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MOLECULAR BIOLOGY OF PYRIDINE NUCLEOTIDE AND NICOTINE BIOSYNTHESIS

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1. ABSTRACT

Nicotinamide adenine dinucleotide (NAD) is a ubiquitous coenzyme in oxidation-reduction reactions. Recent animal and fungal studies show that it also plays important roles in transcriptional regulation, longevity, and age-associated diseases. NAD is synthesized *de novo* from aspartic acid in *E. coli* or from tryptophan in animals, by way of quinolinic acid. Although the number of biochemical studies on NAD is very limited, a bioinformatic search of genome databases suggests that Arabidopsis (dicots) synthesizes NAD from aspartic acid whereas rice (monocots) may utilize both aspartate and tryptophan as starting amino acids. The salvage pathway recycles the breakdown products of NAD metabolism. In tobacco, an intermediate in the *de novo* NAD synthetic pathway supplies the pyridine ring moiety of nicotine alkaloids. Gene expression studies in tobacco suggest that part of the NAD pathway is coordinately regulated with nicotine biosynthesis.

2. INTRODUCTION

For a long time, nicotinamide adenine dinucleotide (NAD) has been known as a coenzyme that is inter-converted between its oxidized and reduced forms without net consumption. NAD is converted to its reduced form NADH mainly in catabolic reactions (1). Conversion of one molecule of sucrose to 4 pyruvate molecules during glycolysis produces 4 NADH. Subsequently the complete oxidation of one pyruvate molecule in the citric acid cycle (also known as the tricarboxylic acid cycle and the Krebs cycle) leads to the formation of 4 molecules of NADH. NADH is mostly oxidized by the mitochondrial electron transfer chain. The ratio of NAD to NADH in cells is often considered to reflect the intracellular redox state and regulate many metabolic enzymes, including the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and the pyruvate dehydrogenase complex in the citric acid cycle.

NAD also regulates gene expression by modulating activities of some animal transcription factors (2). NADH enhances the heterodimerization of BMAL1 and BMAL2, the transcription factors involved in the circadian clock (3), and their DNA binding activity, whereas NAD attenuates these activities. The binding of the co-repressor CtBP to transcriptional repressors is reported to be regulated by NAD and NADH (4).

In addition to the above roles in energy metabolism and transcription, NAD is utilized irreversibly as a substrate by several distinct classes of enzymes. First, ADP-ribosyl transferases transfer the ADP-ribose moiety of NAD to an acceptor protein, leading to marked changes in the target protein function (5). The diphteria toxin is a typical prokaryotic enzyme of this class, but eukaryotic cells also possess similar transferases. Second, NAD is cleaved by NAD glycohydrolases/ADP-ribosyl cyclases to form cyclic ADP-ribose, an effector molecule regulating cellular calcium homeostasis (6, 7). Third, NAD is also consumed in a deacetylation reaction catalyzed by the Silent Information Regulator 2 (Sir2) family enzymes (2, 8). This unique class of enzymes couples the NAD cleavage and the deacetylation of substrate (such as acetylated histones) to the formation of *O*-acetyl-ADP-ribose (9).

Recent emerging interest in Sir2 and its homologues stems from their suspected roles in lifespan extension. In the budding yeast, calorie restriction extends lifespan by increasing the activity of Sir2 (10); increased dosage of a *Caenorhabditis elegans* Sir2 homologue

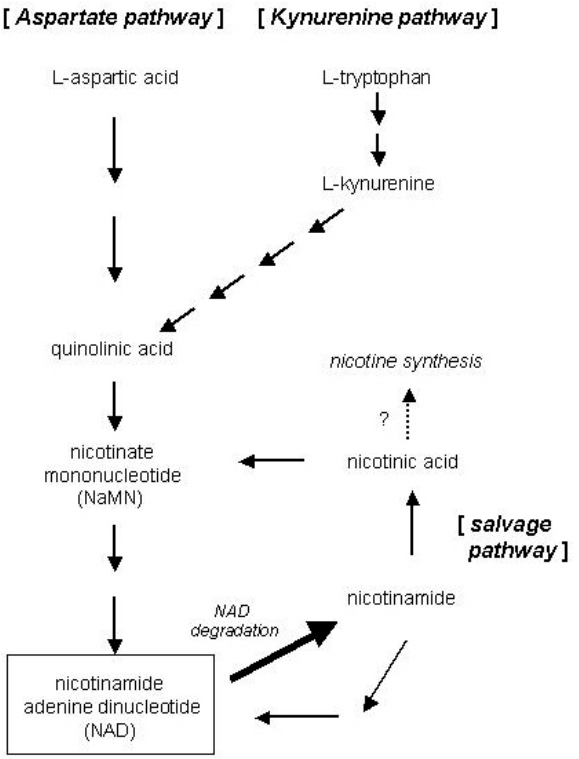

Figure 1. Overview of NAD biosynthesis. Two *de novo* pathways, the aspartate pathway and kynurenine pathway, converge at quinolinic acid, and subsequently use three common steps to synthesize NAD. After the degradation of NAD by several enzymes, two *salvage* pathways are possible to recycle nicotinamide back to the *de novo* pathway. The pyridine moiety of nicotine is derived from nicotinic acid or its derivative in tobacco. All the major pathways reported in several organisms are summarized in this figure

