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Hydrolysis of the phosphoanhydride linkage of cyclic ADP-ribose by the Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase

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ABSTRACT

Cyclic ADP-ribose (cADPR) metabolism in mammals is catalyzed by NAD glycohydrolases (NADases) that, besides forming ADP-ribose, form and hydrolyze the N^1-glycosidic linkage of cADPR. Thus far, no cADPR phosphohydrolase was known. We tested rat ADP-ribose/CDP-alcohol pyrophosphatase (ADPRibase-Mn) and found that cADPR is an ADPRibase-Mn ligand and substrate. ADPRibase-Mn activity on cADPR was 65-fold less efficient than on ADP-ribose, the best substrate. This is similar to the ADP-ribose/cADPR formation ratio by NADases. The product of cADPR phosphohydrolysis by ADPRibase-Mn was N^1-(5-phosphoribosyl)-AMP, suggesting a novel route for cADPR turnover.

1. Introduction

Cyclic ADP-ribose (cADPR) is a universal Ca^{2+} regulator formed by enzymes of the ADP-ribosyl cyclase family [1] including *Aplysia* soluble ADP-ribosyl cyclase [2], the mammalian membrane-bound NAD glycohydrolases (NADases) CD38 [3–9], BST-1/CD157 [10,11], and the mitochondrial NADase [12]. They form a glycosidic linkage between the adenine N^1 and the distal-ribose C1 atoms of NAD, converting it to cADPR and nicotinamide. For CD38, the most studied mammalian ADP-ribosyl cyclase, this is a quantitatively minor activity, compared to the hydrolysis of NAD to ADP-ribose. Those enzymes catalyze also the hydrolysis of cADPR to ADP-ribose, the only known pathway for cADPR turnover.

Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase (ADPRibase-Mn) (EC 3.6.1.53) hydrolyze the phosphoanhydride linkages of ADP-ribose, CDP-alcohols and ADP with decreasing efficiencies, requiring low micromolar Mn^{2+} concentrations not substituted by Mg^{2+} [13]. It may constitute a metallophosphoesterase family of its own, specific to vertebrates, plants and algae. In rodents, it is expressed preferentially in immune cells [14], with a possible signalling role inferred from the roles of its expression profile neighbors, i.e. the genes displaying the most similar expression profiles [14,15], and from the activatory effect of its best substrate, ADP-ribose, on TRPM2 ion channels [16,17]. Like ADP-ribose, cADPR is a TRPM2 (co)activator [18]; therefore, we decided to test cADPR as an ADPRibase-Mn ligand and substrate.

2. Materials and methods

2.1. Materials

cADPR, D-xylulose-5-phosphate and thiamine pyrophosphate were from Sigma, and NAD and D-ribose-5-phosphate were from Boehringer (Roche). cADPR was dissolved in 50 µl water per 0.5-mg vial and frozen at −20 °C until used. When indicated, to remove ADP-ribose contamination, 50 mM Tris–HCl (pH 7.5) substituted for water, and the solution was treated with 50 ng of venom phosphodiesterase and 60 ng of alkaline phosphatase for 4 h at 25 °C followed by overnight incubation at 4 °C. This left cADPR intact

Abbreviations: ADPRibase-Mn, Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase; cADPR, cyclic ADP-ribose; DMSO, dimethylsulfoxide; NADase, NAD glycohydrolase; pRib-Ado, N^1-(5-phosphoribosyl)-adenosine; pRib-AMP, N^1-(5-phosphoribosyl)-adenosine 5′-monophosphate; pRib-ATP, N^1-(5-phosphoribosyl)-adenosine 5′-triphosphate; Rib-Ado, N^1-(ribosyl)-adenosine; Rib-AMP, N^1-(ribosyl)-adenosine 5′-monophosphate

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but converted ADP-ribose to adenosine, ribose and phosphate. cADPR was purified by HPLC (see below).

Standard N^1-(5-phosphoribosyl)-adenosine 5′-monophosphate (pRib-AMP) was prepared by dilution of 1 μl of 20 mM cADPR in 125 μl of dimethylsulfoxide (DMSO) containing 1 mg of *tert*-butoxide, and incubation for 60–120 min at 37 °C. This treatment hydrolyzes the phosphoanhydride linkage of cADPR to pRib-AMP [19]. In our hands, it gave full conversion of contaminant ADP-ribose to AMP and partial conversion of cADPR to pRib-AMP (HPLC analysis). Potassium *tert*-butoxide was from Aldrich and DMSO (99.9%) was from Sigma.

