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PREPARATIVE ISOELECTRIC FOCUSING AND SOME PROPERTIES OF SOLUBILIZED ADENYLATE CYCLASE FROM RAT BRAIN

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Summary

A new procedure for the purification of rat brain adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) is presented. The enzyme solubilized in Lubrol PX was purified either by molecular sieving or by hydrophobic chromatography, followed by a preparative isoelectric focusing step. For this purpose, a new isoelectric focusing technique was developed which allows a good resolution of adenylate cyclase in a short period of time. When resolved by this procedure, the enzyme migrated as a single molecular species with a p*I* of 6.3. When isoelectric focusing was performed in the presence of EGTA, two distinct peaks of activity could be detected at p*I* 6.1 and 7.3. This suggests that adenylate cyclase consists of two subunits held together by divalent ions.

It is shown that the purified adenylate cyclase has a smaller sedimentation coefficient and is less hydrophobic than the native one. We conclude that the adenylate cyclase containing complex was at least partially disaggregated by this procedure.

Introduction

Adenylate cyclase is a unique membrane-bound enzyme which transduces the message of a number of stimuli in the cell by catalysing the conversion of ATP to cyclic AMP. Solubilization of adenylate cyclase from mammalian cells can be achieved by treatment of the membrane fraction with non-ionic detergents [1,2]. Recent studies indicate, however, that the solubilization of the

enzyme does not liberate the individual component of the transducing system but a complex detergent-phospholipid-protein structure [3,4]. This fact, as well as principally the high thermolability of adenylate cyclase have greatly hindered the study of its molecular characteristics.

Isoelectric focusing is known to split non-covalent complexes held together by low energy interactions. During the transient state, molecules held together by such bonds, have the time to split apart. It is then expected, that this technique is the method of choice for the purification of the enzyme, if it can be carried out rapidly at low temperature. The isoelectric focusing of adenylate cyclase is, however, further complicated by the size of the enzyme and its hydrophobicity [5]. It is known, in fact, that high molecular weight proteins, such as myosin, focalize on polyacrylamide gels in the form of a broad band, probably due to incomplete dissociation of aggregates and perhaps to precipitation of the molecule [6].

We describe a method of isoelectric focusing by which we overcame those difficulties and which allowed, for the first time a good resolution of the enzyme.

Isoelectric focusing is a high resolution technique. However, particularly in the case of adenylate cyclase, which represents no more than 0.005% of total membrane proteins [7], this technique has to be combined with other purification methods.

A two step purification scheme of brain adenylate cyclase is presented, which combines gel filtration and isoelectric focusing, which leads to an increase of 300 times of the specific activity of the enzyme.

Materials and Methods

150—200 g male Wistar rats were used. Cyclic [^{3}H]AMP (specific activity 25 Ci/mmol) and α[^{32}P]ATP were from the Radiochemical Centre, Amersham, England. ATP, cyclic AMP, creatine phosphate, creatine kinase (EC 2.7.3.2) were from Sigma Chemical Co; bovine serum albumin was from Poviet Producters; Dowex AG50 WX8 (200—400 mesh) from Biorad; aluminium oxide Woelm neutral activity, grade 1, was from ICN Pharmaceuticals; theophylline, glycerol, NaF were from Merck; Sephadex G-15 from Pharmacia; Ultrogel ACA-22 was from I.B.F.; ampholines from LKB; Diaflo ultrafiltration membranes (PM-10) from Europe Amicon B.V.; and Lubrol PX was from ICI England.

The washed cerebral particles of rat brain were prepared and solubilized by Lubrol PX according to Johnson and Sutherland [8]. The solubilized enzyme was separated from the insoluble particulate fraction by centrifugation at 100 000 $\times g$ for 30 min. This fraction will be designated as Lubrol supernatant. In some cases, the enzyme was preactivated prior to solubilization by incubation during 15 min at room temperature in the presence of 10 mM NaF according to Bradham [9].

Adenylate cyclase. This was assayed by measuring the formation of cyclic [^{32}P]AMP from α[^{32}P]ATP according to Krishna et al. [10]. The cyclic AMP produced was isolated according to Salomon et al. [11].

