Nicotinamide Riboside and Nicotinic Acid Riboside Salvage in Fungi and Mammals

QUANTITATIVE BASIS FOR Urh1 AND PURINE NUCLEOSIDE PHOSPHORYLASE FUNCTION IN NAD\(^+\) METABOLISM\(^*\)

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NAD\(^+\) is a co-enzyme for hydride transfer enzymes and an essential substrate of ADP-ribose transfer enzymes and sirtuins, the type III protein lysine deacetylases related to yeast Sir2. Supplementation of yeast cells with nicotinamide riboside extends replicative lifespan and increases Sir2-dependent gene silencing by virtue of increasing net NAD\(^+\) synthesis. Nicotinamide riboside elevates NAD\(^+\) levels via the nicotinamide riboside kinase pathway and by a pathway initiated by splitting the nucleoside into a nicotinamide base followed by nicotinamide salvage. Genetic evidence has established that uridine hydrolase, purine nucleoside phosphorylase, and methylthioadenosine phosphorylase are required for Nrk-independent utilization of nicotinamide riboside in yeast. Here we show that mammalian purine nucleoside phosphorylase but not methylthioadenosine phosphorylase is responsible for mammalian nicotinamide riboside kinase-independent nicotinamide riboside utilization. We demonstrate that so-called uridine hydrolase is 100-fold more active as a nicotinamide riboside hydrolase than as a uridine hydrolase and that uridine hydrolase and mammalian purine nucleoside phosphorylase cleave nicotinic acid riboside, whereas the yeast phosphorylase has little activity on nicotinic acid riboside. Finally, we show that yeast nicotinic acid riboside utilization largely depends on uridine hydrolase and nicotinamide riboside kinase and that nicotinic acid riboside bioavailability is increased by ester modification.

NAD\(^+\) and its phosphorylated and reduced derivatives are essential co-enzymes for hydride transfer enzymes central to intermediary metabolism. NAD\(^{-}\) is also a consumed substrate of three classes of enzymes, which produce ADP-riboseyl products plus nicotinamide (Nam)\(^4\) (1). Sirtuins utilize the ADP-ribose moiety of NAD\(^+\) to accept the acetyl modification of lysine, thereby producing a deacetylated protein plus Nam and a mixture of 2'- and 3'-acetylated ADP-ribose (2–4). Such reactions are important for chromatin silencing (5) and regulation of transcription factors and enzymes, thereby controlling a variety of genomic transactions (6), metabolic switches (7, 8), and lifespan (9–11). ADP-ribose transferases and polyADP-ribose polymerases utilize NAD\(^+\) to add ADP-ribose as a post-translational modification and/or to form ADP-ribose polymers (12, 13). Finally, cyclic ADP-ribose synthases produce and hydrolyze the calcium-mobilizing compound, cADP-ribose (14, 15). Thus, via pleiotropic ways and means, NAD\(^+\) is a central mediator of cellular and organismal metabolism and signaling.

Although co-enzymatic NAD\(^+\) functions do not necessitate ongoing NAD\(^+\) synthesis, the activities of the NAD\(^+\)-consuming enzymes mandate either ongoing de novo or salvage synthesis (see Fig. 1). In yeast, de novo synthesis from tryptophan maintains intracellular NAD\(^+\) at ~0.8 mM, at which concentration cells grow well but perform Sir2-dependent gene silencing poorly and have relatively short replicative life spans (16). However, supplementation with 10 \(\mu\)M nicotinamide riboside (NR) more than doubles intracellular NAD\(^+\) and doubles replicative longevity (16). NR is imported into yeast cells by a specific, pH-dependent NR transporter, Nrt1, with a \(K_m\) of 22 \(\mu\)M (17).

