Molecular Identification of Human Glutamine- and Ammonia-dependent NAD Synthetases

CARBON-NITROGEN HYDROLASE DOMAIN CONFERS GLUTAMINE DEPENDENCY

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NAD synthetase catalyzes the final step in the biosynthesis of NAD. In the present study, we obtained cDNAs for two types of human NAD synthetase (referred as NADsyn1 and NADsyn2). Structural analysis revealed in both NADsyn1 and NADsyn2 a domain required for NAD synthesis from ammonia and in only NADsyn1 an additional carbon-nitrogen hydrolase domain shared with enzymes of the nitrilase family that cleave nitriles as well as amidases to produce the corresponding acids and ammonia. Consistent with the domain structures, biochemical assays indicated (i) that both NADsyn1 and NADsyn2 have NAD synthetase activity, (ii) that NADsyn1 uses glutamine as well as ammonia as an amide donor, whereas NADsyn2 catalyzes only ammonia-dependent NAD synthesis, and (iii) that mutant NADsyn1 in which Cys-175 corresponding to the catalytic cysteine residue in nitrilases was replaced with Ser does not use glutamine. Kinetic studies suggested that glutamine and ammonia serve as physiological amide donors for NADsyn1 and NADsyn2, respectively. Both synthetases exerted catalytic activity in a multimeric form. In the mouse, NADsyn1 was seen to be abundantly expressed in the small intestine, liver, kidney, and testis but very weakly in the skeletal muscle and heart. In contrast, expression of NADsyn2 was observed in all tissues tested. Therefore, we conclude that humans have two types of NAD synthetase exhibiting different amide donor specificity and tissue distributions. The ammonia-dependent synthetase has not been found in eucaryotes until this study. Our results also indicate that the carbon-nitrogen hydrolase domain is the functional domain of NAD synthetase to make use of glutamine as an amide donor in NAD synthesis. Thus, glutamine-dependent NAD synthetase may be classified as a possible glutamine amidase in the nitrilase family. Our molecular identification of NAD synthetases may prove useful to learn more of mechanisms regulating cellular NAD metabolism.

The coenzyme NAD has a role in the majority of metabolic redox reactions and represents an essential component of metabolic pathways in all living cells. In a number of signaling pathways, NAD also serves as a precursor of potent calcium-mobilizing agents such as cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (1) and serves as a substrate for post-translational modifications of protein, mono- (2–4) and poly(ADP-ribosyl)ations (5). Depletion of cellular NAD by poly-(ADP-ribose)transferase activation in response to DNA damage results in cell death (6). Increased NAD synthesis has been shown to extend life span in yeast (7) and in Caenorhabditis elegans (8) via activation of an NAD-dependent histone deacetylase, silent information regulator 2 (Sir2) (9). The cellular level of NAD may modulate the sensitivity of cells to apoptotic responses through deacetylation of the p53 tumor suppressor by a human homologue of Sir2 (10). Recent publications have demonstrated that fluctuation of the NAD level in cells seems to have significant impact on their physiology. Despite these significant effects of NAD levels on cellular functions, mechanisms regulating cellular contents of NAD through metabolic events remain to be established.

NAD biosynthesis is accomplished through either de novo or salvage pathways (11, 12). These two pathways converge at the level of an intermediate nicotinic acid mononucleotide (NaMN), 1 which is then converted into nicotinic acid adenine dinucleotide (NaAD) through the action of NaMN adenyltransferase and, lastly, into NAD by NAD synthetase (Fig. 1). Although most of the genes involved in both pathways have been identified in prokaryotes (13), little is known of those genes, including that of NAD synthetase in eucaryotes, except for nicotinamide mononucleotide adenyltransferase (14) and quinolnic acid phosphoribosyltransferase (15) genes.

