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Structural and Functional Characterization of Human NAD Kinase

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NADP is essential for biosynthetic pathways, energy, and signal transduction. Its synthesis is catalyzed by NAD kinase. Very little is known about the structure, function, and regulation of this enzyme from multicellular organisms. We identified a human NAD kinase cDNA and the corresponding gene using available database information. A cDNA was amplified from a human fibroblast cDNA library and functionally overexpressed in *Escherichia coli*. The obtained cDNA, slightly different from that deposited in the database, encodes a protein of 49 kDa. The gene is expressed in most human tissues, but not in skeletal muscle. Human NAD kinase differs considerably from that of prokaryotes by subunit molecular mass (49 kDa vs 30–35 kDa). The catalytically active homotetramer is highly selective for its substrates, NAD and ATP. It did not phosphorylate the nicotinic acid derivative of NAD (NAAD) suggesting that the potent calcium-mobilizing pyridine nucleotide NAADP is synthesized by an alternative route.

The importance of NAD and NADP as electron carriers in energy metabolism has long been known. A large number of studies have also established the role of these coenzymes in signal transduction pathways (reviewed in Ref. 1). NAD is a substrate for two different types of covalent protein modifications. Poly(ADP-ribosyl)ation participates in the regulation of a variety of nuclear processes including base excision repair and transcription control (1–3). MonoADP ribosylation represents a mechanism for enzyme regulation in the cytosol (4) and in mitochondria (5) and does also influence extracellular processes (6). Furthermore, NAD and NADP can be converted into the calcium mobilizing agents cADPR, cADPRP, or NAADP (7, 8). It has been suggested that NAD kinase could convert NAAD, a physiological intermediate of NAD synthesis, to NAADP, a highly potent intracellular calcium-mobilizing agent (1, 9–11). The cyclic nucleotides (cADPR and cADPRP) are synthesized by ADP ribosyl cyclase (7–12). There is evidence that this enzyme may also synthesize NAADP by catalyzing the exchange of nicotinamide for nicotinic acid in NADP (7, 8). However, this reaction requires a low pH and a rather high concentration of nicotinic acid. Moreover, in higher eukaryotes the catalytic activity of known ADP-ribosyl cyclases has been localized to the cell surface (7, 8). Therefore, it is reasonable to expect that an alternative biosynthetic pathway for the generation of NAADP may exist.

In energy metabolism NADP is primarily required in its reduced form as an electron donor for biosynthetic reactions such as fatty acid or steroid synthesis. Importantly, this coenzyme is also an essential constituent of cellular defense mechanisms against oxidative stress. NADPH is the coenzyme for many enzymes participating in detoxification reactions including glutathione reductase (13), thioredoxin reductase (14) and cytochrome P_{450} reductase (15). The consequences of a diminished NADPH concentration in mitochondria were shown recently (16). An elevated level of reactive oxygen species, DNA fragmentation, lipid peroxidation, and mitochondrial damage accompanied by a significant reduction of the cellular ATP concentration was observed when expression of the mitochondrial NADPH producing isoform of isocitrate dehydrogenase was decreased.

The multiple functions of NADP in energy metabolism, signaling pathways and detoxification reactions imply that regulation of its cellular concentration is critical for cell survival. The final reactions leading to NAD(P) synthesis are well known (17). However, only very recently the first molecular characterization of an enzyme of this pathway from a multicellular organism was presented, namely, for the human nicotinamide mononucleotide adenylyl transferase (18, 19). This enzyme catalyzes the formation of NAD or NAAD from

Abbreviations used: cADPR(P), (2′-phospho) cyclic ADP-ribose; NAAD(P), nicotinic acid adenine dinucleotide (phosphate); NMN(AT), nicotinamide mononucleotide (adenylyl transferase).

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ATP and NMN or NaMN, respectively. Primary structures of NAD kinase from a few prokaryotes and the yeast *Saccharomyces cerevisiae* where also established only very recently (20–22). The enzyme transfers a phosphate group from ATP to NAD. This reaction represents the only known way to generate NADP both in prokaryotic and eukaryotic cells.

