Human CD38 is an authentic NAD(P)$^+$ glycohydrolase

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The leucoyte surface antigen CD38 has been shown to be an ecto-enzyme with multiple catalytic activities. It is principally a NAD$^+$ glycohydrolase that transforms NAD$^+$ into ADP-ribose and nicotinamide. CD38 is also able to produce small amounts of cyclic ADP-ribose (ADP-ribosyl cyclase activity) and to hydrolyse this cyclic metabolite into ADP-ribose (cyclic ADP-ribose hydrolase activity). To classify CD38 among the enzymes that transfer the ADP-ribosyl moiety of NAD$^+$ to a variety of acceptors, we have investigated its substrate specificity and some characteristics of its kinetic and molecular mechanisms. We find that CD38-catalysed cleavage of the nicotinamide-ribose bond results in the formation of an E·ADP-ribosyl intermediary complex, which is common to all reaction pathways; this intermediate reacts (1) with acceptors such as water (hydrolysis), methanol (methanolysis) or pyridine (transglycosidation), and (2) intramolecularly, yielding cyclic ADP-ribose with a low efficiency. This reaction scheme is also followed when using nicotinamide guanine dinucleotide as an alternative substrate; in this case, however, the cyclization process is highly favoured. The results obtained here are not compatible with the prevailing model for the mode of action of CD38, according to which this enzyme produces first cyclic ADP-ribose which is then immediately hydrolysed into ADP-ribose (i.e. sequential ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities). We show instead that the cyclic metabolite was a reaction product of CD38 rather than an obligatory reaction intermediate during the glycohydrolase activity. Altogether our results lead to the conclusion that CD38 is an authentic 'classical' NAD(P)$^+$ glycohydrolase (EC 3.2.2.6).

INTRODUCTION

The human cell-surface antigen CD38 is a 46 kDa type II glycoprotein with a single transmembrane domain [1]. Its expression is widely used as a phenotypic marker of differentiation or activation of human T and B lymphocytes [2–4]. The high sequence similarity between ADP-ribosyl cyclase from *Aplysia californica* and CD38 [5] led to the finding that this antigen, for which no biological activity is yet known, is an ectoenzyme that catalyses the cleavage of the nicotinamide-ribose bond in NAD$^+$ (reviewed in [2–4]). In contrast with the invertebrate enzyme, which transforms NAD$^+$ exclusively into cyclic ADP-ribose (cADPR), human and murine CD38 were reported to be endowed with at least three different catalytic activities: NAD$^+$ glycohydrolase (NADase) and ADP-ribosyl cyclase activities that convert NAD$^+$ into nicotinamide and respectively ADP-ribose and cADPR, and a cADPR hydrolase activity producing ADP-ribose from cADPR [6–10]. CD38 is prevalently an NADase and only very little cADPR is formed during the transformation of NAD$^+$, i.e. less than 1 % of reaction products [6–9,11]. The low yield of the cyclic metabolite, which is thought to be an endogenous regulator of the Ca^{2+}-induced Ca^{2+}-release process mediated by the ryanodine receptor [12–14], was generally attributed to the 'multifunctionality' of CD38 [3,6,10,15,16]. Thus it was proposed that the measurable NADase activity of CD38 resulted from the addition of its ADP-ribosyl cyclase and cADPR hydrolase activities, with cADPR as a reaction intermediate that is quickly turned over [6,10,15,16]. This assumption strongly questioned the real catalytic activity of the 'classical' NADases (EC 3.2.2.5 and 3.2.2.6) which, in mammals, constitute a heterogeneous family of enzymes (in terms of molecular mass and catalytic properties) [17]. The question arose whether the NADases were also multifunctional enzymes whose ADP-ribosyl cyclase and cADPR hydrolase activities had been overlooked. Indeed, Kim et al. [18] found a canine spleen NADase that possessed also cADPR-producing and -degrading activities. Recent studies by Schuber's group established that the well-known calf spleen ecto-NADase was also able to catalyse the transformation of NAD$^+$ into small amounts of cADPR and to hydrolyse cADPR; however, their studies on the cyclization mechanism, which excluded cADPR as a kinetically competent reaction intermediate in the transformation of NAD$^+$ into ADP-ribose [19], did not support the mechanism that was suggested for CD38 [20]. The present situation is therefore rather confusing and one can therefore wonder whether CD38 and the classical ecto-NADases are the same enzymes or whether they constitute subclasses of a superfamily of enzymes that might differ in some aspects of their molecular mechanism. Related to this question is the observation that mammalian NADases have a much wider tissue and cellular distribution [17] than originally thought for CD38, although this latter point is rapidly evolving [3,9,21–24]. In the absence of structural data for NADases, it was therefore of importance to characterize more accurately the catalytic properties of CD38 in accordance with criteria used to classify the different NADases [17]. From our studies it seems likely that human CD38 is an

Abbreviations used: ϵNAD$^+$, nicotinamide 1,N^6-adenine dinucleotide; cADPR, cyclic ADP-ribose (ADP-cyclo[N^1, C-1″]-ribose); NGD$^+$, nicotinamide guanine dinucleotide; cGDPR, cyclic GDP-ribose (GDP-cyclo[N^7,C-1″]-ribose); INH, isonicotinic acid hydrazide; hy^{4}PyAD$^+$, 4-hydrazinocarbonylpyridine adenine dinucleotide; araF-NAD$^+$, nicotinamide 2′-deoxy-2′-fluoroarabinoside adenine dinucleotide; NADase, NAD$^+$ glycohydrolase.