lengthens the lifespan of the worm (8); and a human homologue SIRT1 promotes cell survival by negatively regulating the p53 tumor suppressor (11). Although plant Sir2 homologues have not been characterized functionally, resveratrol, a polyphenol found in red wine, is attracting wide attention as a potent activator of the human SIRT1 (12).

The NAD-consuming enzymes generate nicotinamide as the breakdown product of NAD metabolism. Nicotinamide is recycled back to the NAD biosynthetic pathway. When nicotinic acid is supplied exogenously to cultured cells or organisms in the culture medium or as a food ingredient, it is likely to be converted to NAD via this *salvage* pathway (13).

In this chapter, we will review the *de novo* and salvage pathways and enzymes involved in NAD biosynthesis. Because the number of biochemical studies on NAD biosynthesis is very limited in plants, findings from non-plant organisms are included where appropriate. We also summarize the biosynthesis of nicotine, which is derived from a part of the NAD pathway in tobacco.

3. *DE NOVO* NAD BIOSYNTHESIS IN BACTERIA: ASPARTATE PATHWAY

NAD is synthesized *de novo* from amino acid precursors in two alternate pathways (Figure 1; 14-16). In bacteria, L-aspartic acid is the starting amino acid (Figure 1). The first enzyme in this pathway, L-aspartate oxidase (EC 1.4.3.16), oxidizes the amino group of L-aspartate to the imino group, forming α-iminosuccinic acid. *E. coli* L-aspartate oxidase loosely binds cofactor FAD (17). Interestingly, two alternate electron acceptors, molecular oxygen and fumarate, can be used to oxidize and regenerate a reduced form of FAD that is formed during the reaction (18).

In the next step, α-iminosuccinate is condensed with glyceraldehyde 3-phosphate (also called dihydroxyacetone phosphate) and cyclized, with the removal of phosphate and two water molecules, to produce quinolinic acid which possesses the pyridine ring. The enzyme that catalyzes this step, quinolinate synthase, does not require any cofactors. Co-fractionation of the enzyme activities of L-aspartate oxidase and quinolinate synthase during the purification of *E. coli* extracts indicated a possible association of the two enzymes *in vivo* (19) but the presence of such an enzyme complex has not been unequivocally demonstrated.

The third step is catalyzed by quinolinic acid phosphoribosyl transferase (EC 2.4.2.19; QPT). QPT is a homodimer of 36-kDa subunits in *E. coli* and forms nicotinic acid mononucleotide (NaMN) from quinolinic acid and phosphoriboxyl pyrophosphate (20). As described below, the biosynthetic steps subsequent to the formation of quinolinic acid, and the enzymes involved, are conserved in the aspartate and kynurenine pathways.

In the following penultimate step in NAD biosynthesis, nicotinic acid mononucleotide adenyltransferase (EC 2.7.7.18; NaMN-AT) converts NaMN to nicotinic acid adenine dinucleotide (NaAD) by utilizing ATP as a cofactor. Bacteria NaMN-ATs catalyze the adenylation of NaMN much faster than the adenylation of nicotinamide monocucleotide (NMN) (21).

The last step is catalyzed by NAD synthase (EC 6.3.1.5) which converts NaAD to NAD in the presence of ATP and ammonia. During the reaction, the acyladenylation of NaAD is followed by the displacement of AMP by ammonia, thus producing the amide.

4. *DE NOVO* NAD BIOSYNTHESIS IN ANIMALS: KYNURENINE PATHWAY

In contrast to prokaryotes, mammals and fungi couple tryptophan catabolism with NAD biosynthesis (Figure 3). In mammals, L-tryptophan is mostly degraded through the "kynurenine pathway", which generates quinolinic acid and other biologically active compounds. The first reaction is catalyzed by tryptophan 2, 3- dioxygenase