Sucrose density gradient ultracentrifugation. Linear density gradients (5 ml)

were prepared from 3 and 10% sucrose solutions exactly according to Guillon et al. [12], except that Triton X-100 was replaced by Lubrol PX. Samples of the solubilised enzyme which had been previously purified by isoelectric focusing (see below) were separated from glycerol and excess ampholines by quick sieving on a small Sephadex G-15 column (9 ml bed volume) (PD10 Pharmacia Sweden).

Preparative isoelectric focusing. This was performed at 1—2°C by a modification of the technique of O'Brien et al. [13] as follows. The Sephadex G-15 column which serves as a solid support was equilibrated with 10% glycerol containing 2% of carrier ampholites and Lubrol PX at a final concentration of 1%. The sample was loaded in the same mixture. Focusing was carried out at 900 V constant for 6.5 h followed by 1100 V for 60 more min. The initial amperage increased from 20 to 22 mA to about 50 mA after 0.5 h and then decreased steadily during the following 6 h to 3.0 mA. Experiments during which isoelectric focusing was interrupted after a 4-h run, showed that the pH gradient was already established. 2 h were necessary for its stabilization. Product of voltage and time totalled 6900 V h. 1% NaOH and 1% H_3PO_4 were used as anolyte and catolyte, respectively. The run was interrupted routinely after 7.5 h and the gradient was eluted with buffer at a flow rate of 60 ml/h.

Hydrophobic chromatography was carried out on a dodecyl Sepharose column [14] according to Homcy et al. [4].

Ultrogel ACA-22 gel filtration chromatography was performed as described by Welton et al. [3]. K_D determinations were made according to Laurent and Killander [15]. Molecular weights were calculated according to Bon et al. [16]. The column was calibrated using thyroglobulin, β-galactosidase, ferritin and catalase as markers.

Protein concentration was determined according to the method of Lowry et al [17]. When determinations were done in the presence of detergent, the samples were centrifuged before the measurements. After isoelectric focusing the ampholines were eliminated by extensive dialysis against a 9 M urea solution.

Results

Purification by Ultrogel ACA-22 chromatography

Washed particles were preactivated and solubilized as described under Methods. The Lubrol supernatant was chromatographed on an Ultrogel ACA-22 column in the presence of 0.01% Lubrol PX (Fig. 1A). The enzyme eluted in the included volume as a single peak in front of the bulk of proteins with a K_D of 0.19. The specific activity was increased 4 times by this procedure (Table I). Similar results were obtained for the non-preactivated enzyme.

Sedimentation analysis

The sedimentation profile of the solubilized adenylate cyclase was determined by centrifugation through sucrose density gradients made up in H_2O and ^2H_2O. The standard sedimentation coefficient of the enzyme purified by molecular sieving chromatography was significantly higher than that of the non-purified one (10.3 and 8.8 S, respectively). The partial specific volumes of both

