Unifying mechanism for *Aplysia* ADP-ribosyl cyclase and CD38/NAD⁺ glycohydrolases

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Highly purified *Aplysia californica* ADP-ribosyl cyclase was found to be a multifunctional enzyme. In addition to the known transformation of NAD⁺ into cADP-ribose this enzyme is able to catalyse the solvolysis (hydrolysis and methanolysis) of cADP-ribose. This cADP-ribose hydrolase activity, which becomes detectable only at high concentrations of the enzyme, is amplified with analogues such as pyridine adenine dinucleotide, in which the cleavage rate of the pyridinium-ribose bond is much reduced compared with NAD⁺. Although the specificity ratio $V_{\max}/K_m$ is in favour of NAD⁺ by 4 orders of magnitude, this multifunctionality allowed us to propose a 'partitioning' reaction scheme for the *Aplysia* enzyme, similar to that established previously for mammalian CD38/NAD⁺ glycohydrolases. This mechanism involves the formation of a single oxocarbenium-type intermediate that partitions to cADP-ribose and solvolytic

products via competing pathways. In favour of this mechanism was the finding that the enzyme also catalysed the hydrolysis of NMN⁺, a substrate that cannot undergo cyclization. The major difference between the mammalian and the invertebrate enzymes resides in their relative cyclization/hydrolysis rate-constant ratios, which dictate their respective yields of cADP-ribose (ADP-ribosyl cyclase activity) and ADP-ribose (NAD⁺ glycohydrolase activity). For the *Aplysia* enzyme's catalysed transformation of NAD⁺ we favour a mechanism where the formation of cADP-ribose precedes that of ADP-ribose; i.e. macroscopically the invertebrate ADP-ribosyl cyclase conforms to a sequential reaction pathway as a limiting form of the partitioning mechanism.

Key words: cADP-ribose, cyclic ADP-ribose hydrolase, hydrolysis, methanolysis, NGD⁺.

INTRODUCTION

cADP-ribose, a new metabolite of NAD⁺ discovered in the late 1980s by Lee and collaborators [1], has attracted considerable attention because of its intracellular Ca²⁺-mobilizing properties that are distinct from those of inositol 1,4,5-trisphosphate (for review see [2]). The first known enzyme able to convert NAD⁺ into cADP-ribose was detected in invertebrates such as sea urchins [1]. Subsequently, the ADP-ribosyl cyclase from *Aplysia californica*, which is a soluble protein, was purified [3,4], cloned [5] and its three-dimensional structure determined [6]. Besides this enzyme, a cADP-ribose hydrolase that converts the cyclic metabolite into ADP-ribose has also been found in invertebrates, but much less is known about this activity [7].

In mammalian systems, the situation appeared to be quite different. First, the cloning of the *Aplysia* ADP-ribosyl cyclase revealed a striking sequence identity of this enzyme with CD38, a lymphocyte antigenic marker for which no catalytic activity was hitherto known [5]. Then an NAD⁺ glycohydrolase, purified from canine spleen microsomes, was demonstrated to be a multifunctional enzyme. Besides its major NAD⁺ glycohydrolase activity, this enzyme was also able to convert NAD⁺ into cADP-ribose and to hydrolyse this molecule into ADP-ribose [8]. The same multifunctionality was established for human CD38 [9] and later for bovine spleen NAD⁺ glycohydrolase [10,11], an archetypal representative of this class of enzymes (EC 3.2.2.6). Some topological and mechanistic problems were raised with regard to the biosynthesis of cADP-ribose in mammals. Thus CD38 and NAD⁺ glycohydrolases, which are ecto-enzymes, transform NAD⁺ only into small proportions of cADP-ribose (less than 2% of the reaction products). Moreover, no intracellular equivalent of the invertebrate ADP-ribosyl cyclase, which was given

to convert NAD^+ quasi-exclusively into cADP-ribose, has been described to date. The multifunctionality of CD38 was first invoked to explain the limited formation of the cyclic metabolite. A sequential mechanism was proposed according to which cADP-ribose, after its formation from NAD^+ , does not accumulate because it is quickly turned over; accordingly the NAD^+ glycohydrolase activity of CD38 represents the sum of the ADP-ribosyl cyclase and cADP-ribose hydrolase activities [9, 12]. In favour of this mechanism was the observation that nicotinamide-guanine dinucleotide (NGD^+), an alternative substrate which leads to the non-hydrolysable cGDP-ribose, was predominantly transformed by CD38 into the cyclic reaction product [13]. The study of the molecular mechanism of bovine spleen NAD^+ glycohydrolase, which we have since demonstrated to be equivalent to CD38 [14, 15], led us to propose a different reaction scheme. Accordingly, NAD^+ , after cleavage of its nicotinamide-ribose bond, generates an oxocarbenium-type intermediate which then partitions between two competing pathways (Scheme 1): a reversible intramolecular reaction leading to cyclization (step k_3) and a macroscopically irreversible intermolecular hydrolysis reaction (step $k_{\text{H}_2\text{O}}$), generating ADP-ribose [11]. This mechanism, which links the low formation of cADP-ribose by CD38/ NAD^+ glycohydrolases to an inefficient cyclization step relative to the capture of the oxocarbenium ion by a water molecule, was recently borne out by careful kinetic studies with a recombinant human CD38 [16].