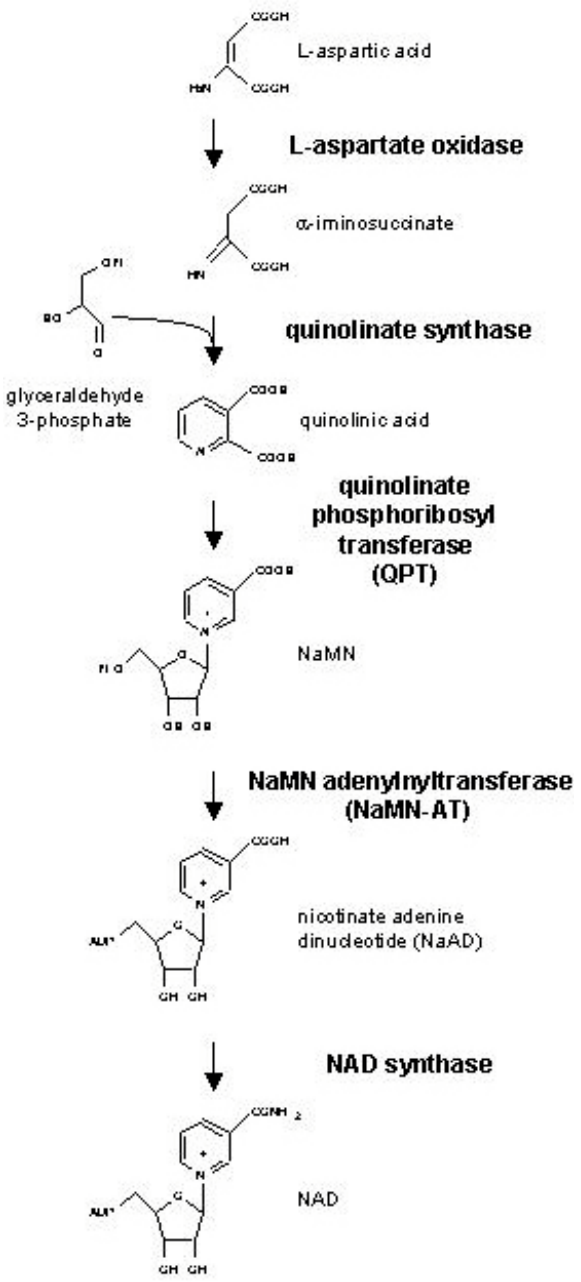

Figure 2. The aspartate pathway

(EC 1.13.11.11), which oxidatively cleaves the pyrrole ring of tryptophan, forming *N*-formylkynurenine. The rat liver enzyme is composed of two pairs of identical subunits, contains two heme molecules per protein, and is regulated allosterically by tryptophan at neutral pH (22, 23). The rat liver enzyme is induced by both the substrate tryptophan and glucocorticoid hormones, and is the rate-limiting enzyme of tryptophan degradation in the liver (24, 25).

Mammalian cells also ubiquitously express indoleamine 2, 3-dioxygenase (EC 1.13.11.42), which requires superoxide anion radical as a substrate and cofactor for maximal activity and degrades a variety of indole compounds, including D-tryptophan, 5-hydroxytryptophan, and serotonin (26, 27). The rabbit enzyme is a monomeric glycoprotein containing protoheme IX as the sole prosthetic group. Its expression is not controlled by tryptophan levels but is induced upon infection and by interferon γ, pointing to an immunoregulatory function for indoleamine 2, 3-dioxygenase (28-30).

N-formylkynurenine is converted to quinolinic acid in four enzymatic steps, by way of kynurenine. Arylformamidase (EC 3.5.1.9) hydrolyzes *N*-formylkynurenine to kynurenine and formate. Two enzyme forms which differ in molecular weight have been reported in various organisms, and *Drosophila melanogaster* and *Streptomyces parvulus* possess both forms, although kinetic parameters appear not to vary considerably among the two forms (31, 32).

L-Kynurenine is then hydroxylated by kynurenine 3-monooxygenase (EC 1.14.13.9). The enzyme is a mitochondrial FAD-dependent monooxygenase that uses NADPH as an electron donor and incorporates molecular oxygen into 3-hydroxykynurenine and water (33-35). In the next step, 3-hydroxykynurenine is converted to 3-hydroxyanthranilic acid by the pyridoxal-5'-phosphate-dependent enzyme kynureinase (EC 3.7.1.3) which is a homodimer (36). The last enzyme leading to quinolinate formation is 3-hydroxyanthranylate 3, 4-dioxygenase (EC 1.13.11.6). The enzyme contains non-heme ferrous ion, and converts 3-hydroxyanthranilic acid to an unstable aliphatic compound, α-amino-β-carboxymuconic acid ε-semialdehyde, which spontaneously cyclizes to quinolinic acid (37, 38).

The steps from quinolinic acid to NAD are conserved among prokaryotes and eukaryotes, and catalyzed by similar enzyme sets. One notable variation is found in the properties of NAD synthases; *E. coli* and *B. subtilis* enzymes require ammonia for the amino donor (39, 40) whereas the enzymes from yeast, human erythrocyte and rat liver can utilize glutamine, as well as ammonia (41, 42).