The activity of NAD kinase has been assigned to the cytosol (23), although in yeast a mitochondrial form may exist which accepts both NAD and NADH as substrate (24). The enzyme appears to be regulated by the redox state of the cell, because NADPH inhibits the enzyme's catalytic activity (25). For plants, sea urchin eggs and human neutrophils a stimulation of NAD kinase activity by calcium/calmodulin was reported (26–28).

Given the important role of NADP(H) and thus of NAD kinase in a variety of vital processes as well as its potential function as a messenger enzyme we aimed our study at identifying the primary structure of human NAD kinase and the functional characteristics of this enzyme. So far, very limited information, including essentially only some kinetic and physical characteristics of partially purified preparations from pigeon, rabbit or bovine liver (25, 29–31), has been available for this enzyme from multicellular organisms. Here we describe the cloning and functional overexpression of the human enzyme in *Escherichia coli.* Tissue-specific expression of the human NAD kinase was established by Northern blot analysis. Kinetic and structural properties of the enzyme were determined and were found to be similar to the endogenous NAD kinase which we partially purified from bovine liver. The mammalian enzymes exhibited substantial differences to their counterparts from lower organisms.

MATERIALS AND METHODS

Amplification and cloning of a cDNA encoding human NAD kinase. Cytoplasmic RNA from SV-40 transformed lung fibroblasts (Wi 38) was isolated as described in Ref. (32). Reverse transcription of 3 μg RNA was performed in RT buffer (Promega). The reaction mixture contained 250 μM dNTP, 40 U RNase inhibitor (Life Technologies), and 140 U M-MLV reverse transcriptase (Promega) and was incubated at 37°C for 1 h. RNA was then digested by adding 40 U RNase H (Takara). The primers for amplification of NAD kinase cDNA were 5′-AAC GGC ATC AGT GTT TTT CTG-3′ and 5′-GAT TCG GGC CTG GAT AGG-3′. PCR was carried out for 35 cycles with an annealing temperature of 56°C. The PCR product was cloned into the 2.1-TOPO vector (Invitrogen) and transformed into *E. coli* cells according to the manufacturer's protocol. For expression cloning the cDNA encoding NAD kinase was amplified with a primer containing a *Bam*HI recognition site. The PCR product was phosphorylated, restricted with *Bam*HI and cloned into the pQE30 expression vector (Quiagen) previously digested with *Sma*I and *Bam*HI. Thereby, the recombinant protein was provided with an N-terminal 6× His-tag. The vector construct was transformed into *E. coli* (JM 109) cells.

DNA sequencing. DNA sequencing was performed with a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Sequi Therm Excel II, Biozyme). Gels were run and analyzed on a DNA sequencer model 4000L LI-COR (MWG-Biotech).

Expression and purification of recombinant human NAD kinase. For overexpression the vector construct was transformed into *E. coli* (M15) cells. An overnight culture was transferred to 500 ml LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. Overexpression was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 2 mM at an optical density of 0.6 (600 nm) of the cells. After 4 h of expression the cells were harvested and sonicated in lysis buffer (10 mM Tris–HCl, pH 7.8). The cell lysate was incubated for 5 min at 60°C. After centrifugation the supernatant was applied to a DEAE column. The column was washed with lysis buffer containing 100 mM NaCl and elution was carried out with a gradient from 100 mM to 1 M NaCl in lysis buffer. Fractions containing NAD kinase were pooled and loaded onto a nickel–nitrilotriacetic acid column (Qiagen). The column was consecutively washed with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.5), with buffer A containing 2 M NaCl and with buffer A containing 2 mM imidazole. Elution was carried out with a gradient from 2 to 50 mM imidazole in buffer A.

Partial purification of NAD kinase from bovine liver. Bovine liver tissue was homogenized in a Waring blender with a buffer containing 20 mM Na_2CO_3, 20 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, pH 7.5. The homogenate was centrifuged for 3 h at 30,000*g* and the supernatant was incubated for 5 min at 60°C. After centrifugation the supernatant was saturated with 60% ammonium sulfate. The pellet was diluted with 10 mM Tris–HCl, pH 7.8 and dialyzed against the same buffer. This fraction was further purified by DEAE chromatography as described above for the recombinant enzyme. For some experiments further purification was afforded by affinity chromatography using a Cibacron blue resin.

