Guanylate cyclase from the Rat Renal Medulla

PHYSICAL PROPERTIES AND COMPARISON WITH ADENYLATE CYCLASE*

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Guanylate cyclase from the rat renal medulla is found in both the soluble and particulate fractions of the cell. Sucrose density gradient centrifugation and gel filtration in H2O and D2O indicate that the enzyme from the soluble cell fraction has the following properties: s20,w 6.3 S; Stokes radius, 54 Å; partial specific volume, 0.75 ml/g; mass, 154,000 daltons; f/f0, 1.4; axial ratio (prolate ellipsoid), 7. The addition of 0.1% Lubrol PX to this fraction activates the enzyme and changes the physical parameters to: s20,w 5.5 S; Stokes radius, 62 Å; partial specific volume, 0.74 ml/g; mass, 148,000 daltons; f/f0, 1.6; axial ratio (prolate ellipsoid), 11. These findings show that detergent activates the enzyme by changing its conformation and not simply by dispersing nonsedimentable membrane fragments. The dimensions of this guanylate cyclase in detergent are very similar to those of detergent-solubilized adenylate cyclase from the same tissue (Neer, E. J. (1974) J. Biol. Chem. 249, 6527-6531).

Guanylate cyclase can be solubilized from the particulate cell fraction with 1% Lubrol PX but has properties quite different from those of the guanylate cyclase in the soluble cell fraction. It is a large aggregate with a value of s20,w of about 10 S, Stokes radius of 65 Å, and a mass of approximately 300,000 daltons. However, the peaks of guanylate cyclase activity in column effluents and sucrose density gradients are very broad indicating a mixture of different size proteins.

The conditions used to solubilize guanylate cyclase from the particulate fraction also solubilize adenylate cyclase, and the two activities can be separated on the same sucrose gradient.

Studies of this sort require a rapid, accurate guanylate cyclase assay. We have developed an assay for guanylate cyclase activity which meets these criteria by adapting the competitive protein binding assay for guanosine cyclic 3':5'-monophosphate originally described by Murad et al. (Murad, F., Manganiello, V., and Vaughn, M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 736-739).

Guanylate cyclase catalyzes the formation of guanosine cyclic 3':5'-monophosphate from GTP. The enzyme exists in two forms, particulate and soluble, but the proportion of each varies from tissue to tissue. In most mammalian tissue, 80 to 90% of guanylate cyclase activity does not sediment on centrifugation at 100,000 x g (1), although there are exceptions such as the rat small intestine in which virtually all of the activity is membrane-bound (1).

The activity of both the particulate and the soluble forms of the enzyme can be increased with nonionic detergents (1, 2). There are a number of possible ways in which detergent could activate the nonsedimentable guanylate cyclase. It could break up membrane vesicles, making the enzyme more available to substrate. It could form micelles into which the enzyme inserts and becomes activated by the hydrophobic, membrane-like environment. A hydrophobic environment might also be provided by binding of a large amount of detergent to the surface of the molecule. Finally, the detergent could activate by binding at a few specific sites causing a conformational change in the enzyme. These possibilities can be distinguished from one another by examining the hydrodynamic properties of nonsedimentable guanylate cyclase in the presence and absence of detergent. The results can give clues to the interaction between the enzyme and hydrophobic cell components.

In this paper we describe studies with guanylate cyclase from the rat renal medulla as well as comparisons of the soluble enzyme with the enzyme solubilized from the membranous fraction by Lubrol PX, a nonionic detergent. Finally, we compare the physical properties of guanylate cyclase to adenylate cyclase solubilized from this tissue.

Because studies of this type require that many samples be assayed, a fast, reliable, and inexpensive assay is essential. We have adapted the assay for intracellular cyclic GMP described by Murad et al. (3) and modified by Illiano et al. (4) for the measurement of guanylate cyclase activity in vitro. The basis of the assay is the competition between radioactive and
nonradioactive cyclic GMP\(^1\) for binding sites on cyclic GMP-binding protein from lobster tail muscle.

