Kinetic competence of the cADP-ribose–CD38 complex as an intermediate in the CD38/NAD⁺ glycohydrolase-catalysed reactions: implication for CD38 signalling

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CD38/NAD⁺ glycohydrolase is a type II transmembrane glycoprotein widely used to study T- and B-cell activation and differentiation. CD38 is endowed with two different activities: it is a signal transduction molecule and an ectoenzyme that converts NAD⁺ into ADP-ribose (NAD⁺ glycohydrolase activity) and small proportions of cADP-ribose (cADPR; ADP-ribosyl cyclase activity), a calcium-mobilizing metabolite, which, ultimately, can also be hydrolysed (cADPR hydrolase activity). The relationship between these two properties, and strikingly the requirement for signalling in the formation of free or enzyme-complexed cADPR, is still ill-defined. In the present study we wanted to test whether the CD38–cADPR complex is kinetically competent in the conversion of NAD⁺ into the reaction product ADP-ribose. In principle, such a complex could be invoked for cross-talk, via conformational changes, with neighbouring partner(s) of CD38 thus triggering the signalling phenomena. Analysis of the kinetic parameters measured for the CD38/NAD⁺ glycohydrolase-catalysed hydrolysis of 2'-deoxy-2'-aminoribo-NAD⁺ and ADP-cyclo[N1,C1']-2'-deoxy-2'-aminoribose (slowly hydrolysable

analogues of NAD⁺ and cADPR respectively) ruled out that the CD38–cADPR complex can accumulate under steady-state conditions. This was borne out by simulation of the prevalent kinetic mechanism of CD38, which involve the partitioning of a common E·ADP-ribosyl intermediate in the formation of the enzyme-catalysed reaction products. Using this mechanism, microscopic rate conditions were found which transform a NAD⁺ glycohydrolase into an ADP-ribosyl cyclase. Altogether, the present work shows that if the cross-talk with a partner depends on a conformational change of CD38, this is most probably not attributable to the formation of the CD38–cADPR complex. In line with recent results on the conformational change triggered by CD38 ligands [Berthelie, Laboureaux, Boulla, Schuber and Deterre (2000) Eur. J. Biochem. 267, 3056–3064], we believe that the Michaelis CD38–NAD⁺ complex could play such a role instead.

Key words: cADP-ribose analogues, ectoenzymes, kinetics.

INTRODUCTION

CD38 is a type II plasma membrane-associated glycoprotein endowed with catalytic and signalling properties (for reviews, see [1,2]). This antigenic marker of lymphoid cells, which acts as an ectoenzyme in a variety of cells, converts NAD⁺ overwhelmingly into ADP-ribose (NAD⁺ glycohydrolase activity) and into minor proportions (<2%) of cyclic ADP-ribose (cADPR; ADP-ribosyl cyclase activity) [2]. This latter cyclic metabolite is of particular interest because it is a potent inducer of Ca²⁺ mobilization from intracellular stores via inositol 1,4,5-trisphosphate-independent mechanisms (for reviews, see [3,4]). CD38 also serves as a receptor which can regulate cellular events, such as growth, differentiation and apoptosis [5]. It should be noted that in most cases these effects have been obtained by *in vitro* ligation of cellular CD38 with monoclonal antibodies. Amongst the many systems described to date, the association of CD38 with the antigen receptor of T- and B-lymphocytes has been particularly well studied (for review, see [6]). In B-lymphocytes, it was found that CD38 functions as a co-receptor for the B-cell receptor (BCR) and that its signal transducing properties relied on cross-talk with that receptor's own signalling complex [7].

The important questions that remain to be resolved, pertaining

to the mechanism of CD38 signalling, are the following: (1) is there a correlation between the catalytic activity of CD38 and its signalling properties and how can extracellularly formed cADPR affect intracellular events?; and (2) how does CD38, whose short intracellular domain lacks consensus sequences, cross-talk with its partners such as BCR? Related to the first question, the groups of De Flora [8] and Lee [9], based on experimental and structural data, have suggested that CD38 could function as a channel/transporter for catalytically formed cADPR leading to increased intracellular Ca²⁺ levels. However, Lund et al. [10] have shown that CD38 signalling in B-cells was independent from its catalytic activity. Thus A20 cells expressing CD38 mutants, which had a much reduced catalytic activity, were still fully competent in signalling as measured by the formation of IL2; such results seemingly rule out, at least in this model system, that the cross-talk of CD38 with BCR requires the formation of free cADPR. Similarly, in T-lymphocytes the formation of extracellular cADPR by CD38 did not correlate with intracellular Ca²⁺ signalling [11].

The kinetic and molecular mechanisms of CD38 have been elucidated [12–15]. The catalytic conversion of NAD⁺ follows a partitioning mechanism involving the formation of a stabilized ADP-ribosyl oxocarbenium ion intermediate which can react either with water or intramolecularly with position N1 of the

Abbreviations used: BCR, B-cell receptor; cADPR, cADP-ribose; 2'-NH₂-NAD⁺, 2'-deoxy-2'-aminoribo-NAD⁺; 2'-NH₂-ADP-ribose, ADP-2'-deoxy-2'-aminoribose; 2'-NH₂-cADPR, ADP-cyclo[N1,C1']-2'-deoxy-2'-aminoribose; C_f, forward commitment.

