SOLUBILIZATION BY PROTEOLYSIS OF AN ACTIVATED FORM OF RAT LIVER MEMBRANE GUANYLATE CYCLASE

Marie-Lise LACOMBE, Rosine HAGUENAUER-TSAPIS, Dominique STENGEL, Abderraouf BEN SALAH and Jacques HANOUNE

Unité de Recherches INSERM U-99, Hôpital Henri Mondor, 94010 Créteil, France

Received 20 May 1980

1. Introduction

Guanylate cyclase has been found associated with both the supernatant and particulate fractions of almost all animal tissues examined [1–4]. Among the activators of the enzyme described [1,2], none has been clearly proved to be of physiological importance [2]. The membrane-bound guanylate cyclase activity from rat liver was markedly increased by proteolysis [5], while the activity of the cytosolic form remained unchanged or inhibited. Here we report that this proteolytic activation is accompanied, as well, by the solubilization of the enzyme as indicated by its presence in the 165 000 × g supernatant. The adenylate cyclase of the same preparation although activated by proteolysis [6–8] remains membrane-bound. The solubilized guanylate cyclase (app. M_r ~140 000) thus appears as the hydrophilic moiety of the membrane enzyme which can easily be split off from its hydrophobic anchor by proteolysis.

2. Materials and methods

Purified proteases, nucleotides and other chemicals were obtained as in [5,8].

2.1. *Preparation and proteolysis of plasma membranes*

Rat liver plasma membranes were prepared according to [5,9]. Proteolysis of liver membrane suspensions (2 mg membrane protein/ml, 10 mM Tris–HCl, pH 7.5) by trypsin (0–25 μg enzyme/mg membrane protein) or α-chymotrypsin (0–250 μg enzyme/mg membrane protein) was performed at 30°C for up to 25 min as indicated in the figure legends. The reaction was stopped by the addition of a 5-fold excess (w/w) of soybean trypsin inhibitor. The soybean trypsin inhibitor, although not altering the activity of the cyclizing enzymes, was capable of completely blocking both the activation and solubilization processes. After proteolysis, the membrane suspension was divided by centrifugation (Spinco rotor 50 Ti, 165 000 × g_{av}, 1 h or Beckman Airfuge, 120 000 × g_{av}, 11 min) into a supernatant fraction and a membrane containing pellet. The pellet was resuspended in the initial volume of buffer. Prior to or following proteolysis, the membrane suspension and the supernatant and resuspended pellet fractions were analyzed for protein [10], guanylate cyclase or adenylate cyclase activities.

2.2. *Guanylate and adenylate cyclase assays*

Guanylate cyclase activity was determined and cyclic GMP isolated as in [11], with some minor modifications [12]. Unless otherwise indicated in the figure legends, the reaction mixtures (50 μl total vol.) contained 50 mM Tris–HCl (pH 7.5), 1 mM [α-^{32}P]-GTP (1–3 × 10^6 cpm), 1 mM cyclic [8-^{3}H]GMP (10^4 cpm), 5 mM DTT, 1.5 mM $MnCl_2$ and a regenerating system (10 mM creatine phosphate and 1 IU phosphocreatine kinase). Incubations were initiated by the addition of aliquots of the enzyme solution to be assayed and continued for 20 min at 30°C. Under these experimental conditions, the activity of the membrane-bound guanylate cyclase was found to be linear as a function of time up to 40 min with up to 10 μg protein/assay. Adenylate cyclase activity was measured as in [8]. The assay mixture (60 μl total vol.) contained 0.5 mM [α-^{32}P]ATP (1–2 × 10^6 cpm), 3 mM $MgCl_2$ or 2 mM $MnCl_2$, 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris–HCl (pH 7.6) an ATP regenerating system consisting of 25 mM phos-

phocreatine and 1 mg/ml creatine phosphokinase. Incubation was initiated by the addition of aliquots of the enzyme solution to be assayed and was performed for 10 min at 30°C.

2.3. *Sucrose density gradient centrifugation*

The proteolytically solubilized guanylate cyclase (0.3 ml containing 0.2 mg protein) was applied to the top of a 4.8 ml gradient prepared from 5% and 20% sucrose solutions in 50 mM Tris–HCl, 1 mM DTT, (pH 7.5) and centrifuged at 4°C in a Beckman SW50.1 rotor at 38 000 rev./min for 17 h. All following steps, including sedimentation coefficient estimation were as in [13].

