Fluorescent Analogs of Cyclic ADP-Ribose: Synthesis, Spectral Characterization, and Use[†]

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ABSTRACT: Cyclic ADP-ribose (cADPR) is a Ca^{2+}-mobilizing cyclic nucleotide derived from NAD^+. Accumulating evidence indicates that it is an endogenous modulator of the Ca^{2+}-induced Ca^{2+} release mechanism in cells. In this study, we show that ADP-ribosyl cyclase catalyzes the cyclization of not only NAD^+ but also several of its analogs with various purine bases (guanine, hypoxanthine, or xanthine) substituting for adenine. Unlike cADPR, the resulting cyclic products are fluorescent. Comparisons with various model compounds indicate that only 7-methyl substituted purine nucleosides and nucleotides are fluorescent, and the pH-dependence of their UV spectra is most similar to that of the fluorescent cADPR analogs, indicating that the site of cyclization of these analogs is at the N7-position of the purine ring. This finding is novel since the site of cyclization is at the N1-position for cADPR as determined by X-ray crystallography. That a single enzyme can cyclize a variety of substrates at two different sites has important implications mechanistically, and a model is proposed to account for these novel catalytic properties. Among the analogs synthesized, cyclic GDP-ribose is highly resistant to hydrolysis, while cyclic IDP-ribose can be readily hydrolyzed by CD38, a bifunctional enzyme involved in the metabolism of cADPR. These unique properties of the analogs can be used to develop fluorimetric assays for monitoring separately the cyclization and hydrolytic reactions catalyzed by the metabolic enzymes of cADPR. The convenience of the method in measuring kinetic parameters, pH-dependence, and modulator activity of the metabolic enzymes of cADPR is illustrated.

Cyclic ADP-ribose (cADPR)[1] is a newly discovered cyclic nucleotide derived from NAD^+ that has potent calcium release activity in a variety of cells [reviewed in Lee et al. (1994c)]. It is emerging as an endogenous modulator of the Ca^{2+}-induced Ca^{2+} release mechanism, a major system for mobilizing internal Ca^{2+} stores (Galione et al., 1991; Lee, 1993; Lee et al., 1995a). It has been shown that together with calmodulin, cADPR can increase the Ca^{2+} sensitivity of the release mechanism by several orders of magnitude (Lee et al., 1994a, 1995a). During the enzymatic synthesis of cADPR by ADP-ribosyl cyclases, the nicotinamide group from NAD^+ is lost and the adenine ring covalently attaches to the anomeric carbon of the terminal ribose (Lee et al., 1989; 1994b). The cyclic structure of cADPR has been confirmed by X-ray crystallography studies, and the bond between the adenine and terminal ribose moieties has been established as occurring via the N1-position of the adenine ring in a β-conformation (Lee et al., 1994b).

In addition to a soluble ADP-ribosyl cyclase from *Aplysia californica* ovotestis two other types of enzymes are known to be involved in the metabolism of cADPR [reviewed in Lee et al. (1995b)]. CD38, a lymphocyte antigen, is a bifunctional enzyme that can both synthesize and hydrolyze cADPR (Howard et al., 1993; Takasawa et al., 1993). There is also a cGMP-activable ADP-ribosyl cyclase described for sea urchin egg extracts (Galione et al., 1993). The *Aplysia* ADP-ribosyl cyclase is unique in that it is monofunctional and produces only cADPR (Lee & Aarhus, 1991). In comparison, other sources of ADP-ribosyl cyclase, such as CD38, also have high cADPR hydrolase activity, and the primary product of incubation with NAD^+ is ADP-ribose (ADPR) (Howard et al., 1993; Takasawa et al., 1993; Kim et al., 1993). Recently, it was shown that ADP-ribosyl cyclases can also cyclize nicotinamide guanine dinucleotide (NGD^+) to produce cyclic GDP-ribose (cGDPR) (Graeff et al., 1994a,b). The demonstrated importance of cADPR in regulating cellular Ca^{2+} necessitates a better understanding of the catalytic mechanism of its metabolic enzymes. In this study, we explore the leniency of the cyclase toward the purine base of its substrate and use it to synthesize a series of fluorescent analogs of cADPR. Spectral characterizations of these analogs indicate that the site of cyclization is at the N7-position of the purine ring instead of the N1-position as in cADPR. That the cyclase can cyclize its substrate at two

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[1] cADPR, cyclic ADP-ribose; cGDPR, cyclic GDP-ribose; cIDPR, cyclic inosine diphosphoribose; cXDPR, cyclic xanthosine diphosphoribose; e-cADPR, cyclic 1,N^6-ethenoadenosine diphosphoribose; e-ADPR, 1,N^6-ethenoadenosine diphosphoribose; ADPR, ADP-ribose; NAD^+, nicotinamide adenine dinucleotide; NGD^+, nicotinamide guanine dinucleotide; NHD^+, nicotinamide hypoxanthine dinucleotide; e-NAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; NXD^+, nicotinamide xanthine dinucleotide; XMP xanthosine 5′-monophosphate; β-NMN, β-nicotinamide mononucleotide; 1-mA, 1-methyladenosine; 1-mG, 1-methylguanosine; 7-mG, 7-methylguanosine; 7-mGMP, 7-methyl-GMP; 1-mI, 1-methylinosine; 7-mI, 7-methylinosine; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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different sites of the purine ring has important implications for the structure of its active site as well as its catalytic mechanism. The utility of these fluorescent analogs for monitoring the cyclization and hydrolysis reaction is also described.

MATERIALS AND METHODS

Recombinant CD38 and ADP-Ribosyl Cyclase. Recombinant human CD38 was produced as previously described (Graeff et al., 1994b; Fryxell et al., 1995). Briefly, the putative intracellular and transmembrane portions of human CD38 were deleted and the four potential N-linked glycosylation sites removed by conservative amino acid substitutions. The remaining DNA sequence was spliced into *Pichia* expression vector pHIL-S1 (Invitrogen Corporation, San Diego, CA) and expressed as a soluble, secreted protein in *Pichia pastoris*. The soluble CD38 from the culture medium was further purified to homogeneity on a cationic ion exchange column (SP 5PW, Waters). The purified preparation appears as a single band on silver stained SDS−PAGE gels. In all respects it behaves the same as the preparation we have used and reported previously (Howard et al., 1993; Graeff et al., 1994b). The soluble ADP-ribosyl cyclase was purified from *Aplysia* ovotestis according to procedures published previously (Lee & Aarhus, 1991).

