Nicotinamide Riboside and Nicotinic Acid Riboside Salvage in Fungi and Mammals

QUANTITATIVE BASIS FOR Urh1 AND PURINE NUCLEOSIDE PHOSPHORYLASE FUNCTION IN NAD$^+$ METABOLISM*[S]

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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-ribosyl 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 μM 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 μM (17).

NR is converted to NAD$^+$ in two steps by nicotinamide riboside kinase (Nrk)-dependent phosphorylation and adenylylation by nicotinamide mononucleotide adenylyltransferase (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 riboside ethyl ester, can function as an NAD$^+$ precursor in yeast, through both an Nrk-dependent and an

[4] The abbreviations used are: Nam, nicotinamide; Nrk, nicotinamide riboside kinase; NR, nicotinamide riboside; NA, nicotinic acid; NaR, nicotinic acid riboside; meNaR, nicotinic acid riboside methyl ester; Urh1, uridine hydrolase; Pnp, purine nucleoside phosphorylase; Meu1, yeast methylthioadenosine phosphorylase; Mtap, human methylthioadenosine phosphorylase; 5FOA, 5-fluoroorotic acid; HPLC, high pressure liquid chromatography.

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 Urh1 is a uridine-specific nucleoside hydrolase (22), our kinetic analysis of recombinant Urh1 demonstrates a 100-fold greater specificity for NR than for uridine. Urh1 also functions *in vitro* and *in vivo* as an NaR hydrolase such that Nrk-independent NaR salvage in yeast principally depends on Urh1.

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 Urh1 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 BsaI restriction sites. The PCR product was digested with BsaI, 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 $A_{(600 \, nm)}$ 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. Urh1 and Pnp1 were then recovered from the flow-through of a second amylose column. Bovine Pnp was purchased from Molecular Probes.

Hydrolysis and/or phosphorolysis 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 phosphorolysis activity of crude extracts and phosphorolysis 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 Urh1, 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
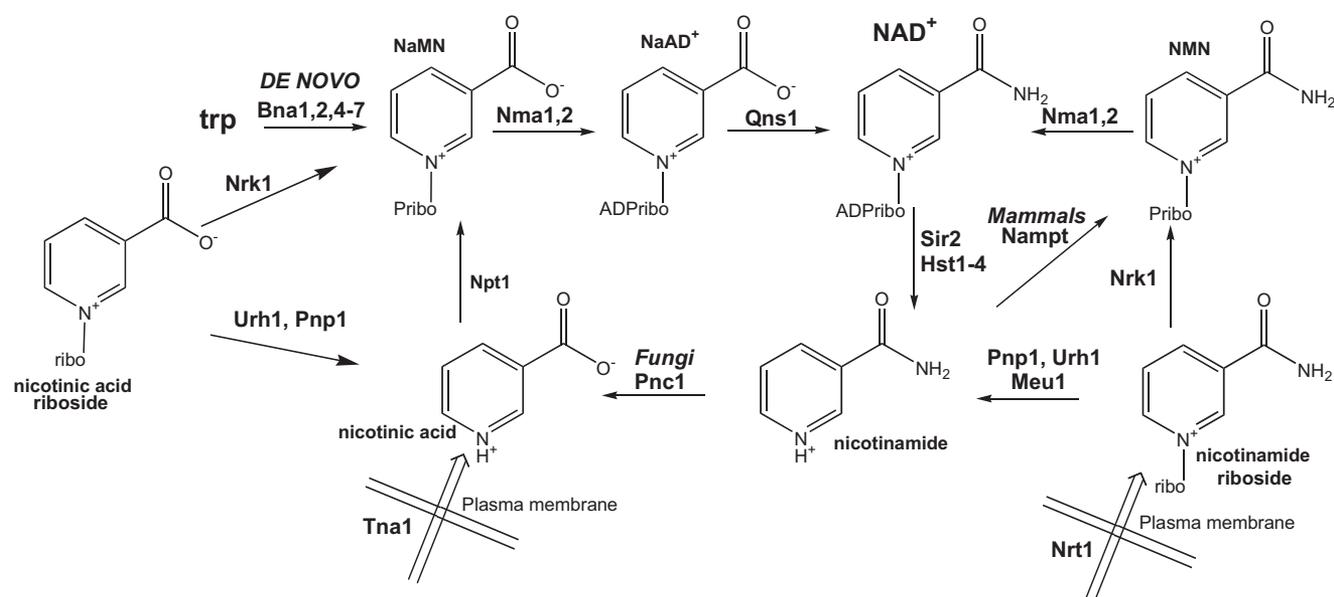
FIGURE 1. **NAD+ biosynthetic pathways in S. *cerevisiae* and mammals.** NAD$^+$ is synthesized from tryptophan by the *de novo* biosynthetic pathway and from vitamin precursors, NA, Nam, and NR, through salvage pathways (1, 44). Salvage pathways in mammals and yeast differ in the utilization of Nam. Yeast convert Nam to NA via the nicotinamidase, Pnc1, whereas mammals convert Nam to nicotinamide mononucleotide (*NMN*) through the activity of the nicotinamide phosphoribosyltransferase, Nampt. In yeast, NR is salvaged via the Nrk1 pathway and the Urh1/Pnp1 pathway with slight flux through Meu1 (16). In yeast, NaR is salvaged by Nrk1 and the Urh1/Pnp1 pathway (20). According to the data in this study, mammalian Nrk-independent NR salvage relies solely on Pnp and, in yeast, Nrk-independent usage of NaR relies principally on Urh1. Urh1 is now classified as an NR hydrolase, with lower activity on uridine and NaR. *ribo*, riboside; *ADPribo*, ADP-ribose.

