Biophysical Chemistry 10 (1979) 217–220


# SELF-ASSOCIATION OF LYOPHILIZED HORSE LIVER ALCOHOL DEHYDROGENASE

J.B. Alexander ROSS
*Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218, USA*

Eddie L. CHANG
*Department of Chemistry, University of Washington, Seattle, Washington 98195, USA*

and

David C. TELLER
*Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA*

Received 2 March 1979

The molecular weights of lyophilized and non-lyophilized horse liver alcohol dehydrogenase have been compared by quasi-elastic light scattering, and ultracentrifugation. Whereas the non-lyophilized enzyme has the expected molecular weight of 78 000, the lyophilized enzyme has an initial molecular weight of about $10^6$ which increases with time by an endothermic process. This result shows that any physical measurement using lyophilized liver alcohol dehydrogenase to investigate the enzyme mechanism, which relies upon the molecular size, will be invalid.

## 1. Introduction

Horse liver alcohol dehydrogenase (ADH) has a broad substrate specificity which includes a large number of aliphatic and aromatic alcohols and their corresponding aldehydes [1]. The functional enzyme has a molecular weight of 78 000, and is composed of two identical subunits [2,3]. Details concerning the three-dimensional structure and kinetic properties have been recently reviewed [4]. Commercial preparations of the enzyme are available either as crystalline suspensions in buffer with alcohol present, or as a dry, essentially alcohol-free, lyophilized powder.

The thermodynamics of binding of oxidized and reduced nicotinamide adenine dinucleotides ($NAD^+$ and NADH, respectively) have been studied using both kinds of enzyme preparations [5,6]. The results are essentially the same. For both enzyme preparations the coenzyme binding is predominantly entropy-drive.

Fernandez et al. [7] used lyophilization to prepare alcohol-free ADH and found 50–70% of the original enzymatic activity after this procedure. They also referred to unpublished data which indicated that neither the kinetic parameters nor apparent mechanism of the alcohol-free enzyme corresponded to that of the alcohol-containing enzyme. Indeed, we have observed that solutions of lyophilized ADH do not keep well compared with those prepared from the alcohol-containing protein; refrigerated samples of the alcohol-free enzyme tend to become opaque within a week's time whereas samples of the unlyophilized enzyme will remain as clear solutions for several weeks. These factors led us to examine the hydrodynamic properties of the alcohol-free and alcohol-containing enzymes by quasi-elastic light scattering (QLS) and ultra centrifugation. As described in this paper, the behavior of these two preparations is remarkably different. Lyophilization of ADH induces a large increase in the rate of self-association. In addition, since the rate of self-association increases with increased temperature, we conclude that this process is endothermic.

## 2. Experimental section

### 2.1. Materials

Horse liver alcohol dehydrogenase was obtained from Sigma Chemical Company (lot 116C-8090) as a 1X crystallized and lyophilized material, and from Boehringer Mannheim (lots 1516242, 1318508) as a crystallized suspension in 10% ethanol buffered at pH 7 with 0.02 M $K_xPO_4$. $NAD^+$ was purchased from the same sources. Other chemicals were reagent grade.

### 2.2. Enzyme preparation

The lyophilized and non-lyophilized preparations were exhaustively dialyzed at an enzyme concentration of 10 mg/ml in a 0.1 M $K_xPO_4$ buffer. Depending upon the experiment, the pH was adjusted with KOH in the range from 6.5 to 8.5. In some cases, the dissolved lyophilized enzyme was incubated with $NAD^+$ for 24 hours before dialysis. The coenzyme concentration was five-fold the maximum possible number of sites, estimated from the dry weight of the enzyme. After dialysis, these samples were treated with activated charcoal to remove any bound nucleotides [6]. Finally, all samples were centrifuged in a Beckman Model L for one hour at 30 000 rpm and 4°C, using a type 65 rotor. For QLS analysis, samples were also filtered through 0.2 $\mu$ Millipore filters, which had been previously freed of detergent by heating in distilled water. The enzyme activity was obtained by the method of Dalziel [8], and one Sigma sample was examined for heterogeneity by sodium dodecyl sulfate (SDS) gel electrophoresis [9].