Preparation of Nicotinamide Xanthine Dinucleotide (NXD^+). The chemical coupling of xanthosine 5′-monophosphate (XMP) with β-nicotinamide mononucleotide (β-NMN) was performed by the method of Prescott and McLennan (1990). XMP (2.5 μmol), β-NMN (7.5 μmol), and $MgCl_2$ (6.5 μmol) were combined in a microfuge tube and evaporated to dryness. The coupling reaction was initiated by adding 10 μL of 1.5 M Hepes-NaOH (pH 6.8) and 10 μL of 1.5 M 1-ethyl-3-(3-(dimethylaminopropyl)carbodiimide hydrochloride and incubated at 37 °C for 18 h. The product, NXD^+, was purified by HPLC using an AG MP-1 column and the trifluoroacetic acid gradient described below.

Synthesis of Fluorescent Analogs of cADPR. The cyclic compounds cADPR, cGDPR, cyclic inosine diphosphoribose (cIDPR), cyclic xanthosine diphosphoribose (cXDPR), and cyclic 1,N^6-ethenoadenosine diphosphoribose (e-cADPR) were prepared by incubation of the *Aplysia* ADP-ribosyl cyclase with NAD^+, NGD^+, nicotinamide hypoxanthine dinucleotide (NHD^+), NXD^+, or nicotinamide 1,N^6-ethenoadenine dinucleotide (e-NAD^+), respectively. The various nicotinamide purine dinucleotides (1 mM) were incubated with the cyclase (1.08 μg/mL) for 18 h at 23 °C. The cyclic products were purified by HPLC and evaporated to dryness.

Spectral Characterization. The UV and fluorescence spectra of various compounds were measured in solutions ranging from 1 to 150 μM and at various pH values. The solutions of different pH were prepared as follows: pH 4, 30.7 mM citric acid and 38 mM dibasic sodium phosphate; pH 7, 20 mM Tris, adjusted with HCl; pH 10.5, 20 mM Tris base. Stock solutions of nucleoside and nucleotide ranged from 1 to 10 mM and were diluted in the appropriate buffer for measurements. Other solutions are described in the figure legends.

HPLC. Compounds were purified, and some reactions were analyzed by an HPLC method described previously (Graeff et al., 1994b). Briefly, the compounds were diluted in 20 mM Tris, pH 7, and injected in 1 mL volumes. The nucleotides were eluted from a 15 × 0.3 cm column packed with AG MP-1 (Bio-Rad). The flow rate was maintained at 1 mL/min. The nucleotides were eluted with a gradient of trifluoroacetic acid starting at 0% B (solvent B is 150 mM trifluoroacetic acid in water, and solvent A is water) and held at 0% for 1 min, increased linearly to 4% from 1 to 6 min, increased linearly to 8% from 6 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. The column was calibrated with standards including NAD^+, cADPR, ADPR, NGD^+, cGDPR, GDPR, NHD^+, cIDPR, IDPR, NXD^+, cXDPR, and XDPR.

Heat-Induced Hydrolysis. A 25 nmol amount of the cyclic compounds was diluted in 500 μL of 20 mM Tris, pH 7, and placed in a boiling water bath for 30 min. The hydrolysis products, nucleoside diphosphoriboses, were purified by HPLC and evaporated to dryness. The reactions went to completion for cADPR, cGDPR, and cIDPR. Only 30% of cXDPR was converted to xanthosine diphosphoribose (XDPR) under these conditions.

Mass Spectrometry. Samples were reconstituted in H_2O (1 μg/mL) and analyzed 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. A 1 μL aliquot of sample was added to a matrix of dithiothreitol/dithioerythritol for positive ion spectra as described previously (Graeff et al., 1994b). Fast atom bombardment by 7 kV argon atoms was used to desorb the preformed ions from the matrix, which was supported on a gold probe held at +8 kV.

Materials. NHD^+, NGD^+, e-NAD^+, 1-methyladenosine, AMP, 1-methylguanosine, 7-methylguanosine, 7-methyl GMP, IMP, 1-methylinosine, 7-methylinosine, XMP, and 7-methylxanthosine were purchased from Sigma. All other compounds used were reagent grade.

RESULTS

Enzymatic Synthesis of Fluorescent Analogs of Cyclic ADP-Ribose. ADP-ribosyl cyclase catalyzes the cyclization of NAD^+ to produce cADPR (Lee et al., 1989). The enzyme is quite lenient with respect to the purine base of its substrate and can be utilized to produce various analogs of cADPR (Graeff et al., 1994b; Walseth & Lee, 1993). In this study, ADP-ribosyl cyclase was purified from *Aplysia* ovotestis and used to produce the cyclized products cADPR, cGDPR, cIDPR, and cXDPR from their respective substrates, NAD^+, NGD^+, NHD^+, and NXD^+. Figure 1 shows representative results of the synthesis of cIDPR from NHD^+ as monitored by HPLC. Incubation of NHD^+ with the cyclase produced a product with an elution time of about 1.4 min later than the substrate. The retention time for NHD^+ is 10.0 ± 0.1 min, and for cIDPR it is 11.4 ± 0.05 min (n = 3, ±SD). The released nicotinamide eluted at about 2 min. Two lines of evidence indicate the product is cyclized IDPR or cIDPR. First, hydrolysis by heat treatment converted it quantitatively to IDPR with an elution time at about 16.2 ± 0.01 min as shown in Figure 1. Second, mass spectrometry analysis of the cyclized product showed a major positive ion of 543 mass units, which is 18 mass units smaller than the 561 mass measured for the positive ion of IDPR. The mass difference is consistent with cIDPR being smaller than its hydrolysis

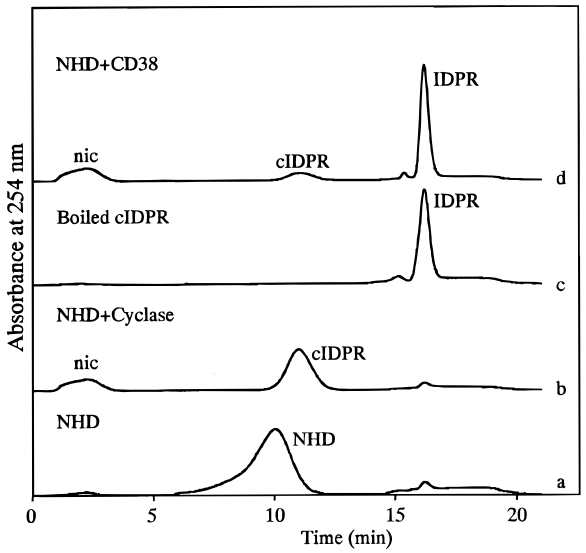

FIGURE 1: Synthesis of cyclic IDP-ribose. cIDPR was prepared by incubating NHD^+ (1 mM) with *Aplysia* ADP-ribosyl cyclase (1.08 μg/mL) in 20 mM Tris, pH 7, overnight at 23 °C. The chromatograms in the figure were displaced along the *Y*-axis to avoid superposition. Trace a represents the starting material. Trace b represents the products of the reaction, cIDPR and nicotinamide (nic). Trace c represents the hydrolysis product of cIDPR (50 μM), IDPR. The peak corresponding to cIDPR in trace b was collected and lyophilized. The reconstituted sample was then treated for 30 min in a boiling water bath. Trace d represents the products formed after incubating 50 μM of NHD^+ with CD38 (0.4 μg/mL) in 20 mM Tris, pH 7, for 30 min at 37 °C, which resulted in formation of both cIDPR (18%) and IDPR (82%).

