D-LACTICODEHYDROGENASE FROM ANAEROBIC YEAST : COMBINATION OF APOENZYME WITH ZINC AND COBALT AND COMPARISON OF RECONSTITUTED ENZYMES.

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The problem discussed here concerns the role and the specificity of the metal involved in a metalloenzyme activity.

It has been shown previously that inactivation by EDTA (ethylenediaminotetracetic acid) of D-lacticodehydrogenase from anaerobic yeast, D-LDH, (Slonimski and Tysarowski, 1958, Labeyrie et al., 1959), is irreversible in spite of elimination of free EDTA (Stachiewicz et al., 1961). We have also shown (Curdel et al., 1959) that the inactivated enzyme can be reactivated by Zn^{++} and Co^{++}. The two metals were the only ones, among some twenty tested, that were capable to reactivate the enzyme. Some of the inactive metals like Ni^{++}, Pb^{++} or Cu^{++} have a higher affinity for EDTA than Zn^{++} or Co^{++} (Schwartzenbach and Freitag, 1959). It was therefore thought that the reactivation involved a specific recombination of metal free apoenzyme with Zn^{++} or Co^{++}, since it could not result from the dissociation of an inactive enzyme - EDTA complex by saturation of free EDTA. These results were confirmed later by an independant study of Boeri et al. (1960), who misinterpreted however certain of our previously published results specially those pertaining to the affinity of metals to EDTA in respect to their capacity in restoring D-LDH activity. The concentration of Zn found by these authors to be necessary to give half the reactivation

(2, 8 μM) compares favorably with the value published in our previous paper; we then found 7 μM to be sufficient to a complete reactivation.

The problem we are dealing with concerns the comparison of enzymes reconstituted from EDTA treated D-LDH and Zn^{++} or Co^{++}. Two questions are considered : 1) Is the reactivation produced by Co^{++} due to impurities of Zinc in Cobalt preparations ? 2) Are the kinetic properties of the reconstituted enzymes different ?

The answer to the first question is negative. A fraction of the reactivation produced by the usual analytical grade reagent (Merck) Co preparations (less than 0, 01 % Zn) is due to contamination because preparations of spectroscopically pure Co^{++} (Matthey Specpure) reactivate to a smaller extent when added at the same concentration. Nevertheless the purest Co preparations used do reactivate the enzyme as shown in fig. 1. It can be seen (fig. 1) that in both cases the relation between the concentration of the metal and the enzyme activity is hyperbolic, that half saturation is attained for 6 μM of Zn^{++} and 20 μM of Co^{++}, and that maximal activity at saturating metal concentration is ca 4 times smaller with Co^{++} than with Zn^{++}. It should be mentioned that the extent of reactivation as compared to the initial activity of D-LDH depends on the delay between the moment when the inactivation is almost complete (< 90 %) and the moment when the metal is added. For instance if the addition of metal follows closely the inactivation (free EDTA being eliminated without dialysis through chelation with an excess of Zn^{++}) the reactivation is complete. If, however, free EDTA is eliminated first through 2 hours dialysis, an excess of Zn restores ca 60 % of the original activity only. The reactivation takes place in 2 minutes in every case.

In order to answer the second question, we compare the K_M and the V_m

values of native enzyme and reactivated enzymes. Michaelis constant for D-lactate of native D-LDH is 1,7 mM. This value is not modified in spite of an extensive inactivation by EDTA. It characterizes also the enzyme first completely inactivated then reactivated by Zn^{++} (fig. 2). However, the Michaelis constant of the Co-reactivated enzyme is quite different and equal to 0,15 mM (fig. 2). The addition of Co^{++} at the same concentration to the native enzyme does not bring any modification of K_M or V_m.

Table 1

Enzyme preparation		K_M	V_m
Native D-LDH		1,65mM	100
Native D-LDH	in the presence of Co 1,5 mM	1,7 mM	
EDTA Inactivated D-LDH*		1,8 mM	10
EDTA Inactivated D-LDH*		-	1
- same sample -	then reactivated by Zn 0,2 mM	1,7 mM	60
" "	then reactivated by Co 1,5 mM	0,15 mM	12

* Two samples inactivated at different extents.

