A NOVEL METHOD FOR THE DETERMINATION OF GUANOSINE 3':5'-CYCLIC MONOPHOSPHATE (CYCLIC GMP)

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Summary ; A sensitive enzymatic procedure for the assay of cyclic GMP (cGMP) has been developed, which utilizes the highly specific interaction of the E. coli polypeptide chain elongation factor Tu (EF-Tu) with GDP. The present method includes the conversion of cGMP to 5'-GMP by cyclic nucleotide phosphodiesterase and the transfer of ^{32}P from [γ-^{32}P]ATP to GMP to yield [β-^{32}P]GDP by ATP: GMP phosphotransferase (GMP kinase). The [^{32}P]GDP formed is incubated with an excess of EF-Tu·GDP in the presence of EF-Ts, and after the exchange reaction is completed, the radioactivity bound to EF-Tu is determined by the nitrocellulose membrane filter technique. This procedure permits the detection of as small as 0.05 pmole of cGMP in several rat tissues with highly reproducible results.

It has been suggested that cGMP, like cAMP, may be involved in a variety of cellular regulatory mechanisms (1,2). At an early stage of induction of cell proliferation by several mitogens, the intracellular concentration of cGMP was elevated as much as 10-fold (3,4). Because of the relatively low levels of cGMP content in tissues, development of a sensitive assay method is required for the study of the physiological function of this nucleotide.

The present paper describes a new sensitive enzymatic method for the determination of cGMP. A unique feature of the assay is the use of EF-Tu of E. coli which posesses a strong and specific affinity toward GDP. The dissociation constant (Kd) of EF-Tu·GDP is in the order of 5×10^{-9} M and the exchange between bound and free GDP occurs very rapidly in the presence of EF-Ts (5,6). The present assay is based on the following three enzymatic steps:

$$\text{cGMP} \xrightarrow{\text{Phosphodiesterase}} \text{GMP} \quad \text{[1]}$$

$$\text{GMP} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightleftharpoons{\text{GMP kinase}} [\beta\text{-}^{32}\text{P}]\text{GDP} + \text{ADP} \quad \text{[2]}$$

$$\text{EF-Tu}\cdot\text{GDP} + [\beta\text{-}^{32}\text{P}]\text{GDP} \xrightleftharpoons{\text{EF-Ts}} \text{EF-Tu}\cdot[\beta\text{-}^{32}\text{P}]\text{GDP} + \text{GDP} \quad \text{[3]}$$

The $[^{32}P]$GDP formed in Reaction 2 can be converted to EF-Tu·$[^{32}P]$GDP which is retained quantitatively on a nitrocellulose membrane filter. The procedure for the determination of cGMP content in rat tissues is also described.

Materials and Methods

Cyclic nucleotide phosphodiesterase [E. C. 3. 1. 4. 17] and GMP kinase [E. C. 2. 7. 4. 8] were purchased from Boehringer. EF-Tu·GDP and EF-Ts were prepared from *E. coli* Q13 cells as described previously (7). [8-^{3}H]cGMP (14 Ci/mmole) was purchased from New England Nuclear and was purified by QAE-Sephadex A-25 column chromatography (8). $[\gamma\text{-}^{32}P]$ATP was prepared by the method of Glynn and Chappell (9) with some modifications.

Assay Procedure for cGMP —— The reaction mixture contained in a final volume of 0.1 ml; 50 mM Tris-HCl buffer (pH 7.6), 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 2 mM EDTA, 0.1 μM $[\gamma\text{-}^{32}P]$ATP (1-3 mCi/μmole), 1 μg of phosphodiesterase, 4 μg of GMP kinase, and a sample containing cGMP to be assayed. The reaction was carried out for 90 min at 0°, after which time the mixture was supplemented with 10 μl of EF-Ts (22 μg/ml) and 5 μl of EF-Tu·GDP (0.5 mg/ml). After further incubation for 2 min at 0°, the reaction was stopped by the addition of 2 ml of a cold solution containing 10 mM Tris-HCl buffer (pH 7.6) and 10 mM magnesium acetate, and the mixture was poured onto a nitrocellulose membrane filter (0.45 μ pore size). The filter was washed twice with the above solution, dried, and counted in a liquid scintilation spectrometer.

