Structural role of disulfide bridges in the cyclic ADP-ribose related bifunctional ectoenzyme CD38

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Abstract **Human CD38, a type II cell surface glycoprotein, is a bifunctional ectoenzyme catalyzing both ADP-ribosyl cyclase and cyclic ADP-ribose (cADPR) hydrolase reactions. It shares a high degree of sequence homology with the cyclase from *Aplysia* species and studies of site-directed mutagenesis have recently demonstrated the importance, but not elucidated the role, of several cysteine residues highly conserved between these proteins. *N*-Ethylmaleimide, iodoacetamide and thiol-oxidizing reagents failed to affect either the cyclase or the weaker hydrolase activity of the *Aplysia californica* protein. Likewise, these reagents did not impair the two activities of CD38 purified from human erythrocytes. β-mercaptoethanol had no effect on the *Aplysia* enzyme activities, while it inactivated both the cyclase and the cADPR hydrolase of CD38 by inducing its extensive oligomerization. In intact erythrocytes the β-mercaptoethanol-dependent enzyme inactivation was completely prevented by prior cross-linking of the membrane proteins with glutaraldehyde. These data demonstrate that none of the cysteine residues plays any direct catalytic role in CD38 and *Aplysia* proteins, and that disulfide bridges are essential for maintaining the monomeric, catalytically active structure of CD38.**

Key words: CD38; Human erythrocyte; ADP-ribosyl cyclase; Cyclic ADP-ribose hydrolase; Disulfide bond; *Aplysia californica*

1. Introduction

CD38 is a 46 kDa cell surface glycoprotein present on early and late differentiation stages of lymphocytes and on a number of other cells [1]. Several studies have highlighted two main features of CD38. First, it behaves as an orphan receptor, as shown by activation and differentiation programs triggered by 'agonistic' monoclonal antibodies that mimic so far unidentified physiologic ligand(s) [2]. A second property of CD38 is its nature of bifunctional ectoenzyme catalyzing both the conversion of NAD^+ to nicotinamide and cyclic ADP-ribose (cADPR), through an ADP-ribosyl cyclase reaction, and the hydrolysis of cADPR to free ADP-ribose (ADPR) [3–7]. Growing interest toward cADPR is justified by its nature of IP_3-independent Ca^{2+} mobilizer in several cell types (see [8] for a review) and compartments [9].

The demonstration of CD38 as a bifunctional enzyme arose from its high degree of amino acid sequence identity [10] with ADP-ribosyl cyclase from *Aplysia californica* [11] and *Aplysia kurodai* [12]. Specifically, 10 cysteine residues are conserved between the *Aplysia* enzymes and human CD38. Two out of six additional cysteines (residues 119 and 201) found in the CD38 sequence, but not in the *Aplysia* protein, were recently reported to be involved in the cADPR hydrolase reaction [13]. Thus, on the basis of elegant site-directed mutagenesis studies, Okamoto and coworkers [13] converted CD38 to a monofunctional enzyme apparently devoid of cADPR hydrolase activity by replacing cysteines 119 and 201. In addition, they reverted the monofunctional cyclase activity of *Aplysia* protein to a CD38-like bifunctional enzyme (i.e. cyclase/hydrolase), by restoring the two cysteines corresponding to residues 119 and 201 of CD38 [13].

Although these studies point to the importance of specific cysteine residues for CD38 catalysis, their redox state and precise role remain controversial. The enzyme activity (measured as disappearance of $1,N^6$-etheno-NAD^+) of CD38 from retinoic acid-differentiated HL-60 cells has been reported to be inhibited by dithiothreitol [4]. Moreover, extensive self-aggregation and inactivation of native erythrocyte CD38, both purified [14] and in situ within membranes [15], take place following exposure to β-mercaptoethanol or to GSH. On the other hand, inhibition of the *Aplysia kurodai* ADP-ribosyl cyclase by dithiothreitol has been reported by Tohgo et al. [13], but not confirmed by Inageda et al. [16]. These data, coupled with our preliminary observation that, despite the absence of cysteines 119 and 201, the *Aplysia californica* enzyme does exhibit a weak cADPR hydrolase activity, suggested to re-investigate the role of cysteines/cystines using both the *Aplysia* protein and human CD38. Our results allow to exclude any direct involvement of cysteine residues in the catalytic mechanism of cyclase and hydrolase activities, while they demonstrate an essential role of disulfide bridges in maintaining the monomeric, catalytically active structure of CD38.

