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The coenzyme-binding characteristics of highly purified preparations of sheep liver cytoplasmic aldehyde dehydrogenase

Graham J. HART* and F. Mark DICKINSON
Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

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The binding of NADH and NAD^+ by cytoplasmic aldehyde dehydrogenase was studied by various direct and indirect methods. At pH 7.0 at 25°C there appears to be approx. 1 binding site for both nucleotides per 200000 daltons of protein, although the NAD^+-binding results are rather uncertain. Estimates of the dissociation constants of the E·NADH and E·NAD^+ complexes under the stated conditions are also presented. Preparations of enzyme are sometimes found to contain significant amounts of very tightly bound NAD^+ and NADH. The implications of these findings are discussed.

Aldehyde dehydrogenases from the livers of several mammalian species, sheep (MacGibbon *et al.*, 1979), rabbit (Duncan, 1977), horse (Feldman & Weiner, 1972) and human (Pietruszko *et al.*, 1977), have been investigated over recent years, and all have been shown to be tetrameric enzymes composed of apparently identical subunits. Kinetic properties have been studied in some detail (see Hart & Dickinson, 1982, and cited references therein), but few studies of the binding of coenzyme to the enzyme have been made. Takahashi & Weiner (1980) and Takahashi *et al.* (1980) have found that the pI5 (mitochondrial) isoenzyme from horse liver has only 2 coenzyme-binding sites/tetramer in the absence of Mg^{2+}, but that in the presence of Mg^{2+} the enzyme dissociates into dimers and displays 2 binding sites/dimer. The change is also accompanied by a 2-fold increase in activity. The sheep liver cytoplasmic enzyme is not affected in this way. The enzyme maintains its tetrameric structure, the NADH-binding capacity is unchanged and the enzyme is severely inhibited by Mg^{2+} (Dickinson & Hart, 1982). The inhibition appears to be due to formation of an E·NADH·Mg^{2+} complex from which NADH dissociates but slowly. Vallari & Pietruszko (1981) found that the E_1 isoenzyme from human liver gives a burst of NADH production in the pre-steady state equivalent to 4 mol of NADH/mol of enzyme. This enzyme is said to be 'not titratable by conventional methods', however. For the sheep cytoplasmic enzyme studies of the effect of disulfiram indicate a maximum of 2.0 active sites/tetramer (Dickinson *et al.*, 1981). Catalytic studies in the stopped-flow apparatus with saturating substrates gave only a burst of approx. 0.7 mol of NADH/mol of enzyme (Hart & Dickinson, 1982). The binding of NADH to the sheep liver cytoplasmic enzyme has been studied by MacGibbon *et al.* (1979). They do not explicitly state the NADH-binding capacity of their enzyme preparations, but it appears from their Fig. 3 that approx. 1 μM-enzyme bound 1.5 μM-NADH with a dissociation constant of 1.1 μM at 25°C. It appears from the above that the NADH-binding capacity of the enzyme is less than one might expect for a tetrameric enzyme composed of very similar or identical subunits. Our purpose in the present work was to study the binding of both NAD^+ and NADH by various methods in an effort to characterize our preparations in this respect.

* Present address: University of Cambridge Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Experimental

Materials

NADH (grade I) and NAD^+ (grade II) were from Boehringer Corp., London W.5, U.K. NAD^+ was further purified before use by chromatography on DEAE-cellulose by the method of Dalziel & Dickinson (1966). Other chemicals were analytical-reagent grade whereever available, obtained from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Aldehydes were redistilled before use.

Cytoplasmic aldehyde dehydrogenase, free from the corresponding mitochondrial enzyme, was prepared as described by Dickinson *et al.* (1981) and was assayed by the method of Hart & Dickinson (1977). Protein concentrations were calculated by using $A^{1\%}_{1cm} = 11.3$ at 280nm, determined from dry-weight measurements and an M_r value of

200000. For most preparations $A_{280}/A_{260} = 1.69$. Before use, enzyme solutions were dialysed against sodium phosphate buffer, pH 7.0 and I 0.1, containing 100 μM-dithiothreitol.

Methods

Glass-distilled water was used throughout.

Determination of coenzyme and substrate concentrations. NAD^+ and NADH were assayed enzymically with yeast alcohol dehydrogenase by the method of Dalziel (1962*a*, 1963) and by using $\varepsilon = 6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ for NADH at 340nm (Horecker & Kornberg, 1948). Aldehyde concentrations were checked by monitoring the loss of NADH at 340nm by using yeast alcohol dehydrogenase and an excess of NADH in 0.2M-sodium phosphate buffer, pH 7.0.

