# Self-aggregation of purified and membrane-bound erythrocyte CD38 induces extensive decrease of its ADP-ribosyl cyclase activity

Elena Zocchi[a], Luisa Franco[a], Lucrezia Guida[a], Lesley Calder[b], Antonio De Flora[a,*]

[a]*Institute of Biochemistry, University of Genoa, and Advanced Biotechnology Center, Viale Benedetto XVI1, 16132 Genoa, Italy*
[b]*Division of Virology, National Institute for Medical Reaearch, Mill Hill, UK*

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**Abstract The transmembrane glycoprotein CD38 is a bifunctional enzyme that catalyzes at its ectocellular domain both the synthesis and the hydrolysis of cyclic ADP-ribose (cADPR). The complete reaction, converting $NAD^+$ to nicotinamide and ADP-ribose, reproduces an $NAD^+$glycohydrolase (NADase) reaction. CD38 purified from human erythrocyte membranes has been recently shown to undergo stable oligomerization induced by either $NAD^+$ or β-mercaptoethanol. We demonstrate that oligomerization is also triggered by reduced glutathione (GSH) and that the GSH-induced self-aggregation of purified CD38 is accompanied by extensive and comparable decrease of its ADP-ribosyl cyclase and NADase activities. GSH-induced oligomerization of CD38 and strong enzyme inactivation take place also in situ on erythrocyte membranes.**

*Key words:* CD38; Erythrocyte; Oligomerization; ADP-ribosyl cyclase; $NAD^+$ glycohydrolase; Enzyme inactivation

## 1. Introduction

Human CD38, a type II transmembrane glycoprotein of 46 kDa predominantly expressed on the activated phenotypes of mononuclear and lymphocyte cell types [1–3], has been recently shown to be a bifunctional enzyme catalyzing both the synthesis of cyclic ADP-ribose (cADPR) from $NAD^+$ and the hydrolysis of cADPR to ADP-ribose (ADPR) [4–8]. The two enzyme activities are located at the ectocellular, C-terminal region of CD38[4–6] and are defined as ADP-ribosyl cyclase (cyclase) and cADPR hydrolase (hydrolase). The combination of these two activities reproduces an apparent $NAD^+$ glycohydrolase (NADase) reaction and it is likely that several known NADases are, in fact, ADP-ribosyl cyclases [9].

CD38 is believed to be involved in the reception/transduction processes of signals eliciting the activation and proliferation of lymphoid cells [1]. The observation that 'agonistic' monoclonal antibodies (MoAbs), reactive against the ectocellular domain of CD38, stimulate the proliferation of $CD38^{+ve}$ T and B lymphocytes [3], strongly supports this notion. However, it is not known whether the mechanisms of these cellular processes are related to cADPR formation. This $NAD^+$ metabolite is a potent $Ca^{2+}$-mobilizer from intracellular stores both in invertebrate as well as in vertebrate cells [10–13] and perturbations of intracellular $Ca^{2+}$ concentrations are known to be implicated in several mechanisms of cell activation/proliferation. The ectoenzymatic nature of CD38, however, poses a topological obstacle to the possible involvement of extracellularly produced cADPR in intracellular $Ca^{2+}$ mobilization [12].

We have recently demonstrated that human CD38, purified to homogeneity from erythrocyte membranes, undergoes a stable self-aggregation when is incubated with its substrate $NAD^+$ and/or β-mercaptoethanol [14]. Since receptor dimerization or oligomerization upon interaction with specific ligands is a widely known pre-requisite for subsequent internalization [15,16], susceptibility of CD38 to self-aggregation might represent a means for its intracellular import and metabolism of cADPR. Therefore, we investigated (a) the relationship between CD38 self-aggregation and levels of its intrinsic cyclase activity, (b) the occurrence of CD38 oligomerization within human erythrocyte ghosts taken as a model of cell membrane. Red cell ghosts are more suitable than lymphocyte membranes to study the enzyme activities of CD38 because, although CD38 has a much lower density on erythrocyte than on lymphocyte membranes, no other enzyme activities degrading $NAD^+$ or ADPR are present on the erythrocyte membrane [17]. The results reported in this paper indicate that oligomerization is paralleled by extensive enzyme inactivation, both with purified and with membrane-bound CD38.