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authentic ecto-NAD(P)$^+$ glycohydrolase, belonging to the EC 3.2.2.6 class of nucleosidases that hydrolytically cleave both NAD^+ and $NADP^+$.

EXPERIMENTAL

Chemicals

All chemicals used were from Sigma (Saint Quentin Fallavier, France).

Source of CD38

Intact human B Daudi cells were used as a source of CD38. This cell line expresses CD38 highly and lacks other ectoenzymes, such as nucleotide pyrophosphatase, that also metabolize NAD^+. Most studies reported here were performed on CD38 purified by immunoprecipitation (see below), preliminary experiments having shown that CD38 ligation with antibodies did not alter its enzymic activity. To check the purity of this preparation, an immunoprecipitate prepared from biotinylated Daudi cells was subjected to SDS/PAGE, blotted on nitrocellulose and labelled with avidin: one major band at 45 kDa was obtained, in agreement with similar preparations reported previously [25]. A second source of CD38 was a recombinant fusion protein between the extracellular portion of human CD38 and mouse CD8α [26] kindly provided by Dr. Banchereau and Dr Brière (Schering Plough, Dardilly, France). This preparation consists of a supernatant of transfected COS cells and was estimated as 95% pure by SDS/PAGE stained with Coomassie Blue. The specific activities, at pH 7.4 and 37 °C, were: 5 m-unit per 10^6 Daudi cells, 0.3 m-unit for an immunoprecipitate prepared from 10^6 Daudi cells and 10 m-unit/mg of protein for the recombinant CD38. One unit of CD38 NADase activity is defined as the amount able to hydrolyse 1 μmol of NAD^+/min.

Cell culture and CD38 immunopurification

Daudi cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 i.u./ml penicillin and 50 μg/ml streptomycin, at a density of $(0.5–1) \times 10^6$ cells/ml. For CD38 immunopreparation, 10^8 cells were washed twice in PBS and incubated for 30 min at 4 °C in 1 ml of lysis buffer [20 mM Tris (pH 7.5)/1 mM EDTA/140 mM NaCl/1% (v/v) Nonidet P40 0.5% aprotinin/1 mM PMSF]. Cell debris was removed by centrifugation at 13000 ***g*** for 15 min and the supernatant was precleared by incubation for 40 min at 4 °C with 10 mg of Protein A immobilized on Q Sepharose CL-4B. The supernatant was then incubated with 1% (v/v) CD38-specific monoclonal antibodies BB51 (ascites fluid kindly provided by Dr. L. Boumsell) [27] at 4 °C for 2 h. Protein A–Sepharose (10 mg) was then added to the reaction mixture, followed by further incubation at 4 °C for 1 h. Immune complexes were washed three times with lysis buffer and once with buffer A (see below), before being used in enzymic assays.

Enzymic assays and HPLC analysis of reactions products

Reactions were performed in buffer A [50 mM Hepes (pH 7.4)/150 mM NaCl/1 mM $CaCl_2$/0.5 mM $MgCl_2$/5 mM KCl/1 mM Na_2HPO_4] at 37 °C. At given times 50 μl aliquots were taken and centrifuged briefly (5000 ***g*** for 10 s). For reactions with recombinant CD38 protein, aliquots were frozen in liquid N_2. Samples were then diluted by addition of 500 μl of the starting HPLC buffer (see below) and filtered on 0.2 μm (pore size) cellulose filters before injection. For methanolysis and transglycosidation experiments, chromatography was performed on a 300 mm × 3.9 mm μBondapak C_{18} column (Waters), at a flow rate of 1 ml/min, with a Beckman system equipped with a Gold-166 spectrophotometric detector set at 260 nm. Compounds were eluted isocratically with 10 mM ammonium phosphate buffer, pH 5.6, containing a given percentage of acetonitrile [i.e. 1.5% (v/v) for the elution of products obtained by hydrolysis and methanolysis of NAD^+, nicotinamide 1,N^6-adenine dinucleotide (ϵ-NAD^+), cADPR and nicotinamide guanine dinucleotide (NGD^+); 2% (v/v) for the elution of the products obtained by transglycosidation with isonicotinic acid hydrazide (INH); and 2.5% (v/v) for the elution of the products obtained by transglycosidation with 3-aminopyridine and 3-acetylpyridine].

To assay the hydrolytic capacity of the CD38 preparations, at least four aliquots of the reaction medium were sequentially taken, analysed by HPLC and the slopes of the substrate disappearance or product formation progression curves were calculated (means ± S.D.). Reaction products were identified by coelution with authentic samples. The mixture of α- and β-methyl ADP-ribose was prepared by chemical solvolysis of NAD^+; β-methyl ADP-ribose was obtained by the treatment of NAD^+ with purified calf spleen NADase in the presence of methanol, as reported previously [28]. Kinetic parameters (K_m, V) were obtained by analysis, with a non-linear regression program, of the initial rates plotted against substrate concentrations, with using at least five data points.