Rat ADPRibase-Mn was expressed from plasmid pGEX-6P-3-rADPRMn and purified as described [14]. Snake venom phosphodiesterase (from *Crotalus durissus*) was from Boehringer, alkaline phosphatase (grade I from calf intestine) was from Roche, and AMP deaminase (from *Aspergillus* sp.), transketolase (from baker's yeast) and glyceraldehyde 3-phosphate dehydrogenase (from rabbit muscle) were purchased from Sigma. Other (bio)chemicals were as described [14].

2.2. ADPRibase-Mn incubations

Assay mixtures contained 50 mM Tris–HCl (pH 7.5 at 25 °C), 100 μM $MnCl_2$, 0.1 mg/ml BSA, cADPR as indicated and an appropriate ADPRibase-Mn concentration. The formation of pRib-AMP was measured by HPLC after defined incubation lengths at 37 °C terminated by injection in the column (see below). Initial rate assays were performed under conditions of linearity with incubation length and ADPRibase-Mn amount, and blanks without ADPRibase-Mn were run in parallel.

2.3. HPLC

A 15 cm × 0.4 cm octadecylsilica column (Kromasil 100, Teknokroma, Spain) was used with a 1 cm × 0.4 cm guard column of the same material, under the control of a Hewlett-Packard HP1100 chromatograph equipped with a diode array detector.

For analytical purposes, samples were analyzed by ion-pair reverse-phase HPLC monitored at 260 nm and 310 nm. On selected occasions, the diode array detector was programmed to record full ultraviolet–visible spectra. The column was equilibrated in 33.5 mM sodium phosphate, pH 8.5, 20 mM tetrabutylammonium bromide and 10% (vol/vol) methanol. After sample injection (20 μl), the elution was run at 1 ml/min with a 10-min linear gradient of 33.5–62 mM phosphate, followed by an isocratic wash with 62 mM phosphate (the pH and the rest of components of the initial mobile phase were kept constant). The use of a mobile phase at pH 8.5 allowed for the specific detection of compounds containing N^1-(ribosyl)-adenosine (Rib-Ado), like cADPR and pRib-AMP, as at pH 8.5 (but not e.g. at pH 7.0) solutions of those compounds show ε_{310} much higher than plain adenosine derivatives like ADP-ribose or AMP [19–21].

For cADPR purification, the HPLC column was equilibrated in water, and a 15-μl sample of treated cADPR (see Section 2.1) was chromatographed at 0.5 ml/min in water with monitoring at 260 nm and 310 nm. cADPR (retention time 4 min) was recovered at the column outlet at a concentration of 0.5 mM. It was judged 99.9% pure from analytical HPLC chromatograms at 260 nm.

2.4. Enzymatic assays of AMP and D-ribose-5-phosphate formed by heating pRib-AMP, the product of hydrolysis of cADPR by ADPRibase-Mn

AMP was measured by enzyme end-point assay in reaction mixtures of 0.6 ml containing 10 mM sodium citrate, pH 6.5, and 33 mM KCl. After recording a baseline at 265 nm, the assay was initiated by addition of 1 μl of AMP deaminase (freshly prepared dissolving 10 mg in 0.1 ml 1 M KCl) and the decrease of A_{265} concomitant to the conversion of AMP to IMP ($\varepsilon = 8.1\ mM^{-1}\ cm^{-1}$) was recorded until a new baseline was reached. The increase of A_{265} caused by the enzyme itself was determined and used to calculate the true decrease due to AMP deamination.

