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Evidence that the slow conformation change controlling NADH release from the enzyme is rate-limiting during the oxidation of propionaldehyde by aldehyde dehydrogenase

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The displacement of NADH from the aldehyde dehydrogenase·NADH complex by NAD^+ was followed at pH 7.0, and the data were fitted by a non-linear least-squares iterative procedure. At pH 7.0 the decay constants for the dissociation of NADH from aldehyde dehydrogenase·NADH complexes ($1.62 \pm 0.09\ s^{-1}$ and $0.25 \pm 0.004\ s^{-1}$) were similar to the values previously determined by MacGibbon, Buckley & Blackwell [(1977) Biochem. J. **165**, 455–462] at pH 7.6, and apparent differences between these values and those reported by Dickinson [(1985) Biochem. J. **225**, 159–165] are resolved. Experiments at low concentrations of propionaldehyde show that isomerization of a binary *E·NADH complex is part of the normal catalytic mechanism of the enzyme. Evidence is presented that the active-site concentration of aldehyde dehydrogenase is halved when enzyme is pre-diluted to low concentrations before addition of NAD^+ and substrate. The consequences of this for the reported values of $k_{cat.}$ are discussed. A general mechanism for the aldehyde dehydrogenase-catalysed oxidation of propionaldehyde which accounts for the published kinetic data, at concentrations of aldehyde which bind only at the active site, is presented.

INTRODUCTION

The cytoplasmic aldehyde dehydrogenase from sheep liver has been shown to operate by a compulsory-ordered mechanism with NAD^+ as the leading substrate (MacGibbon *et al.*, 1977*a*; Bennett *et al.*, 1982; Hart & Dickinson, 1982). The mechanism shown in Scheme 1 was proposed by Bennett *et al.* (1982) for the oxidation of propionaldehyde at pH 7.6, on the basis of detailed pre-steady-state studies of NADH formation (monitored by absorbance, or by nucleotide and protein fluorescence) and proton release. Scheme 1 is a slightly simplified version of Scheme III of Bennett *et al.* (1982), omitting an isomerization of the E·NAD^+ complex.

In Scheme 1 an isomerization (k_{+3}) of the E·NAD^+· propionaldehyde ternary complex (accompanied by the loss of a single proton) controls the rates of the proton and NADH bursts. Hydride transfer is a fast reversible step, since an isotope effect is not observed for the burst in NADH production (MacGibbon *et al.*, 1977*b*) or proton release (Bennett *et al.*, 1982) and the reaction can be easily reversed by acylating the enzyme·NADH complexes (MacGibbon *et al.*, 1977*b*) with anhydrides such as acetic anhydride (Hart & Dickinson, 1978). Displacement of NADH from the enzyme is a two-step process, involving an isomerization step before the NADH can be released (MacGibbon *et al.*, 1977*c*). It has been assumed (Bennett *et al.*, 1982) that this isomerization step lies on the reaction pathway, mainly because at high concentrations of propionaldehyde (around 20 mM) the steady-state rate apparently becomes equal to the rate constant for isomerization of the *E·NADH complex. In order to account for the $k_{cat.}$ value of $0.08\ s^{-1}$ per active site reported at low propionaldehyde concentrations (200 μM) by MacGibbon *et al.* (1977*a*), it had to be assumed that some other step before this slow isomerization is also partly rate-limiting in the steady-state phase of the reaction at low propionaldehyde concentrations. This step would presumably be acyl-enzyme hydrolysis, which can be quite slow, particularly at acid pH (Buckley & Dunn, 1982), and may be rate-limiting in the steady state for propionaldehyde at pH values below 7 (Motion, 1986).

On the basis of the NADH-displacement experiments and pre-steady-state studies carried out at pH 7.0, Dickinson (1985) and Dickinson & Haywood (1986) have questioned the correctness of the published decay constants for the displacement of NADH from the

$$E \underset{k_{-1}}{\overset{k_{+1}NAD^+}{\rightleftharpoons}} E\cdot NAD^+ \underset{k_{-2}}{\overset{k_{+2}ALD}{\rightleftharpoons}} E\cdot NAD^+\cdot ALD \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} {}^*E\cdot NAD^+\cdot ALD \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}}$$

$$^*E\cdot NADH\cdot ACYL \xrightarrow{k_{+5}} {}^*E\cdot NADH \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} E\cdot NADH \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} E + NADH$$

Scheme 1.