In the present study, we have re-examined some aspects of the mechanism of *Aplysia* ADP-ribosyl cyclase. We have thus established that a highly purified form of this enzyme is also endowed with intrinsic NAD⁺ glycohydrolase and cADP-ribose hydrolase activities. These activities are unmasked at higher enzyme concentrations, especially when using poorly hydro-

Abbreviations used: PyAD⁺, pyridine adenine dinucleotide; NGD⁺, nicotinamide–guanine dinucleotide.

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Scheme 1 Reaction mechanism of CD38/NAD⁺ glycohydrolases

CD38/NAD⁺ glycohydrolases catalyse the cleavage of the nicotinamide bond of NAD⁺ leading to the formation of an E · ADP-ribosyl intermediary complex (where E is the enzyme). This intermediate then partitions between competing intramolecular (ADP-ribosyl cyclase) and intermolecular (solvolysis, transglycosidation) pathways [11,14,16] (where x is the substituent of the pyridine). Note that the solvolysis reactions (hydrolysis and methanolysis) are macroscopically irreversible.

lysable pyridinium analogues of NAD⁺. Moreover this enzyme is also able to catalyse the methanolysis of both NAD⁺ and cADP-ribose. Altogether, these reactions are very similar to the ones described for bovine and human CD38/NAD⁺ glycohydrolases. We therefore propose a unifying mechanism for the invertebrate and mammalian enzymes, the major difference being their relative efficiencies in favouring the intramolecular reaction leading to the cyclic reaction product.

EXPERIMENTAL

Materials

[adenosine-U-¹⁴C]NAD⁺ (567 Ci/mol) was from NEN (Boston, MA, U.S.A.). NAD⁺, NGD⁺, 1,N⁶-ethenoNAD⁺ and cADP-ribose were from Sigma (L'Isle d'Abeau Chesnes, France). Pyridine adenine dinucleotide (PyAD⁺) was prepared according to [17]. Hydroxylapatite (Bio-Gel HTP) and Affi-Gel Blue were obtained from Bio-Rad (Ivry-sur-Seine, France). DEAE-cellulose (DE 53) was from Whatman Laboratories (Maidstone, Kent, U.K.) and CM-Sepharose CL-6B was from Pharmacia Biotech (Uppsala, Sweden).

Purification of *A. californica* ADP-ribosyl cyclase

ADP-ribosyl cyclase was purified, with some modifications, according to published procedures [3,4,18]. The soluble fraction (0.9 g) obtained from ovotestis of *A. californica* that was provided generously by Dr Bernard Poulain (CNRS-Neurochimie, Strasbourg, France) was applied to a 5 × 23-cm CM-Sepharose CL-6B column. The enzyme was eluted (flow rate, 1 ml/min) with a 0–1 M gradient of NaCl in 10 mM potassium phosphate buffer, pH 7.4 (buffer A). After dialysis against buffer A the enzyme was then purified further on a 2.5 × 60-cm DEAE-cellulose column, equilibrated and eluted with the same buffer. In some experiments the cyclase was also chromatographed on Affi-Gel Blue. Briefly, a 1 × 2.5-cm column was washed with buffer A containing 1 M NaCl and then equilibrated with the same buffer. About 100 µg of the enzyme were loaded at 0.2 ml/min and the enzyme was eluted with a linear 0–1 M gradient of NaCl in buffer A. The protein concentration was determined by the BCA (bicinchoninic acid) protein assay from Pierce using BSA as standard.

Gel electrophoresis

SDS/PAGE was performed according to standard methods and the gels were silver-stained using a kit based on the technique of Heukeshoven and Dernick [19].

Enzyme assays

Cyclase activity was determined routinely using either the cyanide-addition assay [20] for ADP-ribosyl cyclase activity or continuous fluorometric assays with NGD⁺ as substrate by estimating the appearance of cGDP-ribose (emission at 410 nm, excitation at 310 nm) [13] for GDP-ribosyl cyclase activity: NGD⁺ (final concentration 50 µM) was incubated at 37 °C in buffer A in the presence of about 20 m-units of enzyme. To determine the kinetic parameters, ADP-ribosyl cyclase and cADP-ribose hydrolase activities were measured using, respectively, 2.5–20 µM NAD⁺ in the presence of [adenosine-¹⁴C]NAD⁺ (220 000 d.p.m.) and 0.025–2.25 mM cADP-ribose in 50 mM potassium phosphate buffer, pH 7.4, incubated at 37 °C in the presence of, respectively, 2 and 200 m-units of purified ADP-ribosyl cyclase. At different time points, aliquots were analysed using HPLC as described below.

Methanolysis catalysed by ADP-ribosyl cyclase

The nucleotides (NAD⁺, PyAD⁺ and cADP-ribose; final concentration 20 µM) or NGD⁺ (100 µM) were incubated at 37 °C in buffer A, in the presence of 0–2 M methanol (final volume 200–500 µl) and given amounts of ADP-ribosyl cyclase. At different time points, aliquots were analysed by HPLC as described below. The β-methyl ADP-ribose and β-methyl GDP-ribose standards were prepared as published previously [11,21].

