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Effects of Deamido-NAD$^+$ on Self-splicing of Primary Transcripts of Phage T4 Thymidylate Synthase Gene

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Effects of deamido-NAD$^+$ on self-splicing of primary transcripts of the phage T4 thymidylate synthase gene (*td*) was investigated. The self-splicing was not affected by deamido-NAD$^+$ at concentrations up to 2 mM. However, it began to decrease at 5 mM and the formation of splicing products such as the linear intron, intron-exon 2 and exon 1-exon 2, was slightly reduced. At 20 mM the self-splicing activity was almost completely abolished. This analog of the coenzyme NAD$^+$ inhibits the self-splicing of *td* intron RNA although it does not possess a guanidine group in its structure. The analysis of inhibitory concentrations and structural examination suggests that the key structural features of deamido-NAD$^+$ responsible for the inhibition of splicing may be the ADP-ribose moiety.

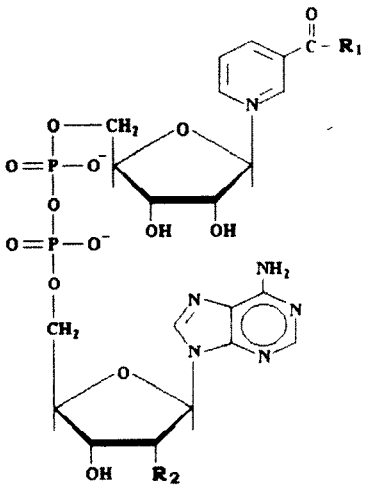
The T4 phage thymidylate synthase gene (*td*), which can undergo self-splicing *in vitro* in the absence of any protein factors or energy source, contains a group 1 intron (Chu et al., 1984). Like the intron in the nuclear large rRNA gene of *Tetrahymena thermophila* (Cech et al., 1983), the *td* intron is processed from a precursor RNA *via* a series of transesterification mechanisms (Chu et al.,1986).

The self-splicing reaction of group I introns is inhibited by a number of small molecules. The guanosine analogs deoxyguanosine and dideoxyguanosine (Bass and Cech, 1986), the amino acid arginine (Yarus, 1988), and the antibiotics streptomycin (von Ahsen and Schroeder, 1991), viomycin and di-β-lisylcapreomycin (Wank et al., 1994) competitively inhibit of the self-splicing via their guanidino groups, which they have in common with the cofactor guanosine. However, the pseudosaccharide lysinomycin (Rogers and Davies, 1994), which does not contain a guanidino group, also inhibits the self-splicing by a competitive interaction. Aminoglycoside antibiotics such as neomycin, gentamycin, kanamycin and tobramycin (von Ahsen et al., 1991), spectinomycin (Park et al., 2000; Park and Sung, 2000), tetracycline and pentamidine (Liu et al., 1994), which do not have guanidino groups are non-competitve inhibitors of the group 1 intron splicing.

Deamido-NAD$^+$ is an analog of the coenzyme NAD$^+$ that mediates various oxidation-reduction reactions when present as a domain group in dehydrogenases (Buggs, 1997). During this reaction, NAD$^+$ accepts a hydride ion from the substrate molecule undergoing the oxidation. The poly (ADP-ribose), a byproduct of NAD$^+$ in the poly (ADP-ribose) polymerase reaction, is thought to be involved in DNA replication and repair as well as cell division and differentiation (Ueda and Hayaishi, 1985; Lautier et al., 1993).

NAD$^+$ has been also considered to be a remnant of the transition from the earlier "RNA world" since it contains RNA components in its structure. FMN, another coenzyme that mediates important oxido-reduction reactions, has been shown to act as a competitive inhibitor of the *td* intron (Kim and Park, 2000).

Because of its structural resemblance to the coen-



Compounds	R_1	R_2
NAD	NH_2	OH
Deamido-NAD	OH	OH

Fig. 1. Chemical structures of NAD$^+$ and deamido-NAD$^+$.

