Regulation of NAD$^+$ glycohydrolase activity by NAD$^+$-dependent auto-ADP-ribosylation

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NAD$^+$ glycohydrolase (NADase; EC 3.2.2.5) is an enzyme that catalyses hydrolysis of NAD$^+$ to produce ADP-ribose and nicotinamide. Its physiological role and the regulation of its enzymic activity have not been fully elucidated. In the present study, the mechanism of self-inactivation of NADase by its substrate, NAD$^+$, was investigated by using intact rabbit erythrocytes and purified NADase. Our results suggest that inactivation of NADase was due an auto-ADP-ribosylation reaction. ADP-ribosylated NADase of rabbit erythrocytes was deADP-ribosylated when incubated without NAD$^+$, and thus enzyme activity was simultaneously restored. These findings suggest that reversible auto-ADP-ribosylation of NADase might regulate the enzyme's activity *in vivo*.

INTRODUCTION

NAD$^+$ glycohydrolase (NADase; EC 3.2.2.5), the enzyme that catalyses the hydrolysis of NAD$^+$ to nicotinamide and ADP-ribose, is present ubiquitously from bacteria to mammals [1–10]. Most eukaryotic NADases are membrane-associated and the activity of the enzyme is exclusively localized at the outer surface of the membrane [6–10]. Ecto-NADase of rabbit erythrocytes can be solubilized by treatment with a bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), suggesting that NADase might be anchored to the cell membrane via glycosylphosphatidylinositol (GPI) [11]. Previously we have purified this GPI-anchored NADase with an apparent molecular mass of 65 kDa [12]. We showed that macrophage cell lineages express high concentrations of NADase whereas other tissues express different concentrations of the enzyme [6,13,14]. The physiological role and the regulation of GPI-anchored NADase are still unknown.

NAD$^+$-dependent disappearence of cellular NADase activity was first observed by Lieberman working with suspension cultures of intact mouse fibroblast cells [15]. Removal of NAD$^+$ caused a rapid reappearance of the enzyme activity. Therefore NADase activity might be regulated by its substrate. This observation has been confirmed with NADases from various sources, although the inactivation seems to differ qualitatively between the NADases investigated [16–18]. We investigated the mechanisms involved in the inactivation of NADase because of the possible importance of NAD$^+$-dependent inactivation of NADase in cellular regulation. We observed that the self-inactivation reaction occurred on the cell surface of intact rabbit erythrocytes and with the purified enzyme, indicating that NAD$^+$-dependent inactivation of NADase is most probably caused by auto-ADP-ribosylation. The ADP-ribosylation proceeded reversibly in intact erythrocytes.

MATERIALS AND METHODS

Materials

NAD$^+$, ATP, ADP-ribose, 1,N^6-etheno-NAD$^+$ and Cibacron Blue-agarose were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). Omnisorb cells were purchased from Calbiochem (La Jolla, CA, U.S.A.). [*adenylate*-^{32}P]NAD$^+$ (1000 Ci/mmol) was obtained from Amersham (Bucks., U.K.) and [*adenine*-2,8-^{3}H]NAD$^+$ (4 Ci/mmol) from Du Pont (Boston, MA, U.S.A.). PI-PLC from *Bacillus cereus* was purchased from Boeringer-Mannheim (Mannheim, Germany). Snake-venom phosphodiesterase was obtained from Worthington (Freehold, NJ, U.S.A.). Polyethyleneimine cellulose thin-layer chromatography plates were purchased from Schleicher & Schuell (Dassel, Germany). An NADase-specific antiserum was raised by immunizing goats with purified NADase from rabbit erythrocytes, and anti-(NADase IgG) was purified by using a Protein G–agarose column (Genex, Gaithersburg, MD, U.S.A.).

Purification of NADase from rabbit erythrocytes

PBS-washed rabbit erythrocytes were incubated with bacterial PI-PLC in PBS for 30 min as described previously [12]. The supernatant was applied to a column of Cibacron Blue-agarose. NADase was eluted with 0.5 M KCl. The purity was determined by SDS/PAGE.

