Purification and kinetic properties of two soluble forms of calmodulin-dependent cyclic nucleotide phosphodiesterase from rat pancreas

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The calmodulin-dependent cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase (EC 3.1.4.17) activity of rat pancreas was purified 280-fold by affinity chromatography on calmodulin–Sephadex 4B. It then accounted for 15% of the total cytosol cyclic GMP nucleotide phosphodiesterase activity, in the presence of Ca^{2+} , and represented a minor component of proteins specifically adsorbed by the column. This activity was resolved on a DEAE-Sephacel column into two fractions, termed P_I and P_{II} , on the basis of their order of emergence. After this step, P_I and P_{II} were purified 5650- and 3700-fold respectively. The molecular weight of P_I was 175 000 and that of P_{II} was 116 000, by polyacrylamide-gradient-gel electrophoresis. Both forms of phosphodiesterase could hydrolyse cyclic AMP and cyclic GMP, although P_{II} displayed a higher affinity toward cyclic GMP than toward cyclic AMP. P_I and P_{II} exhibited negative homotropic kinetics in the absence of calmodulin. Upon addition of calmodulin, both enzymes displayed Michaelis–Menten kinetics and a 5–9-fold increase in maximal velocity, at physiological concentrations of cyclic GMP and cyclic AMP. When a pancreatic extract freshly purified by affinity chromatography was immediately analysed by high-performance gel-permeation chromatography on a TSK gel G3000 SW column, P_{II} represented as much as 78% of the eluted activity. This percentage decreased to 52% when the sample was stored at 0°C for 20 h before analysis, suggesting that P_{II} , possibly predominant *in vivo*, was converted into the heavier P_I form upon storage.

Cyclic nucleotides and Ca^{2+} have been implicated as secondary messengers in pancreatic exocrine secretion. Cyclic nucleotide phosphodiesterases (EC 3.1.4.17) could play an important role in regulating the extent and duration of cyclic AMP and cyclic GMP action in this gland (for review, see Schulz & Stolze, 1980; Christophe *et al.*, 1981). In a preliminary study on a rat pancreas crude homogenate, Ca^{2+} was not found to stimulate the hydrolysis of cyclic nucleotides (Rutten *et al.*, 1973), at variance with the situation prevailing in rat brain extracts (Kakiuchi & Yamazaki, 1970; Lin *et al.*, 1974). Subsequently, three forms of cyclic nucleotide phosphodiesterase were partially purified from the rat pancreatic cytosol by DEAE-cellulose chromatography and Biogel A 0.5 m filtration, but Ca^{2+} effects were not tested or considered as negligible (Terai *et al.*, 1976). In 1977, we succeeded in isolating one peak of phosphodiesterase activity that was stimulated 3–5-fold by purified calmodulin

in the presence of Ca^{2+} by submitting the soluble fraction of rat pancreas homogenate to gel filtration on Sephadex G-200 (Vandermeers *et al.*, 1977). The present investigation describes the partial purification and kinetic characteristics of two forms of Ca^{2+} –calmodulin-dependent cyclic nucleotide phosphodiesterase in rat pancreatic cytosol.

Materials and methods

Materials

Cyclic [8- ^3H] AMP (sp. radioactivity 20 Ci/mmol) and cyclic [8- ^3H] GMP (sp. radioactivity 17.2 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and purified by using the same Dowex AG 1 (X2) columns as those used for the phosphodiesterase assay. *Crotalus atrox* 5'-nucleotidase (Sigma grade IV) was purified 40-fold by gel filtration on Sephadex G-75. Calmodulin from bovine pancreas was

prepared as described previously (Vandermeers *et al.*, 1977). The calmodulin–Sephadex 4B affinity gel used for the purification of phosphodiesterase was prepared by the method of Kümel *et al.* (1979) starting from 20 g of Sepharose slurry and 20 mg of calmodulin.

