Interaction between Dehydrogenases and a New NAD⊕-Isomer

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A new NAD⊕-isomer was prepared, in which the D-ribose of the adenosine moiety was sub-
stituted by the enantiomeric L-ribose. As compared to nicotinamide-adenine-dinucleotide (NAD⊕)
and NADH the coenzyme isomer (D,L)-NAD⊕ and its dihydroform (D,L)-NADH are far less
tightly bound to lactate dehydrogenase and alcohol dehydrogenase from horse liver. In the presence
of the second substrate (D,L)-NAD⊕ and (D,L)-NADH act as hydrogen acceptor and hydrogen
donator, respectively, with lactate dehydrogenase and alcohol dehydrogenases from horse liver and
yeast. Compared to NAD⊕ and NADH the Michaelis constants are always increased, the catalytic
constants (V/E_t) were found to be decreased except for the dihydroform reacting with alcohol
dehydrogenase from liver.

Introduction

In a recent paper the preparation and charac-
teristics of coenzyme analogues of NAD were de-
scribed where the ribose of the adenosine mono-
phosphate moiety was replaced by *n*-alkyl groups [1].
These analogues showed marked differences in
their reactions with dehydrogenases as compared
to NAD⊕ and NADH. To explain these, the pos-
sibility was discussed that upon complex formation
the hydrophobic alkyl-chains are repelled by hydro-
philic amino acid residues of the dehydrogenases
and, thus, interfere with the incorporation of the
adenine moiety. Recent X-ray diffraction studies
support this view by showing for different dehydro-
genases a basically hydrophobic adenosine binding
site where the $C_{2'}$-hydroxy group of the adenosine
ribose moiety forms a hydrogen bridge with an
aspartate residue of the proteins [2-4].

In order to investigate the competing effects of
hydrophobic binding forces and hydrogen bridges
in complex formation, an NAD⊕-isomer was syn-
thesized with the enantiomere L-ribofuranose sub-
stituting the D-ribose of the adenosine moiety.

Methods

Materials and general procedures

Crystalline alcohol dehydrogenase from horse
liver and from yeast (alcohol:NAD⊕ oxidoreduc-
tase, EC 1.1.1.1), lactate dehydrogenase from pig
heart (L-lactate:NAD⊕ oxidoreductase, EC 1.1.1.27),

NAD⊕ and NADH were purchased from Boehringer
Mannheim. The preparation of L-AMP was de-
scribed previously [10].

The hypochromicity of (D,L)-NAD⊕ and (D,L)-
NADH was measured by means of difference spectra
as described previously [5]. A similar arrangement
was used to measure spectral changes caused by
complex formation between (D,L)-NADH and de-
hydrogenases [5]. The concentration of the proteins
was 0.1 mM. The concentration of (D,L)-NADH
was 0.5 mM when measuring the complexes of alco-
hol dehydrogenases from horse liver and from yeast
and 1 mM when the complex of (D,L)-NADH with
lactate dehydrogenase was investigated.

UV-absorption spectra and difference spectra
were recorded on a Cary 14 spectrophotometer at
25 °C.

Fluorescence spectra were obtained using the
Perkin Elmer Fluorescence Spectrophotometer
MPF 4 (the concentration of the enzymes was
52 μM and the concentration of (D,L)-NADH was
42 μM).

The dissociation constants of the binary com-
plexes formed by (D,L)-NAD⊕ and (D,L)-NADH
with lactate dehydrogenase and alcohol dehydro-
genase from liver were estimated by means of
fluorescence titration according to Velick [6]. The
concentration of the proteins was 30 μM in 0.2 M
glycine/NaOH buffer pH 9.5. The concentration of
(D,L)-NAD⊕ was varied between 37 and 1360 μM,

the (D,L)-NADH concentration between 4 and 168 μM. The excitation wavelength was 290 nm and the emitted protein fluorescence was measured at 340 nm. The dissociation constant of the NAD⊕ complex with horse liver alcohol dehydrogenase was measured by the same method, using a protein concentration of 10 μM and 1−91 μM NAD⊕. The dissociation constant of NAD⊕ from lactate dehydrogenase was obtained according to Stinson and Holbrook[7] from competition between NADH and NAD⊕ for the nucleotide binding sites of the enzyme.