mM in vitamin-free medium and that genetic deletion of *nrk1*, *urh1*, *pnp1*, and *meu1* eliminates NR utilization (16). Although the expression of Mtap on a multicopy vector failed to restore NR utilization to an *nrk1 urh1 pnp1 meu1* strain, human Pnp allowed the yeast strain to increase intracellular NAD$^+$ from 0.85 to 1.34 mM with the addition of 10 μM NR. By comparison, a yeast strain with an intact Nrk-independent salvage pathway (genotype *nrk1*) increased NAD$^+$ levels from 0.67 to 1.87 mM (Fig. 2*A*).

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 *MEU1* on NR-promoted gene silencing, whereas no effect of *meu1* deletion was observed in an assay of NAD$^+$ levels (16). The gene silencing assay utilizes a yeast strain in which the *URA3* gene is integrated at a Sir2-silenced telomeric locus. Because *URA3* expression confers sensitivity to 5-fluoroorotic acid (5FOA), *URA3* silencing can be scored by resistance to 5FOA (34). In Fig. 2*B*, all strains exhibited 5FOA-resistant growth when supplemented with 10 μM 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. nrk1* or *urh1 pnp1 meu1*) exhibited 5FOA-resistant growth with 1 or 10 μM NR. However, the strain with *nrk1* deleted along with deletion of *urh1 pnp1* and *meu1* remained 5FOA-sensitive with the addition of NR to 10 μM. Although expression of human Pnp restored convincing 5FOA-resistant growth, the Mtap construct was incapable of contributing to gene silencing (Fig. 2*B*). 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 Phosphorolytic and Sensitive to a Specific Inhibitor of Pnp—Phosphorolysis of NR has been reported to be a function of purine nucleoside phosphorylase (23, 35), although other investigators have claimed that NR phosphorolysis 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 phosphorylase 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. 