Kinetic properties of highly purified preparations of sheep liver cytoplasmic aldehyde dehydrogenase

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The kinetic properties of highly purified preparations of sheep liver cytoplasmic aldehyde dehydrogenase (preparations that had been shown to be free from contamination with the corresponding mitochondrial enzyme) were investigated with both propionaldehyde and butyraldehyde as substrates. At low aldehyde concentrations, double-reciprocal plots with aldehyde as the variable substrate are linear, and the mechanism appears to be ordered, with NAD^+ as the first substrate to bind. Stopped-flow experiments following absorbance and fluorescence changes show bursts of NADH production in the pre-steady state, but the observed course of reaction depends on the pre-mixing conditions. Pre-mixing enzyme with NAD^+ activates the enzyme in the pre-steady state and we suggest that the reaction mechanism may involve isomeric enzyme– NAD^+ complexes. High concentrations of aldehyde in steady-state experiments produce significant activation (about 3-fold) at high concentrations of NAD^+ , but inhibition at low concentrations of NAD^+ . Such behaviour may be explained by postulating the participation of an abortive complex in product release. Stopped-flow measurements at high aldehyde concentrations indicate that the mechanism of reaction under these conditions is complex.

Over the past 10 years there have been several studies of the kinetic properties of aldehyde dehydrogenases from various types of mammalian liver (Feldman & Weiner, 1972; Sidhu & Blair, 1975; Eckfeldt & Yonetani, 1976; MacGibbon *et al.*, 1977a). These authors have concluded that the mechanism of aldehyde oxidation by NAD^+ and aldehyde dehydrogenase is basically ordered, with NAD^+ being the leading substrate. The reaction is thought to proceed through an enzyme– NAD^+ –aldehyde ternary complex, with NADH release from a product E–NADH complex being at least partially rate-limiting. However, detailed kinetic studies have only been performed with one aldehyde substrate with each enzyme. Since one important test of mechanism is to compare the kinetic parameters for two or more related substrates (Dalziel, 1975), we have investigated in some detail the kinetics of oxidation of both propionaldehyde and butyraldehyde by NAD^+ and highly purified preparations of sheep liver cytoplasmic aldehyde dehydrogenase. Dickinson & Berrieman (1979) have shown that enzyme preparations may often be contaminated by significant amounts of the corres-

ponding mitochondrial enzyme, and that such contamination would produce serious errors in the estimation of initial rates at low concentrations of aldehyde. Accordingly we have used preparations purified from contaminating mitochondrial aldehyde dehydrogenase by pH-gradient ion-exchange chromatography on DEAE-cellulose (Dickinson *et al.*, 1981).

Working with a single high concentration of NAD^+ , MacGibbon *et al.* (1977a) observed that high concentrations of both acetaldehyde and propionaldehyde produced a significant degree of substrate activation, a phenomenon observed also by Greenfield & Pietruszko (1977) working with an isoenzyme of aldehyde dehydrogenase from human liver. The effect of high aldehyde concentration at low concentrations of NAD^+ is not known. Since the activation process would be expected to have a bearing on the overall reaction mechanism, we have investigated the oxidation of high concentrations of aldehydes, and we present the results of such studies using both steady-state and pre-steady-state methods together with those for low concentrations of aldehydes. We use the information obtained to draw some conclusions about the mechanism for aldehyde oxidation by NAD^+ catalysed by cytoplasmic aldehyde dehydrogenase.

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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). Other chemicals were analytical-reagent grade whenever 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 $A_{1\text{cm}}^{1\%} = 11.3$ at 280 nm, determined from dry-weight measurements. Before use, enzyme solutions were dialysed against phosphate buffer, pH 7.0, *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 methods of Dalziel (1962a, 1963) and by using $\epsilon = 6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for NADH at 340 nm (Horecker & Kornberg, 1948). Aldehyde concentrations were checked by following loss of NADH at 340 nm by using yeast alcohol dehydrogenase and an excess of NADH in 0.2 M-phosphate buffer, pH 7.0.

Steady-state kinetics. Aldehyde dehydrogenase activity was determined at 25°C in sodium phosphate buffer, pH 7.0, *I*0.1, by using a recording filter fluorimeter of the type described by Dalziel (1962b). 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% at the higher substrate and coenzyme concentrations, but only to about 15% at the lowest concentrations. At the lowest aldehyde concentrations enzyme concentrations as high as 13 nM were used, so that for butyraldehyde enzyme concentrations reached as high as 10%, whereas for propionaldehyde they reached about 5%, of the total substrate concentration. Generally progress curves were linear for several minutes, except where the aldehyde concentration was much greater than the coenzyme concentration. Here a linear increase in fluorescence lasted for only about 30–45 s.

Before each experiment was begun, air was blown through the cell-housing of the fluorimeter for 2–3 min to displace any aldehyde vapour, and then

assays were performed in the absence of added aldehyde to check for 'blank reactions'. Corrections were made for the observed blank rates, which were negligible at the highest aldehyde concentrations, rising to about 20% at the lowest aldehyde concentrations used. To avoid inaccurate measurements of initial rate owing to cross-contamination by aldehyde, assays were performed in order of increasing aldehyde concentration, and only assays containing the same concentration of aldehyde were placed in the fluorimeter at any one time.