2. Materials and methods

2.1. Materials

ADP-ribosyl cyclase, purified from *Aplysia californica* ovotestis as described previously [17], was a generous gift of Prof. H.C. Lee, Minneapolis, MN. CD38 was purified from human erythrocyte membranes as described [5]. Anti-CD38 monoclonal antibody IB4 [2] was kindly provided by Prof. F. Malavasi, Turin, Italy. Recombinant human CD38 [18] was provided by Dr. W.D. Branton, Minneapolis, MN. Blood samples were obtained from normal volunteers using heparin as anticoagulant. Leukocytes and platelets were removed with a leukocyte removal filter (Sepacell, Asahi Medical Co., Tokyo, Japan). All chemicals were of the highest purity grade available from Sigma.

2.2. Assay of cyclase and hydrolase activities of the Aplysia enzyme

The purified enzyme (0.13 μg/ml) was incubated at 37°C in the presence of 10 mM Tris-HCl, pH 6.5, and 0.1 mM NAD^+ for 15 min (to assay cyclase activity) or 0.1 mM cADPR for up to 5 h (to estimate

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Abbreviations: NGD^+, nicotinamide guanine dinucleotide; GDPR, guanosine diphosphate ribose; cGDPR, cyclic guanosine diphosphate ribose.

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hydrolase activity). At different times, aliquots were withdrawn, deproteinized with TCA (5% final concentration), centrifuged and the excess TCA removed with diethylether. The amounts of nucleotides produced were determined by HPLC analysis as described below.

2.3. Assay of cyclase and hydrolase activities of human CD38

Purified CD38 (0.1 μg/ml) was incubated for up to 4 h at 37°C in the presence of 10 mM Tris-HCl, pH 6.5, 0.05% Triton X-100 and 0.1 mM NGD^+ (for cyclase activity) or 0.1 mM cADPR (for hydrolase activity). Aliquots were withdrawn at different times, deproteinized as above and subjected to HPLC analysis.

2.4. HPLC analyses

These were performed on a Hewlett-Packard HP 1090 instrument. A 5 m, 60 × 4.6 mm, ODS-Hypersil C_{18} reverse phase (Hewlett-Packard) column was used. Solvent A was 0.1 M KH_2PO_4 containing 5 mM tetra-*n*-butylammonium, pH 5.0; solvent B was solvent A containing 30% (v/v) methanol. The solvent program was a linear gradient (at a flow rate of 0.4 ml/min) starting at 100% solvent A and increasing to 100% solvent B in 30 min. The nucleotides were detected by a HP 1040 A diode array spectrophotometric detector set at 260 nm. The elution times (in minutes) of standard nucleotides were: cGDPR 4.5, cADPR 5.6, NGD^+ 8.0, NAD^+ 12, GDPR 16.5, ADPR 23.

2.5. SDS-PAGE

Purified CD38 (10 ng) was subjected to SDS-PAGE and Western blot as in [15]. Saturation of 0.45 μm nitrocellulose membranes (Hybond-ECL, Amersham, Italy) with milk-powder, incubation with anti-CD38 MoAb [2] and immunoenzymatic detection were performed following instructions of the Amersham ECL chemiluminescence assay kit.

2.6. Incubation of intact erythrocytes with glutaraldehyde and β-mercaptoethanol

Washed human erythrocytes, withdrawn as described above, were preliminarily incubated at a 10% hematocrit in 0.15 M NaCl, in the absence or presence of 0.15% (v/v) glutaraldehyde, for 15 min at 25°C. After two washings of the glutaraldehyde-pretreated samples with 10 mM glycine in 0.15% M NaCl and two further washings with 0.15 M NaCl, erythrocytes were incubated at 37°C for 5 or 30 min, respectively, at a 35% hematocrit, in 10 mM Tris, pH 6.5, containing 0.15 M NaCl, 5 mM glucose (Buffer A) and 0.1 M β-mercaptoethanol (omitted in the control suspensions). Erythrocytes were then washed 5 times with 0.15 M NaCl and submitted to assays of ADP-ribosyl cyclase and cADPR hydrolase activities, using 0.1 mM NGD^+ and 0.1 mM cADPR, respectively, as substrates. The assay mixtures (35% hematocrit) contained Buffer A and 1 mM β-mercaptoethanol and were incubated for 15 and 30 min at 37°C. Analyses of the various metabolites were performed by HPLC on TCA extracts of the supernatants, as described above.

3. Results

The ADP-ribosyl cyclase from *Aplysia californica* exhibits also a weak cADPR hydrolase activity, accounting for nearly 1% of the cyclase, that could be detected by properly prolonging the time of incubation (not shown).

We next addressed the question of whether any of the 10 cysteine residues present in the *Aplysia* protein is directly involved in the catalytic mechanism of the cyclase/hydrolase reactions. As shown in Table 1, neither the thiol-modifying reagents NEM and iodoacetamide nor thiol-oxidizing compounds like H_2O_2 and Cu^{2+}-*o*-phenanthroline, affected either catalytic activity detectably. β-Mercaptoethanol (β-ME), as high as 100 mM, was ineffective, thus ruling out any important role of disulfide bonds in maintaining an active conformation of the invertebrate protein.