NR is converted to NAD\(^+\) in two steps by nicotinamide riboside kinase (Nrk)-dependent phosphorylation and adenyllylation by nicotinamide mononucleotide adenyllyltransferase (18). Additionally, NR is split into Nam plus a ribosyl product for NAD\(^+\) synthesis through Nam salvage. Genetic analysis indicates that enzymes initially characterized for splitting other nucleosides, namely uridine hydrolase (Urh1), purine nucleoside phosphorylase (Pnp1), and methylthioadenosine phosphorylase (Meu1), are responsible for Nrk-independent NR utilization (16, 19). Nicotinic acid riboside (NaR), synthesized from nicotinic acid ribosideethyl ester, can function as an NAD\(^+\) precursor in yeast, through both an Nrk-dependent and an...
Nrk-independent pathway (20). Human Nrk1 and Nrk2 are closely related ATP- and GTP-dependent metabolite kinases, which can function in place of yeast Nrk1 (18). Both enzymes have approximately equal activity in phosphorylation of NaR and NR and exhibit a crystallographically defined nucleoside-binding site with spatial complementarity to both substrates (20).

As newly discovered eukaryotic NAD⁺ precursors, NR and NaR have the potential to be important mammalian nutritional supplements and/or drugs (19, 21). However, problems remain in understanding the nature of NR and NaR utilization in fungi and mammalian systems. Here we demonstrate that human Pnp but not human Mtap functions in two different yeast assays for NR utilization. Confirming the genetic results, we demonstrate that NR cleavage in mammalian systems is entirely phosphate-dependent and is sensitive to a specific inhibitor of Pnp. We purified yeast Pnp1 to conduct comparative enzymology with yeast and bovine enzymes. Although both enzymes convert NR to Nam with a specificity constant that is 8–13% as great as the corresponding inosine reaction, bovine but not yeast Pnp is also an NaR phosphorylase. Despite a recent claim that Uh1 is a uridine-specific nucleoside hydrolase (22), our kinetic analysis of recombinant Uh1 demonstrates a 100-fold greater specificity for NR than for uridine. Uh1 also functions in vitro and in vivo as an NaR hydrolase such that Nrk-independent NaR salvage in yeast principally depends on Uh1.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Plasmids**—Construction of yeast strains has been described (16, 17). A complete strain and plasmid list is found in the supplemental data. Yeast media were prepared as described (16).

For yeast expression, human PNP and MTAP cDNAs were cloned under the control of the yeast PNP1 promoter. The yeast PNP1 promoter was amplified using primers 14093 and 14094, which appended HindIII and KpnI restriction sites. Human PNP and MTAP coding sequences were amplified from cDNAs purchased from ATCC and Origene using the primer pair 14091 and 14092 and the primer pair 14095 and 14096, respectively, which appended KpnI and EcoRI restriction sites. The promoter and cDNA products were digested with KpnI and then ligated, and the resulting linear products were gel-purified. Plasmid pRS327 and the linear products were then digested with HindIII and EcoRI and ligated to obtain pPAB011 and pPAB008 for yeast expression of PNP and MTAP, respectively.

For bacterial expression, Pnp1 and Uh1 were fused to maltose-binding protein (MBP). The PNP1 coding sequence was amplified from DNA from yeast strain BY4742 using primers 14083 and 14084, which appended EcoRI and Bsal restriction sites. The PCR product was digested with Bsal, filled in with Klenow fragment, and then digested with EcoRI. The pMAL-c2x vector (New England Biolabs) was digested with XmnI and EcoRI and ligated with the blunt to EcoRI insert to generate plasmid pPAB006. The pMAL-URH1 plasmid pPAB003 was generated similarly, initiated with primers 14082 and 14085. DNA primer sequences are provided in the supplemental data.

**Enzyme Expression, Purification, and Kinetics**—TB1 Escherichia coli transformants carrying pPAB003 or pPAB006 were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature for 8 h. Cells (1 liter at an A₆₀₀nm of 3.8) were lysed by sonication, and the MBP fusion proteins were purified by amylose affinity chromatography and eluted with maltose according to the New England Biolabs protocol. Maltose was removed by hydroxylapatite chromatography. MBP was cleaved from the enzymes of interest with factor Xa. Uh1 and Pnp1 were then recovered from the flow-through of a second amylose column. Bovine Pnp was purchased from Molecular Probes.