## 2. Materials and methods

### 2.1. Materials

[$^{32}$P]Adenylate $NAD^+$ was purchased from NEN, Italy, and purified by HPLC on a PL SAX 1000, 50×5 mm anion exchange column (Hewlett Packard) as follows: solvent A was distilled water; solvent B was 0.15 M TFA; the solvent program was a gradient starting at 100% A, linearly increasing to 15% B in 20 min, then linearly increasing to 100% B in 10 min, at a flow rate of 0.5 ml/min.

Standard cyclic ADP-ribose and ADP-ribosyl cyclase purified from *Aplysia californica* ovotestis were kindly provided by Prof. H.C. Lee, Minneapolis, MN. Anti-CD38 monoclonal antibody IB4 [18] was a generous gift by Prof. F. Malavasi, Turin, Italy. CD38 was purified from solubilized erythrocyte membranes as described in [5]. White erythrocyte ghosts were prepared from freshly drawn human citrated blood, after removal of leukocytes and platelets, as described [17].

Nitrocellulose sheets (0.45 μm) for Western blotting and the peroxidase-conjugated anti-mouse Ig kit for immunoenzymatic detection of CD38 were obtained from BioRad, Italy. $^{125}$I-Labelled sheep anti-mouse Ig antibody was purchased from Amersham, Italy.

### 2.2. Fluorimetric detection of cyclic GDP-ribose (cGDPR) production

The assay was performed essentially as in [9] in a Perkin Elmer model LS 50 B luminescence spectrometer at 37°C under continuous stirring. Production of cGDPR from nicotinamide guanine dinucleotide (NGD) by purified CD38 was monitored continuously at 410 nm emission wavelength (excitation at 300 nm).

### 2.3. SDS-PAGE

Purified CD38 (0.2 μg) or white erythrocyte membranes (60 μg) were subjected to SDS-PAGE on 7–12% gradient gels according to [19]. Western blot was performed according to [20] for 3 h at 2°C on

*Corresponding author. Fax: (39) (10) 35-4415.


*SSDI* 0014-5793(95)00005-4

0.45 μm nitrocellulose membranes. Saturation of nitrocellulose with gelatin and incubation with the anti-CD38 MoAb [18] were performed following instructions of the BioRad immunoperoxidase assay kit. Immunodetection of the CD38 protein band was obtained with a peroxidase-conjugated (BioRad) or a $^{125}$I-labelled (Amersham) anti-mouse antibody for the purified CD38 and the erythrocyte membranes, respectively.

### 2.4. Quantitation of [$^{32}$P]ADPR and [$^{32}$P]cADPR produced from [$^{32}$P]NAD$^+$ by purified CD38

Purified CD38 was incubated with [$^{32}$P]NAD$^+$, under the conditions described in the legend to Fig. 3, and TCA extracts at the various times were analyzed on a 3 μm, 60×4.6 mm, ODS Hypersil $C_{18}$ reverse phase column (Hewlett Packard) with the following analysis: solvent A was distilled water; solvent B was 0.1 M $KH_2PO_4$ containing 5 mM Pic A reagent (Millipore, Italy) and 30% methanol, with the pH adjusted to 5.0. The solvent program used (at a flow rate of 0.5 ml/min) was a gradient starting at 100% solvent A for 5 min, then linearly increasing to 10% B in 15 min, then linearly increasing to 80% B in 30 min. This analysis allows a good separation of [$^{32}$P]cADPR not only from [$^{32}$P]NAD$^+$ and [$^{32}$P]ADPR, but also from the trace amounts of [$^{32}$P]orthophosphate that remain after HPLC purification of [$^{32}$P]NAD$^+$ (see above).

The specific activity of [$^{32}$P]NAD$^+$ ($10^5$ cpm/nmol) was calculated from the integrated area (Hewlett Packard 79996A analytical workstation) and the radioactivity of the NAD$^+$ peak at zero time.

