Enzymatic Synthesis of Cyclic GDP-ribose
A PROTOCOL FOR DISTINGUISHING ENZYMES WITH ADP-RIBOSYL CYCLASE ACTIVITY*

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Cyclic nucleotides such as cAMP and cGMP are second messengers subserving various signaling pathways. Cyclic ADP-ribose (cADPR), a recently discovered member of the family, is derived from NAD⁺ and is a mediator of Ca²⁺ mobilization in various cellular systems. The synthesis and degradation of cADPR are, respectively, catalyzed by ADP-ribosyl cyclase and cADPR hydrolase. CD38, a differentiation antigen of B lymphocytes, has recently been shown to be a bifunctional enzyme catalyzing both the formation and hydrolysis of cADPR. The overall reaction catalyzed by CD38 is the formation of ADP-ribose and nicotinamide from NAD⁺, identical to that catalyzed by NADase. The difficulties in detecting the formation of cADPR have led to frequent identification of CD38 as a classical NADase. In this study, we show that both ADP-ribosyl cyclase and CD38, but not NADase, can cyclize nicotinamide guanine dinucleotide (NGD⁺) producing a new nucleotide. Analyses by high performance liquid chromatography and mass spectrometry indicate the product is cyclic GDP-ribose (cGDPR) with a structure similar to cADPR except with guanine replacing adenine. Compared to cADPR, cGDPR is a more stable compound showing 2.8 times more resistance to heat-induced hydrolysis. These results are consistent with a catalytic scheme for CD38 where the cyclization of the substrate precedes the hydrolytic reaction. Spectroscopic analyses show that cGDPR is fluorescent and has an absorption spectrum different from both NGD⁺ and GDP, providing a very convenient way for monitoring its enzymatic formation. The use of NGD⁺ as substrate for assaying the cyclization reaction was found to be applicable to pure enzymes as well as crude tissue extracts making it a useful diagnostic tool for distinguishing CD38-like enzymes from degradative NADases.

The release of Ca²⁺ from internal stores is important to cell signaling for a variety of cellular events including secretion, muscle contraction, and fertilization. The release process is mediated by two major mechanisms, the inositol 1,4,5-trisphosphate pathway and the Ca²⁺-induced Ca²⁺ release mechanism. Inositol 1,4,5-trisphosphate has been recognized as a messenger of internal Ca²⁺ release for more than 50 years and yet having no apparent biological function. The similarity of the overall reactions catalyzed by ADP-ribosyl cyclase and CD38, but not NADase, can also be functionally distinguished from classical NADase using an alternate substrate, nicotinamide guanine dinucleotide (NGD⁺). ADP-ribosyl cyclase and CD38, but not NADase, cyclize NGD⁺ to cyclic GDP-ribose (cGDPR). The structure of this new cyclic pyridine nucleotide is the same as cADPR except the adenine is replaced by guanine. Spectroscopic analyses show that catalyzed by NAD⁺, the structure was first deduced from indirect measurements (3) and has now been definitively confirmed by x-ray crystallography, which also provides detailed information on the site of cyclization and the conformation of the molecule.

The synthesis and degradation of cADPR are catalyzed by ADP-ribosyl cyclase and cADPR hydrolase, respectively. ADP-ribosyl cyclase converts NAD⁺ to cADPR stoichiometrically and its activity has been detected in a wide variety of tissues from invertebrates to mammals (9–11). In most cases the enzyme is membrane bound but a soluble form has been purified to homogeneity from Aplysia ovotestis as a protein of 29-kDa (9). Similar to synthesis enzymes in other signaling pathways, the activity of ADP-ribose cyclase appears to be also tightly regulated. In the case of sea urchin eggs, the enzyme is activated by a cGMP-dependent mechanism, most likely involving protein phosphorylation (12). The degradation enzyme, cADPR hydrolase, which hydrolyzes cADPR to ADP-ribose (ADPR), is also an ubiquitous enzyme (11). Recently, a novel class of enzymes has been identified that catalyze both the formation and hydrolysis of cADPR (13–15). A member of this class, CD38, is a differentiation antigen of B lymphocytes which shares a 70% sequence homology with the Aplysia ADP-ribose cyclase (14, 16). The overall reaction catalyzed by CD38 is the conversion of NAD⁺ to ADPR and nicotinamide, the same reaction as catalyzed by NAD⁺ glycohydrolase (NADase), a hydrolytic enzyme known for more than 50 years and yet having no apparent biological function. The similarity of the overall reactions catalyzed by these two types of enzymes raises an intriguing possibility that some signaling enzymes like CD38 may have been mistaken as degradative NADases.