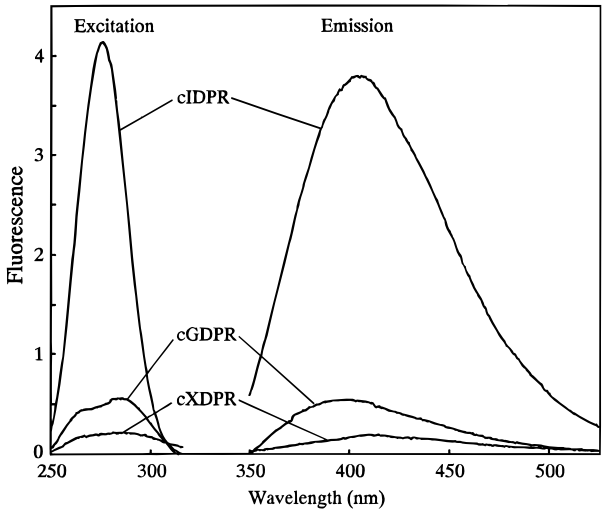

FIGURE 2: Fluorescence spectra of the cyclic analogs of cADPR. Excitation was set at 280 nm and emission at 410 nm. All the cyclic nucleotides were at 2 μM in 20 mM Tris, pH 7.0.

product, IDPR, by one water molecule. Further support for the product being cIDPR comes from the use of CD38, a bifunctional enzyme that catalyzes both the synthesis and hydrolysis of cADPR (Howard et al., 1993; Takasawa et al., 1993). Treatment of NHD^+ with CD38 produced both cIDPR and IDPR, with the latter being the dominant product (Figure 1).

Spectral Characteristics of the Analogs and the Site of Cyclization. In contrast to cADPR, the cyclic analogs synthesized from NHD^+, NGD^+, and NXD^+ are fluorescent. The excitation and emission spectra are shown in Figure 2. The excitation maxima are at 275 nm for cIDPR, 285 nm for cGDPR, and 288 nm for cXDPR. The emission maxima

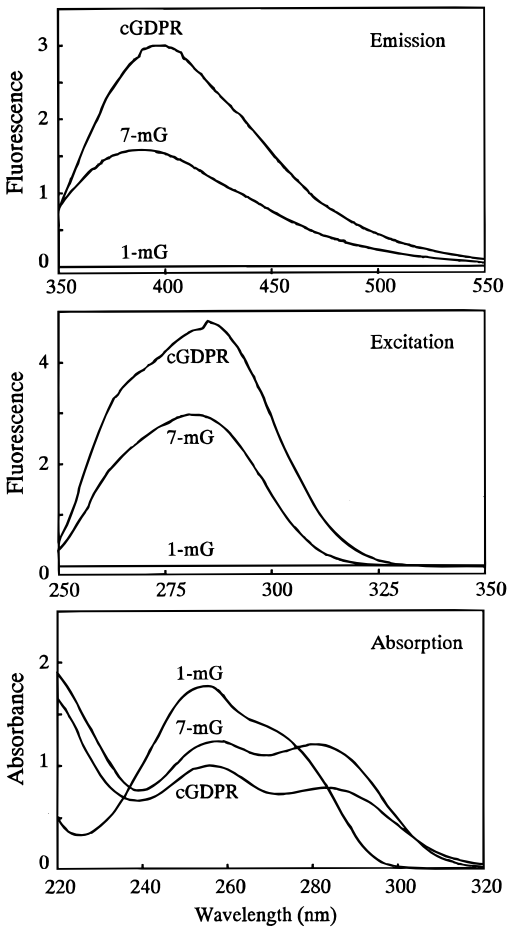

FIGURE 3: Spectral comparisons of cGDPR with 1- and 7-methylguanosine (7-mG). Excitation was set at 300 nm and emission at 410 nm. All nucleotides were at 20 μM in 20 mM Tris, pH 7.0. No fluorescence was detected for 1-methylguanosine (1-mG). The UV spectra were measured at pH 7.0 in 20 mM Tris, with 100 μM 1-mG, 91 μM 7-mG, and 110 μM cGDPR.

are at 404 nm for cIDPR, 400 nm for cGDPR, and 412 nm for cXDPR. The most fluorescent analog is cIDPR which has 7.3 times higher fluorescence than the same concentration of cGDPR and 18.9 times higher fluorescence than cXDPR. None of the fluorescent analogs released Ca^{2+} at 1 mM concentrations nor did they antagonize cADPR in the Ca^{2+} release assay (data not shown).

The cyclic structure of cADPR has been determined by X-ray crystallography, and the site of cyclization was shown to be at the N1-position of adenine ring (Lee et al., 1994b). It is expected that the site of cyclization of the fluorescent analogs would also be at N1. However, spectral characterization of the analogs described below indicates, instead, it is likely to be at the N7-position of the purine ring. Fluorescence and ultraviolet light (UV) absorption spectra of the fluorescent analogs and related compounds were compared. Representative results are presented only for cGDPR. Of all the N1, N7, and unsubstituted guanine nucleotides tested, only the N7-substituted nucleotides or nucleosides are fluorescent. Figure 3 shows the excitation, emission, and UV spectra of 7-methylguanosine and compares them with those of cGDPR. It is clear that spectrally cGDPR is very similar to 7-methylguanosine. It is also shown that 1-methylguanosine is not fluorescent and has a very different UV spectrum, as compared to cGDPR and 7-methylguanosine.