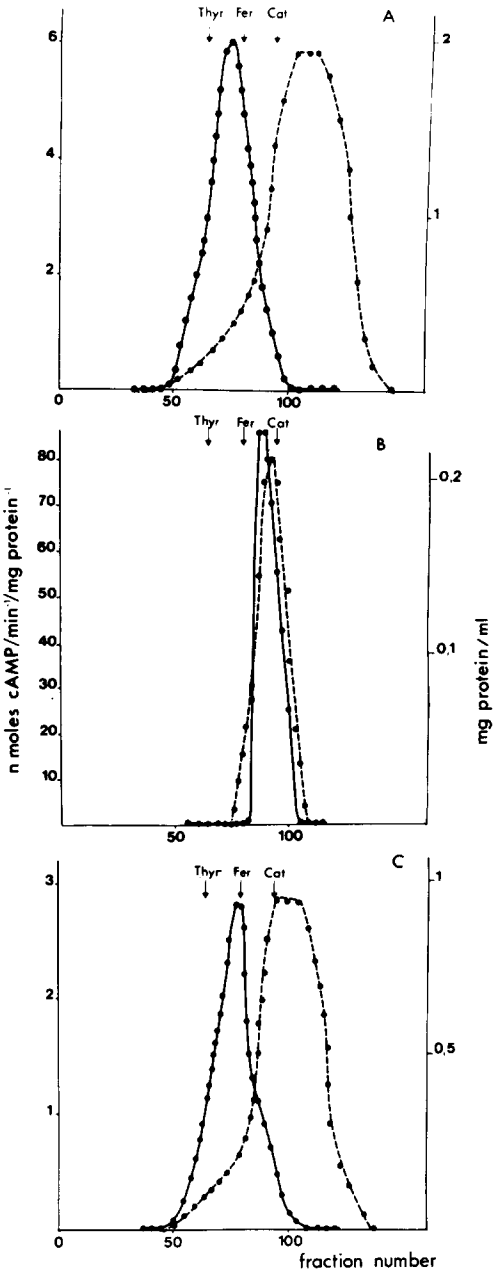

Fig. 1. Ultrogel ACA-22 elution profile of brain adenylate cyclase. (A) Washed particles were preactivated and solubilized. The Lubrol supernatant (4 ml) was applied to a column of Ultrogel ACA-22 (2.5 × 40 cm) and eluted with a buffer containing 10 mM Tris-HCl (pH 7.5), dithiothreitol 3 mM and Lubrol PX 0.01%. The column was run at a constant flow rate of 6 ml/h. Fractions of 1 ml were collected and assayed for adenylate cyclase activity. The reaction mixture contained Tris-HCl 50 mM pH 7.5; 9 mM $MgCl_2$; 1 mg/ml bovine serum albumin; 3 mM dithiothreitol, 22 mM theophylline; 0.5 mM ATP; $\alpha[^{32}P]$-ATP (150—200 cpm/pmol), Lubrol PX 0.1%; 20 mM creatine kinase and 10—20 μg of enzyme protein in a final volume of 100 μl. Incubations were carried out at 22°C for 3 min. The reaction was stopped by addition of 100 μl of a solution containing 1.4 mM cyclic AMP; 5 mM ATP; 2% sodium dodecyl sulfate and cyclic [8-^{3}H]AMP. (B) Solubilized adenylate cyclase was purified by isoelectric focusing on a column

TABLE I

PURIFICATION OF RAT BRAIN ADENYLATE CYCLASE

Washed particles were obtained preactivated and solubilized. Adenylate cyclase was assayed at 22°C either in the absence (A) or in the presence (B) of 0.3 mM EGTA.

Step	Protein (mg)	Total activity (nmol/min) A	Specific activity (nmol/min per mg protein)		Yield (%)
			A	B	
Solubilized washed particle	980.4	500	0.51	0.26	100
Lubrol extract	486.11	350	0.72	0.39	70
A_cA_{22} Ultrogel chromatography	39.76	136	3.42	1.80	27.2
Isoelectric focusing	0.228	40	174.92	80.40	8

TABLE II

PHYSICAL PARAMETERS OF SOLUBILISED RAT BRAIN ADENYLATE CYCLASE AT DIFFERENT STAGES OF PURIFICATION

Values given are means ± S.D. for the number of determinations shown in parenthesis.

Parameters	Solubilised non-purified enzyme	Enzyme purified by		
		A_cA_{22} Ultrogel chromatography	Hydrophobic chromatography	Isoelectric focusing
Apparent sedimentation coefficient measured in H_2) gradient, Sapp (S) *	8.3 ± 0.1 (6)	9.7 ± 0.1 (6)	8.8 ± 0.1 (6)	8.4 ± 0.1 (5)
Apparent sedimentation coefficient measured in ^2H_2O gradient, Sapp (S) *	7.4 ± 0.1 (8)	8.9 ± 0.1 (5)	8.4 ± 0.1 (5)	8.3 ± 0.1 (6)
Standard sedimentation coefficient $S_{20,w}$ (S) **	8.8	10.3	9.0	8.5
Partial specific volume $\bar{v}$ (ml/g) **	0.78 ± 0.01 (10)	0.78 ± 0.01 (10)	0.77 ± 0.01 (8)	0.75 ± 0.01 (7)

* In each run β-galactosidase, catalase and alcohol dehydrogenase, used as standards were layered on top of the gradient together with the adenylate cyclase preparation in a total volume of 300 µl. Assay conditions and values of the hydrodynamic parameters of the standard proteins were those used by Meunier et al. [23].

** Calculated according to Meunier et al. [23].

preparations was 0.78 ml/g (Table II). Identical results were obtained whether basal or preactivated enzyme was used. The molecular weight of the adenylate cyclase purified by Ultrogel ACA-22 chromatography was 330 000.