In this study, we show that metabolic enzymes of cADPR can be functionally distinguished from the classical NADase using an alternate substrate, nicotinamide guanine dinucleotide (NGD⁺). ADP-ribosyl cyclase and CD38, but not NADase, cyclize NGD⁺ to cyclic GDP-ribose (cGDPR). The structure of this new cyclic pyridine nucleotide is the same as cADPR except the adenine is replaced by guanine. Spectroscopic analyses show that catalyzed by NAD⁺, the structure was first deduced from indirect measurements (3) and has now been definitively confirmed by x-ray crystallography, which also provides detailed information on the site of cyclization and the conformation of the molecule.

The abbreviations used are: cADPR, cyclic ADP-ribose; cGDPR, cyclic GDP-ribose; ADPR, ADP-ribose; GDP, GDP-ribose; NAD⁺, nicotinamide adenine dinucleotide; NGD⁺, nicotinamide guanine dinucleotide; NADase, NAD⁺ glycohydrolase; HPLC, high pressure liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

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that cGDP is fluorescent, while neither the substrate, NGD, nor the hydrolysis product, GDP-ribose (GDP), is. This provides a convenient way of monitoring the cycling reaction continuously. The results are consistent with the cyclization of the substrate, either NGD' or NAD', being an obligatory first step catalyzed by CD38. The use of NGD' can be a diagnostic tool for distinguishing this class of signaling enzymes from the degradative NADase and should be useful in clarifying the confusion in classifying these enzymes.

**Experimental Procedures**

**Aplysia ADP-riboyl Cyclase**—The enzyme was purified from *Aplysia californica* as described previously (9). This enzyme is stable to storage at -20 °C (at a protein concentration of 64 μg/ml) and can be frozen and thawed with minimal loss of activity. Incubations were conducted at ambient room temperature (from 23 to 25 °C). The purified cyclase was diluted with a medium containing 20 mM Tris-HCl, pH 7.0, and the cyclase. Plastic tubes were used for incubation to minimize absorption of the enzyme by the tubes. The reactions were terminated by either quick freezing in liquid nitrogen or by adding 20 mM HCl. Prior to HPLC analysis, the acid-treated samples were neutralized with Tris base.

**NADase and Nucleotide Pyrophosphatase**—The NADase from *Neurospora crassa* was purchased from Sigma and used in incubations at a concentration of 20.7 μg/ml. Nucleotide pyrophosphatase (type III, from *Crotalus atrox* venom) was purchased from Sigma and used in incubations at 720 μg/ml. Incubations were maintained at 37 °C and contained 1 mM NAD' or NGD', 20 mM Tris-HCl, pH 7.0, and either NADase or nucleotide pyrophosphatase. The reactions were terminated by the addition of 20 mM HCl. Prior to HPLC analysis the samples were neutralized with Tris base.

**Membrane Extracts from Brain and Heart**—5 g of frozen dog brain (Pel-Freez) was homogenized in a glass homogenizer in 15 ml of buffer containing 340 mM glucose, 20 mM Hepes, 1 mM MgCl₂, 10 mM mercaptoethanol, 50 mM Na₂SO₄, 500 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The homogenate was centrifuged in a microfuge for 5 min, and the pellets were resuspended in 20 ml of the same buffer, homogenized, and centrifuged at 230,000 × g for 30 min. The pellet was resuspended in the original volume of buffer. Incubations contained either 1 mM NAD' or NGD', 5 mM Tris, 5 mM Pipes, 5 mM Hepes, pH 7.0, and brain membranes (1.35 mg of protein/ml) and were maintained at 37 °C. A membrane fraction of dog heart was prepared as described previously (17) and incubated as described for brain membranes at a concentration of 0.88 mg of protein/ml. The membrane fraction from rat heart represented the washed pellet from the 102,000 g centrifugation. The homogenate was centrifuged in a microfuge for 1 min when incubated with 1.1 μg/ml cyclase. The conversion of NAD' to cADPR with the release of nicotinamide was completely converted to cGDP and nicotinamide by 60 min when incubated with 0.88 mg of proteid/ml. The membrane fraction from rat heart was incubated at ambient room temperature (from 23 to 25 °C). The purified cyclase was maintained at 37 °C and there was no apparent difference in the activity of this material, which was used for most of the experiments presented in this paper, and that of the same material further purified to homogeneity on a cationic exchange column (SP 5PW, Waters). The purified preparation appears as a single band on silver-stained SDS-polyacrylamide gel electrophoresis gels. In all respects it behaves the same as the preparation we have used and published previously (14). Purified CD38 was diluted to final protein concentrations of 0.05 to 7.1 μg/ml for incubations with NGD' or 40 to 55 μg/ml for incubations with NAD' at 37 °C as described above for the incubation with the cyclase.