### 2.5. Assay of NADase and ADP-ribosyl cyclase activities of erythrocyte membranes

Following pre-incubation with or without GSH, as described in the legend to Fig. 4, aliquots were withdrawn at different times and membranes washed in 10 mM Tris-HCl, pH 6.5, resuspended in the same buffer at 1.5 mg/ml and incubated further at 37°C in the presence of 1 mM NAD$^+$. At zero time and after 15 and 30 min incubation, aliquots were withdrawn, briefly centrifuged to remove the membranes and the supernatants immediately frozen at −20°C. The NAD$^+$, ADPR and cADPR concentrations in the supernatants were determined by HPLC analysis as in [5].

### 2.6. Acetylcholinesterase (Achase) activity of erythrocyte membranes

This was determined as in [21].

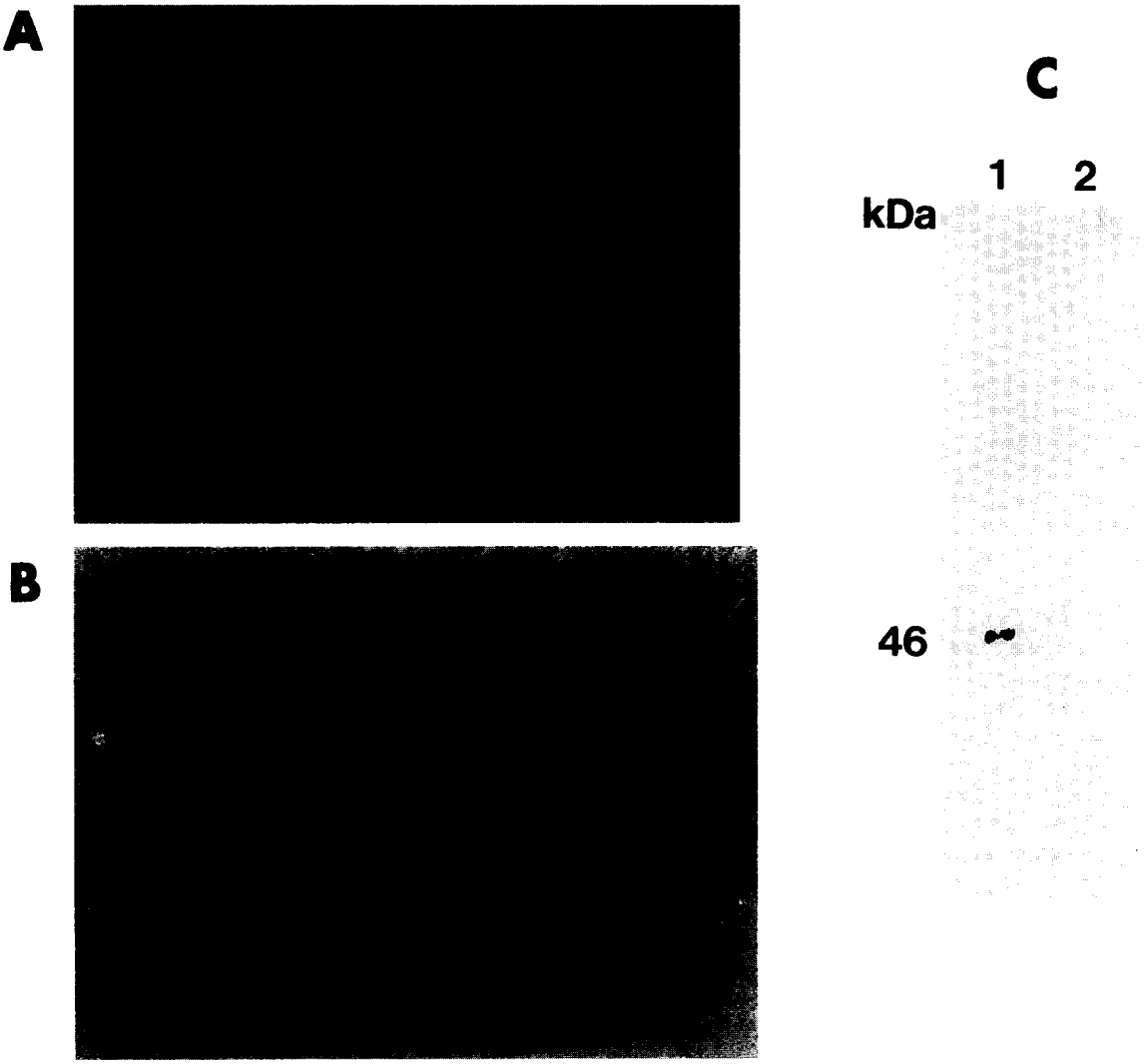


Fig. 1. Transmission electron microscopy and immunoblotting of purified CD38 incubated with GSH. Purified CD38 (0.2 μg) was incubated at 37°C in the absence (control) or presence of 25 mM GSH. After 60 min incubation, the mixtures were divided in two parts. One part was spotted onto a grid, negatively stained with 1% sodium silicotungstate and analyzed by transmission electron microscopy at a 150,000-fold magnification (see [14] for details). (A) Control. (B) GSH-incubated CD38. Bar = 50 nm. The other part of each mixture was subjected to SDS-PAGE and Western blot. Immunoenzymatic detection was performed as described in section 2. (C) Lane 1, control; lane 2, GSH-incubated CD38.

