Kinetic Properties of Aldehyde Dehydrogenase from Sheep Liver Mitochondria

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The kinetics of the NAD^+ -dependent oxidation of aldehydes, catalysed by aldehyde dehydrogenase purified from sheep liver mitochondria, were studied in detail. Lag phases were observed in the assays, the length of which were dependent on the enzyme concentration. The measured rates after the lag phase was over were directly proportional to the enzyme concentration. If enzyme was preincubated with NAD^+ , the lag phase was eliminated. Double-reciprocal plots with aldehyde as the variable substrate were non-linear, showing marked substrate activation. With NAD^+ as the variable substrate, double-reciprocal plots were linear, and apparently parallel. Double-reciprocal plots with enzyme modified with disulfiram (tetraethylthiuram disulphide) or iodoacetamide, such that at pH 8.0 the activity was decreased to 50% of the control value, showed no substrate activation, and the plots were linear. At pH 7.0, the kinetic parameters $V_{\max}$ and $K_m \text{NAD}^{++}$ for the oxidation of acetaldehyde and butyraldehyde by the native enzyme are almost identical. Formaldehyde and propionaldehyde show the same apparent maximum rate. Aldehyde dehydrogenase is able to catalyse the hydrolysis of *p*-nitrophenyl esters. This esterase activity was stimulated by both NAD^+ and NADH , the maximum rate for the NAD^+ stimulated esterase reaction being roughly equal to the maximum rate for the oxidation of aldehydes. The mechanistic implications of the above behaviour are discussed.

The metabolism of ethanol to acetate by liver cells is catalysed by two separable NAD^+ -linked dehydrogenases (Racker, 1949). The first enzyme, alcohol dehydrogenase (EC 1.1.1.1), is cytoplasmic in origin and the kinetics and mechanism of the horse liver enzyme have been well studied (Dalziel, 1975; Brändén *et al.*, 1975). Information about aldehyde dehydrogenase (EC 1.2.1.3) is, by contrast, sparse. Feldman & Weiner (1972) and Eckfeldt & Yonetani (1976a) have studied kinetic and other properties of isoenzymes from horse liver and Sidhu & Blair (1975a,b) have studied some properties of an isoenzyme from human liver. One noteworthy finding has been that these preparations exhibited esterase activity towards *p*-nitrophenol esters. However, the subcellular origin of some of these enzymes has not always been clearly established and the preparations from human liver were not homogeneous. Crow *et al.* (1974) have established that aldehyde dehydrogenase activity in sheep liver is distributed about equally between mitochondria and cytoplasm and they have purified enzyme preparations from both sources. Kitson (1975) in studying the effects of disulfiram (tetraethylthiuram disulphide) on these preparations has provided evidence that the mitochondrial and cytoplasmic enzymes are different. MacGibbon *et al.* (1977a,b) have studied the cytoplasmic enzyme in some detail and have concluded that the dissociation of the terminal $\text{E} \cdot \text{NADH}$ complex is an important rate-limiting step in aldehyde oxidation. A similar

conclusion was reached by Eckfeldt & Yonetani (1976a) with the F1 isoenzyme from horse liver. Both groups of workers suggest that the catalytic mechanism is basically ordered with NAD^+ combining before the aldehyde substrate and the reaction proceeding through an enzyme· NAD^+ ·aldehyde ternary complex. However, MacGibbon *et al.* (1977a) observed marked substrate activation by high substrate concentrations and concluded that this arose because of the operation of an alternative route to the ternary complex via an enzyme·aldehyde complex. The substrate activation has not been studied in detail, but it clearly has an important bearing on proposed mechanisms of catalysis. It is noteworthy that Greenfield & Pietruszko (1977) have observed similar substrate activation with an isoenzyme of aldehyde dehydrogenase from human liver. These observations suggest that mammalian aldehyde dehydrogenases may share this property.

Little detailed kinetic information is available on aldehyde dehydrogenases of established mitochondrial origin. It is therefore difficult to make comparisons of the characteristics of cytoplasmic and mitochondrial enzymes to assess the role and relative importance of the two enzymes. Further, it is not known if the sheep liver mitochondrial enzyme exhibits the same type of substrate activation that is observed with the cytoplasmic enzyme at high concentrations of aldehyde, or whether it exhibits esterase activity towards *p*-nitrophenyl esters. The purpose of

the present work was to provide detailed kinetic information for the sheep liver mitochondrial enzyme with preparations that have already been characterized in some detail (Hart & Dickinson, 1977).