3*A*, the addition of 50 mM phosphate to this reaction restored enzymatic activity to a V_{max} of 4.7 $\pm$ 0.2 nmol/μg/h, indicating that all detectable NR to Nam cleavage in mouse liver is phosphorolytic. Despite the crude system, the K_m of this reaction, 510 $\pm$ 70 μM, 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, phosphorolytic conversion to hypoxanthine occurred with a specific activity of 650 $\pm$ 10 pmol/μg/h. NR to Nam conversion at 500 mM was ~3.5 times slower with a specific activity of 180 $\pm$ 20 pmol/μg/h. Immucilin-H is a specific inhibitor of Pnp with a K_i of 56 pM for the human enzyme (31). As shown in Fig. 3*B*, inosine to hypoxan-
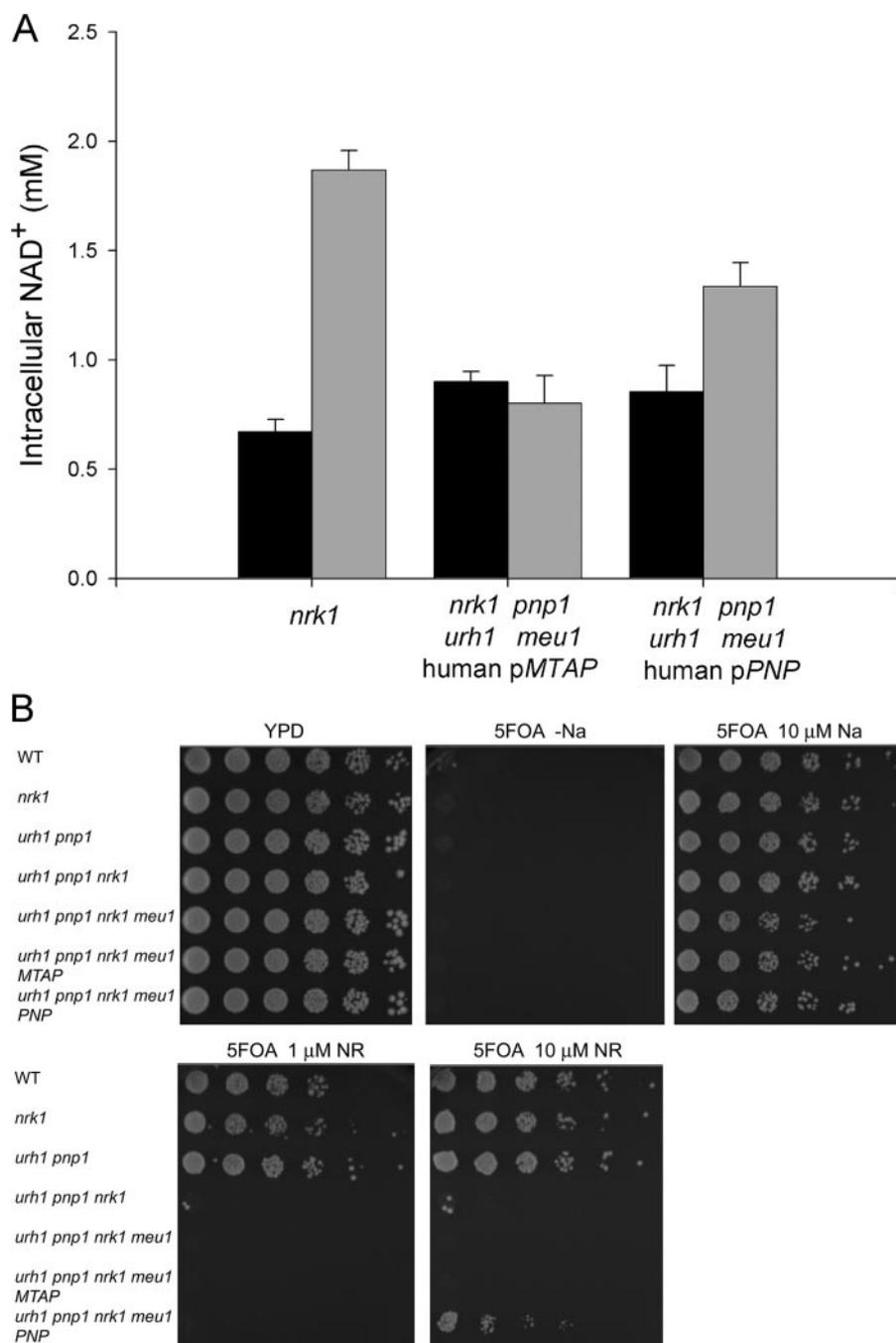
FIGURE 2. **Human Pnp but not Mtap functions in yeast NR utilization.** *A*, intracellular NAD$^+$ concentrations of the indicated genotypes were measured in NA-free SDC media (*black bars*) and in NA-free SDC media plus 10 μM NR (*gray bars*). *pMTAP* and *pPNP* indicate the addition of a pRS327 plasmid encoding a human cDNA fused to the yeast *PNP1* promoter. Expression of human Pnp allows Nrk1-independent NR utilization. *B*, all strains are gene silencing-deficient on medium without NA and are gene silencing-proficient on medium with 10 μM NA. 10 μM NR restores gene silencing to wild-type (*WT*) yeast but not yeast strains lacking both NR salvaging pathways. Human Pnp but not Mtap provides a partial rescue of the gene silencing deficiency, indicating function in NR salvage.

