Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 19, pp. 2267-2272, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00393-2

ENZYMATIC CYCLIZATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADP)

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ABSTRACT: The ADP-ribosyl cyclase of *Aplysia californica* catalyzed the conversion of NADP, 3'-NADP and 2',3'-cyclic NADP into their corresponding cyclic nucleotides (established by NMR, UV, and MS analyses). Cyclic-ADP-ribose-phosphate (cADPRP) was more potent than cADPR in mobilizing calcium in the rat brain microsomal system; the EC_{50} for cADPRP and cADPR were 9.2 μM and 22 μM respectively.

Cyclic ADP-ribose (cADPR) is a metabolite of NAD and its synthesizing enzyme, ADP-ribosyl cyclase, has been shown to be widely distributed among mammalian, amphibian, avian, and marine invertebrate tissues.[1] Biological studies have shown that cADPR may play an important role as a second messenger analogous to inositol triphosphate (IP_3) in Ca^{2+} signaling but targeting a different intracellular Ca^{2+} channel, possibly the ryanodine receptor.[2] The structure of cADPR was established as shown[3,4] and recently has been confirmed by chemical synthesis.[5]

Three types of enzyme systems are now known for the biosynthesis of cADPR from NAD (cyclase), and the hydrolysis of cADPR to ADP-ribose (ADPR) (hydrolase). (1) A membrane-bound NAD glycohydrolase was purified to homogeneity from canine spleen microsomes. The ratio of NADase:cyclase:hydrolase activities for this enzyme was found to be 100:2:30.[6] (2) The human CD38 antigen and its rodent analogs also possess cyclase and hydrolase activities. An ectoenzyme was purified to homogeneity from solubilized human erythrocyte membranes and exhibited a similar ratio of enzyme activities (100:1:10).[7] (3) In contrast, the enzyme derived from the ovotestis of *Aplysia californica* is rich in cyclase and has little NADase and hydrolase activities. This enzyme is now commercially available from Sigma in a highly purified state[8] and has been used for the laboratory preparation of cADPR.

In 1991, Lee and Aarhus[9] incubated NADP with the *Aplysia* cyclase and then assayed for the possible production of the cADPRP using the calcium release assay. On the assumption that the cADPR analogs can also induce calcium release, they surmised that NADP was not cyclized by the *Aplysia* enzyme. Very recently, Inageda et al.[10] showed that nicotinamide was released after exposure of NADP or $1,N^6$-etheno-β-NAD to the *Aplysia* enzyme, but unfortunately the products of these reactions were not isolated and characterized. We recently found that the *Aplysia* cyclase catalyzed the transformation of $1,N^6$-etheno-β-NAD into a novel cyclic nucleotide whose glycosyl linkage is attached onto the N-1 position of the etheno-adenine nucleus, corresponding to the N-7 position of the adenine ring.[11] This alternative mode of cyclization indicated that the *Aplysia* cyclase has a more relaxed substrate specificity than was previously thought and prompted us to reexamine its biosynthetic versatility.

Since NADP is widely distributed among mammalian tissues, we exposed NADP to the *Aplysia* cyclase and found it to be readily cyclized to form cADPRP in yields and rates comparable to that of NAD. In a representative experiment, NADP (10 mg, 13.4 μmol) was incubated with 10 units of the *Aplysia* cyclase in 10 mL of 10 mM phosphate buffer (pH 7.0) at 23°C for 3 h. Approximately 70% of NADP was converted into cADPRP and nicotinamide accompanied by a trace amount of ADPRP. The reaction mixture was concentrated under reduced pressure and then chromatographed over an HPLC Synchropak AX-100 ion-exchange column (250 X 4.6 mm i.d., 5 μM). The column was eluted with a linear gradient of 0-1.2 M NaCl in 0.1 M KH_2PO_4 at a flow rate of 1 mL/min for 16 min, followed by isocratic elution using 1.5 M NaCl in 0.1 M KH_2PO_4 until 22 min at a flow rate of 1.2 mL/min. The HPLC elution profile is shown in Fig. 1A. Fractions containing cADPRP (retention time 13.2 min) were combined and desalination was achieved by passing the column eluent through a small charcoal column (0.4 g of activated charcoal, granular 20-30 mesh). 95% of the cADPRP was adsorbed onto the column, which was washed with 20 mL of deionized water to remove any residual salt. cADPRP was eluted from the column with an ethanol-water-ammonia mixture (60 mL of ethanol containing 0.1 mL of concentrated ammonia, made up to 100 mL with water). Fractions containing UV absorbance at 260 nm were combined and concentrated to dryness under reduced pressure. The recovery yield of this step was 75%. The concentrated cADPRP solution was then further purified using a Waters Nova-pak C18 column, which was eluted isocratically using 3 mM TFA at a flow rate of 0.5 mL/min. The retention time of cADPRP was 5.4 min. Pure cADPRP was obtained as a white solid in 55% total yield (based on 25% of NADP recovered). ^{1}H NMR (300 MHz, D_2O, pH 3.5) δ 4.08-4.22 (2H, m), 4.38-4.55 (4H, m), 4.57-4.67 (1H, m), 4.75-4.90 (2H, m), 5.68-5.78 (1H, m), 6.20 (1H, d, J=3.9 Hz), 6.27 (1H, d, J=5.8 Hz), 8.45 (1H, s), 9.08 (1H, s); ^{13}C NMR (300 MHz, D_2O, pH 3.5) δ 66.3, 71.5, 73.5, 76.7, 78.4, 86.0, 86.1, 90.2, 90.8, 97.2, 121.3, 143.7, 147.2, 148.8, 151.1; UV (H_2O, pH 6.0) λ_{max} 256 nm; MS (ESI) m/z 620 $(M-H)^+$, 642 $(M-2H+Na)^+$.

