Interaction between Dehydrogenases and a New NAD®-Isomer

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NAD®-Isomer, Dehydrogenase, Complex Formation

A new NAD®-isomer was prepared, in which the d-ribose of the adenosine moiety was substituted 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 donor, 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) were found to be decreased except for the dihydroform reacting with alcohol dehydrogenase from liver.

Introduction

In a recent paper the preparation and characteristics of coenzyme analogues of NAD were described where the ribose of the adenosine monophosphate moiety was replaced by n-alkyl groups. These analogues showed marked differences in their reactions with dehydrogenases as compared to NAD® and NADH. To explain these, the possibility was discussed that upon complex formation the hydrophobic alkyl-chains are repelled by hydrophilic 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 dehydrogenases a basically hydrophobic adenosine binding site where the C₂-hydroxy group of the adenosine ribose moiety forms a hydrogen bridge with an aspartate residue of the proteins.

In order to investigate the competing effects of hydrophobic binding forces and hydrogen bridges in complex formation, an NAD®-isomer was synthesized with the enantiomere L-ribofuranose substituting the d-ribose of the adenosine moiety.

Methods

Materials and general procedures

Crystalline alcohol dehydrogenase from horse liver and from yeast (alcohol:NAD® oxidoreductase, 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 described previously.

The hypochromicity of (d,l)-NAD® and (d,l)-NADH was measured by means of difference spectra as described previously. A similar arrangement was used to measure spectral changes caused by complex formation between (d,l)-NADH and dehydrogenases. The concentration of the proteins was 0.1 mM. The concentration of (d,l)-NADH was 0.5 mM when measuring the complexes of alcohol 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 32 μM and the concentration of (d,l)-NADH was 42 μM). The dissociation constants of the binary complexes formed by (d,l)-NAD® and (d,l)-NADH with lactate dehydrogenase and alcohol dehydrogenase from liver were estimated by means of fluorescence titration according to Velick. 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.

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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 from competition between NADH and NAD® for the nucleotide binding sites of the enzyme.

NAD® was purified by ion exchange chromatography (Dowex 1x8, Cl−-form, 100–200 mesh, 20×2 cm)7,8. Lactate dehydrogenase was purified by chromatography on Sephadex G25 coarse (40×1.6 cm), containing 15% charcoal Nuchar C190 (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)-NAD® 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)-NAD® 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 monophosphate9,10 and 251 mg nicotinamide mononucleotide11 using dicyclohexylcarbodiimide in aqueous pyridine12.

(d,l)-NAD® was isolated and purified by ion exchange chromatography on Dowex 1×8 formate form as described previously13. 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); αS +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 G1013. 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 dihydro compound show no differences to the respective spectra of NAD® and NADH14. 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

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**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)-NAD 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)-NAD 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. 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 and NADH (Table I).

### Table I. Dissociation constants of the binary complexes of (d,l)-NAD and (d,l)-NADH.

<table>
<thead>
<tr>
<th>Coenzyme or coenzyme-analogue</th>
<th>Lactate dehydrogenase K [μM]</th>
<th>Alcohol dehydrogenase from horse liver K [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>(d,l)-NAD</td>
<td>2500</td>
<td>1000</td>
</tr>
<tr>
<td>NADH</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>(d,l)-NADH</td>
<td>160</td>
<td>50</td>
</tr>
<tr>
<td>NMNPRH</td>
<td>250 b</td>
<td>—</td>
</tr>
</tbody>
</table>

* a NMNPRH, Nicotinamide-ribofuranosyl-5'-pyrophosphate-5'-ribofuranose.

The redox equilibrium constant was determined for the alcohol dehydrogenase system. \( K_{eq} \) was found to be \( 2 \times 10^{-11} \) M and is of the same order of magnitude as the equilibrium constant of NAD at 18, 19.

The Michaelis constants and the maximal turnover numbers of (d,l)-NAD 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 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 and (d,l)-NADH with NAD 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 must occur upon unfolding of the coenzyme isomer.
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\textsuperscript{\textregistered} to the unfolded NAD\textsuperscript{\textregistered}, 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\textsuperscript{\textregistered}. Arranging the L-ribose in such a way that its hydroxy groups have the same position as in the ribose of NAD\textsuperscript{\textregistered} alters the position of the adenine ring. Upon the formation of binary dehydrogenase complexes with (d,l)-NADH or (d,l)-NAD\textsuperscript{\textregistered} a simultaneous hydrogen bridging over the C\textsubscript{2}'-hydroxy group of the adenosine ribose and the specific adenine binding by hydrophobic interactions are for steric reasons impossible.

Whether 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 chromophoric groups, which causes the energy transfer from tryptophane to the dihydronicotinamide ring in the binary complexes of NADH, does not occur in the (d,l)-NADH complexes. Previous investigations of Woenckhaus\textsuperscript{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\textsubscript{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-\(\beta\)-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-\(\beta\)-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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<table>
<thead>
<tr>
<th>Coenzyme or coenzyme analogue</th>
<th>Lactate dehydrogenase [(K_m), (V/\text{Et}) \text{[mm]} \text{[min}^{-1})]</th>
<th>Alcohol dehydrogenase from yeast [(K_m), (V/\text{Et}) \text{[mm]} \text{[min}^{-1})]</th>
<th>Alcohol dehydrogenase from horse liver [(K_m), (V/\text{Et}) \text{[mm]} \text{[min}^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD\textsuperscript{\textregistered}</td>
<td>0.1 15000</td>
<td>0.2 35000</td>
<td>0.03 300</td>
</tr>
<tr>
<td>(d,l)-NAD\textsuperscript{\textregistered}</td>
<td>0.7 800</td>
<td>5 1400</td>
<td>0.3 100</td>
</tr>
<tr>
<td>NMNPR\textsuperscript{\textregistered}</td>
<td>6 500</td>
<td>10 1000</td>
<td>4 100</td>
</tr>
<tr>
<td>NADH</td>
<td>0.01 140000</td>
<td>0.05 150000</td>
<td>0.008 4000</td>
</tr>
<tr>
<td>(d,l)-NADH</td>
<td>0.2 42000</td>
<td>0.7 10700</td>
<td>0.3 13500</td>
</tr>
<tr>
<td>NMNPRH \textsuperscript{\textregistered}</td>
<td>0.1 9600</td>
<td>2 10000</td>
<td>0.3 2300</td>
</tr>
</tbody>
</table>

\textsuperscript{a} NMNPR\textsuperscript{\textregistered}, nicotinamide-ribofuranosyl-5'-pyrophosphate-5''-ribofuranose.
\textsuperscript{b} NMNPRH \textsuperscript{\textregistered}, dihydro-NMNPR.
\textsuperscript{c} Data from ref. 13.