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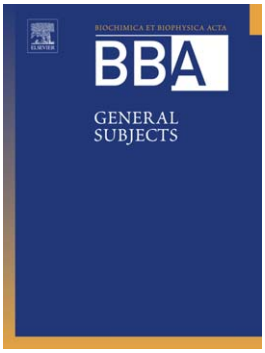

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FOOTNOTES

This paper is dedicated to María Antonia Günther-Sillero, teacher (of some of us), colleague, and friend – of all.

Abbreviations:
DTT, DL-dithiothreitol; FPC, full progress curve (assay); GSSG, oxidized glutathione; IRD, initial rate discontinuous (assay); NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NDP, nucleoside 5´-diphosphate; TRPM, transient receptor potential melastatin

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Abstract

Free ADP-ribose is a putative second messenger and also a potentially toxic compound due to its non-enzymic reactivity towards protein side chains. ADP-ribose hydrolysis is catalysed by NDP-sugar/alcohol pyrophosphatases of differing specificity, including a highly specific, low-K_m ADP-ribose pyrophosphatase. In humans, a submicromolar-K_m ADP-ribose pyrophosphatase has been purified from placenta, while recombinant NUDT9 has been described as a similarly specific enzyme with a nudix motif, but with a 10^2-10^3 higher K_m. Here, a comparative study of both proteins is presented showing that they are in fact enzymically indistinguishable; crucially, they both have submicromolar K_m for ADP-ribose. This study firmly supports the view that the ADP-ribose pyrophosphatase present in human tissues is a product of the *NUDT9* gene. In addition, this study reveals previously unknown properties of both enzyme forms. They display the same, differential properties in the presence of Mg^{2+} or Mn^{2+} as activating cations with respect to substrate specificity, ADP-ribose saturation kinetics, and inhibition by fluoride. Treatment with H_2O_2 alters the Mg^{2+}/Mn^{2+} responses and increases the K_m values for ADP-ribose, changes that are reversed by DTT. The results are discussed in relation to the proposed roles for ADP-ribose in oxidative/nitrosative stress and for ADP-ribose pyrophosphatase as a protective enzyme whose function is to limit the intracellular accumulation of ADP-ribose.

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1. **Introduction**

In mammals, free ADP-ribose can be formed from NAD either directly by the action of NAD glycohydrolases, or indirectly as a consequence of the metabolic turnover of NAD-dependent poly(ADP-ribose), protein-bound mono(ADP-ribose) or cyclic ADP-ribose [1, 2]. On the one hand, free ADP-ribose is potentially toxic, due to its ability to form non-enzymic adducts with proteins that can lead to glycoxidation products [2-5], while, on the other hand, it is a ligand of certain ion channels that can open in response to ADP-ribose-binding [6-19].

The turnover of free ADP-ribose itself is believed to be catalysed by ADP-ribose pyrophosphatases that convert ADP-ribose to AMP and ribose 5-phosphate. Enzymes with ADP-ribose pyrophosphatase activity have been identified both by their purification from tissue extracts and by heterologous expression of cDNA sequences containing the so-called nudix box, a hallmark of many hydrolases active on nucleoside diphosphate-X compounds [20, 21].

In rat liver extracts, four ADP-ribose hydrolases, different from the broad-specificity nucleotide pyrophosphatase or phosphodiesterase I enzymes, are known with diverse specificities for ADP-ribose and other NDP-X compounds that depend, in part, on the nature of the necessary activating cation (Mg^{2+} or Mn^{2+}) used for the assays [22, 23]. ADPRibase-I (cytosolic) and ADPRibase-m (mitochondrial) are specific ADP-ribose pyrophosphatases, that, in the presence of Mg^{2+}, hydrolyze only ADP-ribose and the non-physiological, very close analog IDP-ribose. Although their substrate specificity is less strict when assayed in the presence of Mn^{2+}, it seems clear that Mg^{2+} must be the usual physiological cation. In addition, these highly specific enzymes show a very low (sub)micromolar K_m for ADP-ribose in the presence of Mg^{2+}, which makes them good candidates for playing a major role in the turnover of free ADP-ribose. ADPRibase-II (cytosolic) is a Mg^{2+}-dependent ADP-sugar pyrophosphatase, also less strict in its NDP-X specificity when assayed with Mn^{2+}. Finally, ADPRibase-Mn is not activated by Mg^{2+}, and shows a strict requirement for Mn^{2+}, which can be fully satisfied by 20 µM of this cation. This enzyme shows an unusual specificity for ADP-ribose and CDP-alcohols, as it hydrolyzes ADP-ribose and structural derivatives, IDP-ribose, CDP-glycerol, CDP-choline and CDP-ethanolamine, but not other non-reducing ADP-sugars or CDP-glucose. So far, these rat liver ADPRibases have not been molecularly identified as either nudix or non-nudix proteins.

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In human tissue extracts, two types of ADP-ribose hydrolase have been identified: ADP-sugar pyrophosphatases from erythrocytes and placenta, functionally related to rat ADPRibase-II [24, 25], and an ADP-ribose pyrophosphatase from placenta that closely resembles rat ADPRibase-I and ADPRibase-m in its strict substrate specificity and submicromolar K_m for ADP-ribose (˜ 0.5 µM) in the presence of Mg^{2+} [25]. No human equivalent of rat ADPRibase-Mn has been found so far.

Cloning and expression of human cDNAs coding for proteins with nudix motifs have revealed two ADP-ribose hydrolases: NUDT5 [26, 27], an ADP-sugar pyrophosphatase similar to those purified from human erythrocytes, placenta and rat liver, and NUDT9 [9, 28, 29], a specific ADP-ribose pyrophosphatase with strict specificity for ADP-ribose, like the specific pyrophosphatase purified from human placenta [25], and rat liver ADPRibase-I [23] and ADPRibase-m [22]. The enzymic features of the specific ADP-ribose pyrophosphatases from human and rat tissue extracts were comparable to those of recombinant NUDT9 except that, whereas the former displayed (sub)micromolar K_m values for ADP-ribose in the presence of Mg^{2+} [22, 23, 25], NUDT9 was reported as having a much greater K_m value of 30–180 µM ADP-ribose [9, 28-30]. The marked discrepancy between the K_m values of purified tissue and recombinant human ADP-ribose pyrophosphatases prompted speculation that either the saturation kinetics of the NUDT9 enzyme were strongly influenced by its recombinant character (with additional peptide sequences coming from the expression vector), or that the submicromolar-K_m, placental ADP-ribose pyrophosphatase was derived from a so-far unidentified human gene different from *NUDT9* and *NUDT5*. Aiming to solve this question, we have carried out a parallel enzymic characterization of the specific ADP-ribose pyrophosphatase from human placenta [25] and a recombinant NUDT9 protein expressed from a cDNA clone [28].