**EXPERIMENTAL PROCEDURES**

**Preparation of Guanylate Cyclase**—Rats of either sex were killed by asphyxiation in CO\(_2\). The kidneys were immediately removed and chilled. All subsequent steps were carried out at 4\(^\circ\)C. The cortex was dissected away, the medulla was minced with a razor blade and homogenized in 5 volumes of 0.05 M Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol with a Dounce all glass homogenizer (loose pestle). The homogenate was centrifuged at 100,000 \(\times g\) for 45 to 60 min. The pellet was washed, centrifuged twice more with the same buffer, and suspended in 1% (w/v) Lubrol PX, \(^*\) 0.075 M sucrose, 0.1 M Tris-HCl, pH 7.6, 10 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol. This mixture was centrifuged at 100,000 \(\times g\) for 45 to 60 min. Deviations from this basic procedure will be described where appropriate.

**Assay of Guanylate Cyclase**—The guanylate cyclase assay consisted of two parts, the synthesis of cyclic GMP from GTP, and the measurement of the amount of cyclic nucleotide produced with the lobster tail binding protein.

Cyclic GMP binding protein was prepared from lobster tail muscle according to the procedure of Kuo and Greengard (5) through the ammonium sulfate fractionation. The dialyzed binding protein preparation was divided into portions and stored at -70\(^\circ\)C. There was no detectable loss in binding activity of a preparation over 3 to 4 months. The amount of cyclic GMP needed to saturate the binding sites on the protein was determined for each batch.

The reaction mixture for cyclic GMP synthesis contained 0.5 mM GTP, 5 mM MnCl\(_2\), 4 mg/ml of creatine kinase, 8 mM creatine phosphate, 5 mM theophylline, 0.8% bovine serum albumin, 0.05 to 0.1 M Tris-HCl, pH 7.6, 10 to 100 \(\mu\)g of test protein in a final volume of 75 \(\mu\)l. The reaction took place at 37\(^\circ\)C for 10 min and was stopped by chilling, adding 150 \(\mu\)l of 0.1 M sodium acetate buffer, pH 4.0, and immediately boiling for 30 s. Cyclic [\(^3\)H]GMP (15 to 40 pmol, 3 to 5 Ci/mmol from New England Nuclear or Amersham/Searle) was added to each tube followed by 500 \(\mu\)g of lobster tail binding protein. The binding proceeded at 4\(^\circ\)C for 60 to 90 min. Then 0.9 ml of saturated, ice-cold (NH\(_4\))\(_2\)SO\(_4\) was added. After 5 min the samples were filtered over Millipore AA filters in a 30-sample Millipore sampling manifold, and washed with 20 ml of ice-cold saturated (NH\(_4\))\(_2\)SO\(_4\) solution. The filters were blotted on the bottom and put into scintillation vials with 1 ml of 10% sodium dodecyl sulfate for 10 min. Fifteen milliliters of 80 to 150 cpm, and cpm..s is the number of counts bound in the assay sample. The calculation was made as follows:

\[
\text{Fractional displacement} = \frac{\text{cpm}_{\text{max}} - \text{cpm}_{\text{bound}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{basal}}}
\]

where \(\text{cpm}_{\text{max}}\) is the number of counts bound to the filter without added unlabeled cyclic GMP, \(\text{cpm}_{\text{basal}}\) is the number of counts bound in the presence of a 10,000-fold excess of unlabeled cyclic GMP. Fractional displacement was the most convenient way to express the data because the absolute number of counts bound without added unlabeled nucleotide varied from assay to assay. The range of this variation, using 500 \(\mu\)g of binding protein, was 1,200 to 1,800 cpm in the presence of detergent and 1,600 to 2,400 cpm without it. The calculation was made as follows:

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\]

\(^*\)Lubrol PX was the kind gift of ICI, America, Inc.

The product formed in the guanylate cyclase assay can be entirely destroyed by cyclic 3':5'-nucleotide phosphodiesterase (beef heart, Sigma). Guanylate cyclase assays were prepared and incubated in the routine way (except that theophylline was omitted) using supernatants, homogenates, and pellets with and without 0.1% Lubrol PX. The reactions were stopped by boiling, 0.06 \(\mu\)g of phosphodiesterase was added, and the mixture was incubated again at 30\(^\circ\)C for 15 min. The amount of unlabeled cyclic GMP present was assayed with the binding protein. No cyclic GMP was found in any of the phosphodiesterase-treated tubes although controls containing heat-inactivated phosphodiesterase were also assayed using [\(^3\)H]GTP as substrate and measuring the amount of cyclic [\(^3\)H]GMP formed. The radioactive product purified by Dowex 50 chromatography followed by descending chromatography on ECTEOLA paper (Whatman). The procedure used was analogous to that described for adenylate cyclase (6). The activity measured was the same as that obtained with the protein binding assay.