(pH range 3.5—10). The most active fractions were pooled and chromatographed on a ACA-22 Ultrogen column (2.5 × 40 cm) which was eluted and assayed exactly as described under (A). (C) Adenylate cyclase was preactivated solubilized and purified on a dodecyl Sepharose column. The purified enzyme was subsequently chromatographed on a ACA-22 Ultrogel chromatography column as described under (A). The elution positions of thyroglobulin, ferritin and catalase are demonstrated on the figures. ●———●, specific activity of the enzyme; ●- - - - -●, mg protein/ml. cAMP, cyclic AMP.

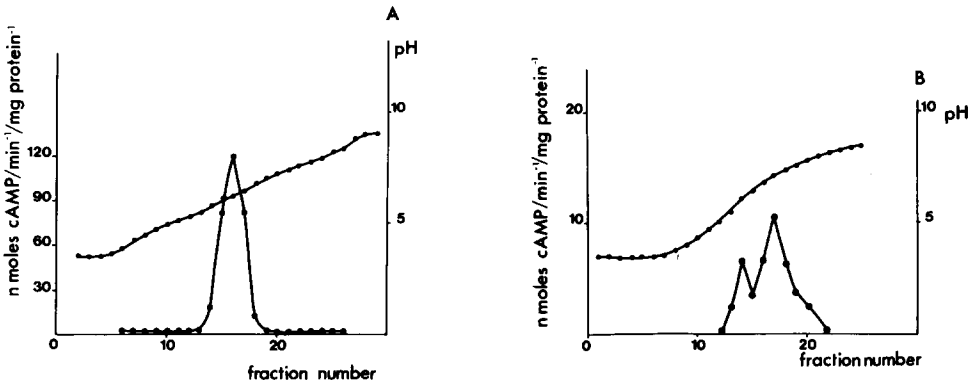

Fig. 2. Isoelectric focusing of adenylate cyclase. Washed particles were preactivated and solubilized. The soluble enzyme was chromatographed on a ACA-22 Ultrogel column as described under Fig. 1A. The most active fractions of the run were either pooled and loaded on an isoelectric focusing column (pH range 3.5—10) which was run as described (A) or adjusted to 1 mM EGTA and loaded on a isoelectric focusing column equilibrated with 1 mM EGTA, 1% Lubrol PX and 2% ampholines (B). When this last procedure was used, 1% NaOH containing 1 mM EGTA was continuously pumped through the cathode at 60 ml/h. Focusing was carried out at 900 V constant for 5 h followed by 1 h at 1100 V constant. In both cases, at the end of the run the fractions were eluted and assayed for adenylate cyclase activity as described under Methods and Fig. 1A in the absence of added Lubrol PX. Similar results were obtained whether the column was eluted through the acidic or through the basic end. Fraction volume was 1 ml. The points plotted on the figure represent the activity of the enzyme. cAMP, cyclic AMP.

Preparative isoelectric focusing

We used isoelectric focusing to further purify the enzyme. Preactivated adenylate cyclase, which had been previously resolved on a Ultrogel ACA-22 column was focalized according to the method described. The activity of the enzyme focalized as a single sharp peak at a position corresponding to a p*I* 6.3—6.4 (Fig. 2A). Similar results were obtained in six independent experiments.

Identical results were obtained when carrier ampholites of narrow pH range (4—8) were used and also when the column was eluted either through the acidic or through the basic end. In the absence of detergent, the isoelectric focusing profile of the enzyme was diffuse and exhibited a maximum of activity between p*I* 6.1 and 6.5.

As can be seen from Table I the enzyme was purified up to 50 times by the isoelectric focusing step alone. The activity of the so purified enzyme was inhibited up to 50% by 0.3 mM EGTA (Table I).

Physical characteristics of purified adenylate cyclase

After isoelectric focusing, adenylate cyclase was rechromatographed on a Ultrogel ACA-22 column (Fig. 1B).The activity eluted as a peak with a K_D of 0.34.

The sedimentation profile of the purified enzyme was determined as described previously. As can be seen from Table II adenylate cyclase after isoelectric focusing had a constant sedimentation coefficient and a partial specific volume which were lower than those of the native solubilized enzyme (8.5 S and 0.75 ml/g). The molecular weight of the purified enzyme was 220 000.