Enzyme assay. NAD kinase activity was assayed by measuring the increasing absorbance at 340 nm caused by the reduction of NADP to NADPH by glucose-6-phosphate dehydrogenase. The reaction was carried out in a solution containing 100 mM Tris–HCl, pH 7.8, 100 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 2 mM NAD, 1 mM glucose 6-phosphate, and 1 U glucose-6-phosphate dehydrogenase. Required changes, e.g., for measurements in the presence of substrate analogs, are indicated in the legends. When desired Ca^{2+} or calmodulin were added to final concentrations of 1 mM and 250 units/ml, respectively.

If substrate analogs were used, nucleotides were analyzed using a 125 × 4-mm polyethylenimine column (Nucleosil 4000-7 PEI, Macherey-Nagel) operated on an HPLC system (Äkta purifier, Amersham–Pharmacia). Elution was performed isocratically in buffer A (25 mM NaH_2PO_4/Na_2HPO_4/acetic acid, pH 4.5) for 4 min followed by a 15-min gradient to 70% buffer B (25 mM sodium phosphate, pH 7.4, 2 M NaCl) at a flow rate of 1 ml/min.

Size-exclusion chromatography. Gel filtration was conducted using a Superdex 200 HR 10/30 column (Amersham–Pharmacia) which was run on the Äkta purifier system. The buffer contained 50 mM Tris–HCl, pH 7.8, 500 mM NaCl. For molecular weight estimation of endogenous and recombinant NAD kinase the following proteins were used for calibration: thyroglobulin (100 μg), ferritin (20 μg), glutamic acid dehydrogenase (100 μg), alcohol dehydrogenase (500 μg), bovine serum albumin (200 μg), and cytochrome *c* (20 μg). Elution of the proteins was monitored at 280 nm. Elution of NAD kinase was detected by assaying eluted fractions for enzyme activity.

Northern blot analysis. The cDNA probe was obtained by PCR of an internal fragment encompassing nucleotides 563–962 of NAD kinase cDNA. Two hundred nanograms of the PCR product was labeled with 60 μCi α-dCTP for 4 h at 37°C and purified by gel filtration. The blot containing RNAs from human tissues (OriGene) was prehybridized for 1 h at 60°C and hybridized with the cDNA probe for 14 h at 60°C. After washing according to Ref. (32) the blot was exposed to an X-ray film.

Kinetic measurements. The kinetic parameters of recombinant human NAD kinase were determined assuming Michaelis–Menten

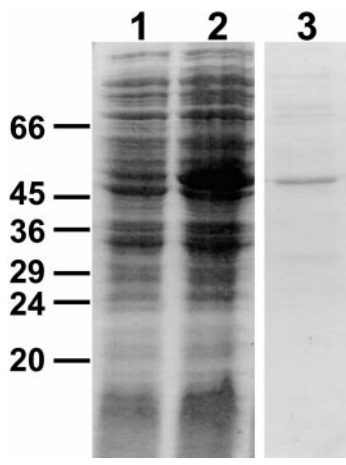

FIG. 1. Overexpression of recombinant human NAD kinase in *Escherichia coli.* Transfected cells were grown and expression induced as described under Materials and Methods. Following induction for 0 (lane 1) or 3 h (lane 2) a sample of the bacterial pellet was treated with sample buffer and subjected to SDS–PAGE. The sample represented by lane 3 contained recombinant human NAD kinase purified by Ni^{2+}–NTA and DEAE chromatography as described under Materials and Methods.

kinetics. Maximal velocity (V_{max}) and K_M values for NAD and ATP were calculated by nonlinear regression analysis of the data using the program SigmaStat. Experiments were conducted in assay medium described above at 30°C.

The dependence of enzyme activity on the temperature was determined using the same medium. The influence of pH was tested at 30°C using Mes–HCl at pH 5.5–7.0 instead of Tris–HCl as buffer.