Table 1: UV Absorption and Fluorescence Emission Maxima of Selected Purine Nucleosides and Nucleotides

	absorption maxima (nm)			emission maxima (nm)[a]		
	pH 4	pH 7	pH 10.5	pH 4	pH 7	pH 10.5
AMP	258	258	258	NF[b]	NF	NF
1-mA	257	257	258	NF	NF	NF
cADPR	257	257	259	NF	NF	NF
GMP	252	252	253	NF	NF	NF
1-mG	255	255	255	NF	NF	NF
7-mG	257	257	**275**[c]	379	389	408
7-mGMP	257	257	**282**	377	380	409
cGDPR	257	256	**286**	393	400	415
IMP	248	248	249	NF	NF	NF
1-mI	249	249	249	NF	NF	NF
7-mI	253	264	**264**	390	388	388
cIDPR	253	265	**269**	405	404	403

[a] The emission spectra were measured at an excitation wavelength of 300 nm. [b] NF, nonfluorescent. [c] Numbers in bold type are values of UV absorption maxima that exhibit a red shift at higher pH.

Further support of the N7-linkage comes from the pH dependence of the UV absorption maximum as shown in Table 1. The UV absorption maximum of cGDPR is shifted from 257 nm at pH 4 to 286 nm at pH 10.5, which is similar to that displayed by 7-methylguanosine (7-mG, shifted from 257 to 275 nm) and 7-methyl-GMP (7-mGMP, shifted from 257 to 282 nm). In contrast, 1-methyl guanosine (1-mG) and the unsubstituted GMP show no such pH-dependent shift in their UV absorption maxima. The shift in the absorption maximum of cGDPR is primarily due to the pH-dependent decrease in absorbance at 257 nm (see spectrum in Figure 3). Very little shift was observed for cADPR ($\leq$2 nm), which is consistent with the site of cyclization being at N1, since the unsubstituted AMP and 1-methyladenine also display no shift. Similar to cGDPR, cIDPR shows a pH-dependent shift of absorption maximum from 253 to 269 nm. The UV spectrum of cIDPR has a single peak between 240 and 300 nm, and this absorption peak shifts to a longer wavelength with increasing pH. No shift was seen with either 1-methylinosine or IMP. On the other hand, 7-methylinosine displays a pH-dependent spectral shift similar to that seen in cIDPR (Table 1).

Corresponding to the shifts in the UV spectra, the cyclic analogs also display changes in fluorescence as shown in Figure 4. As the pH increases, the fluorescence of cIDPR increases whereas that of cGDPR decreases and that of cXDPR shows little change (not shown). In each case, the pH-dependence of the fluorescence of the respective 7-substituted derivative is the same as the cyclic analog, further demonstrating the similarity between them. On the other hand, none of the 1-substituted derivatives is fluorescent, irrespective of the pH. The pH-dependent changes in the fluorescence of the cyclic analogs are completely reversible, suggesting that they are due to changes in the protonation state of the compounds.

All of these results are consistent with the site of cyclization of these fluorescent analogs being at the N7-position of the purine ring. Their proposed structures are shown in Figure 5. The N7-linkage would give the purine ring a positive charge, making the compounds zwitterionic. When the phosphate group is neutralized at acidic pH, the compounds would be cationic. This is consistent with the free acid of cIDPR displaying a prominent positive ion mass

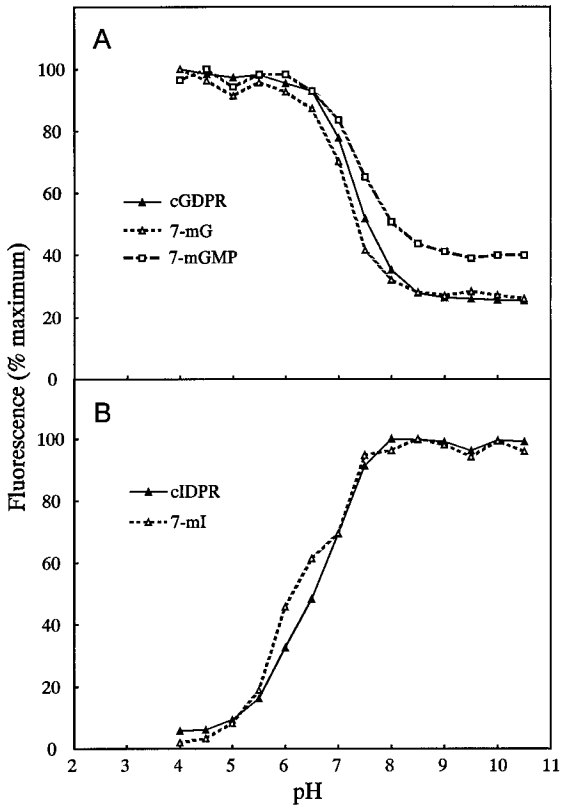

FIGURE 4: The pH-dependence of fluorescence of purine nucleosides and nucleotides. The fluorescence was excited at 280 nm and measured at 410 nm. The concentrations of cIDPR, 7-methylinosine (7-mI), cGDPR, 7-methylguanosine (7-mG), and 7-methyl-GMP (7-mGMP) were between 2 and 4 μM, and the buffers used were as follows: pH 4−5.5, 25 mM sodium acetate adjusted with acetic acid; pH 6−7, 25 mM Hepes adjusted with NaOH; and pH 7.5−10.5, 20 mM Tris adjusted with HCl. The fluorescence intensity was expressed as percent of the maximum for each substance.

spectrum (data not shown). The measured mass of 543 of the positive ion of cIDPR is also identical to the calculated mass based on the structure shown in Figure 5. An N7-linkage of chemically synthesized cGDPR has previously been proposed (Gu & Sih, 1994).

More supporting evidence that ADP-ribosyl cyclase can cyclize the substrate at N7 comes from the use of an analog of NAD^+ which has the N1-position blocked. As shown in Figure 6, the cyclase readily converted etheno-NAD^+ (e-NAD^+) to a metabolite that has an elution time on HPLC, consistent with it being cyclic etheno-ADP-ribose (e-cADPR). Similar to the results shown in Figure 1, CD38, on the other hand, produced mainly etheno-ADP-ribose (e-ADPR) from the substrate instead, as is expected from the hydrolytic activity of the enzyme. Therefore, when the N1-position of the adenine is blocked by the etheno group, the cyclase can still cyclize the e-NAD^+. The only available site, in this case, is the N7-position.