The modification of the kinetic constants of EDTA inactivated, then Co-reactivated D-LDH rules out the interpretation of inactivation as resulting only from a fixation of EDTA on the native enzyme. It adds also a new argument against the hypothesis of Zn impurities as being responsible for reactivation produced by Co preparations. It seems reasonably certain that we are dealing here with Zinc-apoenzyme and Cobalt apoenzyme complexes, the first being non distinguishable from the native D-LDH. The half reactivation concentration corresponds probably to the dissociation constants of these complexes. It is interesting that the Co-D-LDH comple-

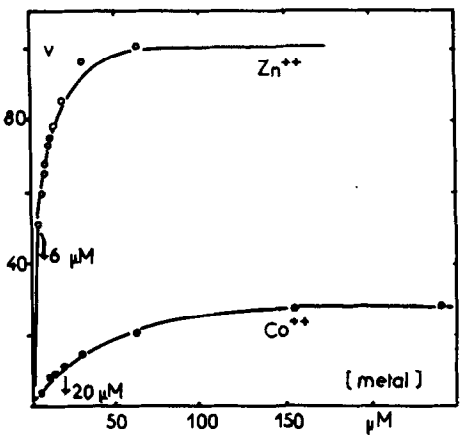

Figure 1

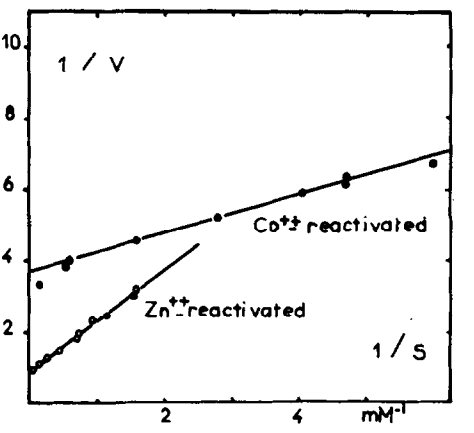

Figure 2

Fig. 1 : Reactivation by Zn and Co of EDTA inactivated D-lacticodehydrogenase.

D-LDH is inactivated (to 90 %) by EDTA 20 µM in K, Na phos. buffer, pH 7,2 during 1 hour, at 30°C. Aliquotes of the inactivated enzyme were preincubated 3 minutes at 30°C with variable amounts of the metal ion in presence of the same buffer, 70 mM, and 26-DPIP* 1,7 µM. D-lactate (15 mM) was then added and the reaction followed at 600 mµ. The velocity is constant in time.

Fig. 2 : Comparison of kinetic constants relative to (1) Zn^{++} and (2) Co^{++} reactivated EDTA inactivated enzyme.

D-LDH is inactivated (to 99 %) as previously and divided in two equal parts. One is preincubated with 0,2 mM Zn^{++}, the other with 1,5 mM Co^{++}. Aliquotes of each of them are added in spectrophotometric cells to the following reaction mixture : Na, K phos. buffer 70 mM, 26 DPIP* 1,7 µM and variable concentrations of D-lactate.

* 26 dichlorophenolindophenol.

xes has a definitely lower K_M and V_m values.

It has been shown for the native D-LDH that the K_M is ca 300 times higher than the dissociation (K_s) of the native enzyme-D-lactate complex (Labeyrie and Stachiewicz, to be published). The difference of K_M values relative to Co^{++}-apoenzyme and Zn^{++}-apoenzyme complexes therefore cannot be interpreted as resulting from different substrate binding capacities. The correct interpretation will be made only when the exact signification of K_M will be known. The metal may be involved in electron transfer between the enzyme bound substrate and the acceptor.

References

Boeri E., Cremona T. and Singer T. P., Biochem. and Biophys. Research Comm. 2, 298 (1960).

Curdel, A., Naslin, L., and Labeyrie, F., Compt. rend. Acad. Sci. 249, 1959 (1959)

Dixon, M. and Webb, E. C., Enzymes, Longmans, Green and Co., London (1958).

Labeyrie, F., Naslin, L., and Slonimski, P. P., Biochim. Biophys. Acta, 34, 262, (1959).

Schwartzenbach, G., and Freitag, E., Helv. Chem. Acta, 234, 2615 (1959)

Slonimski, P. P., and Tysarowski, W., Compt. rend. Acad. Sci., 246, 1111 (1958).

Stachiewicz, E., Labeyrie, F., Curdel, A., and Slonimski, P. P., Biochim. Biophys. Acta, accepted for publication.