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*A*, 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 MgCl$_2$. 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
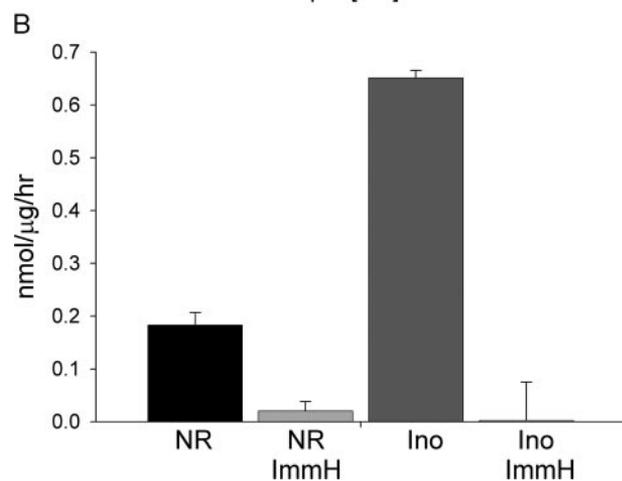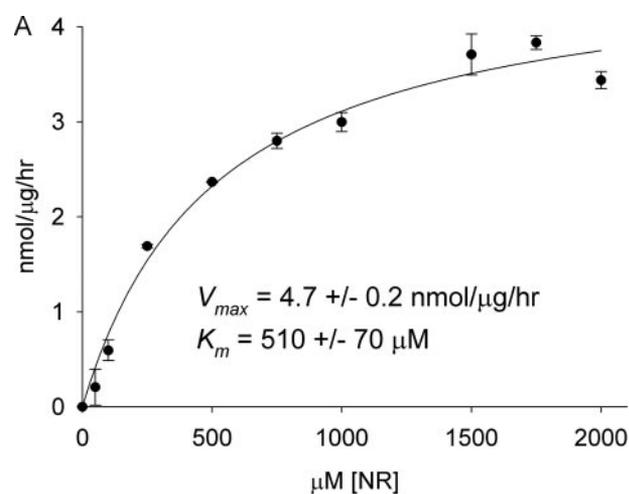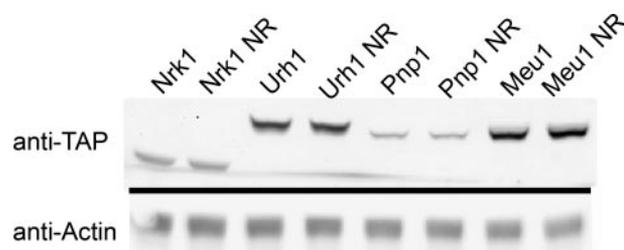
FIGURE 4. **Relative protein abundance of NR salvage enzymes.** Yeast strains, containing tandem affinity purification-tagged NR salvage enzymes, were grown in synthetic media without or with the addition of 100 μM NR. The relative abundance of the proteins, Meu1 > Urh1 > Nrk1 > Pnp1, was determined by Western blotting with an anti-tandem affinity purification antibody CAB1001 (Open Biosystems). Half of the blot was probed with anti-actin antibody as a loading control.

FIGURE 3. **Mammalian NR phosphorylase activity is Pnp.** *A*, crude mouse liver NR phosphorylase activity. *B*, specific activity of CACO-2 cell lysate phosphorolysis of inosine (*Ino*) and NR without and with immucillin-H (*ImmH*) inhibition.

TABLE 1

Kinetic parameters of Pnp1, bovine Pnp and Urh1

	K_m	k_{cat}	k_{cat}/K_m
	μM	s⁻¹	s⁻¹M⁻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

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 phosphorylized 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 μM was only 34% of the increase in NAD⁺ levels with provision of 10 μM 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 μM and a k_{cat} of 1.8 s⁻¹. The resulting specificity constant of 11,000 s⁻¹M⁻¹ is 130-fold lower than that for NR and on a par with the activity of Urh1 for uridine.