D-ribose-5-phosphate was measured by kinetic assay of its reaction with 35 μM D-xylulose-5-phosphate catalyzed by transketolase in 0.6-ml mixtures containing 25 mM glycylglycine, pH 7.3, 5 mM $MgCl_2$, 0.22 mM thiamine pyrophosphate, 5 mM sodium arsenate, 1 mM NAD, 0.1 mg/ml bovine serum albumin, 5 μg/ml transketolase (dissolved in 250 mM glycylglycine, pH 7.3) and 60 m-unit D-glyceraldehyde-3-phosphate dehydrogenase (pre-assayed under these conditions with 80 μM D-glyceraldehyde-3-phosphate as substrate). Transketolase forms one mole of D-glyceraldehyde-3-phosphate per mole of D-xylulose-5-phosphate either by monosubstrate reaction or by bisubstrate reaction with D-ribose-5-phosphate [22,23]. The formation of D-glyceraldehyde-3-phosphate is coupled to its arsenate and NAD-dependent oxidation catalyzed irreversibly by D-glyceraldehyde-3-phosphate dehydrogenase. Under the chosen conditions, the initial rate of NAD reduction, started by addition of D-xylulose-5-phosphate and recorded at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$), was linearly related to the concentration of D-ribose-5-phosphate present in the mixture at least in the range 0-25 μM. The concentration of D-ribose-5-phosphate formed by heating the reaction product of ADPRibase-Mn was thus determined in a sample volume that would give an analyte concentration within the linearity range, and a calibration plot with standard amounts of D-ribose-5-phosphate 0-25 μM was obtained for each assay session. In this system, D-ribose did not react as, when added up to a concentration of 1 mM, it did not alter appreciably the slope recorded at 340 nm with D-xylulose-5-phosphate alone.

All the samples and standards were assayed in duplicate. Since the preparation of the samples for AMP and D-ribose-5-phosphate assays involved incubation in a boiling water bath for 15 min, the recovery of the two compounds was evaluated: 101 ± 1% of AMP, but only 63 ± 2% of D-ribose-5-phosphate ($n = 3$) was recovered after the treatment. Therefore, the measurements of D-ribose-5-phosphate were corrected as needed.

2.5. Structural models and AutoDock simulations

The coordinates of rat ADPRibase-Mn, modelled by homology to the X-ray structure of its zebrafish ortholog, were taken from the SWISS-MODEL repository (accession code q5m886) [24,25]. The ADPRibase-Mn model was prepared for docking as described [14], including two Zn^{2+} ions, present in the zebrafish protein, which form a dinuclear center typical of the metallophosphoesterase superfamily. The preparation of the structure of ADP-ribose and its docking to the ADPRibase-Mn model with AutoDock [26,27] has been also reported [14]. The structure of cADPR was generated with Marvin 5.0.1 (updated 2008; ChemAxon, Budapest, Hungary, http://www.chemaxon.com) and its partial Gasteiger charges were calculated with AutoDockTools [28] (total charge: 2−). To overcome the limitation of AutoDock in treating flexible cyclic and macrocyclic ligands, like cADPR, an approach for introducing ring flexibility in docking with AutoDock 3.0.5 was implemented at source code level in AutoDock 4.0.1. Briefly, the ring was broken between the C4 and C5 atoms of N^1-ribose, and a modified potential function was applied between them to bias the conformational search toward ring closure [29]. The two ribose rings of cADPR were modelled as rigid moieties to reduce the conformational search space. Otherwise, the parameters to run cADPR docking simulations with the modified version of AutoDock 4.0.1 were as described for ADP-ribose docking [14].

3. Results

3.1. cADPR docking to a model of ADPRibase-Mn and molecular-dynamics simulation

To test cADPR as ADPRibase-Mn ligand, we used a computational approach. The active center of ADPRibase-Mn, as indicated by the binding of P_i in the X-ray structure and of ADP-ribose in a docked model, is a pocket that is partly closed by two amino acid-loops (36–41 and 196–211) and that contains the dimetallic center in the bottom [14]. After a docking simulation with AutoDock, cADPR fit also into this site. Whereas the adenosine and the ribose moieties of ADP-ribose protrude towards opposing sides [14], cADPR remained in a more retracted position with the adenine centered within the pocket, above the pyrophosphate group (Fig. 1). The pyrophosphate groups of cADPR and ADP-ribose occupy practically the same position, with the phosphate esterified to the northern ribose (the one attached to the N1 of adenine) being coordinated to both metallic ions. This confirmed that the ADPRibase-Mn complex with cADPR could represent an enzyme-substrate complex for hydrolysis of the phosphoanhydride linkage, like with ADP-ribose.