Experimental

Materials

NADH (grade I) and NAD⁺ (grade II) were from Boehringer Corp. (London), London W.5, U.K. NAD⁺ was further purified before use by chromatography on DEAE-cellulose by the method of Dalziel & Dickinson (1966a). *p*-Nitrophenyl acetate was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. [²H]Acetaldehyde (99% ²H by n.m.r.) was a product of Aldrich Chemical Company Inc., Milwaukee, WI, U.S.A., and was used as supplied. Other chemicals were analytical-reagent grade whenever available, from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Aldehydes except [²H]acetaldehyde were redistilled before use.

Aldehyde dehydrogenase was prepared and assayed as described previously (Hart & Dickinson, 1977). Enzyme solutions were dialysed against phosphate buffer, pH 7.0, 1.0, containing 100 μ M-dithiothreitol before use. For experiments with nitrophenyl esters, dithiothreitol was removed by overnight dialysis against the same buffer but without added dithiothreitol.

Methods

Dialysis tubing was boiled in 10 mM-EDTA, pH 7.0, and washed well with water before use. All solutions were prepared in glass-distilled water.

Determination of coenzyme and substrate concentrations. NAD⁺ and NADH were assayed enzymically with yeast alcohol dehydrogenase by the methods described by Dalziel (1962a, 1963) and by using $\epsilon = 6.22 \times 10^3$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for NADH at 340 nm (Horecker & Kornberg, 1948). A value of $\epsilon = 9.6 \times 10^3$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ was used for *p*-nitrophenol at 400 nm, pH 7.0 (Eckfeldt & Yonetani, 1976a). Aldehyde concentrations (except formaldehyde) were checked by following loss of NADH at 340 nm by using yeast alcohol dehydrogenase and an excess of NADH in 0.1 M-phosphate buffer, pH 7.0.

Steady-state kinetics. Aldehyde dehydrogenase activity was determined at 25°C by using a recording filter fluorimeter of the type described by Dalziel (1962b). At pH 6.0, 7.0 and 8.0, sodium phosphate buffers, 1.0, were used, but at pH 9.0 NaHCO₃/Na₂CO₃ (10 mM total carbonate) buffer was used, containing sufficient Na₂HPO₄ to achieve 1.0. Solutions of purified NAD⁺ containing phosphate

buffer were adjusted to the required pH value before use, and an allowance was then made for the phosphate concentration when making up assay mixtures.

As reported briefly on a previous occasion (Hart & Dickinson, 1977) lag phases are observed in enzyme assays and measured rates are those that are achieved once the lag phase is over. These rates are maintained for several minutes. In the present work, this phenomenon has been studied further (see below), but rates were measured in the same way. For kinetic work assays were performed at least in duplicate, and measured rates generally agreed to within 5%. Assays were performed in the absence of added aldehyde to check for 'blank reactions'. Normally these were negligible, although on occasions, at the lowest aldehyde concentrations, a correction of about 10% was made for the observed blank reaction. Blank reactions were minimized by performing assays in order of increasing aldehyde concentration, and only assays containing the same concentration of aldehyde were placed in the fluorimeter at any one time. It was found that, if an assay mixture containing a high concentration of aldehyde (1 mM) was placed in the fluorimeter in an uncapped cuvette with one containing no added aldehyde for about 2 min, cross-contamination by aldehyde led to a very high apparent control rate (about 10-times normal) in the assay with no added aldehyde when enzyme was added. Thus to avoid inaccurate measurements of initial rate at low aldehyde concentrations, these assays must not be performed with assay mixtures containing high concentrations of aldehyde also being present in the instrument.

Esterase activity was determined at 25°C with *p*-nitrophenyl acetate as substrate by following the production of *p*-nitrophenol at 400 nm in a Cary 14 u.v. spectrophotometer. Stock *p*-nitrophenyl acetate solutions were made in acetone, the final acetone concentration in the assay being less than 1%. Control experiments show that acetone at this concentration has no effect on the activity of the enzyme.