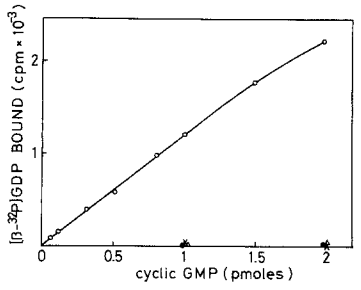
Preparation of Tissue Samples —— Male Wistar rats weighing 100 to 150 g were anesthetized with diethyl ether and the tissues were

rapidly removed and frozen in liquid nitrogen. Frozen tissues (100 to 200 mg) were powdered, and homogenized in 1.5 ml of ice-cold 6 % trichloroacetic acid. A tracer amount of [^{3}H]cGMP (0.013 μCi) was added to each tube as recovery marker and the homogenate was centrifuged for 15 min at 10,000 x g. The precipitate was discarded and the supernatant was extracted three times with 5 ml of water-saturated diethyl ether.

The sample was then applied to an alumina column (0.8 x 2 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.6) and the column was washed with 8 ml of the same buffer. Under these conditions, cGMP passed through the column, while GMP and other 5'-nucleotides are firmly retained (10). The eluate was directly applied to a column of QAE-Sephadex A-25 (0.6 x 1.5 cm) to adsorb cAMP and cGMP. The column was eluted first with 8 ml of 60 mM ammonium formate (pH 9.0) and then with 6 ml of 80 mM ammonium formate (pH 6.0). The latter fraction containing cGMP was concentrated by lyophilization and the residue was dissolved in a small volume of 5 mM Tris-HCl buffer (pH 7.6). The recovery of cGMP to this step, calculated from the amount of [^{3}H]cGMP added as recovery marker, was approximately 70-80 %. The protein content of the tissue samples was determined by the method of Lowry et al. (11).

Results

As shown in Fig. 1, a good proportionality was obtained between the amount of cGMP added and the radioactivity recovered as EF-Tu·GDP, and as small as 0.05 pmole of cGMP could be determined when [γ-^{32}P]ATP of the specific radioactivity of 1.4 mCi/μmole was used. The higher sensitivity of the assay is expected by the use of [γ-^{32}P]ATP of a higher specific radioactivity. The figure also shows that the assay is dependent on the each step of three enzymatic reactions. It was noted, however, that there was some assay blank in the



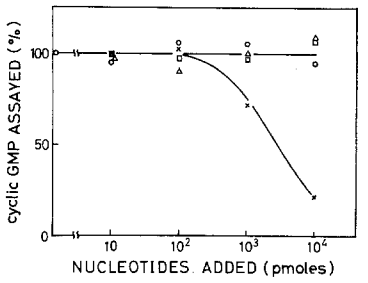


Fig. 1. Standard curve for the determination of cGMP. The assay for cGMP was carried out as described in "Materials and Methods" using known amounts of cGMP and [γ-^{32}P]ATP of the specific radioactivity of 1.4 mCi/μmole. In the complete system (○), and in the system without phosphodiesterase (●), the blank (about 200 cpm) in the absence of added cGMP was subtracted. The assay without GMP kinase (△) or EF-Tu·GDP plus EF-Ts (x) gave no blanks.

Fig. 2. Influence of other cyclic nucleotides on the assay for cGMP. cAMP (○), cUMP (△), cCMP (□) or cIMP (x) was added to the standard assay mixture containing 1.0 pmole of cGMP. Values are given as per cent of the radioactivity obtained in the absence of other cyclic nucleotides.

absence of cGMP and phosphodiesterase which increased substantially by the prolonged incubation in the last step, i. e., the EF-Ts-mediated exchange of EF-Tu·GDP with [^{32}P]GDP. This blank was minimized by shortening the time of incubation of the exchange reaction to 2 min. As reported previously, the equilibrium between bound and free GDP was established within one min at 0° in the presence of a sufficient amount of EF-Ts (7).