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Scheme 1 Minimal kinetic mechanism for the reactions catalysed by CD38/ NAD^+ glycohydrolases

CD38/ NAD^+ glycohydrolases catalyse the cleavage of the nicotinamide-ribose bond in NAD^+ leading to the formation of an $\text{E} \cdot \text{ADP-ribose}$ intermediary complex (where E is the enzyme). This intermediate partitions between two competing pathways: an intramolecular reaction between ribosyl C-1' and N-1 of the adenine ring that yields cADPR and a macroscopically irreversible hydrolysis reaction giving ADP-ribose [12–15]. The putative kinetically competent complexes in the conversion of NAD^+ into ADP-ribose under steady-state conditions are boxed.

adenine ring to produce cADPR (Scheme 1). The poor efficiency of this latter step versus hydrolysis was given as an explanation for the modest formation of cADPR [13]. In contrast with the hydrolytic step, which is macroscopically irreversible, cADPR can reversibly bind to CD38 and undergo hydrolysis. This step explains why CD38 is also considered to be a cADPR hydrolase. Regardless of the low production of cADPR by CD38, and the problems inherent to its uptake by cells, analysis of the catalytic scheme (Scheme 1) indicates that the formation of the cyclic metabolite could still be of importance and needed in cross-talk leading to signalling events [7,10], provided that the CD38–cADPR complex is kinetically competent in the conversion of NAD^+ into ADP-ribose. This possibility could be invoked in cases where CD38 in this complex (boxed in Scheme 1) adopts a specific conformation that is recognized by its partners, such as BCR [7,10]. In favour of such a possibility, we have recently shown that ligands, such as NAD^+ , were able to trigger conformational changes in human CD38 [16]. From a kinetic perspective, this hypothesis requires that during the catalytic cycle cADPR is released very slowly from the CD38–cADPR complex, which accumulates under steady-state conditions, and that the fate of the cyclic metabolite is overwhelmingly via hydrolysis (Scheme 1, $k_5 < k_{-4}$). To explain the modest formation of free cADPR by CD38, step k_{-4} must allow its conversion back into the intermediary ADP-ribosyl oxocarbenium ion, which is then, ultimately and irreversibly, converted into ADP-ribose (step k_3). According to this hypothesis, the signalling trigger would not be free cADPR, but its transient complex with CD38 en route to the hydrolysis step. We have tested this possibility by studying the hydrolysis of cADPR, by manipulating the kinetic constants in Scheme 1 using an analogue of NAD^+ and by performing kinetic simulation studies on reaction progress curves. Our conclusion is that the CD38–cADPR complex is not kinetically competent in the CD38-catalysed conversion of NAD^+ into ADP-ribose and is probably not involved in its signalling functions; this raises again the question of the relationship between the formation of cADPR and the functional role of CD38.

EXPERIMENTAL

Materials

NAD^+ , ADP-ribose, cADPR, AMP, nucleotide pyrophosphatase from *Crotalus atrox* venom and Chaps were purchased from Sigma (L'Isle d'Abeau Chesnes, France). ADP-ribosyl cyclase was purified, with some modifications as described previously [16a,16b,16c] from *Aplysia californica* ovotestis obtained from

Dr Bernard Poulain (CNRS-Neurochimie, Strasbourg, France). The enzyme was stored at -20°C in 10 mM potassium phosphate buffer, pH 7.4. NAD^+ glycohydrolase from bovine spleen was purified routinely as described previously [17]. The enzyme (specific activity 140 units/mg) was stored at -20°C in 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) Chaps. 2'-Deoxy-2'-aminoribo- NAD^+ (2'- NH_2 - NAD^+) was prepared as described previously [18].

Synthesis of ADP-cyclo[N1,C1']-2'-deoxy-2'-aminoribose (2'- NH_2 -cADPR) and ADP-2'-deoxy-2'-aminoribose (2'- NH_2 -ADP-ribose)