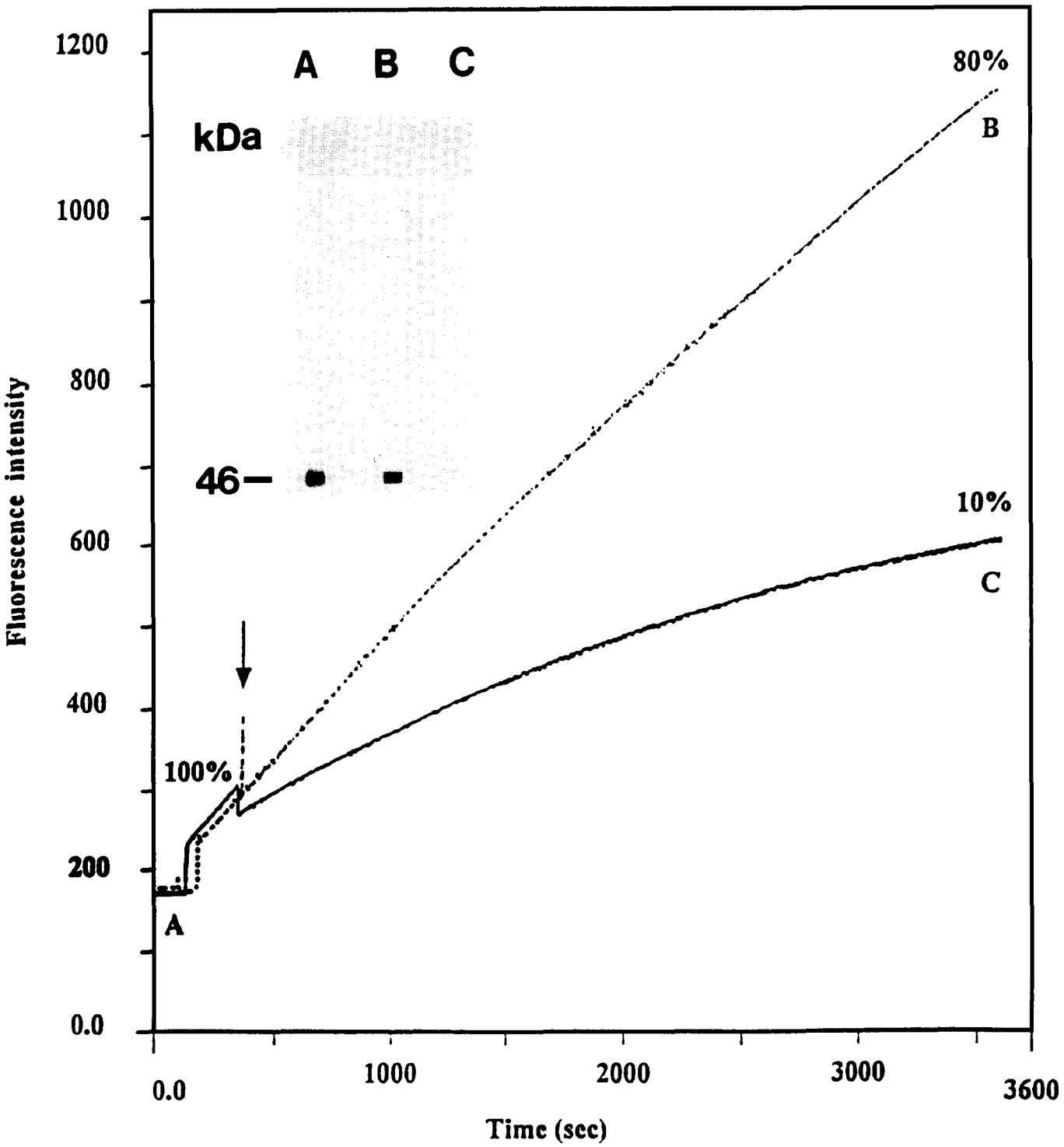


Fig. 2. Production of cGDPR by purified CD38 in the absence or presence of GSH and Western blot analysis of the protein before and after incubation. Purified CD38 (0.2 μg per assay) was incubated at 37°C in a fluorimetric cuvette in 2 ml of 2 mM Tris-HCl, pH 6.5, 0.01% Triton X-100 and 25 mM NGD (buffer), under continuous stirring. The trace shows the production of cGDPR (see section 2). The arrow indicates addition of 100 μl buffer (B) or 100 μl 0.5 M GSH (C). At zero time and after 60 min incubation with or without GSH, the samples were rapidly frozen, lyophilized and subjected to SDS-PAGE and Western blot. Immunoenzymatic detection of CD38 was carried out as described in section 2. Inset: (A) zero time; (B) 60 min incubation with NGD; (C) 60 min incubation with NGD and GSH. Result from a representative experiment are shown for the sake of clarity. Variability in five different experiments never exceeded 9%.

## 3. Results

In a previous paper, we demonstrated that purified human CD38 undergoes self-aggregation upon incubation with $NAD^+$ and/or β-mercaptoethanol (β-ME) [14]. Evidence for protein aggregation was obtained by two different approaches: (a) disappearance of the monomeric 46 kDa protein band detectable by SDS-PAGE, due to formation of large aggregates unable to

