Formation of Nicotinamide from Nicotinuric Acid by Peptidylglycine α-Amidating Monooxygenase (PAM): A Possible Alternative Route from Nicotinic Acid (Niacin) to NADP in Mammals

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Peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3) is a copper and zinc-dependent enzyme that generates neuropeptides having a C-terminal amide functionality by oxidative removal of the two carbons from a glycine-extended precursor.[1,2] The process probably occurs in all animals[3] and requires two steps (Figure 1). The first involves ascorbate and copper-dependent aerobic hydroxylation of the glycine α-carbon with retention of configuration by peptidylglycine α-hydroxylating monooxygenase (PHM). The second step, catalyzed by peptidyl-amidoglycolate lyase (PAL), is zinc-dependent and resembles a retro-aldol reaction to form the primary amide and glyoxylate. In mammals, RNA splicing and posttranslational modifications lead to bifunctional PAM as well as monofunctional PHM and PAL proteins.[2] An X-ray analysis of the core of PHM with a bound substrate provides an intriguing picture of its catalytic machinery, which has two copper atoms about 11 Å apart in the active site.[4,5] Recently, it has become clear that PAM substrate specificity for the *N*-acyl moiety on the glycine extends beyond peptides to fatty acids and other lipids.[6,7] For example, the natural sleep-inducing substance, oleamide,[8] may be generated in mammals by PAM cleavage of *N*-oleylglycine.[6a] Since a host of carboxylic acids can be conjugated to glycine in vivo by acyl-CoA:glycine *N*-acyltransferase (ACGNAT, EC 2.3.1.13)[9] and nicotinic acid (niacin, vitamin B3) (**1**) is known to be rapidly converted in the liver to nicotinuric acid (**2**),[10] it was of interest to determine whether **2** could be transformed to nicotinamide (**3**) by PAM. In the present study, we describe this conversion as well as preliminary labeling studies with rats to evaluate the importance of this pathway in mammals for the formation of NAD(P).[11]

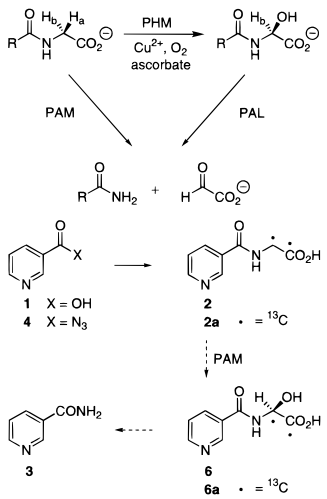

Figure 1. Function of amidating enzymes and structures of nicotinic acid derivatives.

Recombinant type A rat medullary carcinoma PAM was purified[6b,12] and shown to cleave **2** to **3** and glyoxylate. Production of the glyoxylate was determined spectrophotometrically after formation of its 1,5-diphenylformazan derivative,[6b,13] and nicotinamide formation was shown by HPLC analysis.[13] Oxygen consumption was measured with an electrode[6b] to provide kinetic information for the initial oxidative step ($K_{M,\ app} = 1.9 \pm 0.14$ mM; $V/K_{app} = 4.3 \pm 0.24 \times 10^3\ M^{-1}\ s^{-1}$). Under similar conditions, the V/K_{app} values for other PAM substrates are $4.6 \pm 0.087 \times 10^4\ M^{-1}\ s^{-1}$ for D-Tyr-Val-Gly, $1.7 \pm 0.055 \times 10^5\ M^{-1}\ s^{-1}$ for *N*-lauroylglycine, and $6.2 \pm 0.12 \times 10^3\ M^{-1}\ s^{-1}$ for *N*-benzoylglycine. To examine this process by NMR spectrometry, [*glycyl*-1,2-$^{13}C_2$]-nicotinuric acid **2a** was synthesized by coupling the corresponding labeled glycine (99 atom % ^{13}C) to nicotinic acid azide (**4**).[15] Reaction of PAM and **2a** in deuterated buffer (pD 5.6) with copper and ascorbate under aerobic conditions was followed by HMQC NMR analysis.[16] These spectra show that the only detectable labeled species are the starting material **2a** and the hydrate of [1,2-$^{13}C_2$]-glyoxylate. However, the same reaction in the presence of the known PAL inhibitor, 2,4-dioxo-5-acetamido-6-phenylhexanoic acid (*N*-Ac-Phe-pyruvate) (**5**),[17]

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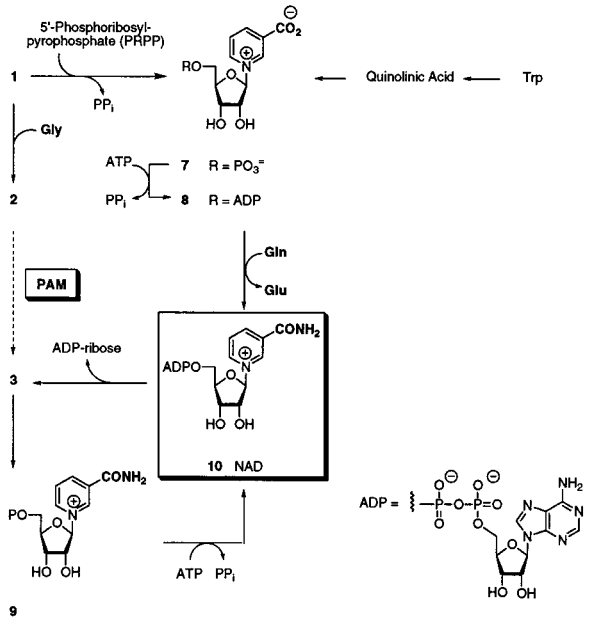

Figure 2. Mammalian metabolic pathways to NAD (**10**) and its derivatives.

results in accumulation of the [*glycyl*-1,2-$^{13}C_2$]-hydroxyglycine intermediate **6a**. This compound spontaneously fragments to glyoxylate and **3** under slightly basic conditions and is converted to these products by PAL activity in PAM when inhibitor **5** is absent.