Approximately 200 nmol of 2'- NH_2 - NAD^+ , was incubated at 37°C in 250 μl of 50 mM potassium phosphate buffer, pH 7.4 (buffer A) in the presence of *A. californica* ADP-ribosyl cyclase (2 units) to produce nicotinamide, 2'- NH_2 -cADPR and a small proportion (<10%) of 2'- NH_2 -ADP-ribose. After approx. 20 min the conversion was essentially complete (as assessed by HPLC analysis of aliquots) and the reaction mixture was centrifuged at 10000 *g* for 10 min at 4°C in a Microcon microconcentrator (10 kDa cut-off, Amicon) [13]. The analogue was purified by HPLC on a 250 mm $\times$ 4.6 mm YMC C_{18} column (A.I.T. Chromato, Le Mesnil le Roi, France) under isocratic elution conditions (0.7 ml/min) using 10 mM ammonium acetate, pH 3.5, containing 0.5% (v/v) methanol as elution buffer. The peaks, detected by their UV absorbance at 260 nm, containing the analogue (elution time of approx. 20 min at 20°C) were collected, pooled and the eluent removed by freeze-drying. Similarly, 2'- NH_2 -ADP-ribose was obtained by incubating approx. 200 nmol of 2'- NH_2 - NAD^+ in the presence of purified bovine spleen NAD^+ glycohydrolase (1 unit) in buffer A containing 0.5% (w/v) Chaps (buffer B). The identity of the purified reaction products was confirmed by electrospray ionization MS operated in the negative-ion mode. 2'- NH_2 -cADPR and 2'- NH_2 -ADP-ribose gave the expected molecular mass at m/z 539.1 [$\text{M} - \text{H}$] $^-$ and 557.2 [$\text{M} - \text{H}$] $^-$ respectively. Moreover, as expected [19], 2'- NH_2 -cADPR was resistant to the action of *C. atrox* dinucleotide pyrophosphatase.

Enzyme assays

To determine the kinetic parameters K_m and $V_{\max}$ 3–75 μM 2'- NH_2 - NAD^+ was incubated at 37°C in the presence of 12 m-units of NAD^+ glycohydrolase in 300 μl of buffer B. For a given substrate concentration, initial rates were calculated from progress curves established by analysis of aliquots withdrawn at five different time points (from 0 to 15 min). The reaction was stopped by addition of the aliquot to ice-cold 10% (v/v) perchloric acid (2% final concentration). After vortex-mixing, the pH of the supernatant was adjusted to 6–7 with 3.5 M K_2CO_3 and the mixture was centrifuged [20]. The precipitated KClO_4 was eliminated by centrifugation and the supernatant analysed by HPLC (see below). The ratio $V_{\max}/K_m$ was determined (see below) from progress curves (≥ 8 time points) obtained at 37°C with 20 μM cADPR or 2'- NH_2 -cADPR incubated in the presence of 12.4 and 625 m-units of enzyme in buffer B (100 μl final volume) respectively. The specific activity of the NAD^+ glycohydrolase preparation (i.e. $V_{\max}$ for NAD^+) was estimated by following the hydrolysis of 1 mM NAD^+ , during incubation with the enzyme at 37°C in buffer B (500 μl final volume).

Methanolysis catalysed by NAD^+ glycohydrolase

2'- NH_2 - NAD^+ and 2'- NH_2 -cADPR (50 μM final concentration) were incubated at 37°C in buffer B, in the presence of 0–2 M methanol (200 μl final volume) and 15 and 500 m-units of NAD^+

glycohydrolase respectively. At different time points, aliquots were analysed by HPLC as described below.

Analysis of the reaction products by HPLC

Product analysis was performed by HPLC on a 300 mm × 3.9 mm μ Bondapak C₁₈ column (Waters). The compounds were eluted isocratically (1 ml/min) at 20 °C with a 10 mM ammonium phosphate buffer, pH 5.5, containing 1.2% (v/v) acetonitrile and detected by UV absorbance at 260 nm. Standards were injected to identify and calibrate the different reaction products.

Kinetic analysis

The initial rates of substrate conversion were calculated from the data points obtained by HPLC using a linear regression program. Kinetic parameters ($V_{\max}$ and K_m) were determined from the plots of the initial rates as a function of substrate concentration (data points ≥ 8) using a non-linear regression program (GraphPad, Prism). To gain access to the specificity constants (i.e. the ratio $V_{\max}/K_m$ of the kinetic parameters) reactions were run under experimental conditions where $[S]_0 \ll K_m$. Their values were calculated from the progress curves, monitored by HPLC, using the equation: $[S] = [S]_0 \exp(-V_{\max} \times \text{time}/K_m)$ [21]. Progress curve simulations were carried out using the DynaFit program [22].

RESULTS AND DISCUSSION

We have recently shown by molecular cloning that bovine spleen ecto-NAD⁺ glycohydrolase is the bovine equivalent of CD38 [23]. Moreover, the kinetic and molecular mechanisms for this enzyme have been particularly well studied and were found to be in excellent agreement with those recently established for human CD38 [14,15]. The kinetic competence of the CD38–cADPR complex in the transformation of NAD⁺ into ADP-ribose and its potential relevance in the signalling functions of CD38 have been investigated using highly purified bovine spleen ecto-NAD⁺ glycohydrolase and the results analysed according to Scheme 1.