Fluorometric assay of NADase and GDP-ribose cyclase activities

NADase activity was also assayed fluorometrically with ϵ-NAD^+ as substrate as previously described [29]. Briefly, ϵ-NAD^+ was added to the assay medium (buffer A, 2 ml final volume) containing CD38 (cells, immunoprecipitate or recombinant) at 37 °C in a thermostatically controlled fluorimeter (Perkin-Elmer, Bois d'Arcy, France) cuvette. The fluorescence emission at 410 nm (excitation at 300 nm) was then followed. To calculate the initial rates, the maximal fluorescence was obtained by the addition of 0.1 unit of *Crotalus atrox* venom pyrophosphatase (1 unit is the amount able to hydrolyse 1 μmol of NAD^+/min). The GDP-ribosyl cyclase activity of CD38 was assayed similarly, by estimating the appearance of the highly fluorescent cyclic GDP-ribose (emission at 410 nm, excitation at 300 nm) [30,31].

Slow-binding inhibition of CD38 by nicotinamide 2′-deoxy-2′-fluoroarabinoside adenine dinucleotide (araF-NAD^+)

Hydrolysis of ϵ-NAD^+ was followed by the continuous fluorometric assay, as described above, in the presence of various concentrations of araF-NAD^+. About 15–25 points obtained from the progress curve were taken at times corresponding to equal increments of fluorescence (F) and were analysed, as described previously [32], by fitting to the equation:

$$F = v_s t + (v_0 - v_s)(1 - \exp(-kt))/k + F_0 \quad (1)$$

Use of a non-linear regression program yielded the different parameters, i.e. v_0 initial rate; v_s, steady-state rate; k, the apparent first-order constant for reaching the steady-state enzyme–inhibitor complex; and F_0, the initial fluorescence. The kinetic parameters k_{off}, k_{on} and $K_i (= k_{off}/k_{on})$ were calculated from the plot of k against inhibitor concentrations according to the equation:

$$k = k_{off} + k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

Protein assay

Protein concentration was calculated with the Bio-Rad Bradford protein assay (Ivry sur Seine, France), with BSA as standard.

RESULTS

For the present studies we used immunopurified CD38, originating from Daudi cells, and the recombinant form expressed in mammalian cells. Both human CD38 sources had similar catalytic properties, which were compared with features that are characteristic of 'classical' NADases [16].

Substrate specificity

The kinetic parameters of NAD^+ hydrolysis catalysed by CD38 were first determined, followed by the exploration of the substrate specificity of the enzyme by using β-NAD^+ and analogues (Table 1). The K_m found with native CD38, i.e. 46 μM, is somewhat higher than that (15 μM) obtained with a non-glycosylated recombinant form of CD38 [33]. The enzyme also hydrolytically cleaved $NADP^+$ and ϵ-NAD^+, a fluorescent analogue of NAD^+, with relative V_{max} similar to those found previously [11]; the K_m for ϵ-NAD^+ was, however, somewhat lower than that for β-NAD^+. In contrast, 3-acetylpyridine adenine dinucleotide, with a K_m in the upper μM range, was a poorer substrate for CD38. Nevertheless under saturating conditions this pyridinium analogue of NAD^+ was hydrolysed almost as fast as NAD^+. These results are reminiscent of the mammalian NADases (ED 3.2.2.6), which, in their vast majority, also hydrolyse $NADP^+$ and pyridinium analogues of NAD^+ [17,34,35], as opposed to most other NAD^+-metabolizing enzymes, such as ADP-ribosyltransferases, which recognize only NAD^+ as substrate.

Alkaline inactivation

A striking feature that discriminates mammalian NADases of different origins is the occurrence of paracatalytic inactivation under alkaline conditions [36,37]. Thus, certain NADases, e.g. from mouse, rat and rabbit, when incubated at pH 8 or higher, undergo irreversible inactivation in the presence of NAD^+, whereas at more acidic pH values the glycohydrolase reaction goes to completion [37]. As shown in Figure 1(A), human CD38 undergoes such self-inactivation at alkaline pH. Although the hydrolysis of NAD^+ is practically linear with time at pH 6.5 and 7.5, it decreases progressively at pH 8.5. This was not due to product inhibition of the reaction, because the addition of fresh enzyme restored the reaction with a similar initial velocity (Figure 1A, arrow). Moreover, preincubation of the enzyme at pH 8.5 for 30 min in the absence of NAD^+ had no effect on its subsequent activity (results not shown). Therefore the inactivation of CD38 is due to both NAD^+ turnover and alkaline pH and thus, according to the Green and Dobrjansky classification [37], this enzyme belongs to the category of the 'self-inactivating' NADases. Recently, Han et al. [36] gave some convincing evidence in an erythrocyte NADase, i.e. an NADase that operates via a distinct mechanism [17], that this phenomenon might be due to auto-ADP-ribosylation. CD38 was indeed found to be ADP-ribosylated [38], but this modification could not be corre-