Isoelectric focusing in the presence of EGTA

In some experiments, the isoelectric focusing column was equilibrated before the run with the above mentioned carrier ampholyte mixture containing 1 mM EGTA. 1% NaOH containing 1 mM EGTA was pumped continuously through the cathode (60 ml/h) during the whole procedure. By this method, preactivated adenylate cyclase was resolved into two peaks of activity with a p*I* of 6.1 and 7.2—7.3, respectively (Fig. 2B). However, by this procedure, isoelectric focusing led to an increase of only 3—5 times in the specific activity of adenylate cyclase in each peak. Identical results were obtained in four independent experiments. The enzyme purified by this method was too unstable to allow further sizing of the subunits.

Hydrophobic chromatography

A 4 times purification of the enzyme could be obtained by this procedure. The isoelectric focusing profile of the preactivated adenylate cyclase which had been previously purified by dodecyl Sepharose chromatography was indistinguishable from that of the enzyme which had been resolved by conventional gel chromatography.

Physical characteristics of adenylate cyclase after hydrophobic chromatography

Ultrogel ACA-22 chromatography of the enzyme purified on a hydrophobic column showed a small but reproducible shift of the elution peak with respect to that of native solubilized adenylate cyclase. In six independent experiments, the purified enzyme eluted with a K_D of 0.22 (Fig. 1C). The standard sedimentation coefficient was 9.0 S and the partial specific volume 0.77 ml/g. Identical results were obtained for the basal and the preactivated enzyme. The molecular weight of the adenylate cyclase purified by hydrophobic chromatography was 280 000.

In view of these results, we compared some molecular properties of the hydrophobically purified and the native enzyme.

The thermal stability of the membrane bound as well as of the solubilized adenylate cyclase was compared to that of the enzyme purified on a dodecyl Sepharose column. For this purpose the enzyme preparation was incubated for different time ranges at 22 or 37°C prior to the assay as described under Fig. 3.

Membrane-bound adenylate cyclase was stable for 20 min at 22 and 37°C (Fig. 3A). In the presence of 0.5% Lubrol PX, which solubilizes the enzyme, adenylate cyclase was rapidly inactivated at both temperature. The preactivated enzyme was significantly more stable then the basal one, particularly at 22°C (Fig. 3). The thermal inactivation curves were non linear which suggests the coexistence of more than one form of adenylate cyclase of different thermostability (Fig. 3).

Under the conditions used in this study, hydrophobic chromatography separates free detergent from the enzyme. In the absence of added Lubrol, the stability of the hydrophobically purified adenylate cyclase (non preactivated and preactivated) was similar to that of the membrane bound enzyme (Fig. 3). In the presence of added Lubrox PX (0.5% final concentration) the slopes of the thermal inactivation curves of the purified enzyme were identical to those of the solubilized non purified one (compare Figs. 3A and B).

