Purification of an enzyme aggregate containing 3′,5′-cyclic-nucleotide phosphodiesterase and nucleotidase

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Several steps of purification (octyl-Sepharose chromatography, Blue Sepharose 6B chromatography and sucrose density gradient centrifugation) led to a highly purified aggregate of the enzymes, 3′,5′-cyclic-nucleotide phosphodiesterase (PDE) and nucleotidase. The purified enzyme aggregate showed an *S* value of 7.3 (SE ± 0.3, $n = 10$). Further analysis by SDS-polyacrylamide gel electrophoresis (PAGE) revealed two proteins near 67 and 60 kDa. Dissociation of the 7.3 S enzyme aggregate showed a 3.6 S PDE form and a nucleotidase form at 4.2 S. Additionally, higher *S* value forms of the nucleotidase up to 17 S have been observed. Apparently, they had formed by self-association. SDS-PAGE of the 17 S nucleotidase form showed only one band at 67 kDa. This was taken as evidence for the homogeneity of the 17 S nucleotidase form and the self-association of the nucleotidase after dissociation from the 7.3 S enzyme aggregate. Furthermore, from this it could be concluded that the 67 kDa protein of the 7.3 S enzyme aggregate should be identified with the nucleotidase, and thus the 60 kDa band represents the PDE.

3′,5′-Cyclic-nucleotide phosphodiesterase *Nucleotidase* *Enzyme cluster* *Cyclic AMP metabolism*

1. INTRODUCTION

Cyclic AMP is known as an intracellular mediator for various hormones [1]. The inactivation and degradation of cyclic AMP to the nucleotide 5′-AMP is catalysed by 3′,5′-cyclic-nucleotide phosphodiesterase (PDE).

Very efficient degradation of cAMP via 5′-AMP and adenosine to the nucleoside inosine has been observed in the microsomal fraction of bovine adrenal cortex. The enzymes which catalyse the sequential steps (cAMP ⟶ 5′-AMP ⟶ adenosine ⟶ inosine) are in close physical proximity, as demonstrated, and form a multienzyme sequence [2]. Additionally, it was shown that in the soluble fraction of the cell homogenate, cAMP was degraded in a similar way [3,4]. The existence of the enzyme aggregates was mainly demonstrated by kinetic experiments.

Here, a soluble enzyme cluster from bovine adrenal cortex containing PDE associated with nucleotidase was purified using octyl-Sepharose chromatography, Blue Sepharose chromatography and sucrose density gradient centrifugation. The obtained soluble enzyme complex was resubmitted to sucrose density gradient centrifugation and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2. MATERIALS AND METHODS

2.1. *Isolation of the 7.3 S soluble enzyme aggregate*

The 135000 × *g* supernatant (57.6 mg/ml) from homogenized bovine adrenal cortex, prepared as described [2], was chromatographed on octyl-Sepharose [3]. The PDE activity, which eluted firstly with decreasing ammonium sulfate concentration (peak 1), was pooled, dialysed against 10 mM Pipes (pH 6.3), 5 mM NaCl and concentrated (4–5 mg/ml). This protein (1 ml) was loaded on a Blue Sepharose 6B column (1 × 3.8 cm). Chromatography was performed according to Morill et al. [5] with the modification that the elution buffer was 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM cAMP and 0.2 mM cGMP.

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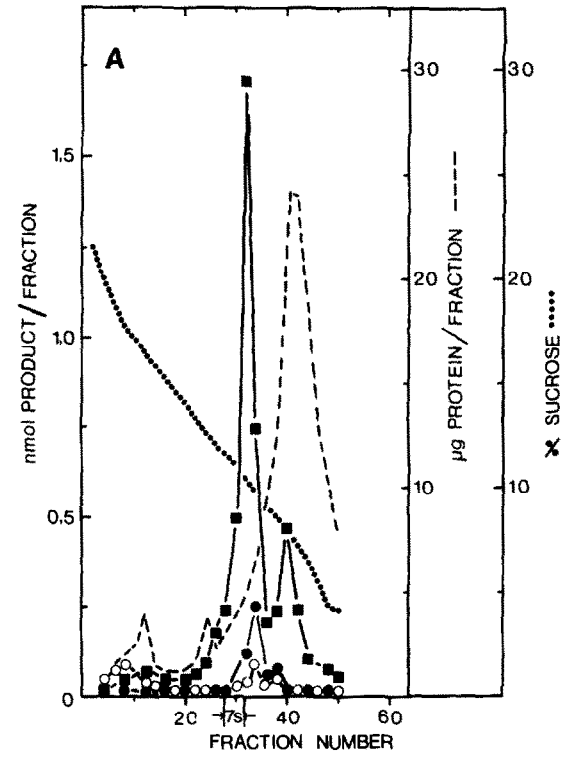