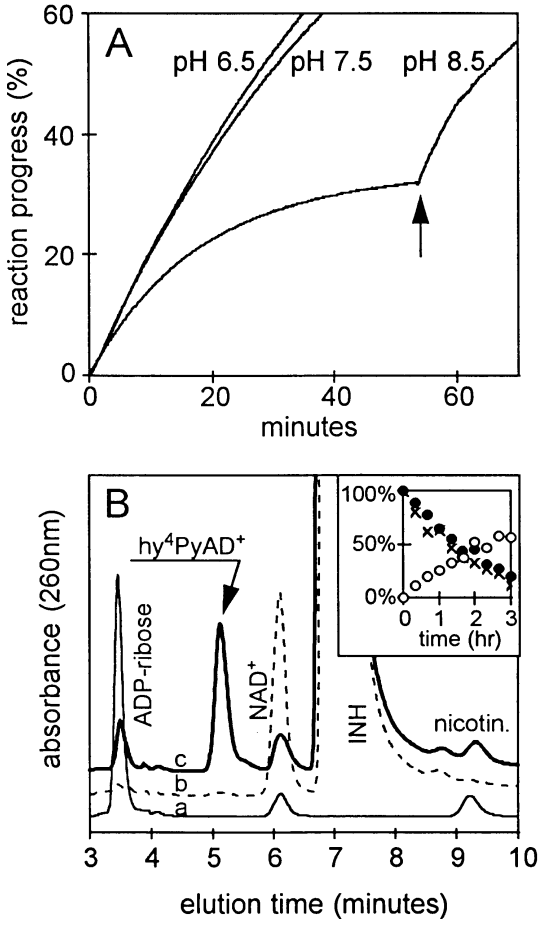


Figure 1 Self-inactivation and INH sensitivity of CD38

(**A**) The NADase activity of immunopurified CD38 was assayed fluorometrically at 37 °C with 100 μM ϵ-NAD^+ in buffer A (see the text) adjusted to pH 6.5, 7.5 or 8.5, as indicated. In buffer A, Hepes was replaced by 50 mM Pipes (pH 6.5) or 50 mM Tris (pH 7.5 and 8.5). At pH 8.5 an identical quantity of enzyme was added at 55 min (arrow). (**B**) Immunopurified CD38 was incubated at 37 °C in buffer A with 200 μM NAD^+ in the absence (trace a) or in the presence (traces b and c) of 25 mM INH. HPLC analyses were performed on aliquots taken at 0 h (trace b) or 3 h (traces a and c). The transglycosidation reaction product (elution time 5.2 min) was eluted with authentic hy^{4}PyAD$^+$ [35]. Inset: progress curves of percentage NAD^+ disappearance (●) and hy^{4}PyAD formation (○). For comparison the progress curve is given for the disappearance of NAD^+ in the absence of INH (×).

Table 1 Kinetic characteristics of CD38 towards NAD^+ and analogues

The maximal rates are given as a percentage of the V_{max} observed for NAD^+ (100% = 0.3 nmol/min with an immunoprecipitate obtained from 10^6 Daudi cells). Abbreviations: ac^{3}PyAD$^+$, 3-acetylpyridine adenine dinucleotide; n.d., not detected.

Substrates ...	β-NAD^+	$NADP^+$	ϵ-NAD^+	ac^{3}PyAD$^+$	α-NAD^+	cADPR	NGD^+
K_m (μM)	46 ± 4 (8)	65 ± 19 (7)	7 ± 1 (6)	851 ± 37 (4)	–	224 ± 9 (4)	1.6 ± 0.4 (5)
Relative rate (%)	100	70	29	95	n.d.	16	39

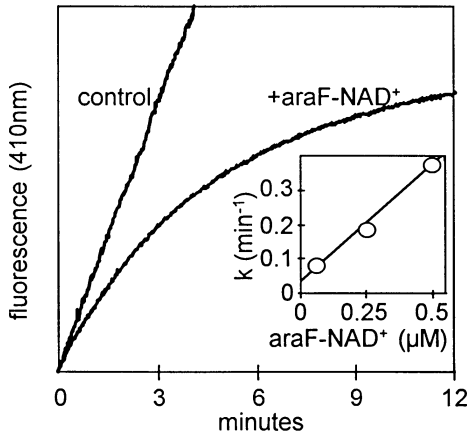


Figure 2 Slow-binding inhibition of CD38 by araF-NAD$^+$

Assays were run fluorometrically at 37 °C in buffer A containing 20 μM ϵ-NAD$^+$ and 0–0.5 μM araF-NAD$^+$. The representative progress curves show the increase in fluorescence at 410 nm ($F - F_0$) observed after the addition of CD38 to an assay run in the absence (control) or presence of 0.25 μM inhibitor. The analysis of such curves, by fitting them to eqn. (1) (see the Experimental section), allowed the calculation of k. Inset, plot of k against inhibitor concentration yielding k_{off} (intercept) and k_{on}, which can be calculated from the slope [see eqn. (2)].