Analysis of the reaction products by HPLC

Product analysis was performed by HPLC on a 300 × 3.9-mm µBondapak C₁₈ column (Waters) on aliquots of the reaction medium centrifuged at 10 000 g for 10 min at 4 °C in a Microcon microconcentrator (10-kDa cut-off; Amicon) [11]. The com-

pounds were eluted isocratically with a 10 mM ammonium phosphate buffer, pH 5.5, containing 1.2% (v/v) acetonitrile, detected by their UV absorbance at 260 nm and/or by radio-detection (Flo-one, Packard Instruments). Standards were injected to determine the elution times and to identify the different reaction products.

Kinetic analysis

The initial rates of substrate transformation were calculated from a polynomial regression curve. Kinetic parameters were determined from the plot of initial rate as a function of substrate concentration using a non-linear regression program (GraphPad Prism).

RESULTS

When using large quantities of purified *A. californica* ADP-ribosyl cyclase to transform slow-reacting NAD^+ analogues into their corresponding cyclic derivatives we uncovered an apparent NAD^+ glycohydrolase activity, i.e. formation of ADP-ribose, which otherwise remains largely unnoticed. This observation prompted us to study the capacity of this enzyme to catalyse alternative pathways that might be useful in further delineating its molecular mechanism.

Aplysia ADP-ribosyl cyclase is also a cADP-ribose hydrolase

In Figure 1 are represented typical progress curves obtained by *Aplysia* ADP-ribosyl cyclase-catalysed transformations of NAD^+ . When using intermediate amounts of enzyme (50 m-units; Figure 1A) the substrate was essentially transformed into cADP-ribose and only limited amounts of ADP-ribose were measured (less than 10% of the reaction products under these experimental conditions). This is in agreement with several authors who have also observed the limited contribution of a hydrolytic activity to the overall reaction [3,22,23]. However,

Figure 2 Double reciprocal plot of the *A. californica* ADP-ribosyl cyclase-catalysed hydrolysis of cADP-ribose

Initial rates of the hydrolysis of cADP-ribose were measured in a 50 mM potassium phosphate buffer, pH 7.4, at 37 °C.

with higher amounts of cyclase, the situation was quite different (Figure 1B); in this case, after a transient formation cADP-ribose declined with the concomitant formation of ADP-ribose, indicative of a hydrolytic step via either NAD^+ glycohydrolase or cADP-ribose hydrolase pathways, or by a combination of both.

The occurrence of a hydrolytic transformation of cADP-ribose into ADP-ribose by *Aplysia* ADP-ribosyl cyclase was then studied directly and confirmed when using high-enough concentrations of a highly purified enzyme (see below). The kinetic parameters for this newly described cADP-ribose hydrolase activity of the *Aplysia* enzyme were determined under steady-state conditions. At pH 7.4, a K_m value of $1.04 \pm 0.15 \text{ mM}$ ($\pm \text{S.D.}$) was found (Figure 2) and the maximal rate ($V_{\max}$) was $3.34 \pm 1.15 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. In comparison, the

Figure 1 Transformation of NAD^+ catalysed by *A. californica* ADP-ribosyl cyclase

NAD^+ (300 μM) and [*adenosine- ^{14}C*] NAD^+ (220 000 d.p.m.) in 50 mM potassium phosphate buffer, pH 7.4, were treated with 50 m-units (A) or 1 unit (B) of highly purified *A. californica* ADP-ribosyl cyclase at 37 °C. At the given times, aliquots were taken, treated with 5% trichloroacetic acid and analysed by radio-HPLC. The reaction products formed are given as percentages of the total counts.

Figure 3 SDS/polyacrylamide gel of purified *A. californica* ADP-ribosyl cyclase

A heat-denatured sample of purified enzyme (2 μ g) was submitted to electrophoresis under reducing conditions on a 12% polyacrylamide gel. The band was revealed by silver staining. The positions of the molecular-mass markers (kDa) are indicated.

following values were determined for NAD^+ : $K_m = 4.6 \pm 0.4 \mu\text{M}$ and $V_{\max} = 465 \pm 57 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. It appears therefore that *Aplysia* ADP-ribosyl cyclase is a bifunctional enzyme that can synthesize and hydrolyse cADP-ribose. This cyclase/hydrolase ratio was not affected by temperature (down to 4 $^{\circ}\text{C}$) and by pH (5–8; results not shown). It should be stressed that, as noted before [24], the Michaelis constants (K_m) found in the literature for NAD^+ and some other substrates of the cyclase vary greatly and the value determined here is among the lowest. The reason for this unusual scatter in published data is unknown, but might reflect the mode of preparation of the *Aplysia* enzyme and its propensity to oligomerize [25].