NAD⊕ was purified by ion exchange chromatography (Dowex 1×8, Cl⊖-form, 100−200 mesh, 20×2 cm)[7, 8]. Lactate dehydrogenase was purified by chromatography on Sephadex G 25 coarse (40× 1.6 cm), containing 15% charcoal Nuchar C 190 (acid treated).

The kinetic studies with (D,L)-NAD⊕ were performed in 0.2 M glycine/NaOH buffer pH 9.5 at 25 °C. The concentration of the other substrates were: lithium lactate = 50 mM; ethanol = 430 mM; (D,L)-NAD⊕ was varied between 0.1 mM to 1 mM. The enzymatic reaction was initiated by addition of 100 μg alcohol dehydrogenase from yeast, 50 μg alcohol dehydrogenase from horse liver or 36 μg lactate dehydrogenase per ml test volume.

The kinetic studies with (D,L)-NADH were performed in 0.2 M phosphate buffer pH 7 at 25 °C. The concentrations of the other substrates were: sodium pyruvate = 2.5 mM; acetaldehyde = 29 mM; (D,L)-NADH was varied between 0.3 and 1 mM. The enzymatic reaction was started by addition of 0.7 μg lactate dehydrogenase or alcohol dehydrogenase from yeast, or 10 μg alcohol dehydrogenase from horse liver per ml assay mixture.

Synthesis of (D,L)-NAD⊕ and (D,L)-NADH

Nicotinamide-1-β-D-ribofuranosyl-[(adenine-9-yl)-1-β-L-ribofuranosyl]pyrophosphate, (D,L)-NAD⊕ was prepared by the condensation of 98 mg β-L-adenosine monophosphate[9, 10] and 251 mg nicotinamide mononucleotide[11] using dicyclohexylcarbodiimide in aqueous pyridine[12].

(D,L)-NAD⊕ was isolated and purified by ion exchange chromatography on Dowex 1×8 formiate form as described previously[13]. The yield was 75 mg (D,L)-NAD⊕. The light-absorption maximum in 0.2 M glycine/NaOH buffer pH 9.5 is 259 nm (ε 17700); a_D^{20} +2.8° (c 1.5 in water).

(D,L)-NADH was prepared by enzymatic reduction of (D,L)-NAD⊕ and purified by exclusion chromatography on Sephadex G 10[13]. Light absorption maxima in 0.2 M glycine/NaOH buffer pH 9.5 259 and 340 nm (ε 14300 and 6200 respectively).

Results

The UV spectra of (D,L)-NAD⊕ and its dihydrocompound show no differences to the respective spectra of NAD⊕ and NADH[14]. Splitting the pyrophosphate bond of the coenzyme isomer (D,L)-NAD⊕ causes an increase in the extinction at the absorption maximum by 11% while with (D,L)-NADH an increase by 7.5% at 258 nm is observed.

In the fluorescence spectra as well, (D,L)-NADH shows no differences as compared to the isomer NADH. Upon excitation at a wavelength of 340 nm the maximum of the fluorescence emission spectrum is at 456 nm. Measuring the intensity of the emitted
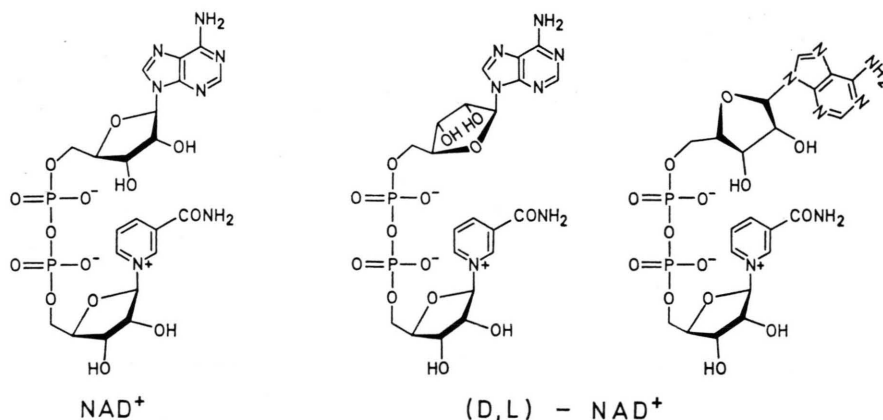

Fig. 1. Diagrammatic view of NAD⊕ and two possible orientations of (D,L)-NAD⊕.

light at 455 nm the fluorescence excitation spectrum shows two maxima at 260 and 340 nm.