**The Ca²⁺ Release Assay**—Homogenates of sea urchin egg (Strongylocentrotus purpuratus) were prepared as described previously (20). Frozen egg homogenates (25%) were allowed to stand at 17 °C for 20 min and diluted to 2% with a medium containing 250 mM N-methylglucamine, 250 mM potassium gluconate, 20 mM Hepes, 1 mM MgCl₂, 2 units/ml of creatine kinase, 8 μM phosphocreatine, 0.5 mM ATP, and 5 μl of trizma buffer, pH 7.2, adjusted with acetic acid. The homogenates were diluted to 2.5% and finally 1.25% with the medium described and were incubated at 17 °C for 1 h between dilutions. Ca²⁺ release was measured spectrophotometrically in 1.25% homogenates with an excitation wavelength of 490 nm and emission wavelength of 533 nm. The measurements were done in a cuvette maintained at 17 °C and the homogenate was continuously stirred. The volume of homogenate used was 0.2 ml, and additions were usually made in 2-μl volumes. The Ca²⁺ release activities of the samples were calibrated by comparisons to those of the cADPR standards.

**HPLC Analysis**—The reaction of the ADP-riboyl cyclase, cADPR hydrolysis by NADase were monitored by HPLC and quantified by comparisons with calibration curves of known standards.

**Mass Spectroscopy**—Analyses were conducted in the mass spectrometry facility of the Department of Biochemistry, University of Minnesota, St. Paul, using a Kratos Analytical Instruments analyzer equipped with a fast atom bombardment source. Samples were dissolved in water at 1 μg/ml and a 1-pl aliquot was added to a matrix of either dithiothreitol/dithioerythritol for positive ion spectra or triethanolamine for negative ion spectra. The samples were desorbed by the electric field for 1.5 s. The ions that adhered to the matrix were detected with a fast atom bombardment source and separated on a 15 cm × 3.9 cm column packed with AG MP-1 (Bio-Rad) similar to that described previously (3, 9). The flow rate was maintained at 1 ml/min and the nucleotides were eluted with a gradient of trifluoroacetic acid starting at 0% B (solvent A is water, solvent B is 100 mM trifluoroacetic acid in water, solvent A is water) and held at 0% for 1 min, increased linearly to 4% from 1 min to 6 min, increased linearly to 8% from 6 min to 11 min, increased linearly to 16% from 11 to 13 min, stepped to 100% from 13 to 13.1 min, and held at 100% until 17 min. All of the nucleotides of interest eluted by 15.8 min. The column was calibrated with nicotinamide, NAD', cADPR, ADPR, GDP, NGD', and GMP as standards. NGD' was purchased from Sigma and further purified by using the same HPLC conditions described above. The peak corresponding to NGD' was collected, evaporated to dryness, reconstituted in water, and adjusted to pH 7 with Tris base. The purified NGD' was stable to freezing and thawing. The molar extinction coefficients of NGD', cGDP, and GDP, were determined by total phosphate analysis using GMP as standard as described previously (21).

**Results**

**Cyclization of NGD' Catalyzed by ADP-riboyl Cyclase**—HPLC analyses of the reaction catalyzed by the purified ADP-riboyl cyclase from *Aplysia* using 1 mM NAD' as substrate is shown in Fig. 1A. After 3 min (3') of incubation, the amount of NAD' (peak eluted at 4.9 ± 0.2 min, n = 8) present in the mixture was reduced by 35% compared with that at the beginning (0') of the reaction. The peak at 9.4 ± 0.2 min (n = 8) was identified as cADPR by its Ca²⁺ releasing activity. The conversion of NAD' to cADPR with the release of nicotinamide (peak at 1.5 ± 0.1 min, n = 6) has been shown to be stoichiometric and there is essentially no further metabolism of cADPR to ADPR (9).

Fig. 1B shows that the *Aplysia* ADP-riboyl cyclase can also use NGD' as substrate. After incubating 1 mM NGD' (peak eluted at 8.52 ± 0.03 min, n = 4) with the enzyme for 20 min (20'), nicotinamide and an additional product eluting at 11.24 ± 0.06 min (n = 6) were formed. The identification of this product as cGDP is provided below. As shown in Fig. 1B, the NGD' was completely converted to cGDP and nicotinamide by 60 min when incubated with 1.1 μg/ml cyclase. The conversion of

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**Fig. 1.** Cyclization of NAD⁺ and NGD⁺ by ADP-ribosyl cyclase. A, NAD⁺ (1 mM) was incubated with the *Aplysia* ADP-ribosyl cyclase (150 ng/ml) at room temperature. Samples were analyzed by HPLC immediately after mixing (0') and after 3 min (3') of incubation. Nic is nicotinamide. The inset shows kinetic analyses of the cyclization reaction. The concentration of NAD⁺ varied from 3.75 to 150 μM with 6.4 mg/ml cyclase and NGD⁺ varied from 0.3 to 100 μM with 32 ng/ml enzyme. The times of incubation were adjusted such that less than 22% of the substrate was converted. The units for the y axis are (μmole/mg protein/min)² and for the x axis are millimolar. B, NGD⁺ (1 mM) was incubated with the cyclase (1.1 μg protein/ml). Samples were analyzed by HPLC immediately after mixing (0') and after 20 min (20') and 60 min (60') of incubation.

