Structural properties of long- and short-chain alcohol dehydrogenases

Contribution of NAD⁺ to stability

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Structural studies were undertaken on long-chain and short-chain alcohol dehydrogenases (from horse liver and *Drosophila* respectively). Far-u.v. c.d. measurements were used to estimate the secondary structure contents of the enzymes. For the horse liver enzyme, the results agree well with the X-ray data; for the *Drosophila* enzyme (for which a crystal structure is not yet available), the results are in good agreement with those obtained by applying a range of structure-prediction procedures to the amino acid sequence of this enzyme. The conformational stabilities of the two enzymes were investigated by studying the unfolding brought about by guanidinium chloride (GdnHCl) by using activity and c.d. measurements. The unfolding of the *Drosophila* enzyme was analysed in terms of a two-state model; the presence of the substrate NAD⁺ leads to considerable protection against unfolding. By contrast, the unfolding of the horse liver enzyme shows a plateau effect at intermediate concentrations of GdnHCl, indicating that a two-state model is not appropriate in this case. NAD⁺ affords little, if any, protection against unfolding for the horse liver enzyme.

INTRODUCTION

Alcohol dehydrogenases (ADH; EC 1.1.1.1) are enzymes that occur widely across the range of living organisms, where they are important for the detoxification and metabolism of ethanol and other alcohols. They catalyse the oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones, with the concomitant reduction of NAD⁺. They can be grouped into two main families (Jörnvall *et al.*, 1981, 1984), which appear to be unrelated at the sequence level. Long-chain ADHs are characterized by a preference for primary alcohols (Dalziel & Dickinson, 1966), by a requirement for Zn²⁺ ions (Dunn & Hutchinson, 1973) and by hydride transfer with 4-*pro-R* stereospecificity (You, 1982). In contrast, short-chain ADHs prefer secondary alcohols (Sofer & Ursprung, 1968; Winberg *et al.*, 1982), have no requirement for metal cofactors (Chambers, 1984) and show 4-*pro-S* stereospecificity (Benner *et al.*, 1985). It is clear from these observations that the catalytic mechanisms of the two types of ADH must be different, although the overall reactions are the same.

Long-chain alcohol and polyol dehydrogenases have approx. 350 amino acid residues per subunit, and examples include the familiar mammalian liver ethanol dehydrogenase, as well as ADHs from birds, plants, yeast and bacteria. Mammalian sorbitol dehydrogenase is also a member of the long-chain ADH family (Jörnvall *et al.*, 1981, 1984). The short-chain ADHs have been found in mammals, insects and bacteria. These enzymes have approx. 250 amino acid residues per subunit, and examples include *Drosophila* ADH (Thatcher, 1980), bacterial ribitol, glutamate and 20 β -hydroxysteroid dehydrogenases (Jörnvall *et al.*, 1984; Marekov *et al.*, 1990), and mammalian 17-hydroxysteroid (Peltoketo *et al.*, 1988) and 15-hydroxy-prostaglandin (Krook *et al.*, 1990) dehydrogenases.

The three-dimensional structure of the long-chain ADH from horse liver is known in great detail from crystallographic and sequence studies (Eklund *et al.*, 1976; Jörnvall, 1970; reviewed in

Eklund & Brändén, 1987). It is reasonable to assume that the other members of the homologous long-chain ADH family have similar topologies. The short-chain family is as yet much less well characterized, although suitable crystals of *Drosophila* ADH have recently been obtained, and diffraction data are currently being collected (E. Gordon & L. Sawyer, personal communication). In the absence of a crystal structure, it is of interest to explore predicted structures derived from sequences and spectroscopic data. A secondary-structure prediction for ADH from *Drosophila melanogaster* (Thatcher & Sawyer, 1980) gives a strong suggestion that the *N*-terminal half of the enzyme folds into the alternating α -helix/ β -strand structure characteristic of nucleotide-binding domains [reviewed in Richardson (1981)]. The *C*-terminal half does not appear to have an alternating α / β structure.