Gel electrophoresis. SDS–polyacrylamide gel electrophoresis was conducted according to Ref. (33) using 10% polyacrylamide slab gels.

Protein determination. Protein concentrations were determined using either the biuret procedure or the BCA assay (Pierce) and bovine serum albumin as standard.

RESULTS

To identify the primary structure of human NAD kinase the two initially available protein sequences from prokaryotic NAD kinases (20) were used for a BLAST search of human EST sequences deposited in available data bases (34). The sequences showed similarity to several eukaryotic proteins of unknown function. A human hypothetical protein, FLJ13052 (Accession No. NP_075394), exhibited significant homology to the sequences used for comparison. In the meantime, the sequences from *E. coli* (21) and *S. cerevisiae* (22) have become available. They also exhibit similarity to the human and the other two bacterial sequences. The human cDNA sequence encoding this protein was used to generate primers corresponding to the 5′- and 3′-terminal sequences of its putative open reading frame. Using these primers a cDNA fragment with the expected size of about 1400 bp was amplified from a cDNA library prepared from human fibroblasts. This product was cloned and sequenced. The obtained cDNA comprised a sequence with a start codon including a Kozak initiation sequence (ACAATGG) and an in-frame stop codon at nucleotide positions 1339–1341. The sequence was identical to the hypothetical protein FLJ13052 with the exception of three differences. A nucleotide exchange from A to G at position 1212 that does not lead to an amino acid exchange, a nucleotide exchange from C to A at position 786 that would change an amino acid of the protein from asparagine to lysine at position 262 and one missing GAG codon reducing the C-terminal polyglutamate stretch from ten to nine residues.

The protein encoded by the cloned cDNA contains 446 amino acids with a theoretical molecular mass of 49,228 Da. The protein was overexpressed in *E. coli*, using an expression vector that endowed the protein with an N-terminal 6× His tag. The recombinant protein was purified by two chromatographic steps (see Materials and Methods). As shown in Fig. 1, the size of the overexpressed protein, as estimated by SDS–PAGE, coincided with that of the predicted polypeptide. The purified protein catalyzed the synthesis of NADP using NAD and ATP as substrates (see below) and, therefore, represents indeed human NAD kinase.

The sequence of the cloned cDNA was used to search a human genome database (34) in order to obtain information about the chromosomal localization and organization of the gene encoding NAD kinase. It was found that the gene (GenBank Accession No. AL031282) is located on chromosome 1 (1p36.21–36.33). It represents a DNA stretch of about 14 kb length. The coding region is contained in 11 exons consisting of 85–180 bp, the introns being of variable size ranging from 74 to 4641 bp.

Tissue-specific expression of the mRNA coding for the protein was examined by Northern blot analysis (Fig. 2). In most tissues tested a band was detected at approximately 3 kb which corresponds to the NAD

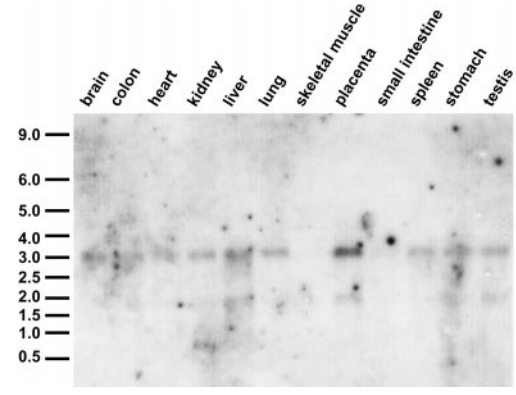

FIG. 2. Expression of NAD kinase mRNA in human tissues. Northern blot analysis was performed as indicated under Materials and Methods using a probe encompassing nucleotides 563–962 of the human NAD kinase cDNA. Each lane of the blot contained 2 μg of mRNA isolated from the indicated tissue.