NAD synthetase catalyzes the conversion of NaAD into NAD, and NH3 or glutamine is used as an amide donor in the following reactions.

\[
\text{NaAD} + \text{NH}_3 + \text{ATP} \rightarrow \text{NAD} + \text{AMP} + \text{PP}, \\
\text{REACTION 1} \\
\text{NaAD} + \text{glutamine} + \text{ATP} \rightarrow \text{NAD} + \text{glutamate} + \text{AMP} + \text{PP}, \\
\text{REACTION 2}
\]

In prokaryotes, two types of NAD synthetase have been reported, a type catalyzes Reaction 1 and is strictly ammonia-dependent, whereas the other synthetase catalyzes both Reactions 1 and 2 and uses both ammonia and glutamine as amide donors. Bacillus subtilis synthetase, a representative of the former, is a protein of 271 amino acid residues (16), and crystal

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB091316 (NADsyn1) and AB091317 (NADsyn2).

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1 The abbreviations used are: NaMN, nicotinic acid mononucleotide; CN-hydrolase, carbon-nitrogen hydrolase; HPLC, high performance liquid chromatography; NaAD, nicotinic acid adenine dinucleotide; kb, kilobase(s).
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Fig. 1. Metabolic pathways of NAD biosynthesis. Nam, nicotinamide; NMN, nicotinamide mononucleotide; NA, nicotinic acid; QA, quinolinic acid; NAPRT, nicotinic acid phosphoribosyltransferase; NaMNAT, nicotinic acid mononucleotide adenyllytransferase; NADsyn, NAD synthetase; QPRT, quinolinic acid phosphoribosyltransferase.

structure analysis of the synthetase revealed residues responsible for binding sites for ATP and NaAD (17). Although one of the latter enzymes, Mycobacterium tuberculosis synthetase of 738 amino acids, has been also reported (18), the structural basis underlying the potential to use glutamine or ammonia of two types of synthetase has not been determined. In eucaryotes, although NAD synthetase activity of the latter type has been reported (19, 20), molecular cloning and characterization have not been done. In the case of the strictly ammonia-dependent NAD synthetase, the counterpart of B. subtilis enzyme, even the presence of the activity has not been demonstrated in eucaryotic organisms.

We now report molecular identification of two human NAD synthetases, a synthetase that use not only ammonia but also glutamine and the other synthetase with strictly ammonia-dependent activity (referred as NADsyn1 and NADsyn2, respectively). To our knowledge, this is the first report demonstrating the presence of the strictly ammonia-dependent NAD synthetase in eucaryotes. We also describe the structural basis underlying the potential of NADsyn1 to use glutamine as an amide donor as well as the distinct distribution of NADsyn1 and NADsyn2 in animal tissues.

EXPERIMENTAL PROCEDURES

Materials—[^15N]Pyruvate (6000 Ci/mmol) was purchased from Amersham Biosciences. NaAD, AMP and inorganic pyrophosphatase were from Sigma. ATP, NAD, L-glutamine, and ammonium chloride were from Oriental Yeast (Tokyo, Japan), Roche Molecular Biochemicals (Basel, Switzerland), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan), respectively. COS-7 cells and a human promyelocytic leukemia cell line HL60 were obtained from Riken Cell Bank (Takushu Science City, Japan). Human glioma cell line LN229 and human hepatocyte cell lines HepG2 and Huh7 were from American Type Culture Collection (Manassas, VA).