Each subunit of the long-chain dehydrogenases (as exemplified by the horse liver enzyme) folds into two domains, with the active site at the interface between the domains [reviewed in Eklund & Brändén (1987)]. One molecule of NAD⁺ binds to this interdomain region and makes contacts with both domains. It thus may be expected that the cofactor could stabilize the enzyme to a variety of insults such as heat or denaturing agents. It is the main aim of the present study to compare the structural properties of long-chain and short-chain ADHs (such as those from horse liver and from *Drosophila*), and the contribution which NAD⁺ makes to the stability of the enzymes against unfolding by guanidinium chloride (GdnHCl).

EXPERIMENTAL

Materials

ADHs from horse liver and from yeast (*Saccharomyces cerevisiae*) were purchased from Boehringer and Sigma respectively. Before use, the crystalline suspension of liver ADH was dialysed for 18 h against 20 mM-Tris/HCl, pH 8.0. Yeast ADH was

resuspended in 20 mM-Tris/HCl, pH 8.0. Both solutions were centrifuged to remove any insoluble material.

ADH from *Drosophila* was purified using a modification of the method of Juan & Gonzalez-Duarte (1980) as described below. It was purified from frozen adult flies from a *Drosophila melanogaster* strain homozygous for the *AdhF* allele. All buffered solutions contained 1% (v/v) propan-2-ol and 0.1 mM-dithiothreitol to stabilize the enzyme. Flies (100 g) were blended with 250 mg of homogenization buffer (2 mM-Tris/HCl, pH 7.5). The slurry mixture was clarified by glass-wool filtration and a 30 min centrifugation at 10000 rev./min. The supernatant was first fractionated with salmine sulphate (0.28%, w/v) and secondly with $(\text{NH}_4)_2\text{SO}_4$. The protein that precipitated between 40% and 70% saturation of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 10× homogenization buffer and applied to a Sephadex G-25 column (2.5 cm × 50 cm). The fractions containing ADH activity were pooled and loaded on a Blue Sepharose column (2.5 cm × 20 cm) equilibrated with 10× homogenization buffer. After being washed with the same buffer, the enzyme was eluted with a pulse of NAD^+ (1 mM in 10× homogenization buffer). The fractions showing ADH activity were pooled, and protein was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation. It was redissolved in storage buffer (20 mM-Tris/HCl, pH 8.6) and applied to a Sephacryl S-200 column (2.5 cm × 100 cm) equilibrated with storage buffer. The peak fractions containing *Drosophila* ADH were pooled, and samples (10 µl) from each were used to assay activity and for SDS/PAGE analysis. Protein was kept at 4 °C in storage buffer which had been purged with nitrogen. The same method was used for the purification of the enzyme from *Drosophila lebanonensis*.

GdnHCl (AristaR grade) was purchased from BDH, Poole, Dorset, U.K. The concentrations of solutions were checked by refractive-index measurements (Nozaki, 1972). Other reagents were of the highest grade commercially available.

Enzyme assays

ADH activity was measured at 25 °C, by monitoring the production of NADH spectrophotometrically at 340 nm. The concentrations of ethanol and NAD^+ were 700 mM and 220 µM respectively in 50 mM-Tris/HCl, pH 8.8. The concentrations of ADH solutions were routinely determined by either spectrophotometric measurements at 280 nm by using published absorption coefficients (Juan & Gonzalez-Duarte, 1980; Fernandez *et al.*, 1962; Sund & Theorell, 1963) or Coomassie Blue binding (Sedmak & Grossberg, 1977). In all cases the validity of the method was checked by amino acid analysis, by using the published composition data for these enzymes.

Unfolding of ADH by GdnHCl

The inactivation of ADH by GdnHCl was measured after incubation of enzyme (typically 20 µg/ml) with GdnHCl in 20 mM-Tris/HCl, pH 8.0, for 15 min at 25 °C. After this time, a sample (100 µl) was taken for assay under the conditions noted above, but including the same concentration of GdnHCl as used in the original incubation mixture. In all cases assays were linear for at least 5 min; data from the first 1 min were used for subsequent analysis. The effect of NAD^+ on the unfolding was studied by adding NAD^+ (final concn. 220 µM) to the enzyme 15 min before the addition of GdnHCl. This mixture was then incubated for a further 15 min under the standard conditions before assay of a sample. Free-energy analysis of the data in terms of the two-state model was done as described by Pace (1986).