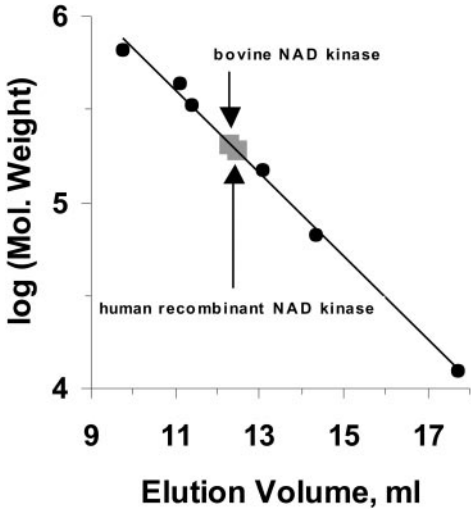

FIG. 3. Molecular weight determination of catalytically active bovine and recombinant human NAD kinases. Gel filtration of partially purified bovine liver or recombinant human NAD kinase was conducted as described under Materials and Methods. The proteins used for calibration (circles) were thyroglobulin (669 kDa), ferritin (440 kDa), glutamic acid dehydrogenase (336 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and cytochrome *c* (12.3 kDa). The molecular weight of NAD kinases was calculated according to the elution volume of fractions containing NAD kinase activity.

kinase cDNA. In several tissues a minor band of approximately 1.8 kb of unknown origin was also labeled. NAD kinase appears to be equally expressed in most tissues. However, no expression of NAD kinase was detected in skeletal muscle and only little in small intestine tissue. In placenta a significantly enhanced expression was observed.

Studies of bacterial NAD kinases revealed that these enzymes consist of polypeptides with a subunit molecular mass of 30–35 kDa (20, 21) as opposed to 49 kDa of the human enzyme. Moreover, the yeast and *E. coli* enzymes exist as hexamers (21, 22), whereas the human protein apparently consists of only four subunits that form the catalytically active enzyme. This conclusion was drawn from gel filtration experiments (Fig. 3). Similar to the recombinant human NAD kinase, the partially purified bovine enzyme also exhibited a native molecular mass of about 200 kDa (Fig. 3). The molecular masses of the recombinant human enzyme and the partially purified bovine liver enzyme were calculated to be about 190 and 210 kDa, respectively.

Analyses of the catalytic properties of the recombinant human enzyme (Table 1) established that the substrate affinities for ATP (K_M = 3.3 mM) and NAD (K_M = 0.54 mM) are similar to values previously reported for the enzyme from bovine liver and other animal tissues (23). The specific activity was calculated to be 6.7 μmol (min $\times$ mg)$^{-1}$. Both the recombinant human enzyme and bovine liver NAD kinase exhibited similar characteristics regarding the dependence of

TABLE 1

Physical and Kinetic Properties of Human NAD Kinase

Subunit molecular mass (calculated)	49,228 Da
Subunit composition (total molecular mass)	Homotetramer (200 kDa)
$K_{M_{ATP}}$	3.3 mM
$K_{M_{NAD}}$	0.54 mM
V_{max}	6.7 μmol (min $\times$ mg protein)$^{-1}$
Temperature optimum	55°C
pH optimum	7.5 (7.0–8.0)

Note. NAD kinase activity was assayed by the enzymatic procedure as described under Materials and Methods.

their catalytic activities on pH and temperature (Table 1). Both enzymes are quite resistant to heat treatment. This is indicated by the rather high temperature optimum of about 55°C (Table 1). Moreover, incubation at 60°C for 5 min resulted in a minimal loss (<5%) of activity. The pH optimum is in the alkaline range (pH 7–8). Under acidic conditions (pH < 5.5), the enzyme is readily inactivated.

Both the human and the bovine enzymes required divalent cations for their catalytic activity. That is, presumably the substrate nucleotides need to be complexes with bound divalent metal ions. The optimal concentration of Mg^{2+}-ions was about 10 mM. Several other metal ions were also efficient in supporting the catalytic activity (Table 2). In fact, zinc and manganese ions were preferred over Mg^{2+}. Copper and nickel ions were not accepted.