Preparation of immunoprecipitates of purified NADase

Purified NADase (100 μg) from rabbit erythrocytes and anti-(NADase IgG) (100 μg) were incubated at 4 °C for 1 h in Dulbecco's PBS containing 2 mM ATP and 2 mM ADP-ribose

Abbreviations used: GPI, glycosylphosphatidylinositol; NADase, NAD glycohydrolase; PI-PLC, phosphatidylinositol-specific phospholipase C.

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(buffer A) plus 10 % (v/v) glycerol. The antibody-bound NADase was treated with 50 μl of 10 % (w/v) heat-inactivated *Streptococcus* sp. cells (Omnisorb cells) at 4 °C for 30 min as previously described [14]. The immunoprecipitates were washed with PBS and resuspended in 500 μl of buffer A containing 10 % (v/v) glycerol.

Assay of NADase activity

NADase activity was measured by the fluorimetric method of Muller et al. [9] after preincubation with NAD^+. For preincubation of intact erythrocytes with NAD^+, rabbit erythrocytes (5×10^5 cells) were incubated at 37 °C for the indicated times with various doses of NAD^+ in 100 μl of buffer A, and the cells were washed with PBS. For preincubation of purified NADase with NAD^+, 10 μl of immunoprecipitate of purified NADase was incubated at 37 °C with various concentrations of NAD^+ for the indicated times in buffer A containing 10 % glycerol and the precipitate of purified NADase was washed with PBS. The preincubated cells or the immunoprecipitates of purified NADase were then incubated in 50 μl of PBS containing 200 μM of $1,N^6$-etheno-NAD^+. After incubation for 10 min at 37 °C, the reaction was stopped with 50 μl of 10 % trichloroacetic acid. The reaction mixture was centrifuged at 10000 ***g*** for 10 min and 80 μl of supernatant was transferred into 0.72 ml of 0.1 M phosphate buffer, pH 7.4. Fluorescence was measured with a fluorimeter (Hitachi F-3010) with excitation at 297 nm and emission at 412 nm. NADase activity was expressed as pmol/min per 10^6 cells.

Identification of ADP-ribosylated NADase by electrophoresis

For purified NADase, NADase-bound Omnisorb cells were incubated with 15 μM [*adenylate*-^{32}P]NAD^+ in buffer A for the indicated times; NADase-bound immunoprecipitates were washed with buffer A, extracted with SDS sample buffer and analysed by SDS/PAGE by the method of Laemmli [19], followed by autoradiography. For rabbit erythrocytes, the cells were incubated with 15 μM [*adenylate*-^{32}P]NAD^+ in buffer A for the indicated times, washed with buffer A and resuspended in lysis buffer [1 % (w/v) Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 0.5 % Nonidet P40, 2 mM ATP, 2 mM ADP-ribose]. After unlysed materials had been removed by centrifugation (600 ***g*** for 10 min), the supernatants were precleared with Omnisorb cells. The cleared lysates were incubated with anti-(NADase IgG) for 4 h at 4 °C and were further incubated with Omnisorb cells for 30 min. Immunoprecipitates were washed with lysis buffer, extracted with Laemmli buffer, and analysed by electrophoresis on 10 % (w/v) polyacrylamide gels followed by autoradiography.

Identification of ADP-ribosylated amino acid residue of NADase

After 1 μg of purified NADase had been incubated with 15 μM [*adenylate*-^{32}P]NAD^+ in buffer A for 1 h, the [^{32}P]ADP-ribosylated NADase was subjected to SDS/PAGE, and then the separated protein was transferred to a poly(vinylidene difluoride) filter. The filters were incubated with water, 10 mM $HgCl_2$, 1 M neutralized NH_2OH or 1 M NaOH at 45 °C for 3 h and then subjected to autoradiography.