C.d. spectra

C.d. spectra of ADH were recorded at 21 °C with a JASCO

J-600 spectropolarimeter. Typically, enzyme concentrations were in the range 50–250 µg/ml, with cell path-lengths in the range 0.02–0.1 cm, and spectra were recorded over the range

Fig. 1. C.d. spectra of ADHs

Spectra were recorded at 21 °C as described in the Experimental section. Enzymes were dissolved in 20 mM-Tris/HCl buffer, pH 8.7. (a), (b) and (c) refer to the enzymes from *D. melanogaster*, *D. lebanonensis* and horse liver respectively.

Table 1. Secondary structures of ADHs

The amounts of secondary structures were estimated from c.d. spectra by the CONTIN procedure. The predicted values were obtained by using the multiple prediction procedure PREDICT (interpreted as shown in Fig. 2). The amounts of secondary structure for the horse enzyme from the crystallographic data are also included for comparison.

Enzyme	Method	α -Helix (%)	β -Strand (%)	Other structures (%)
<i>D. lebanonensis</i> ADH	C.d.	28 ± 1.0	41 ± 1.8	31 ± 1.7
<i>D. melanogaster</i> ADH	C.d.	29 ± 2.1	31 ± 2.6	40 ± 2.0
<i>D. melanogaster</i> ADH	Prediction	29.6	33.5	36.9
Horse liver ADH	C.d.	28 ± 1.3	35 ± 2.5	37 ± 2.2
Horse liver ADH	Prediction	30.8	32.5	36.7
Horse liver ADH	X-ray data	28	34	38

Fig. 2. Secondary structure prediction for *D. melanogaster* ADH

The histograms show the scores of the residues according to the prediction package PREDICT. The interpretation of those scores is shown in lines over the histograms. α -Helix, 29.6%; β -strand, 33.5%; other structures, 36.9%. The interpretation of the results of the prediction procedure was undertaken in the light of the known secondary-structural pattern in liver ADH and other dehydrogenases (Eklund & Brändén, 1987).

250–190 nm. Molar ellipticity values were calculated by using a value of 108 for the mean residue weight (Thatcher, 1980; Jörnval, 1970; Jörnval *et al.*, 1975). The secondary-structure contents were derived by the CONTIN procedure (Provencher & Glöckner, 1981).

Secondary-structure prediction

Prediction of the secondary structure of the *Drosophila* enzyme was made from the amino acid sequences by using a multiple structure-prediction program which combines several published algorithms (Garnier *et al.*, 1978; Lim, 1974; Chou & Fasman, 1974; Nagano, 1973; Burgess *et al.*, 1974; Dufton & Hider, 1977). In one of the methods within the program (Garnier *et al.*, 1978), data on the α -helix content from c.d. can be used to refine the prediction; however, inclusion of such estimates did not appreciably affect the outcome of the predictions.

RESULTS

Purification of *Drosophila* enzyme

The modifications to the previously published procedure (Juan & Gonzalez-Duarte, 1980) have resulted in a considerable (2-fold) increase in yield with a specific activity some 20% higher. These improvements probably result from the omission of several

time-consuming steps and more careful attention being paid to the need to prevent oxidative damage to the enzyme. The preparation was > 98% homogeneous on SDS/PAGE, with a subunit M_r (28000) consistent with the published data (Thatcher, 1980).

Secondary structure of *Drosophila* ADH

The far-u.v. c.d. spectra of *Drosophila* ADH are shown in Fig. 1. The secondary-structure contents (Table 1) were derived by the CONTIN procedure. From the published amino acid sequence of *D. melanogaster* ADH the secondary structure could be predicted as shown in Fig. 2; the percentages of α -helix and β -strand are also included in Table 1, and are seen to be in reasonable agreement with the analysis of the c.d. data.

Secondary structure of horse liver ADH

We have analysed the c.d. spectrum of the horse liver enzyme (Fig. 1) to obtain estimates of secondary structure. The results (Table 1) are seen to be in good agreement with those from crystal structure studies (Eklund & Brändén, 1987) summarized in Table 1, thus confirming the validity of the c.d. approach.

Unfolding of *Drosophila* ADH by GdnHCl

As shown in Fig. 3, the activity of *Drosophila* ADH was lost at relatively low concentrations of GdnHCl. Thus, on incubation with 0.4 M denaturing agent, 50% activity was lost, and essentially all activity had been destroyed at 0.7 M-GdnHCl.