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2. Materials and methods

2.1. Materials

Hydroxyapatite HTA was from BioRad, Ni^{2+}-NTA agarose was from Qiagen, and Q-Sepharose was from Pharmacia. Other chemicals and column chromatography media were from the sources reported elsewhere [25].

Human placenta ADP-ribose pyrophosphatase was purified from isotonic 30 000 x *g* supernatants by ammonium sulphate fractionation (between 60 and 80% saturation) and anion-exchange chromatography as described [25], followed by chromatography on a hydroxyapatite column (3.4 cm x 1.4 cm i.d.) eluted with a 40-ml linear gradient of 100–400 mM potassium phosphate (pH 7.0) with 1 mM DTT.

Human NUDT9 enzyme was expressed in *E. coli* from the plasmid pQE-NUDT9α [28], which contains the human NUDT9α cDNA (accession number BC000542) cloned in the *Sma*I site of pQE-31 (Qiagen), and codes for a NUDT9 enzyme with a N-terminal vector-derived tag sequence MRGSHHHHHHTDPHASSVPRLVPRGS. The enzyme was induced with IPTG and purified from bacterial lysates by chromatography on a Ni^{2+}-NTA agarose column as described [28], followed by chromatography on a Q-Sepharose column (18 cm x 0.9 cm i.d.) eluted with a 100-ml linear gradient of 50–400 mM KCl in 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM DTT.

2.2. Enzyme assays

ADP-ribose hydrolase activity was assayed at 37°C by coupling to alkaline phosphatase and measuring colorimetrically the amount of P_i formed [25, 31]. The standard assay mixture contained, in a volume of 0.1 ml, 50 mM Tris-HCl (pH 7.5), 0.35 mM ADP-ribose, 5 mM $MgCl_2$ (or $MnCl_2$, where indicated), 0.25 µg (0.7 units) alkaline phosphatase, 1 mg/ml bovine serum albumin, and the appropriate amount of enzyme sample. Reaction was stopped and colour developed by addition of 1 ml of standard P_i reagent [23, 25]. Enzyme activities were linear with time and amount of enzyme. Blanks without enzyme and/or substrate were run in parallel.

For the study of saturation kinetics, two assays were used, referred to as initial rate discontinuous (IRD) and full progress curve (FPC) assays. The former gives reliable K_m values above 5 µM; the later allows accurate rate estimates at ADP-ribose concentrations less than 0.5 µM and must be used for more precise K_m determinations when lower values are found. In the IRD assay, assay mixtures were similar to those

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described above, except that the initial reaction volume was 2.8 ml and aliquots of 0.8 ml were withdrawn at different time points and colorimetrically assayed for the amount of P_i formed upon addition of 0.2 ml of a concentrated P_i reagent [25]. In the FPC assay, progress curves were recorded at 37°C and 265 nm with a coupling system of alkaline phosphatase and adenosine deaminase [32]. Reactions were started by the addition of 10–20 µM ADP-ribose and progress curves were recorded beyond the end point. Successive curves, recorded in the same reaction mixture by repetition of the addition of substrate, yielded similar results, indicating the absence of enzyme inactivation or product inhibition during the assay. Estimates of K_m, V_{max}, and their standard errors (S.E.) were obtained by non-linear regression [33].

2.3. Enzyme treatment with H_2O_2

Enzymes samples (2.5 ml) were chromatographed on a PD-10 column (Pharmacia) equilibrated and eluted with 25 mM Tris-HCl (pH 7.5) to remove DTT. The eluate (3 ml) was made 1 mM in H_2O_2, incubated 3 h at 4°C, and chromatographed again on a PD-10 column as before, to remove H_2O_2. Mock-treated samples were prepared in parallel by running PD-10 columns in 25 mM Tris-HCl (pH 7.5), 1 mM DTT, and omitting H_2O_2 treatment.

2.4. Molecular weight determination

Protein molecular weight was assayed by gel chromatography on a Sephadex G-75 column (Superfine grade; 132 cm x 0.95 cm i.d.), calibrated with bovine serum albumin (66 000), ovalbumin (45 000), trypsinogen (24 000) and cytochrome c (12 400). For H_2O_2-treated samples (see Section 2.3), elution was performed with 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA; for untreated or mock-treated samples, elution was performed with the same buffer supplemented with 1 mM DTT.

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3. Results

To ascertain whether the specific ADP-ribose pyrophosphatase purified from human placenta, with a reported submicromolar K_m for ADP-ribose [25], was related to the recombinant NUDT9 nudix hydrolase expressed from a human cDNA, with a reported 10^2–10^3-fold higher K_m [9, 28-30], we carried out a parallel characterization of both with respect to known and novel enzymic properties. The latter included the study of the enzyme responses to Mg^{2+} or Mn^{2+} as activating cation and to oxidative treatment with H_2O_2, which led to previously undescribed, reversible changes in divalent cation specificity. In all aspects considered (see below), tissue and recombinant ADP-ribose pyrophosphatases were enzymically indistinguishable (Fig. 1).

In agreement with previous reports [25, 28, 29], ADP-ribose pyrophosphatase strictly required Mg^{2+} or Mn^{2+} for full activity, which was attained either with =1 mM $MgCl_2$ or with ˜ 50 µM $MnCl_2$, the latter being inhibitory above 50 µM (Fig. 1A, left panels). Other cations were only marginally effective (Co^{2+}, Zn^{2+}) or ineffective (Ca^{2+}) (not shown).

