Nudix Hydrolases That Degrade Dinucleoside and Diphosphoinositol Polyphosphates Also Have 5-Phosphoribosyl 1-Pyrophosphate (PRPP) Pyrophosphatase Activity That Generates the Glycolytic Activator Ribose 1,5-Bisphosphate*

A total of 17 Nudix hydrolases were tested for their ability to hydrolyze 5-phosphoribosyl 1-pyrophosphate (PRPP). All 11 enzymes that were active toward dinucleoside polyphosphates with 4 or more phosphate groups as substrates were also able to hydrolyze PRPP, whereas the 6 that could not and that have coenzyme A (CoA), NDP-sugars, or pyridine nucleotides as preferred substrates did not degrade PRPP. The products of hydrolysis were ribose 1,5-bisphosphate and P_i. Active PRPP pyrophosphatases included the diphosphoinositol polyphosphate phosphohydrolase (DIPP) subfamily of Nudix hydrolases, which also degrade the non-nucleotide substrates. Km and kcat values for PRPP hydrolysis for the Deinococcus radiodurans DR2356 enzyme and for the DIPP subfamily were ribose 1,5-bisphosphate and P_i. Active site mutants of the Caenorhabditis elegans diadenosine tetraphosphate hydrolase had no activity, confirming that the same active site is responsible for nucleotide and PRPP hydrolysis. Comparison of the specificity constants for nucleotide, diphosphoinositol polyphosphate, and PRPP hydrolysis suggests that PRPP is a significant substrate for the D. radiodurans DR2356 enzyme and for the DIPP subfamily. In the latter case, generation of the glycolytic activator ribose 1,5-bisphosphate may be a new function for these enzymes.

The Nudix hydrolases are members of an enzyme family that was named after their ability to hydrolyze predominantly the pyrophosphate linkage in a variety of compounds having the general structure of a nucleoside diphosphate (Npp) linked to another moiety, X, with varying degrees of specificity (1, 2). Thus, nucleoside triphosphates (Npp-p), dinucleoside polyphosphates (Npp-p,

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1-Pyrophosphate (PRPP) Pyrophosphatase Activity That Generates the Glycolytic Activator Ribose 1,5-Bisphosphate*

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gesting that these compounds may be important substrates in vivo. They may be involved in the regulation of vesicle trafficking (14), apoptosis (15), DNA repair (16), and in vacuole biogenesis and environmental stress responses in yeast (17); hence, the DIPP Nuddix hydrolases have also been implicated in these processes.

The ability of this subset of Nudix hydrolases to utilize a sugar pyrophosphate as a substrate prompted us to test another such compound of known biological importance, 5-phosphoribosyl 1-pyrophosphate (PRPP). PRPP is both a substrate and regulator of purine, pyrimidine, and pyridine nucleotide synthesis (18–21); in bacteria and lower eukaryotes it is also a precursor for histidine and tryptophan biosynthesis (22–25). Furthermore, a potential product of pyrophosphatase activity acting upon PRPP is ribose 1,5-bisphosphate (Rib-1,5-P₂), which has recently been shown to be a physiological regulator of glycolysis and the fructose 6-phosphate/fructose 1,6-bisphosphate cycle (26–28). Here, we show that Nuddix hydrolases of the DIPP subfamily and the related Ap₄A hydrolases all exhibit PRPP pyrophosphatase activity, whereas Nuddix hydrolases previously shown to be specific for NDP-sugars, pyridine nucleotides, and coenzyme A are unable to hydrolyze PRPP.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human Aps1 (DIPP-3β) and Aps2 (DIPP-3α) (10), S. cerevisiae Ddp1p Ap₆A hydrolase (YOR163w protein) (11), and Npy1p NADPH pyrophosphatase (YGL067w protein) (29), Caenorhabditis elegans Ap₄A hydrolase (3), African Swine Fever virus g5R protein (13), human NUDT5 ADP-sugar hydrolase (30), NUDT9 ADP-ribose hydrolase (31), and mouse Nudt7 coenzyme A pyrophosphatase (32) were prepared as previously described. Active site mutants of the C. elegans Ap₄A hydrolase (ES5Q and ES6Q) were a gift from H. Abdelghany. The YgdP Ap₄A hydrolase from Salmonella typhimurium was a gift from T. Ismail. Recombinant human DIPP-1, DIPP-2, and -2β were prepared as GST-fusion proteins as described for hAps1 and hAps2 (10). Recombinant human Ap₄A hydrolase, Deinococcus radiodurans coenzyme A pyrophosphatase (DR1184 gene product), and D. radiodurans Ap₄A hydrolase (DR2356 gene product) were prepared by procedures similar to those used for the C. elegans Ap₄A hydrolase. PRPP and all nucleotides were from Sigma. The EnzChek phosphatase assay kit was from Molecular Probes.