Kinetic coefficients in the initial-rate equation (1):

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{[s_1]} + \frac{\phi_2}{[s_2]} + \frac{\phi_{12}}{[s_1][s_2]} \quad (1)$$

were determined as described by Dalziel (1957). In this equation e is the molar concentration of enzyme (calculated by taking the enzyme molecular weight to be 200 000), v is the initial velocity [corrected to an enzyme specific activity of 160 units ($\mu\text{mol}/\text{min}$)/mg in the standard assay], and s_1 and s_2 are, respectively, NAD⁺ and aldehyde. A specific activity of 160 units/mg is the highest that we have obtained, and where necessary observed rates have been corrected to this standard. Most preparations had a specific activity of 110–120 units/mg.

Stopped-flow experiments. These were performed at 25°C in phosphate buffer, pH 7.0, *I*0.1, in an apparatus modified from the design of Gibson & Milnes (1964). The apparatus has been described by Dickinson & Dickenson (1978). The output from the photomultipliers was usually stored by a dual-channel transient signal processor (Barman Electronics, Hull, U.K.) and was then displayed on a Tektronix 546 B storage oscilloscope. Permanent records of the experiments were obtained by output of the contents of the transient-recorder memory to a Servogor 743 chart recorder. Occasionally progress curves were photographed directly from the oscilloscope. Experiments were performed under various pre-mixing conditions, and the progress of the reactions was followed by monitoring (in separate experiments) both fluorescence and absorbance changes. For experiments in the spectrophotometric mode an effective optical path length of 2.2 cm was used.

Results and discussion

Preliminary experiments

Acetaldehyde and propionaldehyde are compared as substrates at pH 7.0 and 25°C in Fig. 1 under conditions of high (425 μM) NAD⁺ concentration. Both aldehydes show the same behaviour, with the reciprocal plots being linear at low aldehyde concentrations and with substrate activation at high aldehyde concentrations. For

Fig. 1. Initial-rate measurements for aldehyde oxidation at pH 7.0, 25°C

Experiments were carried out as described in the text with acetaldehyde (○) and propionaldehyde (●) as substrate and with 425 μM -NAD⁺. The inset shows results at the high substrate concentrations in more detail.

propionaldehyde the results confirm the findings of MacGibbon *et al.* (1977a), working at pH 7.6. For acetaldehyde, however, those authors found non-linearity over almost the entire range of concentration; the plots were concave downwards. Furthermore, at low concentrations the rates for acetaldehyde were much less different from those for propionaldehyde than we have found. These discrepancies might have been caused by contamination of the cytoplasmic enzyme by the mitochondrial form. The latter is much more active at low acetaldehyde concentrations, so that a small amount will produce a disproportionately large effect and would make the measured rates too fast (Dickinson & Berrieman, 1979).

Further work was concentrated on propionaldehyde and butyraldehyde as substrates. The rates with these aldehydes being considerably faster than for acetaldehyde meant that results would be more reliable at low substrate concentrations. There was less problem with 'blank' reactions and enzyme concentrations could be kept lower.

Initial-rate measurements at low substrate concentrations

The results of initial-rate measurements at 25°C in sodium phosphate buffer, pH 7.0, 10.1, with propionaldehyde as substrate are shown in the double-reciprocal plots of Fig. 2(a), with secondary plots of the slopes and intercepts shown in Fig. 2(b). Substrate activation at aldehyde concentrations above about 50 μM and substrate inhibition at low concentrations of NAD⁺ and high concentrations of

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

(a) Primary plot showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the propionaldehyde concentration at four constant NAD⁺ concentrations. The NAD⁺ concentrations (μM) were: ●, 437; ○, 6; ▲, 3; △, 0.9. (b) Secondary plots showing the variation of the intercepts (○) and slopes (●) of the double-reciprocal plots of (a) with the reciprocal of the NAD⁺ concentrations.

aldehyde have been ignored for the drawing of the linear plots of Fig. 2. These phenomena will be considered later. For butyraldehyde entirely similar results were obtained with the concentration ranges NAD⁺ 1–500 μM and butyraldehyde 0.12–60 μM . Substrate activation occurred with 500 μM -

NAD⁺ at butyraldehyde concentrations over 150 μM .

The linear secondary plots permitted the initial-rate parameters of eqn. (1) to be determined (Dalziel, 1957), and these are given in Table 1, together with the values of Michaelis constants for NAD⁺ (ϕ_1/ϕ_0) and for aldehyde (ϕ_2/ϕ_0) and the ratio (ϕ_{12}/ϕ_2). Table 1 further shows values for K_m for NAD⁺, K_m for propionaldehyde and K_{E-NAD^+} (the dissociation constant of the enzyme-NAD⁺ complex), determined in 25 mM-phosphate buffer, pH 7.6, and in 33 mM-sodium pyrophosphate buffer, pH 9.3, by MacGibbon *et al.* (1977a). In aqueous solution, aldehydes exist partially as hydrated forms, but only the unhydrated forms are substrates for aldehyde dehydrogenase (Bodley & Blair, 1971). Accordingly, the parameters ϕ_2 and ϕ_{12} are given after correction for the states of hydration of the aldehyde, which were taken as propionaldehyde (42% hydrated) and butyraldehyde (22.3% hydrated), the values being calculated from the gem-diol dissociation constants given by Bell (1966).