Homogeneity of the ADP-ribosyl cyclase

The enzyme used in the preliminary part of our study was purified from *A. californica* ovotestis according to the published procedures [3,4,18]. However, to ensure the homogeneity of the protein used, additional purification steps were performed such as a DEAE-cellulose column followed by pseudo-affinity chromatography on Affi-Gel Blue [26]. The first step, which is a 'negative' one, i.e. the enzyme is not retained by the gel, eliminated the small contaminating bands visible on denaturing gels. In contrast, the cyclase interacts strongly with the Blue gel and is eluted only by high monovalent-salt concentrations. The enzyme preparation thus obtained gave a single band at ≈ 29 kDa on an overloaded SDS/polyacrylamide gel (Figure 3). Microsequencing of the N-terminal region over 18 residues (IVPTRELENVFLGRXKDY) was in perfect agreement with the known sequence of the cyclase from *A. californica* [5,27] and did not reveal any additional underlying sequence when using up to 1 nmol of protein in the Edman degradation. Electrospray MS also revealed a single-molecular-mass peak of 28694.9 ± 1.3 Da. This value, which was strictly reproducible for two different batches of purified *Aplysia* ADP-ribosyl cyclase, is slightly

inferior to 29539.8 Da, i.e. the theoretical molecular mass expected from the sequence based on the cloning of the *Aplysia* enzyme [5,27]. This might be related to the occurrence of isoforms, most probably at the C-terminus, as described previously by Lee et al. [18]. Heat inactivation of the enzyme was also studied by measuring both ADP-ribosyl cyclase and cADP-ribose hydrolase activities. Incubation at 60 $^{\circ}\text{C}$ over a period of 40 min led to a decrease of activity that followed pseudo-first-order kinetics and in both cases identical slopes were observed ($t_{1/2} \approx 5.75$ min; results not shown). Altogether these studies indicate that we have obtained a homogenous enzyme preparation and that the observed cADP-ribose hydrolase activity is an intrinsic property of the *Aplysia* ADP-ribosyl cyclase and is not due to a contaminating protein present in our preparation.

Further solvolysis reactions catalysed by *Aplysia* ADP-ribosyl cyclase

One of the important issues raised by the finding that *Aplysia* ADP-ribosyl cyclase is also able to hydrolyse cADP-ribose is the following: what are the reaction pathways of this multifunctional enzyme? We reasoned on the reactions illustrated in Scheme 1, which has been established for the related bovine and human CD38/ NAD^+ glycohydrolases [10,14]. For example, is ADP-ribose obtained by the transformation of NAD^+ in the presence of high amounts of the enzyme (Figure 1B) originating from the hydrolysis of transiently formed cADP-ribose or from a competition between the $k_{\text{H}_2\text{O}}$ and k_3 steps? We have addressed this question using three different tools: (i) the transformation of poorly hydrolysable pyridinium analogues of NAD^+ , such as PyAD^+ , (ii) the study of methanolysis of cADP-ribose, NAD^+ and PyAD^+ and (iii) the hydrolysis of NMN^+ .

The interest of PyAD^+ resides in the fact that this pseudo-substrate leads, after the cleavage of its pyridinium-ribose bond, to exactly the same reaction intermediate and products as NAD^+ (i.e. cADP-ribose and ADP-ribose). The major difference between the two dinucleotides is that because of the higher bond energy of the scissile bond in PyAD^+ , the k_2 step should be substantially slowed down. For example, this was the case for bovine CD38/ NAD^+ glycohydrolase where the turnover rate was decreased 500-fold [17]. Thus if cADP-ribose is a reaction intermediate between NAD^+ or its pyridinium analogues and the final hydrolytic product ADP-ribose, one would expect that cADP-ribose originating from PyAD^+ would have a higher probability of getting hydrolysed than when originating from NAD^+ . As a result, using such a poorly hydrolysable analogue we should amplify the importance of the cADP-ribose hydrolase pathway in the catalytic reaction, i.e. more ADP-ribose should be produced at the expense of the cyclic metabolite. This was precisely the case; we found that the *Aplysia* enzyme was able to transform PyAD^+ into both cADP-ribose and ADP-ribose, but at a much lower rate than NAD^+ (≈ 3 orders of magnitude). Moreover with e.g. a 20 μM initial concentration of the analogue, at a 10% reaction progress, the $[\text{cADP-ribose}]/[\text{ADP-ribose}]$ ratio was about 2.2. As expected, this ratio decreased with advancement of the reaction (Figure 4), indicative of a progressive hydrolysis of cADP-ribose. In comparison, under similar conditions when using NAD^+ as substrate, no ADP-ribose was detectable. Importantly, the slow turnover of PyAD^+ indicates that, in analogy to the CD38/ NAD^+ glycohydrolases, the formation of the ADP-ribosyl intermediary complex is also rate limiting in the *Aplysia* ADP-ribosyl cyclase-catalysed transformation of NAD^+ and its pyridinium analogues.

We then studied the methanolysis of cADP-ribose. By analogy to bovine and human CD38/ NAD^+ glycohydrolases [10,14,16],

Figure 4 HPLC elution profile of the products obtained by the *A. californica* ADP-ribosyl cyclase-catalysed hydrolysis of PyAD⁺

PyAD⁺ (20 μ M) was incubated at 37 °C in buffer A (final volume 500 μ l), in the presence of 0.5 unit of enzyme. Product analysis (reaction advancement, 28%) was performed by HPLC as described in the Experimental section. (a) cADP-ribose, (b) ADP-ribose and (c) PyAD⁺.

