Gel-Filtration Analysis of Soluble Adenylate Cyclase from Bovine Corpus Luteum

By JOHN L. YOUNG*† and DAVID A. STANSFIELD‡

**Department of Obstetrics and Gynaecology, University of Dundee, Ninewells Hospital, Dundee DD1 9SY, Scotland, U.K., and*

‡*Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.*

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1. Sepharose 6B gel-filtration analysis of soluble adenylate cyclase from bovine corpus luteum is described. Both zonal and frontal techniques of analysis were used. 2. Under conditions of zonal analysis recoveries of activity were low. It was concluded that dissociation of two or more components of the adenylate cyclase complex was occurring on the column and that the maintenance of the complex was essential for the high-activity state of the catalytic unit. Two peaks of adenylate cyclase activity, of approximate mol. wts. 45000 and 160000 were detected. 3. The theory of frontal analysis (or steady-state gel filtration), applied to the study of the interacting components of the adenylate cyclase complex is discussed, and activity profiles are predicted. Activity profiles obtained experimentally by frontal analysis compared well with the theoretically predicted profile and provide evidence that dissociation of a high-activity complex, with concomitant loss of activity, does occur. Recoveries of activity under conditions of frontal analysis were higher than with zonal analysis. 4. The effects of concentration and removal of detergent on the activity of the soluble enzyme are discussed.

We have previously reported the preparation, in high yield, of soluble adenylate cyclase from the 600g sediment of bovine corpus-luteum homogenate (Young & Stansfield, 1978*a,b*). In the present paper we describe the gel-filtration analysis of soluble adenylate cyclase by both zonal and frontal techniques. The effects of detergent removal and concentration on the enzyme activity are also discussed.

Experimental

Materials

ATP (disodium salt) was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. p[NH]ppG (tetralithium salt) and calibration proteins were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Lubrol-12A9 (also designated PX) was a gift from ICI, Alderley Park, Macclesfield, Cheshire, U.K. Sepharose 6B and Blue Dextran 2000 are products of Pharmacia (G.B.) Ltd., London W.5, U.K. Cyclic AMP-binding protein (bovine adrenal gland), Amberlite XAD-2 and all other chemicals (analytical

grade if available) were obtained from BDH, Poole, Dorset, U.K.

Methods

The washed 600g ($r_{av.} = 23$ cm) sediment of bovine corpus-luteum homogenate was prepared as described previously (Young & Stansfield, 1977). All tissue extract concentrations were equivalent to 75 mg wet wt. of corpus luteum/ml.

p[NH]ppG-treated 600g sediment was prepared by incubation for 20 min at 37°C with 0.1 mM-p[NH]ppG in 40 mM-Tris/HCl (pH 7.5)/6 mM-MgSO₄, followed by washing (twice) with p[NH]ppG-free buffer at 4°C.

Adenylate cyclase of the p[NH]ppG-treated 600g sediment was solubilized by using Lubrol-12A9 (10 g/litre) in 40 mM-Tris/HCl (pH 7.5) containing 6 mM-MgSO₄. Adenylate cyclase of the native (untreated) 600g sediment was solubilized by using Lubrol-12A9 (10 g/litre) in 40 mM-Tris/HCl (pH 7.5) containing 6 mM-MgSO₄ and 5 mM-NaF. The washed 600g sediment was resuspended in the detergent solution at a concentration equivalent to 75 mg wet wt. of tissue/ml, rehomogenized and then gently shaken for 0.5 h at 4°C. The detergent-dispersed preparation was centrifuged at 105000g ($r_{av.} = 5.74$ cm) for 1.5 h at 4°C and the supernatant (soluble enzyme) was collected. Detergent-free soluble enzyme

Abbreviation used: p[NH]ppG, guanosine 5' [β , γ -imido]triphosphate.

† To whom reprint requests should be addressed.