Human NAD kinase appears to be highly selective for its substrates, NAD and ATP. Although ATP could be replaced by dATP, the specific activity using GTP as phosphoryl donor amounted to less than one tenth of that measured in the presence of ATP (Table 3). None of the NAD analogs tested was phosphorylated in the presence of ATP under the assay conditions used. That is, modification of either the purine or the nicotinamide

TABLE 2

Influence of Metal Ions on the Catalytic Activity of Human NAD Kinase

Mg^{2+}	100
Zn^{2+}	350
Mn^{2+}	290
Co^{2+}	70
Ca^{2+}	28
Cu^{2+}	n.d.
Ni^{2+}	n.d.

Note. NAD kinase activity was assayed by the enzymatic procedure described under Materials and Methods in the presence of 1 mM indicated metal ion. The activities (in %) are given related to that measured in the presence of 1 mM Mg^{2+}. In the absence of divalent metal ions, no activity was detected. n.d., no detectable activity.

TABLE 3

Substrate Specificity of Human NAD Kinase

Substrate analog	Relative activity (%)[a]	Inhibition (%)[b]
GTP	7	0
dATP	103	—
3-Acetyl-pyridine adenine dinucleotide	n.d.	30
3-Aldehyde-pyridine adenine dinucleotide	n.d.	32
NAAD	n.d.	37
Nicotinamide guanine dinucleotide	n.d.	23
Nicotinamide hypoxanthine dinucleotide	n.d.	24
ADP-ribose	n.d.	0

Note. NAD kinase activity was assayed by the enzymatic procedure if NAD served as phosphoryl acceptor. HPLC analysis was used, if NAD analogs were to be phosphorylated (see Materials and Methods).

[a] Phosphorylation of 1 mM NAD by 5 mM GTP or dATP or of NAD analogs (at 1 mM) by 5 mM ATP was measured. The values were related to the activity measured in the presence of 1 mM NAD and 5 mM ATP (100%).

[b] The enzymatic NAD kinase assay was performed in the presence of 0.2 mM NAD and 5 mM ATP and the indicated compound at 1 mM concentration.

moiety of NAD is sufficient to prevent phosphorylation of the substrate (Table 3). Nevertheless, as indicated in Table 3, these analogs apparently bind weakly to NAD kinase, because they exerted some inhibition of NAD phosphorylation. These observations also apply to NAAD (Fig. 4) whose phosphorylation would lead to the formation of the potent calcium-mobilizing agent NAADP (9, 10). Phosphorylation of NAAD by NAD kinase was attempted under several conditions including variations of pH or addition of calmodulin in the absence or presence of Ca^{2+}. None of these conditions led to any detectable formation of NAADP.

DISCUSSION

The present study has established the molecular properties of human NAD kinase and has also provided important physical and kinetic characteristics of this enzyme. Although NAD kinase is essential to all organisms and should be highly conserved, our results have revealed significant differences to the enzymes from prokaryotes and unicellular eukaryotes (20–22, 35). First, the human enzyme has a subunit molecular mass of about 49 kDa as opposed to about 30–35 kDa reported for those from prokaryotes. Moreover, subunit molecular masses have been reported for partially purified forms of the enzyme from mammalian and avian liver (23) that are considerably different from the human form. Perhaps, other proteins had been enriched along with (far less amounts of) NAD kinase. In fact, in our studies about 5000-fold enriched bovine liver NAD kinase preparations contained two prominent protein bands at about 35 and 60 kDa (not shown). Second, the mammalian enzyme appears to be a catalytically active homotetramer, rather than a hexamer, which has been reported for the yeast and *E. coli* enzymes. Third, the human enzyme exhibits a very stringent substrate specificity for NAD and ATP. For example, the bacterial and the two isoforms of NAD kinase detected in *Euglena gracilis* readily accepted GTP as phosphoryl donor; one *Euglena* isoform even preferred GTP over ATP (35). In contrast, the human enzyme uses GTP only at a substantially lower rate (<10% compared to ATP). None of the NAD analogs tested served as substrate for phosphorylation by the human enzyme.