Commitment to catalysis of the CD38–cADPR complex

As alluded to in the Introduction section, for CD38–cADPR to be the key complex (boxed in Scheme 1) in the conversion of NAD⁺ into ADP-ribose under steady-state conditions, the cyclization step should be faster than hydrolysis ($k_4 > k_3$) and the off-rate (k_5) should be slow compared with k_{-4} (and k_3). This latter condition is important because it takes into account the fact that ADP-ribose is overwhelmingly the major product of the pathway catalysed by CD38. No transient accumulation of cADPR was measurable during the entire reaction course, up to a complete transformation of NAD⁺ (see, for example, [24]). Situations where the dissociation of one of the reaction products is rate limiting have been well documented in enzymology. In our particular case, where the reaction scheme can also be entered via cADPR (step k_{-5}), if $k_{-4} > k_5$ one expects that on hydrolysis of the cyclic metabolite the CD38–cADPR complex, which now corresponds to a Michaelis complex, has a high commitment to catalysis. Forward commitment (C_f) to catalysis has been defined as the tendency of the enzyme–substrate complex poised for catalysis to continue forward as opposed to its tendency to partition back into free enzyme and substrate [25]. In the case of a high commitment to catalysis $k_{\text{cat}} > k_{\text{off}}$; in Scheme 1 this translates into $k_{-4} > k_5$ for the catalytic pathway leading to hydrolysis of cADPR, because, in the reactions catalysed by

Figure 1 Structure of the 2'-amino analogues of NAD⁺ and cADPR

The pK_a of the 2'-amino group of 2'-NH₂-NAD⁺ is 4.6 (A. L. Handlon and N. J. Oppenheimer, unpublished work).

bovine spleen NAD⁺ glycohydrolase, the hydrolytic step (k_3) is fast compared with the bond cleavage steps [26,27]. This point was borne out in the present work (see below). In favourable cases where k_{cat} is relatively small, commitment to catalysis can be determined experimentally, for example by isotope-trapping under low turnover reaction conditions [28]. This was not possible in the present study because of the binding constant of cADPR (see below), the efficiency of its hydrolysis and the high quantities of enzyme needed. One must therefore rely on extrapolations using the kinetic parameters we have determined previously [24] for the NAD⁺ glycohydrolase-catalysed hydrolysis of cADPR: $K_m = 2.15$ mM and $k_{\text{cat}} = 106$ s⁻¹. These data indicate a fairly weak binding of the cyclic metabolite to the enzyme in comparison with that of NAD⁺ ($K_m = 26$ μ M). Considering these values and a C_f (equal to k_{-4}/k_5) of 10, one can estimate that k_{-5} , the second-order on-rate constant, would be approximately equal to 4.9×10^3 M⁻¹·s⁻¹. This is a very low value compared with the rates of diffusion ($\approx 10^8$ M⁻¹·s⁻¹) that generally characterize the formation of enzyme–substrate complexes, and such a slow binding rate should have been detectable experimentally. In contrast, in the hypothesis of low commitments to catalysis one finds that k_{-5} attains more reasonable values; for example, with C_f equal to 0.1 or 0.001, and k_{-5} equal to 4.9×10^5 or 4.9×10^7 M⁻¹·s⁻¹ respectively. In conclusion, the kinetic parameters found for the hydrolysis of cADPR are more in favour of the formation of a CD38–cADPR complex that shows a low C_f to catalysis. This result also predicts that if this complex is kinetically competent in the conversion of NAD⁺ into ADP-ribose, a transient accumulation of free cADPR should have been measurable.

Studies with slowly hydrolysable NAD⁺ and cADPR analogues

To test the above conclusions, we have studied the conversion of analogues of NAD⁺ and cADPR that permit the manipulation of some crucial kinetic steps in Scheme 1. We have shown previously that replacement of 2'-OH of NAD⁺ by substituents with different electron acceptor/donor characteristics resulted in changes in the nicotinamide-ribose cleavage rates (k_2) whose magnitudes were dependent on the ability of that substituent to stabilize the intermediary oxocarbenium ion [18]. Such analogues could be interesting tools because for the same reactivity reasons that they slow down step k_2 , they could also decelerate step k_{-4} , which corresponds to a bond cleavage involving the formation of the common oxocarbenium ion intermediate. We reasoned that if a CD38–cADPR type of complex is kinetically competent in the transformation of NAD⁺ into ADP-ribose, then a decrease

Table 1 Kinetic parameters of bovine spleen CD38/NAD⁺ glycohydrolase substrates

n.d., not determined.

Substrate	K_m (μ M)	V_{max} (μ mol/min per mg)	V_{max}/K_m (l/min per mg)
NAD ⁺	26 $\pm$ 2.0	140 $\pm$ 20	5.38
2'-NH ₂ -NAD ⁺	5.02 $\pm$ 0.80	2.82 $\pm$ 0.23	0.56
cADPR [*]	2150	210	97 $\times 10^{-3}$
2'-NH ₂ -cADPR	n.d.	n.d.	1.55 $\times 10^{-3}$

* [24].

Figure 2 HPLC elution profile of the reaction products obtained by incubating bovine spleen CD38/NAD⁺ glycohydrolase with 2'-NH₂-NAD⁺

2'-NH₂-NAD⁺ (50 μ M) was incubated at 37 °C in buffer B (final volume of 1 ml) in the presence of 0.3 unit of enzyme. Product analysis (reaction advancement of 74%) was performed by HPLC as described in the Experimental section. a, 2'-NH₂-ADP-ribose; b, 2'-NH₂-cADPR; c, nicotinamide; d, 2'-NH₂-NAD⁺; and x, unknown.

in k_{-4} would increase the probability of dissociation of the cyclic compound (step k_5) and favour the transient production of the free cADPR analogue, which should be easily visualized on chromatograms. Thus the aims of this part of our study were first to verify our ability to manipulate the constants k_2 and k_{-4} with analogues of NAD⁺ and cADPR and then to check whether an increased formation of a cyclic analogue was observed.