Steady-state kinetics. Aldehyde dehydrogenase activity was determined at 25°C in sodium phosphate buffer, pH 7.0 and I 0.1, by using a recording filter fluorimeter of the type described by Dalziel (1962*b*). The solutions of purified NAD^+ were adjusted to pH 7.0 before use, and an allowance was then made for the phosphate concentration when making up assay mixtures. Assays were performed at least in duplicate. Measured rates generally agreed to within 5%. The specific activity of enzyme preparations can be variable, especially, it seems, at certain times of the year. The experiments described in the present paper were obtained with enzyme of specific activity 140–180 units (μmol/min)/mg, which is the best that we can achieve. All binding experiments were conducted with enzyme preparations containing very little or no bound nucleotide (see below). Our enzyme purification procedure (Dickinson *et al.*, 1981) is based on that of Crow *et al.* (1974) and is therefore very similar to that of MacGibbon *et al.* (1979). The modifications that we use are an early protamine sulphate step, which in our hands improves the $(NH_4)_2SO_4$ fractionation, and an extra DEAE-cellulose column step, which involves a pH-gradient elution to remove traces of mitochondrial enzyme. It is not possible to compare the specific activities of the enzyme obtained by us and by MacGibbon *et al.* (1979), partly because of quoted differences in assay procedures, but mostly because they do not record the temperature used for assays. However, it seems unlikely from the above considerations that our enzyme preparations will be inferior to those of MacGibbon *et al.* (1979).

Preparation of anti-enzyme serum. Over a 3-week period a rabbit was given three injections containing a total of 2mg of enzyme previously homogenized with Freund's adjuvant. The injections were made subcutaneously at four sites on the back. Starting at the fourth week, 1mg of enzyme per week was injected intravenously into the ear. The rabbit was bled out at the seventh week, when antibody concentrations were high. Double-diffusion plates were run in 2% agarose in the cold. Precipitin lines developed overnight.

NAD^+-binding studies. The binding of NAD^+ to the enzyme was investigated by using equilibrium dialysis. Mixtures of enzyme and NAD (0.2ml) were made, and were dialysed for 110min against 3ml of 50mM-sodium phosphate buffer, pH 7.0, initially containing approximately the same concentration of NAD^+ as present inside the dialysis sac. The NAD^+ concentrations used were in the range 3–120 μM. To avoid the likely problem of enzyme-catalysed reduction of NAD^+ due to the small amounts of aldehydes that are unavoidably present even in freshly distilled water, an NAD^+-regenerating system was incorporated within the sacs. The system used was sodium pyruvate (1mM) and lactate dehydrogenase (2% of the total protein, w/w). Control experiments showed that this was an efficient NADH-oxidizing system with very low concentrations (0.5 μM) of NADH. A small length (approx. 4cm) of glass rod was also placed in each sac so that the liquid inside the sac formed a thin film, thus decreasing the time required for equilibration, which control experiments showed to be 90–100min. The capped tubes containing the dialysis sacs and the 3ml of buffer/NAD^+ were attached to the spokes of a rotating wheel (approx. 15rev./min) so that the mixtures were inverted, and the contents mixed, once in every rotation. The dialyses were performed at room temperature (21–23°C).

At equilibrium, the dialysis sacs were removed, and the enzyme within them was assayed as a measure of any volume change that may have occurred during the dialysis. Such assays showed volume increases to be less than 10%. In separate experiments aldehyde dehydrogenase was shown to be completely stable at room temperature for 110min under the conditions used (enzyme concentrations 5–11.5mg/ml). Corrections were made to the protein concentrations on the basis of the activity-indicated volume changes. Portions (0.1ml) of the enzyme/NAD^+ mixtures were treated as follows. First 0.1ml of 5% (w/v) trichloroacetic acid was added, and the precipitated protein was removed by centrifugation. The NAD^+ solution was then neutralized by the addition of 0.3ml of 0.2M-sodium phosphate buffer, pH 7.4. These deproteinized NAD^+ solutions from inside the dialysis sacs, and the untreated solutions from outside, were then assayed fluorimetrically for NAD^+ by using a filter fluorimeter. NAD^+ was converted into NADH by ethanol and yeast alcohol dehydrogenase in an assay medium containing 0.5M-glycine/NaOH buffer, pH 10.0, ethanol (0.84M) and yeast alcohol dehydrogenase (17.5nM) in a volume of 4ml. The amount of NAD^+ solution added (15–200 μl) was such that approximately the same increase in

fluorescence was observed in each assay, and assays were made in duplicate.

Corrections were made for the apparent small losses of NAD^+ that occurred during deproteinization, presumably due to entrapping of NAD^+ within the protein pellet, by making mixtures of bovine serum albumin [with the same concentration (w/v) as the aldehyde dehydrogenase used in the binding experiments] and NAD^+, and carrying these through the deproteinization procedure described above. The recovery of NAD^+ from such experiments was 88.4% (mean for three separate experiments, S.D. = 2.6), and all NAD^+ concentrations after deproteinization were corrected by using this factor.

NADH-binding experiments. The binding of NADH to aldehyde dehydrogenase was investigated both by the gel-filtration technique of Hummel & Dreyer (1962) and by fluorescence titrations. For the Hummel & Dreyer (1962) method a thermostatically controlled column (22cm × 1.8cm) of Sephadex G-25 was used, equilibrated with 50mM-phosphate buffer, pH7.0, containing NADH at the required concentration. A 0.9ml enzyme sample (8–12mg) containing NADH at the same concentration was applied to the column, and elution was then continued with the original buffer. The NADH contents of eluted samples were measured spectrophotometrically at 340nm within a few minutes of their being obtained. Fluorimetric titrations were performed at approx. 20°C with the Farrand Mk. I spectrofluorimeter equipped with micro-cell attachment. Excitation was at 350nm and emission at 430nm, and 10nm slits were used. Solutions (0.2ml) were prepared containing increasing concentrations (0–50μM) of NADH in the absence or in the presence (2.0μM) of enzyme, and their fluorescences were determined.

