II. THE DISSOCIATION OF THE ALCOHOL DEHYDROGENASES

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Received May 2, 1968

It has been established that liver and yeast alcohol dehydrogenase (LADH and YADH) can be inactivated by low and high pH, high salt concentrations, or urea, and then reactivated (1,2). To ascertain the nature of the changes involved in the reversible inactivation, molecular weight studies have been carried out on the enzymes, inactivated under the conditions from which activity can be recovered. While urea is known to reversibly dissociate YADH into four subunits (2), the reversible dissociation of LADH and YADH in the present experiments has been mainly established by light scattering measurements.

EXPERIMENTAL AND RESULTS

The solutions of inactivated enzyme were prepared as previously described (1). They were examined in the ultracentrifuge and by light scattering. The latter technique was mainly used, as with care the molecular weight could be measured immediately and subsequent changes with time followed.

The light scattering measurements were performed in a Brice-Phoenix light scattering photometer, using a 50 ml. cylindrical cell and the associated diaphram system. Light of

5461Å was employed. Solutions were clarified by spinning for 3 hr. in a preparative ultracentrifuge at 70,000 x g or by passing through a 100 mμ millipore filter. The solutions were then introduced into the cell which had been cleaned by refluxing in acetone vapor. An enzyme concentration range of 0.5 - 2.0 mg/ml was used. The refractive index increment dn/dc was found at 23°C to be 0.20 for the protein in phosphate buffer, while in urea and guanidine-HCl it was 0.14 and 0.06 respectively. It was determined in a Brice-Phoenix differential refractometer using the mercury green line of 5461Å. Table I lists the results obtained. The molecular weights were calculated using the dissymmetry method (3). The error in measurements was approximately 10%.

LADH: The molecular weight of native enzyme is 84,000 (4). Whether the enzyme is free or mainly as binary complex with NADH, the light scattering molecular weight agrees with the accepted value, and shows that the presence of the coenzyme, neither aggregates nor dissociates LADH. At acid pH, or in the presence of urea or guanidine-HCl, dissociation into two, four or perhaps more subunits is indicated. At acid pH, reproducibility of the molecular weights was difficult to achieve due to dissociation being accompanied by aggregation. The situation was complicated as both these processes depended on time as well as pH, temperature, and enzyme concentration. Aggregation from the species measured occurred more readily at pH 4.0 than at pH 2.5, where the subunits appeared more stable. The fact that more activity can be recovered at pH 4 than at pH 2.5, also suggests enzyme-forms that cannot revert to active enzyme. The molecular weight of 320,000 at pH 4.0 results from aggregation caused by standing and ultracentrifugation used to remove turbility. At alkali pH aggregation occurs rapidly (reactivation is difficult), and the

TABLE 1

Molecular Weight from Light Scattering

	pH	Additions	n_o	dn/dc	$\bar{M}w \times 10^{-3}$
LADH					
	7.3	-	1.334	0.20	95
	"	NADH (140μM)	"	0.21	94
	2.5	-	"	0.167	41
	"	-	"	"	50
	"	-	"	"	48
	4.0	-	"	0.20	11
	"	-	"	"	21
	"	-	"	"	19
	"	-	"	"	320*
	12.0	-	"	0.057	400
	7.1	8M Urea	1.407	0.14	14
	"	8M Urea + 0.1M ME**	"	0.11	11
	"	4M Guanidine-HCl	1.381	0.06	16
YADH					
	7.3	-	1.334	0.20	190
	2.5	-	"	0.167	46
	12.0	-	"	0.06	455
	7.1	3M-Guanidine-HCl	1.381	0.06	39

* After spinning the solution for three hours in a preparative ultracentrifuge at 70,000 × g.
** β-mercaptoethanol.

high molecular weight at pH 12.0 demonstrates this. Table 1 also shows that in the presence of urea, the molecular weight measured is independent of whether mercaptoethanol is present or not.

YADH: As with LADH, aggregation is found at alkali pH. At acid pH or in the presence of guanidine-HCl dissociation into four subunits occurs. The latter result agrees with the reversible dissociation of YADH into four subunits by urea (2).

Using a Spinco Model E Ultracentrifuge, some experiments were also carried out at 20°C to determine sedimentation coefficients for the species present in solution. The ultracentrifuge was fitted with phase-plate Schlieren optics and a RTIC unit. However, the ultracentrifuge technique did not prove satisfactory due to aggregation often resulting from the centrifugal gradient, enzyme concentration and time used for experiments. Thus a comparison of native with inactive enzyme showed aggregation of LADH at pH 4.0 (6.1S; native 5.1S) and of YADH at pH 2.5. In agreement with the light scattering results, a smaller molecular size was indicated for LADH at pH 2.5 (3.2S).