Hydrolysis and/or phosphorylase of NR, uridine, NaR, and NaR methyl ester (meNaR) were measured spectroscopically at 269, 280, 260, and 260 nm, respectively, as described (23, 24). Phosphorolysis was measured in 50 mM sodium phosphate, pH 7.0, and hydrolysis was measured in 50 mM Tris-HCl, pH 7.0. Phosphorolysis of inosine was measure using the coupled xanthine oxidase procedure (23). Hydrolysis and phosphorylase activity of crude extracts and phosphorylase activity of NaR by Pnp1 were measured by strong anion exchange HPLC separation using a sodium phosphate gradient as mobile phase (20). Reactions at all substrate concentrations were performed alongside no-enzyme controls to account for nonenzymatic nucleoside degradation. All reactions were conducted at room temperature. Data were fit and analyzed using Sigma Plot.

**Nucleosides**—Inosine was purchased from Sigma. NR was produced both enzymatically and chemically as described (16, 25). Immucilin-H was a generous gift of Vern L. Schramm. Syntheses of NaR and meNaR and their verification by NMR are described in the supplemental data. The identities of all nucleosides were determined by strong anion exchange HPLC and matrix-assisted laser desorption mass spectrometry.

**RESULTS AND DISCUSSION**

**Human Pnp but Not Mtap Functions in NR Utilization**—As shown in Fig. 1, Qns1-independent NR utilization depends on Nrk1 (18). However, when Nrk1 is deleted, NR can be converted to NAD⁺ via nucleoside splitting and Nam salvage (16). By genetic criteria, the enzyme with the greatest apparent role in Nrk1-independent NR utilization is Uh1, which has homologs in a limited number of fungi, protista, and eubacteria but not animals. The two other enzymes in yeast, Pnp1 and Meu1, the yeast homologs of mammalian Pnp and Mtap (26, 27), play a moderate and minor role, respectively, in Nrk1-independent NR utilization (16). Pnp is an extensively studied enzyme (28) whose human mutation produces symptoms similar to that of severe combined immunodeficiency (29, 30).

Indeed, because Pnp is critically important for T cell function, it is a target for immunosuppressive drugs (31). Mtap is required for the recycling of methylthioadenosine to maintain cellular S-adenosylmethionine (32), and its deletion is common in tumor cells (33).

As an initial assay of whether Pnp or Mtap might function in NAD⁺ metabolism, human cDNAs encoding Pnp and Mtap were cloned into the pRS327 vector under the control of the yeast PNP1 promoter. We previously showed that the addition of 10 μM NR increases intracellular NAD⁺ from ~0.8 to ~2.0
mM in vitamin-free medium and that genetic deletion of \( \text{nrk1} \), \( \text{urh1}, \text{pnp1} \), and \( \text{meu1} \) eliminates NR utilization (16). Although the expression of Mtap on a multicopy vector failed to restore NR utilization to an \( \text{nrk1} \text{urh1} \text{pnp1} \text{meu1} \) strain, human Pnp allowed the yeast strain to increase intracellular NAD\(^+\) from 0.85 to 1.34 mM with the addition of 10 \( \mu \)M NR. By comparison, a yeast strain with an intact Nrk-independent salvage pathway (genotype\( \text{nrk1} \)) increased NAD\(^+\) levels from 0.67 to 1.87 mM (Fig. 2A).

We previously demonstrated that provision of NR improves Sir2-dependent telomeric gene silencing (16). This assay is more sensitive than a measurement of intracellular NAD\(^+\) because it detects the slight but reproducible effect of \( \text{MEU1} \) on NR-promoted gene silencing, whereas no effect of \( \text{meu1} \) deletion was observed in an assay of NAD\(^+\) levels (16). The gene silencing assay utilizes a yeast strain in which the \( \text{URA3} \) gene is integrated at a Sir2-silenced telomeric locus. Because \( \text{URA3} \) expression confers sensitivity to 5-fluoroorotic acid (5FOA), \( \text{URA3} \) silencing can be scored by resistance to 5FOA (34).