Results and discussion

Purity of the enzyme preparations

Immunodiffusion plates of enzyme against anti-enzyme serum showed only one precipitin line (Fig. 1), suggesting that the enzyme is of high purity. As the test is independent of any of the fractionation methods used in the isolation procedure, it is valuable.

Isoelectric focusing on polyacrylamide-gel plates gave results that were very similar to those obtained by Agnew *et al.* (1981). Protein and activity staining gave no evidence for the presence of inactive protein or for the presence of the mitochondrial enzyme. The latter result is expected, since our procedure incorporates a pH-column step designed to remove this contaminant (Dickinson *et al.*, 1981). The cytoplasmic enzyme focused sharply, and it appeared that the band was actually composed of two or perhaps three close-running bands. Agnew *et al.*

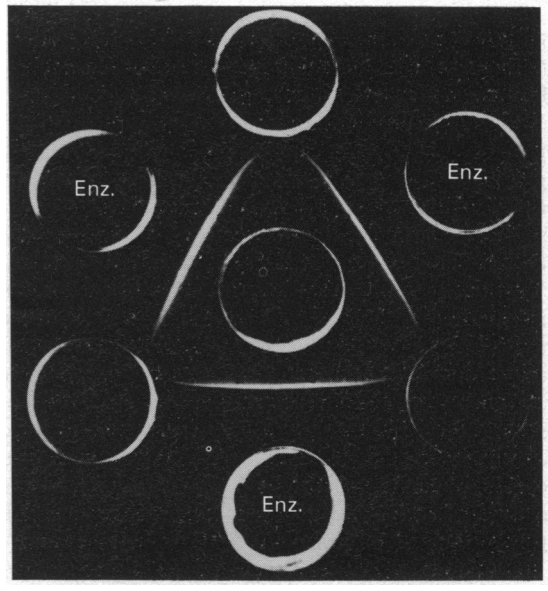

Fig. 1. *Enzyme–antibody reaction on a double-diffusion plate*

The antiserum was placed in the centre well and highly purified sheep liver cytoplasmic aldehyde dehydrogenase in the three alternate outer wells that are marked.

(1981) found that their preparations showed two bands very close together. The bands are so close together and their pI values so very similar that there appears to be no immediate prospect of separating them. Isoelectric-focusing experiments were also conducted on centrifuged extracts of a fresh liver homogenate made in iso-osmotic sucrose. A major band of activity was found at the same point on the gels as purified enzyme, leading us to suppose that the isolated enzyme had not suffered substantial modification. Electrophoresis of purified enzyme in 0.1% sodium dodecyl sulphate on 14cm × 12cm slab gels (10% acrylamide) in the buffer systems described by Laemmli (1970) suggests that modification of the enzyme by partial proteolysis has not occurred. Loading of 5–50μg of protein showed only one band present (M_r approx. 50000; see also MacGibbon *et al.*, 1979), with no trace of any minor bands of either higher or lower M_r values.

Greenfield & Pietruszko (1977) successfully used an AMP–Sepharose 4B affinity column as a step in the purification of human liver aldehyde dehydrogenase. We do not use such a step in our procedure, but we have applied preparations of enzyme (purified up to the Sephacryl column stage; see Dickinson *et al.*, 1981) to an AMP–Sepharose 4B column under the conditions used by Greenfield & Pietruszko (1977). The enzyme was adsorbed and eluted in high yield, but with little (15%) or no increase in

purity on different occasions. Insofar as this is an affinity-chromatography step, it provides evidence for the purity of the preparation.

Attempts were made to protect the enzyme from possible attack by cellular proteinases by including phenylmethanesulphonyl fluoride (200mg/ml) in buffers used in the preparation. This was unsuccessful, because the enzyme progressively lost activity; the enzyme is inactivated by the reagent. When a 4mg/ml solution of purified enzyme was mixed with 1mM-phenylmethanesulphonyl fluoride at pH7.0 at 0°C, the enzyme lost 75% of the initial activity within 1h and was then stable for a further 3h. Addition of a further 1mM-phenylmethanesulphonyl fluoride served to inactivate the enzyme completely in the next hour. It is to be noted that the enzyme was quite unaffected by the small volumes of ethanol that were added as solvent with the phenylmethanesulphonyl fluoride.

The sensitivity of our enzyme to phenylmethanesulphonyl fluoride is noteworthy because this reagent has been used very successfully to protect yeast aldehyde dehydrogenase from proteolysis during purification (Bostian & Betts, 1978; Clark & Jakoby, 1970*a*,*b*). The fact that the yeast enzyme is activated by univalent cations (Steinman & Jakoby, 1967) whereas the liver cytoplasmic enzyme is not, although it is strongly inhibited by bivalent cations (Dickinson & Hart, 1982), also points up to the clear difference between these enzymes.