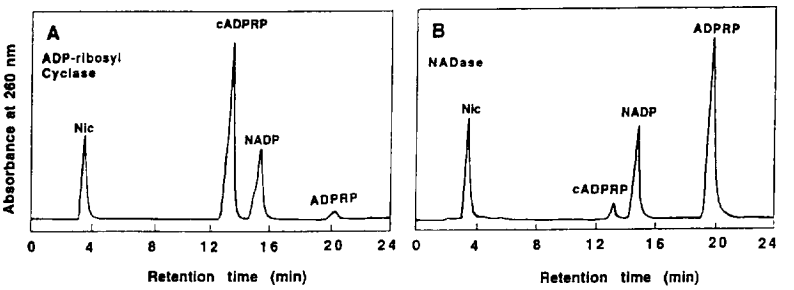
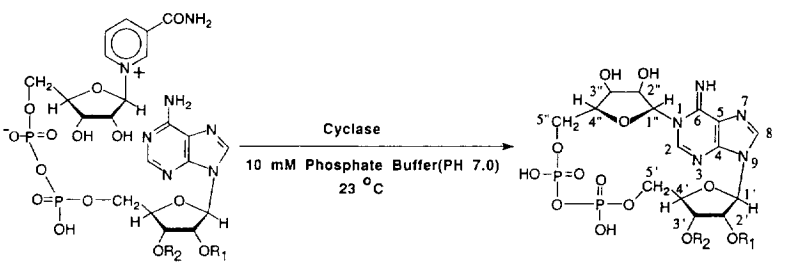


Figure 1. Cyclization of NADP by *Aplysia* cyclase and pig brain NADase. (A) NADP (1.34 mM) was incubated with the *Aplysia* cyclase (0.5 μg/mL) at 23°C for 3 h. (B) NADP (2.0 mM) was incubated with pig brain NADase (8.5 mg/mL) at 23°C for 20 min. Samples were analyzed by ion-exchange-HPLC as described in the text.

With cADPRP preparations, we found it more difficult to desalinate than cADPR using the reverse phase C18 HPLC column. However, inorganic salts were removed from cADPRP by first adsorption of the cyclic nucleotide onto charcoal, and then eluted off the column using ethanolic NH_3. To assure that the 2'-phosphate in NADP did not migrate during the conditions of incubation, we exposed 3'-NADP and 2',3'-cyclic NADP to the *Aplysia* cyclase. Both substrates were likewise cyclized to yield their corresponding cyclic nucleotides, whose retention times on ion-exchange and reverse-phase HPLC columns (3'-cADPRP: 15.2 and 5.6 min; 2',3'-cADPRP: 14.0 and 5.8 min respectively) and physical properties were found to be different from that of cADPRP.



NAD	$R_1 = R_2 = H$	cADPR
NADP	$R_1 = -P(=O)(OH)-OH$ $R_2 = H$	cADPRP
3'-NADP	$R_1 = H$ $R_2 = -P(=O)(OH)-OH$	3'-cADPRP
2',3'-Cyclic NADP	$R_1R_2 =$ 	2',3'-cADPRP

The ^{1}H NMR (Fig. 2) and ^{13}C NMR spectra (not shown) of cADPRP, 3'-cADPRP, and 2',3'-cADPRP are very similar to that of cADPR indicating the identity of the skeletal structures. This assertion is further supported by their 2D-COSY, UV, and mass spectral data. These results show that the *Aplysia* cyclase can readily accept substitutions in the adenyl-ribosyl moiety of the pyridine nucleotide molecule.

