PREPARATION OF A STABLE, HIGHLY-ACTIVE SOLUBILIZED ADENYLATE CYCLASE FROM RAT CEREBELLUM

Derek N. MIDDLEMISS and Trevor J. FRANKLIN

Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG, England

Received 24 April 1975

1. Introduction

The rate of conversion of ATP to cyclic AMP is enhanced by adenylate cyclase (EC 4.6.1.1), an enzyme first described by Rall and Sutherland [1]. Purification of the enzyme from mammalian tissues has been hampered by the fact that it is tightly associated with the plasma membrane [2].

Levey [3] described the use of the non-ionic detergent Lubrol-PX to solubilize adenylate cyclase from mammalian tissues. Attempts to purify this detergent solubilized enzyme have been hindered by the low specific activity of the enzyme in some tissue [4,5] and its inherent instability in the absence of detergent [5–7].

The central nervous system in mammals has the highest activity of adenylate cyclase [6,8] and therefore provides an attractive source material for the purification of the enzyme. The present study reports the partial purification of membranes from rat cerebellum, the subsequent solubilization and stabilization of the adenylate cyclase contained in these membranes and the purification resulting from the passage of the solubilized material through Sepharose 6B. We have been able to obtain a preparation of high specific activity that shows excellent stability in the presence of the non-ionic detergent Lubrol-PX.

2. Materials and methods

Adenylate cyclase activity was measured using a modification of the method of Johnson and Sutherland [6]. Approximately 20 μg of protein was added to 900 μl of a buffer containing 50 mM Tris–HCl pH 7.4, 10 mM KCl, 5 mM $MgCl_2 \cdot 6\,H_2O$, 0.25 mM 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-s-triazolo (1,5-a) pyrimidine (ICI 63, 197 - a potent phosphodiesterase inhibitor), 3 mM dithiothreitol, 0.1% (wt/vol) Lubrol-PX (I.C.I. Limited), 0.1 mM Tris-ATP and an ATP regenerating system consisting of 10 mM creatine phosphate and 15 μg/ml creatine kinase (Boehringer-Mannheim). All incubations were carried out at 37°C for 10 min. The reaction was terminated by the addition of 100 μl of 300 mM HCl and heating at 100°C for 3 min. After neutralization of the reaction mixture with 20 μl of 2 M Tris, the cyclic AMP content of suitable aliquots of the incubation mixture was measured by the method of Brown et al. [9] using cyclic AMP binding protein (British Drug houses).

Protein concentrations of all samples excepting material eluted from the Sepharose 6B columns were determined on material precipitated and washed with 10% trichloroacetic acid by the method of Lowry et al. [10] using bovine serum albumin as standard. Protein concentrations of the Sepharose 6B column eluate were too low in relation to substances which interfere with the development of the colour in the Lowry method and were, therefore, determined by a fluorimetric method [11] using the solubilized membrane proteins as standards.

Inorganic phosphorus was estimated using the method of Yoda and Hokin [12]. Phospholipid content was taken as inorganic phosphorus content X 25.

Solubilized adenylate cyclase was prepared at 4°C as follows: A washed particulate preparation was obtained by low speed centrifugation of an homogenate