2.4. *AcA 34 Ultrogel column filtration*

The proteolytically solubilized guanylate cyclase preparation containing 1 mg protein in 1 ml was layered onto an AcA 34 Ultrogel column (1.6 X 40 cm) pre-equilibrated with 50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT and 0.1 M NaCl. The column was then eluted at 4°C with the same buffer at 6 ml/h flowrate. Fractions of 0.3 ml were collected. Calibrating proteins detection and Stokes radius estimation were as in [13].

3. Results

Solubilization of the membrane-bound guanylate cyclase was found to depend upon the trypsin concentration although not following a course parallel with activation (fig.1a,b). While half-maximal activation was obtained with 1.25 μg trypsin/mg membrane protein, virtually no guanylate cyclase was released from the membrane. Maximal activation (3-fold) occurred with 5 μg trypsin/mg protein and maximal solubilization (~60%) with 10 μg trypsin/mg protein. At high concentrations, trypsin inhibited guanylate cyclase, probably due to proteolytic degradation of

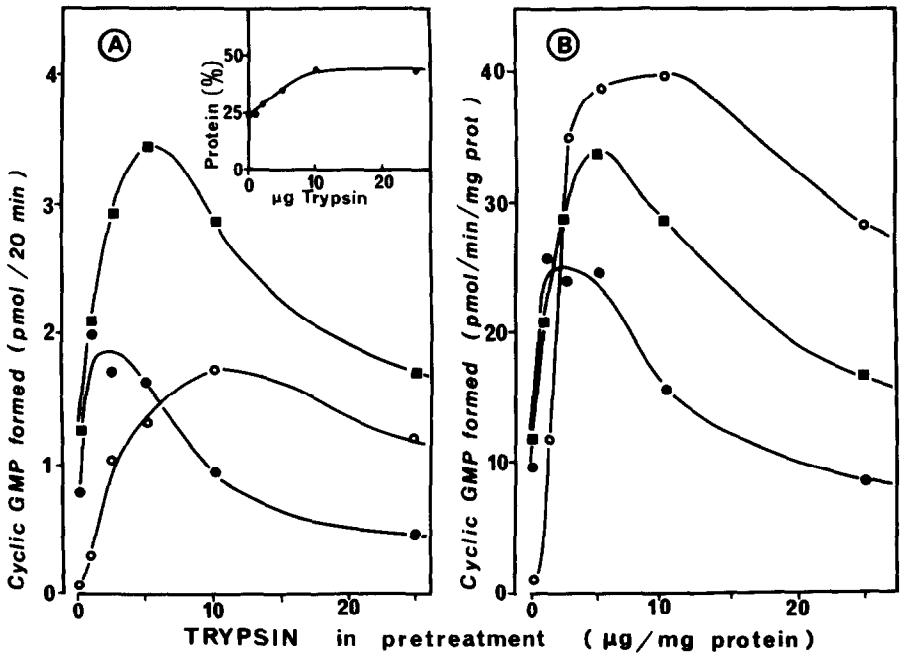

Fig.1. Effect of increasing concentrations of trypsin upon activation and solubilization of guanylate cyclase from rat liver plasma membranes. Membranes (2 mg protein/ml) were preincubated as in section 2 in the presence of increasing concentrations of tyrpsin for 5 min at 30°C. The supernatant (○) and resuspended pellet (●) fractions and the initial membrane solution (■) were either assayed for protein content or assayed after a 10-fold dilution for guanylate cyclase activity (30 μl aliquots) as in section 2. Each value results from triplicate determinations and agrees within ±5%. Results are expressed in (A) as pmol cyclic GMP formed/assay at 30°C in 20 min, and in (B) as pmol formed . min^{-1} . mg $protein^{-1}$. The inset of (A) depicts the amount of protein which is recovered in the supernatant-fraction during the same pretreatment. It is represented as percent of the total protein content of the initial membrane solution.

the catalytic site. At all concentrations the sum of the guanylate cyclase activities of the supernatant and resuspended pellet fractions corresponded to that of the original membrane suspension. Preincubation of the membranes in the absence of trypsin neither altered nor solubilized the guanylate cyclase activity but did solubilize ~20% of the membrane protein (fig.1a, inset). Trypsin treatment solubilized almost 50% of the membrane protein. In fig.1b, the results are expressed as specific activity in the different fractions. The specific activity of guanylate cyclase of the supernatant was increased 1.4-fold as compared to the corresponding activated membrane, and 3.3-fold as compared to the initial membrane activity.