Analysis of products released from ADP-ribosylated NADase of rabbit erythrocytes

After erythrocytes had been incubated with 15 μM [*adenylate*-^{32}P]NAD^+ in buffer A for the indicated times, the cells were washed with buffer A. Subsequently, ADP-ribosylated erythrocytes were incubated in the absence of NAD^+, the media were collected, and the concentrates of the media were applied to polyethyleneimine-cellulose TLC plates. The mixture of [*adenylate*-^{32}P]NAD^+, [*adenylate*-^{32}P]ADP-ribose and [^{32}P]AMP was used as standard markers and spotted on to the origin of one lane as described [20]. The samples were resolved with 0.05 M ammonium bicarbonate as a developing solvent, and the plates were dried and autoradiographed.

RESULTS

Effects of NAD^+ on NADase activity of intact rabbit erythrocytes

To study the effect of the substrate NAD^+ on NADase activity of rabbit erythrocytes, cells were incubated with 100 μM NAD^+ for various periods or with various concentrations of NAD^+ for 80 min at 37 °C. The time course of inactivation of NADase is shown in Figure 1. In the absence of NAD^+, NADase activity did not change when incubated at 37 °C for up to 80 min. However,

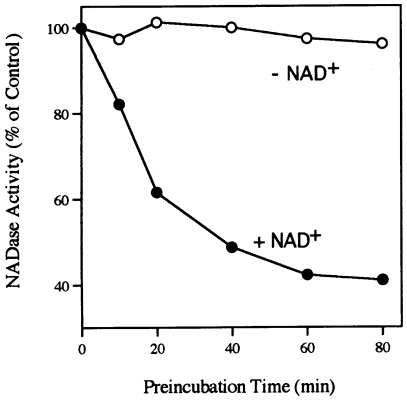

Figure 1 Effect of preincubation of rabbit erythrocytes with NAD^+

Erythrocytes (5×10^5 cells) were incubated with or without 100 μM NAD^+ for various lengths of time at 37 °C and then washed with PBS. NADase activity was assayed as described in the Materials and methods section.

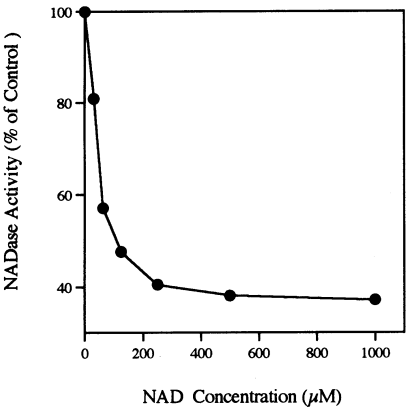

Figure 2 Effect of preincubation of rabbit erythrocytes with various amounts of NAD^+

Erythrocytes (5×10^5 cells) were incubated with various concentrations of NAD^+ for 40 min at 37 °C and then washed with PBS. NADase activity was assayed as described in the Materials and methods section.

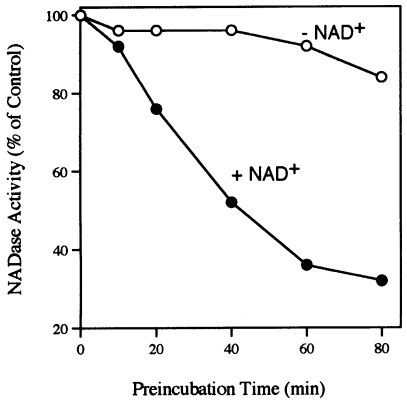

Figure 3 Effect of preincubation of purified NADase with NAD+

Purified NADase was immunoprecipitated with anti-(NADase IgG) as described in the Materials and methods section. The immunoprecipitate was incubated with or without 100 μM NAD+ for various lengths of time at 37 °C and then washed with PBS. NADase activity was assayed as described in the Materials and methods section.