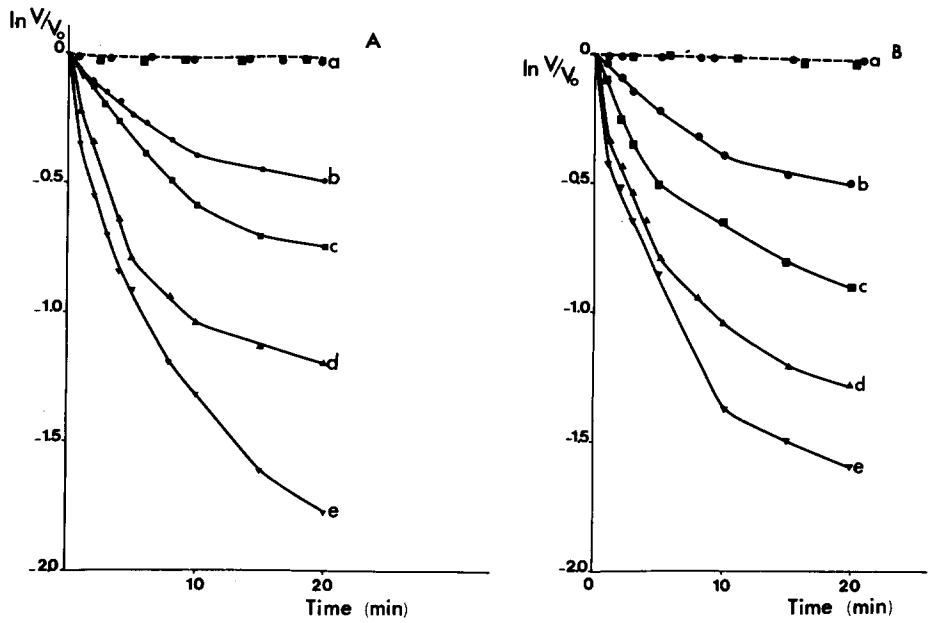

Fig. 3. Thermal inactivation of brain adenylate cyclase. Aliquots of non preactivated (a. c, e) or NaF preactivated (b, d) enzyme preparation containing 200 μg/ml of protein were incubated for various periods of time at 22°C (b, c) or at 37°C (d, e) in the presence of 3 mM, 10 mM Tris-HCl (pH 7.5) in the absence (a) or in the presence (b, c, d, e) of 0.5% Lubrol PX in a total volume of 1 ml. Incubation in the absence of Lubrol PX (a) was performed at 22°C (•) and at 37°C (■). Following incubation for different periods of time, samples were taken and assayed for residual adenylate cyclase activity V at 22°C as described under Fig. 1A in the absence of added Lubrol PX. All values were expressed as percentage of the initial activity of the enzyme preparation V_0, which was taken in all cases as 100%. Data were plotted as logarithmn of V/V_0 as a function of the time of incubation t. The initial specific activities (before thermal inactivation) of the enzyme preparations used were as follows: 0.2 nmol/min per mg protein for non preactivated washed particles; 0.35 nmol/min per mg protein for preactivated washed particles; 1.4 nmol/min per mg protein for non-preactivated hydrophobically purified enzyme; 3.2 nmol/min per mg protein for preactivated hydrophobically purified enzyme. (A) Washed particles, and (B) hydrophobically purified adenylate cyclase.

Kinetics of catalysis

In a first series of experiments a non-preactivated enzyme preparation was used, which had been separated from unbound detergent by chromatography on an Ultrogel ACA-22 column in the absence of Lubrol PX (Fig. 4A). When no detergent was added, the reaction was linear for 15 min. Addition of increasing detergent concentrations to the reaction mixture caused a stimulation of the enzyme activity at concentrations of Lubrol PX between 0.001 and 0.1%. Beyond this concentration practically no further stimulation of the enzyme could be observed. Identical results were obtained when a preactivated preparation was used (results not shown). In parallel a reduction of the time of linearity of the reaction took place which was more important at higher detergent concentrations.

In a second series of experiments, we used solubilized adenylate cyclase which had been purified by chromatography on a dodecyl Sepharose column. In the absence of added detergent, the reaction rate of the enzyme was linear

