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CONVERSION OF BASAL 6.0 S ADENYLATE CYCLASE INTO 7.4 S BY GUANYL NUCLEOTIDE TREATMENT OF MEMBRANE BOUND ENZYME*

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Summary : Membrane-bound adenylate cyclase from pig kidney medulla was pretreated with either guanyl-5'-yl-imidodiphosphate (Gpp(NH)p) or NaF and solubilized in the presence of Triton X100 (0.7 % v/v). Pretreatment by Gpp(NH)p, but not by NaF, shifted the standard sedimentation constant of the solubilized enzyme from 6.0 S to 7.4 S. This effect depended on the Gpp(NH)p concentration used and correlated to the nucleotide-induced increase in adenylate cyclase catalytic activity. Both the 6.0 S and 7.4 S activity peaks had similar Stokes radii.

Reconstitution of guanyl nucleotide-sensitive adenylate cyclase was obtained by mixing catalytic units and nucleotide binding proteins previously separated by various techniques (1-4). It has been suggested that the resulting activated adenylate cyclase might be a complex composed of a catalytic unit and a nucleotide-binding protein, but there is controversy about the molecular size of the latter. The components characterized respectively by Pfeuffer (1) and Ross et al (3) had molecular weights of 42,000 and 130,000. On the other hand, it had been respectively shown that activation of membraneous adenylate cyclase by Gpp(NH)p [1], a non hydrolyzable GTP analogue, is at least partially preserved during membrane solubilization by non ionic detergents (5). The same applies to NaF-stimulated-adenylate cyclase (6). The results of the experiments reported in this article indicate that pretreatment of pig kidney membranes by Gpp(NH)p but not by NaF increased the standard sedimentation constant of solubilized adenylate cyclase.

Materials and Methods : Sources of the most materials used were specified in an earlier report (7). Gpp(NH)p was obtained from Boehringer Mannheim and D_2O from

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Enzyme : Adenylate cyclase or ATP pyrophosphate-lyase (cyclizing) (EC 4.6.1.1.)

[1] Abbreviations used :
Gpp(NH)p = Guanyl-5'-yl imidodiphosphate
Cyclic AMP = Adenosine 3' - 5' cyclic monophosphate

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C.E.A. (Saclay, France). Pig kidney medullary plasma membranes were prepared as already described (8). Membranes (6 mg protein/ml) were incubated in the presence of either Gpp(NH)p (from 1 nM to 0.1 mM) for 30 min at 30°C or of NaF (10 mM) for 10 min at 4°C. Control membranes were incubated under the same conditions, except for the presence of either Gpp(NH)p or NaF. Adenylate cyclase was solubilized by incubating membranes (2.6 mg protein/ml) for 45 min at 4°C in a medium containing 1.8 mM Tris-HCL, pH 8, 1 mM $MgCl_2$, 0.4 mM EDTA, 0.7 % (v/v)Triton X100 and 300 mM NaCl. The sample was then centrifuged at 20,000 xg for 5 min at 4°C and the supernatant collected. As previously described, more than 95 % of adenylate cyclase activity from detergent-treated membranes were recovered from the supernatant fraction under these conditions (7).

Sucrose density gradient ultra-centrifugation of soluble extracts: Linear gradients (6.2 ml) were prepared using an ISCO gradient former from 3 and 10% or 3 and 15 % sucrose solutions in H_2O or D_2O (final concentration : 98 %). Sucrose solutions were buffered with 100 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 0.02 % (w/v) NaN_3, 0.1 % (v/v) Triton X100 and 0.1 % (v/v) β-mercapto ethanol. 300 µl aliquots of solubilized membranes were layered on top of the gradient together with cytochrome C, alcohol deshydrogenase, catalase and β-galactosidase. Each tube was supplemented with 5 ml of vaseline oil and centrifugation was carried out in a Spinco model L 265B at 41,000 rpm at 4°C (Rotor SW41 Ti) for 7 hours. 200 µl fractions were successively collected from the gradients using an ISCO density gradient fractionator. Sedimentation constant, calibration curves were determined according to Martin and Ames (9). Partial specific volume was calculated as described by Meunier et al (10).

Gel filtration of solubilized material : Ultrogel ACA_{34} or Sepharose 4B columns (1.6 x 40 cm) were equilibrated with an elution buffer identical to the one used for the sucrose density gradients except for the sucrose concentration which was 75 mM. An aliquot of solubilized material (1 ml) was layered on the top of the column together with the following marker molecules : β-galactosidase, catalase, cytochrome C, alcohol deshydrogenase, tritiated water and phage T4. Flow rate was 10 ml per hour. Fraction volume was 1 ml. Stokes radii determinations were performed according to Laurent and Killander (11).