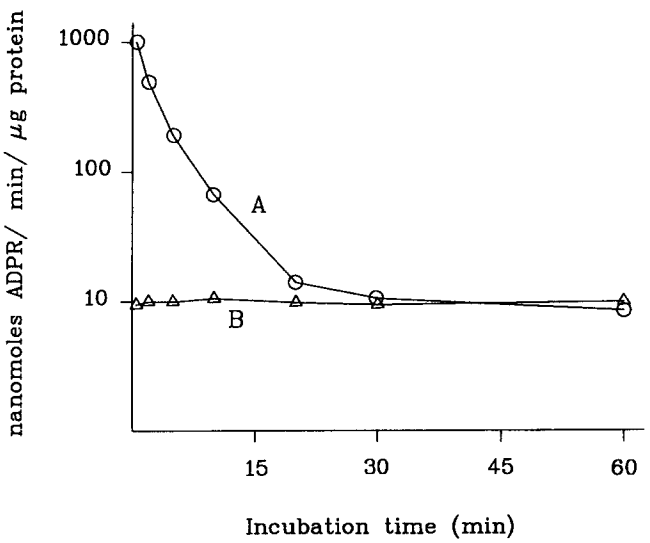


Fig. 3. Production of ADP-ribose from $NAD^+$ by either native or GSH-preincubated purified CD38. Purified CD38 (30 ng) was incubated in 20 μl of 5 mM Tris-HCl, pH 6.5, 0.05% Triton X-100, without (A) or with (B) 0.2 mM GSH. After 45 min at 37°C a sample of each incubation was diluted $10^4$ times in the same, homologous buffer and 200 μl aliquots further incubated in the presence of 0.2 mM GSH and 0.1 mM [$^{32}$P]NAD ($10^5$ cpm/nmol). At the times indicated, aliquots were withdrawn, TCA (10%, final concentration) was added to stop the reaction together with 10 pmol standard, unlabelled cADPR, the TCA extracted with ether and samples were subjected to HPLC analysis as described in section 2. Fractions were collected every 30 s and the radioactivity co-eluting with the ADPR and cADPR peaks was counted. For the sake of clarity, only the NADase activity of both incubation mixtures is shown. Cyclase activity was approximately 1/100 of the value of the NADase at each time point. Results of a representative experiment are shown, out of four different ones in which variability never exceeded 13%.

enter the stacking gel, and (b) transmission electron microscopy of negatively stained incubation samples [14].