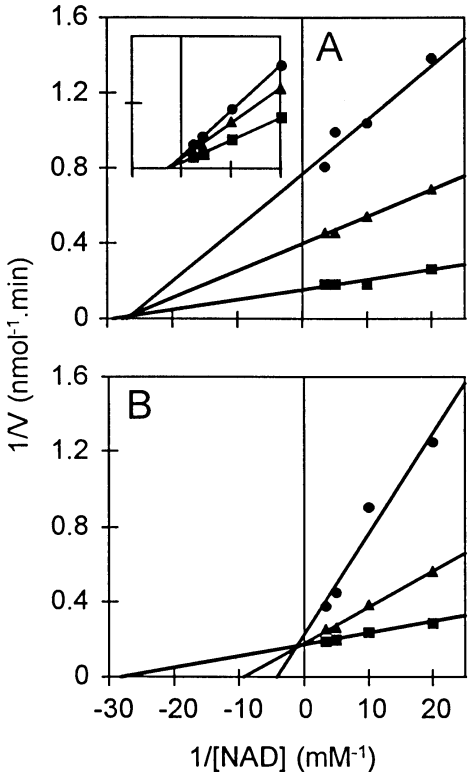


Figure 3 Inhibition of CD38 NADase activity by reaction products

(**A**) Assays were run with CD38 from Daudi cells, at 37 °C, in buffer A containing various concentrations of NAD$^+$, in the presence of 0 (■), 1 (▲) or 3 (●) mM nicotinamide. Double-reciprocal plots of the NADase activity assayed by HPLC are shown. Similar results were obtained with recombinant CD38 assayed fluorimetrically. Insert, cADPR hydrolase activity was assayed under the same conditions. Symbols: ■, control; ▲, 1 mM nicotinamide; ●, 2 mM nicotinamide. Ordinate unit, 10 nmol^{-1} · min; abscissa unit, 10 mM^{-1}. (**B**) Assays were run with CD38 from Daudi cells, at 37 °C, in buffer A containing various concentrations of NAD$^+$, in the presence of 0 (■), 5 (▲) or 10 (●) mM ADP-ribose. Double-reciprocal plots of the NADase activity assayed by HPLC are shown. Similar results were obtained with recombinant CD38 assayed fluorimetrically.

lated with its alkaline inactivation (V. Berthelier and P. Deterre, unpublished work).

Slow-binding inhibition of CD38 by araF-NAD$^+$

One of the most potent inhibitors known for mammalian NADases is araF-NAD$^+$, an arabino analogue of NAD$^+$, which behaves as a reversible slow-binding inhibitor [32]. This molecule showed similar inhibitory characteristics when tested on CD38. As shown in Figure 2, when the assay was started with the enzyme, the presence of araF-NAD$^+$ caused a progressive increase in inhibition until a steady state was reached asymptotically. The transient pre-steady state was of the order of minutes. Analysis of the kinetic data (Figure 2, inset), gave the following results: $k_{on} = (10.20 \pm 0.01) \times 10^4$ M$^{-1} \cdot$ s^{-1} (association rate), $k_{off} = (1.72 \pm 0.05) \times 10^{-4}$ s^{-1} (dissociation rate), $t_{\frac{1}{2}} = 67 \pm 2$ min (half-life of the E.I complex) and $K_i = 1.69 \pm 0.05$ nM ($n = 3$). These results are in the range of those found for the inhibition of bovine NADase by araF-NAD$^+$ [32]. A similar kinetic mechanism can be proposed for CD38 that favours a slow interconversion between E and E·I, the enzyme–inhibitor complex, as opposed to a mechanism involving a slow transconformation of E·I into a tighter final complex, E·I* [32].

The arabino analogues of NAD$^+$ are the only documented inhibitors of NADases that give this unusual inhibition pattern; the similarity of inhibition found for CD38 is therefore very striking and highly significant in terms of likeness of reaction mechanism and active-site topologies between the two classes of enzyme. Moreover, araF-NAD$^+$ is, to our knowledge, the most powerful inhibitor so far described for CD38 and should be an interesting molecular tool to modulate its cellular activity.

Transglycosidation reactions catalysed by CD38 and kinetic mechanism

One of the best characterized features of mammalian NADases is the ability of most of these enzymes to catalyse a pyridine-base exchange reaction [17]. Pyridinium NAD$^+$ analogues are produced with retention of configuration and some can be used as coenzymes for dehydrogenases [39]. We observed that CD38 was capable of catalysing such transglycosidation reactions in the presence of 3-acetylpyridine, 3-aminopyridine (results not shown) and INH (Figure 1B). This last pyridine allows a classification of NADases [40]. Some are inhibited by INH without leading to the formation of the analogue and have been called 'INH-sensitive' [40]; in contrast, the 'INH-insensitive' NADases catalyse the formation of 4-hydrazinocarbonylpyridine adenine dinucleotide (hy^{4}PdAD$^+$). The fact that the rate of NAD$^+$ disappearance remains unaffected in the presence of INH (Figure 1B, inset) and that the analogue is formed, indicates that CD38 can be categorized as an 'INH-insensitive' NADase, as is the human spleen NADase [40]. The ability of human CD38 to catalyse transglycosidation with these different pyridines indicates that this reaction might be relevant to the physiological role of CD38. Our observation extends the work by Aarhus et al. [41] showing that, under acidic pH, the transglycosidation of NADP$^+$ in the presence of nicotinic acid yields nicotinic acid adenine dinucleotide phosphate (NAADP$^+$), a metabolite able to release intracellular Ca^{2+} [42,43]. The occurrence of transglycosidation reactions suggests a mechanism of action for CD38 that implies the formation of an E·ADP-ribosyl reaction intermediary com-