In Fig. 2B, all strains exhibited 5FOA-resistant growth when supplemented with 10 \( \mu \)M nicotinic acid (NA), indicating strong gene silencing, and showed complete 5FOA sensitivity on NA-free plates, indicating poor silencing. The wild-type strain and the strains lacking only one of the NR-salvaging pathways (i.e. \( \text{nrk1} \) or \( \text{urh1} \text{pnp1} \text{meu1} \)) exhibited 5FOA-resistant growth with 1 or 10 \( \mu \)M NR. However, the strain with \( \text{nrk1} \) deleted along with deletion of \( \text{urh1} \text{pnp1} \text{meu1} \) remained 5FOA-sensitive with the addition of NR to 10 \( \mu \)M. Although expression of human Pnp restored convincing 5FOA-resistant growth, the Mtap construct was incapable of contributing to gene silencing (Fig. 2B). Thus, of the two human enzymes homologous to yeast enzymes, which participate in Nrk-independent NR salvage, we demonstrate a role for Pnp but not Mtap as an NAD\(^+\) biosynthetic enzyme.

**Mammalian NR to Nam Conversion Is Phosphorylolytic and Sensitive to a Specific Inhibitor of Pnp**—Phosphorylolytic of NR has been reported to be a function of purine nucleoside phosphorylase (23, 35), although other investigators have claimed that NR phosphoryloly is a function of a different enzyme (36). Given our discovery of yeast Urh1 as an apparent NR (16) and NaR (20) hydrolytic enzyme, we wished to test whether mammalian NR-splitting extracts contain any hydrolase activity and whether the phosphoryloly activity is sensitive to a specific inhibitor of Pnp. We prepared a cellular lysate from 13 mouse livers and recovered the majority of NR-splitting activity in the 60\% ammonium sulfate pellet fraction. When this enzyme fraction was dialyzed against phosphate-free buffer (50 mM Tris-HCl, pH 7.0) and assayed in the same buffer, no NR to Nam conversion was observed despite increasing the amount of enzyme fraction by 100-fold and extending incubation to overnight. As shown in Fig. 3A, the addition of 50 mM phosphate to this reaction restored enzymatic activity to a \( V_{max} \) of 4.7 ± 0.2 nmol/\( \mu \)g/h, indicating that all detectable NR to Nam cleavage in mouse liver is phosphorylolytic. Despite the crude system, the \( K_m \) of this reaction, 510 ± 70 \( \mu \)M, is within 2-fold of the \( K_m \) of bovine Pnp (Table 1).

Nucleoside to nucleobase conversion was also tested in soluble extracts of human CACO-2 cells, an intestinal epithelial cell line. At 500 mM inosine, phosphoryloly conversion to hypoxanthine occurred with a specific activity of 650 ± 10 pmol/\( \mu \)g/h. NR to Nam conversion at 500 mM was ~3.5 times slower with a specific activity of 180 ± 20 pmol/\( \mu \)g/h. Immucillin-H is a specific inhibitor of Pnp with a \( K_i \) of 56 pm for the human enzyme (31). As shown in Fig. 3B, inosine to hypoxan-
thine conversion and NR to Nam conversion were exquisitely sensitive to inhibition by 100 nM immucillin-H. This result, in combination with data from Figs. 2 and 3, indicate that Pnp is the apparently unique Nrk1-independent NR salvage enzyme in mammalian cells.

**Urh1 Is Expressed at a Higher Level than Pnp1**—Previously, we demonstrated that Urh1 makes a greater contribution to Nrk1-independent NR salvage than does Pnp1 or Meu1 (16). To determine whether this is due, in part, to increased protein levels, we measured the steady-state levels of endogenously expressed, C-terminally epitope-tagged Urh1, Pnp1, Meu1, and Nrk1 (37) by Western blot of cells grown in the presence and absence of 100 μM NR. As shown in Fig. 4, the addition of NR did not alter expression of any of the NR salvage enzymes. Earlier, we showed that in the Nrk1-independent NR salvage pathway, the rank order of contributions is Urh1 > Pnp1 > Meu1. However, the rank order of steady-state expression is Meu1 > Urh1 > Nrk1 > Pnp1. Thus, although the greater role of Urh1 than Pnp1 in cellular NR to nicotinamide salvage may be attributable, in part, to greater expression level, the lesser role of Meu1 in NR salvage is despite high expression.