Subsequent experiments demonstrated that the concentrations of $NAD^+$ and β-ME could be decreased to 10 μM without impairing the oligomerization process (not shown). In addition, reduced glutathione (GSH) was found to be as powerful as β-ME in inducing CD38 self-aggregation, as shown both by electron microscopy and by SDS-PAGE followed by immunoblotting (Fig. 1).

In order to follow the cyclase activity of CD38 during the process of GSH-induced aggregation, we took advantage of a recently described fluorimetric assay for the detection of cyclase activity. This assay is based on the fluorescent properties of cyclic GDP-ribose (cGDPR), which is produced from the non-fluorescent NGD by *Aplysia* cyclase, as well as by human recombinant CD38 [9]. Moreover, cGDPR, unlike cADPR, is a very poor substrate for the hydrolase activity of CD38 [9], and GDPR is not fluorescent: thus, the fluorimetric detection of cGDPR production from NGD represents a convenient assay for determination of the cyclase activity only.

When purified CD38 was incubated with NGD at 37°C, the rate of production of cGDPR, as determined by its fluorescence emission at 410 nm, was almost linear (Fig. 2, curve B): the slope of the curve after 60 min incubation was 80% of that recorded during the first minutes after addition of substrate. On the other hand, upon addition of 25 mM GSH, the rate of cGDPR production decreased progressively (Fig. 2, trace C) and after 60 min the cyclase activity was reduced to 10% of the control.

In parallel experiments, performed on the cyclase from *Aplysia californica* [22], GSH had no effect on the production of cGDPR (not shown). This rules out any quenching of the cGDPR fluorescence by GSH and any inactivation of the cyclase.

In the same experiment illustrated in Fig. 2, immunoblot analyses of CD38 were carried out on samples withdrawn before and after incubation with or without GSH. As shown in Fig. 2 (inset), a 46 kDa band was present at zero time (A) and its intensity was comparable after 60 min incubation with NGD (B), in agreement with the limited decay of the cyclase activity. No 46 kDa band was detectable after 60 min incubation with NGD and GSH (C), indicating that, concomitant with extensive cyclase inactivation, all CD38 had aggregated (see also Fig. 1).

The experiment shown in Fig. 2 cannot be performed with $NAD^+$ as substrate, because cADPR is not fluorescent, nor with a mixture of NGD and $NAD^+$ as substrates, because a decreased rate of cGDPR production might be due to competition between the two substrates for the enzyme's active site [9] rather than to CD38 oligomerization. Therefore, an alternative approach was followed. Purified CD38 was pre-incubated for 45 min with or without GSH, i.e. under conditions resulting in oligomerization or maintenance of the 46 kDa monomeric form, respectively. Both samples were then diluted $10^4$ times and their rates of NADase and cyclase activities were determined using $NAD^+$ as substrate. The protein concentration in this assay was kept very low (3 pM) in order to prevent CD38 from aggregating too fast during the assay, i.e. in the presence of $NAD^+$. As shown in Fig. 3, in the pre-aggregated sample (B), the rates of NADase and cyclase activities were constant at 1% approximately of the starting level observed with monomeric CD38 (A). On the contrary, the enzyme activities of sample A decreased markedly (100 times) during the first 20 min incubation and remained constant thereafter (30–60 min), at about the same value as in the pre-aggregated sample.

These results indicate that the intrinsic NADase and cyclase activities of oligomerized CD38 are around 1% of those displayed by monomeric CD38. Conversion of the monomeric to the aggregated species, occurring during the assay itself because of the presence of GSH and $NAD^+$, is accompanied by a concomitant and progressive drop of enzyme activities, i.e. from 100% to 1%.

Finally, we investigated if aggregation of CD38 and enzyme inactivation could take place also in situ on erythrocyte membranes in the presence of GSH. Thus, erythrocyte membranes were incubated at 37°C in the absence or presence of 25 mM GSH, and NADase and ADP-ribosyl cyclase activities were determined at various times of incubation.