(the term detergent-free ignores the possibility of protein-bound detergent) was prepared by using Amberlite XAD-2 (Beckman *et al.*, 1974; van Heyningen, 1976). The resin was added to the soluble enzyme preparation at a concentration of 100g/litre and then the mixture gently shaken for 1 h at 4°C. The supernatant was recovered after allowing the resin to settle out. The soluble enzyme fractions were concentrated by using Centriflo membrane cones (CF50A; Amicon Corp., Lexington, MA, U.S.A.) or vacuum dialysis by using Visking membranes.

Adenylate cyclase activity was assayed as described previously (Young & Stansfield, 1977, 1978a). The total assay incubation volume of 1 ml contained 40 mM-Tris/HCl (pH 7.5), 6 mM-MgSO₄, 1 mM-ATP, 6.7 mM-caffeine and 500 µl of enzyme preparation (see legends for variable additions). All assays of 600g-sediment enzyme and of soluble enzyme contained tissue extract equivalent to 37.5 mg wet wt. of tissue/ml of incubation medium. Incubation was carried out at 37°C for 10 min. All assays were performed in duplicate, unless otherwise stated, and the mean and range of duplicate values are shown.

The cyclic AMP produced was measured by a competitive protein-binding technique (Young & Stansfield, 1977) using a partially purified cyclic AMP-binding protein from bovine adrenal glands. Standard assay mixtures used to obtain dose-response curves for cyclic AMP were supplemented with the appropriate concentrations of incubation components to compensate for any effect of these substances on the binding assay.

Downward-flow gel filtration was carried out at 4°C on columns of Sepharose 6B. The eluent buffer was 40 mM-Tris/HCl (pH 7.5) containing 6 mM-MgSO₄ and Lubrol-12A9 (1 g/litre). A constant flow rate of 8 ml/h was maintained with a peristaltic pump. The column size used was either 52 cm long × 1.6 cm diameter or 52 cm long × 0.9 cm diameter (see Figure legends) and the corresponding fraction sizes were 2.3 and 0.928 ml. Portions (500 µl) of each fraction were assayed for adenylate cyclase activity. The detection limit of the method is indicated on the Figures by a horizontal broken line. The columns were calibrated for molecular-weight determination with purified proteins of known molecular weight. The void volume and total volume of the columns were assessed with Blue Dextran 2000 (labelled BD in Figures) and ¹²⁵I respectively.

Protein was measured by the method of Lowry *et al.* (1951) after treatment of the tissue fraction with 1 M-NaOH and 3 min immersion in a boiling-water bath. Bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) was used as the standard. The appropriate corrections were made for the effects of detergent and other buffer constituents on the protein determination.

Results and Discussion

Gel filtration of soluble adenylate cyclase

(1) *Zonal analysis.* Zonal analysis is defined as gel filtration of small samples whose components are separated into more or less discrete zones. Preliminary investigations of the behaviour of soluble adenylate cyclase on Sepharose 6B were performed with detergent-free concentrates. It was found that (a) the adenylate cyclase activity was eluted with the void volume of the column, indicating a molecular weight $>1 \times 10^6$, higher than had been reported for the adenylate cyclase of other tissues (Levey, 1970; Forte, 1972; Swislocki & Tierney, 1973; Neer, 1974, 1976; Ryan & Storm, 1974; Queener *et al.*, 1975; Varimo & Londesborough, 1976; Dufau *et al.*, 1977; Haga *et al.*, 1977; Hanski *et al.*, 1977) and (b) the recovery of activity, with respect to the activity in the soluble enzyme preparation before concentration, was low. Recoveries obtained in three experiments were 9.5, 15.6 and 5.7%. Initially, two possibilities were considered; (i) adenylate cyclase is labile at 4°C, most of the activity being lost during the 18 h required to complete the chromatographic run and assays, and (ii) removal of detergent followed by concentration results in aggregation with concomitant loss of activity.

Stability studies revealed that the activity of the soluble enzyme preparation was not lost on storage at 4°C for 18 h, and therefore possibility (i) does not apply. The adenylate cyclase activity solubilized from luteal-homogenate 600g sediment by Lubrol-12A9 was stable at 4°C irrespective of whether the 600g sediment had been (a) pretreated with p[NH]ppG, (b) solubilized in the presence of NaF or (c) subjected to a combination of treatments (a) and (b); 93–110% of the activity was recovered at the end of the storage period.