5. *DE NOVO* DNA BIOSYNTHESIS IN PLANTS: PRECURSOR FEEDING STUDIES

Biochemical and molecular biological studies on NAD biosynthesis in plants have been very limited in number, and have not provided a clear consensus on the *de novo* pathways leading to NAD formation. Initial tracer feeding experiments yielded conflicting results. In germinating corn seeds, only trace levels of [7a-^{14}C]-DL-tryptophan, [^{3}H]-DL-tryptophan, and [^{3}H]-3-hydroxyanthranilic acid were incorporated into niacin (a generic term for nicotinamide and nicotinic acid) and a methyl betaine derivative of nicotinic acid, trigonelline, after several days of incubation (43). [3-^{14}C]-DL-tryptophan was an inactive precursor for trigonelline in garden pea seedlings as well (44). In subsequent experiments, however, significant levels of radioactivity were incorporated into niacin after 48 h of treatment of corn seedlings with [benzene ring-U-^{14}C]-DL-tryptophan and [5-^{3}H]-L-tryptophan, while the tracer incorporation into niacin was negligible from [U-^{14}C]-L-aspartate (45).

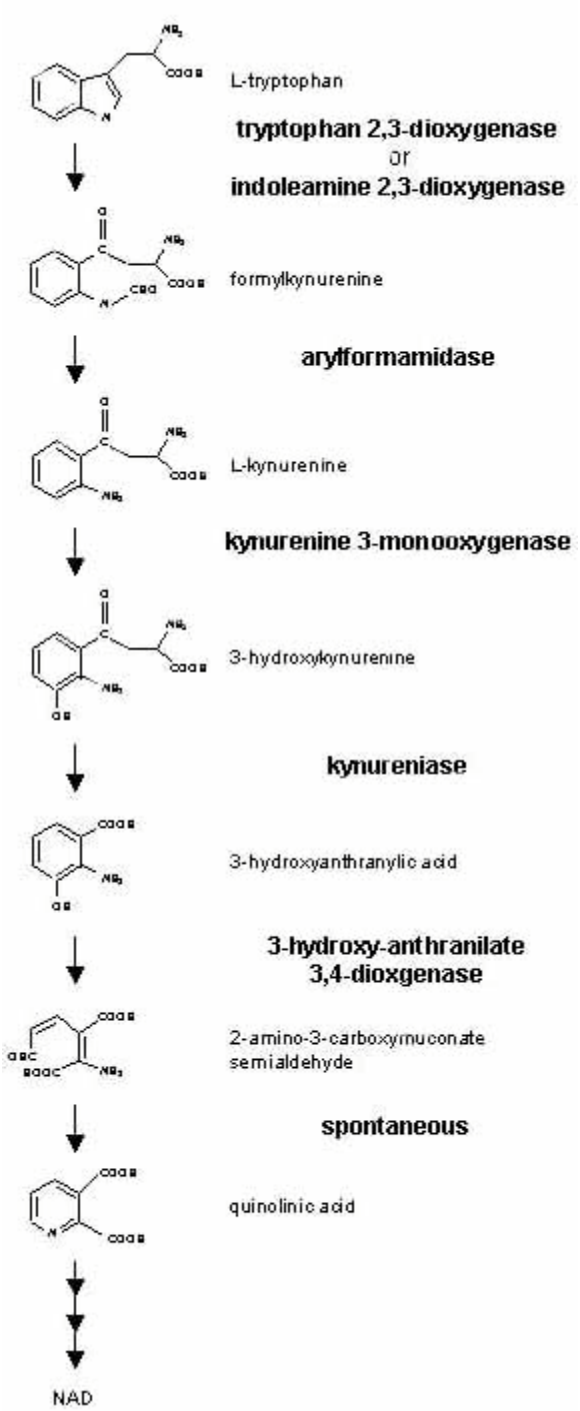

Figure 3. The kynurenine pathway

Biosynthetic pathways of NAD can be inferred from studies on secondary metabolites that presumably utilize part of the NAD pathway. As described later in detail, the nicotinic acid moiety of nicotine is derived from an intermediate of NAD biosynthesis in tobacco and related species. The feeding of [3-^{14}C]-aspartate to *Nicotiana rustica* plants afforded nicotine which was labeled at C-2 and C-3 in the pyridine ring (46). In addition, the feeding of radio-labeled tryptophans to *Nicotiana* species consistently resulted in a negligible incorporation of the label into nicotine (47). Therefore, the nicotinic acid moiety of nicotine appears to be derived from an intermediate in the *de novo* NAD pathway that starts from aspartate.