The time-course of solubilization of guanylate cyclase by trypsin (fig.2) was slow, reaching its maximum after 15 min proteolysis as compared to the activation process, which attained its optimum after 2 min treatment. As in fig.1, the total activity of the supernatant and pellet reflected the activity initially present in the membrane suspension. Furthermore, solubilization occurred linearly with time until the maximum (~70% of the total membrane activity) was attained. Routine solubilization conditions for guanylate cyclase (incubation with 5 μg trypsin/mg membrane protein for 5 min at 30°C) were chosen on the basis of the above results to give a maximum yield of the enzyme with a minimum amount of proteolytic degradation.

As in [6–8], rat liver membrane adenylate cyclase activity was also activated by some proteolytic enzymes, in particular α-chymotrypsin, but not by trypsin. When α-chymotrypsin was used instead of trypsin for proteolysis of rat liver membranes (fig.3), maximal activation of adenylate cyclase (fig.3b) was obtained at 2.5–5 μg enzyme/mg membrane protein while for guanylate cyclase (fig.3a) 25 μg enzyme/mg protein was required. However, up to 80% of the total membrane guanylate cyclase activity could be solubilized by α-chymotrypsin (at 250 μg/mg membrane protein) but none of the adenylate cyclase activity was found in the supernatant when assayed in its basal (fig.3b) or NaF stimulated (not shown), state using either Mg-ATP (fig.3b) or Mn-ATP (not shown).

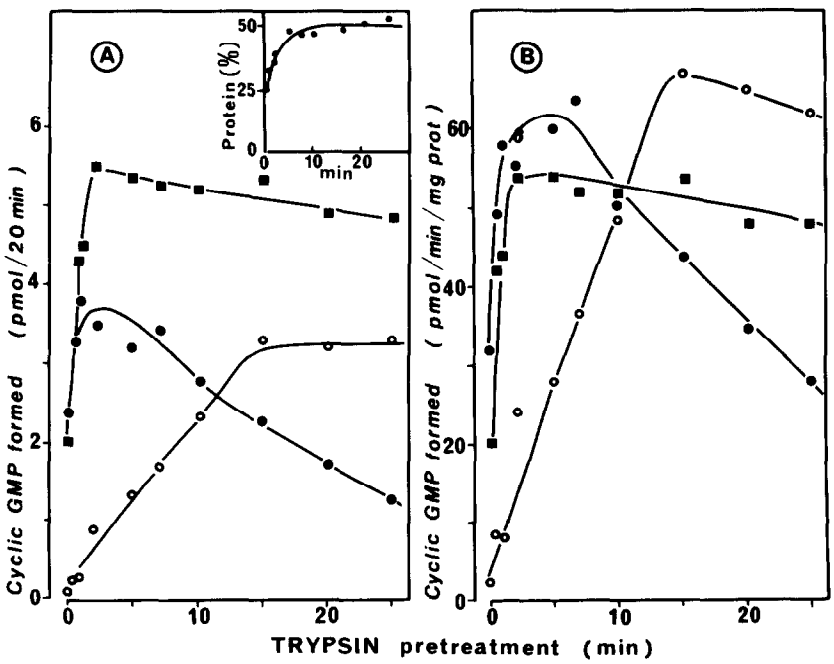

Fig.2. Time course of the trypsin effect upon the activation and the solubilization of guanylate cyclase from rat liver plasma membranes. Membranes (2 mg protein/ml) were preincubated as in section 2 for varying times at 30°C in the presence of 0.5 μg trypsin/mg membrane protein. Preincubation was stopped and tubes were treated as in fig.1 and guanylate cyclase activity of total membrane solution (▪), of the supernatant (○) and of the resuspended pellet (•) was measured as in section 2. Results are expressed in (A) as pmol cyclic GMP formed/assay at 30°C in 20 min, and in (B) as pmol formed . min^{-1} . mg $protein^{-1}$. The inset of (A) depicts the amount of protein recovered in the supernatant fraction during the pretreatment.

