D-LACTIC DEHYDROGENASE FROM ANAEROBIC YEAST : REVERSIBLE DISSOCIATION OF FAD AND Zn BY ACID TREATMENT OF THE HOLOENZYME .

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Studies on D-lactic dehydrogenase of anaerobic yeast (Slonimski et al. 1958, Boeri et al. 1958, Labeyrie et al. 1959) by means of a chelator (Stachiewicz et al. 1961, Curdel et al. 1959, 1961) or by means of an acridine (Baudras et al. 1960, Iwatsubo et al. 1961) suggested that two dissociable cofactors were necessary for the full activity of the enzyme : Zn^{++} and FAD. This suggestion was based on two lines of evidence : (a) Zn^{++}, at low concentration, is the only ion that can reactivate rapidly and completely the EDTA - inactivated enzyme (Curdel et al. 1959) or the FMN - inactivated enzyme. The reactivated enzyme has the same kinetic properties as the native one (K_m, V_m and activation energy (Curdel et al. 1961) ; (b) FAD is the only flavin that can protect the enzyme against quinacrin inactivation and, under certain conditions, can activate it. It has been argued (Boeri et al. 1960, Ghiretti-Magaldi et al. 1961) that the Zn^{++} reactivation of EDTA treated enzyme is not due to the reconstitution of the resolved enzyme but simply to the removal of EDTA from an inactive EDTA - enzyme complex.

The present work proves the correctness of our initial hypothesis. It will be shown that an acid treatment at low temperature in the presence of ammonium sulfate leads to an enzymatically inactive apoenzyme which can be totally reactivated by a simultaneous addition of FAD and Zn^{++}.

D-LDH is prepared from the strain 59 RL5 (Galzy, 1960) of Saccharomyces cerevisiae. The yeast is grown anaerobically and the enzyme extracted by freezing and thawing (Lippincott). It is purified by ammonium sulfate fractionation and heating in the presence of lactate, further fractionation on Sephadex G 75 and on DEAE Sephadex A 50

columns. It is kept precipitated in 0.7 sat. Am S. The apoenzyme is prepared by method similar to the one described by Warburg and Christian (1938) : the 0.7 sat. Am S precipitate is dissolved in buffer, passed or not (according to the experiment) through Sephadex G 50 columns and Am S added to 0.4 sat. This solution, kept at -6°C, is brought to the desired pH by the addition of 5 N HCl (for very low pH, 0.5 to 1, the temperature is -12°C). The protein is precipitated by Am S 0.7 sat., centrifuged and the sediment dissolved at 0°C in phosphate buffer 0.2 M, pH being adjusted to 7.2.

Acid-treated enzyme is inactive in catalyzing the oxidation of D-lactate in the presence of acceptor like 2,6 dichlorophenol indophenol or ferricyanide, if the pH of treatment has been lower than 4. It can be stored at 0°C for several days without showing any "spontaneous" reactivation and without loosing capacity to be reactivated by cofactors. To obtain this, precautions have to be taken to avoid trace-metal contamination. The salts used (even analytical grade) should be recrystallized first in the presence of EDTA, then once again in the absence of EDTA from pyrex-bidistilled water.

The table shows that the resolved enzyme is reactivated by Zn^{++} and FAD.