Expression of NADsyn1 and NADsyn2 in COS-7 Cells—To express NADsyn1 and NADsyn2 as C-terminal-His$_6$-tagged proteins in COS-7 cells, a His$_6$ tag sequence followed by a TGA termination codon was introduced into the pcDNA3 vector (Invitrogen) between XbaI and AgeI cloning sites to obtain the pcDNA3His$_6$ vector. Segments of human NADsyn1 cDNA were PCR-amplified from fetal human brain cDNA (Clontech, Palo Alto, CA) using two sets of primers, 5′-ATG GGC CGG CAG GTG ACC-3′ (sense) and 5′-CAG ACC TGG CAG CAC ATG-3′ (antisense) and 5′-ATG GGC CGG AAA GTG ACC-3′ (sense) and 5′-GAA GCA GCC GGC TCT A3′ (antisense). The PCR products were gel-purified, combined, and amplified using primers (the underlined regions correspond to cloning sites) 5′-AATG CTT GTG ACC ATG GGC CGG CAG GTG ACC-3′ (sense) and 5′-AATG CTT TCT AGA GTG ACC GTC CAC GCC GTC CAG GGA-3′ (antisense), yielding full-length NADsyn1 cDNA. Human NADsyn2 cDNA was amplified from LN229 total RNA treated with deoxyribonuclease I (Nippon Gene, Tokyo, Japan) by reverse transcription-PCR using primers 5′-AATG CTT GTG ACC ATG CAA GCC GTA CAG CCG GCA-3′ (sense) and 5′-AATG CTT TCT AGA GTG TGC AAA CGG CAT CAC-3′ (antisense). Amplified cDNAs were digested with KpnI and XbaI for NADsyn1 and BanHI and XbaI for NADsyn2 and ligated, respectively, into KpnI- and XbaI-digested and BamHI- and XbaI-digested pcDNA3His$_6$. The vector or to express the mutant NADsyn1, in which Cys-175 was replaced with serine (C175S-NADsyn1), was made using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the pcDNA3His$_6$ plasmid vector carrying wild-type NADsyn1 and the oligonucleotide primers 5′-TT TGG AAT GAG ATC aag GAG GAC CTC TGC-3′ and 5′-CCA GAG CTC TCT CGC TCT CTA TCT CTA TCA-3′, where the mutated residues are indicated in lowercase italics. The resultant expression plasmids, purified using a Qiagen plasmid kit (Hilden, Germany), were transfected into COS-7 cells (4.5 × 10$^5$ cells/100-mm dish) using an activated-dendrimer PolyFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions. Forty-eight hours after transfection, the COS-7 cells were washed twice with phosphate-buffered saline and plated by scraping. After the cells were lysed by sonication and centrifugation, recombinant NAD synthetases were purified from the supernatants with His-Bind Resin (Novagen, Madison, WI) according to the manufacturer’s protocol. A cDNA fragment of mouse glutamine-dependent NAD synthetase was amplified from Balb/c mouse kidney total RNA treated with deoxyribonuclease I by reverse transcription-PCR using primers based on the sequence of mouse homologue of human NADsyn1 (see Fig. 7), 5′-ATG GGC CGG AAA GTG ACC-3′ (sense) and 5′-CAG ACC AGG CAG CAC ATG-3′ (antisense). Sequences of expression plasmids or PCR fragments were confirmed by entire sequencing in both directions.

5′-Rapid Amplification of cDNA Ends—Adaptor-ligated double stranded cDNA was synthesized using Marathon cDNA amplification kit (Clontech) from poly(A)$^+$ RNA isolated from HL60 bone marrow micro mRNA purification kit (Amersham Biosciences). The 5′ parts of the NADsyn1 and NADsyn2 cDNAs were amplified with Advantage 2 polymerase mix (Clontech) using sense adaptor primer 5′-CCA TCC TAA TAC GAC TCA TGA TAG GCC-3′ and gene-specific antisense primers 5′-CAG CCC AGC TCC CCG TAG TTAG CCT TC-3′ (NADsyn1) and 5′-CAC CCC AGC AAG TCC CGG GAG ACT GC-3′ (NADsyn2). After nested PCR, the products were subcloned into pcDNA3, and positive clones were isolated and sequenced.