Without detergent, the production of cyclic GMP is a linear function of the amount of protein assayed, as is shown in Fig. 2. In the presence of detergent, the production of cyclic GMP by the supernatant is proportional to the amount of protein, but this is not the case for the pellet or the homogenate. In these studies the amount of protein used was always within the range which gave a linear response.

The amount of cyclic GMP formed increased linearly with time for at least 10 min in all fractions.

Detergent stimulation of guanylate cyclase is not an artifact caused by inhibition of phosphodiesterase activity in the assay as is shown by the following experiments. Reaction mixtures for cyclic GMP synthesis by all fractions, with and without 0.1% detergent, were set up as for guanylate cyclase assays, except that GTP was omitted, and cyclic activity of cyclic [\(^3\)H]GMP caused by the addition of known amounts of unlabeled cyclic GMP.

**Fig. 1** shows a comparison between a theoretical and experimental displacement curve of cyclic [\(^3\)H]GMP from the binding protein. The observed curve, obtained in the presence of all components of the guanylate cyclase assay, including GTP and detergent, always agreed with the theoretical curve within 5%. Therefore, it is apparent that none of the components of the assay system interferes with the displacement of cyclic GMP from the binding protein.

The specificity of the binding protein is the major proof of the identity of the product of the guanylate cyclase assay. In agreement with the findings of Murad et al. (3), ATP, GTP, and 5'-GMP even in 100-fold excess caused negligible displacement of labeled cyclic GMP (data not shown). Cyclic adenosine 3':5'-monophosphate and cyclic guanosine 3':5'-monophosphate compete with cyclic GMP, but 30 times the concentration of the former nucleotides is required for equivalent displacement of cyclic [\(^3\)H]GMP. Cyclic adenosine 2':3'-monophosphate do not compete at all.

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studies theophylline was replaced by 1.6 mM cyclic AMP. The specific method of Krishna et al. (7) and has been described (6). In these detergent. Therefore, the rise in cyclic GMP measured in the presence destruction of cyclic GMP in any cell fraction either with or without GMP expected if there were no breakdown. There was no measurable boiled cell fractions and incubated at 4° gave the amount of cyclic GMP determined with the binding protein assay. Controls made up with exnected if there were no breakdown. There was no measurable sucrose density gradient centrifugation of supernatant guany late cyclase. The sedimentation coefficient and Stokes radius experimentally determined for this form of the enzyme, both in the presence and absence of 0.1% Lubrol PX, are given in Table II.

In the presence of detergent, the sedimentation coefficient becomes smaller by about 1 S compared to that determined without detergent, while the Stokes radius of the enzyme increases from 54 A to 62 A. Such a change could be due to either of two events. First, the enzyme might bind a large amount of detergent. This binding would increase the Stokes radius and would increase the partial specific volume (δ), thus slowing the rate of sedimentation. The mass of the particle would increase. Second, the detergent might cause a conformational change such that the enzyme would become more asymmetric. It would then have a larger Stokes radius and a smaller sedimentation coefficient but there would be no change in its mass or in its partial specific volume. In this case, the enzyme might bind some detergent but in an amount too small to cause a measurable change in these parameters.

To decide between these alternatives, we measured the partial specific volume of soluble guanylate cyclase by comparing the sedimentation of the enzyme in sucrose density gradients made up in H₂O or D₂O with and without detergent. Both with and without Lubrol PX the value for δ is that of a typical soluble protein (see Table II). To exclude the possibility that detergent is released in D₂O, we measured the Stokes radius of guanylate cyclase by gel filtration on columns made up in D₂O, with and without 0.1% Lubrol PX. The results were the same as those obtained in H₂O. This is in agreement with the findings of Clarke (11) who reported that the ratio of [³H]Triton X-100 bound to proteins in D₂O to [³H]Triton X-100 bound in H₂O is 0.78 to 0.92. This difference would not be detectable by the methods used here.

Measurement of δ allows the explicit calculation of the molecular weight of the enzyme. This, too, is unchanged in the presence of detergent. Therefore, we conclude that the changes in Stokes radius and sedimentation coefficient produced by the detergent must be primarily due to a conformational change in the enzyme with unfolding of the polypeptide chain.