Treatment of the enzymes with H_2O_2 (followed by its elimination) dramatically changed the specificity of both ADP-ribose hydrolase activities for the activating divalent cation, giving them a marked preference for Mn^{2+} (but at concentrations higher than before the oxidative treatment) and rendering them virtually inactive with Mg^{2+} (Fig. 1A, right panels). The change produced by H_2O_2, observed at 5 mM Mg^{2+} or Mn^{2+} as a full inversion of the preference for Mg^{2+} versus Mn^{2+}, was reversed by DTT (Fig. 1B).

The Mg^{2+}-dependent, strict specificity of mammalian ADP-ribose pyrophosphatase for ADP-ribose (or IDP-ribose) was demonstrated earlier using a broad range of NDP-X substrates [9, 23, 25, 28]. Here we checked this Mg^{2+}-dependent specificity, to see if it changed when Mn^{2+} was substituted for Mg^{2+}, using ADP-ribose and a group of four substrates selected because they are attacked by other mammalian ADP-ribose hydrolases and/or by the Mn^{2+}-dependent activity of ADP-ribose pyrophosphatase itself. The absence of Mg^{2+}-dependent activity on ADP-glucose, CDP-choline, UDP-glucose and Ap_2A (Fig. 1C) served to distinguish ADP-ribose pyrophosphatase from ADP-sugar pyrophosphatase [23-27], ADP-ribose/CDP-alcohol pyrophosphatase [23], UDP-glucose pyrophosphatase [34] and nucleotide pyrophosphatase/phosphodiesterase-I [35, 36]. In the presence of 50 µM Mn^{2+}, ADP-ribose pyrophosphatase remained specific for ADP-ribose, whereas with 5 mM Mn^{2+},

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minor but significant activities towards the three ADP-X derivatives were observed, Ap_2A being a better substrate than ADP-ribose under these conditions (Fig. 1C, left panels). Treatment of the enzyme with H_2O_2 also modified this behaviour, since, in addition to the above-mentioned loss of Mg^{2+}-dependent activity (Fig. 1A,B), the 5 mM Mn^{2+}-dependent activity of the treated enzyme was now strictly specific for ADP-ribose (Fig. 1C, right panels).

Saturation curves for ADP-ribose, derived from full progress curves recorded with 5 mM Mg^{2+} as the activating cation, indicated hyperbolic kinetics with submicromolar K_m values near 0.3–0.7 μM for both tissue and recombinant ADP-ribose-pyrophosphatase (Fig. 1D and 2). In the presence of Mn^{2+}, the K_m values for ADP-ribose were several-fold higher. Interestingly, treatment of the enzyme with H_2O_2 increased both the Mg^{2+}- or Mn^{2+}-dependent K_m values (Fig. 1D). In general, the recombinant enzyme showed slightly higher K_m values than the tissue enzyme. Such differences were small (e.g. 0.4 μM for control enzymes in presence of 5 mM Mg^{2+}; Fig. 1D), by no means comparable with the previously reported 10^2–10^3-fold higher K_m [9, 28-30], and were not always observed (e.g. the experiment reported in Fig. 2).

Inhibition by fluoride is a typical feature of nudix hydrolases [21] and has been reported for several ADP-ribose hydrolases [25, 28, 37]. The results in Fig. 1E (left panels) show the expected inhibition of the Mg^{2+}-dependent activity of ADP-ribose pyrophosphatase. However, the Mn^{2+}-dependent activity was not affected by fluoride. Similar results were observed after enzyme treatment with H_2O_2 both for the predominant Mn^{2+}-dependent, and for the minor Mg^{2+}-dependent activities (Fig. 1E, right panels).

Treatment with H_2O_2 did not affect enzyme molecular weight: both ADP-ribose pyrophosphatase from human placenta and recombinant NUDT9, treated and mock-treated, eluted from a Sephadex G-75 column with a V_e corresponding to a protein of 36-37 kDa (not shown).

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4. Discussion

In all the experiments performed (Fig. 1 and 2), there was a striking similarity between the ADP-ribose pyrophosphatase purified from human placenta and that expressed from NUDT9 cDNA, such that both preparations were enzymically indistinguishable from each other. In particular, it must be stressed that, using an appropriate methodology to study saturation kinetics at submicromolar and low micromolar ADP-ribose concentrations [32], the tissue and recombinant untreated enzymes displayed essentially identical, submicromolar K_m values (Fig. 2). We do not have a complete explanation for the high K_m values reported earlier [9, 28-30], although they could be partly due to (i) the use of insufficiently sensitive IRD assay methods, which require high substrate consumption for accurate rate measurements at low ADP-ribose concentrations, rather than the sensitive and robust FPC assay used in this work, and/or (ii) the oxidation of the enzyme with a concomitant increase in K_m value (see below).

The fact that, in all respects considered, each enzyme preparation mimicked the other so well, firmly supports the conclusion that human placenta ADP-ribose pyrophosphatase is a product of the *NUDT9* gene, something that could not be concluded in previous studies due to the high K_m values reported for NUDT9. It has been argued that substrates hydrolyzed by Nudix enzymes with millimolar K_m values may be non-natural substrates [38]. The demonstration that NUDT9 has much higher affinity for ADP-ribose than previously thought reinforces the view that the hydrolysis of this compound is the physiological role of the enzyme.

The *NUDT9* gene seems to be translated into a pre-mRNA that can be processed to give two mature products, named NUDT9α and NUDT9β [28, 29], coding for proteins that in transfected cells are directed to the mitochondria or remain in the cytosol, respectively [29]. The major product of the gene seems to be NUDT9α, although the actual relevance of NUDT9β is controversial: Lin et al. found NUDT9β RNA in several tissues [28], whereas Perraud et al. reported that it is an aberrant minor product [29]. In rat liver, cytosolic and mitochondrial forms of (sub)micromolar-K_m ADP-ribose pyrophosphatases have been demonstrated by subcellular fractionation procedures [22, 23]. The occurrence of ADP-ribose pyrophosphatase in human placenta mitochondria has not been specifically investigated. The enzyme studied in this work was purified from placenta isotonic extracts (prepared in 0.25 M sucrose) that had very low mitochondrial contamination (data not shown) [25]. Further work will be needed to

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ascertain the intracellular location of human ADP-ribose pyrophosphatase in normal cells and the relative contribution of NUDT9α and NUDT9β to the total ADP-ribose pyrophosphatase activity present in human cells.