Results of a typical experiment are shown in Fig. 4. While CD38 enzyme activities kept constant during incubation without GSH (Fig. 4, curve A), a marked decay of both NADase and cyclase activities was observed in the membranes incubated with GSH: after 24 h, levels as low as 4% and 5% of the activities measured at zero time were observed, respectively (Fig. 4, curve B). Western blot analysis of the membranes at the end of the 24 h incubation revealed that the CD38 46 kDa band was present in the control membranes (Fig. 4, inset, A) while it had disappeared from the GSH-incubated membranes (Fig.

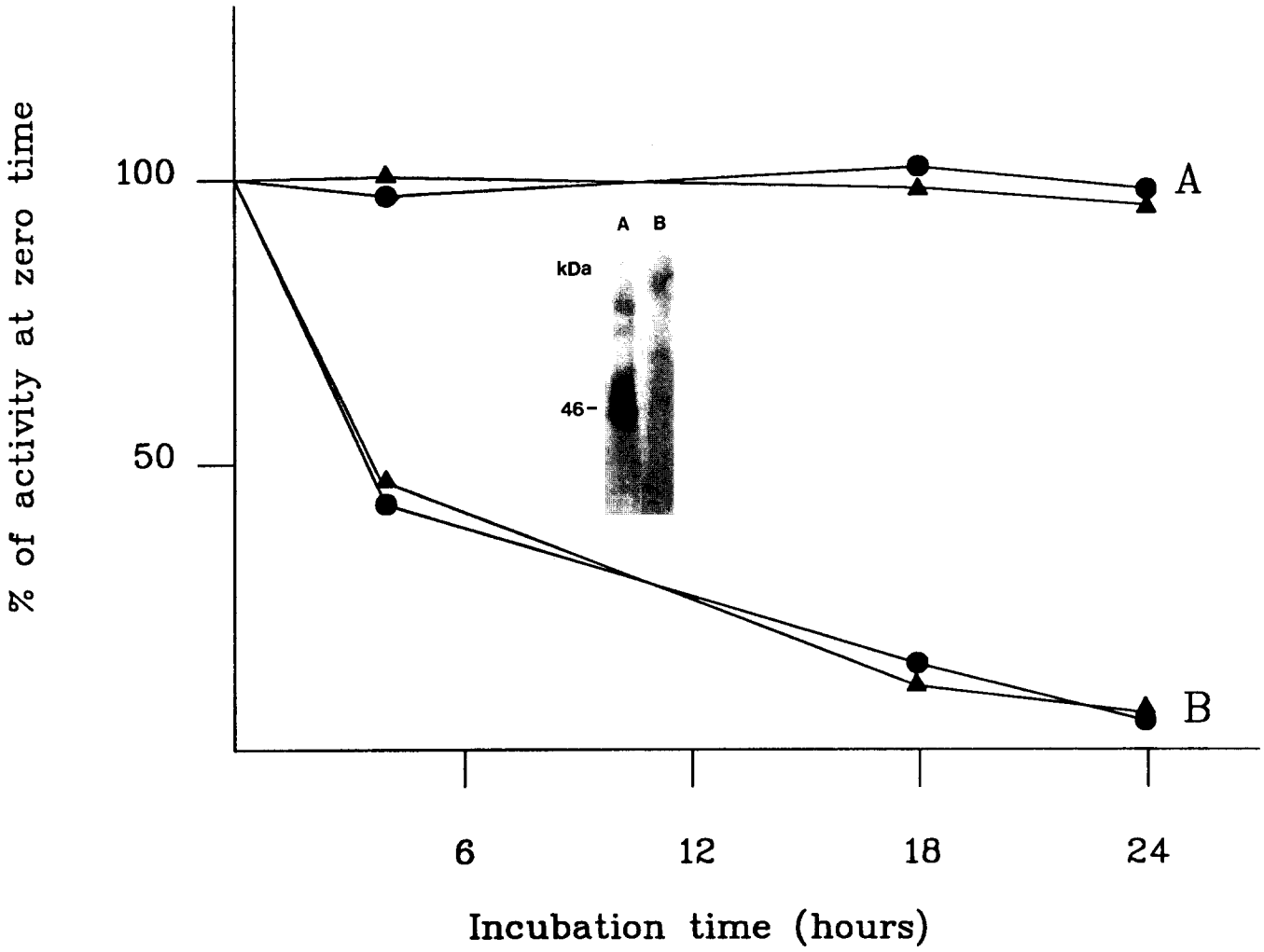


Fig. 4. NADase and ADP-ribosyl cyclase activities of erythrocyte membranes incubated with GSH. Hemoglobin-free erythrocyte ghosts (3 mg/ml) were incubated at 37°C in 10 mM Tris-HCl, pH 6.5, in the absence (A) or presence (B) of 25 mM GSH. At the times indicated, aliquots from the incubation mixtures were withdrawn, the membranes washed three times with saline, and NADase (circles), ADP-ribosyl cyclase (triangles) and Achase activities were determined as in section 2. The mean values from three different experiments are shown: variability never exceeded 6% for each time point. After 24 h the membranes from either incubation mixture were washed and 60 $\mu$g were subjected to SDS-PAGE, Western blot and radioimmunodetection of CD38 as in section 2. The result of a representative experiment is shown in the inset.

4, inset, B). Acetylcholinesterase activity of both control and GSH-treated membranes, measured as a reference enzyme, remained constant throughout the 24-h incubation (not shown).

## 4. Discussion

The purpose of this study was two-fold: (a) to determine if aggregation of CD38 had any effect on its enzymatic activities, particularly on the ADP-ribosyl cyclase activity; and (b) to investigate whether oligomerization could also take place on the erythrocyte membrane. The present findings demonstrate that the GSH-induced aggregation of purified CD38 is accompanied by extensive loss of its cyclase activity, assayed with either NGD or $NAD^+$ as substrate. Specifically, the cyclase activity decreased 10 times (with NGD as substrate, Fig. 2) or 100 times (with $NAD^+$ as substrate, Fig. 3) following addition of GSH. This discrepancy is most likely due to the fact that $NAD^+$ itself is also an efficient inducer of CD38 aggregation [14], while NGD is not (Fig. 2, inset). The apparent NADase activity was also markedly decreased to a comparable extent following aggregation. It is still uncertain whether NADase activity is an intrinsic