Figure 5 HPLC elution profile of the products obtained by the *A. californica* ADP-ribosyl cyclase-catalysed solvolysis of cADP-ribose in water/methanol

cADP-ribose (20 μ M) was incubated at 37 °C in buffer A containing 0.5 M methanol (final volume 200 μ l) in the presence of 30 m-units of enzyme. Product analysis was performed by HPLC as described in the Experimental section. (a) cADP-ribose, (b) ADP-ribose and (c) β -methyl ADP-ribose.

Aplysia ADP-ribosyl cyclase was also found to catalyse the methanolysis of the cyclic metabolite into methyl ADP-ribose. As determined by HPLC, this reaction resulted in the exclusive formation of the β -anomer, i.e. the methanolysis occurred with complete retention of configuration (Figure 5). Working with a 0–2 M range of methanol, two important parameters were established: (i) the product-partitioning ratio, i.e. $K = [\beta\text{-methyl ADP-ribose}][\text{H}_2\text{O}]/[\text{ADP-ribose}][\text{methanol}]$, which indicates the relative reactivity of the $\text{E} \cdot \text{ADP-ribosyl}$ intermediate (where E is

Figure 6 Dependence of the initial rate of cADP-ribose solvolysis on the concentration of methanol

Initial rates of cADP-ribose (20 μ M) solvolysis catalysed by *Aplysia* ADP-ribosyl cyclase (30 m-units) at 37 °C in buffer A containing increasing concentrations of methanol (MeOH).

the enzyme) with water and methanol, and (ii) the effect of methanol on the initial rates of cADP-ribose solvolysis (hydrolysis and methanolysis). Methanolysis/hydrolysis product ratios were found to be constant throughout the time course of the reactions and proportional to the methanol concentrations; the value of K was estimated to be 152 ± 9 ($\pm$ S.D.). This indicates that, on a molar basis, methanolysis is two orders of magnitude faster than hydrolysis and points to the possible occurrence of a stabilized oxocarbenium reaction intermediate in the catalysed reaction [21]. Such a result can be used further to dissect the reaction mechanism of the cADP-ribose hydrolase activity of the enzyme from a phenomenological standpoint. Thus it was of interest to determine whether this very important acceleration of the solvolysis of the ADP-ribosyl intermediate due to methanolysis had an incidence on the turnover of the reaction. With regard to that point, one can predict that if the formation of the ADP-ribosyl intermediate from cADP-ribose is rate limiting (step k_{-3}), the addition of methanol would have no effect on the overall rate of cADP-ribose transformation, whereas if $k_{\text{H}_2\text{O}}$ is rate limiting then the turnover would be accelerated by the solvent [28]. This latter case was observed experimentally, i.e. initial rates of cADP-ribose solvolysis were increased by the presence of methanol and a linear dependence of v_i on the concentration of this solvent was found (Figure 6), suggesting that $k_{-3} > k_{\text{H}_2\text{O}}$ in Scheme 1.

Methanolysis of NAD⁺ catalysed by *Aplysia* ADP-ribosyl cyclase was also studied. Under the experimental conditions used, where little or no hydrolysis was observed in the absence of methanol, the addition of this solvent up to 2 M did not drastically change the product distribution. Thus at 2 M methanol, β -methyl ADP-ribose represented less than 5% of the reaction products.

The *Aplysia*-cyclase-catalysed transformation of PyAD⁺, which leads to the formation of both cADP-ribose and ADP-ribose (see above), represents a favourable case for the study of methanolysis. In the presence of 0.5–2 M methanol, as assessed by HPLC, β -methyl ADP-ribose was also formed, mostly at the expense of cADP-ribose. In this case also, methanolysis was strictly proportional to the concentration of methanol and the partitioning ratio of 200 ± 19 is within the range of the one measured for the methanolysis of cADP-ribose. This is an important observation because it indicates that, in Scheme 1, the

partitioning of the ADP-ribosyl intermediate between water and methanol is independent from its route of formation (steps k_2 or k_{-3}).

ADP-ribosyl cyclase-catalysed hydrolysis of NMN⁺ was also examined because of the great interest of this reaction for the understanding of the molecular mechanism of the enzyme. When using high amounts of enzyme (0.4 unit), we could indeed detect the hydrolysis of this mononucleotide and thus demonstrate that the cyclization step is not compulsory for the transformation of substrates by the *Aplysia* enzyme; this result is in favour of a 'partitioning' mechanism (Scheme 1; see the Discussion). Because NMN⁺ cannot afford a cyclized product ($k_3 = 0$ in Scheme 1) its turnover rate should reflect $\approx k_2 + k_{H_2O}$; we found that for e.g. 1 mM NMN⁺, which is close to the maximal velocity, the rate ratio NAD⁺/NMN⁺ was about 3900. This high value contrasts with the CD38/NAD⁺ glycohydrolase-catalysed reactions, where the observed maximal velocities for NMN⁺ and NAD⁺ are within the same range [16,29]. It also indicates that in the *Aplysia*-enzyme-catalysed transformation of a substrate that cannot engage in a cyclization process, the slow step of the reaction pathway is most probably k_{H_2O} and not the formation of the intermediary complex resulting from the cleavage of the nicotinamide-ribose bond. This was borne out by studying the effect of methanol, which increased the turnover rate of NMN⁺; thus for example at 1 M methanol, the initial rate of NMN⁺ solvolysis was increased 2-fold.