NADH binding

Fig. 2 shows a fluorescence titration of 2.0μM-aldehyde dehydrogenase with NADH at pH7.0 at 25°C. It is clear that saturation was achieved with 25–30μM-NADH. In a similar experiment in which 2.15μM-NADH was added to increasing concentrations (2–35μM) of enzyme, a maximum increase in fluorescence on binding of 0.21×10^{-6} *A* unit was noted. Thus 1μM-enzyme·NADH complex had a fluorescence of 0.098×10^{-6} *A* unit under the conditions of Fig. 2 and, assuming that the intrinsic fluorescence of the complex is independent of saturation, the enzyme exhibited 0.96 NADH-binding sites/molecule. By using the observed fluorescences of the enzyme·NADH complex and free NADH, the points on the titration curve of Fig. 2 were used to estimate the dissociation constant of the enzyme·NADH complex. There was variability in the estimates, but values in the range $2.5 \pm 0.8\mu$M were obtained. Similar titrations have been performed on several different preparations of enzyme with similar results. Apart from one isolated example, where a value of 1.7 binding sites/molecule was obtained, values have fallen in the range 0.9–1.3 binding site/molecule, with estimates for the dissociation constant in the range $3 \pm 1.5\mu$M. The values seem to be similar to those obtained by

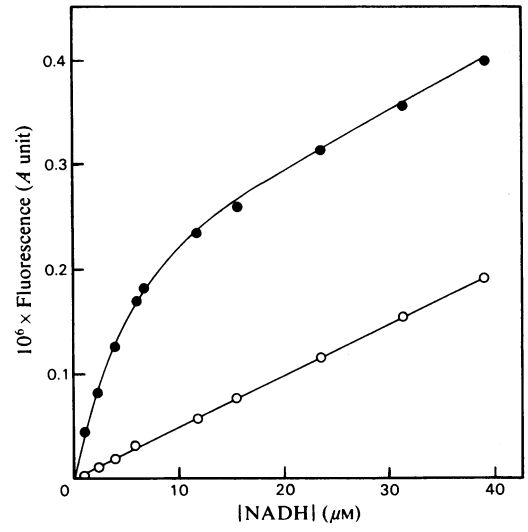

Fig. 2. *Fluorescence titration of aldehyde dehydrogenase with NADH*

Individual mixtures with (●) or without (○) enzyme were prepared in 50mM-phosphate buffer, pH7.0, and their fluorescences determined as described in the text. The enzyme concentration used was 2μM.

MacGibbon *et al.* (1979). The variability in the estimates for the NADH-binding sites is mainly due to variations in estimates of the specific fluorescence of the E·NADH complex. Although the work described in this section on coenzyme binding was done with enzyme preparations that exhibit very little fluorescence at 430nm (indicating very little bound NADH), other preparations made by the same procedure have contained tightly bound nucleotide (see below). It seems likely that even preparations with very low fluorescence may still contain a small concentration of tight-binding sites, and it is likely that these would cause problems in the estimation of the fluorescence and dissociation constant of the enzyme·NADH complex. In this connection it may be noted that, although fluorescence titrations with some enzyme preparations were apparently normal (see, for example, MacGibbon *et al.*, 1979), others yielded anomalous plots on analysis with the method of Stinson & Holbrook (1973). The plots were markedly concave towards the $1/(1-R)$ axis and, more importantly, the extrapolation intersected the $1/(1-R)$ axis and not the $[NADH]/R$ axis (R is the fractional saturation). The second characteristic of the plots was not modified by selecting different values for fluorescence at full saturation. Whether this behaviour is due to different types of NADH-binding site as indicated above is yet to be determined.

Aldehyde dehydrogenases from various sources have M_r values about 200000 and are composed of four similar or identical subunits. Sheep liver cytoplasmic aldehyde dehydrogenase also exhibits these characteristics (MacGibbon *et al.*, 1979), and it is surprising to obtain estimates for numbers of NADH-binding sites that fall very far short of 4/molecule. In order to check the estimates and to avoid errors arising from assumptions concerning the fluorescence of the enzyme·NADH complex (see above), we have used the column method of Hummel & Dreyer (1962). The column of Sephadex G-25 was equilibrated at pH 7.0 at 25°C in 0.1 M-phosphate buffer containing 186 μM-NADH. Under these conditions 82 nmol of enzyme bound 122 nmol of NADH when the estimates were based on the peak in A_{340} of the elution profile and when correction was made for absorbance contributed by the enzyme alone. According to Fig. 2, 186 μM-NADH would be saturating, so that the result indicated 1.5 NADH-binding sites/molecule of enzyme. We have assumed in the calculation that for the enzyme·NADH complex $\varepsilon_{340} = 5.5 \times 10^{-3}\,M^{-1} \cdot cm^{-1}$ (Agnew *et al.*, 1981).