The following materials were purchased as indicated: Sephadex G-75, Sepharose 4B, DEAE-Sephacel (Pharmacia, Uppsala, Sweden); Ultropac TSK-G 3000 SW prepacked column (0.75 cm × 60 cm) (LKB Produkter AB, Bromma, Sweden); dithiothreitol (Sigma, St. Louis, MO, U.S.A.); Dowex AG 1 (X2) and all reagents and stain used for electrophoresis (Serva, Heidelberg, Germany). All other chemicals of analytical grade were supplied by Merck (Darmstadt, Germany) or by Serva.

Purification of calmodulin-binding proteins by affinity chromatography

Male Wistar adult rats (150–200 g) fed *ad libitum* on standard chow were used. Fresh rat pancreas (20 g) was homogenized at 4°C in 100 ml of buffer A (50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl, 1 mM-MgCl₂, 0.1 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl fluoride) supplemented with 2 mM-EDTA and centrifuged thereafter at 105 000 g for 90 min. About 75 ml of clear supernatant was immediately applied to a Sephadex G-75 column (110 cm × 6.5 cm) equilibrated with buffer A enriched with 0.1 mM-EGTA. Fractions (9.5 ml) were collected at a rate of 120 ml/h. The peak eluting with the void volume was adjusted to 0.2 mM-CaCl₂ and applied to a calmodulin–Sephadex 4B column (15 cm × 1 cm) equilibrated with buffer A supplemented with 0.1 mM-CaCl₂. The column was washed with the equilibrating buffer until no further protein was eluted. The calmodulin-binding proteins were eluted, thereafter, with buffer A enriched with 0.1 mM-EGTA in place of the CaCl₂. The pooled fractions were dialysed against buffer A devoid of NaCl and stored at –80°C before further purification.

Assay of phosphodiesterase activity

Enzyme activity was measured by a modification of the radiochemical procedure of Thompson & Appleman (1971) as described previously (Vandermeers *et al.*, 1977) except that (1) a one-step coupled enzymic assay including purified 5'-nucleotidase (EC 3.1.3.5) was used instead of the classical two-stage method, (2) the reaction was terminated by addition of 50 µl of 1 M-HCl followed by 30 µl of 2 M-Tris to readjust the pH to neutrality before purifying the reaction product by chromatography on the anion-exchange resin Dowex AG 1 (X2).

The standard assay mixture contained, in a final volume of 0.4 ml, 0.4–20.4 µM-cyclic AMP or -cyclic

GMP (400 000 c.p.m. of ³H-labelled material), 40 mM-Tris/HCl (pH 7.5), 3 mM-MgCl₂, 0.3 mM-dithiothreitol, 0.1 mM-MnCl₂, 0.2 mM-EGTA or 50 µM-CaCl₂ and 0.2 µM-calmodulin, an appropriate amount of enzyme preparation and 10 units of purified 5'-nucleotidase (1 unit hydrolysed 1 nmol of 5'-AMP per min at 30°C under the conditions of the phosphodiesterase assay). Conversion of cyclic nucleotides into products was not allowed to exceed 20% over the incubation period time (10–90 min at 30°C) and was linear with time under these conditions.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Molecular-weight determination by using polyacrylamide-gradient-gel electrophoresis

Cyclic nucleotide phosphodiesterases were analysed by polyacrylamide-gradient-gel electrophoresis. Linear gradient gels (4.5–30% acrylamide; bisacrylamide/acrylamide, 2.66:100) were cast by the method of Anderson *et al.* (1972) in 8 cm × 0.5 cm tubes using a home-made casting apparatus (roughly a 10 cm × 3 cm column, allowing 14 tubes to be filled upward simultaneously under standard conditions). Gradient-gel electrophoresis was performed in 0.09 M-Tris/0.08 M-boric acid (pH 8.4) at 150 V for 15 h at 4°C. The electrophoretic gels were either stained for protein with Coomassie Blue or sliced into about 1.7 mm segments (41 slabs for a 7 cm gel length). Each gel segment (about 30 mg) was suspended with 170 µl of buffer A (50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl, 1 mM-MgCl₂, 0.1 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl fluoride) with 50 µM-CaCl₂ and bovine serum albumin (0.1 mg/ml). After 4 h at 0°C, the whole medium was assayed for phosphodiesterase activity in the presence of calmodulin. Shrinkage of the gel occurring during staining was corrected when estimating the electrophoretic mobility of standard proteins.