6. *DE NOVO* NAD BIOSYNTHESIS IN PLANTS: ASPARTATE PATHWAY

Some of the discrepancies in the classical feeding studies may be reconciled when genome sequences from two model plants, *Arabidopsis thaliana* and *Oryza sativa* (rice), are made available. In the Arabidopsis genome and cDNA databases, predicted amino acid sequences with significant homology to L-aspartate oxidase and quinolinate synthase can be found (Table 1). Similarly significant matches were also found for rice homologues of these two enzymes. In addition, Arabidopsis and rice have predicted homologues of three subsequent enzymes that convert quinolinate to NAD (QPT, NaMN-AT, and NAD synthase). Most of these homologous sequences are found in cDNA databases, as well as in genome databases, indicating that these genes are transcribed and not pseudo-genes. Therefore, these two model plants possess the complete enzyme set for the aspartate pathway that is used in prokaryotes. Since some predicted amino acid sequences, although often partial sequences, that are homologous to these enzymes are also found in expressed sequence tag (EST) and cDNA databases of other plant species, the aspartate pathway is predicted to exist in plants in general.

There are some biochemical and molecular studies that support this view. L-Aspartate oxidase was partially purified from cotton callus cells (48). The cotton enzyme accepts L-aspartate with a *K*m value of 0.66 mM but does not oxidize D-aspartate, L-glutamate, or D- and L- alanine. When expressed heterologously in *E. coli*, Arabidopsis and tobacco cDNAs encoding homologues of L-aspartate oxidase and quinolinate synthase rescued the growth of *E. coli* mutants lacking respective enzyme activities (our unpublished results). QPT which has been purified from castor bean endosperm is a homodimer (49). QPT activity is also found in tobacco (50) and tobacco cDNA encoding a QPT homologue can complement an *E. coli* QPT-deficient mutant (51). Enzyme activities of NaMN-AT and NAD synthase have been detected in crude protein extracts from several tissues of tobacco (52-54). The tobacco NAD synthase activity was detected with glutamine or asparagine but not with ammonia. Biochemical studies using purified enzyme preparations are required to determine whether the plant enzymes are similar to prokaryotic or mammalian enzymes.

7. *DE NOVO* NAD BIOSYNTHESIS IN PLANTS: KYNURENINE PATHWAY

A computer homology search of the Arabidopsis genomic database did not detect apparent homologues of mammalian enzymes specifically involved in the kynurenine pathway, except arylformamidase (Table 1). In

Table 1. Putative NAD synthesis enzymes found in the nucleotide databases of Arabidopsis thaliana and Oryza sativa