MATERIALS AND METHODS

Assay of PRPP Pyrophosphatase Activity—Initial screening of enzyme preparations for their ability to release Pᵢ from PRPP was carried out using a phosphomolybdate colorimetric assay (33). Purified enzymes (5 µg) were incubated for 15 min at 37 °C in 50 mM Tris-HCl, pH 8.0, 5 mM Mg acetate, 1 mM dithiothreitol, and 0.2 mM PRPP in a total volume of 200 µl. The reactions stopped by the addition of the molybdate detection reagent. Enzymes testing negative in an initial assay were omitted from further characterizations.

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HPLC Analysis of PRPP Hydrolysis Products—The products of PRPP hydrolysis by the D. radiodurans Ap₄A hydrolase were generated by incubation of 0.2 mM PRPP with 7 µg of enzyme in 50 mM Tris-HCl, pH 8.0, 5 mM Mg acetate, 1 mM dithiothreitol for 10 min at 37 °C.
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Table II

Kinetic constants for substrate hydrolysis by selected Nudix hydrolases

| Enzyme                  | Substrate | Conc. in vivo (S.) | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) | S from \( k_{cat}/K_m \) |
|-------------------------|-----------|-------------------|----------|---------------|------------------|------------------------|
| *D. radiodurans* ApnA hydrolase | PRPP      | \( 10^{-3} \)     | \( 10^{-3} \) | 1.5          | \( 1.5 \times 10^2 \) | 1.5                    |
|                         | ApnA      | \( 10^{-6} \)     | \( 3.0 \times 10^{-5} \) | 0.035 | \( 1.2 \times 10^2 \) | 1.2 \times 10^{-3} |
|                         | PRPP      | \( 10^{-4} \)     | \( 1.3 \times 10^{-4} \) | 0.057 | \( 4.4 \times 10^2 \) | 0.044                  |
| Human ApnA hydrolase    | ApnA      | \( 10^{-6} \)     | \( 5.0 \times 10^{-7} \) | 5.0  | \( 10^7 \)     | 10                     |
| Human DIPP-1            | ApnA      | \( 10^{-5} \)     | \( 5.9 \times 10^{-6} \) | 1.0  | \( 2.6 \times 10^7 \) | 0.26                   |
|                         | PRPP      | \( 10^{-6} \)     | \( 4.2 \times 10^{-9} \) | 0.2  | \( 4.7 \times 10^7 \) | 47.4                   |

enzyme toward ApnA. Compared with the wild type value of 23 s\(^{-1}\), the *C. elegans* E52Q and E56Q mutants have \( k_{cat} \) values for ApnA of 0.0052 and 0.00024 s\(^{-1}\), respectively. They are also completely inactive with PRPP. This establishes that the same active site is responsible for ApnA and PRPP hydrolysis. The same is assumed to hold true for the other enzymes.