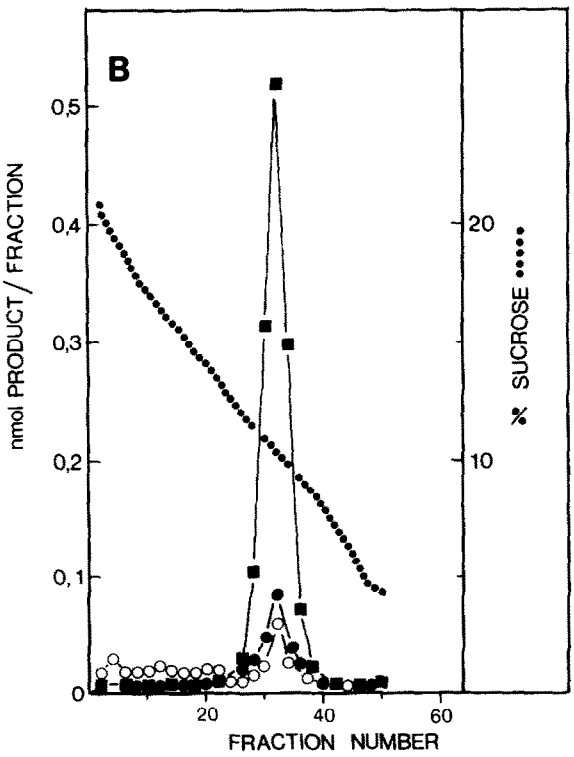

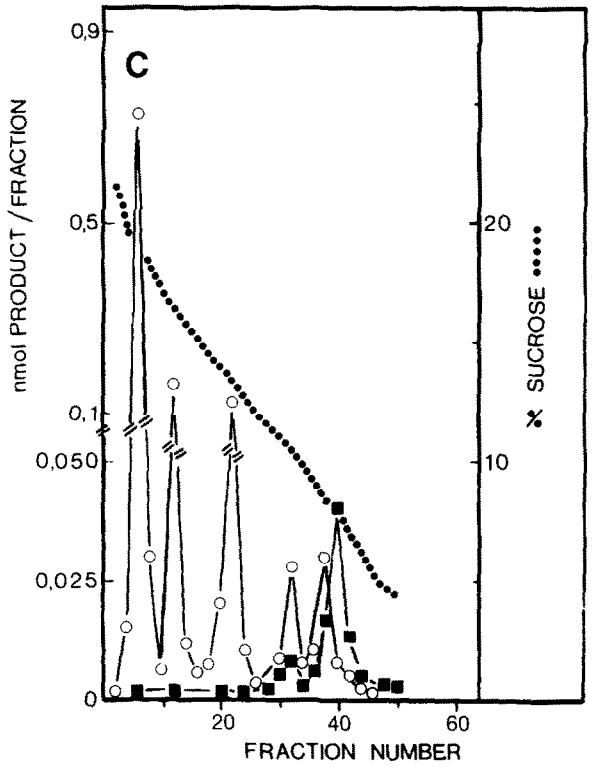

Fig.1. (A) Sucrose density gradient centrifugation of the soluble protein material obtained after octyl-Sepharose chromatography and subsequent Blue Sepharose chromatography. Protein: 200 μl (250 μg) of the concentrated eluates from Blue Sepharose chromatography. Enzyme activities were detected in aliquots of the fractions using 2.8 μM cAMP and 2.8 μM 5′-AMP as substrates. (■) PDE activity (sum of the products derived from cAMP). (●) Adenosine formation derived from cAMP via 5′-AMP. (○) Nucleotidase activity (adenosine formation from 5′-AMP). (– – –) Protein elution curve. (B) Sedimentation velocity profile of the soluble 7.3 S enzyme complex. Protein: 200 μl (20 μg) of the concentrated fractions 28–32 from the sucrose density gradient centrifugation (panel A). Centrifugation was performed after dialysis (four times for 1 h against 50 mM Tris-HCl, pH 7.9) and concentration of the 7.3 S pool. (C) Sedimentation velocity profile of the soluble 7.3 S enzyme complex after dilution. The concentrated 7.3 S pool (panel A) was diluted with 50 mM Tris-HCl (pH 7.9) to a protein concentration of 25 μg/ml and stored at 4°C for 24 h. The protein material was then concentrated and 200 μl (20 μg protein) were layered on the gradient.