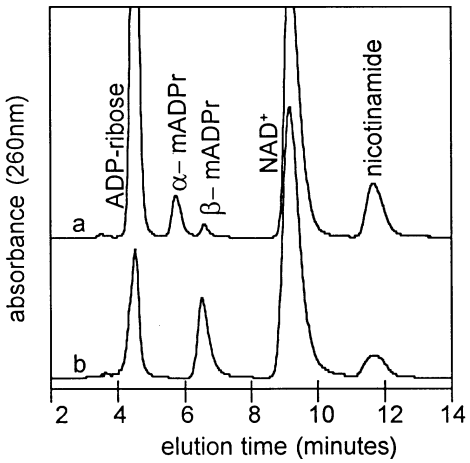


Figure 4 NAD$^+$ methanolysis catalysed by CD38

Trace a, HPLC profile of the products of the non-enzymic solvolysis of NAD$^+$ [500 μM NAD$^+$ in buffer A, incubated at 90 °C and pH 5.0 for 90 min in the presence of 20% (v/v) methanol]. The peaks were identified by co-elution with authentic α- and β-methyl ADP-ribose [28]. Trace b, HPLC profile of the products of NAD$^+$ solvolysis catalysed by a CD38 immunoprecipitate in the presence of 10% (v/v) methanol.

plex, resulting from the nicotinamide-ribose bond cleavage, that can partition between different acceptors such as water and pyridines. In agreement with this mechanism is the observation that nicotinamide is a non-competitive inhibitor of CD38 with a K_i of 0.92 ± 0.16 mM ($n = 4$) and that ADP-ribose is a competitive inhibitor with a K_i of 4.2 ± 0.4 mM ($n = 4$) (Figure 3). Together these results are similar to those found with classical NADases, such as bovine spleen NADase [44]. As already suggested [11], they are consistent with a minimal Ping Pong Bi Bi kinetic mechanism for the reactions catalysed by CD38, in which nicotinamide is the first reaction product released, and which reduces to an ordered Uni Bi mechanism for hydrolysis alone.

NAD$^+$ methanolysis reaction catalysed by CD38

An alternative acceptor for the ADP-ribosyl intermediate occurring along the reaction pathway of NADases is methanol [17,19,28,45], and it has been shown that this alcohol reacts in competition with water leading to the formation of β-methyl ADP-ribose [45]. As illustrated in Figure 4, CD38 was similarly able to catalyse the methanolysis of NAD$^+$ and, by analogy with the NADases studied so far, the reaction, which yielded the β-form of methyl ADP-ribose, also occurred with an exclusive retention of configuration.

We can estimate the relative efficacy of water and methanol in the nucleophilic attack of the ADP-ribosyl intermediate by calculating the partitioning ratio, K [28]:

$$K = ([\text{methyl ADP-ribose}]/[\text{ADP-ribose}]) \times ([H_2O]/[CH_3OH])$$

In the presence of 2.5 M methanol, i.e. 10% (v/v), this ratio was 12 ± 1.7 ($n = 10$). On a molar basis, methanolysis of NAD$^+$ catalysed by CD38 was therefore more than 10-fold faster than hydrolysis: CD38 showed a clear selectivity for the solvent. Importantly, this ratio was found to be constant throughout the time course of the reaction and the extent of methanolysis was strictly proportional to the concentration of methanol (results not shown); this indicates that our experimental conditions were not saturating and that the selectivity of methanol versus water probably reflects more its intrinsic reactivity with the ADP-ribosyl intermediate than a higher affinity for the active site of the enzyme.

Table 2 Reaction products obtained by CD38-catalysed transformation of NGD$^+$ in the presence of methanol

A is defined in eqn. (3). Results are means $\pm$ S.D. for the number of replicates in parentheses.

	Products (% of total)			
[MeOH] (M)	GDP-ribose	Methyl GDP-ribose	cGDPR	A
0	13 ± 1 (9)	–	87 ± 1 (9)	6.7 ± 0.7 (9)
1.25	12 ± 2 (9)	15 ± 3 (9)	73 ± 3 (9)	2.7 ± 0.4 (9)
2.5	11 ± 2 (9)	27 ± 5 (9)	62 ± 4 (9)	1.7 ± 0.5 (9)

Mechanistic study of the ADP(GDP)-ribosyl cyclase reactions

Because of the small quantities of cADPR formed during NAD$^+$ hydrolysis catalysed by CD38 (see the Introduction section), it was difficult to analyse the ADP-ribosyl cyclase activity of the enzyme. In contrast, NGD$^+$, which was shown to be a convenient surrogate substrate to study the formation of cyclized compounds [31,33], was converted in high yields (more than 80% of reaction products, Table 2) into cyclic GDP-ribose (cGDPR). The kinetic parameters (K_m and V_{max}) measured (Table 1) were very similar to those published previously [31,33].