Enzyme Assays—Unless otherwise stated, NAD synthetase activity of the recombinant protein was based on fluorometric measurements of the NAD formed, as described below. Recombinant NADsyn1, the mutant NADsyn1, and NADsyn2 were incubated with glutamine or NH$_4$Cl as indicated in the reaction mixture (50 μM) containing 50 mM HEPES (pH 8.8), 2 mM ATP, 1 mM NaAD, 56 mM KCl, 5 mM MgCl$_2$, and 10 μg of bovine serum albumin. For the assay of NADsyn1 activity, 2 mM diethiothreitol was included in the reaction mixture. When glutamine was used as a substrate for NADsyn1, 50 μM Tris-Cl (pH 7.5) was included instead of 50 mM HEPES (pH 8.8). The reactions were terminated by adding 0.4 μl of 7 N NaOH and then incubated at 37 °C for 30 min to obtain the fluorescent product (21). The fluorescence was measured using 380 nm for excitation and 460 nm for emission by Fluoroskan Ascent FL (Labsystems, Helsinki, Finland). The fluorescence intensity of standard NAD solutions at known concentrations was used to calculate the amount of NAD. NAD synthetase activity was calculated by subtracting the NAD content of enzyme-deficient blanks from the NAD content of the complete reaction mixture.

In some cases NAD synthetase activity was determined by HPLC analysis. After the NAD synthetase reactions had been terminated by a 10-fold dilution with 0.1% trifluoroacetic acid, the reaction products were separated on a reversed phase Cosmosil 5C18-MS column (4.6 × 150 mm, Nacalai Tesque) with 0.1% trifluoroacetic acid as the mobile phase and detected by measuring absorbance at 254 nm.

Kinetic parameters for NAD synthetase reaction were determined as follows by analysis of a Lineweaver-Burk plot of the initial rates of NAD synthesis. In the reaction mixture fixed concentrations of two of the three substrates concentrations of the third substrate varied from 0.1 to 2.5 mM for NaAD, from 0.5 to 30 mM for ATP, from 0.1 to 150 mM for glutamine, and from 1.5 to 30 mM (NADsyn1 and C175S-NADsyn1) or from 0.01 to 0.2 mM (NADsyn2) for NH$_4$Cl. In the reaction catalyzed by NADsyn1, $K_m$ values for NaAD and ATP were determined using glutamine and were measured in reactions catalyzed by NADsyn2 and C175S-NADsyn1, values were determined using NH$_4$Cl. Amounts of NAD formed were determined by the fluorometric method described earlier.

Determination of PP$_i$—NAD synthetase reactions (50 μl) were terminated by adding 8 μl of 10% trifluoroacetic acid. After standing on ice for 15 min and then neutralizing by 1× Tris-Cl (pH 9.0), the reaction mixtures were incubated with or without 2.5 milliunits/μl of pyrophosphatase at 25 °C for 30 min. The reactions were terminated by 10%
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Expression and Functional Characterization of Human NAD Synthetases—We next expressed NADsyn1, NADsyn2, and a mutant NADsyn1 in which Cys-175 was replaced with serine (C175S-NADsyn1) in COS-7 cells as His6-tagged recombinant proteins, and we purified these proteins on nickel chelate resin. SDS-PAGE analysis indicated that the purified wild-type (Fig. 3A, inset) and mutant NADsyn1 (data not shown) have a molecular mass of 80 kDa, in accordance with the value calculated from the deduced sequences, 80.3 kDa. The purified recombinant NADsyn2 appeared as a single band with a molecular mass of 34 kDa (Fig. 3B, inset), slightly larger than the value calculated from the deduced sequence, 30.8 kDa. The difference may depend on the pI of the recombinant protein (pI 5.9).