***Aplysia* ADP-ribosyl cyclase does not catalyse methanolysis of NGD⁺**

NGD⁺ is a useful alternative substrate because its transformation into solvolytic and cyclization reaction products occurs via dead-end (macroscopically irreversible) reactions; i.e. in contrast to cADP-ribose, cGDP-ribose is not enzymically hydrolysable (Scheme 1, $k_{-3} \approx 0$). In this respect, we have demonstrated previously that in bovine and human CD38/NAD⁺ glycohydrolase-catalysed transformations of NGD⁺, methanol was competing efficiently ($K \approx 50$) with the hydrolysis (k_{H_2O}) and the cyclization (k_3) steps and produced β -methyl GDP-ribose at the expense of cGDP-ribose [11,14]. This indicates that the reaction products are derived from a common oxocarbenium ion intermediate via competing intra- and intermolecular reactions [11,16]. In sharp contrast with these enzymes, and with the results obtained above with NAD⁺, the *Aplysia* ADP-ribosyl cyclase transformed NGD⁺ into cGDP-ribose alone; no GDP-ribose was produced and in the presence of up to 2 M methanol no β -methyl GDP-ribose became detectable. These results are important because they indicate that (i) the k_3/k_{H_2O} ratio is high enough in the *Aplysia* enzyme that $k_3/(k_{H_2O} + k_{CH_3OH})$ still macroscopically favours the cyclization reaction, and that (ii) most probably, the formation of ADP-ribose and β -methyl ADP-ribose by the *Aplysia*-enzyme-catalysed solvolysis of NAD⁺ involves a prior formation of cADP-ribose.

DISCUSSION

The major finding of the present work was that *A. californica* ADP-ribosyl cyclase, by analogy with the multifunctional mammalian CD38/NAD⁺ glycohydrolases, was also able to catalyse the solvolysis (hydrolysis and methanolysis) of cADP-ribose. When considering NAD⁺ and cADP-ribose as competing substrates for the active site of the *Aplysia* enzyme, the specificity ratio, i.e. the ratio of the specificity constants V_{max}/K_m , is about 3.2×10^4 in favour of NAD⁺. This very high value, which is due to a concomitant $2 \times \log$ increase of K_m and decrease of V_{max}

values for cADP-ribose as compared with NAD⁺, contrasts with the much lower ratio of 50 established for example for the bovine CD38/NAD⁺ glycohydrolase [10]. From these data we can conclude that the invertebrate ADP-ribosyl cyclase is overwhelmingly a cyclase and that the cADP-ribose hydrolase activity, which becomes detectable only at very high enzyme concentrations (Figure 1B), has most probably no physiological meaning.

Nevertheless, the unravelling of this minor cADP-ribose hydrolase activity is important from a mechanistic standpoint and sheds some new light on the mode of action of the *Aplysia* cyclase. From the literature and the results obtained here we can conclude that the ADP-ribosyl cyclase and mammalian CD38/NAD⁺ glycohydrolases, which are characterized by a great structural similarity [6], have fundamentally the following activities in common: (i) transformation of NAD⁺ into cADP-ribose, albeit with vastly different yields (see the Introduction); we have shown here that this ADP-ribosyl cyclase reaction can also be extended to pyridinium analogues of NAD⁺ such as PyAD⁺; (ii) transglycosidation reactions [30–32]; (iii) hydrolysis of the mononucleotide NMN⁺; (iv) solvolysis of NAD⁺ or its pyridinium analogues, i.e. NAD⁺ glycohydrolase activity; and (v) solvolysis of cADP-ribose, i.e. cADP-ribose hydrolase activity. Interestingly, all of these reactions occur with retention of configuration at the anomeric carbon linked to the nicotinamide or other pyridinium residue, i.e. they generate β -ribosyl derivatives. Two mechanisms have been invoked for the mammalian CD38/NAD⁺ glycohydrolases that could account for their low cyclase activities. The mechanism that was proposed first ('sequential' mechanism) involves an obligatory cyclization of NAD⁺ into cADP-ribose followed by its hydrolysis into ADP-ribose [9]. Accordingly, the observed NAD⁺ glycohydrolase activity of these multifunctional enzymes results from the sequential ADP-ribosyl cyclase and cADP-ribose hydrolase activities. The second mechanism ('partitioning' mechanism), which suggests that a single oxocarbenium-type intermediate partitions to cyclic and other products (solvolysis, transglycosidation), was borne out by experiment [8,11,14,16]. From the results obtained here, we can conclude that this latter mechanism also applies to *A. californica* ADP-ribosyl cyclase and therefore propose for these classes of enzymes a unifying mechanism that is illustrated in Scheme 1. The ratio [cADP-ribose]/[ADP-ribose], which is the main difference between the invertebrate and the mammalian enzymes in terms of outcome of the reaction, resides most probably in the actual values (and/or ratios) of critical rate constants that are dictated by structural contingencies.