To gain a better understanding of the molecular mechanism of this cyclization process, we adopted a strategy that has proved highly informative with bovine NADase: methanolysis [20]. By analogy with NAD$^+$ (see above), CD38 was also able to catalyse the methanolysis of NGD$^+$; importantly, in the presence of increasing concentrations of methanol, there was an increased formation of methyl GDP-ribose, together with a net decrease in cGDPR formation (Table 2). We excluded the possibility that methyl GDP-ribose could arise from the CD38-catalysed solvolysis of cGDPR itself. As expected from its very low rate of hydrolysis [33], we found that, under the same assay conditions, the methanolysis of cGDPR remained negligible, forming less than 3% of the whole NGD$^+$-metabolizing activity (results not shown). The partitioning ratio found for the methanolysis of NGD$^+$ (48 ± 7; $n = 9$) was higher than that found for NAD$^+$ (see above) but was similar to that of bovine NADase [20]. The competition between cyclase and hydrolysis/methanolysis activities was quantified by the ratio, A of the different reaction products:

$$A = [\text{cGDPR}]/([\text{GDP-ribose}] + [\text{methyl GDP-ribose}]) \quad (3)$$

As shown in Table 2, this ratio decreased with increasing concentrations of methanol; i.e. methyl GDP-ribose was produced by CD38 at the expense of cGDPR. This result indicates clearly that the solvolysis and cyclization reactions are in competition for a common intermediate and that cGDPR cannot be a reaction intermediate in the transformation of NGD$^+$ into GDP-ribose.

These results indicate that the molecular reaction mechanism recently proposed for bovine spleen NADase [20] also applies to CD38: i.e. a common ADP (GDP)-ribosyl intermediate formed after the substrate's nicotinamide-ribosyl bond cleavage gives rise to the different reaction products, ADP(GDP)-ribose, methyl-ADP(GDP)-ribose, cADPR (cGDPR) and pyridinium analogues. The difference in yield in the formation of cADPR and cGDPR can be attributed to the intrinsic reactivity (nucleo-

philicity and positioning) of the purine N-positions (N-1 and N-7 respectively) involved in the cyclization reactions within the E·ADP(GDP)-ribosyl complexes [20].

Study of the cADPR hydrolase activity

Confirming earlier results [6,9,10], CD38 is also a cADPR hydrolase. However, cADPR is a relatively poor substrate of CD38 compared to NAD^+ (Table 1). This is also consistent with the conclusion reached above. Indeed, as expected of a mechanism that assumes that cADPR is an obligatory reaction intermediate in the conversion of NAD^+ into ADP-ribose, NAD^+ and cADPR should act as competing substrates for the enzyme. The present finding, which indicates that the specificity ratio V/K_m is in favour of NAD^+ by a factor of 30 (estimated from the results in Table 1), predicts that the cyclic metabolite should accumulate during the major part of the reaction course. The fact that cADPR remains a minor reaction product throughout the conversion of NAD^+ into ADP-ribose [6,8–10] rules out the possibility that the NADase activity of CD38 results from a sequential ADP-ribosyl cyclase/cADPR hydrolase activity. A similar conclusion was previously reached with bovine spleen NADase [19,20].

As with NAD^+, the incubation of cADPR with CD38 in the presence of methanol also yielded β-methyl ADP-ribose (results not shown). Moreover, a partitioning ratio of 14 ± 1.4 ($n = 3$) was found, which is very close to that obtained with NAD^+. These results therefore suggest that the hydrolytic transformation of NAD^+ and cADPR give rise to a common enzyme-stabilized ADP-ribosyl reaction intermediate that can be trapped by acceptors such as water, methanol or pyridines. This conclusion is validated by the observation that nicotinamide is, as observed with NAD^+ (see above), a non-competitive inhibitor of the hydrolysis of cADPR ($K_i = 3 \pm 1$ mM; $n = 4$) (Figure 3A, inset) and by the known ability of CD38 to catalyse the formation of β-NAD^+ in the presence of cADPR and nicotinamide [11]. This latter reaction is equivalent to the transglycosidation of NAD^+, where free nicotinamide can also react with the reaction intermediate to regenerate NAD^+.

DISCUSSION

This work demonstrates that CD38 presents many of the catalytic properties that characterize the well-known 'classical' NADases: transglycosidation, hydrolysis and methanolysis of NAD^+ with retention of configuration, slow-binding inhibition by araF-NAD^+, ADP(GDP)-ribosyl cyclase and cADPR hydrolase activities (summarized in Table 3). These features contrast strikingly with the reactions catalysed by other NAD^+-metabolizing enzymes such as mono-ADP-ribosyl transferases and poly(ADP-ribose) polymerase, which are unable to catalyse the methanolysis of NAD^+ and which transfer the ADP-ribosyl moiety to their acceptors with inversion of configuration [46]. Although some differences exist between the bovine spleen enzyme, which is the best characterized mammalian NADase and the human CD38 molecule (for example the magnitude of the methanol-partitioning ratio and the kinetic parameters of ϵ-NAD^+), they fall within the normal range of differences found in this heterogeneous family of enzymes. We therefore propose that CD38 belongs to the EC 3.2.2.6 class of enzymes, the $NAD(P)^+$ nucleosidases, which, in contrast with the EC 3.2.2.5 nucleosidases, hydrolytically cleave both NAD^+ and $NADP^+$. To alleviate the confusion that is pervasive in the nomenclature of the $NAD(P)^+$glycohydrolase, it should be emphasized that CD38 belongs to a family of enzymes very distinct from another class of NADases, mostly of microbial origin (e.g. *Neurospora crassa*), which hydrolyse only NAD^+ and do not catalyse transglycosidation reactions [17].