Pathway	NAD synthesis enzyme			Arabidopsis homologue in MAtDB			Oryza homologue in Genbank or DDBJ		
	Protein accession number	**Protein name**	**Species**	**Transcript code**	***E* -value**	**Amino identity**	**Sequence accession number**	***E*-value**	**Translated amino acid identity**
[*Escherichia coli*]									
Aspartate pathway	NP_417069	L-aspartate oxidase	*E. coli*	*AT5G14760*	$1e^{-104}$	222/517 (42%)	AAAA01027418	$1e^{-166}$	293/503 (58%)
	NP_415271	quinolinate synthase	*E. coli*	*AT5G50210*	$1e^{-10}$	72/295 (24%)	AAAA01072566	$1e^{-64}$	118/168 (70%)
	NP_414651	QPT	*E. coli*	*AT2G01350*	$2e^{-34}$	94/293 (32%)	AAAA01051622	$2e^{-48}$	116/258 (44%)
	NP_308704	NaMN-AT	*E. coli*	n.s.h.	-	-	AAAA01098297	$9e^{-19}$	62/181 (34%)
	NP_308704	NAD synthase	*E. coli*	*AT1G55090*	$1e^{-5}$	64/255 (25%)	AAAA01059954	$9e^{-14}$	70/239 (29%)
	P21369	nicotinamide deaminase	*E. coli*	*AT3G16190*	$4e^{-8}$	25/92 (27%)	AAAA01049712	$4e^{-43}$	87/199 (43%)
	NP_415451	NaPT	*E. coli*	*AT4G36940*	0.002	78/341 (22%)	AAAA01026118	$2e^{-56}$	134/377 (35%)
[Mammal]									
Kynurenine pathway	XP_192935	tryptophan 2,3-dioxygenase	*Mus musculus*	n.s.h.	-	-	AAAA01028159	$6e^{-14}$	51/126 (40%)
	AAO34405	indoleamine 2,3-dioxygenase	*Homo sapiens*	n.s.h.	-	-	AK110021	$9e^{-25}$	108/403 (26%)
	NP_082103	arylform-amidase	*M. musculus*	*AT5G15860*	$4e^{-14}$	64/239 (26%)	AAAA01021182	$8e^{-22}$	71/237 (29%)
	NP_067604	kynurenine 3-mono-oxygenase	*Rattus norvegicus*	n.s.h.	-	-	AAAA01049201	$1e^{-64}$	143/353 (40%)
	NP_081828	kynureniase	*M. musculus*	n.s.h.	-	-	AK111206	$1e^{-94}$	193/467 (41%)
	NP_036337	3-hydroxy-anthranilate 3,4-dioxgenase	*H. sapiens*	n.s.h.	-	-	AAAA01029387	$7e^{-25}$	58/150 (38%)
	Q15274	QPT	*H. sapiens*	*AT2G01350*	$5e^{-24}$	80/260 (30%)	AAAA01051622	$7e^{-28}$	81/240 (33%)
	AAG33632	NaMN-AT	*H. sapiens*	*AT5G55810*	$5e^{-23}$	58/187 (31%)	AK071224	$3e^{-20}$	46/138 (33%)
	NP_084497	NAD synthase	*M. musculus*	*AT1G55090*	0.0	363/704 (51%)	AK063292	$1e^{-163}$	288/531(54%)
Salvage pathway	P43490	nicotinamide phosphoribosyl transferase	*H. sapiens*	n.s.h.	-	-	n.s.h.	-	-
[Yeast]									
Kynurenine pathway	NP_012612	indoleamine 2,3-dioxygenase	*Saccharomyces cerevisiae*	n.s.h.	-	-	AK110021	$2e^{-32}$	132/538 (24%)
	NP_012475	arylform-amidase	*S. cerevisiae*	*AT1G77670*	$4e^{-63}$	138/383 (36%)	AK119212	$1e^{-60}$	133/383 (34%)
	NP_009454	kynurenine 3-mono-oxygenase	*S. cerevisiae*	n.s.h.	-	-	AAAA01049201	$3e^{-50}$	131/350 (37%)
	NP_013332	kynureniase	*S. cerevisiae*	n.s.h.	-	-	AK111206	$1e^{-90}$	195/474 (41%)
	NP_012559	3-hydroxy-anthranilate 3,4-dioxgenase	*S. cerevisiae*	n.s.h.	-	-	AAAA01029387	$2e^{-29}$	71/173 (41%)
	NP_602317	QPT	*S. cerevisiae*	*AT2G01350*	$3e^{-29}$	90/268 (33%)	AK068240	$2e^{-29}$	90/275 (32%)
	NP_013432	NaMN-AT	*S. cerevisiae*	*AT5G55810*	$9e^{-24}$	60/165 (36%)	AK071224	$2e^{-20}$	43/110 (39%)
	NP_011941	NAD synthase	*S. cerevisiae*	*AT1G55090*	0.0	363/704 (51%)	AK063292	$1e^{-173}$	303/536 (56%)
Salvage pathway	P53184	nicotinamide deaminase	*S. cerevisiae*	*AT3G16190*	$1e^{-6}$	57/213 (26%)	AAAA01049712	$2e^{-24}$	72/196 (36%)
	P39683	NaPT	*S. cerevisiae*	*AT4G36940*	$5e^{-10}$	102/427 (23%)	AAAA01027541	$2e^{-55}$	130/375 (34%)

Genome and EST databases were searched using the BLAST algorithm, and the homologue with the lowest *E*-value is shown for each enzyme. n.s.h.; No significant hit

contrast, significant hits are recognized for these enzymes when the rice genomic database is searched. Rice homologues of tryptophan dioxygenase, arylformamidase, kynurenine 3-monooxygenase, kynureinase, and 3-hydroxyanthranylate 3, 4-dioxygenase show 24-41 % identity with their animal and yeast counterparts over more than 126 amino acid residues. The *E*-values for the similarity are less than $6e^{-14}$, indicating that the homology is highly significant. These rice genes are apparently expressed since EST sequences are found for many of them, and constitute the whole enzyme set leading from tryptophan to quinolinate. The presence of an indoleamine 2, 3-dioxygenase homologue in the rice EST database suggests that the initial oxidative cleavage of tryptophan in rice may be catalyzed by both types of dioxygenases.

Because the two enzymes specific for the aspartate pathway, L-aspartate oxidase and quinolinate synthase, are also encoded in the rice genome, rice appears to possess biosynthetic genes for a complete set of enzymes for both the aspartate and kynurenine pathways. Homologues of tryptophan dioxygenase, kynurenine 3-monooxygenase, kynureinase, and 3-hydroxyanthranylate 3, 4-dioxygenase are found in the EST sequences of corn and barley, but are absent in nucleotide databases of dicotyledonous plants. Although biochemical and genetic evidence is required, it is reasonable to speculate that dicots use the aspartate pathway whereas monocots can utilize both pathways.