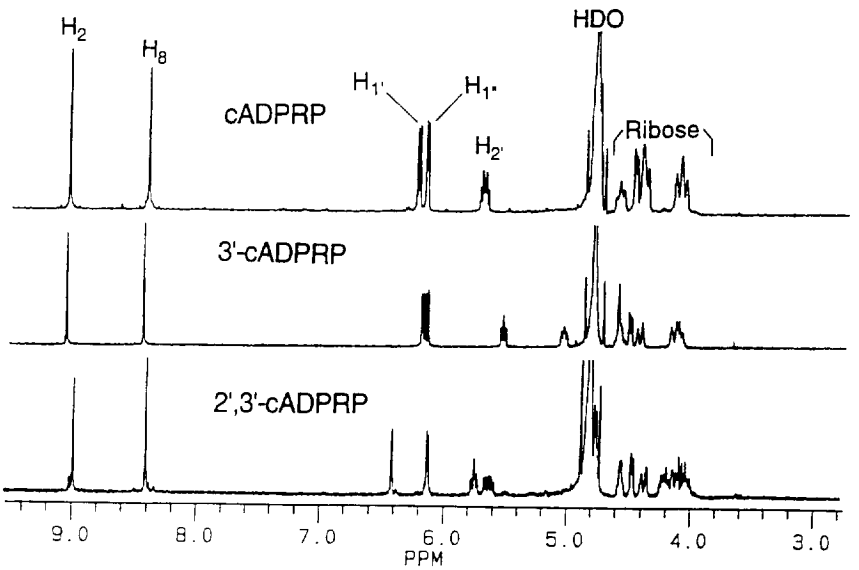


Figure 2. ^{1}H NMR spectra of cADPRP, 3'-cADPRP and 2',3'-cADPRP.

As NADP is widely distributed in mammalian tissues, we raised the question as to whether the $NAD(P)^{+}$ glycohydrolases (EC 3.2.2.6) are also capable of catalyzing the cyclization of NADP. The HPLC profile of Fig. 1B clearly shows the formation of cADPRP from NADP by the pig brain microsomal acetone powder[12] indicating that cADPRP may be likewise formed in mammalian systems. Also, there is suggestive evidence that the lymphocyte antigen CD38 may also be capable of transforming NADP into cADPRP.[10]

These novel cyclic nucleotide analogs were then examined for their abilities to release calcium from rat brain microsomes[13] and compared to authentic cADPR. While 3'-cADPRP and 2',3'-cADPRP, derived from the unnatural substrates 3'-NADP and 2',3'-cyclic NADP, were found to be inactive in inducing calcium release in this system, cADPRP was found to be two times more potent than cADPR with EC_{50} of 9.2 μM and 22 μM respectively. A dose response curve is shown in Fig. 3.

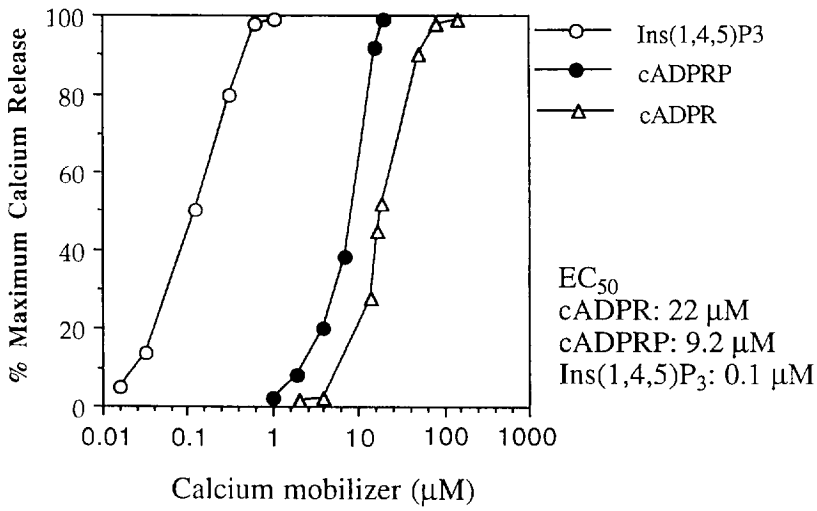


Figure 3. Dose response curve of Ca^{2+} release from rat brain microsomes.

To determine whether cADPRP competes with cADPR for the same binding site, we conducted the following experiments. Addition of cADPRP elicited a rapid Ca^{2+} release and after the Ca^{2+} was resequestered, further additions of cADPRP did not induce more Ca^{2+} release. However, subsequent addition of cADPR or inositol triphosphate (IP_3) induced a significant quantity of Ca^{2+} release. After the microsomes were induced by 100 μM ryanodine,[13] the microsomes became desensitized and did not respond to further additions of cADPRP or cADPR but the microsomes could still respond to IP_3. These results indicate that both cADPRP and cADPR mediated Ca^{2+} release from the same store but probably bind to different sites of the same receptor or calcium channel. Consequently, cADPRP may play an equally important role in calcium modulation as cADPR since it can be biosynthesized from NADP by mammalian enzymes and is more effective than cADPR in mediating calcium release. The physiological relevance of this novel cyclic nucleotide is currently under investigation.

ACKNOWLEDGMENT: This investigation was supported by National Institutes of Health grant GM 331449.

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(*Received in USA* 31 *July* 1995; *accepted* 28 *August* 1995)