and cyclase-independent property of CD38 or rather represents the sum of the cyclase and hydrolase activities. Recent kinetic data obtained with NGD as substrate seem to support the second hypothesis [9], which is also favoured by the following observations: a) throughout the process of GSH-induced aggregation the ratio between NADase and cyclase activities of CD38 remained constant and, b) aggregation induced a comparable decay of cADPR hydrolase activity (Guida, L., unpublished data).

The results shown in Fig. 4 also demonstrate that aggregation of CD38, and the consequent decrease of enzyme activities, can occur in situ on erythrocyte membranes. The process of aggregation is apparently much slower on the membranes as compared to the free protein in solution, taking hours rather than minutes. This difference could be explained by the mobility restrictions imposed on the transmembrane CD38 protein by the lipid bilayer and possibly by cytoskeletal and integral

membrane proteins as well. It may be relevant to this purpose that in other $CD38^{+ve}$ cells like T, B and NK lymphocytes, CD38 is physically and functionally associated with specific surface receptors [18].

As mentioned, this study was carried out with human erythrocyte CD38 because: (a) the red cell membrane lacks other $NAD^+$-degrading enzymes that might interfere with assays of cyclase and NADase activities [17]; and (b) in vitro oligomerization had been observed with the glycoprotein purified from red cell membranes [14]. It is likely that in situ self-aggregation and concomitant enzyme inactivation also take place in the membrane from other $CD38^{+ve}$ cells, notably lymphocytes. Some pathophysiological events, like apoptosis in lymphnodes or extensive cell death, might lead to local increases of extracellular GSH and $NAD^+$ concentrations up to levels (around 10 $\mu$M) that are sufficient to trigger self-aggregation of CD38.

In conclusion, self-aggregation of CD38 seems to be an intrinsic property of this glycoprotein, both purified and membrane-bound. However, the role of such oligomerization and specifically its relationship with CD38-related cellular processes, like activation and proliferation, is still completely unknown. The concomitant marked decay of the cyclase activity might suggest that CD38 oligomerization represents a mechanism designed to down-regulate cADPR-dependent events. Identification of these events and their correlation with CD38 properties, particularly in situ self-aggregation, will be the subject of future research.

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