Working to very low substrate concentrations (approx. 0.2 μM) with aldehyde dehydrogenase leads to certain difficulties. Observed rates are necessarily very slow and difficult to measure, even though the enzyme concentration may approach 10% of the substrate concentration. Blank reduction of NAD⁺ in the absence of added aldehyde becomes more significant, and corrections are often required. These problems lead in some cases to rather poor agreement in estimates of initial rate parameters obtained in full duplicate experiments. This is indicated in Table 1. Nevertheless, in spite of these difficulties our results for propionaldehyde at pH 7.0 are in fair agreement with those of MacGibbon *et al.* (1977a) at pH 7.6

An interesting feature of the results in Table 1 is the relative constancy of the terms ϕ_0 and ϕ_1 on changing aldehyde substrate. There is only a 20% increase in ϕ_0 on passing from propionaldehyde to butyraldehyde, but it seems to be real. MacGibbon *et al.* (1977a) report no effect of changing from propionaldehyde to [1-²H]propionaldehyde on the burst rate constant for NADH production, and they conclude that hydride transfer is not rate-limiting for propionaldehyde oxidation. For butyraldehyde this step may be partly rate-limiting. It is unfortunate that ϕ_1 has proved difficult to estimate accurately. Nevertheless this term seems to be insensitive to change of substrate, as would be expected if the enzyme conformed to a compulsory-type mechanism, with NAD⁺ binding before aldehyde (Dalziel, 1975). Such a mechanism is of course common with the NAD(P)-linked dehydrogenases, and has indeed been proposed for cytoplasmic aldehyde dehydrogenase by MacGibbon *et al.* (1977c). In fact these authors suggest a special case of the compulsory-

Table 1. Initial-rate parameters for aldehyde oxidation at pH 7.0, 25°C

Substrate	ϕ_0 (min)	ϕ_1 ($\mu\text{M} \cdot \text{min}$)	ϕ_2 ($\mu\text{M} \cdot \text{min}$)	ϕ_{12} ($\mu\text{M}^2 \cdot \text{min}$)	$V_{\text{max}} \cdot \left(\frac{1}{\phi_0}\right)$ (min^{-1})	K_m for NAD (μM)	K_m for aldehyde (μM)	ϕ_2^* ($\mu\text{M} \cdot \text{min}$)	ϕ_{12}^* ($\mu\text{M} \cdot \text{min}$)	$\frac{\phi_{12}}{\phi_2}$ (μM)
Propionaldehyde	0.035 (± 0.001)	0.08 (± 0.04)	0.065 (± 0.005)	1.34 (± 0.1)	29	1.9	2.3	0.04	0.78	21
Butyraldehyde	0.043 (± 0.002)	0.11 (± 0.04)	0.008 (± 0.004)	0.17 (± 0.08)	23	2.6	0.19	0.006	0.13	21.3
Propionaldehyde†						2.4	1.4			8.0
Propionaldehyde‡						10.4	5.0			8.4

* Parameters corrected for the degree of hydration of the aldehyde substrate as described in the text.

† Data of MacGibbon *et al.* (1977a) at pH 7.6.

‡ Data of MacGibbon *et al.* (1977a) at pH 9.3.

order mechanism, namely the Theorell–Chance mechanism. Constancy of ϕ_0 as well as ϕ_1 on changing substrate would support such a conclusion (Dalziel, 1957).

For a simple compulsory-order mechanism, $1/\phi_1$ is expected to be equal to the specific rate of combination of NAD^+ with the enzyme (Dalziel, 1957), and this rate must be invariant with change of aldehyde substrate. The mean value for $1/\phi_1$ determined from the data of Table 1 is $10.5 \mu\text{M} \cdot \text{min}^{-1}$, equivalent to $1.75 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$, which is to be compared with a value of $k_{+1} = 1.8 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$ for NAD^+ combination at pH 7.6, determined from protein-fluorescence quenching studies by MacGibbon *et al.* (1977c). An ordered mechanism also predicts that $\phi_{12}/\phi_1\phi_2 = k_{-1}$ (the rate constant for dissociation of NAD^+ from the E– NAD^+ complex). The data of Table 1 enable a mean value for $\phi_{12}/\phi_1\phi_2 = 3.8 \text{s}^{-1}$ to be calculated. MacGibbon *et al.* (1977c) determined $k_{-1} = 2 \text{s}^{-1}$, at pH 7.6. Thus, assuming that there are only small changes in k_{+1} and k_{-1} on changing pH from 7.6 to 7.0, the data appear reasonably consistent with an ordered mechanism with NAD^+ being the first substrate to bind.

For any ternary-complex mechanism, the ratio ϕ_{12}/ϕ_2 should be equal to the ratio k_{-1}/k_{+1} ($=K_{\text{E-NAD}^+}$), and this should be independent of the substrate. The variation in ϕ_{12}/ϕ_2 is small compared with the variation in the individual parameters, and the values are to be compared with $k_{-1}/k_{+1} = 11.1 \mu\text{M}$, at pH 7.6 (MacGibbon *et al.*, 1977c). A direct study of NAD^+ binding to aldehyde dehydrogenase by means of equilibrium dialysis (G. J. Hart & F. M. Dickinson, unpublished work) has yielded a value for $K_{\text{E-NAD}^+}$ of about $7 \mu\text{M}$ at pH 7.0, $I/0.1$. In addition to the ability to oxidize aldehydes in the presence of NAD^+ , aldehyde dehydrogenase is able also to catalyse the hydrolysis of nitrophenyl esters. The esterase activity is stimulated by the presence of coenzymes, and MacGibbon *et al.* (1978) have found that, at pH 7.6 in 25 mM-phosphate buffer, half-maximum stimulation occurs at about $7 \mu\text{M-NAD}^+$.