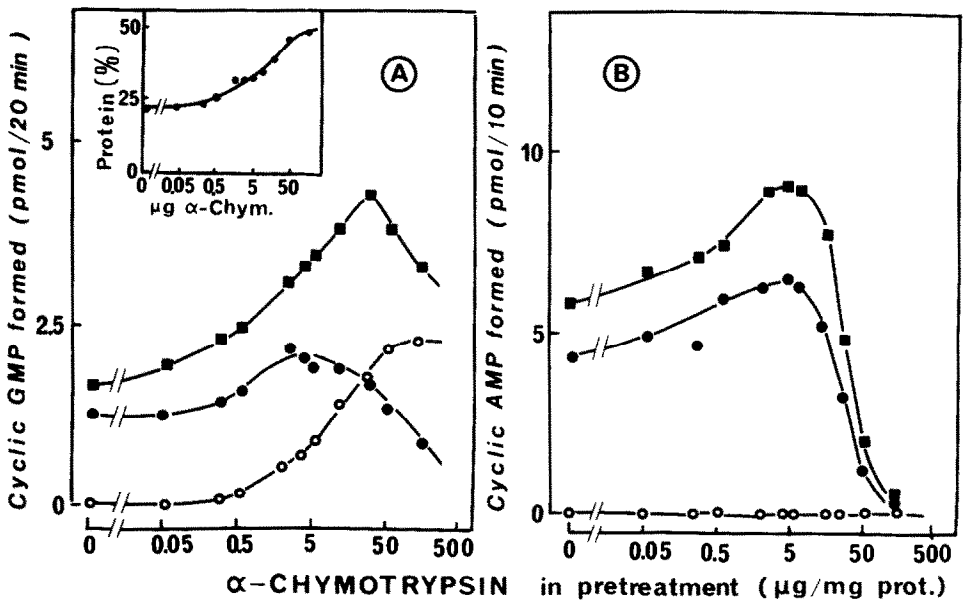

Fig.3. Effect of increasing concentrations of α-chymotrypsin upon activation and solubilization of guanylate cyclase and adenylate cyclase activities of rat liver plasma membranes. Membranes were preincubated as in section 2 in the presence of increasing concentrations of α-chymotrypsin for 5 min at 30°C. Preincubation was stopped and tubes were treated as in fig.1 and section 2. For adenylate cyclase assay enzyme solutions were diluted 2-fold (20 μl aliquots). Guanylate (A) and adenylate (B) cyclase activities of total membrane solution (▪) and of supernatant (○) and resuspended pellet (●) fractions were assayed at 30°C for 20 and 10 min, respectively. Results are expressed as pmol cyclic GMP or AMP formed/assay at 30°C in 20 or 10 min, respectively. The inset of (A) depicts the amount of protein recovered in the supernatant fraction.

The guanylate cyclase solubilized following proteolysis sedimented in a 5–20% linear sucrose gradient giving an $s_{20,w}$ of 6.4 S (fig.4a). Upon gel filtration the enzyme was found to have a Stokes radius of 5.3 nm (fig.4b).

4. Discussion

Proteolytic treatment of plasma membranes is known to selectively activate adenylate cyclase in rat liver [6–8], rat ovary [14], rat cerebral cortical membranes [15] and cultured fibroblasts [16,17]. Rat liver membrane guanylate cyclase was also activated by proteolysis [5]. This activation was clearly restricted to the particulate form of the enzyme; attempts to activate the cytosolic form of the enzyme from the liver [5] have been unsuccessful.

These results demonstrate that the activation of the membrane-bound guanylate cyclase is accompanied, as well, by the solubilization of the enzyme. This is in marked contrast to adenylate cyclase which, although activated by proteolysis, is not solubilized. The solubilized guanylate cyclase could be recovered by centrifugation without the use of detergents. Preliminary biochemical characterization gave an $s_{20,w}$ of 6.4 S and a Stokes radius of 5.3 nm, thus allowing an estimation of an app. M_r ~140 000 (assuming $\bar{v}$ = 0.735 ml/g as for typical globular proteins). Similar molecular weights have been reported for the cytosolic guanylate cyclase from rat liver 150 000 [18], rat renal medulla 154 000 [19], and rat lung 151 000 [20].

