Coenzyme Binding at Different Ionization States of Cytoplasmic and Mitochondrial Malate Dehydrogenase*

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pH-titrations with NADH show two ionizable groups in mitochondrial and cytoplasmic malate dehydrogenase, the first with a pKₐ in the range 6.8 — 8.3 for the mitochondrial and 6.4 — 7.8 for the cytoplasmic enzyme, the second with a lower limit at 10.2 resp. 11. Comparison with bis-(dihydronicotinamide)-dinucleotide and dihydronicotinamide-ribosem-P₄-ribose-pyrophosphate instead of NADH indicates that the second alkaline ionization is caused by a residue placed near the adenine binding site of the active centre of the two isoenzymes. Binding studies with NADH and NAD⁺ give evidence for the participation of a group in the mitochondrial enzyme with pKₐ 6.8, deprotonation of which is necessary for detectable association of NAD⁺. In contrast the fixation of NAD⁺ to the cytoplasmic enzyme is independent of pH.

Introduction

Amino acid residues of dehydrogenases participate in binding of the coenzyme in either protonated or deprotonated form. The formation of binary NADH-enzyme complexes can be monitored by the increase in the intensity of coenzyme fluorescence which is necessary for detectable association of NAD⁺. In contrast the fixation of NAD⁺ to the cytoplasmic enzyme is independent of pH.

Results

The dissociation constant of NADH/mitochondrial enzyme complex increases 20-fold from pH 5.7 to 8.5 (Table I). Between pH 5 and 11 we observed pH-titration curves with changing points of inflection (pHₐ) at different NADH concentrations (Fig. 1), permitting determination of the pKₐ of the dissociable group [3]. We found a pKₐ 6.8 (Fig. 2). The limiting value pHₐ = 8.3 indicates a decrease by a factor of 30 in the affinity of NADH after deprotonation and a shift of the pKₐ of the ligand-free group to 8.3 upon binding (Scheme). The dissociation constants of the protonated and de-
protonated complexes are 0.85 and 26 μM respectively. A second pKₐ, important for the binding of NADH, is found at 10.2. Using the cytoplasmic enzyme a residue with pKₐ 6.4 is involved in NADH binding [3], this pKₐ is shifted to 7.8, and corresponds to 25fold decrease of the affinity in weakly alkaline medium. The second dissociable group shows a pKₐ of approx. 11. Titration curves with NNDH₂ and the mitochondrial enzyme show a pKₐ 6.5, but a second one is not observed, and the analogue is bound by the deprotonated enzyme up to measuring limits. In the corresponding titration curves of the cytoplasmic form with NNDH₂, the pKₐ = 6.4 is shifted to 8.4 upon association of the coenzyme analogue. In the alkaline region a new lower limit at pH 10.2 appears instead of 11. The value 10.2 corresponds to a pKₐ of a free protein group, different to the alkaline one involved in NADH binding. In the case of lactate dehydrogenase from pig heart (EC 1.1.1.27), there is no difference in the pKₐ values of the dissociable groups titrating with NADH [3] or NNDH₂.

In comparison to NADH NMNPRH forms a 10fold weaker complex at pH 6.6 with the mitochondrial, but a 30fold weaker one with the cytoplasmic enzyme (Table). The affinity of the coenzyme fragment to both isoenzymes also decreases between pH 6 and 8; but a second pKₐ is not observed. Per-

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**Table I. Equilibrium constants K(μM).**

<table>
<thead>
<tr>
<th>Enzyme Coenzyme</th>
<th>m-MDH</th>
<th>c-MDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH⁺</td>
<td>NMNPRH⁺</td>
<td>NAD⁺</td>
</tr>
<tr>
<td>pH 5.3</td>
<td>1.25</td>
<td>5.1</td>
</tr>
<tr>
<td>5.7</td>
<td>0.85</td>
<td>5.6</td>
</tr>
<tr>
<td>6.0</td>
<td>1.4</td>
<td>4.3</td>
</tr>
<tr>
<td>6.2</td>
<td>1.4</td>
<td>5.0</td>
</tr>
<tr>
<td>6.6</td>
<td>2.6</td>
<td>5.4</td>
</tr>
<tr>
<td>7.0</td>
<td>4.1</td>
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<td>5.6</td>
<td>10.3</td>
</tr>
<tr>
<td>8.0</td>
<td>11.4</td>
<td>13.0</td>
</tr>
<tr>
<td>8.3</td>
<td>18.5</td>
<td>1200</td>
</tr>
<tr>
<td>8.5</td>
<td>16.0</td>
<td>2000</td>
</tr>
</tbody>
</table>

a Results from fluorescence equilibrium method.

b Results from pH titration method.
Nucleotide fluorescence enhancement = 0.0
\[ K_{d1} = 0.85 \mu M \]
\[ K_{d2} = 26 \mu M \]

Nucleotide fluorescence enhancement = 1.0
\[ K_{d2} = 8.3 \]

Scheme 1. Mechanism to explain the pH-dependence of NADH binding to mitochondrial malate dehydrogenase. The titration of Fig. 1 with \([\text{NADH}] < K_{d1}\) has a pH\(_{1/2}\) which tends to pK\(_{a1}\) (Fig. 2). The titrations of Fig. 1 with \([\text{NADH}] > K_{d2}\) have values of pH\(_{1/2}\), which asymptotically approach pK\(_{a2}\) (Fig. 2). K\(_{d2}\) evaluated from the thermodynamic relation: 
\[ K_{d1} \times K_{a1} = K_{d2} \times K_{a2} \]

H represents the dissociable proton, E the enzyme, and B: the imidazole moiety of the essential histidine of the enzyme.

Discussion

In both the enzymes, dissociable groups are involved in the binding of the coenzyme. The pK\(_{a}\) 6.4 or 6.8 implies a histidine residue [3, 7], the protonated form of which is responsible for the association of the dihydronicotinamide part and in the case of the mitochondrial enzyme, prevents fixation of NAD\(^+\). Anderton found a pK\(_{a}\) 7.1 in inactivation experiments [7], which shifts to 9 in the presence of NADH [8].

A residue with pK\(_{a}\) 10.2 for the mitochondrial and 11 for the cytoplasmic enzyme seems to be essential in the protonated form for the binding of the adenine ring. We failed to detect a second pK\(_{a}\) using the adenine-free coenzyme fragment as a complex ligand. Correspondingly the exchange of adenine in NAD\(^+\) against dihydronicotinamide in NN\(_{2}\)H\(_2\) leads to a different binding behaviour in both enzymes in the alkaline region. These results indicate spatial differences in the structure of the active centres of the two isoenzymes and explain the different behaviour against inactivation by NAD-analogues [9].