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 pK_a 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-ribosyl-P^2-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 pK_a 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 [1]. The complex formation of cytoplasmic and mitochondrial malate dehydrogenase (EC 1.1.1.37) was investigated by fluorescence equilibrium titrations [2], and by the pH-dependence of the dissociation degree in the range pH 5 to 11 [3]. Using bis-(dihydronicotinamide)-dinucleotide ($NNDH_2$), in which adenine of NADH is replaced by dihydronicotinamide, and dihydronicotinamide-ribosyl-P^2-ribose pyrophosphate (NMNPRH), the adenine-free coenzyme fragment, instead of NADH, we tried to draw conclusions for the assignment of dissociable residues to the coenzyme binding.

Materials and Methods

Cytoplasmic and mitochondrial malate dehydrogenase were prepared and assayed as described [4]. $NNDH_2$ and NMNPRH were synthesized [5]. NAD^+ and NADH were commercial products. The enzyme suspensions were dissolved in 20 mM phosphate buffer, pH 6.6 and dialysed against the same buffer. Equilibrium and pH-titrations were performed at 25 °C and analysed as described [2, 3]. The equilibrium constants for malate dehydrogenase/NAD^+ complexes were calculated from the difference of NADH-fluorescence enhancement between pH-titrations with and without NAD^+ at the same pH [6].

Results

The dissociation constant of NADH/mitochondrial enzyme complex increases 20fold from pH 5.7 to 8.5 (Table I). Between pH 5 and 11 we observed pH-titration curves with changing points of inflection ($pH_{1/2}$) at different NADH concentrations (Fig. 1), permitting determination of the pK_a of the dissociable group [3]. We found a pK_a 6.8 (Fig. 2). The limiting value $pH_{1/2} = 8.3$ indicates a decrease by a factor of 30 in the affinity of NADH after deprotonation and a shift of the pK_a of the ligand-free group to 8.3 upon binding (Scheme). The dissociation constants of the protonated and de-

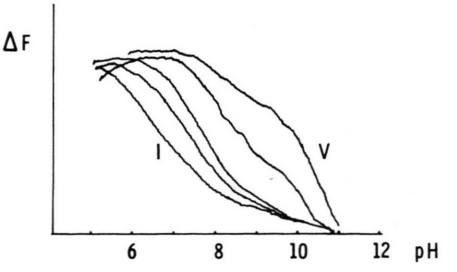

Fig. 1. Variation of the nucleotide fluorescence enhancement (ΔF) with pH of the complex NADH/mitochondrial malate dehydrogenase. The NADH-fluorescence is set zero. After addition of the enzyme the signal of the enhancement is appropriately amplified for each experiment at the starting low pH. From curve I to V the solution contained 0.5, 2, 4.4, 25, 100 μM NADH and 0.1, 0.2, 0.4, 3, 8 μM enzyme.

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Table I. Equilibrium constants $K(\mu M)$.

Enzyme Coenzyme	m-MDH			c-MDH		
	NADH[a]	NMNPRH[a]	NAD^+ [b]	$NNDH_2$ [a]	NMNPRH[a]	NAD^+ [b]
pH 5.3	1.25			5.1		
5.7	0.85			5.6		
6.0			20 000			460
6.2	1.4			4.3		
6.6	1.4	15		5.0	30	
7.0	2.6		2 000	5.4		460
7.4	4.1		1 400	9.5		
7.7	5.6			10.3		
8.0	11.4		1 200	13.0		600
8.3	18.5					
8.5	16.0		1 000			

[a] Results from fluorescence equilibrium method.
[b] Results from pH titration method.

protonated complexes are 0.85 and 26 μM respectively. A second pK_a, important for the binding of NADH, is found at 10.2. Using the cytoplasmic enzyme a residue with pK_a 6.4 is involved in NADH binding [3], this pK_a is shifted to 7.8, and corresponds to 25fold decrease of the affinity in weakly alkaline medium. The second dissociable group shows a pK_a of approx. 11. Titration curves with $NNDH_2$ and the mitochondrial enzyme show a pK_a 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 $NNDH_2$, the $pK_a = 6.4$ 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 pK_a 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 pK_a values of the dissociable groups titrating with NADH [3] or $NNDH_2$.