The identical behaviour of the tissue and recombinant ADP-ribose pyrophosphatases demonstrates that the changes evoked by H_2O_2, particularly the inversion of activating cation preference (Mg^{2+} versus Mn^{2+}), are intrinsic features of a single enzyme and not a consequence of the presence of two ADP-ribose pyrophosphatases in placenta with opposing responses to oxidation, one with a preference for Mg^{2+} and the other for Mn^{2+}. In this respect, it is clear that the Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase previously demonstrated in rat liver is very different from the Mn^{2+}-dependent activity of H_2O_2-treated human ADP-ribose pyrophosphatase.

The functional alterations of ADP-ribose pyrophosphatase treated with H_2O_2 (Fig. 1) were demonstrated after removal of the oxidant by gel filtration; therefore they reflect a modification of the enzyme. The acquisition of a marked selectivity for Mn^{2+} as activating cation, and the increase of the K_m for ADP-ribose were both reversed by DTT, suggesting that the effects of H_2O_2 occur through the oxidation of thiol groups. Human ADP-ribose pyrophosphatase contains three Cys residues (Cys-29, Cys-207 and Cys-347) that are too distant physically to form intramolecular disulfide bridges upon oxidation [39]. The formation of intermolecular disulfides can also be discounted because the native molecular weight of the enzyme was not altered by H_2O_2. However, the involvement of Cys residue(s) in the modulation of ADP-ribose pyrophosphatase behaviour is supported by the in vitro sensitivity of the rat enzyme to GSSG, NO-generating agents and the paracetamol metabolite *N*-acetyl-*p*-benzoquinoneimine, which in every case evoke an increase in the K_m for ADP-ribose [37, 40, 41], and also by the in vivo treatment of rats with acetaminophen which results in the recovery of hepatic enzyme with an increased K_m that can be reverted to normal by DTT in vitro [42]. The oxidation of other amino acid residues, such as Met-216, located within the nudix box, cannot be completely ruled out; indeed, an ADP-ribose pyrophosphatase recently purified from *Arabidopsis thaliana* was found to possess several oxidized Met residues [43]. However, it is known that oxidized Met is only poorly reduced by DTT [44, 45]. Furthermore, it should be noted that the oxidation of ADP-ribose pyrophosphatase might be a complex phenomenon, as there is evidence that the loss of the Mg^{2+}-dependent and the increase of the Mn^{2+}-dependent ADP-ribose pyrophosphatase activities are not obligatorily associated: experiments carried out with

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GSSG showed that the increase of the Mn^{2+}-dependent activity at 5 mM cation occurred without apparent change of Mg^{2+}-dependent activity (results not shown).

Concerning the possible intracellular consequences of ADP-ribose pyrophosphatase oxidation such as are caused in vitro by H_2O_2, it must be emphasized that the concomitant increase of Mn^{2+}-dependent activity occurs only at high Mn^{2+} concentrations that are not expected under intracellular conditions. Therefore, in vivo, the loss of Mg^{2+}-dependent activity most likely would correlate with a net loss of the enzyme's ability to hydrolyze ADP-ribose and the inhibition of the turnover of this metabolite. Nevertheless, it is interesting to note that accumulation of high (mM) levels of Mn^{2+} and activation of Mn-dependent enzymes is a strategy used by certain bacteria to resist oxidative stress [46, 47], so the physiological relevance of this switch does warrant further investigation.

It is known that during oxidative/nitrosative stress there is a consumption of NAD and an increase in the production of ADP-ribose [48-51] which has been proposed to control Ca^{2+} fluxes by mediating in the so-called mitochondrial permeability transition (MPT) [48, 52] and/or by the direct gating of the cell membrane TRPM2 channels [16, 17]. If it actually occurs in vivo, inhibition of ADP-ribose pyrophosphatase during oxidative/nitrosative stress would further contribute to the net increase in ADP-ribose. In this respect, it should be noted that in experiments carried out to demonstrate that ADP-ribose is a transducer between oxidative/nitrosative stress and the opening of TRPM2 channels, H_2O_2 or *N*-methyl-*N´*-nitro-*N*-nitroguanidine (MNNG) were unable to gate the channels in cells overexpressing NUDT9 [17]. Besides confirming the role of ADP-ribose, the results also indicated that, under these conditions, ADP-ribose pyrophosphatase was not inactivated by H_2O_2, at least not to the extent of preventing the rise in ADP-ribose concentration up to the levels needed to gate TRPM2 channels. This does not rule out an inhibitory effect of H_2O_2 as, in cells overexpressing NUDT9, the residual activity of the enzyme could still be sufficient to keep ADP-ribose at low concentration. This could be different in cells with normal enzyme levels. Therefore, the in vivo inhibition of ADP-ribose pyrophosphatase by oxidative stress, and the consequences that could derive from it, are still open questions.

The lack of inhibition of the Mn^{2+}-dependent ADP-ribose pyrophosphatase activity by fluoride, in contrast to the widely demonstrated inhibition of Mg^{2+}-dependent nudix hydrolase activities, tends to support the view that fluoride inhibition of this enzyme family is due to the formation of a tight-binding, Mg^{2+}-containing transition state analog of the form $X–Mg^{2+}(F^–)_3–O–Y$ where, in this case, X is ribose-5-

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phosphate and Y is AMP [53]. Lack of fluoride inhibition in the presence of activating ions other than Mg^{2+} is likely to be commonplace among the nudix hydrolases.

In summary, a low-K_m ADP-ribose pyrophosphatase, such as NUDT9, is well suited to participate in the control of the levels of ADP-ribose as a second messenger, and also to protect cells from the accumulation of this potentially toxic metabolite. On the one hand, ADP-ribose pyrophosphatases terminate the modulation of ADP-ribose-dependent ion channels, because neither AMP nor ribose 5-phosphate is recognized as an activator [16, 18]. On the other hand, the hydrolysis of the pyrophosphate linkage of ADP-ribose diminishes the impact of toxic reactions, because ribose 5-phosphate, the reaction product containing the aldehyde moiety, is much less prone to non-enzymic glycation of proteins and becomes separated from the AMP moiety that can be recognized by many protein sites [2]. Finally, as described by us and by others, ADP-ribose pyrophosphatase itself is a target of cytotoxic agents (this work and [25, 28, 37, 40-42]) some of which are known to increase NAD consumption [48-51]. Determining whether these compounds actually induce ADP-ribose accumulation both by increasing its production and by inhibiting its degradation, and which of the two mechanisms prevails in each case may be important for the development of strategies to prevent or diminish their cytotoxicity.