NGD⁺ to cGDPR and nicotinamide was stoichiometric and no other product, such as guanosine diphosphoribose (GDPR), was formed. The maximal rate of cGDPR formation was about 18 times slower than cADPR formation (Table I). The apparent discrepancy of the peak areas of NGD⁺ at the beginning (0') and cGDPR at the end of the incubation (60') is because the extinction coefficient (at 254 nm) of NGD⁺ is 84% higher than that of cGDPR. These results show that the *Aplysia* enzyme can use both NAD⁺ and NGD⁺ as substrate.

Kinetic analyses were performed on the cyclization reaction using either NAD⁺ or NGD⁺ as substrate. The reactions were monitored by the disappearance of the substrates using the HPLC assay. An example of such an analysis is shown in the inset of Fig. 1A and the average values of the kinetic constants of three determinations are listed in Table I. The enzyme shows about 14-fold lower Kₘ for NGD⁺ than NAD⁺, with Kₘ values of 2.8 and 39 μM for the two substrates, respectively. In contrast, the Vₘₐₓ of the enzyme for NAD⁺ was about 18 times faster than for NGD⁺.

**Identification of Cyclic GDP-ribose**—Various treatments were used to convert cGDPR and NGD⁺ to known products and their retention times on HPLC are summarized in Table II. As shown in Fig. 1B, treatment of NGD⁺ with ADP-ribosyl cyclase resulted in a product with a retention time of 11.24 min, 2.72 min later than that of NGD⁺. This product was not GDPR since hydrolyzing NGD⁺ with Neurospora NADase resulted in a product with a retention time of 15.79 min (see also Fig. 2) which was identified by mass spectroscopy as GDPR as described below. The cyclase product was not GMP either, since the GMP standard had a retention time of 13.20 min which was essentially identical to the retention time of 13.36 min of the product obtained by treatment of NGD⁺ with nucleotide pyrophosphatase. It has previously been shown that cADPR can be quantitatively converted to ADPR by heat treatment (11, 13). Similarly, incubation of the NGD⁺ product after the cyclase treatment in a boiling water bath for 30 min quantitatively converted it to a product with a retention time of 15.79 min, identical to the retention time of 15.79 min of GDPR obtained after the NADase treatment (Table II). That it was indeed GDPR was shown by treatment with nucleotide pyrophosphatase which converted it to a product with retention time of 13.58 min, essentially identical to the retention time of 13.90 min of the GMP standard. Although both cADPR and GDPR could be hydrolyzed by heat the half-time of breakdown of cADPR was found to be 2.8 times faster than GDPR at 85 °C, pH 7.0, indicating that cGDPR is a more stable molecule.

Mass spectroscopy analysis of cGDPR was consistent with a cyclic structure. The m/z value was determined to be 558 for the positive ion, (M + H)⁺, and 556 for the negative ion, (M − H)⁻, indicating the molecule has a molecular weight of 557. The measured m/z value of GDPR, the heat-induced hydrolysis product of cGDPR, was 576 for the positive ion, (M + H)⁺, and 574 for the negative ion, (M − H)⁻, indicating it has a molecular weight of 575. Therefore cGDPR must be a cyclic molecule (18 mass units) less than its hydrolysis product, GDPR, indicating it is a cyclic molecule which by addition of a water molecule produced GDPR. The same kind of reasoning was previously used to demonstrate the cyclic nature of cADPR (3). Fig. 2 shows the chemical structure of cADPR as derived from x-ray crystallography (8). Also shown is the proposed structure of cGDPR. Hydrolysis of the cyclic linkage at the N1 position of the adenine in cADPR converts it to ADPR. A similar reaction would produce GDPR from cGDPR. Unlike cADPR, cGDPR has no Ca²⁺ release activity even at 100 μM, 1000-fold higher than the effective concentration of cADPR, nor did it inhibit Ca²⁺ release induced by cADPR (data not shown). Thus, cGDPR is unlike 8-amino-cADPR, which has been shown to be a specific inhibitor of the Ca²⁺-dependent cADPR-dependent Ca²⁺ release (21, 22).