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

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 μM, 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 μM failed to increase intracellular NAD$^+$, whereas 100 μM NaR increased intracellular NAD$^+$ only 16% as much as did 10 μM NR (Fig. 5A). Surprisingly, 10 μM meNAR increased intracellular NAD$^+$ to a greater degree than did 10 μM NaR. meNaR also produced a dose-dependent increase in intracellular NAD$^+$. Moreover, as shown in Fig. 5B, although 1 μM NR and 1 μM meNAR supported the vigorous growth of a *bna1* mutant, which is deficient in *de novo* NAD$^+$ biosynthesis, 1 μM 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 ~100 times faster than the enzymatic cleavage of the glycosidic bond. The specific enzymatic activity of Pnp1 on meNaR is <100 pmol/min/μg, which is ~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 μM (17) and has the ability to support yeast cell growth at a supplement concentration of 1 μM, 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-
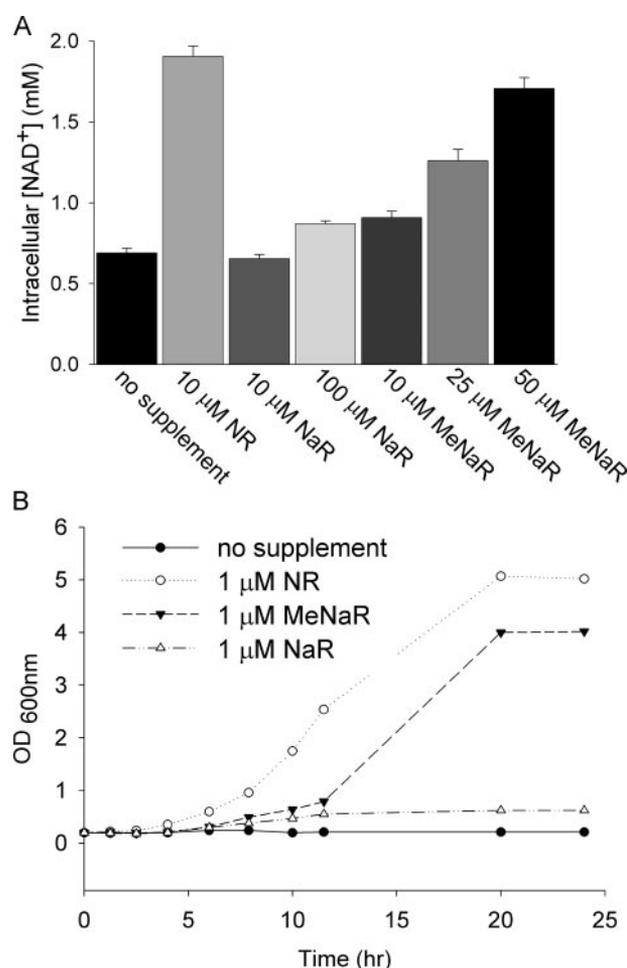
FIGURE 5. **NaR utilization is facilitated by methyl ester modification.** *A*, intracellular NAD$^+$ concentrations of wild-type yeast supplemented with NR, NaR, or meNaR at the indicated concentrations. *B*, *de novo* mutant *bna1* was grown for 18 h in SDC and then grown to exhaustion for 18 h in NA-free SDC. It was then diluted to a starting $A_{(600\,nm)}$ of 0.2 in the specified media. Although 1 μM NR was the best at promoting *bna1* growth, meNaR supported *bna1* growth after a lag period and 1 μM NaR did not.
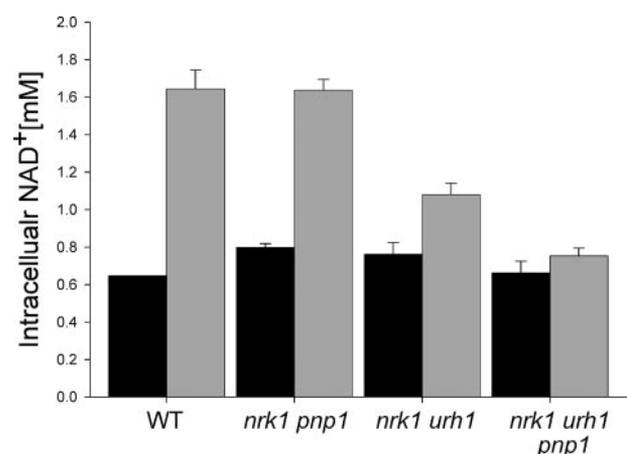
FIGURE 6. **Urh1 is principally responsible for Nrk1-independent NaR/meNaR utilization.** Intracellular NAD$^+$ concentration of yeast strains was supplemented with 50 μM meNaR. The *urh1* deletion from *nrk1* greatly reduces meNaR utilization, which is eliminated by deletion of *nrk1*, *urh1*, and *pnp1*. *WT*, wild type.