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 pK_a is not observed. Per-

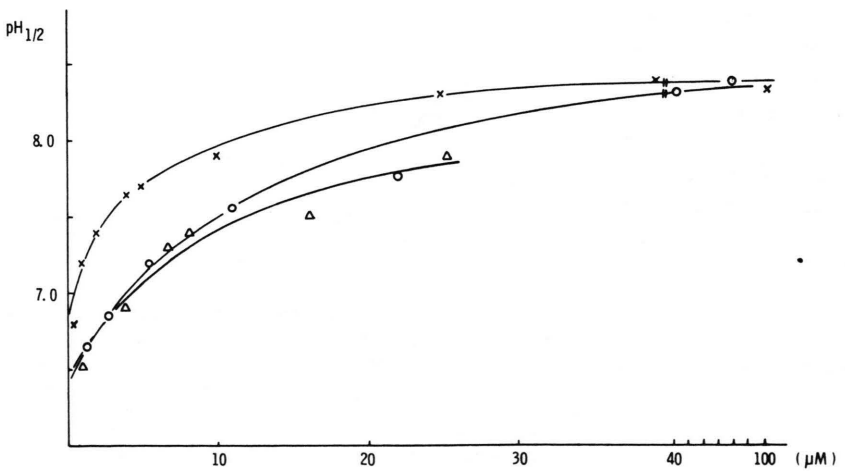
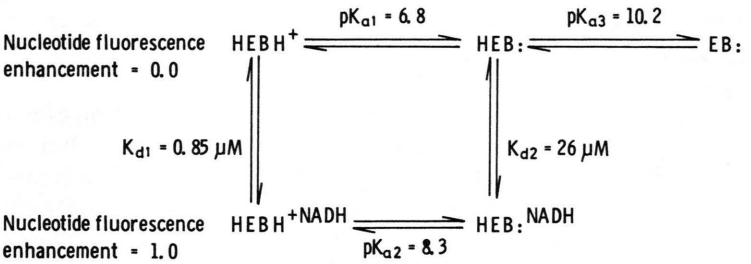


Fig. 2. Variation in the apparent pK_a ($pH_{1/2}$) for the fluorescence enhancement (ΔF) -x- of NADH with mitochondrial malate dehydrogenase against [NADH], -△- of $NNDH_2$ with the mitochondrial enzyme against [$NNDH_2$], -○- of $NNDH_2$ with cytoplasmic malate dehydrogenase against [$NNDH^2$].



Scheme 1. Mechanism to explain the pH-dependence of NADH binding to mitochondrial malate dehydrogenase. The titration of Fig. 1 with [NADH] $\ll K_{d_1}$ has a $pH_{1/2}$ which tends to pK_{a_1} (Fig. 2). The titrations of Fig. 1 with [NADH] $\gg K_{d_2}$ have values of $pH_{1/2}$, which asymptotically approach pK_{a_2} (Fig. 2). K_{d_2} evaluated from the thermodynamic relation: $K_{d_1} \times K_{a_1} = K_{d_2} \times K_{a_2}$. H represents the dissociable proton, E the enzyme, and B: the imidazole moiety of the essential histidine of the enzyme.

forming pH-titrations in presence of NAD^+, the NADH-fluorescence enhancement and hence the degree of NADH-association decreases in the pH-ranges, where NAD^+ is competitively bound. With the mitochondrial enzyme increasing proton concentrations below pH 6.5 lead to a gradual decrease in binding of NAD^+. At this pH the amount of NADH-fluorescence enhancement – in absence or presence of NAD^+ – is unchanged. In the alkaline region, however, a lower NADH-saturation degree is detectable in presence of NAD^+. The dissociation constants for the NAD^+-enzyme complex show a significant decrease with increasing pH (Table). Computer simulations point out, that the best approximation to the experimental curves is maintained when binding of NAD^+ to the mitochondrial enzyme occurs after deprotonation of a group with $pK_a = 6.8$.

With cytoplasmic malate dehydrogenase, the dissociation degree of NADH decreases in the presence of NAD^+ between pH 5 and 11. The dissociation constant for NAD^+, as calculated from the pH titration curves, does not show any essential alterations with pH (Table).

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 NADH against dihydronicotinamide in $NNDH_2$ 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].

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