It is unfortunate that the reaction catalysed by aldehyde dehydrogenase is not readily reversible, so that it is not possible to use other tests of mechanism described by Dalziel (1957). In the presence of acid anhydrides, aldehyde dehydrogenase is partially reversible and will catalyse the oxidation of NADH (Hart & Dickinson, 1978), with a maximum rate of 25min^{-1} , independent of the nature of the anhydride ($\phi_0 = 0.04 \text{min}$). This rate is considerably less than that specific rate of dissociation of NAD^+ from the E– NAD^+ complex calculated from $\phi_{12}/\phi_1\phi_2$ as described above. If we assume that the anhydride-mediated reaction after formation of an E– NADH-acyl complex follows exactly the reverse

course of aldehyde oxidation, this calculation serves only to show that the maximum rate of NADH oxidation is not determined by the rate of dissociation of the terminal E– NAD^+ complex. It tells us nothing about the mechanism of the aldehyde-oxidation reaction.

Initial-rate measurements at high substrate concentrations

At high aldehyde concentrations initial-rate measurements deviate from the simple behaviour described by eqn. (1). At approx. $500 \mu\text{M-NAD}^+$, marked activation occurs with propionaldehyde at concentrations greater than approx. $50 \mu\text{M}$ and with butyraldehyde at concentrations greater than approx. $150 \mu\text{M}$. At low NAD^+ concentrations these effects are reversed and marked substrate inhibition is observed. Data for propionaldehyde which show these effects are shown in Fig. 3. Results with butyraldehyde were very similar. Activation leads to a new maximum rate, which for propionaldehyde is about 3.5-fold and for butyraldehyde about 2.5-fold greater than the maximum rate obtained from the extrapolations of the type shown in Fig. 1.

Substrate activation by high concentrations of propionaldehyde was observed by MacGibbon *et al.* (1977a) at pH 7.6, working at a single high NAD^+ concentration ($360 \mu\text{M}$), the degree of activation (3.3-fold) being very similar to that we have observed under similar conditions. On the basis of their observations, they suggested that the activation occurs because of the occurrence at high

Fig. 3. Variation of the reciprocal specific rate of propionaldehyde oxidation at high substrate concentrations at pH 7.0, 25°C

The NAD^+ concentrations were: $\bullet$, $330 \mu\text{M}$; $\circ$, $33 \mu\text{M}$; $\triangle$, $3.3 \mu\text{M}$; $\blacktriangle$, $1.65 \mu\text{M}$. The insert shows a double-reciprocal plot for the data at $330 \mu\text{M-NAD}^+$.

aldehyde concentrations of an increasing amount of reaction flux via an alternative route to ternary complex through an E-aldehyde complex. Thus the compulsory mechanism operating at the lower aldehyde concentrations is modified under the new conditions and a non-equilibrium random-order addition of substrate becomes apparent. This explanation of the activation seems unlikely in the light of Fig. 3. If high concentrations of aldehyde promote a more efficient pathway via an E-aldehyde complex this should occur more easily at low coenzyme concentrations. The inhibition that we observe at low NAD^+ concentrations does not seem to be compatible with this argument. Further, if, as seems to be the case, $360\text{ }\mu\text{M-NAD}^+$ is effectively saturating, routing substrate combination through an E-aldehyde complex, which reacts more rapidly with NAD^+ than does free enzyme, does not lead to activation. The rate was not significantly limited by the rate of NAD^+ combination at the non-activating aldehyde concentrations.

The activation/inhibition characteristics observed in Fig. 3 are in fact similar to those seen with horse liver alcohol dehydrogenase in the oxidation of cyclohexanol (Dalziel & Dickinson, 1966b), and the explanations of these phenomena by modification of the basic compulsory-order mechanism for that enzyme may perhaps be adopted here. If at high aldehyde concentrations it is possible to form an abortive complex (say of the type E-NADH-aldehyde or E-acyl-aldehyde) and the products leave this complex more rapidly than they do the normal rate-limiting complex (perhaps E-NADH or E-acyl), then activation will occur and will be most marked at high coenzyme concentrations because these conditions are optimal for forming high concentrations of this complex. The inhibition at low coenzyme concentrations and high aldehyde concentrations would arise because, under these conditions, the enzyme will be present largely as free enzyme and as E-aldehyde complex, the latter being formed by breakdown of the abortive complex and possibly by direct reaction of free enzyme and substrate. If the E-aldehyde complex reacts more slowly with NAD^+ than does the free enzyme, inhibition will be expected. Although these explanations seem reasonably satisfactory, it must be admitted that the nature of the abortive complex is uncertain. In fluorescence titrations of the enzyme with NADH, such as those described by Dickinson *et al.* (1981), the shape of the binding isotherm was quite unaffected by the presence of large concentrations (approx. 50 mM) of acetaldehyde. This suggests that a complex of the type E-NADH-acetaldehyde does not form, or if it does that it can only be formed in the course of enzyme turnover.