To investigate whether NADsyn1, NADsyn2, and C175S-NADsyn1 have the predicted enzymatic activities, we incubated the purified recombinant proteins with glutamine or NH4Cl in the presence of NAD and ATP then determined the NAD synthetase activities of the proteins using a fluorometric method. As shown in Fig. 4A, the recombinant wild-type NADsyn1 exhibited almost the same activity with either glutamine or NH4Cl. On the other hand, the recombinant NADsyn2 catalyzed NAD synthesis primarily with NH4Cl (Fig. 4C). In marked contrast with the wild-type NADsyn1, the activity of the mutant NADsyn1 (C175S-NADsyn1) was not detected when glutamine was used as a substrate, whereas the activity remained unaltered with NH4Cl (Fig. 4B). Kinetic analysis indicated that NADsyn1 shows a lower Km for glutamine (1.44 mM) than for NH4Cl (13.1 mM) (Table I). Compared with NADsyn1, NADsyn2 showed a much lower Km for NH4Cl (34 μM)ilter a much higher K_m for glucose (103 μM) (Table I). NADsyn1 and NADsyn2 showed essentially the same K_m values for ATP and NaAD, in the range of those reported for native synthetases (16, 19). Kinetic parameters of the mutant NADsyn1 obtained with NH4Cl did not differ from those of the wild-type NADsyn1, which suggests that disappearance of the glutamine dependence was not because of a drastic change in the tertiary structure of NADsyn1. Compared with NADsyn2 (34 μM), the mutant synthetase exhibited a much higher K_m value for NH4Cl (23.9 mM). With the glutamine preparation,
FIG. 2. Structural analysis of NADsyn1 and NADsyn2. A, domain organization of NADsyn1 and NADsyn2. CN_hydrolase domain, synthetase domain composed of NAD_synthase domain and P-loop motif, and the critical cysteine residue in NADsyn1 at a position of 175 (Cys-175) are shown. 

B, the synthetase domains of NADsyn1 and NADsyn2 (amino acids 357–657 and 52–275, respectively) and B. subtilis NAD synthetase (SWISS-PROT accession number P08164). Amino acid sequences of these proteins are compared with the consensus sequence for NAD_synthase domain (underlined, NAD_synthase; Pfam accession number PF02540). Conserved P-loop motif and residues forming the NaAD binding site are indicated by asterisks and number symbols (#), respectively.

C, the CN_hydrolase domains of NADsyn1 (amino acids 8–296) and NAD synthetases from M. tuberculosis (P71911) and R. capsulatus (Q03638). Amino acid sequences of these proteins are compared with the consensus sequence for CN_hydrolase domain (CN_hydrolase, Pfam accession number PF00795). The critical cysteine residues are indicated by asterisks.

Dashes represent gaps introduced to optimize the alignment. Identical residues are indicated by dots and on dark background when identical among more than three sequences.

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Markers are indicated on the left of the recombinant enzymes. The kinetic parameters were determined as described under “Experimental Procedures.” $K_m$ and $V_{max}$ values represent the mean ± S.D. of three separate experiments.

**TABLE I**

Kinetic parameters in NAD synthetase reactions catalyzed by the wild-type and mutant NADsyn1 and NADsyn2

| Enzymes | Substrates  | $K_m$ (mM) | $V_{max}$ (nmoles/min/mg) |
|---------|-------------|------------|---------------------------|
| NADsyn1 | NaAD        | 0.49 ± 0.06| 0.99 ± 0.30               |
|         | ATP         | 0.089 ± 0.023| 0.61 ± 0.19                |
|         | Glutamine   | 1.44 ± 0.17| 0.70 ± 0.18                |
|         | NH$_4$Cl    | 13.1 ± 1.2 | 1.04 ± 0.18               |
|         | ATP         | 0.12 ± 0.09| 0.53 ± 0.10               |
| C175S   | NaAD        | 0.20 ± 0.04| 0.54 ± 0.10               |
|         | ATP         | 0.12 ± 0.09| 0.53 ± 0.10               |
|         | Glutamine   | 13.1 ± 1.2 | 1.04 ± 0.18               |
|         | NH$_4$Cl    | 23.9 ± 6.7 | 0.98 ± 0.23               |
| NADsyn2 | NaAD        | 0.13 ± 0.02| 1.57 ± 0.32               |
|         | ATP         | 0.40 ± 0.06| 1.62 ± 0.26               |
|         | Glutamine   | 103 ± 17.4| 1.72 ± 0.15               |
|         | NH$_4$Cl    | 0.034 ± 0.01| 1.37 ± 0.52               |