ADPRibase-Mn complexes with docked ligands show the active center in a closed conformation (Fig. 1). To explore whether other conformations could at least theoretically exist, we ran molecular-dynamics simulations with the ADPRibase-Mn/cADPR complex (online Supplementary material). This revealed novel, more open conformations of the active center that may allow for substrate entry (Supplementary Fig. S1). The occurrence of theoretical, open and closed conformations suggest a hypothetical induced fit. Altogether, the results of computational docking and dynamics urged us to test experimentally whether cADPR was an ADPRibase-Mn substrate.

3.2. Conversion of cADPR to pRib-AMP by incubation with ADPRibase-Mn

Incubation of cADPR with ADPRibase-Mn in the presence of Mn^{2+}, produced a compound different to cADPR and ADP-ribose (peak with a retention time of near 9 min in Fig. 2). Contrary to ADP-ribose or AMP, which do not absorb at 310 nm, the unknown product displayed a significant A_{310}/A_{260} ratio (0.15) similar to cADPR (Fig. 3). Since the difference between the ultraviolet spectra of cADPR and ADP-ribose depends on the N^1-glycosidic linkage of the former [21], the unknown product should still contain this linkage. It seemed thus likely that ADPRibase-Mn was hydrolyzing the phosphoanhydride linkage of cADPR, and that the reaction product was pRib-AMP (Fig. 4). The identification of pRib-AMP was confirmed by coelution with the compound produced chemically from cADPR (Fig. 2), and by structural characterization by enzymatic assays. These experiments were performed on standard ADPRibase-Mn reaction mixtures prepared with purified cADPR and incubated at 37 °C until all the substrate was transformed.

To determine the content of terminal phosphate groups in the ADPRibase-Mn reaction product, samples taken from 0.3-ml reaction mixtures containing 30 nmol of hydrolyzed cADPR were incubated with alkaline phosphatase (250 ng of enzyme added, followed by 20-min incubation at 37 °C, in 0.1 ml of 50 mM Tris pH 7.5) and P_i was assayed with an ascorbate-molybdate reagent as described [13]. In this way, 1.91 ± 0.03 mol (n = 3) of P_i was measured per mole of cADPR hydrolyzed by ADPRibase-Mn. In the absence of added alkaline phosphatase, only 0.13 ± 0.04 mol were measured. In contrast to the reaction product, cADPR itself is resistant to alkaline phosphatase. This indicates that ADPRibase-Mn hydrolyzes one of the phosphate linkages of cADPR giving a product with two phosphomonoesterase-sensitive phosphate groups.