There is one further aspect of enzyme activation that merits mention. Inclusion of high concen-

trations (approx. 0.5 M) of ethanol, methanol, acetone etc. in assays results in increased rates of NADH production, and these enhancements are of the order of 1.5–2.0-fold. This is shown in Fig. 4. It might be thought that these effects reflect a general solvent effect on the enzyme and that possibly activation by high aldehyde concentrations is due to the same cause. This does not seem likely, as activation effects are seen with the aldehydes at very low concentrations (approx. $50\text{ }\mu\text{M}$ for propionaldehyde), and similar concentrations of ethanol have no effect on observed rates. On the other hand, it might be possible for ethanol, methanol etc. to mimic the activation behaviour of the aldehydes by forming abortive complexes. Obviously the affinity of the enzyme binding site for these alternative compounds must be much less than for an aldehyde fulfilling the same role. It may also be noted that the inhibition by high aldehyde concentrations at low NAD^+ concentrations (Fig. 3) is not mimicked by ethanol. With $50\text{ }\mu\text{M}$ -propionaldehyde and $2\text{ }\mu\text{M-NAD}^+$ at 25°C , pH 7.0, no significant effect on the rate was noted on including 0.8 M-ethanol in the mixture.

Stopped-flow experiments at low aldehyde concentrations

Initial-rate studies have indicated a compulsory mechanism for aldehyde oxidation at low aldehyde concentrations, with participation of alternative routes involving the formation of an abortive complex and perhaps an E-aldehyde complex at high aldehyde concentrations. To obtain further

Fig. 4. Activation of propionaldehyde oxidation by ethanol and acetone

The rate of propionaldehyde oxidation was measured in the presence of various concentrations of ethanol (●) and acetone (○) at pH 7.0, 25°C . The assays contained $50\text{ }\mu\text{M}$ -propionaldehyde and $100\text{ }\mu\text{M-NAD}^+$.

information, stopped-flow experiments were performed.

Fig. 5 shows the initial changes in A_{340} occurring on mixing $10\mu\text{M}$ -enzyme with $400\mu\text{M}$ - NAD^+ and $80\mu\text{M}$ -propionaldehyde. Fig. 6 shows the observed fluorescence changes on making similar mixtures but with a lower enzyme concentration. Two premixing conditions were used. In one case enzyme was mixed with a solution of $800\mu\text{M}$ - NAD^+ and $80\mu\text{M}$ -propionaldehyde, and in the other case an enzyme solution containing $800\mu\text{M}$ - NAD^+ was mixed with $80\mu\text{M}$ -propionaldehyde. In experiments where enzyme containing $80\mu\text{M}$ -propionaldehyde was mixed with $800\mu\text{M}$ - NAD^+ , progress curves in both spectrophotometric and fluorescence modes were very similar to those when enzyme was mixed with the NAD^+ /aldehyde mixture. This result was also seen at high aldehyde concentrations. Results obtained on mixing enzyme/aldehyde solutions with NAD^+ are not explicitly shown.

The conditions for the experiments for Figs. 5 and 6 approximate very closely to maximum-rate conditions for measurements at low propionaldehyde concentrations (see Fig. 2). Any contribution from pathways or complexes uniquely associated with activation should be negligible, and the pre-steady-state transients are those associated with establishing the non-activated steady-state maximum velocity. In all cases a burst of NADH production occurs before the steady-state rate is established, thus showing that

Fig. 5. Spectrophotometric record of the propionaldehyde- NAD^+ reaction by using the stopped-flow apparatus with $40\mu\text{M}$ -propionaldehyde at pH 7.0, 25°C

The changes in A_{340} were measured after mixing aldehyde dehydrogenase with a solution of NAD^+ and propionaldehyde (●) or aldehyde dehydrogenase/ NAD^+ with propionaldehyde/ NAD^+ (○). The final concentrations were: enzyme, $5\mu\text{M}$; NAD^+ , $200\mu\text{M}$; propionaldehyde, $40\mu\text{M}$. The insert shows first-order plots of the transient phases seen in the early stages of reactions. The derived rate constants are (○) 10 s^{-1} , (●) 7 s^{-1} .

Fig. 6. Fluorimetric record of the propionaldehyde- NAD^+ reaction by using the stopped-flow apparatus with $40\mu\text{M}$ -propionaldehyde at pH 7.0, 25°C

Changes in fluorescence (ΔF) were monitored after the mixing of aldehyde dehydrogenase with NAD^+ /propionaldehyde (●) or aldehyde dehydrogenase/ NAD^+ with NAD^+ /propionaldehyde (○). The final concentrations were: enzyme, $1\mu\text{M}$; NAD^+ , $400\mu\text{M}$; propionaldehyde, $40\mu\text{M}$. One unit of fluorescence is equivalent to the fluorescence of $1\mu\text{M}$ free NADH . The insert shows first-order plots of the transient phases seen in the early stages of reactions. The derived rate constants are (○) 2.4 s^{-1} , (●) 1.7 s^{-1} .

the rate-limiting step occurs after hydride transfer. This observation has been made by MacGibbon *et al.* (1977c). It is not clear at this stage what the rate-limiting step is, but, according to MacGibbon *et al.* (1977b), NADH release from the binary E- NADH complex is a two-stage process at pH 7.6, 25°C , with first isomerization with a rate constant of 0.2 s^{-1} followed by dissociation with a specific rate of 0.8 s^{-1} . Our observed maximum velocities of about 0.45 s^{-1} (Fig. 2, Table 1) at pH 7.0 and 25°C suggest that the reaction does not proceed via a terminal E- NADH complex of the type ultimately obtained on mixing enzyme and NADH . This conclusion is rather different from that of MacGibbon *et al.* (1977a,b), who obtained values of k_{cat} at $50\mu\text{M}$ -propionaldehyde of 0.082 s^{-1} at pH 7.6, 25°C . This rate is some 5-fold lower than that observed by us, and the disparity is difficult to explain unless, and this seems unlikely, the specific activities of the enzyme preparations are very different.