**Urh1 Prefers NR to Uridine by 100-fold**—Prior to the identification of the URH1 gene in 2001, a uridine nucleosidase activity purified from baker’s yeast was described. This activity possessed a $K_m$ for uridine of 0.9 mM and showed strong discrimination against other nucleosides including thymidine, cytidine, adenosine, inosine, and guanosine (24). URH1 was subsequently cloned, and its biochemical activity was ascribed to that of the uridine nucleosidase without any characterization of the recombinant enzyme (38, 39). Urh1 was recently classified as a uridine-preferring pyrimidine nucleoside hydrolase in a study that investigated pyrimidine versus purine nucleoside specificity (22). However, given our genetic assignment of Urh1 as an NR (16) and NaR (20) hydrolase, it was important to characterize pyrimidine versus pyridine nucleoside specificity.

Urh1 activity was unaffected by EDTA or the addition of MgCl₂. Urh1 hydrolyzed uridine to uracil at high substrate concentrations. As shown in Table 1, the $K_m$ value for uridine was 1.6 mM, with a $k_{cat}$ of 20 s⁻¹. However, despite a gene and enzyme name suggesting that uridine is the preferred substrate, Urh1 prefers NR by greater than 100-fold by depression of the $K_m$ to 16 μM and maintaining $k_{cat}$ at 23 s⁻¹. The actual contributions of Urh1 to uridine and NR flux remain unknown because the concentrations of these substrates are unknown. Thus, it is conceivable...
that uridine hydrolysis at high cellular concentrations of uridine is an unavoidable consequence of NR hydrolysis at low cellular concentrations of NR. It is also conceivable that Urh1 is tuned to the cellular concentrations of NR and uridine.

**Yeast and Bovine Pnp Prefer Inosine to NR by 10-fold**—Although terms such as “uridine hydrolase” and “purine nucleoside phosphorylase” suggest a simplified set of reactions in which single enzymes have only one or two substrates, in fact, multiple enzymes, expressed at different levels, participate in the consumption of multiple competitive substrates. Bovine and human Pnp are enzymes with well characterized specificity for guanosine and inosine (40). Bovine and bacterial Pnp have been reported to exhibit a 50-fold preference for inosine over NR (23). We purified yeast Pnp1 to measure the phosphorylase activity of recombinant Pnp1 and bovine Pnp on both inosine and NR and to place the kinetic constants of these enzymes on a common scale with Urh1 and the human Nrk enzymes (20).

As shown in Table 1, yeast Pnp1 exhibited a specificity constant for NR greater than 9% of the corresponding value for inosine with the difference mediated by a 31-fold disadvantage in $K_m$ offset by a 2.9-fold advantage in $k_{cat}$. Bovine Pnp exhibited similar discrimination. NR was phosphorylated at 13% of the inosine specificity constant with the difference mediated by an 18-fold disadvantage in $K_m$ offset by a 2.2-fold advantage in $k_{cat}$.

The absolute specificity constants (Table 1) and relative protein expression levels (Fig. 4) of Urh1 and Pnp1 can also be compared. Urh1 possesses greater than 100-fold more activity on NR than does Pnp1 and is expressed at a higher level. Thus, the fact that Pnp1 appears to be responsible for 25–35% of Nrk1-independent NR salvage suggests that in vivo Urh1 activity might be attenuated by competition for other substrates.

**NaR Is a Hydrolysis Substrate of Urh1 but a Poor Substrate of Yeast Pnp1**—Our previously work showed that NaR, synthesized from NaR ethyl ester, can serve as an NAD$^+$ precursor in a manner that depends on the Nrk1 and the Urh1/Pnp1 pathways (20). However, the increase in NAD$^+$ levels provided by this precursor supplied at 10 $\mu$M was only 34% of the increase in NAD$^+$ levels with provision of 10 $\mu$M NR. We also demonstrated that human Nrk1 and Nrk2 phosphorylate NaR and NR with equal efficiency (20). These data indicated that NaR is a substrate of Pnp1 and/or Urh1. Moreover, there is a suggestion in the data that NaR is either transported less efficiently than NR or split less efficiently by the enzymes of the Urh1/Pnp1 pathway.