Use of Fluorescent Analogs for Monitoring the Cyclization Reaction. Since only the cyclic analogs are fluorescent (except e-ADPR) and neither their substrates nor hydrolysis products are fluorescent, the cyclization reaction can be continuously monitored by simply measuring the fluorescence of the cyclic product. Figure 7A shows the fluorescence increases produced during incubations of 0.54 μg of ADP-ribosyl cyclase/mL with 30 μM NHD^+, NGD^+, or NXD^+. To avoid the potential interference by the 280 nm absorption of the enzyme, an excitation wavelength of 300

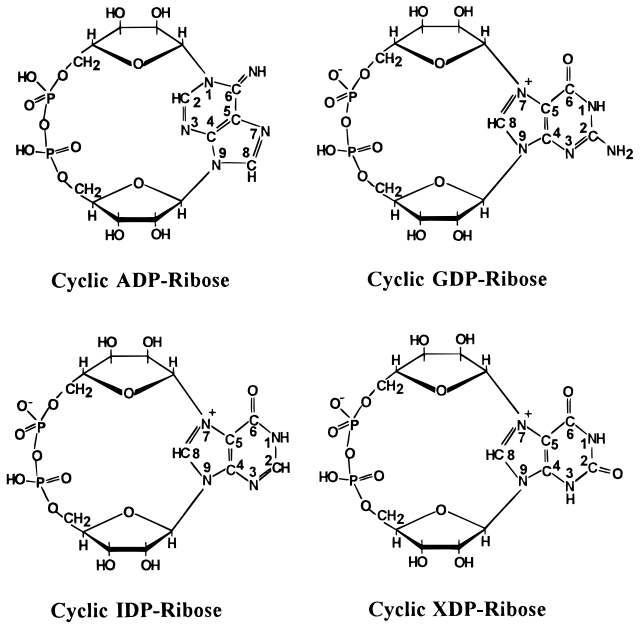

FIGURE 5: The proposed structures of the fluorescent analogs of cADPR. The structure of cADPR is based on X-ray crystallography (Lee et al., 1994b). The site of cyclization is at the N1-position of the adenine ring which is in the *syn*-configuration. The site of cyclization in the analogs is at the N7-position instead. The purine ring in these analogs should be in the *anti*-configuration. The analogs should also be zwitterions at neutral pH with the positive charge at N7 and a negative charge at the phosphate.

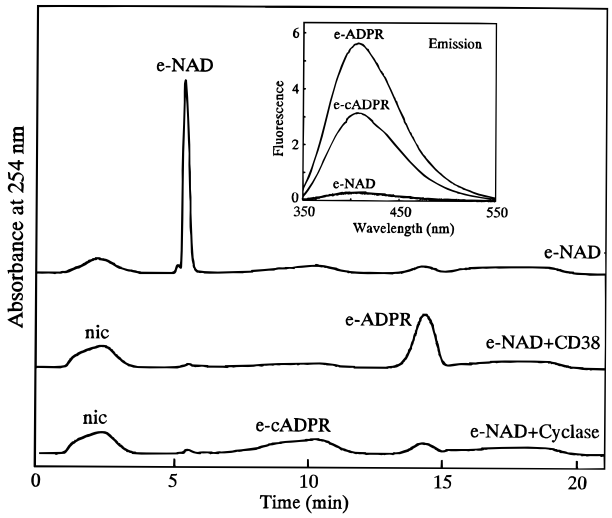

FIGURE 6: Reaction products of 1,N^6-etheno-NAD$^+$ incubated with CD38 and ADP-ribosyl cyclase. 1,N^6-Etheno-NAD$^+$ (e-NAD$^+$) (12 μM) was incubated with either CD38 (0.2 μg/mL, 37 °C) or the cyclase (0.27 μg/mL, 23 °C) at pH 7 for 30 min and analyzed by HPLC. The peaks corresponding to cyclic 1,N^6- etheno-ADP-ribose (e-cADPR) and 1,N^6- etheno-ADP-ribose (e-ADPR) were collected. The inset shows the emission spectra of e-ADPR, e-cADPR, and 1,N^6- etheno-NAD$^+$ (e-NAD$^+$), all at 1.3 μM in 20 mM Tris, pH 7. The excitation wavelength used was 300 nm.

nm was used for monitoring the fluorescence of the analogs. The maximal fluorescence increase was highest with NHD$^+$ as substrate, although the rate of increase was slowest. The plateau reached with each substrate is the point at which the substrate is exhausted. The fluorescence can be calibrated by known concentrations of the analogs. Using the calibration curves, the rates of production of cXDPR, cGDPR, and cIDPR were determined to be 50.0 ± 1.6, 36.3 ± 3.1, and 4.6 ± 0.2 μmol/mg/min (n = 3, ±SD) at room temperature,

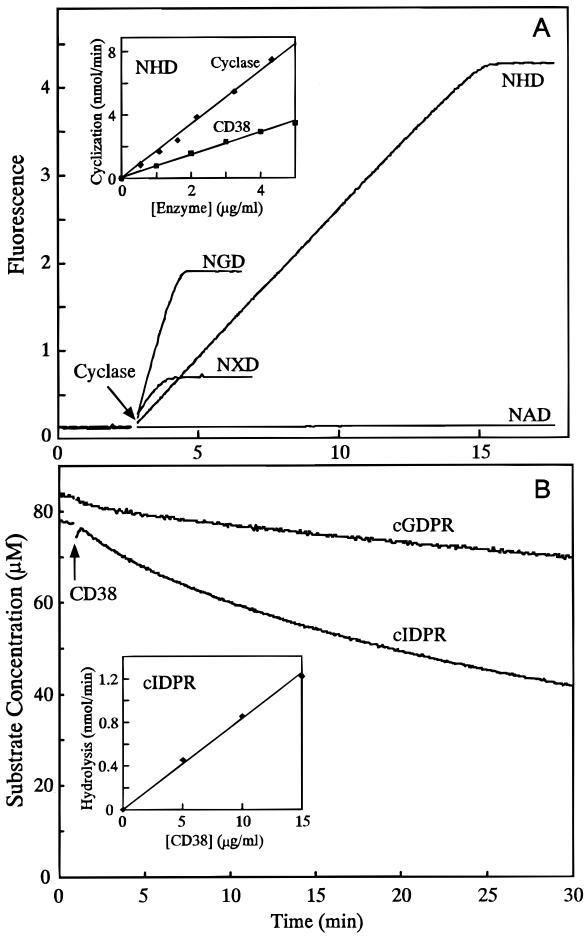

FIGURE 7: Fluorescence assay of ADP-ribosyl cyclase and CD38. A. ADP-ribosyl cyclase (0.54 μg/mL) was incubated with 30 μM of one of the following: NAD$^+$, NGD$^+$, NHD$^+$, or NXD$^+$ in 20 mM Tris, pH 7, at room temperature and monitored fluorimetrically with an excitation wavelength of 300 nm and an emission wavelength of 410 nm. The reactions were started with the addition of enzyme. No fluorescence was detected with NAD$^+$ as substrate. The inset shows the dependence of the rate of cyclizing NHD$^+$ (30 μM) on the cyclase and CD38 concentration under the same experimental condition. The cyclization rate was determined by calibrating the observed fluorescence increase with known concentrations of cIDPR. B. The hydrolysis of cIDPR (78 μM) or cGDPR (84 μM) was monitored by the decrease in fluorescence following addition of CD38 (5 μg/mL). The inset shows the initial rate of hydrolysis of cIDPR is proportional to the concentration of CD38.

respectively, from the rates of the fluorescence increase. The rates of production of cIDPR by the cyclase and CD38 are linearly dependent on the enzyme concentration as shown in the inset of Figure 7A for both the *Aplysia* cyclase and CD38.