Upon addition of (D,L)-NADH to lactate dehydrogenase or to alcohol dehydrogenase from either yeast or horse liver qualitatively similar changes in the short wavelength UV spectrum are observed as upon addition of NADH. Difference spectra between the enzyme-dihydrocoenzyme complex and the unmixed components show a hypochromic effect in the region of adenine absorption. This effect is only clearly distinguishable at high (D,L)-NADH concentrations. In the region of the dihydronicotinamide absorption the spectral changes in the difference spectra are minute.

In contrast to NADH, the coenzyme fluorescence of (D,L)-NADH is not affected upon formation of binary complexes with lactate dehydrogenase or alcohol dehydrogenases from yeast or liver. The fluorescence excitation spectra of the complexes, which were obtained by excitation between 250 and 390 nm and measuring the coenzyme fluorescence at 450 nm, are in all cases the summation of the individual fluorescence of (D,L)-NADH and the protein. The same holds for the fluorescence emission spectra of the complexes between 390 and 510 nm, which were obtained by excitation at 340 nm.

The protein fluorescence, however, of the enzymes is reduced upon formation of the binary (D,L)-NADH complexes, as is the case with NADH. The protein fluorescence was determined at 340 nm while exciting with light of a wavelength of 290 nm. The binding of the oxidized coenzyme isomer to these dehydrogenases goes along with a reduction of the protein fluorescence too.

The decrease in protein fluorescence with increasing amounts of added coenzyme analogue was not exactly proportional to the saturation of the coenzyme binding sites of the proteins [15-17]. Since the saturation of the coenzyme binding sites could not be exactly measured by an independent method we were not able to establish the occurrence of geometric quenching without any doubt. The quenching of protein fluorescence was used for the determination of the dissociation constants of the binary complexes of lactate dehydrogenase and alcohol dehydrogenase from horse liver. The binary complexes of the coenzyme isomers are far more dissociated than the complexes of NAD$^{\oplus}$ and NADH (Table I).

Table I. Dissociation constants of the binary complexes of (D,L)-NAD$^{\oplus}$ and (D,L)-NADH. The dissociation constants were measured at pH 9.5.

Coenzyme or coenzyme-analogue	Lactate dehydrogenase K [μM]	Alcohol dehydrogenase from horse liver K [μM]
NAD$^{\oplus}$	200	10
(D,L)-NAD$^{\oplus}$	2500	1000
NADH	6 [b]	—
(D,L)-NADH	160	50
NMNPRH [a]	250 [b]	—

[a] NMNPRH, Nicotinamide-ribofuranosyl-5′-pyrophosphate-5″-ribofuranose.
[b] Data from ref. 13.

(D,L)-NAD$^{\oplus}$ acts as hydrogen acceptor in the presence of the second substrate with lactate dehydrogenase and alcohol dehydrogenase from yeast and horse liver. (D,L)-NADH donates hydrogen in the presence of the second substrate and the enzyme.

The redox equilibrium constant was determined for the alcohol dehydrogenase system. K^{eq} was found to be 2×10^{-11} M and is of the same order of magnitude as the equilibrium constant of NAD$^{\oplus}$ [18, 19].

The Michaelis constants and the maximal turnover numbers of (D,L)-NAD$^{\oplus}$ and (D,L)-NADH were measured with lactate dehydrogenase and the two alcohol dehydrogenases. They are compiled in Table II and are compared with the constants determined under identical conditions for NAD$^{\oplus}$ and NADH and the adenine-free coenzyme fragment nicotinamide-β-D-ribofuranosyl-5′-pyrophosphate-5″-ribofuranose [13].

Discussion

The similarities which are exhibited by (D,L)-NAD$^{\oplus}$ and (D,L)-NADH with NAD$^{\oplus}$ and NADH in their physico-chemical properties are the results of a folding of the coenzyme isomers in aqueous solution. The folded conformation explains the hypochromic effect of UV-absorption as well as the energy transfer from the adenine ring to the dihydronicotinamide ring in the fluorescence excitation spectrum [20]. A change in the position of the hydroxy groups of the L-ribose and a twisting in the plane of the sugar ring do not influence the base stacking noticeably.