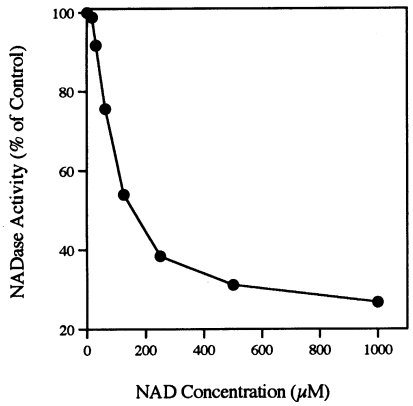

Figure 4 Effect of preincubation of purified NADase with various amounts of NAD+

Purified NADase was immunoprecipitated with anti-(NADase IgG) as described in the Materials and methods section. The immunoprecipitate was incubated with various concentrations of NAD+ at 37 °C for 40 min and then washed with PBS. NADase activity was assayed as described in the Materials and methods section.

as shown in Figure 1, preincubation with NAD+ decreased the enzymic activity by more than 60% at the end of the 80 min reaction. The concentration-dependent decrease in NADase with an IC_{50} of 105 μM is shown in Figure 2. The above results suggest that NADase is inactivated by its substrate NAD+.

Effects of NAD+ on purified NADase activity

To determine whether purified NADase is also inactivated by NAD+, purified NADase from rabbit erythrocytes was incubated with 100 μM NAD+ for various periods or with various concentrations of NAD+ for 80 min. As with the NADase activity of erythrocytes, the purified enzyme was also inhibited by NAD+ in time-dependent (Figure 3) and concentration-dependent manners with an IC_{50} of 165 μM (Figure 4).

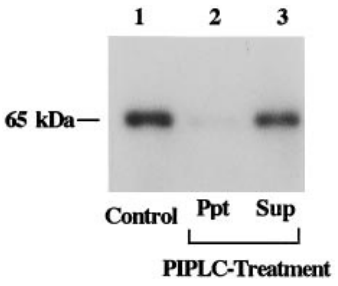

Figure 5 Radiolabelling of 65 kDa NADase by [*adenylate*-^{32}P]NAD+ in rabbit erythrocytes

Erythrocytes (10^6 cells) were incubated with [*adenylate*-^{32}P]NAD+ for 1 h and an aliquot of the cells was lysed in lysis buffer (lane 1). The resuspended cells were further incubated with 10 ng of PI-PLC at 37 °C for 20 min and the reaction mixture was separated from the cells by rapid centrifugation (600 ***g*** for 10 min). The pellet was lysed in lysis buffer (lane 2). The supernatant-containing reaction mixture (lane 3) and lysates were immunoprecipitated with anti-(NADase IgG) and subjected to SDS/PAGE as described in the Materials and methods section.

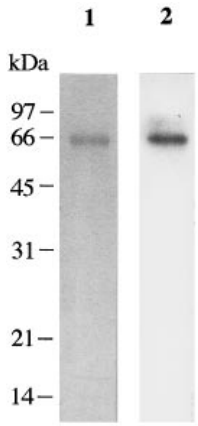

Figure 6 Purified NADase from rabbit erythrocytes and auto-ADP-ribosylation of the purified enzyme

NADase was purified to homogeneity and the purified enzyme was incubated with [*adenylate*-^{32}P]NAD+ as described in the Materials and methods section. The protein was separated by SDS/PAGE and revealed with Coomassie Blue (lane 1). Subsequently, the gel was dried and autoradiographed (lane 2).

NAD-dependent modification of a 65 kDa protein in rabbit erythrocytes

When rabbit erythrocytes were incubated with [*adenylate*-^{32}P]NAD+, immunoprecipitated with anti-(NADase IgG) and then analysed by SDS/PAGE, a radiolabelled 65 kDa protein was observed (Figure 5, lane 1). When the cells were treated with PI-PLC and immediately centrifuged at 600 ***g*** for 10 min, most of the radiolabelled protein was recovered from the supernatant (Figure 5, lanes 2 and 3). These results indicated that the 65 kDa GPI-anchored protein was modified by NAD+ in rabbit erythrocytes.