The fluorescence time courses shown in Figure 7A provide a very convenient method for determining the K_m values of the cyclase (Graeff et al., 1994b). Using calibration curves, the fluorescence time courses can be converted to concentration time courses. Derivatization of the curves converts them to curves representing the rate of production of the cyclic analog versus time. The concentration of the substrates remaining at each time point can be calculated by subtracting the concentration of the cyclic product from the starting substrate concentration. The K_m values can then be determined by a reciprocal plot of the rate verses substrate concentration. Using this method K_m's for NXD$^+$, NGD$^+$, and NHD$^+$ were determined to be 6.2 ± 1., 1.7 ± 0.2, and

Table 2: Kinetic Constants for the ADP-Ribosyl Cyclase with NAD^+or Its Analogs As Substrate

	K_m (μM)	V_{max} (μmol/mg/min)
NAD^+	39 ± 1^b	500 ± 60^b
NGD^+	1.7 ± 0.2	36.3 ± 3.1
NHD^+	0.5 ± 0.3	4.6 ± 0.2
NXD^+	6.2 ± 1.4	50.0 ± 1.6
e-NAD^+	6.2 ± 1.7	94.4 ± 4.2

[a] Kinetic constants for the NAD^+ analogs were determined using the fluorimetric method described in the text. Values are mean ±SD of three determinations. [b] Data from Graeff et al., 1994b.

0.53 ± 0.25 μM ($n = 3$, ±SD), respectively. For comparison, the K_m of the *Aplysia* cyclase for NGD^+ was previously determined to be 2.8 ± 0.2 μM by using an HPLC method (Graeff et al., 1994b). A summary of the kinetic data for ADP-ribosyl cyclase is provided in Table 2.

A fluorimetric assay can also be developed for monitoring the cADPR hydrolase activity. Figure 7B shows the time courses of hydrolysis of cGDPR and cIDPR following the addition of CD38. The hydrolysis was monitored as a decrease in the fluorescence of the cyclic substrate as it was converted to its nucleoside diphosphoribose. Very little hydrolysis of cGDPR was seen over the 30 min incubation, consistent with previous results showing that cGDPR is very resistant to hydrolysis as measured by HPLC (Graeff et al., 1994b; Lee et al., 1995b). In contrast, cIDPR was readily hydrolyzed by CD38 and the rate of hydrolysis was proportional to the concentrations of CD38 used as shown in the inset of Figure 7B. Using a similar procedure for determining the K_m values for the cyclase reaction described above, the fluorimetric method of the hydrolase gave a K_m value of 2.7 ± 0.8 mM ($n = 3$, ±SD) for the hydrolysis of cIDPR by CD38. These results show that the best substrate to use for the fluorimetric measurement of the cyclase reaction is NGD^+ because the product, cGDPR, is resistant to hydrolysis and therefore the hydrolase activity would not interfere with the assay. On the other hand, the ready hydrolysis of cIDPR by CD38 makes it best suited for monitoring the hydrolase reaction.

The fluorimetric method also provides a very convenient way for searching for specific inhibitors of the cyclase and the hydrolase. This is illustrated in Figure 8. Since CD38 also uses NAD^+ as substrate, high concentrations of NAD^+ should competitively inhibit the cyclization of NGD^+. Indeed, the addition of 0.4 mM NAD^+ during the cyclization reaction produced about a 3-fold reduction of the rate of formation of cGDPR from 2.9 to 1.1 nmol/min. The inhibition appears to be competitive as indicated by the double-reciprocal plot shown in Figure 8A. A K_i value of 12 μM for NAD^+ can be determined from the plot. Nicotinamide at 20 mM inhibited the cyclase even more as shown in Figure 8B. The accompanying kinetic analysis reveals a complex inhibition of NGD^+ by nicotinamide. In the presence of 2 mM of nicotinamide, the K_m value for NGD^+ increased from 1.9 to 3.7 μM while the V_{max} value decreased from 19.3 to 14.3 μmol/min/mg. The complex inhibition of nicotinamide on ADP-ribosyl cyclase has previously been reported (Inageda et al., 1995) and may be related to nicotinamide being able to drive the reaction backward, thus regenerating NAD^+ (Kim et al., 1993).

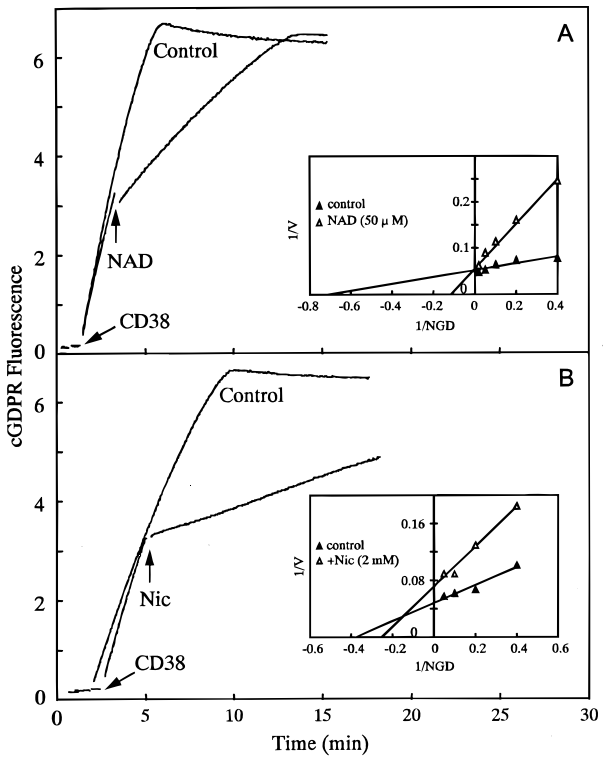

FIGURE 8: The use of the fluorimetric assay as a convenient way to screen for inhibitors of the cyclization activity of CD38. NGD^+ (140 μM) was incubated with CD38 (11 μg/mL) in 50 mM Hepes, pH 7.0, and at 37 °C and the fluorescence at 410 nm (excitation at 300 nm) was monitored (Control). A. When indicated, 0.4 mM NAD^+ was added. The inset shows a double-reciprocal plot of the reaction rate (μmol/min/mg) versus NGD^+ concentration (μM) in the presence and absence of 50 μmol of NAD^+. B. When indicated, 20 mM nicotinamide (Nic) was added. The inset shows a double reciprocal plot of the reaction rate (μmol/min/mg) verses NGD^+ concentration (μM) in the presence and absence of 2 mmol of nicotinamide.