As shown in Table 1, Urh1 hydrolyzes NaR to NA with a $K_m$ of 150 $\mu$M and a $k_{cat}$ of 1.8 $s^{-1}$. The resulting specificity constant of 11,000 $s^{-1}M^{-1}$ is a 30-fold lower than that for NAD$^+$ and on a par with the activity of Urh1 for uridine.

Yeast Pnp1 showed no NaR phosphorylase activity in 30-min continuous spectroscopic assays. However, HPLC runs of the endpoints of the assays indicated low level enzymatic

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**TABLE 1**

| Kinetic parameters of Pnp1, bovine Pnp and Urh1 |
|-----------------------------------------------|
| $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}M^{-1}$) |
|----------------|---------------------|-----------------------------|
| **Pnp1**       |                     |                             |
| Inosine        | 33.0 ± 5.2          | 3.8 ± 0.1                   | 115,000                     |
| NR             | 1,020 ± 140         | 11. ± 0.6                   | 11,000                      |
| NaR            | 3,000 ± 1,000       | 0.13 ± 0.02                 | 40                          |
| **Bovine Pnp** |                     |                             |
| Inosine        | 51.0 ± 5.1          | 14 ± 3.8                    | 275,000                     |
| NR             | 900 ± 100           | 31.2 ± 2.2                  | 35,000                      |
| NaR            | 900 ± 230           | 4.4 ± 0.6                   | 4,900                       |
| **Urh1**       |                     |                             |
| Uridine        | 1,600 ± 500         | 20.0 ± 4.4                  | 125,000                     |
| NR             | 16.0 ± 2            | 23.4 ± 0.8                  | 1,430,000                   |
| NaR            | 150.0 ± 40          | 1.8 ± 0.9                   | 12,000                      |
| MeNaR          | 45.0 ± 15           | 2.3 ± 0.2                   | 51,000                      |
consumption of NaR. To measure this activity, we ran 18-h end point assays and analyzed products by HPLC. As shown in Table 1, the measured \( K_m \) for NaR is 3 mM, and the \( k_{cat} \) is 0.13 s\(^{-1}\). The resulting specificity constant for yeast Pnp1 for NaR phosphorolysis, 40 s\(^{-1}\)M\(^{-1}\), compares poorly with that of Urh1 for NaR hydrolysis. These data suggest that Pnp1 may play a negligible role in NaR utilization.

Bovine Pnp proved to be over 120-fold more active on NaR than yeast Pnp1. Moreover, as shown in Table 1, the \( K_m \) of bovine Pnp for NaR, 900 \( \mu \)M, is nearly identical to its \( K_m \) for NR, such that discrimination in favor of NR is driven by the \( k_{cat} \) term. Although the expression levels of Pnp and Nrk isozymes in mammalian systems are likely to be cell type-specific and regulated, our data support the prediction that NR and NaR utilization in mammals depends on both enzyme systems.

**meNaR Is a Better NAD\(^+\) Precursor than NaR**—We prepared NaR enzymatically from nicotinic acid mononucleotide (16) and chemically from meNaR (see supplemental data). We found that the pure, chemically or enzymatically synthesized NaR provided at 10 \( \mu \)M failed to increase intracellular NAD\(^+\), whereas 100 \( \mu \)M NaR increased intracellular NAD\(^+\) only 16% as much as did 10 \( \mu \)M NR (Fig. 5A). Surprisingly, 10 \( \mu \)M meNaR increased intracellular NAD\(^+\) to a greater degree than did 10 \( \mu \)M NaR. meNaR also produced a dose-dependent increase in intracellular NAD\(^+\). Moreover, as shown in Fig. 5B, although 1 \( \mu \)M NR and 1 \( \mu \)M meNaR supported the vigorous growth of a bna1 mutant, which is deficient in de novo NAD\(^+\) biosynthesis, 1 \( \mu \)M NaR did not. These data suggest that the methyl ester modification of NaR facilitates nucleoside transport and/or enzymatic cleavage of the methyl ester base from the ribose.