Further examination of Figs. 5 and 6 shows that the courses of reaction observed by spectrophotometric and fluorimetric changes are different. The bursts seen in the fluorescence mode are considerably slower than those seen spectrophotometrically. The lower enzyme concentration for the fluorimetric experiments has no effect on the

fluorimetric results apart from altering the apparent amplitude of the burst and changing the steady-state rate. This has been established by using the same enzyme concentration as was used in the spectrophotometric measurements. The lower enzyme concentration was adopted for fluorimetric measurements to avoid corrections for fluorescence quenching at later stages of the reaction. Evidently the steady state is not established until about 2 s after mixing (see Fig. 6), although the spectrophotometric changes seem to indicate that this occurs earlier. The transients in A_{340} that are associated with the fluorescence change are obviously difficult to detect. Study of the spectrophotometric changes at different wavelengths might bring rewards in this area. It seems that the initial NADH-containing complex detected by A_{340} has either little or no fluorescence and that subsequent reaction of this complex leads to a second NADH-containing fluorescent complex. Ternary NADH-containing complexes with severely quenched fluorescence have been observed for glutamate dehydrogenase, lactate dehydrogenase and yeast alcohol dehydrogenase (di Franco & Iwatsubo, 1972; Whitaker *et al.*, 1974; Dickinson & Dickenson, 1978).

The magnitude of the bursts observed in the A_{340} measurements of Fig. 5 indicate that with enzyme pre-mixed with NAD^+ 0.86 mol of NADH/mol of enzyme is formed rapidly, whereas for enzyme not pre-mixed with any substrate 0.53 mol of NADH/mol of enzyme is formed. These measurements assume $\epsilon_{340} = 6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and have been repeated several times with different preparations of enzyme, with similar results. Such calculations from the fluorimetric results cannot easily be made, because the fluorescence-enhancement factor of the complex in question is not known. If an enhancement of 10-fold is assumed in the fluorescence of bound NADH, which is roughly what we observe in conventional fluorescence titrations of the enzyme with NADH, then values similar to those given above are found. Our detailed studies (G. J. Hart & F. M. Dickinson, unpublished work) of coenzyme binding by these preparations indicate 1–1.5 coenzyme-binding sites/molecule of enzyme. Inactivation by disulfiram indicates a maximum of 2 active sites/molecule of enzyme (Dickinson *et al.*, 1981). This reason for these rather low values for this tetrameric enzyme are not yet clear, but it is apparent that a large proportion of the coenzyme-binding sites are involved in the transients of Figs. 5 and 6.

From the point of view of elucidating the mechanism of catalysis, a most interesting result shown in Figs. 5 and 6 is that pre-mixing the enzyme with NAD^+ enhances the activity of the enzyme towards the transient phase(s). The rate of the burst is enhanced, and so is its magnitude. The NAD^+

concentration used in these experiments (200 μM final) is saturating, so that the rate of the burst in the unpre-mixed case is not determined by the rate of reaction of enzyme with NAD^+ . That this NAD^+ concentration is saturating with respect to the process in question is to be expected from the measurements of MacGibbon *et al.* (1977c), and was confirmed by us by using 3-fold higher NAD^+ concentration. The shape of the progress curve was quite unchanged. We may also note that the activation by pre-mixing with NAD^+ is a fairly rapid process. In some fluorescence experiments enzyme was mixed with NAD^+ and after a short period this mixture was mixed with a solution of propionaldehyde in the stopped-flow apparatus. The mixing in the stopped-flow apparatus was performed as early as 7 s after pre-mixing enzyme and NAD^+ . Even at this stage the course of the subsequent reaction was unchanged from what was observed after pre-mixing enzyme and NAD^+ for up to 40 min.

It appears that pre-mixing with NAD^+ activates the enzyme with respect to the transient phase of the reaction. Pre-mixing with aldehyde has no observed effect. This course of events evidently favours a compulsory-order mechanism with NAD^+ binding first, but it does not favour a simple compulsory mechanism, since with a saturating concentration of NAD^+ the course of reaction should be the same whether or not the enzyme is pre-mixed with NAD^+ . We suggest that the results are consistent with a compulsory mechanism in which the binary E-NAD^+ complex isomerizes to a new form before reaction with aldehyde can occur. According to MacGibbon *et al.* (1977b), the binding of NADH to the enzyme is a two-stage process involving isomerization of the first complex. We have confirmed that the NADH-binding reaction is biphasic with our preparations of enzyme (Dickinson *et al.*, 1981). If the above interpretation is correct, then the rate of the burst in unpremixed cases is at least partly determined by the rate of isomerization of the E-NAD^+ complex. All problems are not resolved by this scheme, however. MacGibbon *et al.* (1977b) did not observe a biphasic reaction of enzyme with NAD^+ , using protein fluorescence quenching to follow the reaction. Rather they observed a straightforward second-order reaction of enzyme and NAD^+ , with a specific rate similar to that deduced by us from initial-rate measurements (see above) assuming a simple compulsory-order mechanism. [The fact that $\phi_1 = 1/k_{+1}$ apparently is not a great problem, for it may be explained if the specific rate of isomerization of the first E-NAD^+ complex is substantially faster than the rate of dissociation of the complex (Dalziel, 1963).] However, the finding of 1–1.5 coenzyme-binding sites/molecule does not leave much room for explaining the effects of