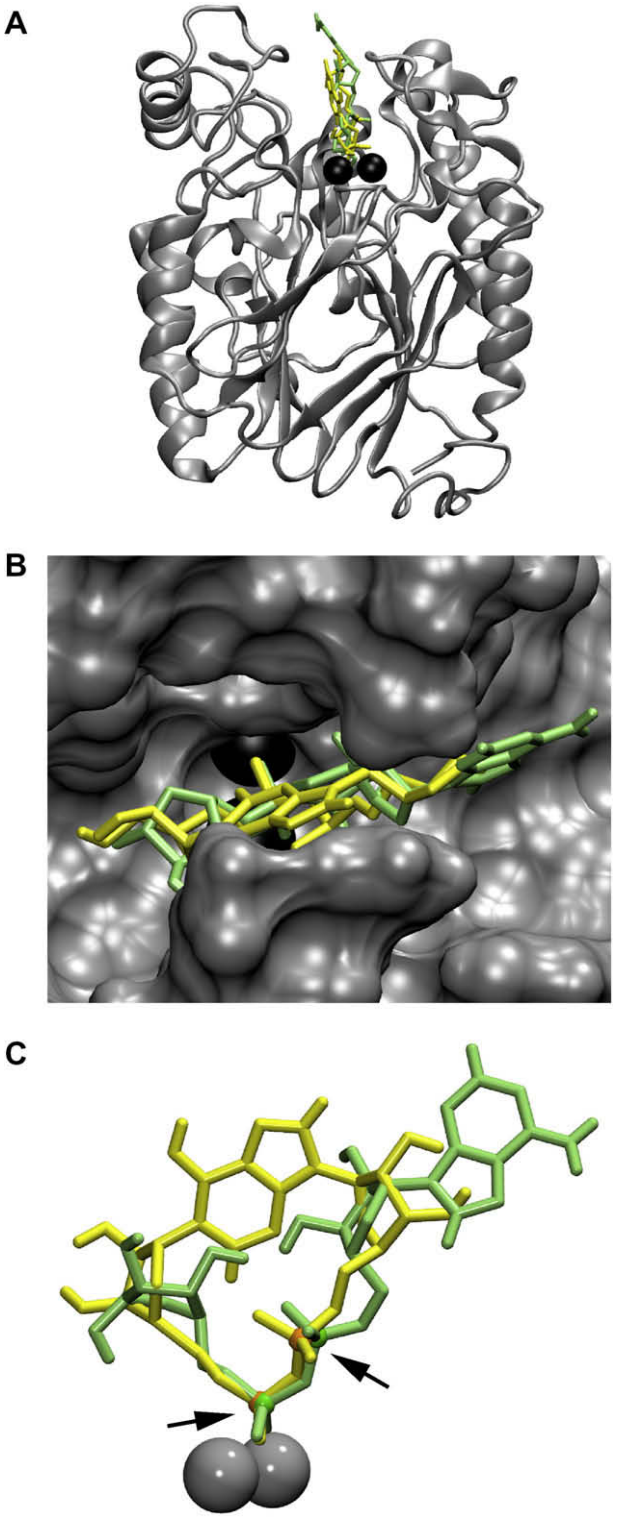

Fig. 1. ADPRibase-Mn complexes with cADPR and ADP-ribose. Both ligands were docked to the same ADPRibase-Mn model with AutoDock. The figure shows the overlapped structures of cADPR (yellow) and ADP-ribose (green). Black or grey spheres correspond to the ions in the dimetallic center. The docking of ADP-ribose is as reported [14]. (A) Full view. (B) Molecular surface of the active center as viewed from above in A. (C) Overlapped structures of cADPR and ADP-ribose in the active center showing the coincidence of the respective pyrophosphate groups in the lower part (P atoms of cADPR and ADP-ribose are represented by the small orange and green balls, respectively, and are marked by arrows).

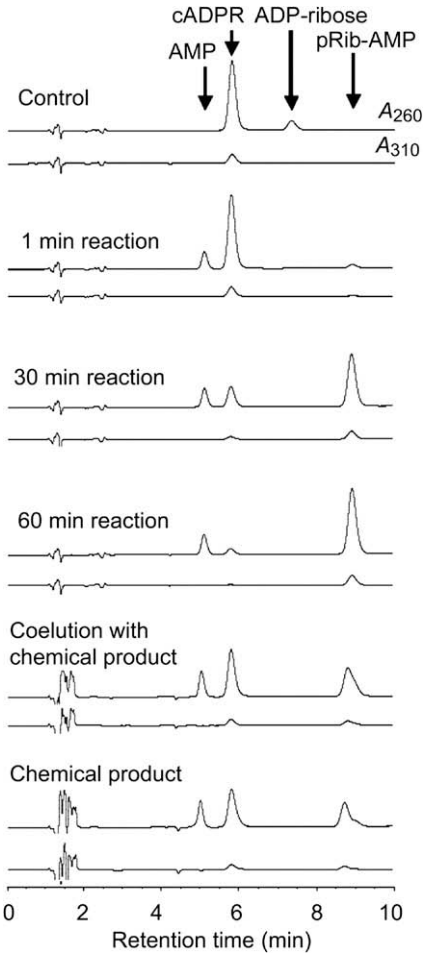

Fig. 2. HPLC detection of the hydrolysis product of cADPR by ADPRibase-Mn: coelution with pRib-AMP produced by chemical hydrolysis of cADPR. A reaction mixture of ADPRibase-Mn (10 μg/ml) with 100 μM commercial cADPR containing a 18% contamination by ADP-ribose (control) was incubated under standard conditions. Samples of 20 μl were taken after 1-min, 30-min and 60-min incubations and were analyzed by HPLC. For identification of the ADPRibase-Mn product as pRib-AMP, another sample of reaction mixture incubated for 75 min was coeluted with a sample of chemically hydrolyzed cADPR in a 1:3 proportion (see Section 2.3).