The column experiments were repeated on several occasions with different preparations of enzyme with broadly similar results to those described above. Thus we have observed values in the range 0.9–1.5 NADH-binding sites/molecule in the presence of 200 μM-NADH. Estimates of binding were usually higher when based on the trough of elution profiles, and here we have values in the range 1.2–1.8 binding sites/molecule. We conclude that the larger values found in the troughs are due to greater experimental errors. Certainly the trough region is more diffuse and the changes in absorbance are much smaller than in the peak region. Small errors in base-line will have a greater effect on calculations based on these fractions.

A few column experiments were conducted as above but with NADH concentrations in the range 10–200 μM. The results were few and there was some scatter, but there seemed to be only small changes in the amount of NADH bound at the lower NADH concentrations. The results were not detailed or accurate enough to provide an estimate of the dissociation constant of the enzyme·NADH complex. They did, however, serve to show that 200 μM-NADH is effectively saturating and were consistent with a dissociation constant in the range 2–10 μM.

To obtain an independent estimate of the dissociation constant of the enzyme·NADH complex, we used NADH as a product inhibitor of the aldehyde-oxidation reaction. Rather limited experiments were conducted by varying the substrate concentration with or without inhibitor (Fig. 3) or with a fixed substrate concentration and varying the concentrations of inhibitor (not shown). The experiments were limited because of the nature of the assay. In working to very low NAD^+ concentrations a sensitive fluorimetric method for detecting NADH is required. When NADH is then added as a product inhibitor the very low rates are measured against a very high background fluorescence. This severely restricts the range of NADH concentrations that can be used. Fig. 3 shows that the inhibition is competitive, and calculations based on the change in slope indicate a value of $K_{E\cdot NADH} = 4.9\,\mu M$ at pH 7.0 at 25°C. The Dixon plot of data from the alternative experiment gave $K_{E\cdot NADH} = 5\,\mu M$. MacGibbon *et al.* (1977*a*) also used product-inhibition data, observing competitive behaviour of NADH towards NAD^+ but obtaining a value of $K_{E\cdot NADH} = 1.2\,\mu M$ at pH 7.6.

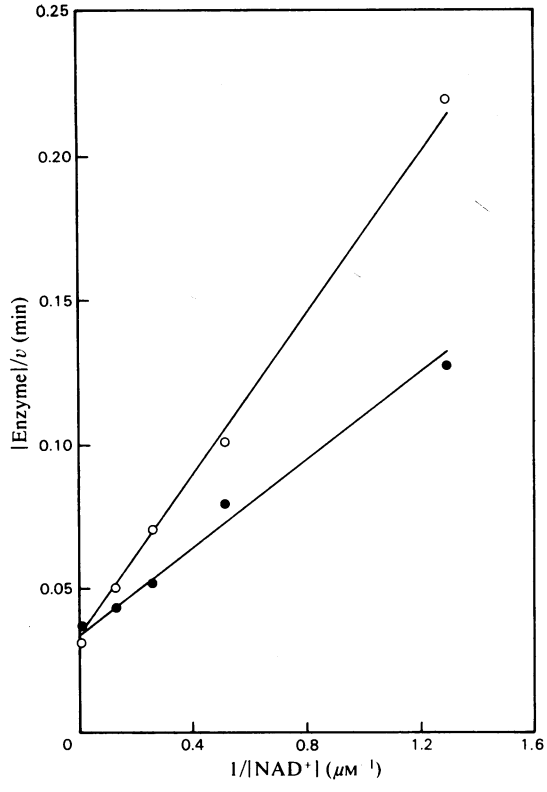

Fig. 3. *Product-inhibition studies of aldehyde dehydrogenase with NADH*

Double-reciprocal plots are shown for propionaldehdye oxidation in the presence (○) and in the absence (●) of NADH (4 μM). The aldehyde concentration was 50 μM, and the experiments were performed in 50 mM-phosphate buffer, pH 7.0, at 25°C.

NAD^+ binding

The extent of NAD^+ binding by the enzyme at various concentrations of NAD^+ was examined by equilibrium dialysis. The results were obtained with several enzyme preparations and are recorded in Fig. 4, where a large scatter is evident. The main problem with the measurements is that in proportion to protein the preparations bind only small quantities of NAD^+. There are also other difficulties. The limited amount of enzyme available dictated the use of small samples (0.2ml) for dialysis. The manipulations involved were awkward to perform and are likely to involve substantial error. The results are certainly not good enough to characterize NAD^+ binding to the enzyme. Nevertheless it does seem significant that even at high NAD^+ concentrations the observed saturations never exceeded 1.1 (i.e. 1.1 binding sites/molecule) and that these values are similar to those obtained for NADH binding. If it is assumed that the binding of NAD^+ is hyperbolic, then inspection of the data of Fig. 4 suggests a maximum of 1.2–1.5 sites/molecule with a nominal value for $K_{E\cdot NAD^+}$ of about 7.0μM. Clearly, there are likely to be substantial errors associated with either or both of these estimates. In support of the assumption, it may be noted that detailed kinetic studies (MacGibbon *et al.*, 1977*a*; Hart & Dickinson, 1982) gave rise to linear Lineweaver–Burk plots with NAD^+ as the variable substrate. A further point concerning Fig. 4 suggests that inactivation of the enzyme with disulfiram does not materially alter the NAD^+-binding characteristics of the enzyme. This supports earlier observations (Dickinson *et al.*, 1981) made by using fluorescence titrations, where both NAD^+ and NADH binding were seen to be largely unaffected by disulfiram inactivation.