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References

[1] H. Hilz, ADP-ribose. A historical overview, Adv. Exp. Med. Biol. 419 (1997) 15-24.

[2] D. Cervantes-Laurean, E.L. Jacobson, M.K. Jacobson, Glycation and glycoxidation of histones by ADP-ribose, J. Biol. Chem. 271 (1996) 10461-10469.

[3] I. Just, P. Wollenberg, J. Moss, K. Aktories, Cysteine-specific ADP-ribosylation of actin, Eur. J. Biochem. 221 (1994) 1047-1054.

[4] E.L. Jacobson, D. Cervantes-Laurean, M.K. Jacobson, Glycation of proteins by ADP-ribose, Mol. Cell. Biochem. 138 (1994) 207-212.

[5] E.L. Jacobson, D. Cervantes-Laurean, M.K. Jacobson, ADP-ribose in glycation and glycoxidation reactions, Adv. Exp. Med. Biol. 419 (1997) 371-379.

[6] A. Zahradnikova, J. Bak, L.G. Meszaros, Heterogeneity of the cardiac calcium release channel as assessed by its response to ADP-ribose, Biochem. Biophys. Res. Commun. 210 (1995) 457-463.

[7] Y.G. Kwak, S.K. Park, U.H. Kim, M.K. Han, J.S. Eun, K.P. Cho, S.W. Chae, Intracellular ADP-ribose inhibits ATP-sensitive K^+ channels in rat ventricular myocytes, Am. J. Physiol. 271 (1996) C464-468.

[8] M. Wilding, G.L. Russo, A. Galione, M. Marino, B. Dale, ADP-ribose gates the fertilization channel in ascidian oocytes, Am. J. Physiol. 275 (1998) C1277-1283.

[9] A.L. Perraud, A. Fleig, C.A. Dunn, L.A. Bagley, P. Launay, C. Schmitz, A.J. Stokes, Q. Zhu, M.J. Bessman, R. Penner, J.P. Kinet, A.M. Scharenberg, ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology, Nature 411 (2001) 595-599.

[10] Y. Sano, K. Inamura, A. Miyake, S. Mochizuki, H. Yokoi, H. Matsushime, K. Furuichi, Immunocyte Ca^{2+} influx system mediated by LTRPC2, Science 293 (2001) 1327-1330.

[11] B. Bastide, K. Snoeckx, Y. Mounier, ADP-ribose stimulates the calcium release channel RyR1 in skeletal muscle of rat, Biochem. Biophys. Res. Commun. 296 (2002) 1267-1271.

[12] P.L. Li, D.X. Zhang, Z.D. Ge, W.B. Campbell, Role of ADP-ribose in 11,12-EET-induced activation of K_{Ca} channels in coronary arterial smooth muscle cells, Am. J. Physiol. Heart Circ. Physiol. 282 (2002) H1229-1236.

ACCEPTED MANUSCRIPT

[13] B. Campo, A. Surprenant, R.A. North, Sustained depolarization and ADP-ribose activate a common ionic current in rat peritoneal macrophages, J. Immunol. 170 (2003) 1167-1173.

[14] A. Gerth, K. Nieber, N.J. Oppenheimer, S. Hauschildt, Extracellular NAD^+ regulates intracellular free calcium concentration in human monocytes, Biochem. J. 382 (2004) 849-856.

[15] F.J. Kühn, A. Lückhoff, Sites of the NUDT9-H domain critical for ADP-ribose activation of the cation channel TRPM2, J. Biol. Chem. 279 (2004) 46431-46437.

[16] F.J. Kühn, I. Heiner, A. Lückhoff, TRPM2: a calcium influx pathway regulated by oxidative stress and the novel second messenger ADP-ribose, Pflugers Arch. 451 (2005) 212-219.

[17] A.L. Perraud, C.L. Takanishi, B. Shen, S. Kang, M.K. Smith, C. Schmitz, H.M. Knowles, D. Ferraris, W. Li, J. Zhang, B.L. Stoddard, A.M. Scharenberg, Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels, J. Biol. Chem. 280 (2005) 6138-6148.

[18] M. Kolisek, A. Beck, A. Fleig, R. Penner, Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels, Mol. Cell 18 (2005) 61-69.

[19] A. Gasser, G. Glassmeier, R. Fliegert, M.F. Langhorst, S. Meinke, D. Hein, S. Krueger, K. Weber, I. Heiner, N. Oppenheimer, J.R. Schwarz, A.H. Guse, Activation of T cell calcium influx by the second messenger ADP-ribose, J. Biol. Chem. 281 (2006) 2489-2496.

[20] M.J. Bessman, D.N. Frick, S.F. O'Handley, The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes, J. Biol. Chem. 271 (1996) 25059-25062.

[21] A.G. McLennan, The Nudix hydrolase superfamily, Cell. Mol. Life Sci. 63 (2006) 123-143.

[22] D. Bernet, R.M. Pinto, M.J. Costas, J. Canales, J.C. Cameselle, Rat liver mitochondrial ADP-ribose pyrophosphatase in the matrix space with low K_m for free ADP-ribose, Biochem. J. 299 (1994) 679-682.

[23] J. Canales, R.M. Pinto, M.J. Costas, M.T. Hernández, A. Miró, D. Bernet, A. Fernández, J.C. Cameselle, Rat liver nucleoside diphosphosugar or diphosphoalcohol pyrophosphatases different from nucleotide pyrophosphatase or

ACCEPTED MANUSCRIPT

phosphodiesterase I: substrate specificities of Mg^{2+}-and/or Mn^{2+}-dependent hydrolases acting on ADP-ribose, Biochim. Biophys. Acta 1246 (1995) 167-177.