\( K_m \) and \( k_{cat} \) values for PRPP were determined for one prokaryotic ApnA hydrolase, one eukaryotic ApnA hydrolase, and one eukaryotic ApnA hydrolase/DIPP, using a continuous spectrophotometric assay. *D. radiodurans* DR2356 ApnA hydrolase is an enzyme that hydrolyzes a variety of dinucleoside and dinucleoside polyphosphates, including p1A, p2A, ApnA, Ap4A, and Ap5A. It has no activity toward diphosphoinositol polyphosphates; however, it readily hydrolyzes PRPP with a \( K_m \) of 1 mM and a \( k_{cat} \) of 1.5 s\(^{-1}\) (Table II). For comparison, \( K_m \) and \( k_{cat} \) values for ApnA were determined to be 30 mM and 0.035 s\(^{-1}\), respectively, resulting in similar specificity constants \((k_{cat}/K_m)\) for both substrates (Table II). Human ApnA hydrolase also hydrolyzed PRPP with a \( k_{cat} \) of 0.057 s\(^{-1}\) and a \( K_m \) of 0.13 mM (Table II). In this case, however, the specificity constant with PRPP was some 20,000-fold lower than that found with ApnA as substrate. Finally, human DIPP-1 was the most efficient of the three enzymes at PRPP hydrolysis in vitro with a \( k_{cat} \) of 1.0 s\(^{-1}\) and a \( K_m \) of 0.38 mM (Table II). The specificity constant with PRPP of 2,800 was 325- and 18,000-fold lower than those previously measured with ApnA and PP-InsP\(_5\), respectively (5). The physiological significance of these data is discussed below.

Identification of Rib-1-P as the Product of PRPP Hydrolysis—Because all Nudix hydrolases cleave pyrophosphate linkages, it was anticipated that they would remove the \( \beta \)-phosphate from the pyrophosphate moiety attached to the ribose C1. Using the *D. radiodurans* DR2356 ApnA hydrolase as an example enzyme, the products of hydrolysis were first separated by anion-exchange HPLC, fractions collected and incubated with alkaline phosphatase, and the Pi released dete-
rated by anion-exchange HPLC, fractions collected and incubated with alkaline phosphatase, and the Pi released determined colorimetrically. Two products, A and B, were observed to co-chromatographed with Pi and PPi, respectively (Fig. 1). Peak area integration showed the ratio of phosphate released of product B was subjected to TLC before and after acid treatment. Acid removes phosphate from the anomeric C1 but not from C5, and ribose and derivatives with an unesterified C1 hydroxyl can be detected after TLC by AgNO\(_3\) treatment (38). Fig. 2 shows the TLC plate after AgNO\(_3\) treatment. It can clearly be seen that product B was not detected by AgNO\(_3\) before acid treatment because it has an esterified C1-OH (lane C); however, after acid treatment, product B generated a visible spot that co-chromatographed with Rib-5-P and acetylated PRPP (lane F). This indicates that product B must be Rib-1,5-P\(_2\). This identification was confirmed by positive ion electrospray mass spectrometry. B had a mass of 333 Da, correspond-
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Fig. 3. A model of PRPP in the substrate-binding site of the C. elegans Ap4A hydrolase. PRPP was modeled into the substrate-binding site of the C. elegans Ap4A hydrolase (PDB entry 1KT9) using Turbo-Frodo software and the PRPP coordinates taken from the ternary complex of hypoxanthine guanine phosphoribosyltransferase-PRPP-9-deazaguanosine (PDB entry 1FSG). The positions of Tyr76 and Tyr121, which bind one adenine ring between them in the previously described binary complex, and of Glu23, one of the catalytic Glu residues in the loop-helix-loop active site motif, are indicated. Phosphoryl groups of PRPP are shown as 5-P, P, and Pβ.