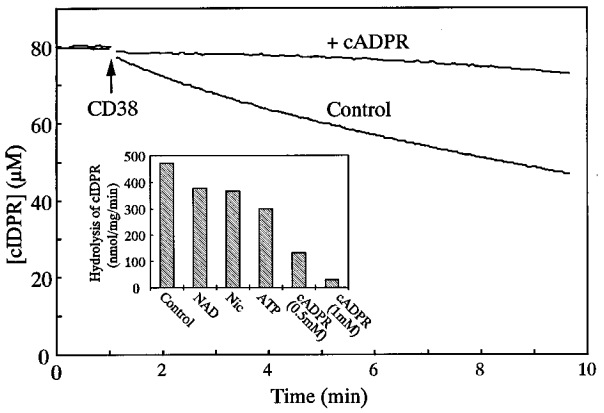

FIGURE 9: The use of the fluorimetric assay for screening for inhibitors of the hydrolytic activity of CD38. The hydrolysis reaction catalyzed by CD38 (11 μg/mL) was monitored by the decrease in cIDPR (80 μM) fluorescence (Control). The reaction was done at pH 7.0, 50 mM Hepes, and at 37 °C. When indicated, cADPR was present as an inhibitor at 1 mM prior to the addition of CD38. The inset shows the effects of NAD^+ (0.5 mM), nicotinamide (Nic, 20 mM), ATP (4 mM), and two concentrations of cADPR (0.5 and 1 mM) on the hydrolytic reaction of CD38. The experimental conditions were the same as those described above.

Figure 9 shows that cADPR is effective in competing with cIDPR as a substrate for the hydrolase reaction. At 0.5 mM, cADPR reduced the rate of hydrolysis of cIDPR by about 4-fold as shown in the inset of Figure 9. It is of interest to

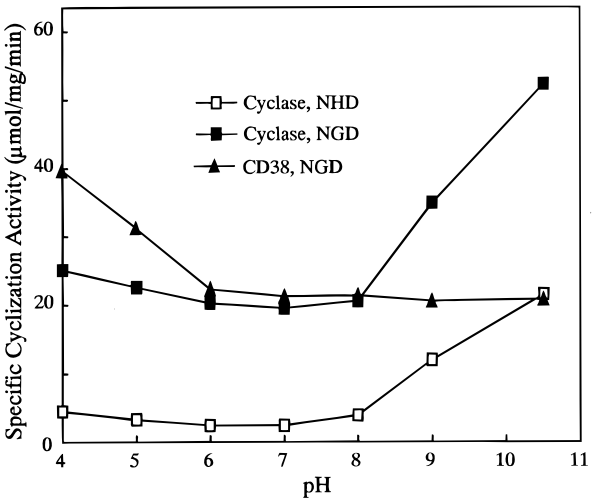

FIGURE 10: The pH-dependence of the cyclization reaction catalyzed by ADP-ribosyl cyclase and CD38. The specific activity of the cyclization reaction catalyzed by CD38 (0.4 μg/mL) was measured with the fluorimetric assay using NGD^+ (150 μM, filled triangles) as substrate. The cyclase (0.54 μg/mL) reaction was measured using either NHD^+ (300 μM, open squares) or NGD^+ (150 μM, filled squares) as substrate. An excitation wavelength of 300 nm and an emission wavelength of 410 nm were used. The reactions were done at 23 °C for the cyclase and at 37 °C for CD38 with the following buffers: pH 4 or 5, 25 mM sodium acetate adjusted with acetic acid; pH 6 or 7, 25 mM Hepes adjusted with NaOH; pH 8, 9, or 10.5, 20 mM Tris adjusted with HCl.

note that NAD^+ and nicotinamide, although very effective in inhibiting the cyclase reaction, have only slight effects on the hydrolase activity.

As shown in Figure 4, the fluorescence of cIDPR and cGDPR show an opposite dependence on pH. It would be expected that the fluorimetric method may not be suitable for investigating the pH dependence of the cyclase reaction. This was found not to be the case. The cyclase was incubated with either NHD^+ or NGD^+ at various pH. The resulting increases in fluorescence were calibrated at the same pH values as the incubation using known concentrations of the respective cyclic nucleotides. As shown in Figure 10, the specific cyclase activity as determined by the fluorimetric method using the two nucleotides shows the same dependence on pH, even though the fluorescence of their cyclic products have quite different pH-dependencies (Figure 4). These results validate the use of the fluorimetric method for investigating the pH-dependence of the cyclase activity. This method was applied to CD38, and the pH-dependence of its activity was found to be opposite to that of the cyclase. The cyclization activity of CD38 is higher at acidic pH than at alkaline pH, while the opposite is true for the cyclase activity.

DISCUSSION

ADP-ribosyl cyclase is used in this study to synthesize a series of analogs of cADPR. These analogs are novel not only because they are fluorescent but also because the site of cyclization in them is different from that of cADPR. X-ray crystallography of cADPR definitively establishes the site of cyclization at the N1-position of the adenine ring (Lee et al., 1994b). Spectral characterizations indicate none of the N1-substituted compounds is fluorescent whereas all of the N7-substituted nucleotides are fluorescent, strongly suggesting that the linkage site in these analogs is at the N7-position of the purine base. Further support of an N7 linkage comes