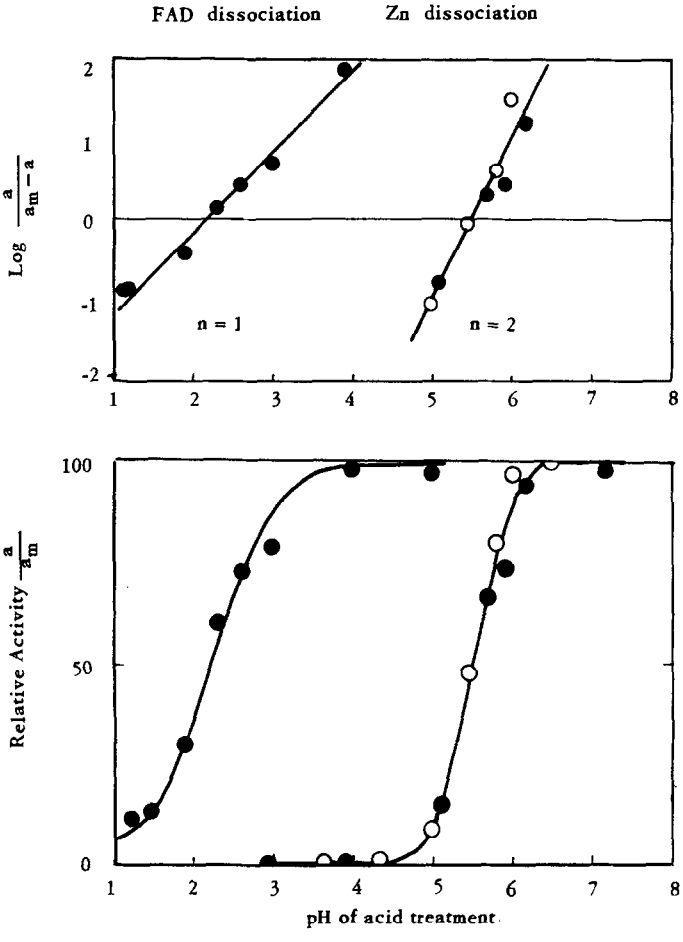
	Exp. 1			Exp. 2		
pH during acid precipitation	1.5	2.3	3.5	3.9	5.4	6.5
Acid-treated enzyme	0	5	5	13	124	223
" " " +FAD	35	37	10	78	179	250
" " " $+Zn^{++}$	14	100	162	234	278	226
" " " $+FAD+Zn^{++}$	116	195	275	252	260	243

The figures show the activity of aliquots of the treated enzyme, neutralized, then incubated 15 min., with or without 20 μM Zn^{++} and with or without 200 μM FAD.

At pH 6.5, the treatment does not lead to any inactivation and the addition of FAD and Zn^{++} does not change the activity. There is no dissociation of the enzyme. Between pH 6.5 and 4, the addition of Zn^{++} alone is sufficient to restore completely the activity. Below pH 4, Zn^{++} alone

restores much less activity than FAD and Zn^{++} added together. In this zone, flavin dissociates. As a result of very acid treatment the mixture Zn^{++}+FAD does not lead to a complete reactivation (40 % only for the pH 1.5). This is probably due to an irreversible denaturation of the apoenzyme.

Influence of the pH of acid-precipitation of DLDH on FAD or Zn^{++} dissociation.



Determination of the number of protons involved in Zn^{++} and FAD dissociation.

Dissociation curves.

a : activity of the acid-treated enzyme passed (●) or not (○) through Sephadex.

a_m : activity maxima of the same enzyme resaturated (see text).

The duration of the acid treatment (ca. 10 min.) is sufficient to attain, at every pH studied, the equilibrium of Zn^{++} dissociation. The rate of reactivation by Zn^{++} is proportional to the metal concentration. Under our conditions, with 20 µM Zn^{++}, maximal activity is attained in less than 15 min. It can be concluded therefore that the plot of the ratio : activity of acid-treated enzyme / activity of the same enzyme saturated with Zn^{++},

as a function of pH of the treatment, corresponds to the dissociation curve of Zn^{++} from the enzyme. Half dissociation is obtained at pH 5.5 (figure). An analogous plot of the ratio : activity of acid-treated enzyme saturated with Zn^{++} 20 μM/activity of the same enzyme saturated with Zn^{++} 20 μM and FAD 200 μM shows a half dissociation value of pH 2.2 and corresponds to the dissociation of the flavin. Experiments on the specificity of Zn^{++} and FAD in restoring the activity of the resolved enzyme will be reported separately (Curdel et al., to be published).

The pK value of 5.5 for Zn^{++} dissociation relative to the simultaneous dissociation of two protons suggests that the cation is linked to two dissociated imidazole groups pertaining to two different histidine residues. The number of coordination of zinc is 4, disposed in a tetrahedric configuration ; the two other coordination positions are probably shared by the substrate (which completely protects against EDTA) and possibly by the flavin. The very low pK for flavin dissociation (like in TPNH cytochrom c reductase) corresponds to one proton dissociation probably relative to the pyrophosphate group.

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