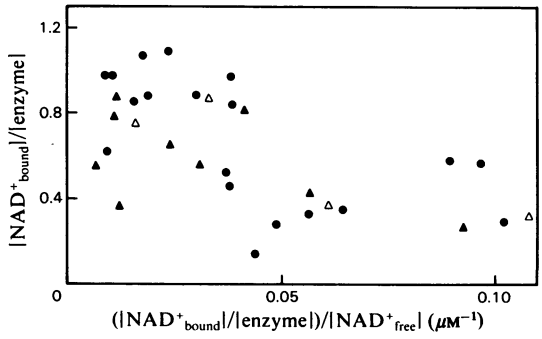

Fig. 4. *NAD^+ binding to aldehyde dehydrogenase in equilibrium-dialysis experiments*

Scatchard plots are shown for the binding of NAD^+ to native enzyme (●) and to enzyme treated with 0.7mol of disulfiram/mol of enzyme (▲) or with 1.0mol of disulfiram/mol of enzyme (△).

In an effort to obtain another estimate of the dissociation constant of the E·NAD^+ complex, NAD^+ was used to compete with NADH in fluorescence titrations such as those shown in Fig. 2. The assumption that NAD^+ and NADH bind to the same sites is justified by Fig. 3. In these circumstances it is expected that:

$$K_{E\cdot NADH,app.} = K_{E\cdot NADH}\left(1+\frac{[NAD^+]}{K_{E\cdot NAD^+}}\right)$$

Our data appear in Fig. 5 and seem to satisfy the relationship. A value of $K_{E\cdot NAD^+} = 19\mu M$ at pH7.0 at 25°C was obtained. Similar values have been obtained by other methods. Kinetic measurements indicated 21μM at pH7.0 at 25°C (Hart & Dickinson, 1982) and 8μM at pH7.6 at 25°C (MacGibbon *et al.*, 1977*a*). Stopped-flow studies of NAD^+ binding and dissociation gave 11μM (MacGibbon *et al.*, 1977*b*), and activation of the esterase activity of the enzyme by NAD^+ required 7μM-NAD^+ for half-maximal effect (MacGibbon *et al.*, 1978).

Presence of bound nucleotide in some enzyme preparations

Systematic examination of the properties of many preparations of highly purified sheep liver cytoplasmic aldehyde dehydrogenase has shown that some preparations exhibit fluorescence characteristics of a type indicating the presence of bound

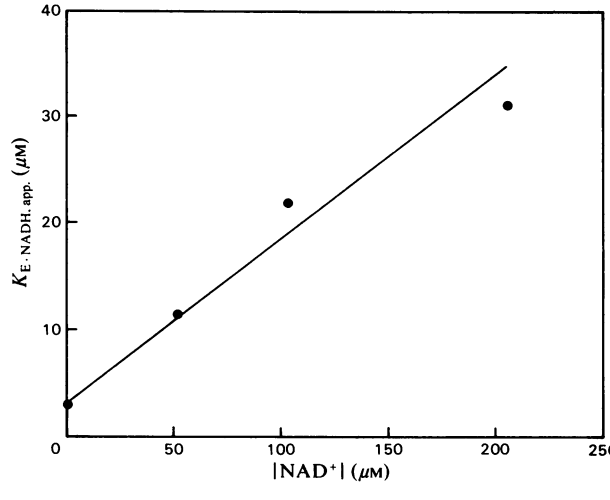

Fig. 5. *Competition of NAD^+ and NADH in fluorescence titrations of aldehyde dehydrogenase*

The titrations were performed as described for Fig. 2 except that in some cases NAD^+ was added at the stated concentrations. The apparent dissociation constants for the enzyme–NADH complex, $K_{E\cdot NADH,app.}$, were determined as described in the text.

NAD(P)H. Emission spectra recorded for a number of enzyme preparations are shown in Fig. 6, and it is clear that the spectra are not of equal amplitude and that the shapes of the spectra are not always the same. The cause of this variability is not known, but all of these samples were made by our usual procedure.

At this stage one supposes that the variability in fluorescence of the preparations indicates the presence of an impurity. It is, however, remarkable that the preparations contain bound nucleotide, because the purification procedure is lengthy, involving several precipitations by $(NH_4)_2SO_4$, several lengthy dialyses and three column fractionation steps. One would certainly expect any freely dissociable NAD(P)H (with $K_{E\cdot NADH} = 2–5\,\mu M$; see above) to be lost in these procedures, and it is evident therefore that the nucleotide is very firmly bound. This view was confirmed by passage of 0.8ml of a 10mg/ml enzyme solution through a 1cm × 10cm column of gel-filtration medium (Bio-Gel P-6) or by dialysis of a similar sample versus 3 × 1000ml of 50mM-phosphate buffer, pH7.0, for 3 days. Neither treatment resulted in any significant change in fluorescence of the enzyme sample.