Specific initial rates for enzyme assays were calculated on a molar basis, using an enzyme mol.wt. of 200 000 (Hart & Dickinson, 1977).

Assays using high concentrations of enzyme. These were performed both in a stopped-flow apparatus and a conventional spectrophotometer to check for possible bursts of NADH or *p*-nitrophenol production.

Stopped-flow experiments were performed at 25°C using an apparatus based on the design of Gibson & Milnes (1964). The apparatus was washed in 30 mM-EDTA for at least 2–3 h before use. This was necessary to prevent losses of enzyme activity during the course of the experiments. This was presumably due to contact of the enzyme with the stainless-steel valves, blocks etc. If this precaution was taken, enzyme

recovered from the apparatus at the end of the work retained full activity. The rate of formation of NADH in a 2cm light-path was followed at 340nm after mixing of a solution containing NAD⁺ (2mM) and acetaldehyde (100mM) in sodium phosphate buffer, pH 7.0, I 0.1, with a solution of enzyme (10 μ M) in the same buffer. Changes of transmittance were recorded by a Tektronix 546B storage oscilloscope.

The spectrophotometric experiments were performed in a Zeiss PMQ II spectrophotometer equipped with a Vitatron UR40 lin-log chart recorder. For dehydrogenase activity, A_{340} was monitored after the addition of acetaldehyde to a 1cm-light-path microcuvette containing NAD⁺ (1mM) and enzyme (3 μ M) in sodium phosphate buffer, pH 7.0, I 0.1, in a final volume of 1ml. Final aldehyde concentrations used were 50mM and 50 μ M. The production of *p*-nitrophenol was followed at 400nm after the addition of *p*-nitrophenyl acetate (1mM final concn.) to a solution of enzyme (1.9 μ M) in phosphate buffer, pH 7.0, I 0.1. Final volume was 1ml. Experiments were also performed in which NAD⁺ (300 μ M) or NADH (200 μ M) had been added to the cuvette before the addition of ester. Control experiments in the absence of enzyme showed that, over the time-period monitored in the burst studies, blank hydrolysis of ester was negligible, and no corrections were made to the rates obtained in the presence of enzyme.

Results and Discussion

Typical progress curves for assays of sheep liver mitochondrial aldehyde dehydrogenase at pH 8.0 in the recording fluorimeter are shown in Fig. 1. It is evident that the progress curves show marked lag phases and that the length of the lag phase is much shortened by increasing the enzyme concentration 10-fold. This concentration effect explains why the lag phase is not observed at all at the much higher enzyme concentrations used in the stopped-flow experiments (see below). The lag phases observed in the fluorimetric assays are not peculiar to pH 8.0 for the same effects have been observed at pH 7.0, 9.0 and 9.8. The only effect of increasing the pH in this range is to increase the steady-state rate some 6-fold. The steady rates achieved in assays at the end of the lag phases are constant for several minutes and are thus easy to measure accurately. Measured rates are directly proportional to enzyme concentration at least over the concentration range 5–3000nM.

Fig. 1 shows that the lag phases at low enzyme concentrations are effectively eliminated if the enzyme is preincubated with NAD⁺ for 6min, under the conditions of the normal assay, before initiation of the reaction by the addition of acetaldehyde. Preincubation of the enzyme with acetaldehyde under the

Fig. 1. Progress curves from fluorimetric assays of mitochondrial aldehyde dehydrogenase

Normal assays were carried out as described in the text with reactions being initiated by the addition of (●) 3 nM-enzyme or (△) 30 nM-enzyme. In the third case the assay (○) was initiated by the addition of acetaldehyde after the enzyme (3 nM) had been preincubated at 25°C for 6 min 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 μ M-NADH.

conditions of the assay, or preincubation in the absence of either acetaldehyde or NAD⁺ for the same period, has no such effect, and the lag phases are not decreased when the missing components of the assay are added. One may further note that addition of the products of reaction, NADH and acetate, to assays, either together or separately and at concentrations that had been achieved at the end of the lag phases in the assays of Fig. 1, has no effect on reaction progress curves. Further, the addition of 1 mM-EDTA, 100 μ M-dithiothreitol or 1 mg of bovine serum albumin to assays is also ineffective in decreasing the lag phases.