Auto-ADP-ribosylation of NADase

NADase was purified to homogeneity, and the purified enzyme was incubated with [*adenylate*-^{32}P]NAD+. The protein was separated by SDS/PAGE and revealed with Coomassie Blue (Figure 6, lane 1). The gel was dried and autoradiographed (Figure 6, lane 2). The purified enzyme was radiolabelled possibly because

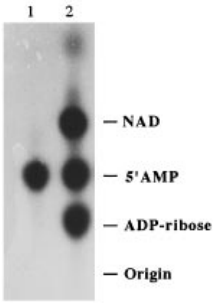

Figure 7 Release of [^{32}P]AMP by treatment of [^{32}P]ADP-ribosylated NADase with snake venom phosphodiesterase

Purified NADase (10 μg) was incubated with 15 μM [*adenylate*-^{32}P]NAD$^+$ at 37 °C for 1 h and precipitated with 10% (w/v) trichloroacetic acid. After centrifugation (10000 ***g*** for 10 min), the precipitate was washed with 4% trichloroacetic acid and dissolved in 10 μl of 0.2 M Tris/HCl, pH 9.0. The sample was further incubated with 0.1 unit/ml snake venom phosphodiesterase in the presence of 6 mM $MgCl_2$ at 37 °C for 30 min. The reaction mixture (5 μl) was applied to polyethyleneimine-cellulose TLC plates and developed with 0.05 M ammonium bicarbonate. After development, the plates were dried and autoradiographed as described in the Materials and methods section. Lane 1, assay; lane 2, standard markers.

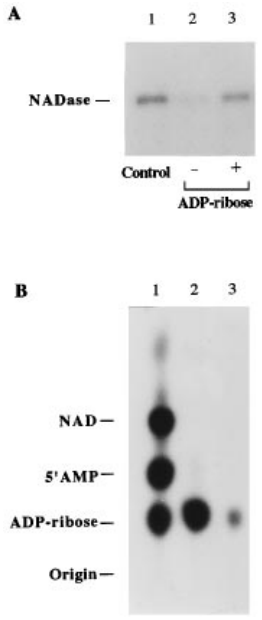

Figure 9 Release of [^{32}P]ADP-ribose from [^{32}P]ADP-ribosylated NADase in rabbit erythrocytes

Erythrocytes (10^6 cells) were incubated with 15 μM [*adenylate*-^{32}P]NAD$^+$ for 1 h as described in Figure 5. After being washed with buffer A, the cells were immediately lysed (**A**, lane 1) or further incubated at 37 °C for 40 min with (**A**, **B**; lane 3) or without (**A**, **B**; lane 2) 2 mM ADP-ribose in PBS. (**A**) The cells were lysed and then subjected to SDS/PAGE and autoradiography. (**B**) The reaction mixture was analysed by TLC as described in the legend to Figure 6. Lane 1 represents standard markers.

of its auto-ADP-ribosylation. To examine whether the radioactivity of the [*adenylate*-^{32}P]NAD$^+$ incorporated into NADase was due to ADP-ribosylation, the radiolabelled NADase was treated with snake venom phosphodiesterase, and radioactive materials released were analysed by TLC. As shown in Figure 7, the major material was identified as [5′-^{32}P]AMP, suggesting that the NAD$^+$-dependent modification of NADase was due to mono-ADP-ribosylation. The possibility that the auto-ADP-ribosylation of NADase was due to non-enzymic association of ADP-ribose seems unlikely because the labelling reactions used in these experiments were performed in the presence of 2 mM ADP-ribose. Furthermore NADase was not significantly labelled when [^{32}P]NAD was replaced with [^{32}P]ADP-ribose, and NADase was not inactivated by incubation with ADP-ribose (results not shown). Auto-ADP-ribosylation of NADase may be due to covalent trapping of NAD$^+$ in the active site of the enzyme during catalysis. The finding that an excess of unlabelled NAD$^+$ did not reduce a significant amount of radioactivity from the [^{32}P]ADP-ribosylated NADase ruled out this possibility (results not shown). These results suggest that the nature of auto-ADP-ribosylation process of NADase might be due to enzyme-catalysed covalent modification.