Swislocki & Tierney (1973) reported aggregation, with concomitant loss of activity, of soluble adenylate cyclase after removal of detergent and concentration, and other investigators have reported the aggregation of soluble adenylate cyclase after removal of detergent (Johnson & Sutherland, 1973; Neer, 1974; Haga *et al.*, 1977). The effect of removing Lubrol-12A9 from the soluble luteal enzyme ranged from a slight loss (24%) of activity to a nearly 3-fold enhancement of activity (results not shown). Thus there were no substantial losses of activity due to removal of detergent.

When the detergent was not removed from the soluble enzyme and when gel chromatography of a concentrate was carried out in the presence of detergent, two peaks of adenylate cyclase activity were detected (Fig. 1), with no detectable activity in the void volume of column. Although the cyclase had apparently not aggregated when concentrated in the presence of detergent the recovery of activity was

Fig. 1. Sepharose 6B chromatography of a concentrate of soluble enzyme derived from p[NH]ppG-treated 600g sediment; chromatography with Lubrol-12A9 (1g/litre) in the eluent buffer

Soluble adenylate cyclase was prepared from p[NH]ppG-treated 600g sediment as described under 'Methods'. 10ml of the soluble enzyme preparation, containing activity equivalent to 11400pmol of cyclic AMP/10min, was concentrated to 1ml by using a Centriflo membrane cone and then chromatographed on a column (1.6cm diameter×52cm long) of Sepharose 6B.

again low; for two experiments the recoveries were 10 and 10.2%. The effect of concentration was therefore investigated. It was found (Table 1) that concentration of both detergent-containing and

detergent-free soluble enzyme preparations resulted in large losses of activity. There was no activity in the ultrafiltrate and activity could not be regained by adding back the ultrafiltrate to the concentrate (results not shown). The solubilization of adenylate cyclase activity was not improved by the inclusion of 1mM-dithiothreitol, and dithiothreitol did not prevent loss of activity during concentration. The effect of 1mM-dithiothreitol was to inhibit adenylate cyclase activity slightly during assay.

However, when detergent-containing soluble luteal-enzyme preparation which had not been concentrated was chromatographed in the presence of detergent, recoveries of activity were of the same order, two experiments giving 11.5 and 2% respectively; two peaks of eluted activity were again observed (Fig. 2). It was concluded that activity might have been lost during gel filtration because of dissociation of two or more components of the adenylate cyclase complex, the association of which is necessary for maintaining a high-activity state of the catalytic unit. Queener *et al.* (1975) reported that the soluble adenylate cyclase of renal cortex disaggregated with loss of activity during gel filtration, and that disaggregation could be prevented by the inclusion of NaF in the elution buffer. However, the inclusion of 5mM-NaF in the buffer used to elute solubilized detergent-containing unconcentrated adenylate cyclase (prepared in the presence of 5mM-NaF) of bovine corpus luteum, did not prevent the loss of activity during gel filtration (results not shown).

Pre-gel-filtration activity could not be regained

Table 1. Effect of a 10-fold concentration of the soluble enzyme followed by dilution in buffer before assay, and the effect of 1mM-dithiothreitol

Twelve tissue fractions were prepared from the p[NH]ppG-treated 600g sediment: (1) 600g sediment resuspended in 40mM-Tris/HCl (pH7.5)/6mM-MgSO₄ (buffer A); (2) 600g sediment resuspended in buffer A containing 1mM-dithiothreitol; (3) 600g sediment dispersed in buffer A containing Lubrol-12A9 (10g/litre); (4) 600g sediment dispersed in buffer A containing Lubrol-12A9 (10g/litre) and 1mM-dithiothreitol; (5) and (6) are the 105000g supernatants of fractions (3) and (4) respectively; (7) and (8) are the Amberlite XAD-2-treated supernatants of fractions (5) and (6) respectively; (9), (10), (11) and (12) are 10-fold concentrates of (5), (6), (7) and (8) respectively, diluted with the appropriate buffer to the original volume before assay.