[24] J.S. Kim, W.Y. Kim, H.W. Rho, J.W. Park, B.H. Park, M.K. Han, U.H. Kim, H.R. Kim, Purification and characterization of adenosine diphosphate ribose pyrophosphatase from human erythrocytes, Int. J. Biochem. Cell Biol. 30 (1998) 629-638.

[25] J.M. Ribeiro, A. Carloto, M.J. Costas, J.C. Cameselle, Human placenta hydrolases active on free ADP-ribose: an ADP-sugar pyrophosphatase and a specific ADP-ribose pyrophosphatase, Biochim. Biophys. Acta 1526 (2001) 86-94.

[26] L. Gasmi, J.L. Cartwright, A.G. McLennan, Cloning, expression and characterization of YSA1H, a human adenosine 5'-diphosphosugar pyrophosphatase possessing a MutT motif, Biochem. J. 344 (1999) 331-337.

[27] H. Yang, M.M. Slupska, Y.F. Wei, J.H. Tai, W.M. Luther, Y.R. Xia, D.M. Shih, J.H. Chiang, C. Baikalov, S. Fitz-Gibbon, I.T. Phan, A. Conrad, J.H. Miller, Cloning and characterization of a new member of the Nudix hydrolases from human and mouse, J. Biol. Chem. 275 (2000) 8844-8853.

[28] S. Lin, L. Gasmi, Y. Xie, K. Ying, S. Gu, Z. Wang, H. Jin, Y. Chao, C. Wu, Z. Zhou, R. Tang, Y. Mao, A.G. McLennan, Cloning, expression and characterisation of a human Nudix hydrolase specific for adenosine 5'-diphosphoribose (ADP-ribose), Biochim. Biophys. Acta 1594 (2002) 127-135.

[29] A.L. Perraud, B. Shen, C.A. Dunn, K. Rippe, M.K. Smith, M.J. Bessman, B.L. Stoddard, A.M. Scharenberg, NUDT9, a member of the Nudix hydrolase family, is an evolutionarily conserved mitochondrial ADP-ribose pyrophosphatase, J. Biol. Chem. 278 (2003) 1794-1801.

[30] L.A. Rafty, M.T. Schmidt, A.L. Perraud, A.M. Scharenberg, J.M. Denu, Analysis of *O*-acetyl-ADP-ribose as a target for Nudix ADP-ribose hydrolases, J. Biol. Chem. 277 (2002) 47114-47122.

[31] B.N. Ames, Assay of inorganic phosphate, total phosphate and phosphatases, Methods Enzymol. 8 (1966) 115-118.

[32] A. Miró, M.T. Hernández, M.J. Costas, J.C. Cameselle, Enzyme saturation and inhibition kinetics studied from multiple progress curves recorded spectrophotometrically from single reaction mixtures for ADP-ribose pyrophosphatase, J. Biochem. Biophys. Methods 22 (1991) 177-184.

ACCEPTED MANUSCRIPT

[33] W.W. Cleland, The statistical analysis of enzyme kinetic data, Adv. Enzymol. Relat. Areas Mol. Biol. 29 (1967) 1-32.

[34] T. Yagi, E. Baroja-Fernández, R. Yamamoto, F.J. Muñoz, T. Akazawa, K.S. Hong, J. Pozueta-Romero, Cloning, expression and characterization of a mammalian Nudix hydrolase-like enzyme that cleaves the pyrophosphate bond of UDP-glucose, Biochem. J. 370 (2003) 409-415.

[35] A. Guranowski, A. Sillero. Enzymes cleaving dinucleoside polyphosphates, in: A.G. McLennan (Ed.) Ap_4A and other dinucleoside polyphosphates, CRC Press, Boca Raton, FL, 1992, pp. 81-134.

[36] M. Bollen, R. Gijsbers, H. Ceulemans, W. Stalmans, C. Stefan, Nucleotide pyrophosphatases/phosphodiesterases on the move, Crit. Rev. Biochem. Mol. Biol. 35 (2000) 393-432.

[37] A. Fernández, J.M. Ribeiro, M.J. Costas, R.M. Pinto, J. Canales, J.C. Cameselle, Specific ADP-ribose pyrophosphatase from *Artemia* cysts and rat liver: effects of nitroprusside, fluoride and ionic strength, Biochim. Biophys. Acta 1290 (1996) 121-127.

[38] M.Y. Galperin, O.V. Moroz, K.S. Wilson, A.G. Murzin, House cleaning, a part of good housekeeping, Mol. Microbiol. 59 (2006) 5-19.

[39] B.W. Shen, A.L. Perraud, A. Scharenberg, B.L. Stoddard, The crystal structure and mutational analysis of human NUDT9, J. Mol. Biol. 332 (2003) 385-398.

[40] J.M. Ribeiro, J.C. Cameselle, A. Fernández, J. Canales, R.M. Pinto, M.J. Costas, Inhibition and ADP-ribose pyrophosphatase-I by nitric-oxide-generating systems: a mechanism linking nitric oxide to processes dependent on free ADP-ribose, Biochem. Biophys. Res. Commun. 213 (1995) 1075-1081.

[41] J.M. Ribeiro, A. Agudo, M.J. Costas, J.C. Cameselle, Rat liver ADP-ribose pyrophosphatase-I as an in vitro target of the acetaminophen metabolite *N*-acetyl-*p*-benzoquinoneimine, Biochim. Biophys. Acta 1336 (1997) 403-408.

[42] J.M. Ribeiro, M.J. Costas, J.C. Cameselle, ADP-ribose pyrophosphatase-I partially purified from livers of rats overdosed with acetaminophen reveals enzyme inhibition in vivo reverted in vitro by dithiothreitol, J. Biochem. Mol. Toxicol. 13 (1999) 171-177.

[43] K. Olejnik, E. Kraszewska, Cloning and characterization of an *Arabidopsis thaliana* Nudix hydrolase homologous to the mammalian GFG protein, Biochim. Biophys. Acta 1752 (2005) 133-141.

ACCEPTED MANUSCRIPT

[44] M.J. Davies, The oxidative environment and protein damage, Biochim. Biophys. Acta 1703 (2005) 93-109.