The fractions which contained the copurifying PDE and nucleotidase were pooled, dialysed against 50 mM Tris-HCl (pH 7.9) and concentrated (1050–1400 μg/ml). 200 μl (210–280 μg protein) were subjected to sucrose density gradient centrifugation at 38000 rpm for 18 h (4°C) in a Beckman SW 40 rotor. The sucrose gradient (5–20%, w/w) contained 50 mM Tris-HCl (pH 7.9). Elution was performed from the bottom of the tube, and 252 μl fractions were collected. The 7.3 S peak was pooled, dialysed four times for 1 h against 50 mM Tris-HCl (pH 7.9) and concentrated in an ultrafiltration apparatus (Amicon/filter YM5) to a protein concentration of 100–125 μg/ml.

2.2. *SDS-PAGE*

SDS-PAGE was performed according to Studier [6] at pH 8.8 using a 15% polyacrylamide, 0.1% SDS resolving gel. Proteins were revealed by silver staining [7].

2.3. *Enzyme activity determination*

PDE, nucleotidase and deaminase were assayed as described in [2,8]. The radiolabelled compounds were obtained from the Radiochemical Centre, Amersham.

3. RESULTS

After octyl-Sepharose chromatography, as described above, the main parts of PDE and copurified nucleotidase were adsorbed to the Blue Sepharose matrix and then co-eluted by the special elution buffer, as mentioned in section 2. At this step, deaminase, part of the nucleotidase and a small amount of the PDE were separated by washing the column.

The PDE and nucleotidase activity which was desorbed by the special elution buffer revealed two PDE peaks after centrifugation on a linear sucrose density gradient. The sedimentation coefficients were determined to 7.3 S (SE ± 0.3, n = 10) and 3.6 S (SE ± 0.3, n = 10) according to Martin and Ames [9]. These S values correspond to 125–145 and 51–62 kDa, respectively (fig.1A). The 7.3 S peak contained the major part of the PDE and copurified nucleotidase activity. It shows a clear kinetic advantage effect concerning adenosine formation from cAMP. The 3.6 S peak exhibited only a small part of the PDE activity and very little nucleotidase activity at 4.2 S.

The 7.3 S PDE showed a biphasic velocity-substrate curve. Assuming a low- and high-K_m enzyme, as discussed earlier [10], the low-K_m value was 0.3 μM and the high-K_m value 60 μM (spec. act. 75 pmol/mg per min and 15 nmol/mg per min, respectively; substrate, cyclic AMP). The copurified nucleotidase showed ordinary Michaelis-Menten kinetics and a K_m value of 2.2 μM (spec. act. 7.1 nmol/mg per min; substrate, 5′-AMP). The 3.6 S PDE form showed only the low-K_m PDE activity.

When the 7.3 S peak was resubmitted to sucrose density gradient centrifugation immediately after the first run, PDE and the major part of the copurified nucleotidase sedimented at 7.3 S (fig.1B). This peak showed two bands at 67 and 60 kDa when subjected to SDS-PAGE (fig.2). If, however, the 7.3 S peak was diluted, stored at 4°C for 24 h and then centrifugated, the elution pattern showed most of the PDE activity at 3.6 S. The

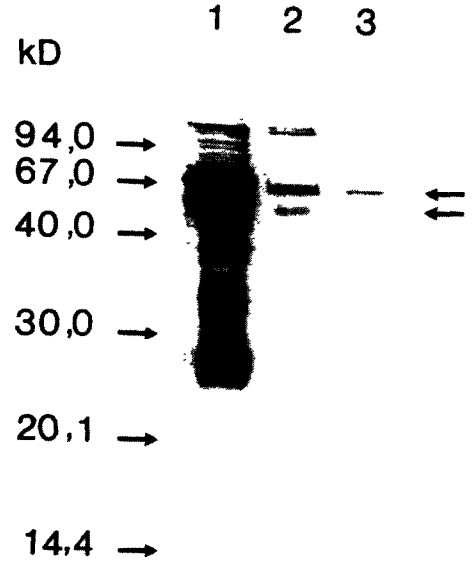

Fig.2. SDS-PAGE. Lanes: 1, peak 1 of the octyl-Sepharose chromatography; 2, 7.3 S form after recentrifugation (fig.1B); 3, 17 S nucleotidase peak (fig.1C). Standard proteins used were: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

nucleotidase was found at 4.2 S and additionally at the high S values: 7.3, 11.5, 14.4 and 17 S (SE ± 0.3, n = 5) (fig.1C).