The above experiments suggest that the activation effects seen in assays can be specifically attributed to the binding of NAD⁺ to the enzyme to cause conversion of the enzyme into a more active form. As the period of preincubation needed to abolish the lag phase may be several minutes, the activation process must involve one or more slow steps. Since, at a high and constant NAD⁺ concentration, the duration of the lag phase is clearly dependent on enzyme concentration, the results suggest that the observed activation of the enzyme may occur by a process involving more than one molecule of enzyme. Ultracentrifuge experiments in the absence of

Scheme 1. Possible mechanism for aldehyde dehydrogenase

coenzyme did not, however, provide any evidence for aggregation of enzyme molecules (Hart & Dickinson, 1977). Whether the activation involves the combination of NAD^+ at catalytic or regulatory sites is not known, but it is of interest that a likely mechanism of catalysis (Scheme 1) does not involve free enzyme in the catalytic cycle.

The results of detailed kinetic studies of the sheep liver mitochondrial enzyme with acetaldehyde as substrate are shown in Fig. 2(a). The experiments were made in phosphate buffer, pH 7.0, 1.0 M with independent variation of coenzyme and substrate concentrations. A secondary plot of the intercepts of Fig. 2(a) is shown in Fig. 2(b) together with alternative plots of the data of Fig. 2(a). Kinetic experiments with $[^2\text{H}]$ -acetaldehyde as substrate gave results that were identical within experimental error to those for acetaldehyde. The marked curvature of the plots in Fig. 2(a) and the apparent strict linearity of the alternative plots in Fig. 2(b) make a striking contrast. The same behaviour is seen in the results of kinetic studies with butyraldehyde as substrate as is evident from Figs. 3(a) and 3(b).

Non-linear double-reciprocal plots with aldehyde as the variable substrate are also seen at other pH values and with other aldehydes (Figs. 4a and 4b). Under standard assay conditions with rate-limiting concentrations of substrate the rate of reaction increases markedly with increasing chain length of the substrate alkyl group. Fig. 4(b) also shows a double-reciprocal plot for the enzyme-catalysed

hydrolysis of *p*-nitrophenyl acetate. Over the accessible range of concentration, this plot appears linear, but the low solubility of *p*-nitrophenyl acetate and the low sensitivity of the assay procedure prevent a proper test of this being made.

Whatever the cause of the curvature of Figs. 2(a), 3(a), 4(a) and 4(b), an important consequence of this behaviour is that the enzyme retains a relatively high activity at very low substrate concentrations. This is, no doubt, of physiological importance [in man the normal acetaldehyde concentration in the blood is reported to be about $10\ \mu\text{M}$ (Lundquist, 1975)], but it means that a proper kinetic study of the enzyme requires the use of very sensitive fluorimetric methods even though a consequence of using the necessarily small enzyme concentrations is that lag phases are encountered in the assays.

Non-linear double-reciprocal plots have also been observed by Greenfield & Pietruszko (1977) for one of the isoenzymes of aldehyde dehydrogenase from human liver, and biphasic kinetics have been observed for aldehyde dehydrogenases from bovine and pig brain (Erwin & Deitrich, 1966; Duncan & Tipton, 1971). Linear kinetics, however, have been observed by Feldman & Weiner (1972) and Eckfeldt & Yonetani (1976a) for enzymes from horse liver. These results are also somewhat different from those reported by MacGibbon *et al.* (1977a) for the cytoplasmic enzyme from sheep liver. These workers found that the double-reciprocal plots for propionaldehyde were linear to $50\ \mu\text{M}$, but deviated to faster rates

Fig. 2. Initial-rate measurements for acetaldehyde-NAD⁺ reaction

(a) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the acetaldehyde concentration at four constant NAD⁺ concentrations. The NAD⁺ concentrations (μM) were: $\circ$, 485; $\bullet$, 89.0; $\square$, 16.0; $\blacksquare$, 4.0. (b) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the NAD⁺ concentration at several constant acetaldehyde concentrations. The acetaldehyde concentrations (μM) were: $\bullet$, 15000; $\circ$, 3000; $\blacktriangle$, 1500; $\triangle$, 150; $\blacksquare$, 75; $\square$, 37.5; $\blacktriangledown$, 15; $\diamond$, 7.5. The Figure also shows the variation of the intercepts (∇) of (a) with the reciprocal of the NAD⁺ concentration.