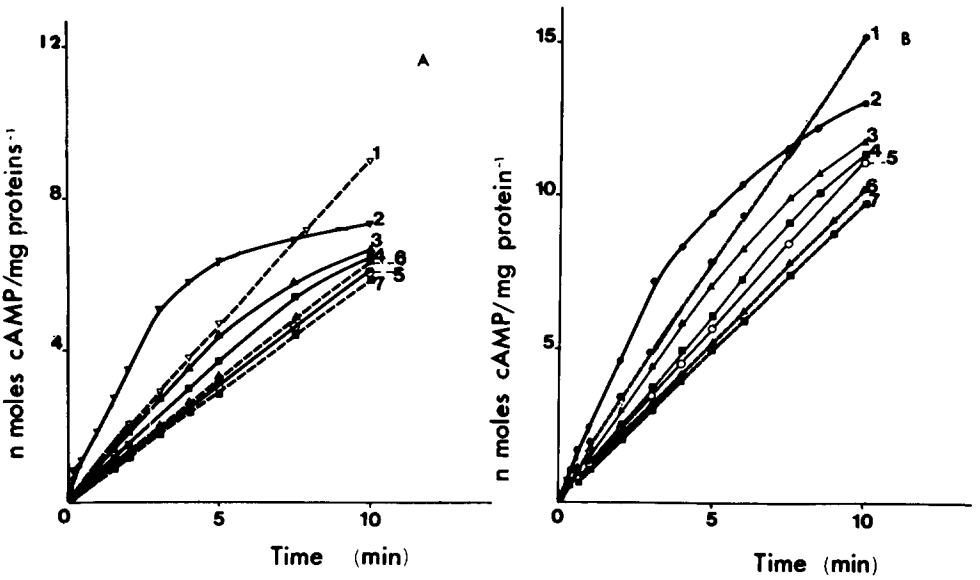

Fig. 4. Time course of adenylate cyclase. (A) Non preactivated solubilized adenylate cyclase separated from free detergent by chromatography on a Ultrogel ACA-22 column (2.5 × 40 cm) equilibrated with 10 mM Tris-HCl (pH 7.5), 3 mM dithiothreitol. (B) Non-preactivated adenylate cyclase purified by dodecyl Sepharose chromatography. Adenylate cyclase assays were performed as described under Fig. 1A in the absence (5) or in the presence of added Lubrol PX at a final concentration of 0.001% (4, 7), 0.01% (3, 6) and 0.1% (1, 2) in the presence (- - - - -) (1, 6, 7) or in the absence (———) (2, 3, 4, 5) of 10 mM NaF. The incubations were carried out at 22° C and were interrupted at different time points between 0 and 10 min. cAMP, cyclic AMP.

for 15 min (Fig. 4B). Addition of Lubrol PX to the assay mixture caused similar effect to those observed for the enzyme which has been partially purified by molecular sieving chromatography (Fig. 4B).

Influence of NaF on enzyme activity

Two effects of F^- on the activity of adenylate cyclase were observed. First, as shown in Fig. 4 the presence of 10 mM NaF prevented the destabilization of the enzyme by increasing detergent concentrations. Second, membrane-bound adenylate cyclase was maximally activated 1.5—2-fold by NaF. After solubilization, the enzyme was inhibited by this effector. As shown in Fig. 4 this inhibition was of the same order whether adenylate cyclase had been purified or not by hydrophobic chromatography and whether Lubrol was present in the assay or not.

Purification scheme

In an attempt to determine whether the method we devised could be used to purify brain adenylate cyclase we worked out the following protocol. The brains of six rats were homogenized and washed particles were obtained, preactivated with 10 mM NaF and solubilized as described in Methods. The 100 000 × *g* supernatant (Lubrol supernatant) (60 ml) was concentrated 6 times on an Amicon PM10 filter and loaded on an Ultrogel ACA-22 gel filtra-

tion column (2.5 × 40 cm) which was eluted as described in Fig. 1. The active fractions from this column were pooled, concentrated to 5 ml on an Amicon PM10 filter and loaded on an isoelectric focusing column equilibrated with carrier ampholites of a range of pH 3.5—10. After the run, the fractions with a p*I* of 6.3 and 6.4 were pooled. As shown in Table I this resulted in an increase of specific activity of approx. 300-fold with a final yield of 8%.

Discussion

In the present study we examined the ability of isoelectric focusing to resolve adenylate cyclase from brain cortex in an enzymatically active form. Previous studies by Francks and Malamud [18] had shown that the enzyme focalizes diffusely in polyacrylamide gels. The very broad zone of activity that these authors obtained at the end of their run made determination of p*I* difficult. Such a focusing profile is, however, not unique to adenylate cyclase but a quite common phenomena when isoelectric focusing of large molecules is performed in polyacrylamide gels [6] and is due most probably to sieving effects and subsequent trailing of the proteins. We overcame this inconvenience using an anticonvectant with a large pore size (Sephadex G-15). This allowed us to obtain, for the first time, a sharp focusing profile of adenylate cyclase at p*I* 6.3. As is shown in the preceding section, the obtaining of a sharp peak is dependent on the presence of large quantities of detergent which prevents aggregation and precipitation of the enzyme.

Using Sephadex G-15 as an anticonvectant has the inconvenience that small carrier ampholyte species (10^3 daltons and less) may be displaced during the elution. Our results clearly demonstrate that this was not the case during our experiments. Thus, the p*I* we found can be considered reliable within the margin of error of the method.

Isoelectric focusing performed by the method we describe, is completed in a relatively short time. This is a great advantage over the classical preparative focusing method on a sucrose gradient, in the course of which the enzyme is totally inactivated [3].