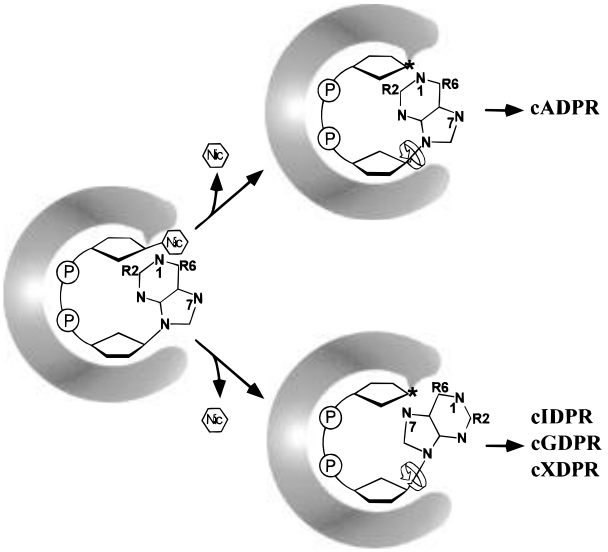

FIGURE 11: A model of the catalysis by ADP-ribosyl cyclase. The substrate is bound to the active site of the cyclase in a folded conformation such that the terminal ribose is in close proximity to the purine ring. This is followed by the release of the nicotinamide (Nic) and the formation of an intermediate characterized by having the anomeric carbon of the terminal ribose in an activated state (*). The purine ring of the substrate is proposed to have freedom of rotation around the C1′-N9 bond. When the ring is in the *syn*-orientation, attack of the activated ribosyl carbon by N1 of the adenine ring (R_6: $-NH_2$) would lead to formation of cADPR. For the other analogs, the keto group at the 6-position (R_6: $=O$) may reduce the reactivity of N1, allowing N7 to link with the terminal ribose as the purine rotates to the *anti*-orientation. The result is the formation the fluorescent analogs, cIDPR, cGDPR, or cXDPR, having the site of cyclization at the N7-position instead of the N1-position in cADPR.

from the similarities of the UV spectra and the pH-dependence of the fluorescent analogs to those of the N7-substituted compounds.

The novel fact that ADP-ribosyl cyclase can cyclize a variety of substrates at two different sites has important implications about the enzymatic mechanism, which is depicted in Figure 11. The crystal structure of NAD^+ shows that it is a long linear molecule with the adenine ring and the anomeric carbon of the terminal ribose separated by about 12.3 Å (Saenger et al., 1977). In order to cyclize the molecule, the cyclase must bind NAD^+ in a folded conformation such that the adenine ring can be brought into close proximity of the ribose. The active site of the cyclase is thus depicted in Figure 11 as a pocket, and NAD^+ is bound in a folded conformation. Since the cyclase can use various nicotinamide purine dinucleotides as substrates, the active site must not have high recognition specificity for the base, which is therefore shown as positioned toward the opening of the active pocket. The next step of the catalysis is likely to be the release of the nicotinamide group (Nic) and the formation of an intermediate characterized by having the anomeric carbon of the terminal ribose in an active state (*). The intramolecular attack by the N1 of the adenine would result in the formation of cADPR. This mode of catalysis is consistent with the cyclization linkage being in the β-conformation as determined by X-ray crystallography (Lee et al., 1994b) since a double inversion at the anomeric carbon would result in a β-linkage.

The alternate pathway depicted in Figure 11 shows that intramolecular attack by N7 of the base would result in the

formation of the N7-linked fluorescent analogs as described in this study. The fact that the cyclase can cyclize its substrates at two sites indicates that the base must be able to rotate around the N9−C1′ bond. This is consistent with the active site not recognizing the base with high specificity since rigid binding to the recognition site would make rotation of the base impossible. The N7 attack should have the conformational advantage since the base is in the *anti*-orientation, the same as in NAD^+ (Saenger et al., 1977), whereas for the N1 attack, the base must rotate from the *anti*- to the *syn*-orientation. That N1-linkage is preferred in the formation of cADPR would indicate that it is either chemically or sterically more reactive than N7. It is likely that the presence of the keto group at the 6-position of all the fluorescent analogs reduces the reactivity of N1 and allows the cyclization to occur at N7 instead. That N1 in the adenine is more reactive than N7 in the fluorescent analogs is consistent with the rate of formation of cADPR being more than 20 times faster than cGDPR (Graeff et al., 1994b). These differences in the point of attack may also reflect the differences of binding of NGD^+, NXD^+, and NHD^+ compared to NAD^+. After all, the K_m's of the former group range from 0.5 to 6 μM whereas that of NAD^+ is 39 μM.

A scheme similar to that shown in Figure 11 can also account for the bifunctionality of the catalysis of CD38. Attack of the activated intermediate by water would produce ADP-ribose instead of cADPR. Thus, one needs to further propose that the active site in CD38 is more accessible to water, perhaps due to a slightly different conformation of the protein. Indeed, a single mutation of one of the 10 cysteine residues is sufficient to eliminate the hydrolytic activity of CD38 (Tohgo et al., 1994). The fact that CD38 can bind and hydrolyze cADPR indicates the active site must recognize the cyclic conformation of the molecule, consistent with the scheme proposed in Figure 11.

Examination of the structures of all the fluorescent compounds described in this study indicates that the most important features necessary for fluorescence are having a keto group at C6 and aliphatic groups that link to N7 and N9. For example, 1-methylguanosine has a C6 keto but has no substitution at N7, and it is not fluorescent. The N7 aliphatic group is not sufficient for fluorescence either, since caffeine (1,3,7-trimethylxanthine), which has an N7 aliphatic linkage and a C6 keto but lacks an N9 ribose, is also nonfluorescent. The 2-position of the base is also important since the substitution of H (cIDPR) by either NH_2 (cGDPR) or O (cXDPR) results in reduction of fluorescence. Thus, cIDPR, with no substitution at C2 is the most fluorescent, while cGDPR, which has an amino substitution, is less fluorescent. The least fluorescent is cXDPR, which has a keto group in the 2-position. Other functional groups on the cyclic nucleotides do not appear to be important for fluorescence. For example, the phosphate groups are not important because 7-methylinosine and 7-methylguanosine are fluorescent. The cyclic structure is also not critical because noncyclic compounds, such as 7-methylguanosine, are also fluorescent, which is a property that has been used previously to estimate base-stacking in the cap structure of mRNA (Nishimura et al., 1980).

The use of these fluorescent analogs for monitoring the cyclase and hydrolase activities is demonstrated in this study. This highly convenient fluorimetric method allows continuous and separate monitoring of the cyclization and hydrolysis reactions. With this assay, it is no longer necessary to purify the substrate and products of the reactions. The fact that cGDPR is very resistant to hydrolysis makes the assay employing NGD^+ ideally suited for monitoring the cyclization without much interference from the hydrolase reaction. Indeed, the NGD^+ assay should be applicable even in crude extracts where the hydrolase activity is likely also to be present (Graeff et al., 1994b). On the other hand, as shown in this study, cIDPR can be readily hydrolyzed by CD38 and thus provide an independent assay for the hydrolase. With this fluorimetric assay, kinetic parameters of the cyclase and hydrolase as well as their pH-dependence can be easily measured. The convenience of this assay should greatly facilitate investigations of the regulation of cADPR metabolizing enzymes and the search for specific inhibitors or activators for the enzymes.

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