The molecular-weight markers were thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), phosphorylase *b* (94 000) and bovine serum albumin (67 000) (Pharmacia calibration kits).

Results

Purification of calmodulin-dependent cyclic nucleotide phosphodiesterase

Table 1 summarizes the data for a typical preparation of the enzyme extracted from 20 g of pancreas.

Gel filtration of the pancreas supernatant on Sephadex G-75 in the presence of EGTA was aimed

Table 1. *Summary of purification of calmodulin-dependent cyclic nucleotide phosphodiesterase from rat pancreas*
 Enzyme activity was measured in the presence of saturating concentration of calmodulin and Ca^{2+} with $0.4 \mu\text{M}$ -cyclic GMP. One unit is the amount of protein hydrolysing 1 nmol of cyclic nucleotide per min at 30°C . Values marked with * represent 15% of the measured activities, since the total phosphodiesterase activity eluted from the calmodulin-Sephacel column was made of 85% of calmodulin-independent and 15% of calmodulin-dependent activity.

Steps	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Crude extract (from 20 g of tissue)	5.9*	0.006*	100	1
Sephadex G-75 gel filtration	5.2*	0.024*	88	4
Calmodulin-Sephacel affinity chromatography	3.3	1.68	56	280
DEAE-Sephacel chromatography				
P_I	0.49	17.3	8.2†	2883†
P_II	0.47	10.9	7.9†	1817†

† Assuming that P_I and P_II accounted for 51 and 49% respectively of the calmodulin-dependent activity in the crude extract as in the DEAE-Sephacel eluate, the actual purification was 5653-fold for P_I and 3708-fold for P_II .

at eliminating calmodulin as well as proteolytic proenzymes such as trypsinogens. Including EGTA in the buffer during the initial steps served to dissociate calmodulin from its target proteins and prevented Ca^{2+} -dependent proteolysis.

The most efficient purification step was the calmodulin-Sephacel 4B column chromatography, which achieved a 70-fold purification, when one considers that only 15% of the total activity applied to the column was contributed by calmodulin-sensitive phosphodiesterase. At that stage, the actual purification factor was 280-fold.

The material purified by affinity chromatography was further fractionated by DEAE-Sephacel chromatography. Two peaks of calmodulin-dependent phosphodiesterase activity, termed P_I and P_II , were eluted at NaCl concentrations of around 80 and 175 mM respectively (Fig. 1). The fractions exhibiting the highest stimulatory factor in the presence of calmodulin (≥ 4 for P_I and ≥ 3 for P_II) were pooled for further analysis (i.e. 0.49 unit under peak P_I and 0.47 unit under peak P_II). The total calmodulin-dependent phosphodiesterase activity recovered was 1.82 nmol of cyclic GMP hydrolysed/min. This activity, which represented 15% of the total phosphodiesterase activity in the crude soluble extract (see the results of the affinity chromatography), was now subdivided into two fractions containing almost the same total enzymic activity (51% for P_I compared with 49% for P_II), which were purified 5650- and 3700-fold respectively, with an overall yield of 16%.