**Cyclization of NGD⁺ Catalyzed by CD38**—The cyclization reactions catalyzed by CD38 were compared using either NAD⁺ or NGD⁺ as substrate. Fig. 3A shows the HPLC analyses of the reaction products following a 15-min (15') incubation of 1 mM NAD⁺ with CD38. The disappearance of NAD⁺ was accompanied mainly by the accumulation of ADPR. The amounts of cADPR produced were so small that it was not apparent in the chromatogram. However, using the more sensitive Ca²⁺-release assay, a time dependent accumulation of cADPR could definitely be demonstrated as shown in the inset of Fig. 3A. To validate that the Ca²⁺ release was due to cADPR generation from NAD⁺, the release was shown to be inhibited by a 10-fold excess of 8-amino-cADPR, a competitive inhibitor of the cADPR-dependent Ca²⁺ release (21, 22). The initial rate of cADPR production was 0.88 μmol/mg protein/min, which is 111 times less than the ADPR production rate of 98 μmol/mg protein/min. This rate of cADPR formation is an underestimate since CD38 catalyzes both the formation and hydrolysis of cADPR and thus the cADPR detected represents the net cADPR synthesized. Direct measurement of cADPR hydrolysis using 1 mM cADPR as substrate revealed a hydrolytic rate of 6.6 μmol/mg protein/min. It thus appears that CD38 is a rather complicated enzyme and its reaction rate is fastest when measured as ADPR production from NAD⁺ and lowest as cADPR production from NAD⁺, while an intermediate rate is measured as hydrolysis of cADPR.

In striking contrast, incubation of CD38 with 1 mM NGD⁺...
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Table I

| Enzyme | Substrate | Concentration range | Method | \( K_m \) (\( \mu M \)) | \( V_{max} \) (\( \mu mol/\mu g/min \)) |
|--------|-----------|---------------------|--------|-----------------|-----------------------------|
| Cyclase | NAD | 3.75 to 150 | HPLC | 39.0 ± 1.0 | 500.0 ± 60.0 |
|         | NGD | 0.3 to 100 | HPLC | 2.8 ± 0.2 | 27.7 ± 6.4 |
|         | NGD | 100 | UV | ND | 34.2 ± 0.5 |
|         | NGD | 2 to 40 | Initial rate | 2.2 ± 0.2 | 45.0 ± 0.6 |
|         | NGD | 0.3 to 40 | Slopes | 1.8 ± 0.5 | 98.0 ± 2.0 |
| CD38   | NAD | 1 to 100 | HPLC | 14.8 ± 0.6 | 98.0 ± 29.0 |
|         | NGD | 0.3 to 100 | HPLC | 2.2 ± 0.1 | 16.7 ± 2.0 |
|         | NGD | 100 | UV | ND | 19.2 ± 1.5 |
|         | NGD | 2 to 40 | Initial rate | 1.0 ± 0.1 | 29.4 ± 0.6 |
|         | NGD | 0.3 to 40 | Slopes | 1.1 ± 0.6 | 19.0 ± 2.2 |

* ND, not determined.
* Determined by fluorimetric assay.

Table II

Retention times of NGD' and products derived from it

| Sample | Treatment | Conversion | Retention time (min) |
|--------|-----------|------------|---------------------|
| NGD' standard | | | 8.52 ± 0.03 |
| GMP standard | | | 13.20 ± 0.2 |
| NGD' | ADP-ribosyl cyclase | NGD' → cGDPR | 11.24 ± 0.06 |
| NGD' | NADase | NGD' → GDPR | 16.79 ± 0.01 |
| NGD' | Nucleotide pyrophosphatase | NGD' → GMP | 13.50 ± 0.00 |
| cGDPR | 30 min boiling | cGDPR → GDPR | 16.79 ± 0.01 |
| Boiled GDP | Nucleotide pyrophosphatase | GDP → GMP | 13.58 ± 0.02 |

* 1 ms NGD was incubated with 1.1 \( \mu g/ml \) ADP-ribosyl cyclase for 10 min at pH 7.0 and 20–23°C.
* 1 ms NGD was incubated with 20.7 \( \mu g/ml \) Neurospora NADase for 1 to 10 min at pH 7.0 and 37°C.
* 1 ms NGD was incubated with 720 \( \mu g/ml \) nucleotide pyrophosphatase for 1 to 10 min at pH 7.0 and 37°C.
* 1 ms cGDPR was incubated for 30 min in boiling water at pH 7.0.
* 1 ms boiled cGDPR (30 min at pH 7.0) was incubated with 720 \( \mu g/ml \) nucleotide pyrophosphatase for 30 min at pH 7.0 and 37°C.

Fig. 2. The structures of cyclic ADP-ribose and cyclic GDP-ribose. The structure for cADPR is based on x-ray crystallographic results (8). The proposed structure for cGDPR is based on HPLC and mass spectroscopy analyses.

resulted in a much larger accumulation of cGDPR with only a 4 times that of GDPR. The inset of Fig. 3B shows an example of the kinetic analysis of the rate of disappearance of either NAD' or NGD' using CD38, and the average values of the kinetic constants obtained from three determinations are listed in Table I. The \( K_m \) value using NAD' as substrate was 14.8 \( \mu M \), while that for NGD' was 2.2 \( \mu M \), about 7 times lower. The maximal rate of CD38 for the NAD' reaction was slower than the cyclase by about 5-fold, but the \( V_{max} \) for the NGD' reaction was about the same for the two enzymes.