Since human CD38 has six additional cysteine residues which are missing or otherwise replaced in the *Aplysia* protein [10,11], the same experiments designed to elucidate the role of sulfhydryl vs. disulfide groups were performed also with CD38 purified from human erythrocyte membranes. However, its comparatively higher cADPR hydrolase than cyclase activity [5] suggested to use NGD^+ as substrate of the cyclase [18,19]. In fact, NAD^+ is eventually converted by CD38 to ADPR through cADPR as a transient intermediate, thereby reproducing an 'NADase' reaction that leads to underestimating the intrinsic cyclase activity. NGD^+, on the contrary, proved in preliminary experiments to be converted to cGDPR to a much higher extent than to GDPR (78 ± 2% cGDPR vs. 12 ± 2% GDPR formed, respectively), this indicating a much lower susceptibility of cGDPR than of cADPR to hydrolysis by native CD38. This finding, which is quite similar to those previously reported with a recombinant soluble form of human CD38 [18], allowed to measure the cyclization reaction without appreciable interferences by the hydrolase activity. Thus, the intrinsic cyclase activity of purified CD38 was estimated to be 2.8 mol cGDPR/min/mg and the ratio of cyclase to hydrolase activity was accordingly much higher (around 1) than previously estimated with NAD^+ as substrate, i.e. 1/10 [5].

As shown in Table 1, none of the thiol-modifying or thiol-oxidizing reagents affected either the cyclase or the hydrolase activity of CD38. On the other hand, unlike what was observed with the *Aplysia* protein, addition of β-mercaptoethanol (β-ME) resulted in a concentration-dependent loss of both enzyme activities. This is in agreement with our previous results on the purified and on the membrane-bound human CD38 [14,15] and also with the reported inhibition of the NADase activity of CD38 by DTT [16], that is in fact the time-dependent loss of the combined cyclase and hydrolase activities of this glycoprotein. That inactivation by β-ME was actually accompanied by self-aggregation of CD38 was shown by disappearance of the monomeric 46 kDa immunoreactive protein band (Fig. 1). When intact native erythrocytes, that possess both cyclase and cADPR hydrolase ectoenzyme activities [20], were incubated with 100 mM β-ME, a progressive inactivation was observed (Table 2). Preliminary treatment of intact erythrocytes with glutaraldehyde at concentrations not impairing CD38 enzyme

Table 1
Effect of thiol-modifying an thiol-oxidizing reagents on ADP-ribosyl cyclase and cADPR hydrolase activities of the *Aplysia californica* protein and human CD38

	Activities (%)			
	Aplysia protein		Human CD38	
Reagent (mM)	Cyclase	Hydrolase	Cyclase	Hydrolase
–	100	100	100	100
NEM (1)	99	99	98	99
Iodoacetamide (1)	98	97	97	97
H_2O_2 (10)	97	96	99	98
Cu^{2+}-*o*-phenanthroline (0.05)	102	100	101	100
β-mercaptoethanol (10)	100	99	81	83
β-mercaptoethanol (100)	99	98	20	27

The *Aplysia* protein (0.13 μg/ml) was pre-incubated 30 min at 37°C in 10 mM Tris-HCl, pH 6.5, containing the compounds indicated at the concentrations shown between parentheses. CD38 purified from human erythrocytes membranes [5] was pre-incubated at 0.1 μg/ml in the same conditions, but with the addition of 0.05% Triton X-100. After pre-incubation, the mixture were assayed for cyclase and hydrolyse activities as described in section 2. Results are expressed as percentage of enzyme activities detected without any thiol reagent. Results of a representative experiment are shown, out of five ones in which variability never exceeded 7%.

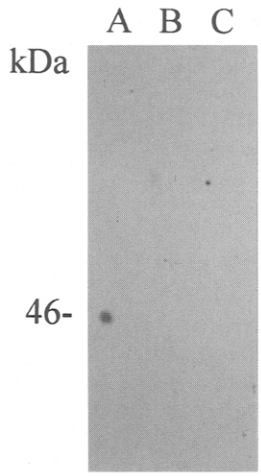

Fig. 1. SDS-PAGE and immunoblotting of human CD38 incubated with β-mercaptoethanol. Purified CD38 (0.1 μg/ml) was incubated for 30 min at 37°C without (lane A) and with 10 mM (lane B) and 100 mM (lane C) β-mercaptoethanol, in the same conditions as in the legend to Table 1. The samples were then submitted to SDS-PAGE followed by Western blotting and immunoassay, as described in section 2.

activities (e.g. 0.15% (v/v), as shown in Table 2), conferred complete stability of cyclase and hydrolase activities upon further exposure of the cells to otherwise inactivating 100 mM β-ME (Table 2). These data indicate that the process of enzyme inactivation by β-ME requires two sequential steps, the first being disulfide bond reduction, followed by oligomerization of active monomeric CD38 to yield low activity aggregates.