Physical properties of the two calmodulin-dependent phosphodiesterases

The molecular weight of P_I and P_II phosphodiesterases eluted from DEAE-Sephacel was estimated by gradient-polyacrylamide-gel electrophoresis by reference to a standard curve obtained with known proteins (Fig. 2). With phosphodi-

Fig. 1. *DEAE-Sephacel chromatography of calmodulin-dependent phosphodiesterases from rat pancreas*
 The column ($10 \text{ cm} \times 0.7 \text{ cm}$) was pre-equilibrated with buffer A without NaCl and gradient elution was carried out with 200 ml of buffer A enriched with a linear 10–350 mM-NaCl gradient (—). Fractions (1.8 ml) were assayed for phosphodiesterase activity as described in the Materials and methods with $0.4 \mu\text{M}$ -cyclic GMP in the presence of $50 \mu\text{M}$ - CaCl_2 and $0.2 \mu\text{M}$ -calmodulin (●) or of $50 \mu\text{M}$ - CaCl_2 alone (○). The fractions under each bar were pooled separately.

esterase P_I , 74% of the enzymic activity migrated to a position corresponding to a mol.wt. of 175 000 and 26% was distributed between the origin and the main band. Phosphodiesterase P_II exhibited a single band of activity corresponding to a mol.wt. of 116 000.

The molecular weights of P_I and P_II were also estimated by high-performance gel permeation on a column of TSK Gel G3000 SW calibrated with known standard proteins. The apparent mol.wt. was 200 000 for P_I and 135 000 for P_II .

This gel-permeation chromatography, when directly performed on the total extract obtained by

Fig. 2. Polyacrylamide-gradient-gel disc electrophoresis of phosphodiesterases P_I and P_{II} eluted from DEAE-Sephacel

Electrophoresis, staining and phosphodiesterase assay were carried out as described in the Materials and methods section. The location of P_I and P_{II} phosphodiesterases on the log (molecular weight) curve is indicated by the arrows. Abbreviation used: LDH, lactate dehydrogenase.

affinity chromatography, revealed, in addition, that the lower-mol.wt. P_{II} form could undergo partial conversion into the higher-mol.wt. P_I form on storage *in vitro* at 0°C, since (1) when immediately analysed (Fig. 3a), 78% of the phosphodiesterase activity was eluted with a mol.wt. of 135 000 and 22% with a mol.wt. of 200 000 and (2) when the same analysis was performed on a preparation stored over ice for 20 h (Fig. 3b), 52% of the activity still emerged with a mol.wt. of 135 000, but the mol.wt.-200 000 component then represented as much as 48% of the collected activity. The latter distribution was similar to that recorded by DEAE-Sephacel chromatography.

Catalytic properties of the two calmodulin-dependent phosphodiesterases

The specific activities of the purified enzymes were 17.3 and 10.9 nmol of cyclic GMP hydrolysed/mg of protein for P_I and P_{II} respectively (Table 1). Both enzymes hydrolysed cyclic GMP and cyclic AMP at similar rates (Fig. 4, Table 2). In the presence of EGTA or of Ca^{2+} alone, Lineweaver-Burk plots were not linear (Fig. 4); apparent K_m values were 4–15-fold lower at low cyclic nucleotide concentration (0.4–1.4 μM) than at a substrate concentration above 4 μM (Table 2). When calmodulin and Ca^{2+} were added, both enzymes followed Michaelis-Menten kinetics (Fig. 4). At low substrate concentration, a moderate decrease in apparent K_m values (2.9- and 4.3-fold with cyclic AMP and cyclic

Fig. 3. TSK-G 3000 SW high-performance gel-permeation chromatography of calmodulin-dependent phosphodiesterases

The material freshly purified by affinity chromatography from 13.2 g of rat pancreas was divided into two parts. One part was analysed immediately (a) and the second part after storage for 20 h at 0°C (b). The column (0.75 cm × 60 cm) was equilibrated and eluted with buffer A. The flow rate was 3 ml/h and the fraction size was 0.32 ml. Phosphodiesterase activity was assayed as described in the Materials and methods section with 0.4 μM -cyclic GMP in the presence of 50 μM - CaCl_2 and 0.2 μM -calmodulin.

GMP respectively) resulted from the interaction of P_I with calmodulin, but the K_m values for P_{II} were hardly affected. An increased maximal velocity of both P_I and P_{II} was also observed: 8.6-fold for cyclic AMP and 7.4-fold for cyclic GMP with P_I , the corresponding stimulatory factors being 5.4- and 4.6-fold respectively for P_{II} .