**meNaR Is a Better Urh1 Substrate than NaR**—Data indicating that meNaR is a better NAD\(^+\) precursor than NaR prompted us to test whether the major Nrk-independent NR/NaR salvage enzyme, Urh1, prefers meNaR to NaR. Remarkably, as shown in Table 1, the methyl esterified pyridine nucleoside is preferred by Urh1 by a factor of 5.4-fold. On the other hand, Pnp1 had little detectable activity on meNaR. We were unable to measure specific kinetic constants for the cleavage of meNaR by Pnp1 because the nonenzymatic conversion of meNaR to NaR was \( \sim \)100 times faster than the enzymatic cleavage of the glycosidic bond. The specific enzymatic activity of Pnp1 on meNaR is \(<\)100 pmol/min/\( \mu \)g, which is \( \sim \)25% less than the specific activity of Pnp1 on NaR.

Although NR makes use of a specific nucleoside transporter with a \( K_m \) for NR of 22 \( \mu \)M (17) and has the ability to support yeast cell growth at a supplement concentration of 1 \( \mu \)M, NaR is a poor supplement, which depends on synthetic ester modifications to improve availability to yeast. These data suggest that a distinction be drawn between NR and NaR as yeast NAD\(^+\) precursors. Although both compounds function as salvageable metabolites, NR has the characteristics of a yeast vitamin, whereas meNaR has the characteristics of a low potency provitamin, which can be utilized once it gains entry to cells.

**Nrk1 and Urh1 Are Responsible for in Vivo meNaR Utilization**—Based on the protein expression data in Fig. 4 and the kinetic data in Table 1, we hypothesized that Urh1 is responsible for the majority of Nrk-independent NaR/meNaR utilization. We tested this hypothesis by measuring the meNaR-dependent NAD\(^+\) increase in wild-type yeast and in strains deleted for specific components of NaR salvage (Fig. 6). As suspected, deleting *pnp1* in the *nrk1* background had no detectable
effect on meNaR utilization, indicating that Pnp1 does not play a major role in meNaR utilization. On the other hand, deleting urh1 in the nrk1 background lowered the meNaR-dependent NAD+ increase from 1.2 to 0.21 mM. This result indicates that Urh1 is responsible for the majority of NAD-independent meNaR utilization. Finally, the triple deletion of nrk1 urh1 pnp1 abolished the NAD+ increase from meNaR, demonstrating that Pnp1 has a minor role in meNaR utilization in the absence of Urh1, consistent with the kinetic data.

Conclusions—We have undertaken to discover and dissect all components of eukaryotic NAD+ salvage pathways and have discovered unanticipated features and complexity (16–18, 20, 41–43). Here we clarified several features of fungal and mammalian salvage of NR and NaR. First, we provide evidence that Pnp but not MtpA has the characteristics of the mammalian NR phosphorylase, and we see no evidence of a mammalian NR hydrolytic activity. Second, we establish that the greater role in yeast of Urh1 than Pnp1 in NR and NaR metabolism is dually driven by higher expression and higher intrinsic activity. Third, we discover that yeast Urh1 is a highly specific NR hydrolase with lower activity on uridine and NaR. This has broad implications on the function of homologous enzymes, which have been termed pyrimidine nucleoside hydrolases (22). Fourth, we extend knowledge on mammalian Pnp activity, showing that both NR and NaR are alternative substrates for this enzyme. Finally, we reveal that the yeast NAD+ precursor activity of NaR depends largely on ester modification of the acid group, which promotes salvage by Urh1. In the future, we plan to determine to what degree NR utilization by particular mammalian cells depends on the Nrk and the Pnp pathways and to dissect the cellular basis for NR and NaR as intracellular metabolites.

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