[45] R.A. Houghten, C.H. Li, Reduction of sulfoxides in peptides and proteins, Methods Enzymol. 91 (1983) 549-559.

[46] D.G. Kehres, M.E. Maguire, Emerging themes in manganese transport, biochemistry and pathogenesis in bacteria, FEMS Microbiol. Rev. 27 (2003) 263-290.

[47] D. Ghosal, M.V. Omelchenko, E.K. Gaidamakova, V.Y. Matrosova, A. Vasilenko, A. Venkateswaran, M. Zhai, H.M. Kostandarithes, H. Brim, K.S. Makarova, L.P. Wackett, J.K. Fredrickson, M.J. Daly, How radiation kills cells: survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress, FEMS Microbiol. Rev. 29 (2005) 361-375.

[48] C. Richter, Control of the pro-oxidant-dependent calcium release from intact liver mitochondria, Redox Report 2 (1996) 217-221.

[49] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 3rd edn, Oxford University Press, Oxford, 1999.

[50] S.W. Yu, H. Wang, M.F. Poitras, C. Coombs, W.J. Bowers, H.J. Federoff, G.G. Poirier, T.M. Dawson, V.L. Dawson, Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor, Science 297 (2002) 259-263.

[51] L. Du, X. Zhang, Y.Y. Han, N.A. Burke, P.M. Kochanek, S.C. Watkins, S.H. Graham, J.A. Carcillo, C. Szabo, R.S. Clark, Intra-mitochondrial poly(ADP-ribosylation) contributes to NAD^+ depletion and cell death induced by oxidative stress, J. Biol. Chem. 278 (2003) 18426-18433.

[52] M. Ziegler, New functions of a long-known molecule. Emerging roles of NAD in cellular signaling, Eur. J. Biochem. 267 (2000) 1550-1564.

[53] A.S. Mildvan, Z. Xia, H.F. Azurmendi, V. Saraswat, P.M. Legler, M.A. Massiah, S.B. Gabelli, M.A. Bianchet, L.W. Kang, L.M. Amzel, Structures and mechanisms of Nudix hydrolases, Arch. Biochem. Biophys. 433 (2005) 129-143.

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LEGENDS TO THE FIGURES

Fig. 1. Functional identity of human ADP-ribose pyrophosphatase purified from placenta and expressed from pQE-NUDT9α: responses to Mg^{2+} and Mn^{2+} as activating cations and effects of treatment with H_2O_2. Enzyme samples obtained in DTT-containing buffers were mock-treated (control) or treated at 4°C for 3 h with 1 mM H_2O_2, as described in Section 2.3. Enzyme activity was determined by the standard assay (except in panel D) with the specifications indicated below. Data in each panel are from one experiment representative of at least three experiments done with two or more different enzyme preparations. Activities are expressed relative to that of the control sample assayed with ADP-ribose (0.35 mM) in the presence of 5 mM $MgCl_2$ (around 30 mU/ml and 50 mU/ml for tissue and recombinant enzyme, respectively).

(A) Change of divalent cation specificity by H_2O_2 treatment. Activities were measured in the presence of the indicated concentrations of $MgCl_2$ (open squares) or $MnCl_2$ (closed squares).

(B) Reversibility by DTT of the change of divalent cation specificity. After mock- or H_2O_2-treatment, samples were divided in two halves, one of which was pre-incubated at 4°C for 2 h with 1 mM DTT prior to assaying for activity.

(C) Effect of H_2O_2 treatment on substrate specificity. Assays were carried out with 0.35 mM of the indicated compound in the presence of the indicated activating cation.

(D) Effect of H_2O_2 treatment on the saturation kinetics for ADP-ribose. Assays were done with the indicated activating cation. K_m values above 5 µM were derived from IRD assays; those below 5 µM from FPC assays (see Section 2.2). Results are expressed as ± S.E. for the non-linear regression adjustment [33].

(E) Inhibition by fluoride of the Mg^{2+}- but not the Mn^{2+}-dependent activity. Assays were done in the presence of 5 mM $MgCl_2$ (open squares), 50 µM $MnCl_2$ (closed triangles) or 5 mM $MnCl_2$ (closed squares), and the indicated KF concentration.

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Fig. 2. Substrate saturation curves showing the submicromolar K_m values of human ADP-ribose pyrophosphatase purified from placenta and expressed from pQE-NUDT9α. Rates at different substrate concentrations were derived from full progress curves (FPC assay; see Section 2.2). To facilitate comparison between the saturation curves, the Y-axis upper border was fixed at the V_{max} value in each panel. The K_m values obtained by non-linear regression were 0.58 ± 0.05 μM for the tissue, and 0.67 ± 0.07 μM for the recombinant enzyme. The inserts show Eadie-Hofstee plots of the same data.