### 2.3. Quasi-elastic light scattering

QLS is a relatively new spectroscopic tool which measures the correlation function of coherently scattered light. For a monodisperse solution of macromolecules, the correlation function is a single exponential whose characteristic decay time $\tau$, is related to the macromolecular diffusion coefficient, $D$, by the equation

$$\tau^{-1} = 2DK^2 ,$$

where $|K| = (4\pi n/\lambda)\sin(\theta/2)$ is the scattering vector, $\lambda$ is the wavelength of incident radiation, $n$ the index of refraction of the solution, and $\theta$ is the scattering angle. The $D$ measured by QLS is weighed by the amount of light, $I_i$, scattered by a particular species, $i$,

$$\bar{D} = \sum_i I_i D_i / \sum_i I_i ,$$

and, neglecting concentration effects, is simply

$$\bar{D} = \sum_i c_i M_i D_i / \sum_i c_i M_i \equiv D_z ,$$

where $c_i$ and $M_i$ are the respective concentration and molecular weight of each species. Thus for a polydisperse solution, $\tau$ yields a $Z$-averaged diffusion constant $D_z$. The diffusion coefficients reported here are adjusted to 20°C and pure water ($D_{20,w}$).

The QLS configuration used a Spectra-Physics He-Ne laser in conjunction with a Chen-type clipped correlator. The 256-points of the correlation function were forced-fitted to a three-parameter single exponential by a DEC PDP-12 minicomputer. Further details on QLS may be found in the literature [10,11].

### 2.4. Ultracentrifugation

Sedimentation velocity experiments were performed on a Beckman Model E ultracentrifuge using an ultraviolet absorption optical system, and the data were analyzed as described by Teller [12]. The sedimentation coefficients were adjusted to 20°C and pure water ($s_{20,w}$) in the limit of zero enzyme concentration according to Ehrenberg and Dalziel [2].

### 2.5. Molecular weight calculations

The $Z$-average diffusion coefficient from QLS may be combined with the weight-average sedimentation coefficient to obtain the weight-average molecular weight, $M_w$, by the Svedberg-Einstein relation. Neglecting concentration effects,

$$\frac{s^0 RT}{D_z(1-\bar{v}\rho)} = \frac{(\Sigma_i c_i s_i^0/\Sigma_i c_i)RT}{(\Sigma_i c_i M_i D_i/\Sigma_i c_i M_i)(1-\bar{v}\rho)} ,$$

where $\bar{v}$ is the partial specific volume (0.75 ml/g for ADH), $\rho$ is the solution density, $s^0$ is the weight-average sedimentation coefficient, and

$$s_i^0 = \frac{(1-\bar{v}\rho)}{RT} D_i M_i$$

thus

$$s^0RT/D_z(1-\bar{v}\rho) = M_w \ .$$

## 3. Results and discussion

### 3.1. Aggregation and lyophilization

Samples prepared from Sigma Chemical Co.'s lyophilized enzyme exhibited a strong tendency to aggregate. The aggregation proceeded regardless of pH from 6.5 to 9.0 and ionic strengths from 0.01 to 0.1 M $K_xPO_4$. Addition of $10^{-4}$ M dithiotheritol did not inhibit the process. The use of activated charcoal to remove any dinucleotide did not affect the enzyme. Control samples prepared without charcoal treatment behaved in the same fashion.

At room temperature (22°C), the *initial* diffusion coefficient of lyophilized ADH was approximately $1.5 \times 10^{-7}$ cm$^2$/s, and decreased monotonically by 50% in the interval of several days. Visual inspection showed an accompanying opacity of the enzyme solution with a final falling out of a white precipitate. Even with refrigeration this aggregation process occurred, although at a much slower rate. Ultracentrifuge measurements at 4°C gave a sedimentation coefficient of 5.1 S with an approximate 25% loss of material. The fast sedimenting portion was estimated to have a sedimentation coefficient of 19 S. By contrast, the non-lyophilized enzyme after exhaustive dialysis had a diffusion coefficient of $D_{20,w} = 6.2 \times 10^{-7}$ cm$^2$/s and a sedimentation coefficient of $s^0_{20,w} = 5.1$ S. The diffusion coefficient was constant, even at room temperature, over a period of 24 hours. From the above data we obtained an initial weight-average molecular weight of 78 000 for the non-lyophilized enzyme and roughly $1.2 \times 10^6$ for the lyophilized preparations.

We examined the possibility that some contaminating protease present in the sample might be damaging the lyophilized enzyme during the dialysis. Smaller molecular weight material would be present as a result, and the nicks in the functional enzyme could be suitable sites for self-association. Examination of the sample by SDS gel electrophoresis showed several bands of smaller than 40 000 molecular weight polypeptide chains representing less than 5% of the total protein. However, there was no change in the amount of this material from the beginning of the preparation procedure to the time of making the physical measurements – typically five to six days. The presence of light material in ADH preparations has been previously noted by Dalziel [13].

### 3.2. Lyophilization and enzymatic activity

The enzymatic activity, as measured by the method of Dalziel [8], showed that during storage after dialysis, the number of active sites decreased much more rapidly in lyophilized ADH than in ADH which had not been previously lyophilized. The alcohol-precipitated enzyme maintained a fairly constant activity, kept at 4°C for several weeks in pH 7.4 buffer. In general, the lyophilized enzyme was less active than the alcohol-precipitated enzyme, whether or not it was treated with charcoal.