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zyme NAD$^+$ as an electron carrier in the cellular metabolism, we examined the mechanism by which deamido-NAD$^+$ (Fig. 1) affects the self-splicing of primary transcripts of the phage T4 thymidylate synthase gene *in vitro*.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strains TG1 and HB101 were obtained from Amersham. M13mp8 phage was purchased from Bethesda Research Laboratories and pGEM-2 vectors were from Promega Corp.

Enzymes and chemicals

Restriction enzymes *Eco*RI and *Hind*III were obtained from New England Biolabs.

[α-^{32}P]GTP (>400 Ci/mmol) was obtained from Amersham. Nucleoside triphosphates were obtained from Boehringer Mannheim. T7 RNA polymerase (20 U/µl) was obtained from United States Biochemical and RNasin (40 U/µl) and RQ1 DNase (1 U/µl) from Promega Corp. Deamido-NAD$^+$ was purchased from Sigma Chemical Co.

Construction and preparation of recombinant plasmids

The cloning procedures were as described previously (Chu et al., 1986). The pGEM recombinant containing 390 nt of the 5' exon 1, 1016 nt of the intron and 824 nt of the 3' exon 2 were kindly provided by Dr. Fred Chu. The pGEM recombinant plasmids were transformed into *E. coli* HB101 cells, propagated in the presence of ampicillin and amplified in the presence of chloramphenicol. The promoter alignment of the *td* fragment was determined by 0.8% agarose gel analysis of restriction fragments from pGEM-2 recombinant plasmids.

Synthesis of RNA by in vitro transcription

The pGEM recombinant plasmids were linearized with *Hpa*1 which cuts the *td* fragment once at 520 bp downstream of exon 2. Each linearized recombinant plasmid DNA was used as a template for *in vitro* transcription following deproteination by phenol extraction and ethanol precipitation. The transcription was performed at 30℃ for 40 min in the transcription buffer (40 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM spermidine, and 5 mM NaCl), 10 mM DTT, 1 U/ml RNasin, 0.5 mM of each rNTP, 5 µCi of [α-^{32}P] GTP, and 10 U of T7 RNA polymerase. The RNA synthesis was terminated by the addition of RQ1 DNase to destroy the DNA template. Following the transcription, the synthesized 2.23 kb primary transcript was purified free of proteins, ribonucleotides and salts by passage through a Nensorb^{20} cartridge (Du Pont). The bound RNA was eluted with 20% ethanol from the cartridge, followed by precipitation with 2 volumes of ethanol in the presence of 0.2 M sodium acetate. The RNA precipitate was washed three times with 70% ethanol to remove salts.

in vitro self-splicing reaction

The splicing reaction buffer contained 40 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 µM GTP and 8 nM RNA. The splicing reaction was performed by incubating precursor RNAs of the *td* intron RNA in the presence of varying concentrations of deamido-NAD$^+$ (0.5, 1, 2, 5, 10 and 20 mM) at 58℃ for 10 min. At the end of the incubation, the reaction mixture was centrifuged briefly to collect the moisture, chilled on ice and 5 µl of the sample buffer (95% deionized formamide, 10 mM Na_2-EDTA, and 0.08% xylene cyanol, and 0.08% bromophenol blue) was added.

Splicing products were electrophoresed in a 0.75 mm thick slab gel containing 4% polyacrylamide and 8 M urea in TBE buffer (0.1 M Trizma base, 0.1 M boric acid, and 2 mM Na_2EDTA) and visualized by autoradiography without drying. Autoradiograms were scanned and integrated with a Hoefer image analyzer.

pH effect

To determine the effect of pH on the splicing inhibition of *td* intron by deamido-NAD$^+$, splicing buffers with various ranges of pH values were used: MES (pH 5.5-6.4), MOPS (pH 6.6-7.5) and Tris (pH 7.9). The accumulated splicing products were determined by densitometric analysis with Hoefer image analyzer. The ratio of the E1-E2 ligation product to the total spliced products (I-E2+I-E1+E1-E2+CI+LI) plus the remaining pre-RNA was calculated.

RNA concentration

The RNA concentration was determined by the spectrophotometric method. The extinction coefficient was determined by hydrolyzing RNA to nucleotides and by measuring the A_{260} value of the resulting mixture.