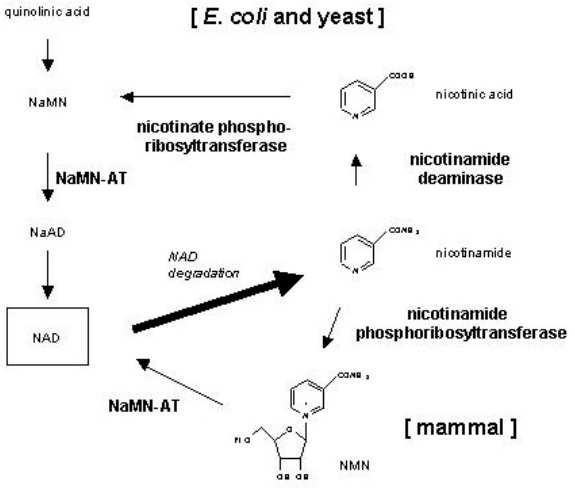

Figure 4. The *salvage* pathways. *E. coli* and yeast recycle nicotinamide into NaMN via nicotinic acid, whereas mammals salvage nicotinamide directly from NAD via NMN. Besides functioning at the penultimate step in the *de novo* pathway, mammalian NaMN adenyltransferase (NaMN-AT) efficiently catalyzes the conversion of NMN to NAD.

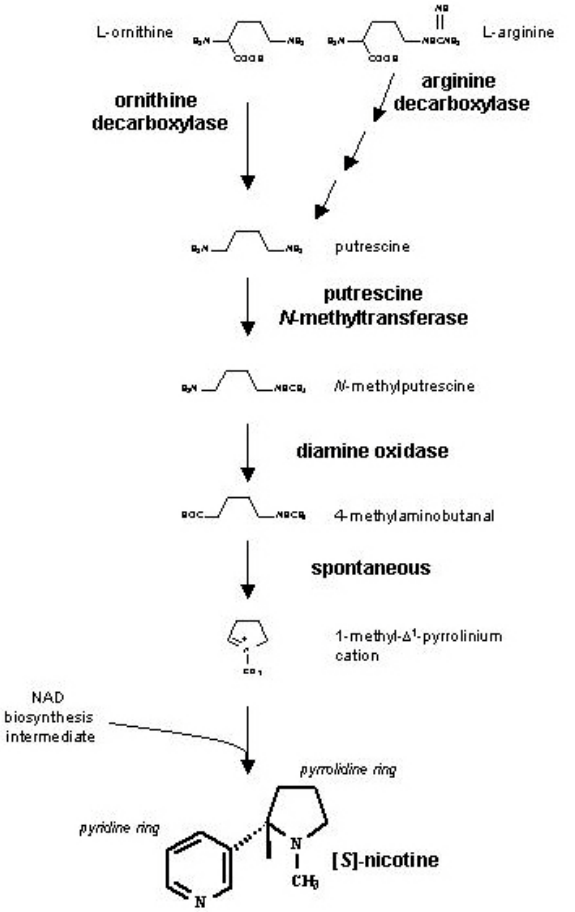

Figure 5. Nicotine biosynthesis in tobacco. The pyrrolidine ring of nicotine is derived from putrescine. Ornithine, arginine, or both serve as the starting amino acid for this symmetrical diamine. Nicotinic acid or its derivative condenses with 1-methyl-Δ^1-pyrrolinium cation to form (*S*)-nicotine.

8. NAD SALVAGE PATHWAY IN BACTERIA, YEAST AND MAMMALS

As described in the Introduction, NAD is utilized as a substrate by several enzymes and is converted to nicotinamide. In the *salvage* pathway of most prokaryotes and yeast, nicotinamide is recycled back to NaMN in two enzymatic steps (Figure 4). The first enzyme nicotinamide deaminase (EC 3.5.1.19) hydrolyzes nicotinamide to nicotinic acid, which is then converted to NaMN by nicotinate phosphoribosyltransferase (EC 2.4.2.11; NaPT). NaPT is classified into three types, according to the requirement of ATP for the enzyme activity (16). In mammals, nicotinamide in the *salvage* pathway is directly converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (EC 2.4.2.12), bypassing nicotinic acid as an intermediate. Mammalian NaMN-AT appears to catalyze two similar conversions of NaMN and NMN to NaDA and NAD, respectively

9. NAD SALVAGE PATHWAY IN PLANTS

Enzyme activities of nicotianamide deamidase and NaPT have been found in crude protein extracts from tobacco, tomato, and castor bean, and a preliminary characterization of tobacco enzymes has been reported (49, 55). Tobacco NaPT activity was stimulated by ATP.

Nucleotide database searches have identified homologues of nicotinamide deamidase and NaPT in several plant species, including Arabidopsis and rice (Table 1). In contrast, mammalian nicotinamide phosphoribosyltransferase does not appear to have significant homologues in the plant DNA databases, nor was its enzyme activity detected in tobacco cell extracts (55). Therefore, *E coli*, yeast, and plants utilize the same NAD salvage pathway.