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 Nrk-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 Mtap 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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DNA Primers used in this study (5' to 3')

14083
CACGGTCTCAATGAGTGATATCTTGAACGTAAGTCAACAACG
14084
GGAATTCCAATTATAATTCCCCCATTACGGCAGC
14082
GCTCTAGAGATGTGCTGTGACTGGGTTCC
14085
CGTGGAAGGATTTCAATGACTGTTAGTAAAATACCCATATGGCTAGATTG
14091
CGGGGTACCGAGACCATGGAGAACGGATACACCC
14092
GGAATTCCAAGGCAGGTCAACTGGC
14093
CGGGGTACCCTTTTAAGTTTATTCGGAGTTTCTCATGTGTTCTAG
14094
CCCAAGCTTCCCTTTTGAACTCCTTGGTAAAGTACG
14095
CGGGGTACCCATGGCCTCTGGCACCAC
14096
GGAATTCCAGTAGCTTTTGGCTTTATAGCGTTTGC

Plasmids used in this study

Name	Insert	Vector	Figures
pPAB003	*URH1*	pMalc2x	Tables 1
pPAB006	*PNP1*	pMalc2x	Tables 1
pPAB008	*Ppnp1-hMTAP*	pRS327	Figure 2
pPAB011	*Ppnp1-hPNP*	pRS327	Figure 2

S. cerevisiae strains used in this study

| Name	Genotype	Figures
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (1)	5a, 6
PAB016	BY4742 *nrk1Δ::HIS3 pnp1Δ::kanMX4* (2)	6
PAB030	BY4742 *NRK1::TAP-HIS3MX6* (2)	4
PAB037	BY4742 *nrk1Δ::HIS3* (2)	2a
PAB038	BY4742 *pnp1Δ::kanMX4 urh1::NAT nrk1Δ::HIS3* (2)	6
PAB040	BY4742 *nrk1Δ::HIS3 urh1Δ::kanMX4* (2)	6
PAB052	BY4742 *pnp1Δ::kanMX4 urh1::NAT nrk1Δ::HIS3 meu1Δ::LEU2 ADH4::URA3-TEL* (2)	2b
PAB053	BY4742 *pnp1Δ::kanMX4 urh1::NAT nrk1Δ::HIS3 meu1Δ::LEU2* (2)	2a
PAB054	BY4742 *URH1:: TAP-HIS3MX6* (2)	4
PAB055	BY4742 *PNP1::TAP- HIS3MX6* (2)	4
PAB056	BY4742 *MEU1::TAP- HIS3MX6* (2)	4
JM84	BY4742 *ADH4::URA3-TEL* (2)	2b
JM85	BY4742 *nrk1Δ::HIS3, ADH4::URA3-TEL* (2)	2b
JM88	BY4742 *pnp1Δ::kanMX4 urh1Δ::NAT ADH4::URA3-TEL* (2)	2b
JM89	BY4742 *nrk1Δ::HIS3 pnp1Δ::kanMX4 urh1Δ::NAT ADH4::URA3-TEL* (2)	2b
KB038	BY4742 *bna1Δ::LEU2* (3)	5b

NaR/meNaR Syntheses

Syntheses of NaR and NaR ethyl ester have been described (4). We modified the procedure by using nicotinic acid methylester instead of nicotinic acid ethylester, to prepare both NaR and NaR methyl ester in the same synthetic sequence. Tetraacetyl ribose was reacted with excess of nicotinic acid methylester in presence of one equivalent of trimethylsilyl triflate to produce crude 2',3',5'-triacetyl NaR methylester triflate, which was deacetylated using three to four equivalents of sodium methoxide in methanol at -20 ^{o}C. At this point, the Yang et al. synthesis had produced crude meNaR, which was purified by HPLC. Our synthesis afforded a mixture of NaR and nicotinic acid methylester (the latter carried over from the first step) obviating the need for ester hydrolysis by pig liver esterase (4). The proton NMR spectrum of the crude product in D_2O exhibited only one methoxy group resonance at 3.94 ppm which increased when the sample was spiked with nicotinic acid methylester. HPLC purification of the crude product afforded pure NaR, the identity of which was confirmed by comparing its NMR spectrum with that of enzymatically prepared NaR.