Among the different options available we chose the 2'-NH₂ substituent, because the analogue 2'-NH₂-NAD⁺ could still be transformed into 2'-NH₂-cADPR (Figure 1) with *A. californica* ADP-ribosyl cyclase. The kinetic parameters of 2'-NH₂-NAD⁺ were determined (Table 1); they indicate that this analogue binds efficiently to bovine spleen NAD⁺ glycohydrolase and, compared with NAD⁺, is hydrolysed at an approx. 50-fold lower maximal rate. These results demonstrate the influence of the 2'-NH₂ substituent on the scissile bond cleavage rate (k_2 step) and can be extrapolated to the cleavage of the C1'-N1 bond in the cyclic analogue. 2'-NH₂-cADPR was thus prepared from 2'-NH₂-NAD⁺, characterized (see the Experimental section) and its kinetic parameters determined. Owing to the high K_m values of the cyclic analogues and the limited amounts available for the 2'-NH₂-containing cyclic analogue, we could not directly determine the kinetic parameters. However, we calculated the specificity constants (V_{max}/K_m) from progress curves obtained at 20 μ M; i.e. under conditions where $[S]_0 \ll K_m$ [21]. The values are given in Table 1 and, as expected from the lower reactivity of the analogue, the ratio (V_{max}/K_m)_{cADPR}/(V_{max}/K_m)_{2'-NH₂-cADPR} is 63.

Figure 3 Progress curve of the conversion of 2'-NH₂-NAD⁺ catalysed by bovine spleen CD38/NAD⁺ glycohydrolase

2'-NH₂-NAD⁺ (50 μ M) was incubated at 37 °C in buffer B (final volume of 1 ml) in the presence of 0.3 unit of enzyme ($[E]_0 = 8.7 \times 10^{-8}$ M). Aliquots were removed at given times and the reaction was quenched by the addition of perchloric acid as described in the Experimental section. Reaction product profiles were obtained by HPLC (see Figure 2) and quantitative estimates (given as the percentage of total products) were deduced from the absorbance peaks at 260 nm (see the Experimental section). (◆) 2'-NH₂-NAD⁺, (▲) 2'-NH₂-cADPR and (●) 2'-NH₂-ADP-ribose. The solid curves represent the best fit of the experimental points to Scheme 1, using the DynaFit program [22], and setting K_5 equal to 5 μ M and 2 mM respectively for 2'-NH₂-NAD⁺ and 2'-NH₂-cADPR (see the text). The following values (s^{-1}) were produced: $k_2 = 1.04$, $k_3 = 8980$, $k_4 = 478$ and $k_{-4} = 1.82$.

In addition, when performed at 200 μ M, this analysis yielded similar values with a ratio of the specificity constants equal to 67. Such a result implies that (1) for both cyclic compounds, even at 200 μ M, $[S]_0$ was still at least one order of magnitude lower than K_m [21], and (2) although we cannot calculate the exact value for the K_m of 2'-NH₂-cADPR, we can estimate that its lower limit is approx. 2 mM (compared with 2.15 mM for cADPR; Table 1). Moreover, we can also conclude that the difference in the V_{max}/K_m values for cADPR and 2'-NH₂-cADPR originates, as expected, from the lower V_{max} for the hydrolysis of 2'-NH₂-cADPR and that (V_{max})_{cADPR}/(V_{max})_{2'-NH₂-cADPR} ≥ 63 . Altogether these results show that the conversion of the analogue 2'-NH₂-NAD⁺ catalysed by CD38/NAD⁺ glycohydrolase will, compared with NAD⁺, decrease k_2 and importantly also k_{-4} by almost two orders of magnitude. The transformation of the analogue 2'-NH₂-NAD⁺ was therefore monitored by HPLC (Figure 2) to investigate the kinetics and extent of 2'-NH₂-cADPR formation. Representative progress curves are given in Figure 3. They show that the NAD⁺ analogue is overwhelmingly hydrolysed into 2'-NH₂-ADP-ribose, and one observes the occurrence of a monotonic increase of 2'-NH₂-cADPR that accounts, throughout the reaction course, for approx. 4.7 $\pm$ 0.3 % ($n = 7$) of the total reaction products. This latter result must be compared with the 2% found for cADPR under similar experimental conditions during the transformation of NAD⁺ [13]. In conclusion, slowing down the step k_{-4} in Scheme 1 did not allow the transient accumulation of free 2'-NH₂-cADPR.

Besides 2'-NH₂-NAD⁺, we have also been looking for other 2'-substituent-bearing analogues (such as N₃ or F), which were also previously shown to be substrates for bovine NAD⁺ glycohydrolase [18] and which might slow down the critical kinetic

Table 2 Kinetic constants for the progress curves simulation of bovine spleen CD38/NAD⁺ glycohydrolase-catalysed conversion of NAD⁺ and 2'-NH₂-NAD⁺ at 37 °C

Sim, simulation.