Fraction	Description	Variables of buffer composition	Adenylate cyclase activity (pmol of cyclic AMP/10min)
1	600g sediment	None	2850 ± 250
2	600g sediment	1 mM-Dithiothreitol	2600
3	600g sediment	Lubrol-12A9	690 ± 30
4	600g sediment	Lubrol-12A9	660
		1 mM-Dithiothreitol	
5	105000g supernatant	Lubrol-12A9	710 ± 30
6	105000g supernatant	Lubrol-12A9	690 ± 10
		1 mM-Dithiothreitol	
7	XAD-2 supernatant	Lubrol-12A9 removed	890 ± 10
8	XAD-2 supernatant	Lubrol-12A9 removed	840 ± 80
		1mM-Dithiothreitol	
9	Concentrate/dilution of 5		270 ± 20
10	Concentrate/dilution of 6		240
11	Concentrate/dilution of 7		325 ± 5
12	Concentrate/dilution of 8		305 ± 5

Fig. 2. Sepharose 6B chromatography of soluble adenylate cyclase derived from *p*[NH]ppG-treated 600g sediment; chromatography without prior concentration of sample and performed in the presence of Lubrol-12A9 (1g/litre). A sample (4ml) of a soluble adenylate cyclase preparation containing activity equivalent to 8480 pmol of cyclic AMP/10 min was chromatographed on a column (1.6cm diameter $\times$ 52cm long) of Sepharose 6B.

by combining the eluted fractions and concentrating the pool by vacuum dialysis (results not shown).

The above data indicate that (i) removal of detergent followed by concentration leads to aggregation and considerable loss of activity, (ii) concentration in the presence of detergent leads to a considerable loss of activity without aggregation and (iii) gel-filtration chromatography of soluble enzyme, without removal of detergent and without prior concentration leads to loss of activity, possibly as a result of dissociation of components of the enzyme complex.

The molecular weights of the two species of adenylate cyclase activity separated by gel-filtration chromatography (Fig. 1) were estimated to be approx. 45000 and 160000; no correction for bound detergent was made. These values compare well with the mol.wts. of 40000 and 157000 found for soluble adenylate cyclase of rat renal cortex by Neer (1974).

(2) *Frontal analysis*. Since it was thought possible that dissociation of high-activity complexes was occurring during gel filtration under conditions of zonal analysis, it was decided to apply the technique of frontal analysis or steady-state gel filtration (Tiselius, 1943; Winzor & Scheraga, 1963, 1964; Burke, 1969) by using large samples of unconcentrated soluble enzyme. The use of gel filtration (molecular sieve chromatography) for the study of interacting protein systems is briefly reviewed by Kellett (1967). The principles of frontal analysis, or steady-state gel filtration, are well described by Burke (1969), who used the technique to study steroid-protein binding.

As a first hypothesis for the study of soluble adenyl-

ate cyclase by frontal analysis, the simplest model proposed assumes that in a soluble preparation of adenylate cyclase there are three molecular species; (i) a high-activity complex (AC) consisting of a catalytic subunit (C) and an activatory subunit (A), (ii) free catalytic subunit of low activity and (iii) free activatory subunit, and that an equilibrium exists such that $AC \rightleftharpoons A + C$. Furthermore, it is assumed that species AC and C correspond to the first and second peaks of activity seen on Sepharose 6B chromatography under conditions of zonal analysis (approx. mol.wts. being 160000 and 45000 respectively). Thus species A and C would be of different molecular weight and therefore of dissimilar mobility during gel filtration.

In zonal analysis, as soon as the sample enters the column the activatory subunit and free catalytic subunit move more slowly than the high-activity complex (AC). The complex, moving into gel not containing species A or C, then dissociates to restore the equilibrium. Dissociation will continue as the complex progresses down the column. It is assumed that the length of the columns used was insufficient to cause complete dissociation of the complex; hence an activity peak corresponding to the complex is still observed.