Calibration proteins : Assay conditions and values of the hydrodynamic parameters of β-galactosidase (E. Coli), alcohol deshydrogenase (yeast) and catalase (beef liver) were those used by Meunier et al (10). Cytochrome C (horse heart) was determined spectrophotometrically by its absorption at 541 nm. Its hydrodynamic parameters were those used by Sober (12) and Margoliash et al. (13).

Adenylate cyclase Assay: Adenylate cyclase activity was measured by conversion of [α-^{32}P]ATP into cyclic [^{32}P]AMP. The incubation medium (50 µl) contained 100 mM Tris-HCl pH 8, 1 mM cyclic AMP, 10 µM [α-^{32}P] ATP (6,000 cpm/pmole), 10 mM $MgCl_2$ or 1.5 mM $MnCl_2$, 0.33 mM EDTA, 1 mg/ml creatine kinase , 10 mM creatine phosphate and 0.08%(v/v) Triton X100 introduced together with the enzyme. The reaction was started by addition of 40 µl of column eluate or gradient fraction and was allowed to proceed for 10-15 min at 30°C. It was stopped as previously indicated (14) and cyclic AMP was separated from [α -^{32}P] ATP as described by Salomon et al (15).

Results and Conclusions : Figure 1 shows sedimentation profiles for adenylate cyclase activities solubilized from control membranes and membranes maximally stimulated by Gpp(NH)p or NaF. Note that firstly, solubilization and ultracentrifugation preserved enzyme activation by Gpp(NH)p or NaF ; secondly, pretreatment by Gpp(NH)p, but not by NaF increased the apparent sedimentation constant of solubilized adenylate cyclase in sucrose-H_2O gradients ; thirdly the Gpp(NH)p -induced increase in the apparent sedimentation constant was less pronounced when the experiment was performed using a sucrose-D_2O gradient. The latter observation suggests that Gpp(NH)p treatment also induced a change in the partial spe-

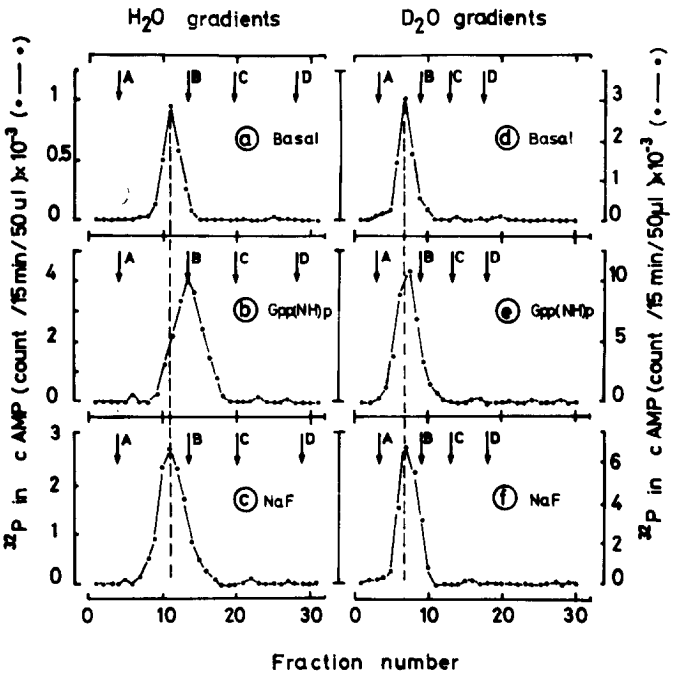

Figure 1 : Sedimentation profiles of adenylate cyclase activity solubilized from control and Gpp(NH)p-or NaF-treated membranes. Pig kidney membranes were incubated in the presence of 0.10 mM Gpp(NH)p (b and e), 10 mM NaF (c and f) as described in Methods, or without effector (a and d). Solubilization was then obtained by the use of Triton X100 (0.7 %) (see Methods). Soluble extracts and protein markers were layered together on 3-15 % sucrose H_2O (left part) or sucrose D_2O (right part) gradients. Centrifugation lasted 7 hours at 4° (41, 000 rpm). Adenylate cyclase activity and protein markers were assayed on each gradient fraction. $MgCl_2$ was the source of divalent cations in the incubation medium. Arrows indicate marker protein activity peaks (A = cytochrome C ; B = alcohol deshydrogenase ; C = catalase ; D = β-galactosidase).