Fig. 4. Lineweaver–Burk double-reciprocal plots of cyclic nucleotide hydrolysis by P_I (a and b) or P_{II} (c and d) phosphodiesterases in the presence (●) or absence (○) of calmodulin. Each point represents the mean of three determinations. The enzyme was assayed as described in the Materials and methods section. The velocity was expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ and the substrate concentration in μM . K_m and V_{max} were calculated by linear regression analysis.

Table 2. Kinetic parameters of cyclic nucleotide phosphodiesterases from rat pancreas

The kinetic constants were calculated by linear regression analysis of the data in Fig. 4 (means of three experiments $\pm$ S.E.M.).

Substrate	Calmodulin	Substrate concentration (μM)	P_I		P_{II}	
			K_m (μM)	V_{max} (nmol/min per mg)	K_m (μM)	V_{max} (nmol/min per mg)
Cyclic AMP	—	0.4–1.4	1.00 ± 0.06	3.09 ± 0.18	5.34 ± 0.69	3.13 ± 0.39
	—	4.0–20.4	4.23 ± 1.07	5.67 ± 0.46	24.97 ± 3.43	9.61 ± 1.18
	+	0.4–20.4	0.35 ± 0.14	26.53 ± 0.77	8.99 ± 0.32	16.86 ± 0.42
Cyclic GMP	—	0.4–1.4	0.69 ± 0.08	2.59 ± 0.17	1.52 ± 0.03	3.36 ± 0.05
	—	4.0–20.4	10.39 ± 0.64	13.54 ± 0.53	8.02 ± 0.75	6.56 ± 0.32
	+	0.4–20.4	0.16 ± 0.10	19.17 ± 0.40	1.73 ± 0.18	15.42 ± 0.30

Discussion

Several mammalian tissues contain multiple forms of cyclic nucleotide phosphodiesterase that can be distinguished by their kinetic properties. One form is a cytosolic enzyme capable of hydrolysing both cyclic nucleotides. This form, generally referred to as

the ‘high- K_m ’ enzyme, is sensitive to calmodulin (Wells & Hardman, 1977), has a greater maximal velocity but also a higher apparent K_m for cyclic AMP than for cyclic GMP. The isolation of calmodulin-sensitive phosphodiesterase(s) has greatly benefited from calmodulin–Sephadex

affinity chromatography (Watterson & Vanaman, 1976; Miyake *et al.*, 1977), which allowed a 3000-fold purification from bovine brain (Morill *et al.*, 1979; Sharma *et al.*, 1980) and a more than 20000-fold purification from bovine heart (Laporte *et al.*, 1979), leading each time to a homogeneous preparation.

In the present work, the two calmodulin-dependent phosphodiesterases were purified 4000–5000-fold from the rat pancreas. $V_{\max}$ values of 15–25 nmol/min per mg of protein were obtained for P_I and P_{II} with both cyclic nucleotides, in the presence of calmodulin (Table 2). Even after complete purification, such specific activities will probably never approach values as high as 150–300 μ mol/min per mg of protein reported for the brain (Morill *et al.*, 1979; Sharma *et al.*, 1980) and heart (Laporte *et al.*, 1979) enzymes. Our values are more comparable with that of 95 nmol/min per mg of protein found for pure calmodulin-independent phosphodiesterase from dog kidney (Thompson *et al.*, 1979).

As previously reported for other calmodulin-dependent phosphodiesterases (Donnelly, 1977; Klee *et al.*, 1979), P_I and P_{II} exhibited negative co-operativity with both cyclic nucleotides, in the absence of calmodulin; the apparent K_m and $V_{\max}$ values increased at supramicromolar substrate concentrations of cyclic AMP and cyclic GMP (Fig. 4, Table 2). Calmodulin suppressed negative co-operativity in P_I and P_{II} at high supraphysiological concentrations of cyclic nucleotides. There is no ready explanation for this observation, but a 'second site' for cyclic nucleotides, present on the same protomer or on another protomer, may not be operative in the oligomer under these conditions (Klee *et al.*, 1979).