In mammals, at least three biochemical routes to nicotinamide adenine dinucleotide phosphate (NADP) have been demonstrated (Figure 2): (a) from tryptophan via quinolinic acid leading to nicotinic acid ribonucleotide (**7**),[18] (b) from **1** via **7** leading to nicotinic acid adenine dinucleotide (**8**), followed by subsequent amido nitrogen transfer from glutamine (Preiss−Handler pathway), and (c) from **3** via nicotinamide ribonucleotide (**9**).[11c] The situation is further complicated because various tissues and organs are able to synthesize their own NAD(P), but **3** is rapidly transported between them. It is known that the liver is involved in conversion of **1**, a very readily absorbed vitamin, to **3**.[11c] However, until the present study, this was believed to occur exclusively by *de novo* conversion of the free acid **1** to NAD(P) via the Preiss-Handler pathway followed by action of NAD(P) glycohydrolase to release free nicotinamide (**3**). It is now clear from the current work that efficient intestinal absorption of the acid **1** and its known rapid conversion in the liver[10] to **2** potentially provides a substrate that PAM in erythrocytes (or other cells) can convert directly to **3**.

As a preliminary study to investigation of in vivo conversion of **2** to NAD(P), we examined the utilization of labeled nitrogen from intravenously administered [*amido*-^{15}N]-glutamine and [^{15}N]-glycine in rats. Although nitrogen from either source can potentially be liberated into the metabolic pool and scrambled, highly efficient labeling of the primary amide nitrogen of NAD(P) by glutamine would support the predominance of the Preiss−Handler pathway, whereas effective labeling by glycine would suggest that PAM conversion of **2** plays an important role. To obtain an independent measure of de novo synthesis of NAD(P), commercially available [$^2H_4$]-nicotinic acid (99 atom % ^{2}H) was intravenously co-administered at the same time as the individual ^{15}N precursors.[19] Unfortunately, this complicated the subsequent analysis because of metabolic deuterium transfer and exchange reactions from the C-4 position of NAD(P) which led to appearance of large amounts of monodeutero and trideutero species. These reactions presumably involve reduction to NAD(P)H followed reoxidation. Since various enzymes dependent on these cofactors can have opposite stereochemical selectivity at C-4, in combination they can introduce hydrogen at C-4 and can transfer the deuterium (via a substrate) to an unlabeled NAD(P).[20] NAD and NAD(P) were isolated from each rat liver by literature procedures[21] followed by HPLC purification. Electrospray mass spectrometric analysis of the NAD showed extensive deuterium labeling (including mono- and trideutero species) in each case and suggested lower levels of nitrogen labeling by both [*amido*-^{15}N]-glutamine and [^{15}N]-glycine. To distinguish between single deutero and ^{15}N species, much higher mass spectral resolution was necessary. Hence each NAD sample was individually hydrolyzed[22] at pH 4 to **3**, which was purified by HPLC and analyzed by high resolution (40 000) mass spectrometry using electron impact ionization. This permitted distinction of ^{15}N and ^{2}H species and showed that labeling of the primary amide nitrogen of NADP by [*amido*-^{15}N]-glutamine is comparable to that by [^{15}N]-glycine.[23] Although the actual significance of these levels of labeling (typical 30% ^{2}H and 1−5% ^{15}N) should not be overemphasized because of possible differences between individual animals and complications due to varying intestinal uptake, transport, and metabolism of the precursors, the results support a possible role for PAM in formation of NADP. In addition, they provide a protocol for further ongoing studies with labeled **2**.

The occurrence of PAM in most mammalian tissues (except the liver) and its ability to transform **2** to **3** via intermediate **6** indicate that this enzyme provides a hitherto unrecognized pathway for utilization of niacin in NAD(P). Since conjugation of acids to glycine is a very common liver function, PAM may play a central role in the formation of many other non-peptide primary amides in mammals.

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Supporting Information Available: Experimental procedures for chemical transformations, animal feeding protocols, and details of NAD isolation and analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) In a typical experiment, a solution of 5 mM [*glycyl*-1,2-$^{13}C_2$]-nicotinuric acid (**2a**), 10 mg/mL bovine catalase, 1 mM $Cu(NO_3)_2$, 6 mM sodium ascorbate, 0.1 mM *N*-Ac-Phe-pyruvate, 0.15 mg/mL PAM in 10 mM NaMES, 4 mM NaCl (D_2O), pD 5.6, total volume 1.6 mL, is incubated at 37 °C for 4 h. PAM is then removed using an Amicon Centricon-30 ultrafiltration device. An aliquot (0.7 mL) of the reaction mixture is analyzed by HMQC NMR using an Inova 600 Varian instrument.

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(18) Although dietary tryptophan can provide up to two-thirds of the human requirement for niacin, the efficiency of conversion is very low. In contrast, nicotinamide (**3**) is very efficiently converted to NAD(P). See ref 11c, page 490.

(19) An indwelling catheter was implanted into the right jugular vein of adult male Sprague Dawley rats. [$^2H_4$]-Nicotinic acid (200 mg/kg) with [^{15}N]-glycine (21 mg/kg) or [*amido*-^{15}N]-glutamine (36 mg/kg) was injected through the catheter. The rats were sacrificed at 6, 12, and 24 h intervals. The liver was dissected out of the animal and immediately frozen in liquid N_2. The livers were stored at −70 °C until analysis, see ref 10c.

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