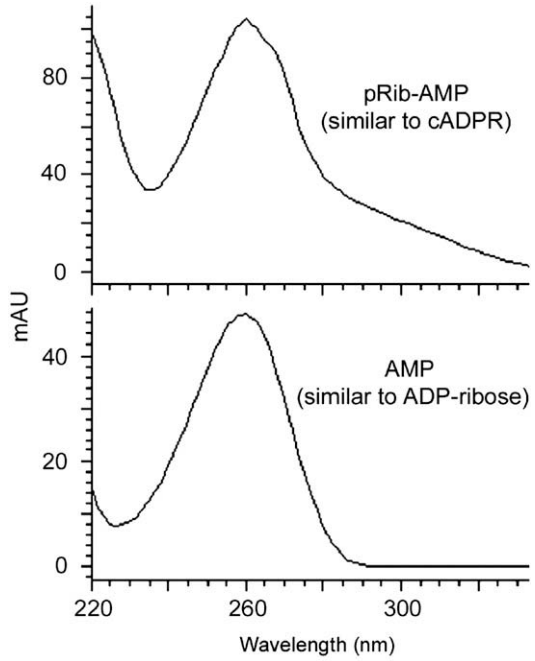

Fig. 3. Ultraviolet spectrum of the ADPRibase-Mn product identified as pRib-AMP. The spectrum of AMP is shown for comparison. Spectra were recorded at pH 8.5 from HPLC peaks (see Fig. 2).

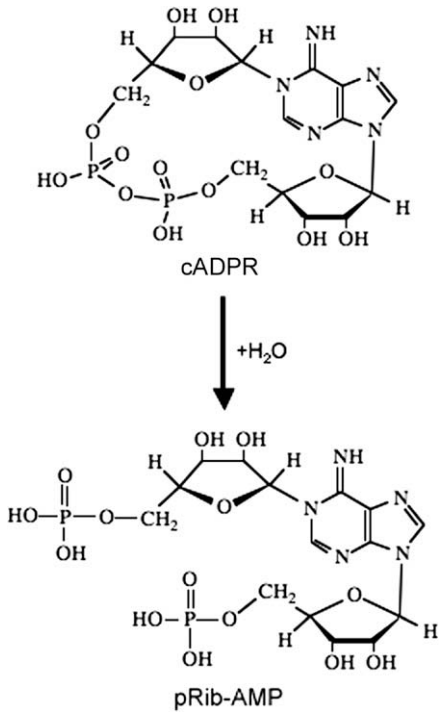

Fig. 4. Phosphohydrolysis of cADPR catalyzed by ADPRibase-Mn. The phosphohydrolytic pattern of the reaction and the identification of the product as pRib-AMP is supported by the demonstration that (i) the product contains two phosphate groups releasable by alkaline phosphatase, while the substrate has none, (ii) the UV spectrum of the product at pH 8.5 is like that of cADPR including the shoulder around 300 nm typical of the N^1-glycosydic linkage with the adenine base, and (iii) the incubation of the product for 15 min in a boiling bath converted it to AMP and D-ribose-5-phosphate.

To determine that the reaction product was a combination of D-ribose-5-phosphate and AMP, 0.4-ml samples of ADPRibase-Mn reaction mixture containing 80 nmol of hydrolyzed cADPR were incubated for 15 min in a boiling bath to hydrolyze the N^1-glycosidic linkage expected to be present in the reaction product as is in cADPR [3]. Enzymatic assays showed the formation of 0.97 ± 0.08 mol AMP and 1.00 ± 0.04 mol D-ribose-5-phosphate (n = 3) per mole of cADPR hydrolyzed by ADPRibase-Mn and then heated. The formation of AMP, not adenosine or ADP, was also confirmed by HPLC assay. This indicates that the phosphoanhydride linkage of cADPR is indeed the one attacked by ADPRibase-Mn and the product is pRib-AMP (Fig. 4).

3.3. Catalytic efficiency of the cADPR pyrophosphatase activity of ADPRibase-Mn

ADPRibase-Mn hydrolyzes ADP-ribose, CDP-choline, CDP-glycerol and CDP-ethanolamine with decreasing catalytic efficiencies as estimated from k_{cat}/K_m ratios derived from saturation parameters [14]. For comparison of cADPR to the other ADPRibase-Mn substrates, saturation curves for cADPR were run with the commercial cADPR preparation contaminated with ADP-ribose. In the HPLC assays, monitored by HPLC at 260 nm and 310 nm, the contaminant ADP-ribose was consumed rapidly (see e.g. Fig. 2) allowing the measurement of linear rates of cADPR hydrolysis without