cific volume of adenylate cyclase which might be due to the binding of additional detergent to the enzyme. Figure 1 further shows that the activities measured in the presence of D_2O are about three times higher than those measured in the presence of H_2O. We have no explanation for this observation. In any case, the relative increases in activity due to Gpp(NH)p or NaF pretreatments were similar in both experimental conditions. The Gpp(NH)p-induced increase in the apparent sedimentation constant of solubilized adenylate cyclase is further evidenced by the experiment shown in Fig. 2. A mixture of equal amounts of adenylate cyclase activities from control and Gpp(NH)p-treated membranes sedimented in two distinct peaks. These peaks corresponded to those obtained when soluble extracts from control and treated membranes were centrifuged separately. Two peaks of activity were also found when soluble extracts of partially stimulated membranes were centrifuged in sucrose density gradients. The relative amounts

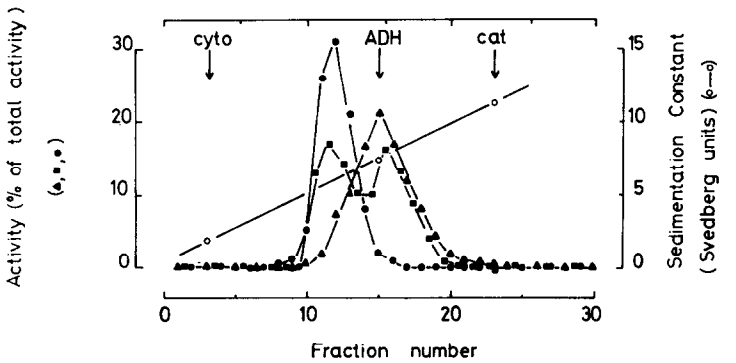

Figure 2 : Resolution in two distinguishable peaks of control and Gpp(NH)p - activated forms of renal adenylate cyclase. Pig Kidney membranes were incubated with or without Gpp(NH)p (0.1 mM) and then solubilized (see Methods). 300 µl of Gpp(NH)p-treated soluble enzyme (▲-▲), untreated soluble enzyme (●-●) and a mixture of these two soluble enzyme preparations (1 vol of Gpp(NH)p-treated enzyme + 7 vol of control enzyme) (■-■) were layered together with marker proteins (cytochrome, alcohol deshydrogenase and catalase) on top of a 3-10 % H_2O sucrose density gradient. After ultracentrifugation (see Methods) gradients were collected and adenylate cyclase activity was measured on each fraction in the presence of 1.5 mM $MnCl_2$. The adenylate cyclase activity in each gradient fraction was expressed as the percent of the total activity recovered on the corresponding gradient. Sedimentation constant calibration curves (o-o) were determined for each gradient (see Methods). Arrows indicate migration peaks of the protein markers used.

of activity included under each peak could be estimated. Fig. 3 shows the parallel evolution of the relative amount of activity recovered in the 7.4 S peak and the increase in solubilized enzyme activity due to Gpp(NH)p treatment of membranes. Mean values of the hydrodynamic parameters of adenylate cyclase solubilized from control and from Gpp(NH)p or NAF-stimulated membranes are given in Table I. Despite the fact that enzyme activation by Gpp(NH)p was preserved during enzyme filtration on Sepharose 4B columns, the increase in the standard sedimentation coefficient and partial specific volume observed with the Gpp(NH)p -activated form of the enzyme is not accompanied by any change in the Stokes radius of the enzyme. The molecular weight of the Gpp(NH)p-activated form of the enzyme was significantly higher than that of the non-activated form. The results are compatible with the hypothesis that the activated form might correspond to a complex, formed between the enzyme and another membrane component under the influence of Gpp(NH)p. The difference observed in molecular weight (40,000) is of the same order of magnitude as the molecular weight of the nucleotide binding protein isolated by Pfeuffer (40,000) (1), but is much less than the molecular weight of the "G/F" factor described by Ross et al (130,000) (3). The difference observed in molecular weight (40,000) is of the same order of magnitude as the molecular weight of the nucleotide binding protein isolated by Pfeuffer