Fig. 3. Initial-rate measurements for butyraldehyde-NAD⁺ reaction

(a) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the butyraldehyde concentration at four constant NAD⁺ concentrations. The NAD⁺ concentrations (μM) were: $\circ$, 490; $\bullet$, 111; $\square$, 32; $\blacksquare$, 18.5. (b) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the NAD⁺ concentration at several constant butyraldehyde concentrations. The butyraldehyde concentrations (μM) were: $\bullet$, 15000; $\circ$, 3000; $\blacktriangle$, 1500; $\blacksquare$, 150; $\square$, 75; $\blacktriangledown$, 37.5; $\diamond$, 15. The Figure also shows the variation of the intercepts (∇) of (a) with the reciprocal of the NAD⁺ concentration.

above this aldehyde concentration, although the plots for acetaldehyde deviated from linearity over almost the entire concentration range studied.

Fig. 4. Initial rate measurements for aldehyde dehydrogenase with different substrates and at different pH values

(a) Variation of the reciprocal of the specific initial rate at 25°C of acetaldehyde oxidation with the reciprocal of the acetaldehyde concentration at several pH values. The pH values were: ○, 9.0; ●, 8.0; □, 7.0; ■, 6.0. The NAD^+ concentration in each case was $485 \mu\text{M}$. (b) Variation of the reciprocal of the specific initial rate for the oxidation of several aldehydes at pH 7.0 and 25°C with the reciprocal of the aldehyde concentration. The aldehydes were: ○, butyraldehyde; ●, propionaldehyde; □, acetaldehyde; ■, formaldehyde. The NAD^+ concentration was $485 \mu\text{M}$. Also shown is the variation of the reciprocal of the specific initial rate at pH 7.0 and

The secondary intercepts plots shown in Figs. 2(b) and 3(b) enable estimates of the maximum rate and the Michaelis constants for NAD^+ to be determined. These are presented in Table 1. With the primary plots of Figs. 2(a) and 3(a) being non-linear, secondary slopes plots are not possible. It is noteworthy that the Michaelis constants for NAD^+ obtained in the present study are significantly greater than those obtained by MacGibbon *et al.* (1977a) for the cytoplasmic enzyme from sheep liver. At pH 7.6, with propionaldehyde as substrate, these workers obtained a value of K_{m,NAD^+} of $2.3 \mu\text{M}$. A similar trend is seen for the horse liver isoenzymes, where the F_1 enzyme, which is probably cytoplasmic in origin (Eckfeldt & Yonetani, 1976a,b; Eckfeldt *et al.*, 1976), has a much lower K_m for NAD^+ than the isoenzyme studied by Feldman & Weiner (1972), which is probably mitochondrial in origin (Eckfeldt & Yonetani, 1976b; Eckfeldt *et al.*, 1976).

In seeking an explanation for the non-linear double-reciprocal plots of Figs. 2(a) and 3(a), two mechanisms suggest themselves. The first of these is a non-equilibrium random-order mechanism whose properties have been discussed at length in the theoretical work of Dalziel (1957) and Pettersson (1969) and which has been suggested as the reason for the activation of sheep liver cytoplasmic aldehyde dehydrogenase at high aldehyde concentrations (MacGibbon *et al.*, 1977a). The second mechanism is a compulsory mechanism with the reaction being diverted via an abortive $\text{E} \cdot \text{NADH} \cdot \text{aldehyde}$ complex at high substrate concentrations. Abortive-complex formation explains in detail substrate activation of horse liver alcohol dehydrogenase at high concentrations of cyclohexanol (Dalziel & Dickinson, 1966c). In the present case closer scrutiny of the results suggests that neither mechanism is satisfactory.