Spectrophotometric Assay of the Cyclization Reaction—Another novel characteristic of cGDPR is that it is fluorescent. The excitation and emission spectra are shown in Fig. 4A. Incubation of NGD' with CD38 resulted a linear increase in absorbance at 300 nm, reflecting the production of cGDPR. The initial rate of the absorbance increase was proportional to the amounts of enzyme from 0.175 to 0.7 \( \mu g/ml \) (data not shown). No increase in absorbance was seen during the conversion of NGD' to GDPR by the Neurospora NADase. Instead, a slight decrease was measured, which is expected from the lower molar extinction coefficient of GDPR as compared with NGD'. Table I shows that the maximal rates of cGDPR synthesis catalyzed by CD38 and the Aplysia cyclase measured using this technique were similar to that measured with the HPLC method, validating the spectrophotometric measurements. The relatively low molar extinction coefficient of cGDPR at 300 nm, however, limits the sensitivity of the assay and precludes the determination of the \( K_m \) values of the enzymes, which, as shown above, are in the low micromolar range.

Spectrofluorimetric Assay of the Cyclization Reaction—Another novel characteristic of cGDPR is that it is fluorescent. The excitation and emission spectra are shown in Fig. 4B and C, respectively. This property of cGDPR is rather unique as NGD', GDPR, neotaminamide, and cADPR were all found to be non-fluorescent (Fig. 4C). At concentrations below about 40 \( \mu M \), the fluorescence intensity of cGDPR at 410 nm (excitation at 300 nm) was linearly related to its concentration. At high-con-
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**Fig. 3. Cyclization of NAD⁺ and NGD' by CD38.** A, NAD⁺ (1 mM) was incubated with the human CD38 (86 ng of protein/ml) at 37 °C. Samples were analyzed by HPLC immediately after mixing (0') and after 15 min (15'). Initial rates, at a given substrate concentration, were proportional to enzyme concentration over a range of protein concentrations from 0.08 to 0.4 pg/ml protein (data not shown). Samples were analyzed by HPLC immediately after mixing (C) and after 3 min (3') of incubation. The inset shows kinetic analyses of the cyclization reaction. The concentration of NAD⁺ varied from 1 to 100 μM and NGD' varied from 0.3 to 100 μM. The times of incubation were adjusted such that less than 22% of the substrate was converted. The enzyme concentration used was 40 ng of protein/ml for NAD⁺ as substrate and NGD' as substrate. The units for the y axis are (micromole/mg of protein/min) and for the x axis are millimolar⁻¹.

In Fig. 3, there was a slight nonlinearity as shown in the inset of Fig. 4C. The fluorescence of cGDPR shows a 2-fold decrease from pH 10 to 3 with a pKₐ value of about 7.1 (data not shown), consistent with titration of the amino group of the guanine ring.

**Fig. 4. Spectroscopic properties of cGDPR.** A, the UV absorption spectra of 100 μM NGD' and cGDPR were measured in a buffer containing 20 mM Tris, pH 7.0. The inset shows the change in absorbance at 360 nm following incubation of 200 ng/ml CD38 and 22.7 μg/ml Neurospora NADase with 100 μM NGD' at room temperature in a buffer containing 20 mM Tris, pH 7.0. B, excitation spectrum of 200 μM cGDPR in the same buffer. The emission wavelength (Em) was set at 410 nm. C, emission spectrum of 200 μM cGDPR with the excitation wavelength (Ex) set at 360 nm. The dependence of the fluorescence at 410 nm (Fluo (410 nm)) on the concentration of cGDPR is shown in the inset.

Fluorescence changes were first converted to concentrations of cGDPR by using a calibration curve. The rate of cGDPR synthesis was obtained by the slopes of the curve determined at 0.5-min intervals (triangles in Fig. 5B) and it decreased as the reaction proceeded. The concentration of NGD' remaining at each time point was calculated by subtracting cGDPR from the starting concentration of the substrate. A rate verses substrate plot of the data is shown in Fig. 5C and a representative reciprocal plot of the data is shown in the inset. The validity of the slope method is illustrated by the consistency of the results obtained with other methods as listed in Table 1.