pre-mixing with NAD^+ by binding to non-catalytic sites and subsequent conformational change in the enzyme. The notion of isomeric E-NAD^+ complexes does at least sit easily with that problem.

Stopped-flow experiments at high aldehyde concentrations

Fig. 7(a) shows the changes in A_{340} on mixing a solution of $10\text{ }\mu\text{M}$ -enzyme with a solution of 66 mM -propionaldehyde and $400\text{ }\mu\text{M}$ - NAD^+ and on mixing a solution of $10\text{ }\mu\text{M}$ -enzyme containing $400\text{ }\mu\text{M}$ -

Fig. 7. Spectrophotometric record of the propionaldehyde- NAD^+ reaction by using the stopped-flow apparatus with 33 mM -propionaldehyde at $\text{pH } 7.0$, 25°C

(a) The changes in A_{340} were measured after mixing aldehyde dehydrogenase with propionaldehyde/ NAD^+ (●) or aldehyde dehydrogenase/ NAD^+ with NAD^+ /propionaldehyde (O). The final concentrations were: enzyme, $5\text{ }\mu\text{M}$; NAD^+ , $200\text{ }\mu\text{M}$; propionaldehyde, 33 mM . (b) Oscilloscope trace showing the change in A_{340} on mixing under the conditions of Fig. 7(a) (O).

NAD^+ with 66 mM -propionaldehyde at $\text{pH } 7.0$, 25°C . Fig. 8(a) shows the fluorescence changes on making the same reaction mixtures. For both spectrophotometric and fluorescence experiments, initiating the reactions by mixing enzyme/aldehyde mixtures with NAD^+ gave very similar results to

Fig. 8. Fluorimetric record of the propionaldehyde- NAD^+ reaction by using the stopped-flow apparatus with 33 mM -propionaldehyde at $\text{pH } 7.0$, 25°C

(a) The changes in fluorescence (ΔF) were measured after mixing aldehyde dehydrogenase with NAD^+ /propionaldehyde (●) or aldehyde dehydrogenase/ NAD^+ with NAD^+ /propionaldehyde (O). The final concentrations were: enzyme $5\text{ }\mu\text{M}$; NAD^+ , $200\text{ }\mu\text{M}$; propionaldehyde, 33 mM . One unit of fluorescence is equivalent to the fluorescence of $1\text{ }\mu\text{M}$ free NADH . The insert shows first-order plots of the transient phases seen in the early stages of reactions. The derived rate constants are (O) 12.6 s^{-1} , (●) 5.1 s^{-1} . (b) Oscilloscope trace showing the changes in fluorescence after mixing under the conditions of Fig. 8(a) (O). The three upper traces were obtained by re-triggering the oscilloscope at the end of the previous scan.

those obtained when enzyme was mixed with NAD^+ /aldehyde mixtures. These results are therefore omitted. The conditions of the experiments correspond to those that give maximal activation (Fig. 3). The experiments involving pre-mixing of enzyme and NAD^+ produce unusual progress curves in both spectrophotometric and fluorescence modes, and photographs of oscilloscope traces are reproduced for inspection in Figs. 7(b) and 8(b). Similar results were obtained by using activating concentrations of the substrates butyraldehyde and acetaldehyde. It is clear that the pre-steady-state changes are complex, and no attempt at detailed interpretation will be made here. It seems likely that much more work will be required to make such an interpretation possible. Several features are noteworthy, however. The activating effect of pre-incubating enzyme and NAD^+ on the subsequent transient phase is still seen in both spectrophotometric and fluorimetric modes. Again the major absorbance changes occur more rapidly than the fluorescence changes. Indeed, in the pre-mixing of enzyme and NAD^+ the initial burst recorded spectrophotometrically now occurs within the dead-time of the instrument. One interesting point is that by both methods of observation the transients are of considerably smaller amplitude than they are at the lower aldehyde concentration. Fig. 7(a) indicates an initial burst of 0.3 mol of NADH /mol of enzyme with both pre-mixing conditions. Unless there is a substantial hypochromic effect on the A_{340} of NADH in the appropriate enzyme complex, it is not clear why the burst should be smaller, especially when high aldehyde concentrations activate the steady-state rate, but it is evident that at least some of the effects of high aldehyde concentrations are manifest within 2–3 ms of mixing. The complexities of the later stages of the transient phases may in part be due to secondary effects of high aldehyde concentration and to the formation of the abortive complex(es) postulated on the basis of the initial-rate experiments. One might see the sharp change in rate at about 300 ms seen in Fig. 7 on mixing the enzyme/ NAD^+ solution with propionaldehyde as a result of activation. The change in rate is of the right order. However, the corresponding fluorescence experiment (Fig. 8) indicates that the steady state is not achieved until after 800–1000 ms. In fact, the change in rate noted on the absorbance trace seems to correspond to completion of the first transient in the fluorescence.