The pathways of the *A. californica* ADP-ribosyl cyclase-catalysed reactions are nevertheless quite interesting within this context. Thus it should be noted that, from a macroscopic point of view, an apparently sequential mechanism could be explained satisfactorily according to Scheme 1 as a limiting form of the partitioning mechanism, provided that $k_3 \gg k_{H_2O}$. This seems to be the case for the *Aplysia* enzyme. Indeed, although methanolysis is much faster than hydrolysis ($k_{CH_3OH}/k_{H_2O} \approx 150$), no β -methyl GDP-ribose was formed from NGD⁺, suggesting that methanol could not compete with the formation of cGDP-ribose and that $k_3 \gg k_{H_2O} + k_{CH_3OH}$. Moreover, only in the transformation of NMN⁺, where solvolysis is the only possible pathway, was the capture of the intermediate by water (step k_{H_2O}) measurable and this was indeed found to be very slow. The fact that this latter step is a slow process in the pathways catalysed by the *Aplysia* enzyme can also be deduced from the increased turnover rate triggered by methanol in the solvolysis of cADP-ribose and NMN⁺. Thus in sharp contrast to the CD38/NAD⁺ glycohydrolases [11], because of the magnitude of k_3 it is

Scheme 2 Partitioning of the ADP-ribosyl intermediate

The putative common ADP-ribosyl oxocarbenium ion intermediate evolves according to two competing pathways. (a) An intermolecular reaction between ribosyl C-1' and water, producing ADP-ribose. This route is highly disfavoured in the *Aplysia* ADP-ribosyl cyclase. (b) An intramolecular reaction between ribosyl C-1' and N-1 of the adenine ring in the correct orientation to yield cADP-ribose. This process is disfavoured in the mammalian CD38/NAD⁺ glycohydrolases.

difficult with the *Aplysia* enzyme to detect the partitioning of the ADP-ribosyl intermediate. It seems therefore that the transformation of NAD⁺ into ADP-ribose, only observed at high concentrations of ADP-ribosyl cyclase, results from the hydrolysis of cADP-ribose, which is formed first and then accumulated rather than being formed from the capture by water of the ADP-ribosyl intermediate originating from NAD⁺. We can thus conclude that the *Aplysia* enzyme catalyses the transformation of NAD⁺ according to an apparent sequential mechanism and that its NAD⁺ glycohydrolase activity is due in fact to a macroscopic succession of ADP-ribosyl cyclase and cADP-ribose hydrolase activities. This conclusion is also in agreement with the observed sequential release of the reaction products nicotinamide and ADP-ribose or cADP-ribose, respectively, in the case of CD38/NAD⁺ glycohydrolases [14,16,28] or the *Aplysia* cyclase [32].

An interesting mechanistic issue is the difference in efficiency of the k_3 and k_{H_2O} steps in the *Aplysia* cyclase and CD38/NAD⁺ glycohydrolases. With NAD⁺ as substrate, the turnover numbers of the ADP-ribosyl cyclase (present study) and e.g. bovine spleen NAD⁺ glycohydrolase [26] are within the same order of magnitude and for both classes of enzymes the slow step in the formation, respectively, of cADP-ribose and ADP-ribose is the formation of the intermediary ADP-ribosyl complex. This latter point is supported by the observation that the turnover rate of both classes of enzymes is much affected by the pK_a of the leaving pyridine, i.e. by the bond energy of the scissile bond (this work and [17]). One would therefore expect that both classes of enzymes are equally efficient in the cleavage of the nicotinamide-ribose bond and this includes NMN⁺. The particularly low rate of solvolysis of the latter mononucleotide by the cyclase, compared with the rate of cyclization of NAD⁺ and to its hydrolysis catalysed by the CD38/NAD⁺ glycohydrolases, is thus instructive. It indicates that when there is no possibility of cyclization, the turnover rate is dictated by the k_{H_2O} step, which is very low for the *Aplysia* enzyme. The same holds true in the hydrolysis of cADP-ribose: in both cases the turnover of the reaction is accelerated by methanol. Thus the characteristic of the *Aplysia* enzyme is that its k_{H_2O} step is much slower than in CD38/NAD⁺ glycohydrolases. Altogether, this means that in the CD38/NAD⁺ glycohydrolases the active site accommodates water molecule(s), which can react easily with the intermediary oxocarbenium to give ADP-ribose and thus efficiently compete with the cyclization process, whereas in the *Aplysia* ADP-ribosyl

cyclase, water is mostly excluded from the active site, leaving the cyclization to take place.

In conclusion, the outcome of the reaction catalysed by the two classes of enzymes must reflect the partitioning of the intermediary complex (Scheme 1), where the fast steps are respectively the intramolecular cyclization in the *Aplysia* ADP-ribosyl cyclase ($k_3 \gg k_{H_2O}$) and the intermolecular reaction with water in the CD38/NAD⁺ glycohydrolases ($k_3 < k_{H_2O}$). Within the intermediary complex of the *Aplysia* enzyme this might be due to a more favourable positioning of the N-1 of adenine with regard to the oxocarbenium ion or, more probably, because the capture by water is a highly disfavoured process, to a better shielding of this latter species from water (Scheme 2). A similar conclusion was reached recently by Munshi et al. [33]. Comparison of three-dimensional structures of the active sites of these enzymes should provide clues along these lines.