When isoelectric focusing was carried out in the presence of EGTA, the activity of the enzyme was resolved into two peaks. These results suggest, that adenylate cyclase consists of two subunits held together by tightly bound divalent ions, such as Ca^{2+}, needed for the integrity of the quaternary structure. This interpretation is consistent with the recent findings of Westcott et al. [19] who separated two forms of brain adenylate cyclase by a procedure involving affinity chromatography in the presence of EGTA.

The physical characteristics of the enzyme at different stages of the purification procedure were determined. The non-purified solubilized enzyme had a high partial specific volume. In our calculations, we used a value of 0.73 ml/g for the partial specific volume of a globular soluble protein [5]. The large difference between this value and the one experimentally determined by us for native adenylate cyclase, shows that the rat brain enzyme is a highly hydrophobic molecule which binds large quantities of detergent. The values determined in this study for the constant sedimentation coefficient and the partial specific volume of the brain enzyme are different from those reported by Neer

[5]. (8.8 S and 0.78 ml/g compared to 8.1 S and 0.79 ml/g obtained by Neer). We postulate that this could be due to the different origin of the enzyme studied (rat and bovine brain) as well as to differences in the detergents used (Lubrol PX and Triton X-100).

After chromatography on a molecular sieving column, the sedimentation coefficient of the enzyme increased markedly. This indicates that the enzyme might have aggregated with other molecules during this procedure. These results are in good accordance with those reported recently by Newby and Chrambach [20] who reported an increase of the molecular radius of the liver enzyme after gel filtration.

The standard sedimentation coefficient of the enzyme purified by isoelectric focusing was smaller than that of the non purified adenylate cyclase. A parallel decrease of the molecular weight and of the partial specific volume of the molecule could be observed. These data show that the purified enzyme was smaller and less hydrophobic than the native one.

The results discussed above show that our method allows the resolution of the adenylate cyclase-containing complex, in a non-denaturating medium. This is a great advantage over recently reported techniques of disaggregation of this enzyme which involve the use of ionic detergents [20].

Hydrophobic chromatography

We examined further, whether hydrophobic chromatography could improve the purification of the enzyme as is the case for cardiac adenylate cyclase [4].

Our results show that although no improvement could be observed in the degree of purification of the enzyme when brain adenylate cyclase was purified by this method instead of classical molecular sieving chromatography prior to the isoelectric focusing step, (1) no significant aggregation of adenylate cyclase took place during hydrophobic chromatography, (2) some small, highly hydrophobic, molecules were separated from the enzyme by this procedure. As adenylate cyclase is solubilized in the form of a complex with other functionally important molecules [3] it was important to determine, in order to establish our definite purification protocol, if any of them was separated from the catalytic unit of the enzyme by hydrophobic chromatography.

Therefore, a comparison of the molecular properties of the partially purified enzyme with those of the membrane bound and detergent solubilised adenylate cyclase was made. Our results, given in detail in the preceding section, demonstrate that the so purified adenylate cyclase was most similar to the native one. 0.3 mM EGTA inhibited the activity of the hydrophobically purified enzyme up to 50% (not shown). Measurements of the calcium-dependent regulatory protein [21] indicate that this molecule is still attached to the hydrophobically resolved enzyme (Pichard, A.L. and Wahrmann, J.P., unpublished results).

Thus, no evidence was obtained that functionally related molecules were separated from the enzyme by hydrophobic chromatography.

We conclude therefore that the hydrophobic purification step which is essential when adenylate cyclase is to be purified further by classical gel chromatography [4] is not a prerequisite for the purification by isoelectric focusing of the enzyme.

Purification

In Table I a purification scheme for brain adenylate cyclase is shown. As can be seen from our data, the enzyme which had been stabilized by preactivation with NaF could be purified 300-fold by sequential Ultrogel chromatography and isoelectric focusing.

This is a great advantage over other existing isoelectric focusing methods some of which inactivate the enzyme completely [3] while others inactivate it up to 40—50% [18].

A similar degree of purification of brain adenylate cyclase had been reported by Stellwagen and Backer [22]. However their method could not be reproduced neither by us nor by several other workers in the field [4,19]. A higher degree of purification has only been reported by Homcy et al. [4] for cardiac adenylate cyclase. Their results suggest that the purified enzyme from heart is much more stable than that from brain.

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