If we ignore the high- K_m component, as it does not operate at physiological concentrations of cyclic nucleotides, the following observations can be made (Table 2). (1) K_m values were only 1.5–5.2-fold higher for cyclic AMP than for cyclic GMP in the submicromolar range, at variance with brain and heart calmodulin-dependent phosphodiesterases, whose K_m for cyclic AMP is 20–30 times higher than that for cyclic GMP (Wang & Waisman, 1980). (2) The apparent K_m values of P_I were 2–5-fold lower in the absence of calmodulin and 10–25-fold lower in the presence of calmodulin than the corresponding values for P_{II} . The $V_{\max}$ values were all very similar. (3) $V_{\max}$ values were increased 5–9-fold by calmodulin, in line with previous data on calmodulin-sensitive phosphodiesterases (Laporte *et al.*, 1979; Morill *et al.*, 1979; Sharma *et al.*, 1980). These $V_{\max}$ values for both substrates were, however, quasi-identical, in contrast with $V_{\max}$ values of brain and heart calmodulin-dependent phosphodiesterases, which are higher for cyclic AMP than for

cyclic GMP (Wang & Waisman, 1980). (4) Basal values for K_m and $V_{\max}$ were identical in P_I and P_{II} , when measured in the presence of EGTA or in the presence of Ca^{2+} alone (without added calmodulin; results not shown).

As revealed by polyacrylamide-gradient-gel electrophoresis, P_I appeared to be partially aggregated (see the Results section), whereas P_{II} exhibited a single band of phosphodiesterase activity. The mol.wt. of 116 000 found for P_{II} (135 000 by HPGPC) agrees with similar values reported for bovine brain (115 800) and heart (114 000) enzymes (Laporte *et al.*, 1978; Sharma *et al.*, 1980). Therefore, it is possible that P_{II} consists in two subunits of mol.wt. 58 000, like heart and brain calmodulin-dependent phosphodiesterases. The mol.wt. of 175 000 (200 000 by high-performance gel-permeation chromatography) found by polyacrylamide-gradient-gel electrophoresis for the 'non-aggregated fraction' of P_I phosphodiesterase could theoretically correspond to a ternary or quaternary complex of a 58 000-mol.wt. subunit. The molecular weight of P_I was too high to be identified with an A_2C_2 complex consisting of two catalytic subunits and two calmodulin molecules (Laporte *et al.*, 1979; Sharma *et al.*, 1980). In addition, the fact that P_I was insensitive to Ca^{2+} alone precludes the presence of endogenous calmodulin.

The P_I and P_{II} components were always present among the calmodulin-dependent proteins eluted from a calmodulin-Sepharose column. P_I never represented more than 20% of total calmodulin-dependent phosphodiesterase activity when care was taken to avoid aggregation phenomena that might occur during storage and/or changes in ionic strength (Fig. 3a). This goal was partly achieved by working (1) rapidly and (2) with the same buffer containing 0.15 M-NaCl, from the initial homogenate to the final gel-permeation analysis on the TSK-G3000 SW column. This low proportion of P_I was increased after storage at 0°C for 20 h (Fig. 3b) or by freezing the sample before analysis on the TSK-G3000 SW column. In line with these observations, the proportion of P_I attained as much as 50% of the calmodulin-dependent phosphodiesterase activity, when the sample was fractionated on a DEAE-Sephacel column, after the partial desalting to 50 mM-NaCl required for chromatography (Fig. 1, Table 1). The presence of a 58 000-mol.wt. component was, however, never observed in our chromatographic eluates, in contrast with the data of Kincaid *et al.* (1981) on bovine brain enzyme. Our results suggest that P_{II} , with a mol.wt. of 116 000 by electrophoresis and 135 000 by gel permeation, might be the predominant species *in vivo*.

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