Results and Discussion

Self-splicing of the *td* intron RNA occurs by an autocatalytic mechanism resembling that of many group 1 introns (Cech, 1987). A number of group 1 introns have been known to be inhibited by small molecules such as antibiotics (von Ahsen et al., 1992), amino acid (Yarus, 1988) and coenzymes (Kim and Park, 2000).

To examine the effect of deamido-NAD$^+$ on the self-splicing of the *td* intron RNA, precursor RNAs were incubated with varying concentrations of deamido-NAD$^+$ under normal splicing conditions as described in Materials and Methods. The autoradiogram shows that the splicing inhibition occurred starting from 5 mM deamido-NAD$^+$ (Fig. 2A). At 5 mM the splicing activity was inhibited by about 20%, demonstrating reduction of

each of splicing products (Fig. 2B). As the concentration of deamido-NAD$^+$ is increased, the formation of I-E2 intermediate, E1-E2 ligation product and linear and circular introns was suppressed accordingly. At 10 mM, very small amounts of the I-E2 intermediate, linear intron and E1-E2 ligation product were detected. At 20 mM no splicing products were observed and complete hydrolysis of the intron RNA occurred.

The results demonstrate that deamido-NAD$^+$, the structural analog of coenzyme NAD$^+$, inhibits the first step of transesterification for the self-splicing of the *td* intron RNA. Such inhibition has been mostly observed with molecules with guanidino residues such as streptomycin (von Ahsen and Schroeder, 1991), arginine (Yarus, 1988), guanosine analogs (Bass and Cech, 1986), and tuberactinomycin peptides (Wank et al., 1994). This is due to competition with the binding of the guanosine cofactor for the G-binding site located in the core of the intron RNA (von Ahsen and Schroeder, 1990). A similar observation has been made with lysinomicin which does not possess a guanidino residue (Rogers and Davies, 1994).

Although deamido-NAD$^+$does not contain a guanidino group this analog exhibits the structural specificity in the splicing inhibition by interfering with the first step of the self-splicing. The nicotinamide coenzyme NAD$^+$ also was shown to be a relatively strong inhibitor compared to deamido-NAD$^+$ (data not shown). The only structure difference between NAD$^+$ and deamido-NAD$^+$ resides in the pyridine ring. The amino group on the pyridine ring of NAD$^+$ is substituted with a hydroxyl group in the latter. This minor change somehow renders NAD$^+$ to act as a stronger inhibitor. Very similar observations have been also made with aminoglycoside antibiotics in which a single substitution of a hydroxyl group in paromomycin with an amino group produces neomycin B (von Ahsen et al., 1992). In fact, neomycin B inhibits the self-splicing of *td* intron RNA at 100-fold lower concentration than that of paromomycin. In general, amino groups favor and hydroxyl groups disfavor the splicing inhibition. The protonated amino groups of these antibiotics have been suggested to interact with the negative charges of the intron RNA backbone.

The effect of pH on the self-splicing inhibition by deamido-NAD$^+$ is shown in Fig. 3.

The splicing activity profile was not affected regardless of the absence or the presence of deamido-NAD$^+$ in the splicing reaction. Thus, the inhibition by deamido-NAD$^+$ was pH independent, indicating the non-ionic nature of deamido-NAD$^+$ in the interaction with the intron RNA. Unlike deamido-NAD$^+$, NAD$^+$ caused the splicing rate to decrease as the pH was raised