**Hydrolysis of NGD' by NADase.** As shown in Fig. 3A, the overall reaction catalyzed by CD38 is the conversion of NAD⁺ to ADPR, a reaction which is classified as catalyzed by NADase. The cyclization reaction of CD38 can only be determined by using the Ca²⁺ release assay or, much more easily, by using NGD' as substrate instead. These results raise an intriguing possibility that all enzymes previously classified as NADases, in fact, may be CD38-like enzymes. This was found not to be the case. As shown in Fig. 6A, incubation of the NADase from *N. crassa*
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Fig. 5. Spectrofluorimetric assay of the cyclization reaction. A, the Aplysia cyclase (0.16 pg/ml) was incubated with 2-40 nM NGD at room temperature in a buffer containing 20 mM Tris, pH 7.0. The resultant fluorescence changes at 410 nm (excited at 300 nm) were measured. The initial rates of fluorescence change were converted to the rates of cGDP synthesis using a calibration curve constructed with known concentrations of cGDP. The inset shows the double reciprocal plot of the rate (V) versus substrate concentration ([NGD]). The units for the y axis are (micromole/mg of protein/min) and for the x axis are millimolar. B, the Aplysia cyclase (0.15 pg/ml) was incubated with 19 nM NGD at room temperature in a buffer containing 30 mM Tris, pH 7.0. Concentrations of cGDP produced and NGD remaining (the two solid curves) were calculated from the fluorescence changes as described in the text. The rates of cGDP production (triangles) at 0.3-min intervals were obtained from the slope of the curve labeled [cGDP]. C, the rate values of Fig. 5B were plotted against [NGD] for each 0.3-min interval. The inset shows the double reciprocal plot of the rate (V) versus [NGD] (S). The units for the y axis are (micromole/mg of protein/min) and for the x axis are millimolar.

with 1 mM NGD produced only GDP with a retention time of 15.7 min. There was no detectable peak at 11.2 min, the retention time of GDP. Similarly, incubation of the NADase with NAD produced only ADPR. None of the assays described in this study, including the Ca2+ release assay, could detect any production of cADPR by the NADase. It is, therefore, not an ADP-ribosyl cyclase. It also does not have cADPR hydrolyzing activity. No ADPR was detected after incubating 1 mM cADPR with the NADase (0.3 unit/ml) for 60 min at pH 7.2 and 37 °C. In comparison, the same NADase preparation at a concentration six times lower hydrolyzed 1.5 mM NAD completely under the same conditions. It is clear that this NADase is not involved

in the metabolism of cADPR.

Cyclization of NGD* Catalyzed by Tissue Extracts—Having established that NGD* is a suitable substrate for monitoring the cyclase reaction catalyzed by pure enzymes such as Aplysia ADP-ribose cyclase and CD38, it was of interest to determine its utility in assaying similar activities in crude tissue extracts. It has been previously shown by the Ca2+ release assay that brain is a rich source of ADP-ribose cyclase and cADPR hydrolase (11). Fig. 6B shows that incubation of a membrane fraction from dog brain with NGD resulted in production of both cGDP and GDPR as determined by HPLC. The production of cGDP could also be detected by the fluorimetric method (data not shown). Unlike the profile seen with CD38, the GDPR appeared to accumulate faster than that of cGDP (Fig. 6B). The initial rates of production of cGDP and GDPR were determined to be 4.9 and 13.3 nmol/mg protein/min, respectively. When half of the NGD* was converted, which took 31 min, the amount of GDPR produced was about 2.7 times that of cGDP.

For comparison, the rate of utilization of NAD* by the same membrane preparation was 4.49 nmol/mg protein/min, which was about 24-fold higher than the 0.18 nmol/mg protein/min for GDPR. The brain membranes also produced as well as hydrolyzed cADPR. The rate of synthesis was measured to be 0.46 nmol/mg protein/min by the Ca2+ release assay and the hydrolysis rate was 127 nmol/mg protein/min. It is clear from these results that the cyclization enzyme is present in crude brain membrane extracts, but the presence of high hydrolysis activity makes it nearly impossible to measure its activity unless

FIG. 6. The hydrolytic and cyclization reactions catalyzed by Neurospora NADase and brain membrane extracts. A, NGD* (1 mM) was incubated with the NADase (21 µg of protein/ml) in 30 mM Tris, pH 7. Samples were analyzed by HPLC immediately after mixing (0') and after 30 min (30') of incubation. Nic is nicotinamide. B, NGD* (1 mM) was incubated with the membrane extracts (1.25 mg of protein/ml) from dog brain in 5 mM Hepes, 5 mM Pipes, pH 7. Samples were analyzed by HPLC immediately after mixing (0') and after 20 min (20') of incubation.
cADPR production is monitored specifically by the highly sensitive Ca\textsuperscript{2+} release assay. The use of NGD\textsuperscript{+} as substrate alleviates this problem and allows the cyclization reaction to be detected readily even in crude tissue extracts by HPLC analysis.