## 4. Conclusions

Bonnichsen [14], in his early work (1950) on purification and isolation of horse liver ADH cautioned, without further comment, that drying destroys the enzyme. Our data, especially the QLS studies, agree that the enzyme is altered by lyophilization. We have shown that a fundamental physical difference between the two kinds of preparations is the high molecular weight of the lyophilized protein: it is not 78 000 but much larger, with an initial molecular weight of about $1.2 \times 10^6$.

This tendency of lyophilized ADH to aggregate would not normally have been suspected in freshly prepared solutions, since fresh samples do not visually scatter an abnormal amount of light. Also, sedimentation velocity measurement by itself may not reveal the self-association problem, because the high molecular weight aggregates are sedimented down very rapidly. Since large particles dominate the intensity of scattered light, QLS enabled us to detect the large, associated ADH colloids. However, if one ingenuously combined the diffusion value from QLS with the *"slow"* (bulk) sedimentation coefficient in the Svedberg-Einstein equation, the resulting molecular weight would be about four times smaller than the molecular weight estimated from using the QLS diffusion coefficient of the lyophilized ADH with the "fast" (aggregate)

sedimentation value. Sedimentation velocity separated the aggregated species from the bulk enzyme (78 000) and most easily measured the latter, whereas QLS measured everything. In fact, the underestimated molecular weight has no meaning since it was the result of combining a measurement of a solution with both aggregates and "monomers" with a measurement of a solution containing only "monomers". Thus, it is unfeasible to use the Svedberg-Einstein equation for systems that aggregate to a high degree of polymerization.

The molecular weight of the aggregates may be estimated from a QLS measurement of the aggregated system along with the "fast" sedimentation value of the large particles keeping three reservations in mind: 1) The "fast" sedimentation coefficient is only an estimate. 2) The distribution of bulk versus aggregates are still different for the two physical methods. 3) No extrapolation to zero concentration of s is possible for the aggregates.

From the thermodynamic binding studies [5,6] it appears that the endothermic process responsible for self-association does not affect the interaction with $NAD^+$ or NADH. Our activity studies indicate that the aggregation reduces the total number of active sites. It is not clear whether the decrease in activity is due to denaturation or to masking of binding sites. At the present time, we do not understand why lyophilization alters the enzyme. The fact that the self-association is endothermic is consistent with a hydrophobic interaction. It is possible that a tightly bound contaminant is adsorbed during lyophilization, or that the drying causes an irreversible change in some portion of the tertiary structure. This change may expose some hydrophobic groups which act as nucleation sites for the intermolecular association. Clearly, what is important about our result with lyophilized ADH is that any physical measurement used to investigate the enzyme mechanism, which relies upon the molecular size, will be invalid.

The authors would like to thank Professor J.M. Schurr for his interest and the loan of his QLS apparatus. This research was partially supported with funds from NSF grant PCM 75-23631 and NIH grant GMI 13401.

## References

[1] H. Sund and H. Theorell, The enzymes, 2nd Ed., Vol. 7 (1963) p. 25.
[2] A. Ehrenberg and K. Dalziel, Acta. Chem. Scand. 12 (1958) 465.
[3] H. Jörnvall, Eur. J. Biochem. 16 (1970) 25.
[4] C.-I. Brändén, H. Jörnvall, H. Eklund and B. Furugren, The enzymes, 3rd Ed., Vol. 11 (1975) p. 103.
[5] F. Schmid, H.-J. Hinz and R. Jaenicke, FEBS 87 (1978) 80.
[6] S. Subramanian and P.D. Ross, Biochemistry 17 (1978) 2193.
[7] V.P. Fernandez, H.R. Mahler and V.J. Shiner, Biochemistry 1 (1962) 259.
[8] K. Dalziel, Acta Chem. Scand. 11 (1957) 13.
[9] U.K. Laemmli, Nature 227 (1970) 680.
[10] B.J. Berne and R. Pecora, Dynamic light scattering (John Wiley, Inc., New York, 1976).
[11] J.M. Schurr, CRC Critical Reviews in Biochemistry 4 (1977) 371.
[12] D.C. Teller, in: Methods in enzymology, Vol. 27D, eds. C.H.W. Hirs and S.N. Timasheff (Academic Press, New York, 1973) p. 346–440.
[13] K. Dalziel, Acta Chem. Scand. 12 (1958) 450.
[14] R.K. Bonnichsen, Acta Chem. Scand. 4 (1950) 715.