The lack of ready correlation between spectrophotometric and fluorescence records of stopped-flow experiments, i.e. Figs. 5 and 6, Figs. 7 and 8, is unlikely to be due to instrumental artefact. The mixing chambers were the same for each type of experiment, and calibration experiments showed that mixing was complete in less than 3 ms. The

instrument has been calibrated several times in the spectrophotometric mode by following the dehydration of carbonic acid with Bromophenol Blue. Strict first-order behaviour with a rate constant of 15.1 s^{-1} at 21°C was obtained. Dalziel (1953) gives 12 s^{-1} at 18°C . The fluorescence results of Fig. 8 may be compared with similar experiments performed by MacGibbon *et al.* (1977c). In the case where enzyme was pre-mixed with NAD^+ the initial burst is first order (Fig. 8), with a rate constant of 12.6 s^{-1} . MacGibbon *et al.* (1977c) obtained 11.7 s^{-1} at pH 7.6. In the experiment where an NAD^+ /aldehyde mixture was mixed with free enzyme (Fig. 8) an initial lag phase was noted, and was also observed by MacGibbon *et al.* (1977c) with the same pre-mixing arrangement but at pH 7.6. The subsequent part of the transient is first-order, with a rate constant of 5.1 s^{-1} . MacGibbon *et al.* (1977c) obtained 4.4 s^{-1} at pH 7.6 with $830 \mu\text{M}$ -propionaldehyde and $40 \mu\text{M}$ - NAD^+ . Agreement was not so good at low propionaldehyde concentrations. In a similar experiment to Fig. 6 where an enzyme/ NAD^+ mixture was mixed with propionaldehyde, MacGibbon *et al.* (1977c) observed a transient characterized by a specific rate of 5 s^{-1} . We obtained a value of 2.4 s^{-1} .

Conclusion

Initial-rate measurements at low aldehyde concentrations are consistent with a compulsory-order mechanism with coenzyme binding first. Stopped-flow measurements at $50 \mu\text{M}$ -propionaldehyde support this view, but, since preincubation of enzyme and NAD^+ activates the enzyme in the pre-steady state, it is suggested that the mechanism might involve isomeric enzyme- NAD^+ complexes. The activation by high aldehyde concentrations seen at high NAD^+ concentrations in initial-rate measurements is thought to be due to participation of an alternative route of product release involving the formation of an abortive complex containing the substrate aldehyde. Stopped-flow measurements at the high aldehyde concentrations certainly support the view that the reaction mechanism is more complex under these conditions, but the actual mechanism is obscure.

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References

- Bell, R. P. (1966) *Adv. Phys. Org. Chem.* **4**, 1–29
- Bodley, F. H. & Blair, A. H. (1971) *Can. J. Biochem.* **49**, 1–5
- Dalziel, K. (1953) *Biochem. J.* **55**, 79–93
- Dalziel, K. (1957) *Acta Chem. Scand.* **11**, 1706–1723
- Dalziel, K. (1962a) *Biochem. J.* **84**, 240–244
- Dalziel, K. (1962b) *Biochem. J.* **84**, 244–255

- Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1538–1543
- Dalziel, K. (1975) *Enzymes*, 3rd Ed. **11**, 1–60
- Dalziel, K. & Dickinson, F. M. (1966a) *Biochem. Prep.* **11**, 84–88
- Dalziel, K. & Dickinson, F. M. (1966b) *Biochem. J.* **100**, 491–500
- Dickinson, F. M. & Dickenson, C. J. (1978) *Biochem. J.* **171**, 629–637
- Dickinson, F. M. & Berrieman, S. (1979) *Biochem. J.* **179**, 709–712
- Dickinson, F. M., Hart, G. J. & Kitson, T. M. (1981) *Biochem. J.* **199**, 573–579
- di Franco, A. & Iwatsubo, M. (1972) *Eur. J. Biochem.* **30**, 517–532
- Eckfeldt, J. H. & Yonetani, T. (1976) *Arch. Biochem. Biophys.* **173**, 273–281
- Feldman, R. I. & Weiner, H. (1972) *J. Biol. Chem.* **247**, 267–272
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Greenfield, N. J. & Pietruszko, R. (1977) *Biochim. Biophys. Acta* **483**, 35–45
- Hart, G. J. & Dickinson, F. M. (1977) *Biochem. J.* **163**, 261–267
- Hart, G. J. & Dickinson, F. M. (1978) *Biochem. J.* **175**, 753–756
- Horecker, B. L. & Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385–390
- MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977a) *Eur. J. Biochem.* **77**, 93–100
- MacGibbon, A. K. H., Buckley, P. D. & Blackwell, L. F. (1977b) *Biochem. J.* **165**, 455–462
- MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977c) *Biochem. J.* **167**, 469–477
- MacGibbon, A. K. H., Haylock, S. J., Buckley, P. D. & Blackwell, L. F. (1978) *Biochem. J.* **171**, 533–538
- Sidhu, R. S. & Blair, A. H. (1975) *J. Biol. Chem.* **250**, 7899–7904
- Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J. & Gutfreund, H. (1974) *Biochem. J.* **139**, 677–697