Constant	NAD ⁺					2'-NH ₂ -NAD ⁺	
	Sim 1	Sim 2	Sim 3	Sim 4	Sim 5	Sim 6	Sim 7
k_1 (M ⁻¹ ·s ⁻¹)	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸
k_{-1} (s ⁻¹)	2600	2600	2600	2600	2600	500	500
k_2 (s ⁻¹)	73	73	73	73	73	1.46	1.46
k_3 (s ⁻¹)	1 × 10 ⁴	1 × 10 ⁴	100	100	100	1 × 10 ⁴	1 × 10 ⁴
k_4 (s ⁻¹)	200	200	1 × 10 ⁴	100	1000	490	490
k_{-4} (s ⁻¹)	110	110	110	110	110	1.70	1.70
k_5 (s ⁻¹)	2.15 × 10 ⁵	10.5	2.15 × 10 ⁵	2.15 × 10 ⁵	500	2.15 × 10 ⁵	0.17
k_{-5} (M ⁻¹ ·s ⁻¹)	1 × 10 ⁸	4.9 × 10 ⁸	1 × 10 ³	1 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁸	79

steps of Scheme 1 even more. However, such NAD⁺ analogues could not be used because their very slow conversion rates by the *A. californica* enzyme [29] precluded significant yields of the cyclic analogues.

The fact that slightly more 2'-NH₂-cADPR was formed compared with cADPR could indicate a modest acceleration in the cyclization step k_4 at the expense of the capture of the oxocarbenium ion by water (step k_3). Two factors could be invoked: (1) because of the 2'-NH₂ substituent, the positioning of the oxocarbenium ion with regard to the N1 of the adenine could be slightly altered favouring the ring closure; and (2) the reactivity of the 2'-NH₂ ribosyl oxocarbenium intermediate is changed and shows a greater selectivity for the N1 of the adenine moiety. To test the selectivity issue, we have also studied the enzymic methanolysis of 2'-NH₂-NAD⁺ and 2'-NH₂-cADPR. Previously we demonstrated, in the solvolysis (reaction with solvent molecules, i.e. water and/or methanol) of NAD⁺ catalysed by bovine spleen CD38/NAD⁺ glycohydrolase, that methanol could compete with water as an acceptor for the ADP-ribosyl oxocarbenium ion yielding methyl ADP-ribose with complete retention of configuration [13,30]. Moreover, a selectivity was observed in favour of methanol that was approx. 50-fold more reactive than water. For the 2'-NH₂-NAD⁺ analogue in the presence of 2 M methanol the product partitioning ratio ($K = [\text{methyl-2'-NH}_2\text{-ADP-ribose}]/[\text{H}_2\text{O}]/[\text{2'-NH}_2\text{-ADP-ribose}]/[\text{CH}_3\text{OH}]$) was 30 ± 1 ($n = 5$) for 2'-NH₂-NAD⁺ and 26 ± 2 ($n = 4$) for 2'-NH₂-cADPR. This decreased selectivity of methanol versus water for the 2'-NH₂ analogues is in line with the lesser stability and greater reactivity of the oxocarbenium ion they generate. Thus a lesser chemical discrimination of the 2'-NH₂ ribosyl intermediate could be invoked to explain an increased cyclization. A further point should be mentioned here; although addition of methanol to the reaction medium increases the rate of solvolysis of the intermediate oxocarbenium ion, no acceleration of the enzyme turnover rate could be observed. This suggests that for both 2'-NH₂-NAD⁺ and 2'-NH₂-cADPR, k_3 is fast relative to the formation of the CD38·2'-NH₂-ADP-ribosyl intermediate.

Progress curve simulations

One of the striking features of the reactions catalysed by the classical CD38/ecto-NAD⁺ glycohydrolases is the low amount of cADPR formed from NAD⁺ and the absence of a transient accumulation of the cyclic metabolite throughout the course of the reaction. This is not always the case. We have shown recently that in the presence of high quantities of *A. californica* ADP-

ribosyl cyclase, which is endowed with a low intrinsic cyclic ADP-ribose hydrolase activity, cADPR is only transiently formed and the final reaction product is ADP-ribose [29]. This apparent sequential reaction pathway could be analysed with the unifying Scheme 1 with $k_4 > k_3$ [29]. Similarly, the presence of CD38-like enzymes showing distinct catalytic features has also been described recently in non-haematopoietic mammalian cells [31–33]. In these cases, the catalysed conversion of NAD⁺ is accompanied by a transient formation of cADPR (up to 50 % of the reaction products; see, for example, [31]), which is ultimately turned-over in favour of ADP-ribose. Such observations are of importance because they point to the possible occurrence of different members of a CD38 family which can be distinguished by their catalytic properties with regard to the formation and eventual hydrolysis of cADPR and probably also by their (sub)cellular/tissular localization (see also, for example, [34–36]).