The results reported here are important for two reasons. First, they have implications for the specificity and physiological function(s) of Nudix hydrolases, particularly the DIPPs and to the ability of the DIPPs to bind diphosphoinositol polyphosphates. This model also explains why the Nudix hydrolases with specificities for NADH, NDP-sugars, and coenzyme A do not accept PRPP as a substrate. These enzymes do not have a second phosphate binding site (like P1) located the required distance away from the catalytic binding site (like P4). Only enzymes able to hydrolyze nucleotide substrates with four or more phosphates in the polyphosphate chain should bind PRPP. These results emphasize the point that certain Nudix hydrolases can also accept non-nucleotide substrates and should prompt the search for other such substrates that satisfy these minimal binding requirements.

Is PRPP a physiologically relevant substrate for any of the enzyme studies here? According to the measured specificity constants, at least for the eukaryotic enzymes, the diadenosine and diphosphoinositol polyphosphates appear to be highly favored over PRPP. However, this does not take into account the relative substrate concentrations in vivo. Literature values for the intracellular concentration of PRPP vary considerably and have been reported in different units. However, taking various measurements for prokaryotes (25, 40, 41) and eukaryotes (20, 26, 42–44) and applying the unit conversion factors of Traut (45) suggests that prokaryotes typically have a PRPP steady-state concentration of around 1 mM, whereas in eukaryotes (excluding erythrocytes) it is 1–2 orders of magnitude lower, although it can be as high as 0.4 mM in mouse fibroblasts deficient in adenine and hypoxanthine phosphoribosyltransferases (43, 45). The measured Km values for PRPP for all three enzymes are, therefore, within acceptable ranges if PRPP were to be considered a physiologically relevant substrate. The intracellular concentration of Ap4A in unstimulated cells is typically 0.1–1.0 μM (46), whereas PP-InsP3 may be in the low micromolar range (47). There are no measurements of cytoplasmic Ap4A in mammalian cells; an estimate of 32 nM in platelets is an intracellular average because the Ap4A is concentrated in the dense granules (48). Indeed, it is likely to be even lower than the sole measured value for Ap4A of 4 nM in Schizosaccharomyces pombe (52). Taking the product of the specificity constant and substrate concentration as a better indication of potential substrate utilization in vivo shows that PRPP hydrolysis is likely to be a much more significant reaction in vivo for the D. radiodurans Ap4A hydrolase than is Ap4A hydrolysis (Table II). In contrast, the human Ap4A hydrolase is more likely to act upon Ap4A than on PRPP. For DIPP-1, PP-InsP3 still appears to be the favored substrate by virtue of its ex-
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extremely low $K_m$ for this compound. Nevertheless, the combined activities of members of the DIPPR subfamily could still have a significant impact on PRPP hydrolysis in vivo in tissues where more than one is expressed.

PRPP pyrophosphatase activity in cell extracts has been detected before. Divalent ion-dependent (49) and -independent (50) activities were ascribed to acid and alkaline phosphatase, respectively. Like the spontaneous degradation of PRPP, these reactions are believed to proceed via 1,2 and 1,5 cyclic derivatives to Rib-1-P and -5-P and ultimately to ribose with Rib-1,5-P$_2$ as a possible minor intermediate in one pathway (37, 38). In view of the established mechanisms of Nudix hydrolases and the fact that Ap±H$_2$O hydrolysis is known to proceed by direct in-line attack of water on the C1- or H9251 phosphoryl group, this molecule is a potent activator of phosphofructokinase.

The rapid rise in Rib-1,5-P$_2$ that occurs in macrophages under hypoxic conditions in parallel with the switch to anaerobic glycolysis is due to a rise in PRPP accompanied by the activation of an unidentified PRPP pyrophosphatase. This activity appears to be divalent ion-independent and may be activated by protein kinase C. Its possible relationship to any of the mammalian Nudix PRPP pyrophosphatases described here is unknown. Nevertheless, it is clear that, at least in mammalian cells, several Nudix hydrolases exist that have the ability to generate Rib-1,5-P$_2$ from PRPP. Like fructose 2,6-bisphosphate, this molecule is a potent activator of phosphofructokinase, also an inhibitor of fructose 1,6-bisphosphatase, and is a potent activator of phosphofructokinase 

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