*a NAD synthetase activity was below the limit of detection, and thus, the $K_m$ value was not determined.*

All these results are consistent with our predictions (i) that both NADsyn1 and NADsyn2 have NAD synthetase activity, (ii) that NADsyn1 utilizes not only ammonia but also glutamine as an amide donor, whereas NADsyn2 is primarily an ammonia-dependent NAD synthetase, and (iii) that Cys-175 in NADsyn1 is essential for the ability to use glutamine as an amide donor. In agreement with our sequence analyses noted above, we therefore conclude that the CN$_2$ hydrolase domain in the N-terminal half of NADsyn1, in particular Cys-175, is responsible for utilization of glutamine as an amide donor and, thus, confers glutamine dependence on the synthetase, whereas the synthetase domain in NADsyn1 and NADsyn2 participates solely in NAD synthesis from ammonia. To further confirm the role of the CN$_2$ hydrolase domain in using glutamine, we made a chimera consisting of the N-terminal region of NADsyn1 and NADsyn2 as well as a construct containing solely the N- or C-terminal half of NADsyn1. However, because of their insufficient expressions, we could not characterize these constructs (data not shown).

We next examined the stoichiometry of the reactions catalyzed by the recombinant synthetases. The purified recombinant NADsyn1 and NADsyn2 were incubated with glutamine and NH$_4$Cl, respectively, in the presence of NaAD and ATP and the reaction products were analyzed by reversed phase HPLC. As shown in Fig. 5, 1 nmol of AMP and PP$_i$ was produced per 1 nmol of NAD synthesized during the reaction catalyzed by each synthetase. These results indicate that amidation of NaAD by the recombinant enzymes is associated with ATP cleavage to AMP and PP$_i$, as noted for the native enzyme (28). Omission of either ATP, Mg$^{2+}$, NaAD, or amide donors from the reaction mixture resulted in a complete loss of NAD synthesis by each enzyme (data not shown).

To examine whether catalytically active forms of human NADsyn1 and NADsyn2 are multimers as native synthetases (16, 19, 20), we fractionated the purified recombinant synthetases by non-denaturing PAGE and determined NAD synthetase activity in gel slices. As shown in Fig. 3, activities of NADsyn1 and NADsyn2 had mobilities consistent with proteins of 500 and 70 kDa, respectively, suggesting that NADsyn1 and NADsyn2 may exist as a homohexamer and a homodimer, respectively.

**Tissue Distribution of NAD Synthetases**—To evaluate the tissue distribution of NADsyn1 and NADsyn2, Northern blot
analyses were done with total RNA from various mouse tissues and several human cell lines. As shown in Fig. 6A, a message of 3.1 kb was detected for NADsyn1, with various intensities. The major sites of NADsyn1 gene expression were the small intestine, kidney, liver, and testis, whereas the skeletal muscle, spleen, lung, heart, and brain showed a weak signal. In the liver and small intestine, an additional signal was observed at 2.1 kb. The NADsyn1 gene was also expressed in human glioma (LN229) and promyelocytic leukemia (HL60) cell lines (data not shown). Although an EST data base homology search did not reveal a human clone with a high degree of similarity to NADsyn2, Northern blot analysis clearly showed that an mRNA species of 1.4 kb is expressed in human cells LN229, HL60, and HepG2 and Huh7 (hepatocyte cell lines) (Fig. 6B). In the mouse, all of the tissues tested expressed the 1.4-kb mRNA, probably representing a mouse homologue of NADsyn2. In the lung and in skeletal muscle, the same 1.4-kb signal was observed after long exposure of the blot (data not shown). In mouse brain and kidney and human cell lines an additional 2.6-kb mRNA species was observed.