The fact that the molecular weight of soluble guanylate cyclase is low and is not changed by detergent shows that detergent activation is not a matter of exposing cryptic enzyme from noncondimentable membrane vesicles.

There has been some controversy over whether nonionic detergents change the conformation of proteins. Kirkpatrick and Sandberg (17) studied the effect of a number of detergents

\[\text{Tris-HCl}.\] A typical example is shown in Table I. The distribution of adenylate cyclase in the same preparation is shown for comparison. These results are qualitative at present since the recovery of the two enzymes is different.

The total activity of adenylate cyclase and guanylate cyclase is similar, the main difference being in the partition between the soluble and particulate forms. The intracellular concentration of cyclic AMP in the kidney is about \(1 \times 10^{-8}\) mol/kg of tissue. Cyclic GMP is about \(3 \times 10^{-8}\) mol/kg of tissue (2, 14–16). This 20- to 30-fold difference in cyclic nucleotide concentration does not seem to be reflected in a difference in activity of the respective cyclase.

Physical Properties of Guanylate Cyclase—Figs. 3 and 4 show the pattern of activity obtained by gel filtration and sucrose density gradient centrifugation of supernatant guanylate cyclase. The sedimentation coefficient and Stokes radius experimentally determined for this form of the enzyme, both in the presence and absence of 0.1% Lubrol PX, are given in Table II.

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Distribution of guanylate cyclase and adenylate cyclase in the rat renal medulla

The renal medulla was homogenized in 0.1% Lubrol PX, 0.075 M sucrose, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.02 M Tris HCl, pH 7.6, and centrifuged at 100,000 × g for 45 min. This gave supernatant 1. The pellet was taken up in the same buffer and centrifuged to give supernatant 2. The remaining pellet was taken up in 1% Lubrol PX, 0.2 M Tris HCl, pH 7.6, and the other components listed above. It was homogenized and centrifuged as before to give supernatant 3. The procedure was repeated to give supernatant 4. The pellet remaining was taken up in the last buffer. All samples were diluted before assay to give a Lubrol PX concentration of 0.1%. Adenylate cyclase was assayed with 7 mM NaF.

| Fraction tested | Guanylate cyclase | Adenylate cyclase |
|----------------|-------------------|-------------------|
|                | Specific activity | Total activity    | Specific activity | Total activity |
|                | pmol cyclic GMP/10 min | g tissue/10 min | pmol cyclic AMP/10 min | g tissue/10 min |
| 0.1% Lubrol PX | 142 | 460 | 65.3 | 500 | 71.0 |
| 0.02 M Tris-HCl | 66 | 620 | 41.0 (63%) | 45 | 3.0 (4%) |
| Homogenate     | 21 | 350 | 7.3 (11%) | 127 | 2.7 (4%) |
| Supernatant 1  | 12 | 1000 | 12.0 (18%) | 735 | 8.8 (12%) |
| Supernatant 2  | 30 | 620 | 18.6 (28%) | 610 | 18.3 (26%) |
| 1% Lubrol PX   | 11 | 710 | 7.8 (12%) | 1630 | 17.9 (25%) |
| Supernatant 3  | 1000 | 12.0 (18%) | 735 | 8.8 (12%) |
| Supernatant 4  | 350 | 7.3 (11%) | 127 | 2.7 (4%) |
| Pellet         | 620 | 18.6 (28%) | 610 | 18.3 (26%) |

*The total recovery of guanylate cyclase activity was 132%. The sum of guanylate cyclase activity in cell fractions was usually more than that measured in the homogenate. The reason for this is not clear. Kimura and Murad have reported similar findings in rat heart (13). The activity of adenylate cyclase is decreased by detergent. The specific activity of the homogenate in Lubrol PX is about 70% of that measured without detergent. Without detergent less than 5% of the total activity is found in a 100,000 × g supernatant.

on the electron spin resonance spectra of spin-labeled red blood cell ghost proteins. They concluded that no detectable conformational change was produced by a nonionic detergent, Triton X-100, in contrast to the measurable changes produced by ionic detergents. The denaturing ionic detergents have been shown by Makino et al. (18) to bind massively and cooperatively to all proteins while the nonionic detergents and the bile salts bind only to proteins with specific hydrophobic sites. It can be seen from our studies that a nonionic detergent, Lubrol PX does lead to a measurable conformational change, presumably because of a small amount of specific binding.