The specificity of the assay was tested by adding 10 to 10,000 pmoles of other cyclic nucleotides to each reaction mixture containing 1 pmole of cGMP. As shown in Fig. 2, the presence of high amounts (10 nmoles per tube) of cAMP, cUMP, and cCMP did not affect the assay. Only with cIMP, a pronounced inhibition was observed when more than 100 pmoles of the nucleotide was added per tube.

Table I shows the cGMP content of several rat tissues determined by the present procedure. As is clear from the table, a good proportionality was obtained between the samples assayed at two different

Table I

Assay of cGMP levels in several rat tissues

Tissues	Sample (µl/tube)		cGMP	
			pmoles/ tube	pmoles/ mg protein
Lung	20		0.88	2.1
	10		0.45	
	10	- phosphodiesterase	0	
	10	+ 1.0 pmole cGMP	1.53	
Liver	40		0.12	0.05
	20		0.05	
	20	- phosphodiesterase	0	
	20	+ 1.0 pmole cGMP	1.05	
Testis	40		0.30	0.10
	20		0.20	
	20	- phosphodiesterase	0	
	20	+ 1.0 pmole cGMP	1.25	
Heart	40		0.66	0.18
	20		0.30	
	20	- phosphodiesterase	0	
	20	+ 1.0 pmole cGMP	1.36	

Preparation of tissue extracts, and purification and assay of cGMP were performed as described in "Materials and Methods." Deletion of phosphodiesterase or addition of authentic cGMP were made as indicated. The values of cGMP per mg of protein in tissue extracts were corrected for the recovery of [^{3}H]cGMP added to the extracts as a recovery marker.

levels. No radioactive material was retained on the nitrocellulose membrane filter when phosphodiesterase is omitted from the incubation mixture, indicating that GMP was completely eliminated from the samples through the two successive column chromatographies. The addition of 1.0 pmole of cGMP to the tissue extracts gave an expected increase in the assay results, indicating that the substances which might interfere the assay are also effectively removed. The values listed in Table I are reasonably comparable with those reported by

other investigators using different procedures (12).

Discussion

The assay for cGMP requires an extremely high degree of sensitivity and specificity since it is present in most tissues in concentrations much lower than those of cAMP. Several assays for cGMP have been developed which include enzymatic recycling (13), radioimmunoassay (14), and competition for binding to (15) or activation of (16) the specific protein kinase. Recently, Schultz et al. (8) reported a new enzymatic method in which GMP derived from cGMP was phosphorylated by [γ-^{32}P]ATP in the presence of GMP kinase, and the [^{32}P]GDP was separated from the remaining [^{32}P]ATP by enzymatic degradation of ATP by myosin and by precipitation of the ^{32}Pi formed.

The present method is similar to that of Schultz et al. (8) in the initial two steps of reactions leading to the formation of [β-^{32}P]GDP but much simpler and more convenient since the radioactive nucleotide is determined directly by binding with EF-Tu. The sensitivity of the present procedure is comparable to that of other assays and the high degree of specificity is assured by the strict substrate specificity of GMP kinase and EF-Tu, respectively, toward GMP and GDP. Especially, EF-Tu binds only with GDP, and much weakly with GTP; no measurable interaction being shown with other nucleotides. In fact,the contamination with other cyclic nucleotides did not influence the assay except for the high amounts of cIMP, the occurrence of which has not yet been found in nature. For the determination of tissue levels of cGMP, however, several interfering substances including GMP have to be removed from the extracts prior to the enzymatic assay. This was accomplished by chromatographic purification of the samples on alumina and QAE-Sephadex column.

The accuracy of the present assay is assured also by the high degree of affinity of EF-Tu to GDP and by the quantitative retention

of the EF-Tu·GDP complex on the nitrocellulose membrane filter. The availability of the highly purified preparation of EF-Tu and EF-Ts in a large quantity (7) and the marked stability of the purified proteins provide an additional advantage to the new procedure reported here.

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