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FIGURE 1

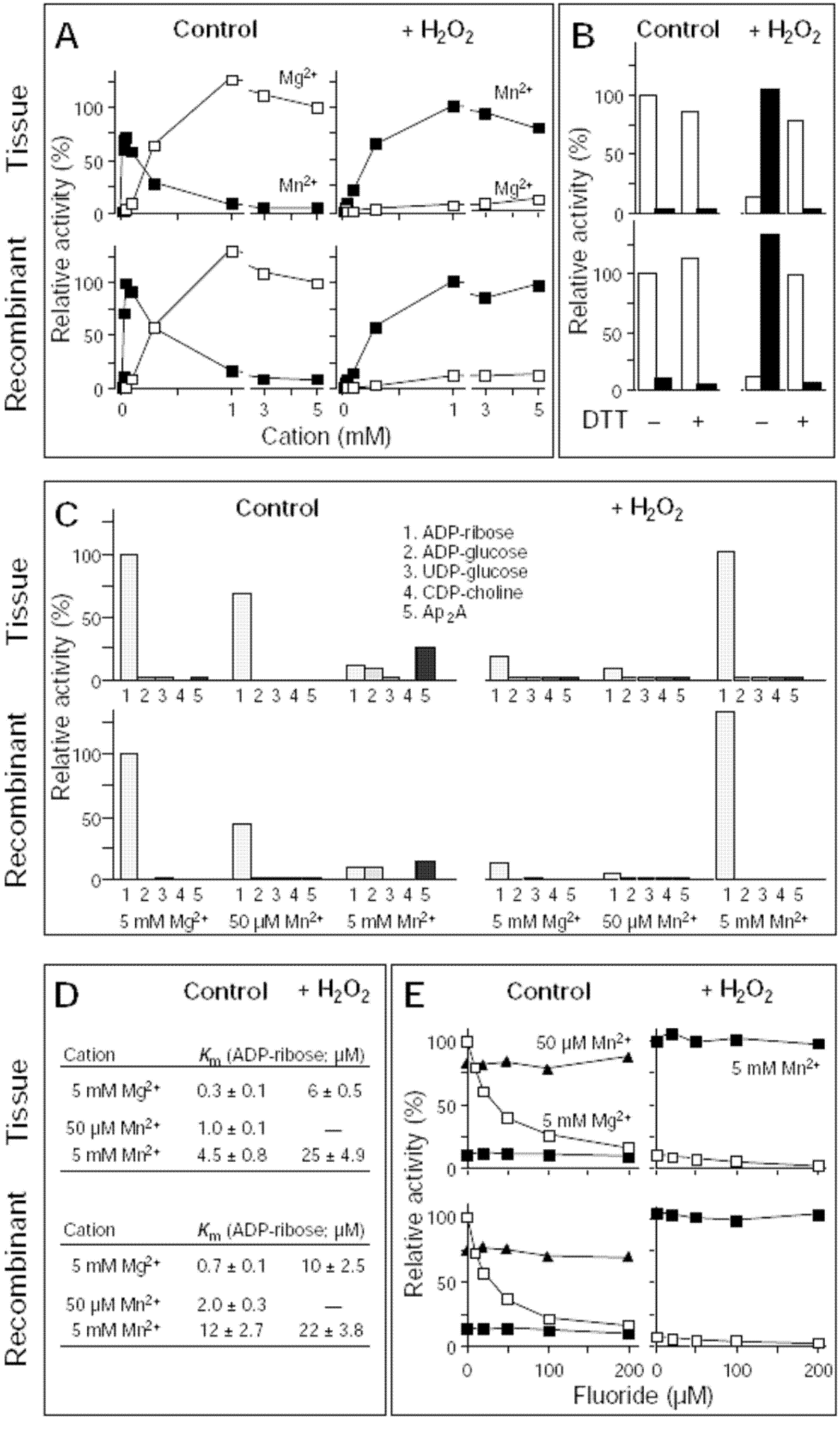
A
Control
+ H2O2
Tissue
Recombinant
Mg2+
Mn2+
Relative activity (%)
Cation (mM)
B
Control
+ H2O2
Relative activity (%)
DTT
C
Control
+ H2O2
1. ADP-ribose
2. ADP-glucose
3. UDP-glucose
4. CDP-choline
5. Ap2A
Relative activity (%)
5 mM Mg2+
50 µM Mn2+
5 mM Mn2+
D
Control
+ H2O2
Cation
Km (ADP-ribose; µM)
5 mM Mg2+ 0.3 ± 0.1 6 ± 0.5
50 µM Mn2+ 1.0 ± 0.1 —
5 mM Mn2+ 4.5 ± 0.8 25 ± 4.9
5 mM Mg2+ 0.7 ± 0.1 10 ± 2.5
50 µM Mn2+ 2.0 ± 0.3 —
5 mM Mn2+ 12 ± 2.7 22 ± 3.8
E
Control
+ H2O2
50 µM Mn2+
5 mM Mn2+
5 mM Mg2+
Relative activity (%)
Fluoride (µM)

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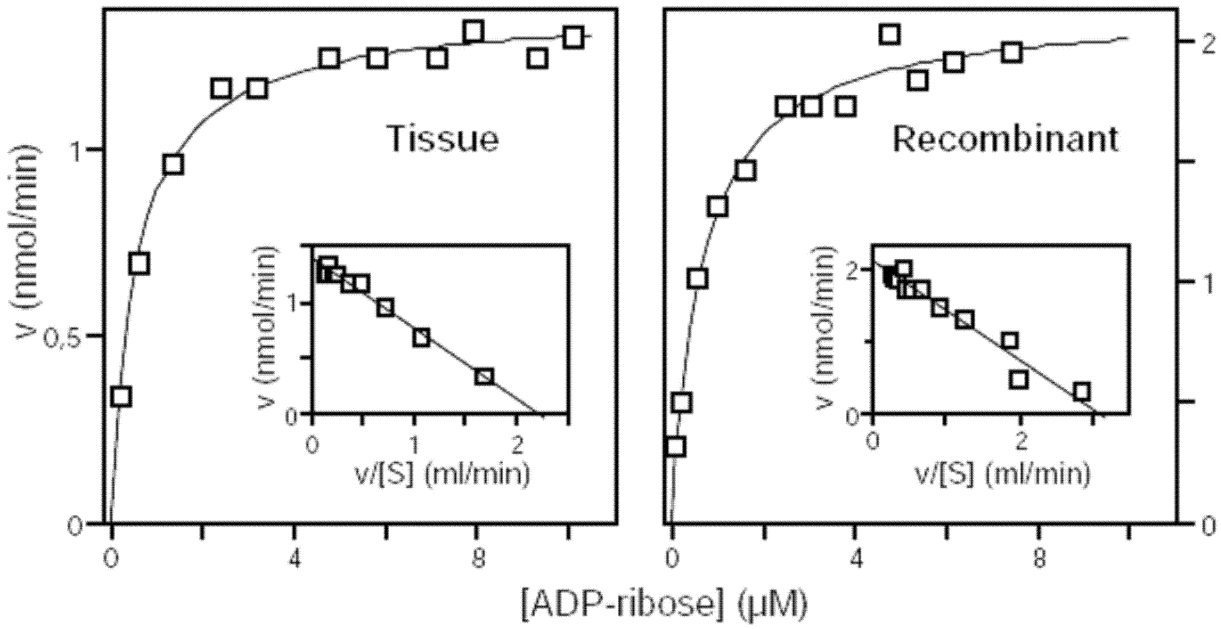
FIGURE 2



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