In frontal analysis, when a large sample of soluble adenylate cyclase is introduced into the column there will be retardation of species C and A relative to AC and dissociation will occur, as before, at the leading edge. However, as more sample enters the gel it would meet high concentrations of both species A and C, which would prevent further dissociation of the complex. The activity of the effluent would therefore gradually increase until it reached a value equal to the activity of the original sample, that is, when a steady state has been reached and the sample is passing through the column unchanged. If the sample is now changed for buffer there will be a fall in eluted activity to a small shoulder or plateau of activity representing low-activity catalytic subunit.

Activity profiles obtained experimentally with large samples of soluble enzyme are shown in Figs. 3 and 4. In Fig. 3 zonal and frontal analysis are compared. With the small sample (2ml) two peaks of activity were evident (Fig. 3a) and the recovery of activity was 24.9%. The larger sample (20ml) gave a small amount of activity in the same position as the first peak obtained with the small sample, followed by a gradual rise of activity to a peak of 175 pmol of cyclic AMP/10 min per 0.5 ml, eluted with approximately the total volume of the column, although a plateau was not attained. The concentration of the activity applied to the column was equivalent to 225 pmol of cyclic AMP/10 min per 0.5 ml. The recovery of activity for the large sample was 57.8%.

Fig. 4 shows the activity profile obtained when a 40 ml sample of soluble adenylate cyclase was

Fig. 3. Sepharose 6B chromatography of small and large samples of soluble enzyme

Adenylate cyclase of p[NH]ppG-treated 600g sediment was solubilized by Lubrol-12A9 as described under 'Methods'. The soluble enzyme was subjected to chromatography on a column (0.9cm diameter $\times$ 52cm long) of Sepharose 6B with two sample volumes: (a) 2ml, (b) 20ml. The soluble enzyme preparation before chromatography contained activity equivalent to 245 ± 15 pmol of cyclic AMP/10min per 500 μ l.

Fig. 4. Frontal (or steady-state) analysis of soluble adenylate cyclase on Sepharose 6B

A sample (40ml) of soluble enzyme prepared from p[NH]ppG-treated, 600g sediment was chromatographed on a column (0.9cm diameter $\times$ 52cm long) of Sepharose 6B. The soluble enzyme preparation before chromatography contained activity equivalent to 78 pmol of cyclic AMP/10min per 500 μ l.

chromatographed on the same column. Several peaks of activity are evident before the activity reaches a plateau, between fractions 50 and 60, of approx. 75 pmol of cyclic AMP/10min per 0.5ml. The concentration of activity applied to the column was 78 pmol of cyclic AMP/10min per 0.5ml and 82.1% of the activity was recovered.

Thus the actual profiles obtained experimentally with large samples were similar to the predicted profile. Indeed, activity did reach a plateau (Fig. 4) and approached values equal to the sample activity. A shoulder of activity on the trailing edge as the sample leaves the column was also observed. Furthermore, recoveries of activity improved with increasing sample size.

The proposed model is more complicated than the system described for the study of steroid-protein binding by Burke (1969), where only two migration rates have to be considered; that is, the steroid-binding proteins and steroid-protein complexes migrate together and the ligand (the steroid) migrates at a lower rate. For the proposed model, the three interacting molecular species migrate at different rates. However, it is possible that more complex interactions than those assumed are occurring if more than three molecular species are involved, all moving down the column at different rates. Such a situation would give multiple peaks of activity, such as were seen (Fig. 4), before a steady state was formed.

The results obtained, although difficult to interpret at this stage, appear to indicate that dissociation of adenylate cyclase complexes does occur during gel filtration, particularly under conditions of zonal analysis, and therefore that the association of two or more components is necessary for the high-activity state. The method of frontal analysis may be useful for the study of the interaction of the three postulated components of the adenylate cyclase complex, the catalytic unit, the guanyl nucleotide-binding component and the hormone receptor.

Ross & Gilman (1977) have suggested that the lability of solubilized adenylate cyclase might be explained by dissociation of two protein components and Pfeuffer (1977) showed that the activity of soluble adenylate cyclase is dependent on the association of two protein fractions, one of which contained the guanyl nucleotide-binding protein.

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