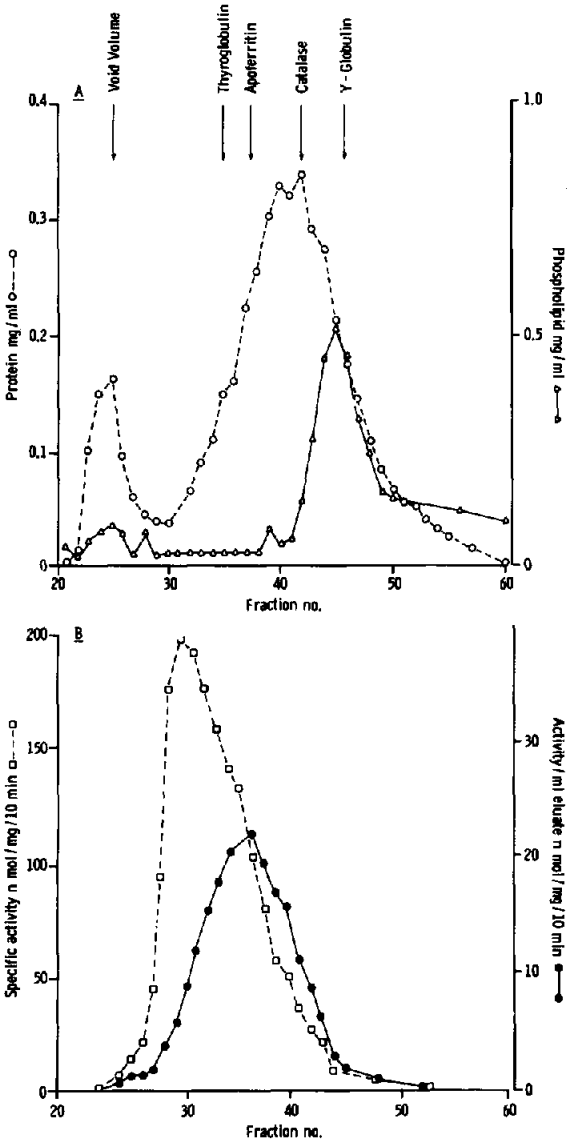

Fig.1. Result of application of 8 ml of solubilized adenylate cyclase to Sepharose 6B column equilibrated with 50 mM Tris–HCl pH 7.4, 250 mM sucrose, 3 mM dithiothreitol, 1% Lubrol-PX. 2.6 ml fractions collected. Protein concentrations were estimated by a fluorimetric method [11]. All adenylate cyclase activity measurements were performed in duplicate. For further details see text.

of the cerebella from 15 to 20 rats (200–500 g, Alderley Park Strain) [6]. This particulate preparation was re-suspended in 18 ml of homogenizing medium [6] and spun at 300 *g* for 5 min. The resulting supernatant was divided into three and each portion carefully layered onto a linear sucrose gradient (vol. 26 ml, 20%–55% sucrose (wt/vol), also containing 100 mM glycylglycine buffer pH 7.4, 2 mM $MgCl_2 \cdot 6\,H_2O$, 1 mM EDTA and 3 mM dithiothreitol). The three gradients were spun at 63 581 g_{av} for 135 min in a Spinco SW 25.1 rotor. The adenylate cyclase activity banded between 36%–45% sucrose. This layer was removed by aspiration, the layers from the three gradients combined and made to 1% with respect to Lubrol-PX by the addition of a suitable volume of 20% detergent solution. After 5 strokes with a loose fitting glass–teflon homogenizer, the preparation was spun at 100 000 g_{av} for 60 min in a Spinco 50Ti rotor. The supernatant was denoted 'solubilized adenylate cyclase'.

Further purification of the solubilized adenylate cyclase was achieved by the application of 8 ml of the material to a Sepharose 6B (Pharmacia) column (2.8 × 35 cm) equilibrated with 50 mM Tris–HCl buffer pH 7.4 containing 250 mM sucrose, 3 mM dithiothreitol and 1% Lubrol-PX. The flow rate was 15 ml/hr and 2.6 ml fractions were collected. The column was calibrated with the proteins indicated in fig.1A. A linear relationship was obtained between the logarithms of the molecular weights of the proteins and the volume of buffer required to elute them from the column. Estimation of the phosphorus content of the fractions showed that most of the phospholipid was divorced from the adenylate cyclase activity (fig.1A).

3. Results

The results of a typical preparation on the sucrose gradient are shown in table 1. Johnson and Sutherland [6] found that the activity of particulate adenylate cyclase from rat cerebellum is markedly stimulated by Lubrol-PX, an observation confirmed by us (not shown) The activity of all samples was, therefore, determined in the presence of 0.1% Lubrol-PX as indicated in Materials and methods. The specific acitivity was increased approximately 4½-fold by this sucrose gradient step.

The purification achieved on a Sepharose 6B column is indicated in fig.1. A mol. wt of 10^6 was

Table 1

	Total protein mg/g wet wt.	% Original activity	Total activity nmol/g wet wt./10 min	% Original activity	Specific activity nmol/mg/10 min
Original homogenate	107.5	100	1100	100	10.2
Before sucrose density gradient	34.0	31.5	415	38	12.2
After sucrose density gradient	13.9	12.9	340	31	24.6
Solubilized adenylate cyclase	7.9	7.4	373	34	47.5

indicated for the solubilized adenylate cyclase in the presence of 1% Lubrol-PX. The highest specific activity achieved was 195 nmol/mg/10 min which represents a 19-fold purification over the detergent-stimulated particulate enzyme.