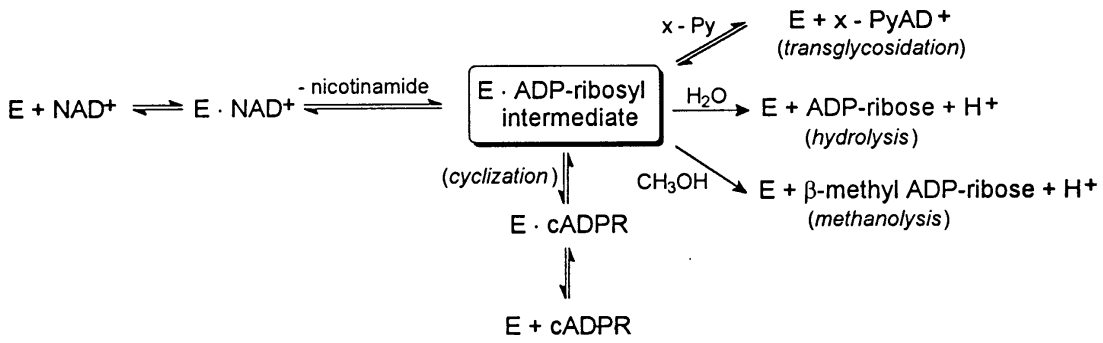
Table 3 Selected CD38 enzymic properties compared with those of classical mammalian NADases

	Human CD38	NADases
Hydrolysis of $NADP^+$?	Yes	Yes [17]
Hydrolysis of ϵ-NAD^+?	Yes	Yes [17]
Transglycosidation with 3-aminopyridine and 3-acetyl pyridine?	Yes	Yes [17,39]
Sensitivity to INH	'INH-insensitive'	'INH-insensitive' (e.g. human) and 'INH-sensitive' [40]
Self-inactivation at alkaline pH (in the presence of NAD^+)?	Yes	Yes/no [17]
Inactivation by thiol-reducing agents?	Yes [48,49]	Yes/no [17]
Inhibition by nicotinamide	Non-competitive	Non-competitive [17]
Inhibition by ADP-ribose	Competitive	Competitive [17,44]
Kinetic mechanism	Ping Pong Bi Bi	Ping Pong Bi Bi [44]
Inhibition by araF-NAD^+	Slow-binding	Slow-binding [32]
Methanolysis of NAD^+?	Yes (retention of configuration)	Yes (retention of configuration) [17,45]
Formation of cADPR?	Yes [6,8–10]	Yes [18,20]
Hydrolysis of cADPR?	Yes [6,8–10]	Yes [18,19]

In the classification of mammalian NADases, human CD38/NADase belongs to the 'INH-insensitive' and 'self-inactivating' category. This probable identity between CD38 and classical NADase is strengthened by the recent finding (D. Cockayne, personal communication) that in $CD38^{-/-}$ knockout mice the tissues that are traditionally considered to be the richest sources of NADase, such as spleen, brain and liver [17], are totally devoid of this enzyme activity.

The similarity between CD38 and bovine spleen NADase allows one to draw some important conclusions about the molecular mechanism of CD38 action. This enzyme, after the cleavage of the nicotinamide-ribose bond of NAD^+, generates an E·ADP-ribosyl intermediate that can then be partitioned between different acceptors, i.e. water (hydrolysis), methanol (methanolysis) and pyridines (transglycosidation). The chemical nature of this intermediate, which in bovine spleen NADase is most probably an oxocarbenium ion [28], remains to be established for CD38. In this respect, the lower apparent selectivity for methanol versus water found in the solvolysis of this intermediary complex could indicate, in the reactions catalysed by CD38, a lesser oxocarbenium ion-like character [28]. However, so far, bovine spleen NADase remains the only mammalian NADase for which such an analysis is available; further work is also needed to assess this feature in CD38 of other species.

One of the most important issues that has been solved in this study pertains to the multifunctionality of CD38. It has often been indicated that the NADase activity of CD38 could in fact result from a sequential combination of ADP-ribosyl cyclase and cADPR hydrolase activities [6,8,10,16,33], and hence that cADPR is the reaction intermediate in the hydrolytic conversion of NAD^+ into ADP-ribose. We have clearly shown that this is not true and that, in this respect, human CD38 functions very similarly to bovine NADase: cADPR is a reaction product whose poor yield is not due to its fast hydrolysis but to the low reactivity, within the active site of CD38, of the N-1 position of the adenine moiety ring of the ADP-ribosyl intermediate (see also [20]). The final reaction scheme catalysed by CD38 is summarized in Scheme 1.



Scheme 1 Reaction mechanism of CD38

Human CD38 catalyses the cleavage of the nicotinamide-ribose bond of NAD$^+$(NGD$^+$) leading to the formation of a E·ADP(GDP)-ribosyl intermediary complex. This intermediate can then partition depending on competing reactions: (1) intermolecular reactions with acceptors such as water (hydrolysis), methanol (methanolysis) or pyridines (transglycosidation), and (2) an intramolecular reaction between position N-1 of adenine (or N-7 of guanine when NGD$^+$ is used as substrate) and C-1′ of the ribosyl moiety, yielding the cyclized products cADP-ribose (cGDP-ribose).

Finally, the finding that CD38 is most probably a classical NADase has an important bearing on the investigation of its physiological function, including recycling extracellular nucleotides [47] or signalling functions [2–4]. It must take into account the important and singular structural and catalytic heterogeneity and the diversified tissue distribution that characterize this class of enzymes.

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