The technique of using NGD\textsuperscript{+} as substrate for monitoring the cyclization reaction was applied to various tissue extracts and the results and comparisons with various pure enzymes are summarized in Fig. 7. At the two extremes, Aplysia ADP-ribosyl cyclase produced only cGDP while Neurospora NADase only GDP. In between these two enzymes, there was a gradual decrease in the ratio of production of cGDP to GDP depending on the enzyme preparations. The rat heart membrane preparation and human CD38 produced about 4–5 times as much cGDP as GDP, whereas heart and brain membrane preparations from dog exhibited predominantly NADase type of activity. It remains to be determined whether the ratios of NADase to cyclase activity measured in these enzyme preparations reflect the intrinsic properties of the CD38-like enzymes present. This is a likely possibility as the activity ratios measured in crude extracts of spleen and red blood cells were shown to be the same as the purified enzymes (13, 15). In any case, the use of NGD\textsuperscript{+} as substrate to reveal the cyclization reaction in crude extracts should provide a very convenient method for monitoring the purification of these enzymes.

**DISCUSSION**

Results presented in this study raise an important issue about classifying the metabolic enzymes of cADPR. One possibility is to put them in a separate class distinct from the classical NADases, as they do exhibit different catalytic properties. Alternatively, one could group all these enzymes into a heterogeneous class. One extreme of this class would be ADP-ribosyl cyclase, catalyzing mainly the formation of cADPR from NAD', while the other extreme would be NADase, producing only ADP. In between these two extremes are the CD38-like enzymes, exhibiting both NADase and the cyclase activities in various proportions depending on the specific source of the enzyme. For example, for CD38 from red blood cells, the ratio of NADase to the cyclase activity is about 100 (15), while for the splenic enzymes, the ratio is about 50 (13). Irrespective of which of these approaches one would like to adopt, it is clear that these enzymes need to be reclassified taking into consideration the discovery of cADPR so as not to create unnecessary confusion with the degradative NADases and obscure their important functions in cellular signaling.

The use of NGD\textsuperscript{+} as a substrate reveals that the cyclization reaction is a dominant reaction catalyzed by CD38. This result clearly shows that CD38 is not an aberrant NADase with a minor side reaction producing small amounts of cADPR. Because of its small size, a protein of about 35 kDa, CD38 is unlikely to have two catalytic sites dedicated for the cyclase and hydrolyase reaction separately. They more likely represent partial reactions of the catalytic process with the cyclization reaction preceding hydrolysis. Further insights into the mechanism of the catalysis can be obtained from the crystal structure of cADPR, which shows that the cyclizing linkage between the ribose and the adenine is in the β configuration (8), the same as the glycosyl linkage of the ribose to the nicotinamide of NAD\textsuperscript{+}, its precursor. The simplest mechanism that can account for the observed catalytic behavior is a double inversion at the anomeric carbon of this ribose. This suggests that the formation of an ADP-ribosylated enzyme intermediate may be the first step of catalysis. Intramolecular attack on the intermediate by the nitrogen at the 1-position of the adenine could then result in cyclization and formation of cADPR. The more probable alternate is a pathway catalyzed by CD38. In this case, the use of NGD\textsuperscript{+} as substrate instead of NAD\textsuperscript{+} the reaction would be very similar except that the activated-cGDP formed could be less susceptible to hydrolysis. The stability of the intermediate would result in cGDP being released mainly as the cyclized product. Indeed, we have shown in this study that the rate of heat-induced hydrolysis of cGDP is about 3 times slower than cADPR, indicating it is a more stable molecule.

The procedure of using NGD\textsuperscript{+} as an alternate substrate described in this study can greatly facilitate the reclassification of the metabolic enzymes of cADPR, since under this condition, the cyclization reaction of these enzymes is specifically manifested. Another novel feature of this procedure is that cGDP is fluorescent allowing continuous monitoring of the cyclization reaction. This greatly simplifies kinetic analyses of the metabolic enzymes of cADPR as illustrated in this study. The utility of the procedure is further demonstrated using crude membrane extracts from brain and heart. The results show that the cyclization activity is widespread among mammalian tissues and that there is a gradation of activity depending on tissues, highest in rat heart and lowest in dog brain. That the procedure is applicable in crude extracts makes it the simplest assay for monitoring the purification of these enzymes.

The difficulties involved in measuring the cyclization reaction are similar to those for assaying adenylyl cyclase activity in complex mixtures. The high hydrolytic activity in the mixture removes and obscures the formation of the cyclic nucleotide. This problem is often avoided in assays of adenylyl cyclase by inclusion of inhibitors of phosphodiesterases. A similar strategy cannot be employed for the CD38-like enzymes because, first, there is no known inhibitor of cADPR hydrolysis, and, second, more importantly, the bifunctional nature of the reaction is part of the catalysis and it may not be possible to...
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block one reaction without affecting the other. The use of an alternate substrate for the cyclization reaction, as demonstrated in this study, is a much better solution.

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