When NAD^+ was included in the incubation mixtures, there was clear evidence for protection against this loss of activity (Fig. 3a). Thus, 50% of activity was retained at 0.6 M-GdnHCl; in the absence of NAD^+ only 10% of activity was retained. The concentration of NAD^+ used in all the protection experiments was 220 μM , a value corresponding to the K_m of NAD^+ for *Drosophila* ADH (Juan & Gonzalez-Duarte, 1981).

The shapes of the curves shown in Fig. 3(a) indicate that a two-state model may be appropriate for the inactivation process. Analysis of the data in terms of this model (Pace, 1986) indicates that the conformational stabilities of the folded states are 13.5 ± 0.73 kJ/mol (3.20 ± 0.17 kcal/mol) and 20.1 ± 0.84 kJ/mol (4.77 ± 0.20 kcal/mol) in the absence and presence of NAD^+ respectively (Fig. 3b), thus indicating an increase in $\Delta G_{(\text{H}_2\text{O})}$ value of the enzyme of about 6.61 kJ/mol (1.57 kcal/mol) when NAD^+ is present in the solution.

The structural changes in *Drosophila* ADH detected by far-u.v. c.d. are shown in Fig. 4(a). It is important to note that the changes in secondary structure occur at somewhat higher concentrations of GdnHCl than do the changes in activity (cf. Fig. 3). This is characteristic of many enzymes, and reflects the greater sensitivity of the active site to small structural perturbations caused by the denaturing agent (Creighton, 1978; Tsou, 1986). However, there is clear evidence for protection by NAD^+ against structural changes in the 0.25–0.5 M region of GdnHCl concentrations, consistent with the activity studies.

Unfolding of horse liver ADH by GdnHCl

In contrast with the situation observed with the *Drosophila* enzyme, it was found that the horse liver ADH retained substantial activity at moderate concentrations of GdnHCl. Thus, as shown in Fig. 5, 50% activity of the horse liver enzyme was retained at 1 M-GdnHCl. Again, in contrast with the *Drosophila* enzyme, the data appeared to show a plateau effect in the 0.5–1.0 M-GdnHCl range, suggesting that a two-state model is inadequate to describe the inactivation process. There is very little protection afforded by NAD^+ at any stage in this inactivation process (Fig. 5).

The changes in secondary structure of the horse liver ADH

Fig. 3. Inactivation of *D. melanogaster* ADH by GdnHCl

Details of the procedures are given in the Experimental section. (a) Primary data and (b) analysis in terms of the two-state model as described by Pace (1986). ●, Enzyme in the presence of NAD^+ ; ○, enzyme in the absence of NAD^+ .

Fig. 4. Structural changes in ADH upon addition of GdnHCl as demonstrated by c.d.

(a) *D. melanogaster*; (b) horse liver (—) and yeast (----).
○, Without NAD⁺; ●, with NAD⁺.

Fig. 5. Inactivation of horse liver (—) and yeast (----) ADH by GdnHCl

Details of the procedures are given in the Experimental section.
●, Enzyme in the presence of NAD⁺; ○, enzyme in the absence of NAD⁺.

are depicted in Fig. 4(b). As with the *Drosophila* enzyme, these changes occur at higher GdnHCl concentrations than do the changes in activity. There is no significant effect of NAD⁺ on the structural changes, consistent with the data from the activity studies. Similar protection experiments with yeast ADH also showed that NAD⁺ provides little protection to this enzyme (results not shown).

DISCUSSION

In this paper we report on the analysis of the secondary structure of the short-chain ADH from *Drosophila*. The far-u.v. c.d. spectrum provides estimates for α -helix and β -sheet which are in good agreement with results of the structure-prediction methods, and the validity of the c.d. approach has been confirmed with reference to the horse liver enzyme, where a crystal structure is available. Previously Benyajati *et al.* (1981) published an amino acid sequence (derived from cDNA sequence) for the *Drosophila* enzyme. Apart from residues 25 and 251, their sequence was identical with that published previously by Thatcher (1980), which was determined by direct protein sequencing. However, Benyajati *et al.* (1981) reported the result of a structure prediction using the Chou-Fasman method as suggesting that the enzyme contained 50% α -helix, a value considerably larger than that reported by Thatcher & Sawyer (1980) using this predictive method. The cause of this discrepancy is not clear, but it should be noted that the results of our multiple prediction studies are closer to the value obtained by Thatcher & Sawyer (1980). Benyajati *et al.* (1981) also mentioned that analysis of the c.d. spectrum gave a value of 50% α -helix, in accordance with their structure-prediction results. We are unable to explain the discrepancy between their value and our values (Table 1), because they did not provide the primary data on which the analysis was based.