For a non-equilibrium random-order mechanism apparent substrate activation would arise because there are two routes through to the catalytic $\text{E} \cdot \text{NAD}^+ \cdot \text{aldehyde}$ complex. One passes through the binary $\text{E} \cdot \text{NAD}^+$ complex and the other through the

Table 1. Kinetic parameters for sheep liver mitochondrial aldehyde dehydrogenase at pH 7.0, 25°C

	Acetaldehyde as substrate	Butyraldehyde as substrate
$V_{\max.} (v/e)$	77 min^{-1}	77 min^{-1}
K_{m,NAD^+}	$36 \mu\text{M}$	$38 \mu\text{M}$

25°C with the reciprocal of the *p*-nitrophenyl acetate concentration for the hydrolysis of *p*-nitrophenyl acetate (▼).

binary E-aldehyde complex. At low substrate concentrations the route via E·NAD⁺ may be favoured, whereas at high substrate concentrations the reaction is forced through the alternative route. If the E-aldehyde complex reacts more rapidly with NAD⁺ than the free enzyme, substrate activation will occur at high aldehyde concentrations and the activation should occur more easily and be more pronounced at the lower NAD⁺ concentrations. Figs. 2(a) and 3(a) provide no evidence that substrate activation occurs more readily at low NAD⁺ concentrations. As indicated above, MacGibbon *et al.* (1977a) suggested the random mechanism to explain substrate activation with the cytoplasmic aldehyde dehydrogenase. Their data, at high concentrations of aldehyde, were, however, confined to a single NAD⁺ concentration.

The abortive-complex mechanism may be discarded for reasons that are, in part, similar to those given above. The abortive complex, E·NADH·aldehyde, should be most in evidence at high substrate and coenzyme concentrations. Thus activation should be most marked at high coenzyme concentrations and be much less evident, or even non-existent, at low coenzyme concentrations. These effects are clearly seen with horse liver alcohol dehydrogenase with cyclohexanol as substrate, where more rapid dissocia-

tion of NADH from the abortive E·NADH·cyclohexanol than from the E·NADH complex explains substrate activation (Dalziel & Dickinson, 1966c). In the present case, the data do not conform to the expected type of behaviour and the mechanism seems unlikely. This conclusion is supported by the results of assays at high enzyme concentrations. Fig. 5 shows that there was no significant accumulation of an NADH-containing enzyme species in the pre-steady state either at high (50 mM) or low (50 μ M) aldehyde concentration. This was a conventional spectrophotometric experiment, but the same result was expected and obtained using stopped-flow spectrophotometric methods. Thus there is no evidence for the accumulation of an E·NADH·aldehyde complex at high substrate concentrations and no direct evidence for an abortive-complex mechanism. One may note that different results were obtained in stopped-flow experiments by MacGibbon *et al.* (1977b) using the sheep liver cytoplasmic enzyme. This does give a burst of NADH production at high substrate and coenzyme concentrations. These observations provide further evidence that the two enzymes have different properties. If alternative-pathway mechanisms are not compatible with the data, more complex mechanisms must be involved. However, it is worthwhile to look at the kinetic data to see if any basic features for the mechanism of catalysis can be deduced.

The results of Table 1 show that the maximum velocity of aldehyde oxidation ($V_{\max}$) is the same for both acetaldehyde and butyraldehyde, and that the K_m for NAD⁺ is also independent of the substrate. Fig. 4(b) suggests that the $V_{\max}$ is also the same for both formaldehyde and propionaldehyde oxidation. These are remarkable facts since at a constant high NAD⁺ concentration, the initial rates at low aldehyde concentration are very dependent on the nature of the aldehyde substrate (Fig. 4b).

Invariance of $V_{\max}$ and K_m values for NAD⁺ on changing substrates are predicted characteristics of a Theorell–Chance-type mechanism; horse liver alcohol dehydrogenase with different primary alcohol substrates provides a well-documented example of this type of behaviour (Dalziel & Dickinson, 1966b). One expectation of this mechanism is that NADH release from the product E·NADH complex should be rate-limiting. However, the experiments of Fig. 5 provide no evidence for the accumulation of NADH-containing species in approaching the steady state at either high or low substrate concentrations. For horse liver alcohol dehydrogenase, stopped-flow experiments do provide firm evidence for the formation of stoichiometric concentrations of the E·NADH complex in the pre-steady state (Shore & Gutfreund, 1970). It is unfortunate that the irreversibility of the aldehyde dehydrogenase reaction means that relationships appropriate to a Theorell–Chance-type, or general compulsory-order, mechanism cannot be

Fig. 5. Acetaldehyde oxidation at high enzyme concentration. The increase in A_{340} was followed after the addition of acetaldehyde to a cuvette containing NAD⁺ (1 mM) and enzyme (3 μ M) in sodium phosphate buffer, pH 7.0, I 0.1, at 25°C. Final aldehyde concentrations were: ●, 50 mM; ○, 50 μ M. The theoretical intercept for a burst of NADH under these conditions would have been 0.075 A unit.

tested (Dalziel, 1957). Lack of a direct estimate for the rate constant for the combination of free enzyme with NAD^+ also means that the equivalence of this quantity with the ratio $V_{\max.}/K_{m,\text{NAD}^+}$ which is required by a compulsory mechanism with NAD^+ binding first cannot be tested either. This latter test could prove decisive, and efforts must be made to provide the necessary data.