As a complement of the above analysis, the time course of the bovine spleen CD38/NAD⁺ glycohydrolase-catalysed conversion of NAD⁺ into ADP-ribose and cADPR was simulated under steady-state conditions using the DynaFit program [22], and Scheme 1 as the minimal kinetic mechanism. Our aim was to find the constraints on some of the kinetic constants that will yield (1) reaction progress curves we have observed experimentally; i.e., no cyclic metabolite accumulation such as in Figure 3, and (2) progress curves with a transient accumulation of cADPR. This approach should also give information on the respective kinetic competence of the two complexes CD38-cADPR and CD38-NAD⁺ (boxed in Scheme 1) in our enzyme-catalysed reaction.

In the first simulation (simulation 1, Table 2), the kinetic constants k_1 and k_{-5} were set as diffusion-controlled limits ($1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) and the other constants were calculated from experimentally determined steady-state parameters, $k_{\text{cat}} = V_{\text{max}}/[E]_0$ and K_m , or were approximated. The values for k_2 and k_{-4} , that correspond to the rate-limiting formation of the ADP-ribosyl oxocarbenium intermediate from NAD⁺ and cADPR respectively [13,26,27] (and see above), were calculated from their respective V_{max} values (Table 1) using 32 kDa as the molecular mass of the enzyme [17,23]. The constants k_{-1} and k_5 were deduced from the approximation that $K_m = K_s$ [26] (see Table 1). As an estimate, the hydrolysis step k_3 was set two orders of magnitude higher than the rate limiting step and $k_4 = 0.02 \times k_3$ to take into account the ratio of the formed reaction products cADPR versus ADP-ribose. We found that k_4 and k_3 could be set over a wider range than given in Table 2; for example, if varied as a pair, when multiplied by 0.1 or by 10 similar results were obtained. With these kinetic parameters the progress curves were obtained by simulation of Scheme 1, in which step k_2 was

Figure 4 Simulation of the progress curves of the catalysed conversion of NAD^+ and $2'\text{-NH}_2\text{-NAD}^+$ with Scheme 1

The simulations were carried out with the DynaFit program [22] and Scheme 1 using the kinetic constants of Table 2 (see the text). The initial concentrations of substrates were set at $50 \mu\text{M}$ and $[\text{E}]_0 (\text{M}) = 1.56 \times 10^{-9}$ (simulation 1), 6.24×10^{-9} (simulations 3–5) and 1.56×10^{-8} (simulations 6 and 7). (◆) NAD^+ (simulations 1, 3–5) or $2'\text{-NH}_2\text{-NAD}^+$ (simulations 6 and 7), (●) ADP-ribose or $2'\text{-NH}_2\text{-ADP-ribose}$, (▲) cADPR or $2'\text{-NH}_2\text{-cADPR}$. Sim, simulation.

considered macroscopically irreversible, in the absence of nicotinamide whose K_i is in the mM range [26]. An example of such a simulation carried out by setting $[\text{S}]_0 = 50 \mu\text{M}$ is shown in Figure 4. The progress curves obtained are in excellent agreement with the ones found experimentally [13]. It should be noted that, although the absolute values of k_1 and k_{-5} are unknown, the simulated curves were not significantly affected by values of k_{on} rates $\geq 10^6$ (results not shown). As expected, throughout the time course of NAD^+ transformation the amount of the E·cADPR complex remained very small, i.e. $< 0.03\%$ of $[\text{E}]_0$.

Simulation 1 corresponds to an example of low C_r to catalysis. In contrast, simulation 2 simulates a case of high commitment with $C_r = 10$ (see above) in which the rate constants k_5 and k_{-5} were changed accordingly (Table 2). We also observed in simulation 2 that the rate of cADPR release from the E·cADPR complex is considerably decreased. Simulations (results not shown) indicate that although the progress curves are comparable with those of simulation 1, they are characterized by a decrease in the formation of free cADPR (0.17% of the reaction products compared with 2% in simulation 1) and the E·cADPR complex is still low compared with the Michaelis complex E· NAD^+ (i.e. $\approx 1.2\%$) and with the free enzyme. With $C_r = 100$ (results not shown), although less cyclic metabolite is formed (approx. three orders of magnitude less than ADP-ribose), the relative importance of the E·cADPR complex remains modest throughout the course of the reaction ($\approx 1.3\%$ of the Michaelis complex).

Altogether, these results indicate that with CD38/ NAD^+ glycohydrolase-catalysed conversion of NAD^+ it is difficult to attain conditions where the E·cADPR complex is formed in significant proportions, even under hypothetical conditions of high commitment for catalysis that impose non-realistic constraints on k_5 and k_{-5} .

