I. REVERSIBLE INACTIVATION OF LIVER AND YEAST ALCOHOL DEHYDROGENASE

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Many enzymes, including certain dehydrogenases, have been reversibly dissociated into inactive subunits (1-6). Dissociation has normally been affected with urea, guanidine, extremes of pH or salts. Reactivation generally required β-mercaptoethanol and neutral pH. In this connection liver and yeast alcohol dehydrogenase (LADH and YADH) are interesting because the native form of these enzymes contain two and four active centres respectively. Difficulties in dissociating or reversing inactivation of the LADH metallo-enzyme has been thought due to irreversible loss of zinc (6). However this does not appear to be the case as the reactivation after inactivation and dissociation of this enzyme shows.

EXPERIMENTAL AND RESULTS

Freshly dialysed solutions (60-100 μM) of the crystalline enzymes (Boehringer, Mannheim, Germany), in 0.1 μ phosphate buffer pH 7.3 were used. In assaying the enzymes (7) it was necessary to dialyse away mercaptoethanol from LADH (but not from YADH), as it is a potent inhibitor of this enzyme (8). Enzymatic activity was assayed in duplicate by withdrawing aliquots before and after inactivation and reactivation. Enzyme inactivation could be achieved by low or high pH, lithium chloride, or urea, and the effect reversed by back-titrating to neutral pH. Thus for acid

inactivation, 50 µl. of LADH (65 µM) were diluted to 3 ml. with 0.1 µ phosphate buffer pH 7.3 and the activity determined. The solution was titrated with H_3PO_4 to pH 2.5 or 4.0. Assay showed that no activity remained. Immediately reactivation was carried out at 23°C by titrating with NaOH to pH 7.5, adding mercaptoethanol and allowing to stand for 1-4 hours. Then over a period of about 20 hr. the mercaptoethanol (0.1 M) was dialysed away using phosphate buffer at 4°C, 73°/o of the initial activity was recovered. Without mercaptoethanol 38°/o of the activity was recovered. Using the above conditions with mercaptoethanol, 29°/o of the activity was recovered if reactivation was carried out for the same time at 0°C instead of 23°C. These inactivations were done in a glass vessel at room temperature. If a cellulose nitrate vessel was used, 85°/o of the initially activity was recovered. The recovery of enzymatic activity also dependedon the concentration of mercaptoethanol. It was only 58°/o with 0.05M mercaptoethanol. These results for LADH are listed in Table 1. As shown, more activity could be recovered after inactivation at pH 4.0 than at pH 2.5, where increased acidity produced further structural change. After inactivation of LADH by acid, the presence of NADH (71 µM) only increased the activity recovered a little (5-8°/o). However the addition of mercaptoethanol (0.15M) in the presence of NADH, resulted in less activity being recovered than if mercaptoethanol was used alone. Under all conditions enzyme concentration was critical. If too high, then enzyme precipitation or aggregation was visible. This was particularly noticeable with high pH, where aggregation occurred readily, and reactivation had to be carried out quickly. Perhaps the increased reactivation given by a container of cellulose nitrate compared with glass, is due to the alkaline nature of the surface of glass.

Table 1

Reactivation of LADH and YADH

Inactivation pH	Inactivation T°C	Reactivation pH	Reactivation T°C	Reactivation [ME*] M	Initial Activity °/o Recovered
LADH					
4.0	23	7.5	23	0.1	73 (85)**
"	"	"	"	-	38
"	"	"	"	0.05	58
"	"	"	0	0.1	29
2.5	"	"	23	"	47
12.0	"	"	"	"	27
"	"	"	"	-	17
7.3	0, 6M LiCl	7.3	20	0.1***	72
"	", "	"	"	***	11
7.1	", 8M Urea	"	"	0.1***	17
"	", "	"	"	- ***	8
YADH					
3.4	23 -	7.5	23	0.15	17
7.3	0, 6M LiCl	7.3	23	0.15***	21

* β-mercaptoethanol
** A cellulose nitrate instead of a glass container was used.
*** Reactivation involved dilution of the inactivated enzyme solution, 6-fold for LiCl, 10-fold for urea.

Temperature was also important, and as listed in Table 1, it was found that inactivation by urea or lithium chloride at pH 7.3 was best carried out at 0° C, and reactivation at room temperature. Dilution was necessary for these reactivations. Although glycerol (10-25°/o) protects LADH and YADH against urea inac-

tivation, nevertheless it did not increase reactivation.

For YADH, the experimental procedure was the same as for LADH, except that the activity recovered with YADH seemed independent of whether mercaptoethanol was dialysed away or not. In Table 1, reactivation of YADH after urea inactivation is not listed as this has been achieved previously (9). No doubt further experiments with YADH could give better conditions for inactivation and reactivation and result in the recovery of more activity.

DISCUSSION

Optimum reactivation depended on pH, temperature, concentration of enzyme, time course of reactivation, container and the presence of mercaptoethanol. More activity was recovered if inactivation was carried out at 0°C and reactivation with mercaptoethanol at 23°C and neutral pH. The period required to produce complete inactivation had to be restricted to a minimum, otherwise a secondary irreversible loss of structure frequently resulted. With high concentration of enzyme, precipitation occurred during reactivation, apparently because of intermolecular aggregation of dissociated protein. With very low enzyme concentration, low reactivation was also found. This seemed due to absorption onto the container surface. Reactivation of LADH after urea inactivation proved most difficult. Prolonged exposure of enzyme to high urea concentrations resulted in irreversible loss of structure. A suitable concentration of enzyme and urea, and a dilution such that the final concentration of urea was not too high to prevent reactivation was necessary.

The reversible inactivation at 0°C by 6M LiCl, parallels the hybridization under neutral conditions of lactic dehydrogenase subunits by saturated sodium chloride at 4°C, or by freezing and

thawing (10). It shows that salt (or urea) can be as important for these processes as high or low pH. The temperature-dependence of the urea experiments indicates that reversible and irreversible inactivation involve hydrophobic bonds with a negative temperature coefficient (11). This also suggests that dissociation into subunits, rather than conformation change alone occurs. The re-activation after inactivation of the alcohol dehydrogenases, suggests that the various methods of inactivation are not releasing the zinc moeity from the protein. In line with this zinc ions neither promoted reactivation nor protected against inactivation. The suggestion that activity cannot be regained after pH 5.0 in-activation, because of zinc removal (13), seems to confuse in-activation with zinc removal, occurring subsequently. Lack of success of previous attempts at LADH reactivation (6, 12) seems due to the conditions necessary being more critical than for many other dehydrogenases. Precipitation of enzyme on adjustment to neutral pH after acid inactivation and consequent lack of rever-sibility, result from the enzyme concentration being too high and bear out the critical nature of enzyme concentration in these processes.

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