Solubilization and activation appeared to be independent processes. Activation always preceded solubilization; it occurred at lower protease concentrations or could occur even without solubilization of the guanylate cyclase. This might indicate that proteolysis triggered a multi-step reaction with activation occurring at an earlier step and solubilization at a later one. Or, proteolysis might act at two different sites, one responsible for activation and the other for solubilization. The membrane guanylate cyclase solubilized by detergents was still activated by proteol-

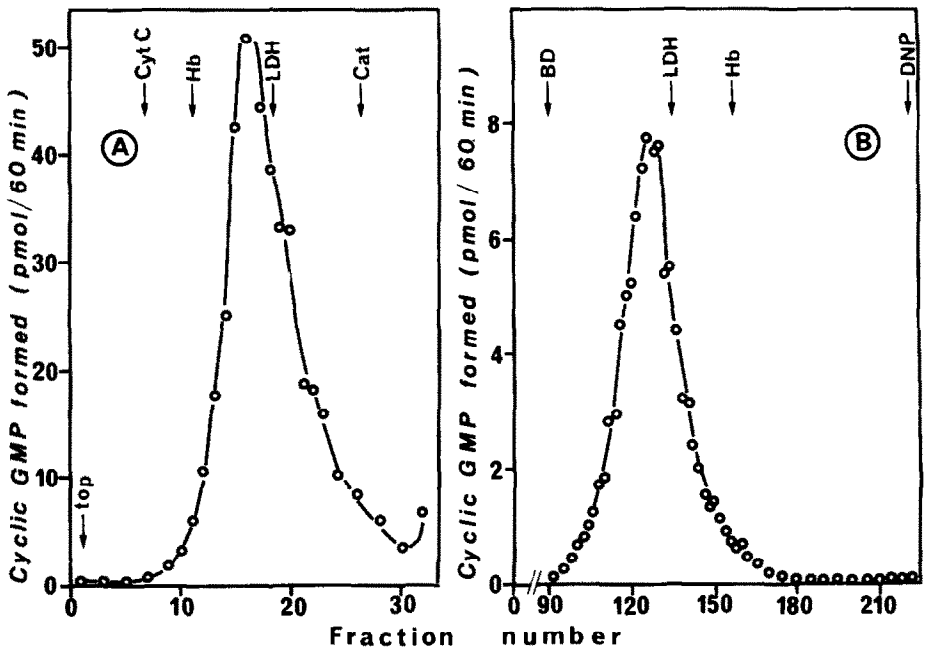

Fig.4. (A,B) Sedimentation in sucrose gradient (A) and elution pattern through an Ultrogel AcA34 column (B) of the proteolytically solubilized guanylate cyclase. (A) Proteolytically solubilized guanylate cyclase was prepared and centrifuged through a 5–20% sucrose gradient as in section 2. Aliquots (30 μl) of each fraction were assayed in duplicate for guanylate cyclase activity as in section 2 in the presence of 5 mM $MnCl_2$ for 60 min at 37°C. Guanylate cyclase activity remained linear even at 37°C, due to the absence of solubilization of any GTPase activity (F. Pecker, S. Lotersztajn, J. H., unpublished). Arrows refer to the position of marker proteins which were run in parallel (Cyt *c*, cytochrome *c*; Hb, human hemoglobin; LDH, lactate dehydrogenase; Cat, catalase). (B) The proteolytically solubilized fraction was prepared and filtered through an AcA 34 Ultrogel column as in section 2. Aliquots (30 μl) of each fraction (0.33 ml) were assayed in duplicate for guanylate cyclase activity for 60 min at 37°C under the conditions in section 2 in the presence of 5 mM $MnCl_2$. Void volume was determined by blue dextran (BD) and total volume by dinitrophenol glycine (DNP). Arrows refer to the position of marker proteins which were eluted in parallel.

ysis [5]. This might suggest that proteolytic activation involves a site or subunit very tightly linked to the catalytic site.