When samples of enzyme dissolved in 50mM-phosphate buffer, pH7.4, were boiled for 2min, the protein was of course precipitated, and the fluorescence of the supernatant was much diminished. In uncorrected measurements the excitation maximum of the supernatant remained at 350nm, but the emission maximum was shifted to 460nm. These characteristics are those shown by NAD(P)H in free solution. Enzymic assay with yeast alcohol dehydrogenase and excess of acetaldehyde at pH6.0 showed that the released nucleotide was NADH and not NADPH. Further analysis of the results showed that preparations of enzyme showing fluorescence readings at 425nm of 0.08×10^{-6} and 0.27×10^{-6} *A* unit for 1mg/ml solutions under the conditions of Fig. 2 released 0.1 and 0.3mol of NADH/mol of enzyme respectively.

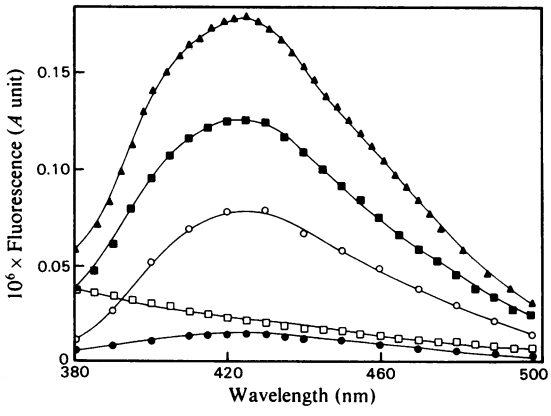

Fig. 6. *Fluorescence spectra of various preparations of aldehyde dehydrogenase and of native and acid-$(NH_4)_2SO_4$-treated aldehyde dehydrogenase*

The spectra of different enzyme preparations (▲, ■, ○ and ●) were determined in a Farrand Mk. I spectrofluorimeter by using an excitation wavelength of 350nm, with 10nm slits. Each spectrum is standardized to a protein concentration of 1mg/ml. A 6μM-NADH solution gave a fluorescence of 0.04×10^{-6} *A* unit under the conditions used. The □—□ spectrum shows the fluorescence spectrum of native preparations (that had given the ■—■ spectrum) after acid-$(NH_4)_2SO_4$ treatment as described in the text.

Assay of the boiled-enzyme supernatants with yeast alcohol dehydrogenase in 0.1M-glycine/NaOH buffer, pH10.0, in the presence of excess of ethanol showed that NAD^+ was also contained in these supernatants (0.2 and 0.35mol/mol). Other samples from enzyme preparations that contained little or no NADH had only very low contents of NAD^+ (<0.05mol/mol). It is noteworthy that the NAD^+ in enzyme samples could not be reduced by addition of acetaldehyde but was reduced by sodium dithionite. NADH in enzyme samples could be slowly oxidized after the addition of acetic anhydride. This compound promotes a partial reversal of the enzyme reaction (Hart & Dickinson, 1978).

As was indicated above, passage of the enzyme preparation through gel-filtration media did not decrease the concentration of bound NADH. Another procedure was then required. Treatment of the enzyme with activated charcoal was, however, rather ineffective. As an alternative, recourse was made to the method of Warburg & Christian (1938), which is traditionally to resolve flavoprotein enzymes into active apoenzyme and free flavin. Samples (7–14mg) of a fluorescent enzyme preparation dissolved in 1ml of 50mM-phosphate buffer, pH7.4, were adjusted to pH2.9 by addition of 0.7ml of saturated $(NH_4)_2SO_4$, pH1.5, and then after 15s the protein was precipitated by addition of 3ml of saturated $(NH_4)_2SO_4$, pH2.9. The precipitated protein was collected by centrifugation, washed once with 2ml of saturated $(NH_4)_2SO_4$, pH2.9, and then dissolved in 50mM-phosphate buffer, pH7.4. Fluorescence measurements on the dissolved protein indicated loss or destruction of bound NADH, as shown in the emission spectra included in Fig. 6. Analysis of a supernatant obtained after boiling the acid-treated preparation for 2min showed no evidence for the presence of NAD^+ (<0.03mol/mol of enzyme). The preparation still retained substantial activity (see below). It seems likely that the loss of NADH was due to exposure to the acid pH.