The values for the sedimentation coefficient and Stokes radius of guanylate cyclase solubilized from the particulate fraction by 1% Lubrol PX are also given in Table II. These values must be viewed as approximations, for the peak of solubilized guanylate cyclase activity is much broader than that of the marker enzymes used in calibration. Because it is so broad, the comparison of sedimentation in H₂O and D₂O
When adenylate cyclase is solubilized with Lubrol PX, about 30% of the activity which does not sediment at 100,000 x g is excluded by Sephalose 4B and sediments more rapidly than the predominant form in a sucrose density gradient (10). It may correspond to the 10 S guanylate cyclase. See also Fig. 5.

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Physical parameter G Guanylate cyclase (10)

| Physical parameter | Soluble, no detergent | Soluble, 0.1% Lubrol PX | Solubilized from particulate form | 0.1% Triton X-100 | 0.1% Lubrol PX |
|--------------------|-----------------------|------------------------|---------------------------------|-------------------|---------------|
| Sedimentation      | 6.3 ± 0.1 (6)*        | 5.5 ± 0.1 (6)*         | 10 ± 2 (3)                      | 5.9 ± 0.2 (14)    | 6.1 ± 0.2 (6) |
| Stokes radius, a (A) | 54 ± 2 (3)*          | 62 ± 2 (5)*            | 65                              | 62 ± 3 (3)        | 64 ± 2 (3)    |
| Partial specific volume, δ (ml/g) | 0.75 ± 0.01 (2) | 0.74 ± 0.01 (2) | 300,000δ | 159,000 | 157,000 |
| Molecular weight   | 154,000               | 148,000                |                                  |                   |               |
| Frictional ratio* (f/fo) | 1.4                  | 1.6                   | 1.6                             | 1.6               | 1.6          |
| Axial ratio* (prolate ellipsoid) | 7                     | 11                    |                                  |                   | 11           |

*The values given are the mean ± 1 S.E. for the number of determinations shown in parentheses except in the case of duplicates where the range of values is given.

** The calculation of δ was made on two pairs of gradients with gradients is not meaningful, and the partial specific volume of solubilized guanylate cyclase could not be measured. The molecular weight given is calculated using a "typical" δ of 0.74 ml/g and is probably underestimated.

Ecology procedure used to solubilize guanylate cyclase from the particulate fraction of the cell also solubilized adenylate cyclase. We were, therefore, able to compare the sedimentation behavior of the solubilized cyclases in the same sucrose gradient. As can be seen in Fig. 5 the two activities are clearly separable. The sedimentation coefficient for adenylate cyclase in the experiment shown was 5.7 S.

The fact that the adenylate cyclase activity sediments as expected from previous experiments (10) shows that the 10 S guanylate cyclase is not the result of nonspecific protein aggregation in the sample.

For purposes of comparison, in Table II we have included the physical parameters for the predominant form of detergent solubilized adenylate cyclase previously reported by one of us (10). There is a striking similarity between adenylate cyclase solubilized with detergent and guanylate cyclase from the cell supernatant analyzed in Lubrol PX.

Ililano et al. (4) have raised the interesting hypothesis that adenylate and guanylate cyclase are the same enzyme which changes its substrate specificity depending on whether or not it is membrane-bound. This hypothesis cannot be rigorously tested until the two activities have been purified to homogeneity and, the proteins have been characterized. However, the fact that adenylate cyclase and guanylate cyclase can both exist in soluble form and that both activities can be found in membrane fractions argues against a change in substrate specificity which depends only on the state of aggregation or on incorporation into a membrane.

There is general agreement that adenylate cyclase is a plasma membrane enzyme. Guanylate cyclase activity has been variably reported to be 90% soluble in some cells and to be 90% particulate in others (1). However, the physical similarities we find between adenylate and guanylate cyclases suggest that perhaps like adenylate cyclase, guanylate cyclase is a membrane-bound enzyme in vivo but differs from adenylate cyclase in the ease with which it is dislodged from the membrane.

An alternative explanation for the two classes of guanylate cyclase activity is that there are in fact two different enzymes presumably with different functions. Which of these alternatives is correct is important to establish because different control mechanisms and different physiological roles would be predicted depending on the conclusions.

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