Sequence Alignment of NADsyn1 Homologues—The deduced amino acid sequences of NADsyn1 and NADsyn2 showed mismatches of three and four amino acids, respectively, compared with those deposited in GenBankTM data base (AK001493 and HSA236885, respectively) (Figs. 7 and 2B). A homology search in a protein data base revealed that NADsyn1 exhibits significant amino acid identity to several eucaryotic putative proteins from the mouse (83%), Saccharomyces cerevisiae (55%), Drosophila melanogaster (53%) and C. elegans (46%) (Fig. 7).

**DISCUSSION**

The present study is the first identification of open reading frames encoding two NAD synthetases, NADsyn1 and NADsyn2, in humans. Heterologous expression of the synthetases indicated that although NADsyn1 utilizes both glutamine and ammonia as amide donors, ammonia may not serve as a physiological amide donor for NADsyn1 in vivo (K_m for NH_4Cl > 10 mM). In marked contrast, NADsyn2 uses ammonia more efficiently than does NADsyn1 (K_m for NH_4Cl = 34 mM) but appears to be unable to use glutamine as a physiological amide donor (K_m for glutamine > 100 mM). Thus, we conclude that NADsyn1 is a glutamine-dependent NAD synthetase, whereas NADsyn2 is a strictly ammonia-dependent synthetase. To our knowledge, this is the first evidence for the presence of an ammonia-dependent NAD synthetase in eucaryotes. Furthermore, by comparing the catalytic activity of NADsyn2, which lacks CN_hydrolase...
domain, with the mutant NADsyn1, in which Cys-175 corresponding to the catalytic cysteine residue in nitrilases (24, 25) was replaced with Ser, we identified the CN_hydrolase domain as the functional domain of NAD synthetase to abstract nitrogen from the amide of glutamine and, thus, to use glutamine as an amide donor.

Identification of the CN_hydrolase domain as the determinant of glutamine dependence means that the homologues of human NADsyn1 homologues were obtained using a BLAST program. Protein sequences included in the comparison (identified as SWISS-PROT codes unless otherwise stated) are NADsyn1 (human) and hypothetical proteins of *Mus musculus* (mouse, GenBank™ accession number AK020264), *Saccharomyces cerevisiae* (P38795), *Drosophila melanogaster* (Q9VYA0), and *Caenorhabditis elegans* (PIR accession number T19420). Identical residues are indicated by dots and on dark background when identical among more than three sequences. The conserved P-loop motif and possible catalytic cysteine residues are shown by asterisks and number symbols (#), respectively. The CN_hydrolase domains are underlined.

**Fig. 7.** Comparison of the amino acid sequence of human NADsyn1 with those of other homologous proteins. NADsyn1 homologues were obtained using a BLAST program. Protein sequences included in the comparison (identified as SWISS-PROT codes unless otherwise stated) are NADsyn1 (human) and hypothetical proteins of *Mus musculus* (mouse, GenBank™ accession number AK020264), *Saccharomyces cerevisiae* (P38795), *Drosophila melanogaster* (Q9VYA0), and *Caenorhabditis elegans* (PIR accession number T19420). Identical residues are indicated by dots and on dark background when identical among more than three sequences. The conserved P-loop motif and possible catalytic cysteine residues are shown by asterisks and number symbols (#), respectively. The CN_hydrolase domains are underlined.
human NADsyn1 found in different species are also glutamine-dependent NAD synthetases. The domain occurring in the NAD synthetase from M. tuberculosis, known to catalyze NAD synthesis with glutamine (18), now provides the previously unrecognized structural basis underlying the glutamine dependence of the synthetase. These results suggest that the glutamine-dependent NAD synthetases can be classified as a possible glutamine amidase into the nitri lase family. In the nitri lases, an invariant cysteine residue has been proposed to act as a nucleophile in the catalytic mechanism, where a nitrite carbon is subjected to a nucleophilic attack by sulfhydryl group in the active site of the enzyme (24, 25). Because the critical cysteine residue in the nitri lases is conserved in these glutamine-dependent NAD synthetases, the cysteine residues in the synthetases probably carry out a nucleophilic attack on a carbonyl carbon of glutamine, abstracting ammonia from glutamine. In the synthetase domains of these synthetases, after adenylation of NaAD in the presence of ATP, the ammonia thus abstracted in the CN-hydrolase domain attacks the adenylated NaAD, resulting in NAD, as proposed (28). X-ray diffraction analysis and a detailed structure-functional analysis of NADsyn1 will give a better understanding of the catalytic mechanism of these glutamine-dependent NAD synthetases.