At present, no crystal-structure information is available for the *Drosophila* enzyme, although preliminary results are encouraging (E. Gordon & L. Sawyer, personal communication). We are therefore at present unable to confirm the validity of the c.d. analysis. The analysis suggests that the enzyme contains significant amounts of both α -helix and β -strand structures. The presence of large amounts of these secondary structures would indicate that a typical NAD⁺-binding site exists in this enzyme; this proposal would be consistent with other data, including the binding of the enzyme to Cibacron Blue (Juan & Gonzalez-Duarte, 1980).

The conformational stability ($\Delta G_{(H_2O)}$) of the *Drosophila* enzyme has been estimated by a linear extrapolation procedure as shown in Fig. 3(b). Although the justification for using this procedure has been the subject of considerable debate [see Pace (1986) and references therein], the value obtained [13.5 kJ/mol (3.20 kcal/mol)] can at least be used as a basis for discussion. It lies at the lower end of the range of values [21–63 kJ/mol (5–15 kcal/mol)] quoted by Pace (1990) for the stabilities of a number of globular proteins. However, it should be noted that in any particular case the exact value can be markedly affected by factors such as temperature, pH and ionic strength, and no attempt was made in the present study to optimize the stability of the enzyme.

The inclusion of NAD⁺ (220 μ M) serves to increase the conformational stability of the *Drosophila* enzyme by 6.61 kJ/mol (1.57 kcal/mol) (Fig. 3b). Although this value may seem low when the number of likely interactions between the enzyme and the substrate is considered, it is of a similar magnitude to other examples of ligand-induced stabilization, e.g. the stabilization of 2.1 kJ/mol (0.5 kcal/mol) afforded to lysozyme by inclusion of the competitive inhibitor (*N*-acetylglucosamine)₃

(Pace & McGrath, 1980). Such data are, in any case, difficult to interpret in detail, because the addition of denaturants perturbs the ligand-binding equilibrium which is coupled to the equilibrium between folded and unfolded states (Shrake & Ross, 1990).

From our data, it appears that the long-chain ADH (horse liver) is more stable to GdnHCl than is the short-chain ADH (*Drosophila*). Moreover, it appears that only the latter enzyme can be stabilized by addition of NAD⁺. The precise structural reasons for this contrasting behaviour await a detailed structure of the *Drosophila* enzyme, but presumably are a consequence of different domain and subunit organizations in the two dimeric enzymes. It can be noted that in preliminary experiments with the tetrameric long-chain enzyme from yeast we have found that NAD⁺ offers no protection against inactivation or unfolding by GdnHCl (Fig. 4b and 5), i.e. a similar result to the horse liver (long-chain) enzyme. For the yeast enzyme, inactivation occurs at lower concentrations of GdnHCl (Fig. 5), consistent with earlier reports (Brändén *et al.*, 1975) which indicate that the yeast enzyme is less stable than the horse liver enzyme towards thermal inactivation. The greater instability of the yeast enzyme is thought to be related to a greater ease of dissociation into monomers. Previous observations on the effect of NAD⁺ on the stability of the yeast and horse liver enzymes have been somewhat contradictory. Thus Sekuzu *et al.* (1957) reported that NAD⁺ (> 1 mM) protected yeast ADH against inactivation by urea, but Wiseman & Williams (1971) found that NAD⁺ at these high concentrations had a destabilizing effect on the enzyme against thermal inactivation.

For the horse liver enzyme, there appears to be at least one intermediate in the unfolding process, as indicated by the plateau region of Fig. 5 (cf. Strambini & Gonelli, 1990). In the plateau region (0.5 M-GdnHCl), there is a 30% loss of activity and a 10% loss of secondary structure (as judged by the ellipticity at 225 nm). The nature of this (these) intermediate(s), including its (their) quaternary structure, requires further investigation.

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