Several membrane bound enzymes and proteins can be solubilized by proteolysis but without activation (reviewed [21]). That proteases liberate a hydrophilic form of guanylate cyclase bearing the catalytic site, suggests that guanylate cyclase is also an amphipathic molecule which largely emerges from the membrane bilayer. Activation and solubilization by proteolysis seem to be rather general properties of the particulate guanylate cyclases since we recently found them with the microsomal enzymes of lung and testis of the rat. That trypsin solubilizes particulate guanylate cyclase from sea urchin sperm was briefly alluded in [22]. A very attractive hypothesis is that the activity of the membrane-bound guanylate cyclase and/or the subcellular distribution of the enzyme could be altered by some endogenous protease(s), in a way similar to the exogenous ones described here. This would imply that there is a direct relationship between the two naturally occurring guanylate cyclases: the membrane bound and the cytosolic forms. Experiments to assess this possibility are now under way.

Acknowledgements

We are greatly indebted to Professor P. Berthelot and Dr L. Aggerbeck for their critical reading of the manuscript and to C. Petit for her skillful secretarial assistance. This investigation was supported by INSERM and by DGRST. R. H.-T. is on leave from the laboratory of Professor A. Kepes (IRBM, Paris).

References

[1] Goldberg, N. D. and Haddox, M. K. (1977) Ann. Rev. Biochem. 46, 823–896.
[2] Murad, F., Arnold, W. P., Mittal, C. K. and Braughler, J. M. (1979) Adv. Cyclic Nucl. Res. 11, 175–204.
[3] Ishikawa, E., Ishikawa, S., Davis, J. W. and Sutherland, E. W. (1969) J. Biol. Chem. 244, 6371–6376.
[4] Kimura, H. and Murad, F. (1975) Life Sci. 17, 837–844.
[5] Lacombe, M. L. and Hanoune, J. (1979) J. Biol. Chem. 254, 3696–3699.
[6] Hanoune, J., Stengel, D., Lacombe, M. L., Feldmann, G. and Coudrier, E. (1977) J. Biol. Chem. 252, 2039–2045.
[7] Lacombe, M. L., Stengel, D. and Hanoune, J. (1977) FEBS Lett. 77, 159–163.
[8] Stengel, D., Lacombe, M.-L., Billon, M.-C. and Hanoune, J. (1979) FEBS Lett. 107, 105–109.
[9] Neville, D. M. (1968) Biochim. Biophys. Acta 154, 540–552.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Levilliers, J., Pairault, J., Lecot, F., Tournemolle, A. and Laudat, M.-H. (1978) Eur. J. Biochem. 88, 323–330.
[12] Lacombe, M. L., Stengel, D., Haguenauer-Tsapis, R. and Hanoune, J. (1979) in: Proteases and hormones (Agarwall, M. K. ed) pp. 227–302, Elsevier/North-Holland, Amsterdam, New York.
[13] Stengel, D. and Hanoune, J. (1979) Eur. J. Biochem. 102, 21–34.
[14] Richert, N. D. and Ryan, R. J. (1977) Proc. Natl. Acad. Sci. USA 74, 4857–4861.
[15] Partington, C. R. and Daly, J. W. (1979) Arch. Biochem. Biophys. 198, 255–262.
[16] Anderson, W. B., Jaworski, C. J. and Vlahakis, G. (1978) J. Biol. Chem. 253, 2921–2926.
[17] Wallach, D., Anderson, W. and Pastan, I. (1978) J. Biol. Chem. 253, 24–26.
[18] Braughler, J. M., Mittal, C. K. and Murad, F. (1979) Proc. Natl. Acad. Sci. USA 76, 219–222.
[19] Neer, E. J. and Sukiennick, E. A. (1975) J. Biol. Chem. 250, 7905–7909.
[20] Garbers, D. L. (1979) J. Biol. Chem. 254, 240–243.
[21] Desnuelle, P. (1979) Eur. J. Biochem. 101, 1–11.
[22] Garbers, D. L., Suddath, J. L. and Hardman, J. G. (1975) Biochim. Biophys. Acta 377, 174–185.