Dissociation of the 7.3 S enzyme complex was accompanied by a considerable decrease in PDE activity. This indicates the instability of the 3.6 S PDE form after dissociation from the 7.3 S enzyme aggregate and, vice versa, the stabilizing effect of the 7.3 S enzyme aggregate. With respect to the dissociated nucleotidase form at 4.2 S, self-aggregation to higher molecular mass forms apparently occurred. This was accompanied by a great increase in nucleotidase activity. SDS-PAGE of the 17 S nucleotidase form showed only one band at 67 kDa which demonstrated the homogeneity of this aggregate.

4. DISCUSSION

The striking copurification of PDE and nucleotidase during different steps of purification was due to a specific association of both enzymes, as demonstrated by the highly purified 7.3 S enzyme complex. An analysis of this complex by SDS-PAGE showed two bands at 60 and 67 kDa (fig.2).

After dissociation of the enzyme complex, different nucleotidase forms arose. An SDS-PAGE analysis of the homogeneous 17 S form showed one band at 67 kDa (fig.2). Therefore, it was concluded that the 60 kDa band of the 7.3 S enzyme aggregate represented the PDE.

As shown by the dissociation experiment of the purified 7.3 S enzyme complex, the 3.6 S PDE form, found at any stage of purification, derived from the 7.3 S enzyme aggregate (fig.1A,C). However, the 3.6 S PDE form showed only low-K_m PDE activity, whereas the 7.3 S complex contained low- and high-K_m PDE activity. The suggestion that the high-K_m PDE is identical with the nucleotidase is incompatible with the result that the high-K_m PDE activity was only slightly reduced by 1 mM ATP, whereas the nucleotidase was completely inhibited.

At present, the problem of the biphasic kinetics of PDE cannot be solved, as stated earlier [11]. Besides, it cannot be excluded that the 7.3 S enzyme complex contained (a) further component(s) which were not detectable in our experiments. In this context, it should be mentioned that an attempt to reconstitute the 7.3 S enzyme aggregate from the dissociated forms was not successful.

However, it is now proved that PDE is rather firmly associated with a nucleotidase even at low protein concentrations. Under the condition of high protein concentration in the cell (e.g. muscle cell 25%, w/w; red blood cell 35%, w/w), such an enzyme cluster is very likely to exist. A review article about enzyme cluster formation and its physiological significance has been published some time ago [12].

The present case leads to the suggestion that the aggregate of PDE and nucleotidase is important for the regulation of adenosine and cAMP concentration. It is suggested that inside the cell adenosine is generated from cyclic AMP via 5′-AMP. Adenosine could then regulate the adenyl cyclase activity by feedback control. An adenosine binding site localized at the cytoplasmic surface of the plasma membrane has been described and was termed 'P-site' [13]. This site inhibits adenyl cyclase activity after binding of adenosine.

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REFERENCES

[1] Sutherland, E.W. (1972) Science 177, 401–408.
[2] Wombacher, H. (1980) Arch. Biochem. Biophys. 201, 8–19.
[3] Wombacher, H. (1980) Mol. Cell. Biochem. 30, 157–164.
[4] Wombacher, H. (1981) J. Cyclic Nucleotide Res. 7, 293–302.
[5] Morill, M.E., Thompson, S.T. and Stellwagen, E. (1979) J. Biol. Chem. 254, 4371–4374.
[6] Studier, F.W. (1973) J. Biol. Chem. 79, 237–248.
[7] Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.
[8] Wombacher, H. (1978) FEBS Lett. 85, 77–80.
[9] Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372–1379.
[10] Wombacher, H. (1982) Biochem. Pharmacol. 31, 3441–3446.
[11] Cheung, W.Y. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16, 357–361.
[12] Wombacher, H. (1983) Mol. Cell. Biochem. 56, 155–164.
[13] Wolff, J., Londos, C. and Cooper, D.M.F. (1981) Adv. Cyclic Nucleotide Res. 14, 199–214.