DISCUSSION

For each of the conditions used for reversible inactivation, the molecular weight measurements established dissociation of liver and yeast alcohol dehydrogenase. However the species involved in reactivation are not necessarily identified. Dissociation of both enzymes by detergents (5,6) and urea (2,7) has been reported. The number of subunits formed were the same as the number of active sites per molecule (four for YADH, and two for LADH) except for the urea inactivation of LADH. In this case, depending on whether mercaptoethanol was presented or not, two and four subunits were reported (7). The present results show dissociation of YADH into four subunits, and LADH into two, four or perhaps more subunits.

That the subunit molecular weights measured are not

necessarily those of the actual forms from which reactivation after inactivation occurs, is indicated by the reduced recovery of activity with standing. Reversible inactivation occurred best at neutral pH, while away from neutrality or if the enzyme concentration was too high, increasing irreversible aggreation resulted. Activity that could not be regained, thus seems due to irreversible structural change in the subunits. These may aggregate directly or may give further breakdown-products which may themselves aggregate. Because zinc did not promote reactivation, loss of zinc from the enzyme is considered part of the subsequent irreversible processes. The metal is regarded as essential for the structure of the subunits reactivating, with reversible inactivation occurring prior to the release of zinc from the subunits. Reversible inactivation appears characterised by dissociation into subunits without loss of zinc. For YADH, reactivation or reversible inactivation involves the 37,000 subunits (2). For LADH the experiments suggest reactivation occurs from the 42,000 or 21,000 species, but these can also break-down further or change irreversibly to forms which can irreversibly aggregate. At pH 4.0, four subunits of 21,000 molecular weight are obtained, while pH 2.5 is considered to stabilise a dimer of 42,000, and at higher pH, larger aggregates are formed. Urea and guanidine-HCl also dissociated LADH into four or more subunits, the molecular weights determined being in this case independent of whether mercaptoethanol is present or not.

The fact that inactivation with urea or lithium chloride occurs readily at $0^{\circ}C$, was already an indication of dissociation into subunits which the molecular weight measurements substantiate. The temperature dependence of reversible inactivation supported

this and indicated that hydrophobic bonds which are more stable at room temperature than lower temperatures, are important in the reversible and irreversible processes involved. Thus room temperature rather than 0°C promotes subunit association to active enzyme, and at 0°C dissociation is promoted and irreversible aggregation during inactivation minimized. That non-polar bonds predominate in holding the subunits in the native enzyme together, was already suggested by the negative temperature coefficient for LADH and YADH inactivation by urea (8). Dissociation by detergents has also been considered as evidence for this (5,6).

While further experiments are necessary to definitely establish the reactivating species, the molecular weight measurements do show, that size as well as shape changes are involved during inactivation. The suggestion from the identity of sedimentation coefficients at pH 3.0 and 7.0, that acid inactivation only involves conformation change (6,9), underlines the limitation due to aggregation of the ultracentrifuge in this situation.

The dissociation of LADH into more than two subunits does raise several problems, as the enzyme contains two C-terminal and two acetylated N-terminal amino acids and is considered to contain two identical polypeptide chains (10-12).

REFERENCES

1. Cheng, L-Y., and McKinley-McKee, J.S., accompanying paper.
2. Ohta, T., and Ogura, Y., J. Biochem. (Japan) 58, 73 (1965)
3. Debye, P., J. Phys. Colloid. Chem., 51, 18 (1947).
4. Ehrenburgh, A., and Dalziel, K., Acta Chem. Scand, 12, 465 (1958).
5. Hersh, R.T., Biochim. Biophys. Acta, 58, 353 (1962).
6. Blomquist, C.H., Arch. Biochem. Biophys., 122, 24 (1967).
7. Drum, D.E., Harrison, J.H., Li, T-K., Bethune, J.L., and Vallee, B.L., Proc. Natl. Acad. Sci., U.S., 57, 1434 (1967).

8. Cheng, L-Y., and McKinley-McKee, J.S., FEBS. 4th Meeting, Abstracts, No. 574, Oslo, 1967.
9. Chilson, O.P., Kitto, G.B., Pudles, J., and Kaplan, N.O., J. Biol. Chem., 241, 2431 (1966).
10. Harris, J.I., Nature, 203, 30 (1964).
11. Brändén, C-I., Arch. Biochem. Biophys., 112, 215 (1965).
12. Jornvall, H., Acta Chem. Scand., 21, 1805 (1967).