The solubilized adenylate cyclase from the sucrose gradient showed no loss of activity when stored at −20°C for several weeks and there was no loss in activity on repeated freeze-thawing. Sucrose is essential for maintaining stability to repeated freeze-thawing. The preparation was also completely stable for at least five days at 4°C.

The adenylate cyclase eluted from the Sepharose 6B column was also stable indefinitely at −20°C. At 4°C, at least 75% of the activity remained after five days in the presence of 250 mM sucrose, 1% Lubrol-PX, 50 mM Tris–HCl pH 7.4 and 1 mM dithiothreitol.

4. Discussion

Previous attempts to purify adenylate cyclase have foundered on the difficult problem of stabilizing the solubilized enzyme. Johnson and Sutherland [6] reported a relatively unstable preparation from rat cerebellum. The stability of their preparation was increased by the inclusion of dithiothreitol and bovine serum albumin in the homogenizing medium. We have found that more extensive purification of the membranes on a sucrose gradient permits the solubilization of adenylate cyclase of very high specific activity and with much improved stability. The membranes which band at these sucrose densities have been associated with synaptic membranes [13]. We have found that the plasma membrane marker enzymes 5′-nucleotidase and alkaline phosphatase are associated with the purified membranes (results not shown). A significant increase in specific activity of adenylate cyclase was achieved by gel filtration in the presence of a buffer, sucrose, dithiothreitol and detergent. About 60% of the activity applied to the column was recovered from the eluate. The stability of the fractions with highest specific activity (180 to 200 nmol/mg/10 min) is adequate for further purification steps to be undertaken. The slight decrease in stability of these fractions as compared to the 'solubilized adenylate cyclase' may derive from their greater dilution and/or from the absence of phospholipid (5,7). The adenylate cyclase from rat cerebellum appears as a single peak in contrast to the results of Neer [14] who described two different molecular weight species from solubilized rat renal medulla. Swislocki and Tierney [7] reported that the stability of a solubilized adenylate cyclase was improved by fluoride and magnesium ions. Our study indicates that a stable, solubilized enzyme can be obtained without recourse to the stimulatory fluoride ion. Thus, our preparation may prove more convenient for further purification. The specific activity of the enzyme eluted from Sepharose 6B is the highest yet described with the possible exception of the 5′-guanylimidodiphosphate stimulated enzyme [15] and should provide a useful preparation for the further study of the effect of activators on the adenylate cyclase system.

Acknowledgements

Dr D. N. Middlemiss is grateful to the Pharmaceuticals Division of Imperial Chemical Industries Limited, for a Postdoctoral Fellowship.

References

[1] Rall, T. W. and Sutherland, E. H. (1962) J. Biol. Chem., 232, 1065–1076.
[2] Perkins, J. P. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G. A., eds.), Vol.3, pp. 1–64, Raven Press, New York.
[3] Levey, G. S. (1970) Biochem. Biophys. Res. Comm. 38, 86–92.
[4] Franks, D. J. and Malamud, D. (1974) Second International Conference on Cyclic AMP. p. 40.
[5] Sulimovici, S., Bartoov, B. and Lunenfeld, B. (1975) Biochim. Biophys. Acta, 377, 454–462.
[6] Johnson, R. A. and Sutherland, E. W. (1973) J. Biol. Chem. 248, 5114–5121.
[7] Swislocki, N. I. and Tierney, J. (1973) Biochemistry, 12, 1862–1866.
[8] Sutherland, E. W., Rall, T. W. and Menton, T. (1962) J. Biol. Chem. 237, 1220–1227.
[9] Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. and Tampion, W. (1971) Biochem. J. 121, 561–562.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Resch, K., Imm, W., Ferber, E., Wallach, D. F. H. and Fischer, H. (1971) Naturwissenschaften, 58, 220.
[12] Yoda, A. and Hokin, L. E. (1970) Biochem. Biophys. Res. Comm. 40, 880–886
[13] DeRobertis, E., Arnaiz, G. R. D. L., Alberici, M., Butcher, R. W. and Sutherland, E. W. (1967) J. Biol. Chem. 242, 3487–3493.
[14] Neer, E. J. (1974) J. Biol. Chem. 249, 6527–6531.
[15] Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. and Rodbell, M. (1974) Proc. Natl. Acad. Sci. USA, 71, 3087–3090.