMP-activated) was not tightly bound to a column of alkyl-agarose of four carbon atoms or less, since relatively low concentrations of KCl were able to elute the enzyme. However, when

layered on alkyl-agarose chains of six carbon atoms or more, it was effectively immobilized, since no significant activity could be detected in the elution buffer during the wash process or by increasing the ionic strength (results not shown).

The following studies were conducted to compare the enzyme in an immobilized state (bound to hexyl-agarose) with free enzyme or enzyme mixed with agarose. In control experiments, it was shown that under our assay conditions the cyclic GMP-activated phosphodiesterase remained bound to the hydrophobic matrix. In particular, this was observed when the suspension did contain the substrate (3 μ M-cyclic AMP or 3 μ M-cyclic GMP) in the enzyme-assay medium: after centrifugation of the gel-enzyme suspension, activities were quantitatively recovered in the pellet and not in the supernatant. A typical experiment with 3 μ M substrate (cyclic AMP or cyclic GMP) is shown in Table 1. Degradation of cyclic AMP (3 μ M) by the immobilized enzyme was activated by 3 μ M-cyclic GMP to a lesser extent than were controls: free enzyme (Table 1) or enzyme mixed with unsubstituted agarose (Fig. 2). In both states, the optimal concentration of cyclic GMP that stimulated cyclic AMP hydrolytic activity was the same: 3 μ M-cyclic GMP at 3 μ M-cyclic AMP (Fig. 2). The kinetic behaviour of cyclic AMP hydrolysis exhibited positive co-operativity (Russell *et al.*, 1973). This feature was maintained, although decreased, when the phosphodiesterase was bound to hexyl-agarose: experiments from three different preparations showed that the Hill coefficient of the cyclic AMP hydrolytic activity always decreased on immobilization. In the experiment shown in Fig. 3(a), the Hill coefficient decreased from 2.17 to 1.33, when the enzyme was bound to hexyl-agarose. At saturating concentrations of cyclic AMP (100 μ M) the activity was somewhat inhibited (in Fig. 3a, activity was 83% of the control), but at micromolar concentrations (up to 10 μ M) no significant change in activity was observed. In the presence of 3 μ M-cyclic GMP, the enzyme always displayed Michaelis-Menten kinetics with respect to cyclic AMP (Russell *et al.*, 1973). Immobilization on hexyl-agarose did not affect this cyclic GMP-dependent shift of the kinetics. However, the apparent K_m increased and V decreased in comparison with the control: values for K_m of 12.5 and 21 μ M and for V of 202 and 186 nmol/min per mg in the free and bound states respectively were calculated from double-reciprocal plots (Fig. 3c).

Cyclic AMP competitively inhibits cyclic GMP hydrolysis by this phosphodiesterase (Russell *et al.*, 1973; Terasaki & Appleman, 1975). At micromolar concentrations of cyclic GMP (substrate), cyclic AMP (0.1 mM) inhibited cyclic GMP hydrolysis to a quite similar extent whatever the enzyme state,

Table 1. *Effect of immobilization on hexyl-agarose of rat liver cyclic GMP-activated phosphodiesterase (peak II')*

The suspension of hexyl-agarose-enzyme was preincubated for 10 min at 30°C in the presence of the assay mixture, including the substrate (3 μ M-cyclic AMP or 3 μ M-cyclic GMP) as described in the Materials and methods section. The enzyme before any addition (control), the enzyme suspension, the pellet and the supernatant collected after centrifugation were tested for phosphodiesterase activity at 3 μ M substrate. Recoveries of enzyme activity in the pellet were respectively 77% and 79% for cyclic AMP (substrate) in basal and cyclic GMP-stimulated assay conditions and 81% for cyclic GMP (substrate). The factor of stimulation by 3 μ M-cyclic GMP is indicated in parentheses, and results are means of quadruplicates.

Enzyme preparation	Addition	Enzyme activity (nmol/min per mg)	
		Cyclic AMP (3 μ M)	Cyclic GMP (3 μ M)
Control	None	1.87	31.03
	Cyclic GMP	35.31 (19)	
Suspension	None	1.82	20.75
	Cyclic GMP	17.95 (9.9)	
Pellet	None	1.35	15.80
	Cyclic GMP	13.21 (9.8)	
Supernatant	None	0.13	1.49
	Cyclic GMP	1.29	

whether mixed with unsubstituted agarose or bound to hexyl-agarose (results not shown). The kinetic parameters of cyclic GMP hydrolysis by this phosphodiesterase were also affected after immobilization: values for K_m of 18 and 25 μ M and for V of 297 and 234 nmol/min per mg in the free and bound states respectively were calculated from double-reciprocal plots (Fig. 3b). Finally, if the ratio of activity on cyclic GMP to that on cyclic AMP (both 3 μ M) always decreased on binding to hexyl-agarose, the enzyme specificity was not affected; cyclic GMP remained the preferred substrate.