We showed that NADsyn1 exerts catalytic activity in a multimeric form. NAD synthetases purified from human erythrocytes and yeast, glutamine-dependent and, thus, expected to possess CN-hydrolase domain, are also multimeric enzymes (19, 20). It has been reported that nitri lase family members form a multimer, probably by subunit contact through highly hydrophobic regions conserved in the CN-hydrolase domain (26) (Fig. 2C). Thus, it appears that the CN-hydrolase domain, including hydrophobic regions, participates in multimer formation of NADsyn1.

Glutamine-dependent yeast NAD synthetase has been reported to have two components, an 80-kDa ammonia-dependent NAD synthetase subunit and an additional 65-kDa subunit, and the latter has been hypothesized to use glutamine as amid donor (19, 20). However, because the yeast homologue of human NADsyn1 with a calculated molecular mass of 80.7 kDa has the CN-hydrolase domain, it seems that the 80-kDa subunit solely represents the yeast synthetase, and it is unlikely that the 65-kDa subunit is required for glutamine-dependent NAD synthetase activity.

The wide variability of NADsyn1 expression revealed by Northern blot analysis may reflect differences in NAD demand among animal tissues. Abundant expression of NADsyn1 was observed in the small intestine, liver, kidney, and testis, whereas skeletal muscle and the heart showed very weak signals. However, nicotinamide mononucleotide adenyltransferase, catalyzing the formation of the substrate of NAD synthetase NaAD (Fig. 1), has been reported to be expressed mainly in skeletal muscle and the heart (14), thus being inconsistent with NADsyn1 expression. This raises the question of how NAD synthesis occurs in these tissues. NADsyn2 is expressed in skeletal muscle and in the heart. In these tissues, gene expression of glutaminase catalyzing the formation of ammonia from glutamine has also been demonstrated (31). Taken together the finding that NADsyn2 could catalyze NAD synthesis using ammonia as an amid donor, NADsyn2 may largely mediate NAD synthesis in these tissues. Alternatively, based on a somewhat higher affinity of nicotinamide mononucleotide adenyltransferase for NMN than for NaMN (14), NAD may also be synthesized via direct conversion of NMN to NAD in the tissues. For a better understanding of the regulation of NAD biosynthesis in higher organisms, including humans, further analyses on quinolinic acid phosphoribosyltransferase and nicotinic acid phosphoribosyltransferase expression in animal tissues are under investigation.

In the present study, we identified glutamine- and ammonia-dependent human NAD synthetases, NADsyn1 and NADsyn2, respectively, with distinct tissue distribution of the synthetases, and we obtained evidence that the CN-hydrolase domain confers glutamine dependence on the former enzyme. Our results suggest that the glutamine-dependent NAD synthetase is classified as a glutamine amidase into the nitri lase family and that the newly identified metabolic pathway involving the ammonia-dependent NAD synthetase plays a role in NAD biosynthesis. These are important clues to better understand detailed structures fundamental to catalytic activity of the enzyme and to elucidate regulatory mechanisms of cellular NAD metabolism.

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