Ternary complex mechanisms predict that primary pots such as those in Figs. 2(a), 2(b), 3(a) and 3(b) should each consist of a set of intersecting lines. Although Figs. 2(a) and 3(a) are strongly curved, they each seem to show a set of lines whose degree of separation is constant. This is confirmed by the fact that in Figs. 2(b) and 3(b) the lines are essentially parallel. It could be argued that there is a degree of divergence in the primary plots, but that it is too small to be detected. This argument cannot be rejected, but it is nevertheless true that over very wide ranges of concentration and with large changes of measured rate, we have not detected any significant degree of divergence in the primary plots. If the plots are accepted as being parallel, then the data indicate that an enzyme-substitution mechanism is in operation, and not a ternary-complex mechanism (Dalziel, 1957). It is noteworthy that for glyceraldehyde 3-phosphate dehydrogenase a group-transfer mechanism has become widely accepted (Harris & Waters, 1976). In view of this, and the supposed similarity of the mechanisms of aldehyde dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (Jakoby, 1963) the mechanism shown in Scheme 1 merits serious consideration.

The basis of the mechanism in Scheme 1 is that the enzyme oscillates between the binary $\text{E}\cdot\text{NAD}^+$ complex and the acyl-enzyme intermediate ($\text{E}\cdot\text{S}\cdot\text{CO}\cdot\text{R}$). For our enzyme there is no direct evidence for the acyl-enzyme, but the facts that the enzyme exhibits esterase activity towards *p*-nitrophenyl esters, and that both the esterase and dehydrogenase activities are inhibited by thiol reagents, are at least consistent with the hypothesis. An alternative type of mechanism where the *gem*-diol form of the substrate (rather than the thiohemiacetal) is dehydrogenated seems unlikely, since chloral hydrate is not a substrate but a strong inhibitor (Crow *et al.*, 1974).

Studies on the esterase activity of our preparation show that it behaves in much the same way as other aldehyde dehydrogenases (Feldman & Weiner, 1972; Sidhu & Blair, 1975a; Eckfeldt & Yonetani, 1976a). The activity is strongly activated by NAD^+ and NADH (Fig. 6), though the effect depends to some extent on the concentration of ester used. The stimulation of activity by low concentrations of coenzyme suggests that a conformational change may occur in the enzyme when coenzyme is bound, the enzyme becoming more active. The mechanism of inhibition by high concentrations of coenzyme is

Fig. 6. Effect of NAD^+ and NADH on the rate of hydrolysis of *p*-nitrophenyl acetate

The variation of the specific initial rate of hydrolysis of *p*-nitrophenyl acetate at pH 7.0 and 25°C with NAD^+ concentration (a) and NADH concentration (b). The *p*-nitrophenyl acetate concentrations (μM) were: ■, 1000; ●, 300; ○, 120.

unknown. High concentrations of NAD^+ (1–5 mM) do not inhibit the dehydrogenase activity.

Experiments at high enzyme concentrations (Fig. 7) indicate that acyl-enzyme hydrolysis is not rate-limiting in either the absence or presence of coenzyme. When considering acetaldehyde oxidation by Scheme 1, the rate of hydrolysis of the $\text{NAD}^+\cdot\text{E}\cdot\text{S}\cdot\text{CO}\cdot\text{CH}_3$ complex is of interest. The lack of a burst of *p*-nitrophenol production (Fig. 7) shows that the rate of hydrolysis of $\text{E}\cdot\text{NAD}^+\cdot\text{acetyl}$ must be considerably greater than the maximum rate of ester hydrolysis at high NAD^+ concentration, which is itself roughly equal to $V_{\max.}$ for acetaldehyde oxidation (cf. Table 1). This step cannot therefore be rate-limiting in aldehyde oxidation. This conclusion is supported by the results in Fig. 5, which show no accumulation of an NADH-containing species in the approach to the steady state of aldehyde oxidation. If the hydrolysis of $\text{E}\cdot\text{NAD}^+\cdot\text{acetyl}$ were rate-limiting, 1 mol of NADH/mol of