4. Discussion

CD38 and CD38-related proteins, previously classified as NAD^+-glycohydrolases [8,21,22], convert NAD^+ to ADPR in two separate but mechanistically related steps, i.e. cyclization and hydrolysis. Several evidences indicate that an ADP-ribosylated enzyme intermediate is formed during catalysis by CD38, thereby leading to cADPR or ADPR formation depending on absence or availability of water, respectively, at the active site [16,18,21,23,24]. Since NAD^+-dependent ADP-ribosylation of proteins can occur on a number of amino acid residues, including cysteines [25], the presence of many cysteine residues in CD38 and in *Aplysia* cyclase might suggest their catalytic involvement through transient ADP-ribosylation. This possibility, however, has been ruled out (Table 1) and ADP-ribosyl amino acid acceptors other than cysteines seem to be involved in both proteins.

Detection of a weak cADPR hydrolase activity in the *Aplysia* cyclase gives further support to the two-step mechanism of catalysis, although this protein is mostly dedicated to cyclization. Moreover, it indicates that residues 95 and 176 in the *Aplysia* protein (lysine and glutamic acid, respectively, as compared to two cysteine residues in the CD38 sequence), although being important in affecting the balance between cyclase and hydrolase activities, as previously shown by site-directed mutagenesis studies [13], do not take part directly in the catalysis.

On the other hand, susceptibility of cyclase and hydrolase activities of human CD38 to β-mercaptoethanol suggests that disulfide bond(s) is(are) somehow required to stabilize a catalytically active conformation. This is apparently a peculiar feature of the 46 kDa monomer since β-ME results in its disappearance ([14,15]; Fig. 1) and, concomitantly, in the loss of both enzyme activities (Table 1). Monomer disappearance and enzyme inactivation may not be strictly comparable from a quantitative standpoint in vitro (compare Table 1 and Fig. 1 at 10–100 mM β-ME), as a result of various experimental conditions like detergent and protein concentration and variable accessibility of substrate(s) to the active site in the aggregates (not shown).

Results on intact native erythrocytes exposed to β-ME are indicative of a condition of partially restricted in situ mobility of CD38 within the membrane, that leads to a delayed inactivation of both cyclase and hydrolase (Table 2), as previously observed also on erythrocyte membranes incubated with GSH [15]. Additional restriction of CD38 lateral diffusion by prior cross-linking of membrane proteins on intact erythrocytes with glutaraldehyde abolishes completely the susceptibility of both ectoenzyme activities to reductive inactivation (Table 2). All these data point to a two-step inactivation (i.e. reduction followed by oligomerization) rather than to direct inhibition by β-ME, and suggest accordingly an important conformational role, rather than direct catalytic involvement, of disulfide bond(s) located in the ectocellular region of CD38. Such localization is also supported by susceptibility to β-ME-induced self-aggregation of a recombinant soluble form of CD38 [18], corresponding to its ectocellular domain (not shown).

On the basis of the present experiments, reductive opening of disulfide bridge(s) by β-ME seems to trigger a drastic change in the conformation of CD38 monomers followed by their stable and apparently non-ordered oligomerization [14]. Identification of the bonds and/or domains responsible for such

Table 2
Effect of glutaraldehyde treatment of intact erythrocytes on the β-mercaptoethanol-induced inactivation of ADP-ribosyl cylase and cADPR hydrolase ectoenzyme activities

Pre-incubated	β-mercaptoethanol (0.1 M, minutes of exposure)	Cyclase (pmol cGDPR/min/mgHb)		Hydrolase (pmol ADPR/min/mgHb)	
–	–	5.53	(100%)	6.79	(100%)
Glutaraldehyde	–	5.43	(98%)	6.60	(97%)
–	5	4.15	(75%)	4.76	(70%)
–	30	1.93	(35%)	2.51	(37%)
Glutaraldehyde	5	5.49	(100%)	6.75	(99%)
Glutaraldehyde	30	5.51	(100%)	6.80	(100%)

Details of incubation are provided in section 2. Results of a representative experiments are shown, out of five different ones in which variability never exceeded 11%.

CD38 self-aggregation will be the subject of further work. However, four leucine zipper-like sequences [26] that occur in the ectocellular portion of CD38 (residues 124–145, 150–164, 208–229 and 271–285, respectively), may be involved in the β-ME-initiated process of oligomerization. The behaviour of *Aplysia* protein, which unlike CD38, is completely refractory to inactivation by β-ME and where only one out of the four mentioned hydrophobic motifs (residues 183–204) is partially conserved [11], is consistent with this hypothesis.

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