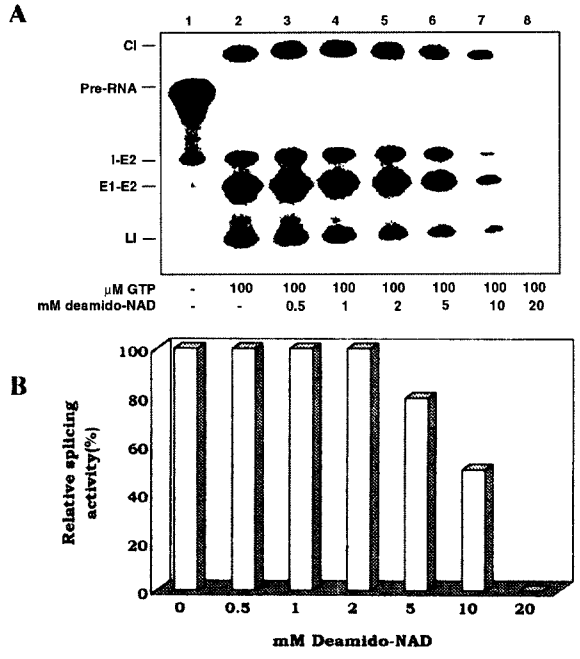

Fig. 2. Inhibition of self-splicing of phage T4 *td* intron RNA by deamido-NAD$^+$. A, Autoradiogram showing splicing activity in the presence of varying concentrations of deamido-NAD$^+$. The precursor RNA of *td* intron was incubated under the splicing conditions with varying concentrations of deamido-NAD$^+$ in the presence of 5 μM $MgCl_2$ and 100 M GTP at 58C for 10 min. The splicing products were separated on a denaturating 8 M urea-4% (w/v) acrylamide gel and revealed by autoradiography. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. B, Splicing rates of the *td* intron RNA as a function of concentrations of deamido-NAD$^+$. The relative inhibition was expressed as the percent reduction in the formation of E1-E2 ligation product in the presence of deamido-NAD$^+$ with respect to that observed in the absence of deamido-NAD$^+$.

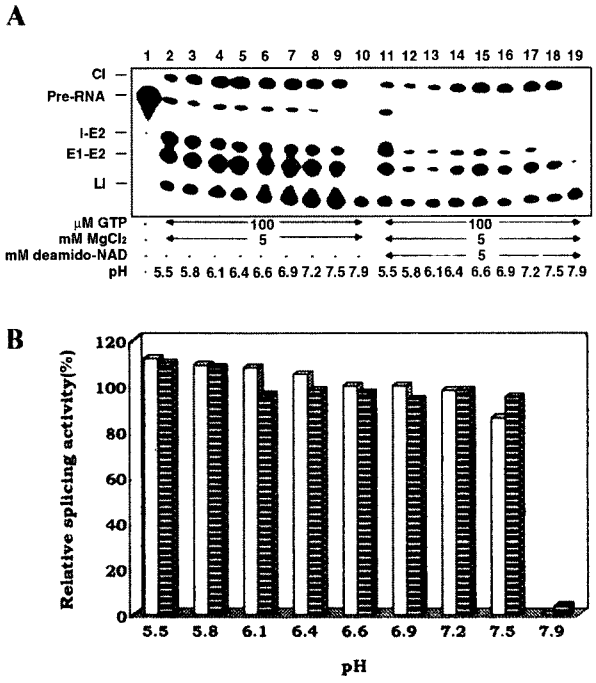

Fig. 3. Effects of pH on splicing inhibition of T4 *td* intron RNA by deamido-NAD$^+$. A, Autoradiogram of splicing products in the absence or presence of deamido-NAD$^+$. B, The relative splicing activity expressed as a function of pH. The band densities on gels were quantitated on a desitometer and the ratio of E1-E2 formation to all splicing products (I-E2+E1-E2+C1+L1+the remaining pre-RNA) is expressed as the percent relative splicing activity. The open and hatched bars represent relative splicing activity in the absence and presence of deamido-NAD$^+$, respectively.

from 5.5 to 7.9 (data not shown). This effect may be associated with the deprotonation of the amino group of the pyridine ring of NAD^+ (Hoch et al., 1998).

Examination of the deamido-NAD^+-intron RNA complex by footprinting or NMR experiments could help to identify specific functional groups involved in the interaction of deamido-NAD^+ with the intron RNA.

The biological significances of the inhibitory actions of coenzyme NAD^+ related to cellular metabolism remain to be seen. To our knowledge, this is the first report which demonstrate that the coenzyme analog acts as an inhibitor of the self-splicing of the group I intron RNA.

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