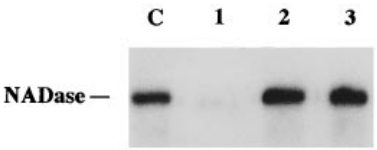

Figure 8 Treatment of [^{32}P]ADP-ribosylated NADase with $HgCl_2$, NH_2OH or NaOH

[^{32}P]ADP-ribosylated NADase was subjected to SDS/PAGE, after which the separated protein was transferred to a poly(vinylidene difluoride) filter. Filters were incubated with water (lane C), 10 mM $HgCl_2$ (lane 1), 1 M neutralized NH_2OH (lane 2), or 1 M NaOH (lane 3) at 45 °C for 3 h, and then subjected to autoradiography.

Auto-ADP-ribosylation of NADase at its cysteine residue

The presence or absence of a modified amino acid in NADase was investigated by the chemical stability of ADP-ribosyl bonds connected to amino acids. ADP-ribosylation reactions are specific for a particular amino acid residue, usually diphthamide (modified histidine), arginine or cysteine [21]. [*adenylate*-^{32}P]ADP-ribosylated NADase, after separation by SDS/PAGE, was transferred to a poly(vinylidene fluoride) filter, and the filter was treated with $HgCl_2$, NH_2OH or NaOH. Treatment with $HgCl_2$, which cleaves the thiol–ribose linkage, released most of the radiolabel from NADase (Figure 8, lane 1). However, the addition of NH_2OH or NaOH, which release ADP-ribose from arginine or histidine residues respectively, did not result in a significant release of ADP-ribose (Figure 8, lanes 2 and 3). Thus these results are consistent with the mono-ADP-ribosylation's occurring at a cysteine residue or cysteine residues of NADase.

Reversible ADP-ribosylation of NADase in intact erythrocytes

When the [^{32}P]ADP-ribosylated cells were further incubated in the absence of [*adenylate*-^{32}P]NAD$^+$, there was a marked decrease in radiolabelled NADase (Figure 9A, lane 2). The lost radioactivity was mostly recovered from the incubation media as [^{32}P]ADP-ribose (Figure 9B, lane 2). Moreover, the observed decrease in [^{32}P]ADP-ribosylated NADase in the absence of [*adenylate*-^{32}P]NAD$^+$ was specifically inhibited by the addition of ADP-ribose to the incubation medium (Figure 9A, lane 3). There might be an enzyme on the cell surface of the erythrocytes

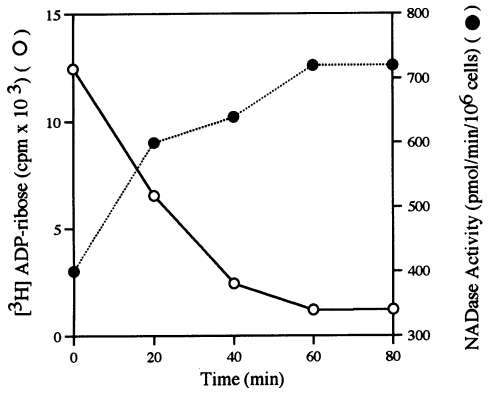

Figure 10 Correlation of de-ADP-ribosylation and recovery of NADase activity

Erythrocytes (10^6 cells) were incubated with 100 μM [*adenine*-^{3}H]NAD$^+$ at 37 °C for 1 h. The cells, after being washed, were incubated in the absence of NAD$^+$. During incubation, the aliquots of the cell suspension were assayed for residual [^{3}H]ADP-ribose and NADase activities as described in the Materials and methods section.

responsible for the removal of ADP-ribose from the modified NADase.