Although the pathways of NAD(P) biosynthesis are well known, physiological degradation of pyridine nucleotides has not been reported. To date, the only known class of enzymes leading to specific degradation of NAD(P) is represented by the bifunctional NAD glycohydrolases/ADP-ribosyl cyclases, the products being ADP-ribose (phosphate), or their cyclized forms (1, 7, 8, 12). However, the presence of an intracellular enzyme of this kind has still to be clearly demonstrated, at least for mammalian cells. In any case, an intracellular activity, if present, is very low. While NAD may also be utilized by ADP-ribosylating enzymes, NADP is not a substrate for these reactions. Consequently, in the absence of a significant degradation process there should be only a minimal requirement to resynthesize NADP under normal physiological conditions. Indeed, NAD kinase activities detected in mammalian tissues are rather low and amount to

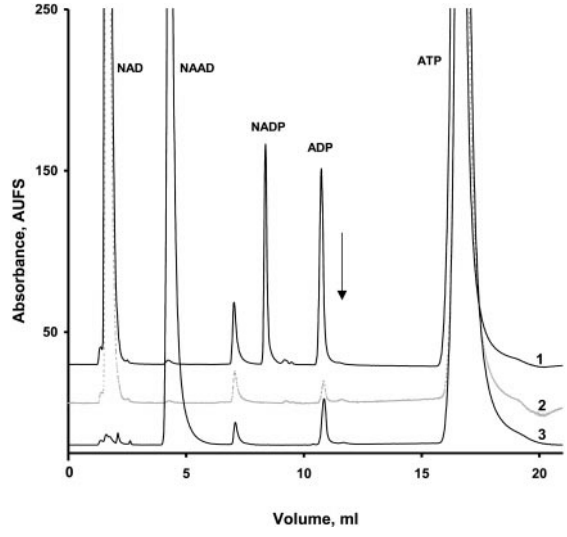

FIG. 4. Human NAD kinase does not phosphorylate NAAD. Recombinant human NAD kinase was incubated in the presence of 5 mM ATP and 1 mM NAD (curves 1 and 2) or 1 mM NAAD (curve 3). Samples were incubated for 1 h (curves 1 and 3) or stopped at zero time (curve 2) and immediately analyzed by HPLC as described under Materials and Methods. The arrow indicates the elution volume of NAADP as determined by separation of standard compounds. Absorbance was measured at 254 nm. The chromatograms were shifted along the ordinate to accommodate all three traces.

only a few nanomoles per hour and milligram of protein, even following initial purification steps (24, 25). This fact was also observed in the course of the present study using bovine liver as enzyme source. On the other hand, the gene encoding NAD kinase appears to be expressed in most human tissues (Fig. 2). It is interesting to note that the expression level is apparently elevated in placenta which would be in accordance with an increased demand for NADP of this rapidly growing tissue. The absence of mRNA encoding NAD kinase in skeletal muscle is in perfect agreement with the well known metabolic specificity of this tissue. That is, in skeletal muscle both the pentose phosphate and the fatty acid synthetic pathways are virtually absent. It is less obvious, why NAD kinase expression in small intestine tissue is very low.

The specific activity of recombinant human NAD kinase amounted to about 6–7 μmol (min $\times$ mg)$^{-1}$, a value that suggests that NAD kinase is indeed a low abundance protein, if one considers the very low activity measured in tissue extracts. The kinetic parameters also indicate that the enzyme may be regulated by substrate availability, because the K_M values for both NAD and ATP (about 0.5 and 3 mM, respectively) are close to the physiological concentrations of these nucleotides.

NAD kinase was found to be unable to catalyze phosphorylation of the physiological intermediate NAAD, at least under the conditions used. This conclusion would clearly imply that another pathway should exist that leads to NAADP synthesis. In this regard it was also intriguing to test the reported stimulatory effect of Ca^{2+}/calmodulin on NAD kinase activity. Besides not promoting NAADP synthesis, these potential activators had also no influence on NADP synthesis. This observation is in apparent contradiction to reports of a stimulation, at least regarding the human enzyme (28). However, it might be possible that the previously noted effect was indirect and perhaps mediated, for example, by a calmodulin-dependent kinase.

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