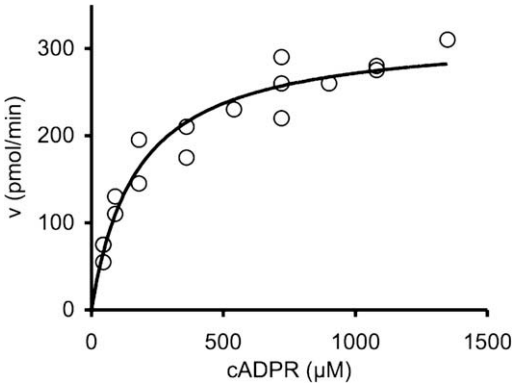

Fig. 5. Saturation kinetics of the phosphohydrolytic activity of ADPRibase-Mn towards cADPR. The results of two different experiments are plotted together and adjusted to the Michaelis–Menten equation by non-linear regression [39].

competition by ADP-ribose. Fig. 5 shows the saturation curve of ADPRibase-Mn with cADPR, which gave a K_m value of 170 ± 30 μM and a k_{cat} of 0.87 ± 0.04 s^{-1}. The resultant catalytic efficiency or cADPR specificity constant, k_{cat}/K_m, is thus near 5000 M^{-1} s^{-1}, 65-fold lower than that of ADP-ribose [14]. This result was confirmed with purified cADPR, devoid of ADP-ribose contamination. In this case, the k_{cat}/K_m ratio was estimated from initial rate measurements at substrate concentrations well below the estimated K_m value, when the enzyme is largely unbound to substrate and the catalytic efficiency or specificity constant is given by $(k_{cat}/K_m) = v/([E]_0[S])$, E_0 being the total enzyme concentration [30]. By this method, the ADPRibase-Mn catalytic efficiency with cADPR as substrate was estimated as 4160 ± 240 M^{-1} s^{-1}, in very good agreement with the result derived from saturation parameters obtained with cADPR contaminated by ADP-ribose.

4. Discussion

This is the first report of the enzymatic formation of pRib-AMP in mammals. In prokaryotes, lower eukaryotes and plants, it is an intermediate of histidine biosynthesis formed by hydrolysis of N^1-(5-phosphoribosyl)-adenosine 5′-triphosphate (pRib-ATP) [20,31,32], but its significance in organisms that do not synthesize histidine is unknown.

To our knowledge, this is also the first report of the enzymatic hydrolysis of the phosphoanhydride linkage of cADPR (Fig. 4), different to the hydrolysis of the N^1-ribosyl linkage of cADPR to ADP-ribose [3–9]. So far, the strategy of phosphohydrolytic turnover seemed inoperant for cADPR, as no enzyme with cADPR phosphohydrolase activity was known. This includes cADPR resistance to broad specificity phosphodiesterases [3,19]. The catalytic efficiency of ADPRibase-Mn for cADPR phosphohydrolysis is low compared to other substrates (ADP-ribose, 328 200 M^{-1} s^{-1}; CDP-choline, 95 000 M^{-1} s^{-1}; CDP-glycerol, 92 400 M^{-1} s^{-1}; CDP-ethanolamine, 29 000 M^{-1} s^{-1}, ADP, 21 400 M^{-1} s^{-1} [14]). However, the ratio of efficiencies for ADP-ribose/cADPR hydrolysis near 100 is like the ratio of ADP-ribose/cADPR formation from NAD by CD38 [3,6,33,34], the major cADPR-forming enzyme in mammals. Therefore, the potential of ADPRibase-Mn for cADPR metabolism should not be disregarded.

CD38 NADases and its products ADP-ribose and cADPR have a role in immune regulation at the interface between the innate and the adaptative systems [35,36]. Concerning ADPRibase-Mn, mouse DNA microarray data indicate its encoding gene *2310004I24Rik* is an "immune gene" [15], and enzyme levels are higher in immune than in non-immune cells from rats [14]. ADPRibase-Mn could be involved in the CD38 network with a role in the turnover of ADP-ribose and cADPR, and in the termination of their effects on TRPM2 channels in immune cells [18]. Moreover, it is worth mentioning that the hydrolysis of cADPR by ADPRibase-Mn could be the first step of a pathway leading to compound(s) (e.g. pRib-AMP, Rib-AMP, pRib-Ado, Rib-Ado) with a potential for cell signaling, as many other adenine-containing derivatives [37,38].

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.023.

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