Next, in analogy to simulation 1, we simulated the progress curves for $2'\text{-NH}_2\text{-NAD}^+$ as substrate. The values for k_{-1} and k_2 were derived from the K_m and V_{max} values of the analogue (Table 1), and k_{-4} from the V_{max}/K_m ratios of the cyclic analogues, with the assumption that their differences originate from their relative V_{max} values (see above). The simulated progress curves (simulation 6, Table 2) are illustrated in Figure 4. They are in excellent agreement with the one found experimentally (Figure 3). The expected proportion of $2'\text{-NH}_2\text{-cADPR}$ was formed; i.e. approx. 4.7% of the reaction products. In contrast with the case with NAD^+ , with $C_r = 10$ (simulation 7), less free cyclic analogue was formed. In both simulations the relative proportions of the E· $2'\text{-NH}_2\text{-cADPR}$ complex remained modest ($< 0.5\%$) and no realistic kinetic conditions could be found, when using the constants derived from the experimental data, under which more free cyclic analogue is accumulated.

Finally, we wanted to investigate the constraints on the kinetic constants that could transform our enzyme into a fully fledged ADP-ribosyl cyclase and/or lead to the transient formation of large amounts of free cADPR. These simulations were carried

out based on Scheme 1, the unifying mechanism for CD38 and ADP-ribosyl cyclases [29]. Essentially two sets of parameters were changed: the relative values of k_3 and k_4 and the K_m value for cADPR. Three examples of progress curve simulations (simulations 3, 4 and 5, Table 2) are given in Figure 4. In simulation 3, when k_2 and k_3 were set to a similar range and k_4 to 100-fold higher, the conversion of NAD^+ resulted overwhelmingly in the formation of cADPR ($\approx 99\%$ of the total products at 90% reaction progress). When k_4 was set equal to k_3 (simulation 4), a concomitant formation of cADPR and ADP-ribose was observed, followed by the slow hydrolysis of the cyclic metabolite. Finally, in simulation 5 the K_m of cADPR was decreased to $5\ \mu\text{M}$ and the ratio k_4/k_3 was set to 10. Under these conditions a transient formation of cADPR was obtained that peaked at approx. 40% of the initial NAD^+ concentration followed by its hydrolysis into ADP-ribose. These simulations give some insights into the kinetic mechanism of the enzymes endowed with appreciable ADP-ribosyl cyclase activity. Simulation 3 mimics *A. californica* ADP-ribosyl cyclases whose overwhelming production of free cADPR is due to an efficient cyclization step (k_4) compared with hydrolysis and with a 2-fold higher K_m for cADPR compared with NAD^+ [29]. Simulations 4 and 5 illustrate the influence of K_m of cADPR. A transient formation of cADPR during the course of the reaction is observed when this metabolite has a low enough K_m to be able to compete with NAD^+ for the active site and be hydrolysed. Thus as the reaction progresses and NAD^+ becomes depleted, the flux of cADPR hydrolysis increases due to decreased competition from the substrate.

Conclusions

In this study we wanted to verify whether the $\text{E}\cdot\text{cADPR}$ complex (Scheme 1) is kinetically competent in the CD38/ NAD^+ glycohydrolase-catalysed conversion of NAD^+ into ADP-ribose. From the K_m value of cADPR, which for the bovine spleen enzyme is almost two orders of magnitude higher than the K_m for NAD^+ (Table 1), we first conclude that the C_r commitment to catalysis of this complex is probably quite low; i.e. during the reaction process the dissociation of $\text{E}\cdot\text{cADPR}$ into free enzyme and cADPR must not represent a rate-limiting step. Moreover, this complex involves only a minor fraction of the total enzyme. In agreement with this assumption, transformation of the slowly hydrolysable analogue $2'\text{-NH}_2\text{-NAD}^+$ into $2'\text{-NH}_2\text{-ADP-ribose}$ was not accompanied by an accumulation of $2'\text{-NH}_2\text{-cADPR}$, indicative of the formation of a slowly dissociable $\text{E}\cdot 2'\text{-NH}_2\text{-cADPR}$ complex. Finally, the progress curves of this catalytic process, which are characterized by the formation of a low and constant proportion of cADPR (i.e. $< 2\%$ of the reaction products [13]), could be successfully simulated using the minimal Scheme 1 and kinetic constants derived from experimental data and applying some constraints on the constants that were not directly available. No plausible kinetic conditions could be found under which the $\text{E}\cdot\text{cADPR}$ complex did accumulate. The main reason appears to be linked to the high K_m of cADPR. Although for bovine spleen CD38/ NAD^+ glycohydrolase this value is in the mM range (see Table 1), in CD38 of other origins, such as from pig or human, cADPR was found to possess a K_m at least one order of magnitude higher than that of NAD^+ [14,37]. Altogether, the present study indicates that during the conversion of NAD^+ into ADP-ribose, the $\text{E}\cdot\text{cADPR}$ complex represents only a minor pathway due to the poor efficiency, compared with the hydrolytic step, of the intramolecular cyclization process leading to the formation of the cyclic metabolite. This would rule out the

necessity for NAD^+ to be first converted into cADPR to engage CD38 into cross-talk with neighbouring partners that might be needed in some signalling processes (see the Introduction section). In contrast, the Michaelis complex $\text{E}\cdot\text{NAD}^+$ (boxed in Scheme 1) could fulfil such a role especially in light of our recent results in favour of a conformational change of human CD38 triggered by the binding of its active-site ligands [16].

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