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REFERENCES

- Clapper, D. L., Walseth, T. F., Dargie, P. J. and Lee, H. C. (1987) *J. Biol. Chem.* **262**, 9561–9568
- Lee, H. C. (1997) *Physiol. Rev.* **77**, 1133–1164
- Hellmich, M. R. and Strumwasser, F. (1991) *Cell Regul.* **2**, 193–202
- Lee, H. C. and Aarhus, R. (1991) *Cell Regul.* **2**, 203–209
- States, D. J., Walseth, T. F. and Lee, H. C. (1992) *Trends Biochem. Sci.* **17**, 495
- Prasad, G. S., McRee, D. E., Stura, E. A., Levitt, D. G., Lee, H. C. and Stout, C. D. (1996) *Nat. Struct. Biol.* **3**, 957–964
- Lee, H. C., Gallione, A. and Walseth, T. F. (1994) *Vitamins Hormones* **48**, 199–257
- Kim, H., Jacobson, E. L. and Jacobson, M. K. (1993) *Science* **261**, 1330–1333
- Howard, M., Grimaldi, J. C., Bazan, J. F., Lund, F. E., Santos-Argumedo, L., Parkhouse, R. M., Walseth, T. F. and Lee, H. C. (1993) *Science* **262**, 1056–1059
- Muller-Steffner, H., Muzard, M., Oppenheimer, N. and Schuber, F. (1994) *Biochem. Biophys. Res. Commun.* **204**, 1279–1285
- Muller-Steffner, H. M., Augustin, A. and Schuber, F. (1996) *J. Biol. Chem.* **271**, 23967–23972
- Zocchi, E., Franco, L., Guida, L., Benatti, U., Bargellesi, A., Malavasi, F., Lee, H. C. and De Flora, A. (1993) *Biochem. Biophys. Res. Commun.* **196**, 1459–1465
- Graeff, R. M., Walseth, T. F., Fryxell, K., Branton, W. D. and Lee, H. C. (1994) *J. Biol. Chem.* **269**, 30260–30267

- 14 Bertheliet, V., Tixier, J. M., Muller-Steffner, H., Schubert, F. and Deterre, P. (1998) *Biochem. J.* **330**, 1383–1390
- 15 Augustin, A., Muller-Steffner, H. and Schubert, F. (2000) *Biochem. J.* **345**, 43–52
- 16 Sauve, A. A., Munshi, C., Lee, H. C. and Schramm, V. L. (1998) *Biochemistry* **37**, 13239–13249
- 17 Tarnus, C. and Schubert, F. (1987) *Bioorg. Chem.* **15**, 31–42
- 18 Lee, H. C., Graeff, R. M., Munshi, C. B., Walseth, T. F. and Aarhus, R. (1997) *Methods Enzymol.* **280**, 331–340
- 19 Heukeshoven, J. and Dernick, R. (1988) *Electrophoresis* **9**, 28–32
- 20 Schubert, F. and Travo, P. (1976) *Eur. J. Biochem.* **65**, 247–255
- 21 Tarnus, C., Muller, H. M. and Schubert, F. (1988) *Bioorg. Chem.* **16**, 38–51
- 22 Jacobson, M. K., Cervantes-Laurean, D., Strohm, M. S., Coyle, D. L., Bummer, P. M. and Jacobson, E. L. (1995) *Biochimie* **77**, 341–344
- 23 Zhang, F. J., Gu, Q. M. and Sih, C. J. (1999) *Bioorg. Med. Chem.* **7**, 653–664
- 24 Migaud, M. E., Pederick, R. L., Bailey, V. C. and Potter, B. V. L. (1999) *Biochemistry*, **38**, 9105–9114
- 25 Munshi, C., Baumann, C., Levitt, D., Bloomfield, V. A. and Lee, H. C. (1998) *Biochim. Biophys. Acta* **1388**, 428–436
- 26 Muller-Steffner, H., Schenherr-Gusse, I., Tarnus, C. and Schubert, F. (1993) *Arch. Biochem. Biophys.* **304**, 154–162
- 27 Glick, D. L., Hellmich, M. R., Beushausen, S., Tempst, P., Bayley, H. and Strumwasser, F. (1991) *Cell Regul.* **2**, 211–218
- 28 Schubert, F., Travo, P. and Pascal, M. (1976) *Eur. J. Biochem.* **69**, 593–602
- 29 Schubert, F., Pascal, M. and Travo, P. (1978) *Eur. J. Biochem.* **83**, 205–214
- 30 Anderson, B. M. (1982) in *The Pyridine Nucleotide Coenzymes* (Everse, J., Anderson, B. and You, K.-S., eds.), pp. 91–133, Academic Press, New York
- 31 Lee, H. C., Graeff, R. M. and Walseth, T. F. (1997) *Adv. Exp. Med. Biol.* **419**, 411–419
- 32 Inageda, K., Takahashi, K., Tokita, K., Nishina, H., Kanaho, Y., Kukimoto, I., Kontani, K., Hoshino, S. and Katada, T. (1995) *J. Biochem. (Tokyo)* **117**, 125–131
- 33 Munshi, C., Thiel, D. J., Mathews, I. I., Aarhus, R., Walseth, T. F. and Lee, H. C. (1999) *J. Biol. Chem.* **274**, 30770–30777

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