We hypothesize that during the reaction, the methyl group of meNaR underwent nucleophilic attack by excess sodium methoxide (Supplementary Figure 1), which resulted in formation of the more thermodynamically stable zwitterionic NaR. Similar deprotection of esters by thiolates is well known and is commonly used as a mild method for converting methylesters into their corresponding carboxylic acids (5). The ethyl group in NaR ethyl ester is hindered enough to prevent this S_N2 reaction and that compound instead undergoes simple transesterification.

To produce stable meNaR, we reverted to using nicotinic acid ethylester in the reaction with tetraacetyl ribose. Deacetylation with sodium methoxide in methanol afforded crude meNaR. The 1H NMR spectrum of the crude product displayed two resonances due to methoxy group protons of meNaR (4.03 ppm) and nicotinic acid methylester (3.94 ppm). meNaR was then isolated from the crude mixture by HPLC.

Both synthetic NaR triflate and meNaR triflate were obtained stereospecifically as β-anomers as evidenced by the lone doublet of the anomeric proton (6.21 ppm for NaR and 6.25 ppm for meNaR).

Supplementary Figure 1
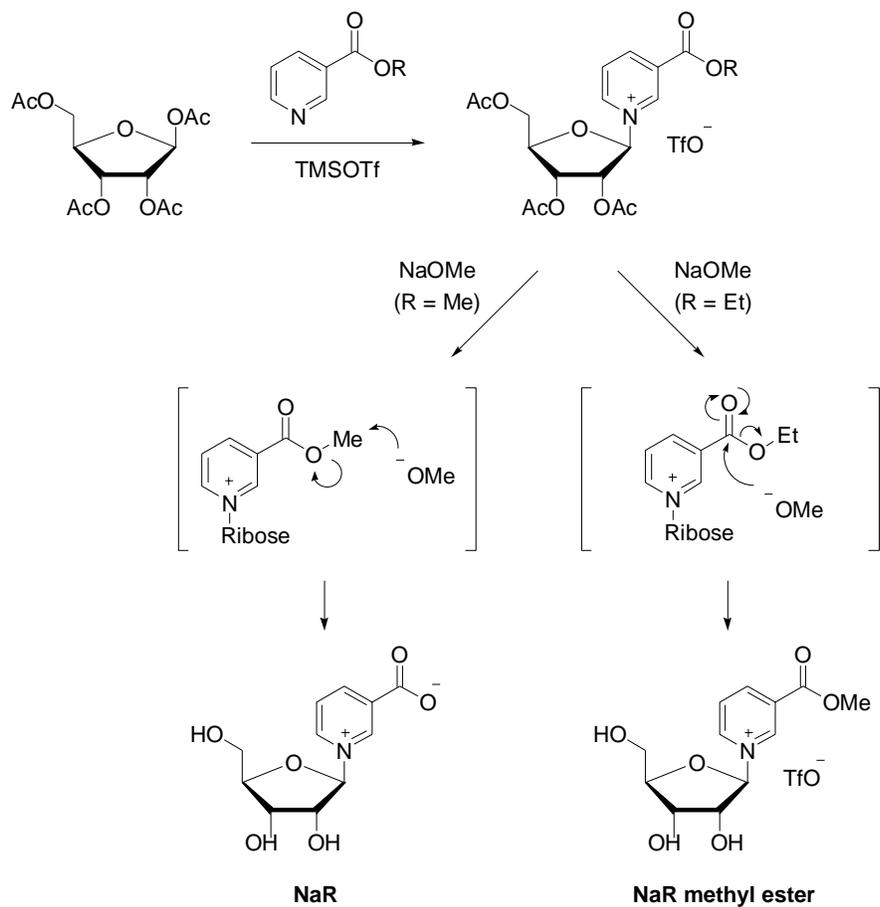
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