Assays of enzyme activity of the low-pH-treated

enzyme led to the further observations shown in Fig. 7. Assays showed pronounced lag phases lasting up to 5 min, which do not occur in assays of native enzyme. The length of the lag was independent of enzyme concentration in the assay mixture. The same result was obtained whether the original enzyme preparation contained high or low amounts of tightly bound nucleotide. The lag phases seen with the low-pH-treated enzyme were specifically abolished by preincubation with NAD^+ for 5 min in assay mixture before initiation of reaction with acetaldehyde (Fig. 7). Preincubation with NADH, either 3 μM or 300 μM, for 5 min before initiation of the reaction with an NAD^+/acetaldehyde mixture had no effect; the reactions then took place as if no preincubation had occurred. When a 7 mg/ml solution of acid-treated enzyme was mixed with 300 μM-NAD^+ for 5 min and then the mixture was passed through a gel-filtration column, the recovered enzyme showed no lag phases in conventional assays. The results suggest a role for NAD^+ in forming an active enzyme conformation, but it may be that the effects are merely an artifact of the low-pH treatment of the enzyme. The rates achieved in assays of the acid-treated enzyme either at the end of the lag phase or after preincubation with NAD^+ indicated that some 30% loss of enzyme activity was incurred on treatment with acid $(NH_4)_2SO_4$ solution. The assay characteristics of the treated enzyne then remained quite unaltered on storage at 0–1°C for 5 days. It is yet to be seen whether adjustments to the isolation procedure can improve the yield of enzyme activity.

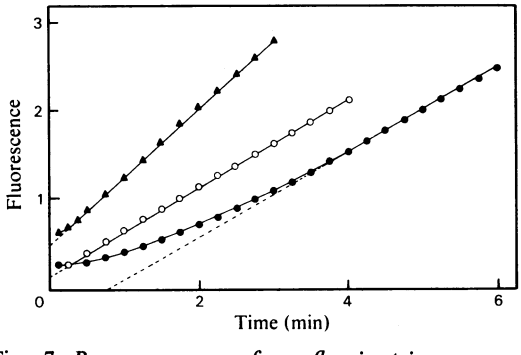

Fig. 7. *Progress curves from fluorimetric assays of aldehyde dehydrogenase*

Standard assays were performed, with the reactions being initiated by the addition of 3.96 nM-enzyme untreated (▲) or 4.26 nM-enzyme that had been treated with acid $(NH_4)_2SO_4$ as described in the text (●). The third assay (○) was initiated by the addition of acetaldehyde after the preincubation for 5 min at 25°C of 4.26 nM acid-$(NH_4)_2SO_4$-treated enzyme in an assay mixture from which acetaldehyde had been omitted. The broken lines are extensions of the linear portions of the progress curves. One unit of fluorescence is equivalent to 1 μM-NADH.

Conclusions

Our best enzyme preparations contain in the region of 1–2 coenzyme-binding sites/molecule of enzyme, with many estimates being at the lower end of the range. Studies of inactivation by disulfiram indicated a maximum of 2 active centres/molecule of enzyme (Dickinson *et al.*, 1981). The results are surprising in view of the tetrameric structure of the enzyme. The situation appears to be quite different from that obtaining with the mitochondrial aldehyde dehydrogenase of horse liver. Here there are apparently 2 NADH-binding sites/tetramer (Eckfeldt & Yonetani, 1976), but in the presence of Mg^{2+} the enzyme dissociates into dimers each exhibiting 2 active sites (Takahashi & Weiner, 1980). In our case treatment with Mg^{2+} inhibits the enzyme and does not effect either the M_r value or the coenzyme-binding capacity (Dickinson & Hart, 1982). A major question is whether our low values stem from the presence of inactive protein or degraded enzyme. We cannot answer this point definitely, but we have no evidence for the presence of inactive protein. The possibly double active band appearing on isoelectric focusing raises the possibility of some degradation, but if there is such the resulting change is really very small. That crude extracts of liver show active enzyme in the same region of isoelectric-focusing plates argues against major degradation. The fact that we have been unable to use the proteinase inhibitor phenylmethanesulphonyl fluoride is, however, unfortunate. Yeast aldehyde dehydrogenase is degraded by intracellular proteinases if an inhibitor is not used (Clark & Jakoby, 1970*a*,*b*; Bostian & Betts, 1978), and we cannot rule out such a change in our case.

If the enzyme is considered to be more or less unchanged throughout the isolation procedure, one is left to consider what possible role a major part of the enzyme protein has. Perhaps the enzyme has a second function or maybe it has been modified by regulatory cellular processes. The finding that certain preparations contain bound nicotinamide nucleotide is of obvious interest here, although the findings that this property is variable and that there has been no obvious correlation between enzyme activity and the amounts of bound nucleotide indicate that what we have here is a minor and variable contamination that contains bound nucleotide. There is, however, one good argument against a dismissal of this kind. Pig liver cytoplasmic aldehyde dehydrogenase has also been found to contain significant amounts of bound nucleotide (A. J. Ramsey & F. M. Dickinson, unpublished work).

This enzyme, however, has a quite different isoelectric point (approx. 6.5–7.0) and has different chromatographic properties from those of the sheep enzyme (it does not bind to DEAE-cellulose, which is used in two important steps in our purification of the sheep enzyme). It seems unlikely that the two enzymes would be contaminated with the same impurity. Whatever the status of this nucleotide-binding protein, it is clear that it is likely to cause difficulties in the measurement of coenzyme-binding capacities of enzyme preparations. It is for this reason that our binding studies were performed on preparations showing very little of this contamination.

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