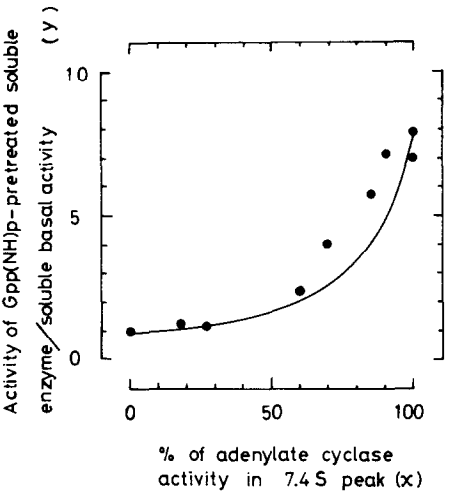

Figure 3 : Conversion of the 6.0 S form of soluble adenylate cyclase into a 7.4 S form by Gpp(NH)p activation. Pig kidney membrane aliquots (2.5 mg/ml) were treated with various concentrations of Gpp(NH)p (1 nM-0.1 mM). The solubilized enzyme present in the supernatant was layered on top of a 3-10 % H_2O sucrose density gradient together with protein markers. After centrifugation (see Methods) adenylate cyclase activity sedimentation profiles and calibration curves were determined for each gradient. 1.5 mM $MnCl_2$ was present in the adenylate cyclase incubation medium. The activity in the 7.4 S peaks was expressed as the % of the total activity included under the 6.0 and 7.4 S peaks and plotted versus the Gpp(NH)p stimulation ratio (•). This ratio was measured by the amount of soluble activity derived from Gpp(NH)p-treated membranes over that of soluble activity from control membranes. The solid line on the graph corresponds to the theoretical relationship which may be expected if it is assumed : 1) that the 7.4 S and 6.0 S forms correspond to the interconvertible forms of the enzyme, and 2) that the relative amounts and activities of the two forms do not change during ultracentrifugation. The relation used is the following :

$$y = 1 + \frac{a - 1}{1 + \left(\frac{1}{x} - 1\right)a}$$

where a = maximal stimulation ratio. The value used for this calculation was 8.

(40,000) (1), but is much less than the molecular weight of the "G/F" factor described by Ross et al (3). These authors (3) also reported that simultaneous restoration of enzyme responses to Gpp(NH)p and NaF was obtained by recombination of soluble adenylate cyclase catalytic unit with the same membrane component. As indicated in Table I, the hydrodynamic parameters of the NaF-activated form of the enzyme are very similar to those of the non-activated form. In addition, Gpp(NH)p but not NaF treatments led to the appearance of enzymatic form of increased molecular weight. These observations argue against the proposal that the NaF-activated form might correspond to a stable association between enzyme and a membrane component of identical or similar size to the component(s) involved in forming Gpp(NH)p-activated adenylate cyclase.

Table I : Molecular parameters of pig kidney adenylate cyclase solubilized from control and from NaF or Gpp(NH)p-treated membranes.

PARAMETERS (a)	Measured in the presence of $MgCl_2$			Measured in the presence of $MgCl_2$ + $MnCl_2$	
	Untreated	Gpp(NH)p treated	NaF treated	Untreated	Gpp(NH)p
Stokes radius, a (nm) (b)	6.5 ± 0.1(17)	6.5 ± 0.2(5)	6.4 ± 0.1(3)	6.6 ± 0.1(3)	-
Apparent sedimentation coefficient measured in H_2O gradient, Sapp (S)	5.9 ± 0.1(10)	7.4 ± 0.1(13)	6.8 ± 0.1(9)	5.9 ± 0.1(6)	7.4 ± 0.1 (8)
Apparent sedimentation coefficient measured in D_2O gradient, Sapp (S)	5.8 ± 0.2(6)	6.5 ± 0.1(5)	5.5 ± 0.3(5)	5.4 ± 0.3(3)	-
Standard sedimentation coefficient S^{20}_{W} (S) (c)	6.1	7.8	6.5	6.0	-
Partial specific volume, $\bar{v}$ (ml/g) (d)	0.755 ± 0.005(13)	0.775 ± 0.005(6)	0.770 ± 0.005(12)	0.745 ± 0.005(9)	-
Detergent protein complex molecular weight, M_R (e)	$185.\ 10^3$	255.10^3	205.10^3	180.10^3	-
Protein molecular weight, M_R (f)	170.10^3	210.10^3	175.10^3	170.10^3	-

(a) Values given are means ± SD for the number of determinations shown in parentheses.
(b) Determined according to Laurent and Killander (13)
(c) Calculated according to Bon et al (16).
(d) Calculated according to Meunier and al (10).
(e) Calculated as recommended by Siegel and Monty (17).
(f) Determined according to Smigel and Fleischer (18).

For these calculations, a value of 0.94 ml/g for the partial specific volume of Triton X100 was used (19). It was assumed that the partial specific volume of the protein component of the detergent-enzyme complex was identical to that found for soluble proteins (0.74 ml/g).

It was recently suggested that the uncoupled form of adenylate cyclase from lymphoma cells could only be revealed in the presence of manganese (3). Addition of Mn^{2+} to the adenylate cyclase assay medium did not alter the sedimentation or elution profile of the activity present in soluble extracts of pig kidney membranes (Table I). However, the activities measured in the presence of Mn^{2+} and Mg^{2+} were higher than those measured in the presence of Mg^{2+} as the sole source of divalent cations. In addition, the relative effect of Gpp(NH)p in the presence of Mn^{2+} was reduced compared to the effect measured in the presence of Mg^{2+} (unshown results).

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