Restoration of the repressed activity of NADase by de-ADP-ribosylation

To study whether the NADase activity could be restored by de-ADP-ribosylation of the enzyme, erythrocyte NADases were ADP-ribosylated by being incubated with NAD$^+$ or [*adenine*-2,8-^{3}H]NAD$^+$ as described above; the cells were then further incubated in the absence of NAD$^+$. Aliquots of the cells were taken and their NADase activities and the remaining [^{3}H]ADP-ribose were measured. NADase activities increased and [^{3}H]ADP-ribose of NADase decreased with incubation time (Figure 10). These results suggest that the activity of ADP-ribosylated NADase in the erythrocytes was restored by de-ADP-ribosylation.

DISCUSSION

Here we present evidence that NAD$^+$-dependent ADP-ribosylation of NADase occurred in intact erythrocytes, causing inactivation of the enzymic activity. This ADP-ribosylation was catalysed by NADase itself, because a similar finding was also observed with purified NADase. ADP-ribosylated NADases were de-ADP-ribosylated in intact rabbit erythrocytes in the absence of NAD$^+$, and NADase activity was simultaneously restored. These results suggest that ecto-NADase in the erythrocytes might be regulated by NAD$^+$-dependent auto-ADP-ribosylation.

We have previously measured the circulating NAD level in the plasma of human, mouse, rat and rabbit [12]. The source of this extracellular NAD$^+$ is not known, but might be from cell lysis. Extracellular NAD$^+$ would not be futilely degraded by ecto-NADases without specific mechanisms to control enzymic activity. The reversible ADP-ribosylation of NADase, described here, certainly could provide one of the regulatory mechanisms of this enzymic activity. Other NADase families, such as ADP-ribosyl cyclase (CD38), ADP-ribosyl transferase and T lymphocyte alloantigen RT6 are also auto-ADP-ribosylated [22–24]. Because all of these enzyme molecules have their catalytic domains on the outer surface of the cells [25–27], their activities might be auto-ADP-ribosylated, preventing them from unneccessarily destroying NAD$^+$ in the extracellular space.

Reversible ADP-ribosylation of NADase was observed only in intact cells (Figure 10), not in the purified enzyme. These results suggest that de-ADP-ribosylation might be mediated by specific enzyme(s) on the cell surface of the erythrocytes. Tanuma and Endo [28] reported that ADP-ribosylcysteine hydrolase activity, which hydrolyses the linkages between ADP-ribose and the cysteine residues of inhibitory GTP-binding protein (G_i), was found in the cytosol of human erythrocytes. It is quite possible that in our study there was a similar enzyme on the cell surface.

Under our experimental conditions, less than 10 μM NAD$^+$ was not sufficient for NAD$^+$-dependent inactivation of NADase. Thus it is conceivable that there are some regulatory cofactor(s) in the extracellular fluid that facilitate auto-ADP-ribosylation of NADase at the physiological concentration of NAD$^+$. Because several forms of ADP-ribosylation factor are regulatory factors for various ADP-ribosylation reactions [29], it is worth pursuing whether ADP-ribosylation factors are also involved in the present auto-ADP-ribosylation of NADase.

ADP-ribosyl transferase catalyses the transfer of ADP-ribose from NAD$^+$ to an acceptor protein. In the absence of a specific acceptor protein, some ADP-ribosyl transferases can catalyse the transfer of ADP-ribose to water, representing an NADase activity. Free amino acids can also act as acceptors for ADP-ribose [30,31]. Because cysteine specifically blocks auto-ADP-ribosylation and NAD$^+$-dependent inactivation of NADase, NADase might have a transferase activity towards free cysteine (results not shown), although further studies are needed to determine whether NADase has an intrinsic transferase activity as well.

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