Differences from the native coenzyme NAD$^{\oplus}$ must occur upon unfolding of the coenzyme isomer

Coenzyme or coenzyme analogue	Lactate dehydrogenase K_m [mM]	V/E_t [min^{-1}]	Alcohol dehydrogenase from yeast K_m [mM]	V/E_t [min^{-1}]	Alcohol dehydrogenase from horse liver K_m [mM]	V/E_t [min^{-1}]
NAD$^\oplus$	0.1	15000	0.2	35000	0.03	300
(D,L)-NAD$^\oplus$	0.7	800	5	1400	0.3	100
NMNPR$^\oplus$ a c	6	500	10	1000	4	100
NADH	0.01	140000	0.05	150000	0.008	4000
(D,L)-NADH	0.2	42000	0.7	10700	0.3	13500
NMNPRH b c	0.1	9600	2	10000	0.3	2300

Table II. Maximal velocities and Michaelis constants of (D,L)-NAD$^\oplus$ and (D,L)-NADH with lactate dehydrogenase and alcohol dehydrogenases from horse liver and yeast. The maximal velocities (V/E_t) are expressed in μmol product/min $\times$ μmol enzyme, the dimension of the Michaelis constants used in this table is mM. The constants of the oxidized coenzymes were measured at pH 9.5, those of the dihydro forms at pH 7.0.

a NMNPR$^\oplus$, nicotinamide-ribofuranosyl-5′-pyrophosphate-5″-ribofuranose.
a NMNPR$^\oplus$, dihydro-NMNPR. c Data from ref. 13.

and binding to the coenzyme binding sites of the dehydrogenases. In this case the absolute spatial conformation of the coenzyme isomer is of importance. Comparing the conformation of the unfolded (D,L)-NAD$^\oplus$ to the unfolded NAD$^\oplus$, one sees that if, the nicotinamide-ribose-pyrophosphate moieties are orientated in the same way, the adenine rings can be arranged in a corresponding position. In this case, the plane of the L-ribose is twisted and the substituents of the sugar have a spatial orientation different from NAD$^\oplus$. Arranging the L-ribose in such a way that its hydroxy groups have the same position as in the ribose of NAD$^\oplus$ alters the position of the adenine ring. Upon the formation of binary dehydrogenase complexes with (D,L)-NADH or (D,L)-NAD$^\oplus$ a simultaneous hydrogen bridging over the $C_{2'}$-hydroxy group of the adenosine ribose and the specific adenine binding by hydrophobic interactions are for steric reasons impossible.

Wether the (D,L)-NADH is bound over the adenine ring or the sugar moiety to the coenzyme binding site can not be differentiated by the data. The hypochromic effect observed in the region of adenine absorption upon complex formation does not prove that the adenine ring after unfolding of the coenzyme interacts with aromatic amino acid residues of the protein. Inserting it into a nonpolar region would explain this effect as well.

The fluorescence spectra show that the specific orientation of chromophorous groups, which causes the energy transfer from tryptophane to the di-hydronicotinamide ring in the binary complexes of NADH, does not occur in the (D,L)-NADH complexes. Previous investigations of Woenckhaus [5] indicate that the binding of the adenine ring to the enzyme is responsible for this energy transfer. Therefore, the adenine moiety in the (D,L)-NADH complexes is not bound in the same manner as in the complexes with NADH. A hydrogen bridge formation in the complexes between the $C_{2'}$-hydroxy group of the L-ribofuranose and the aspartate residues would then be possible. This assumption is supported by the following results:

Complexes of the adenine free NADH fragment dihydronicotinamide-β-D-ribofuranosyl-5′-pyrophosphate-5″-ribofuranose with dehydrogenases show no energy transfer band in the fluorescence excitation spectrum. The fluorescence emission spectra of this coenzyme fragment and (D,L)-NADH in their respective complexes with dehydrogenases are very similar. The Michaelis constants of (D,L)-NADH and dihydronicotinamide-β-D-ribofuranosyl-5′-pyrophosphate-5″-ribofuranose are of the same magnitude, suggesting that the ribose moiety of (D,L)-NADH is bound. In addition, the dissociation constants of the binary lactate dehydrogenase complexes with (D,L)-NADH and the adenine-free NADH fragment are identical within the limits of measurement (s. Table I).

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