Fig. 7. Hydrolysis of *p*-nitrophenyl acetate with high enzyme concentrations

The increase in A_{400} was followed after the addition of *p*-nitrophenyl acetate (1 mM final concentration) to a solution of enzyme ($1.9 \mu\text{M}$) in sodium phosphate buffer, pH 7.0, I 0.1, at 25°C . The cuvette also contained: ●, NAD^+ ($300 \mu\text{M}$); ○, NADH ($200 \mu\text{M}$); ■, no addition. The theoretical intercept for a burst under these conditions would have been $0.077 A$ unit.

active centre should be produced in the first enzyme turnover, although if the equilibrium between the complexes:

is strongly in favour of the first complex, NADH dissociation could be rate-limiting, yet in the approach to the steady state there would be no detectable accumulation of an NADH -containing species. The complete lack of a deuterium isotope effect shows that hydride transfer is not rate-limiting. Another possible rate-limiting step is the nucleophilic attack of the enzyme thiol on the carbonyl carbon of the aldehyde within the $\text{E} \cdot \text{NAD}^+ \cdot \text{aldehyde}$ Michaelis complex to form the thiohemiacetal. In either of the above cases, however, there is no clear reason why the V_{max} values for the oxidation of acetaldehyde, butyraldehyde and the other aldehydes should be the same. If Scheme 1 is correct, the fact that the V_{max} values are constant must arise because one or more steps are insensitive to the nature of the alkyl group. In the same way mechanisms of the type shown in Scheme 1 do not predict that $K_{\text{m}, \text{NAD}^+}$ should be independent of the nature of the substrate (Dalziel, 1957). It may be that, because of certain inequalities in the rate constants, and the fact that certain steps are insensitive to the

Fig. 8. Initial-rate measurements for acetaldehyde oxidation using disulfiram- and iodoacetamide-modified aldehyde dehydrogenase

Variation of the reciprocal of the specific initial rate at pH 7.0 and 25°C , with the reciprocal of the acetaldehyde concentration for: ■, enzyme modified with disulfiram; △, enzyme modified with iodoacetamide; ●, a control sample of enzyme. The NAD^+ concentration was $485 \mu\text{M}$.

nature of the alkyl group, the situation arises, but it is not predicted. As indicated above, a Theorell–Chance-type mechanism gives a more convincing explanation of these phenomena.

Neither Scheme 1 nor the Theorell–Chance mechanism alone explains the non-linear plots seen in Figs. 2(a) and 3(a). It may be that the curvature of the double-reciprocal plots is due to the presence of regulatory sites or non-equivalent active sites, or to negative interactions between active sites. Further work, particularly substrate and coenzyme binding studies will be required to resolve the problem. For rabbit muscle glyceraldehyde 3-phosphate dehydrogenase reciprocal plots with strong downward curvature with respect to NAD^+ have been correlated with negative co-operativity between active centres in the NAD^+ binding reactions (Meunier & Dalziel, 1978). For the present case it is noteworthy that the subunit molecular weight of the enzyme, approx. 50000 (Hart & Dickinson, 1977), is larger than that of most dehydrogenases, and this increase in size may provide regulatory substrate binding sites on the enzyme.

We have reported (Hart & Dickinson, 1977) that aldehyde dehydrogenase is only partially inhibited by disulfiram and iodoacetamide, suggesting that there may indeed be two classes of active centre in the enzyme. Enzyme modified with disulfiram or iodoacetamide, such that in our standard assay at pH 8.0 activity decreased to about 50% of the control value, shows very different kinetic behaviour at pH 7.0 to

the native enzyme. Not only will the activity not normalize when corrections are made for specific activity (this is due to a change in the pH characteristics of the modified enzyme), but the double-reciprocal plots are apparently linear (Fig. 8). This result suggests that treatment of the enzyme with these particular reagents either blocks one type of site, or else abolishes conformational changes that are responsible for the observed activation at high aldehyde concentration.

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