Discussion

The ability of cyclic GMP to stimulate cyclic AMP hydrolysis was first reported in rat liver (Beavo *et al.*, 1971). A soluble form of the phosphodiesterases was later identified as being responsible for this control mechanism (Russell *et al.*, 1973). This enzyme shows positively co-operative behaviour with respect to cyclic AMP and cyclic GMP. When its activity is stimulated by cyclic GMP, cyclic AMP hydrolytic activity displays Michaelis-Menten kinetics, and a decrease in the apparent Michaelis constant for cyclic AMP is observed (Moss *et al.*, 1977). We show here that this enzyme behaves as a hydrophobic protein: it binds to hydrophobic matrices with side chains of at least six carbon atoms. Furthermore, we found that in our assay conditions in the presence of hexyl-agarose the enzyme is irreversibly immobilized. A pronounced decrease of cyclic GMP-dependent activation is observed on immobilization. This is not due to any change in cyclic AMP hydrolytic activity measured

in the absence of cyclic GMP, but rather to a fall in activity determined in its presence.

Hofstee (1973) reported that the binding of a number of proteins to agarose substituted with n-alkylamines did not involve the active centre. In our experiments, enzymic activity remains measurable after immobilization, although it was decreased at any concentration of cyclic GMP and at saturating concentrations of cyclic AMP. However, since activity can be detected, and since a competitive inhibitor of cyclic GMP hydrolysis, cyclic AMP, inhibits equally the soluble and immobilized enzyme, the hydrophobic binding site does not appear to involve the catalytic site. Similar results were obtained with the rat lung soluble guanylate cyclase, where MnATP appeared to be a competitive inhibitor with respect to MnGTP of either form of the enzyme (Garbers, 1978). Several arguments support the hypothesis that the hydrophobic site does not involve the cyclic GMP-activating site, either. First, in the immobilized state, the cyclic GMP-activating site is still accessible as in the free enzyme, since the optimum concentration of cyclic GMP for stimulation is identical in both states. Secondly, immobilization does not appear to prevent cyclic GMP shifting the positively co-operative behaviour with respect to cyclic AMP to Michaelis-Menten kinetics. Since in our assay conditions the immobilization is irreversible, the interaction site between hexyl-agarose and the phosphodiesterase can be distinguished from the cyclic GMP-activating one.

In the free state, theophylline and several purines are much more effective inhibitors of cyclic GMP-stimulated cyclic AMP phosphodiesterase than of

basal enzymic activity. Under such conditions, the factor of cyclic GMP-dependent stimulation rapidly falls (Erneux *et al.*, 1977; Filburn *et al.*, 1978; Oleshansky, 1980). The mechanism by which theophylline inhibits this enzyme is not firmly elucidated, but could involve competitive inhibition of the xanthine at the catalytic site (C. Erneux, unpublished work). This proposal is by no means general, and the present data on hexyl-agarose show that the marked decrease in activity on immobilization (Fig. 2) can be achieved without any direct interaction with the hydrolytic or activating sites. It would be interesting to see if a similar mechanism exists for other hydrophobic substances, proteins or inhibitors.

Fig. 2. Effect of increasing concentrations of cyclic GMP on the hydrolysis of cyclic AMP by the cyclic GMP-activated phosphodiesterase

The enzyme was prepared after the gel-filtration step on Sephacryl S-200 (peak II'). It was immobilized, as described in the Materials and methods section, on hexyl-agarose (O) or unsubstituted agarose (●); cyclic AMP concentration was $3 \mu\text{M}$. Results are expressed as the activation in the presence of cyclic GMP as a percentage of that in its absence.

Fig. 3. Kinetic behaviour of the cyclic GMP-activated phosphodiesterase

The enzyme prepared after gel filtration on Sephacryl S-200 (peak II') was mixed with unsubstituted agarose (●) or immobilized on hexyl-agarose (O). Substrate concentrations ranged from 3 to $100 \mu\text{M}$. (a) Cyclic AMP alone; (b) cyclic GMP alone; (c) cyclic AMP in the presence of or stimulated by $3 \mu\text{M}$ -cyclic GMP. The velocity v is in nmol of cyclic AMP (or cyclic GMP) hydrolysed/min per mg. Results are representative of experiments with three different preparations.

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References

- Beavo, J. A., Hardman, J. G. & Sutherland, E. W. (1971) *J. Biol. Chem.* **246**, 3841–3846
- Erneux, C., Van Sande, J., Dumont, J. E. & Boeynaems, J. M. (1977) *Eur. J. Biochem.* **72**, 137–147
- Erneux, C., Couchie, D. & Dumont, J. E. (1980) *Eur. J. Biochem.* **104**, 297–304
- Filburn, C. R., Colpo, F. & Sacktor, B. (1978) *J. Neurochem.* **30**, 337–346
- Garbers, D. L. (1978) *J. Cyclic Nucleotide Res.* **4**, 271–279
- Hardman, J. G. & Sutherland, E. W. (1969) *J. Biol. Chem.* **244**, 6363–6370
- Hjerten, S. (1973) *J. Chromatogr.* **87**, 325–331
- Hofstee, B. H. J. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1137–1144
- Moss, J., Manganiello, V. C. & Vaughan, M. (1977) *J. Biol. Chem.* **252**, 5211–5215
- Oleshansky, M. A. (1980) *Life Sci.* **27**, 1089–1095
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Russell, T. R., Terasaki, W. L. & Appleman, M. M. (1973) *J. Biol. Chem.* **248**, 1334–1340
- Terasaki, W. L. & Appleman, M. M. (1975) *Metab. Clin. Exp.* **24**, 311–319
- Wells, J. N. & Hardman, J. G. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 119–143
- Wells, J. N., Baird, C. E., Wu, Y. J. & Hardman, J. G. (1975) *Biochem. Biophys. Acta* **384**, 430–442