10. NICOTINE BIOSYNTHESIS IN TOBACCO

Nicotine is the most well known secondary metabolite derived from the NAD pathway. Feeding studies using tobacco plants have consistently demonstrated that radio-labeled quinolinic acid and nicotinic acid are efficiently incorporated into the pyridine ring of nicotine (56-59), indicating that the pyridine ring of nicotine is synthesized from nicotinic acid or its metabolite. Although Leete speculates that nicotinic acid itself condenses with methyl-Δ^1-pyrrolinium cation and serves as the direct precursor of nicotine (47, 56, 60), experimental evidence is still lacking with regard to which intermediate in the NAD pathway is used for nicotine biosynthesis. The pyrrolidine ring moiety of nicotine is derived from the diamine putrescine (Figure 5). The enzymes and the steps leading to the formation of the *N*-methyl-Δ^1-pyrrolinium cation have been reviewed (61).

During the incorporation into nicotine, the C6 tritium label of nicotinic acid is specifically exchanged with unlabelled hydrogen (60). This result was interpreted to suggest that nicotinic acid (or its derivative) is initially oxidized at C6 and then reduced at the same position in the

course of its condensation reaction with *N*-methyl-Δ^1-pyrrolinium cation. In a crude cell extract from tobacco root, a weak activity ("nicotine synthase" activity) that forms labeled nicotine from [2-^{3}H]-nicotinic acid and *N*-methyl-Δ^1-pyrrolinium cation was reported (62). However, we have been unable to detect such nicotine synthase activity using protein preparations from tobacco roots (our unpublished results).

Another alkaloid that is possibly derived from the NAD pathway is damascenine isolated from *Nigella damascena*. 3-Hydroxyanthranilic acid in the kynurenine pathway is conceived as a putative precursor, but [3-^{14}C]-tryptophan was not incorporated into this alkaloid (63).

Nicotianamine was so named after its initial isolation from *Nicotiana tabacum* (64) but subsequent studies showed that this amino acid is widespread in plants (65, 66). Although its common name conjures up a biosynthetic relationship with nicotinic acid metabolism, nicotianamine is synthesized from methionine and is not derived from the NAD pathway (67).

11. PATHWAY REGULATION

In mammals, the kynurenine pathway is initially controlled by the enzymes for tryptophan degradation. Liver tryptophan dioxygenase activity is induced by tryptophan and glucocorticoid hormones, and allosterically regulated by tryptophan levels, while indoleamine 2, 3-dioxygenase activity is induced by infection. We are yet to know whether the presumed kynurenine pathway in rice is regulated at the level of these dioxygenases and whether the aspartate pathway in higher plants in general is subject to any regulation at the transcriptional and enzyme activity levels.

We do know, however, that the aspartate pathway in tobacco is regulated in accordance with nicotine biosynthesis. Nicotine biosynthesis is controlled by two regulatory loci, *NIC1* and *NIC2* (68), is active in young roots, especially at the root tips (69), and is induced in the root by treatment with a plant hormone, methyljasmonate (70, 71). Tobacco QPT mRNA is most abundant in the root and the message level in the root increases after mechanical damage to aerial tissues (51). L-Aspartate oxidase and quinolinate synthase genes are partially controlled by the *NIC* loci, are most active in the tobacco root, and are induced to express by methyljasmonate treatment (our unpublished results). In tobacco leaves where nicotine synthesis does not occur, these genes are expressed at the basal level and do not appear to respond to methyljasmonate (our unpublished results). Therefore, the aspartate pathway in tobacco may be under the same transcriptional control with nicotine biosynthesis only in the cells that synthesize nicotine.

Subcellular compartmentalization may contribute to the control of metabolite flux. Indeed, three core plant enzymes in the aspartate pathway (L-aspartate oxidase, quinolinate synthase, and QPT) were predicted to be distributed to either plastid or mitochondria, based on the subcellular-targetting prediction software Predotar and TargetP. In rice cells where the aspartate and kynurenine pathways may coexist, differential regulation of the two *de novo* NAD pathways is possible at the transcriptional and subcellular levels.

The salvage pathway may be controlled independently from the *de novo* pathways. In yeast, the nuclear nicotianamide level controls the enzyme activity of the NAD-utilizing nucleolar silencing regulator Sir2p, thereby influencing transcriptional regulation. Consistent with the idea that the NAD *salvage* pathway plays an important role in transcriptional silencing at the telomere and rDNA loci, the *salvage* pathway enzyme NaPT is localized primarily in yeast nucleus while the enzymes in the *de novo* pathway are distributed in the cytoplasm (72, 73). It remains to be seen whether the same holds true for higher plants which contain Sir2 homologues in the genome.

12. ACKNOWLEDGMENT

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Note added in proof: The kynurenine pathway is recently found in several bacteria (Kurnasov *et al*, 2003)

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