Key: ALD, aldehyde; *E, enzyme conformer; ACYL, acyl.

enzyme by NAD^+ measured by pH 7.6 (MacGibbon *et al.*, 1977*c*) and the magnitudes of the $k_{cat.}$ values that we have previously reported (MacGibbon *et al.*, 1977*a*).

In the present paper we examine the difference between our data and those of Dickinson (1985). The results provide clear evidence not only that the slow conformational change of the enzyme·NADH complex lies on the catalytic pathway for aldehyde dehydrogenase at low propionaldehyde concentrations, but that under these conditions it is mainly rate-limiting in the steady-state phase of the reaction.

EXPERIMENTAL

NADH (grade III) and NAD^+ (grade III) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), propionaldehyde solutions were prepared as described previously (MacGibbon *et al.*, 1977*b*) and cytoplasmic aldehyde dehydrogenase was prepared as described by MacGibbon *et al.* (1979). The active-site concentration was determined by NADH titration (MacGibbon *et al.*, 1979) or from $V_{max.}$ measured by using 20 mM-propionaldehyde and 1 mM-NAD^+ at pH 7.6 and 25 °C, as described previously (Bennett *et al.*, 1982). Under these conditions $k_{cat.}$ has been reported (MacGibbon *et al.*, 1977*a*) as 0.25 s^{-1} per active site. The rapid kinetic experiments monitoring nucleotide fluorescence were performed on a Durrum–Gibson D110 stopped-flow spectrophotometer essentially as described by MacGibbon *et al.* (1977*b*,*c*). The photomultiplier on the spectrophotometer was calibrated with NADH solutions of known concentration.

The spectrophotometer was interfaced to a Data Laboratories (Mitcham, Surrey, U.K.) DL905 transient recorder. The voltage output from the stopped-flow spectrophotometer was stored as 1024 8-bit points in the transient recorder and displayed on an oscilloscope. The data were then transferred to the memory of a Cromemco CS-2 microcomputer for processing and storage under the control of a FORTRAN program described previously (Hardman *et al.*, 1985).

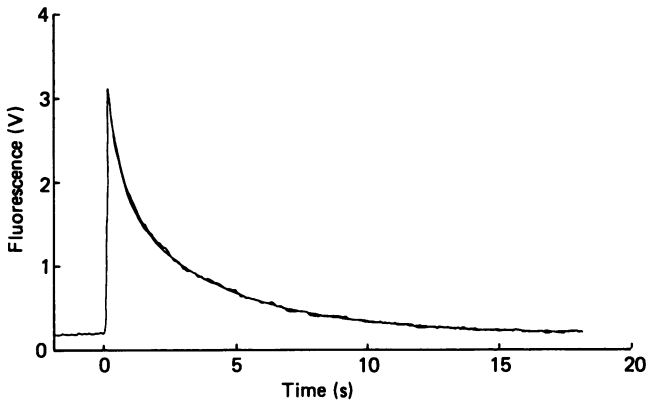

Fig. 1. Biphasic nucleotide-fluorescence decay during NADH displacement

Aldehyde dehydrogenase (9.0 μM in active sites) and NADH (40 μM) in pH 7.0 phosphate buffer ($I = 0.1$ M) from one syringe was rapidly mixed with NAD^+ (2 mM) in a second syringe in the same buffer at 25 °C. The sweep time was 20 s and the voltage setting was 5 V full scale; 52 μM-NADH gave a fluorescence of 6.25 V. The first 100 points represent the pre-trigger level from the previous experiment. The data were fitted as described in the Experimental section, and the reconstructed decay curve is superimposed on the experimental data.

Non-linear least-squares fitting of the data from the single-turnover experiments and the displacement experiments was carried out with a FORTRAN program (Daniel & Wood, 1971; Hardman, 1983) based on the Marquardt (1963) method. Data were fitted to the equation for two exponentials:

$$Y = Y_f + A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} \quad (1)$$

where A_1 and A_2 represent the amplitudes of the two phases and λ_1 and λ_2 represent the corresponding first-order rate constants. In addition, the first-order decay constant for the slower phase of the biphasic nucleotide fluorescence decrease in the displacement experiments was also determined by linear least-squares analysis of $\log(Y - Y_f)$ against time, where Y and Y_f respectively represent the voltage at a given time and the voltage when reaction is complete. The decay constant for the faster phase was then obtained by logarithmic extrapolation and a logarithmic plot of the resulting differences (Gutfreund, 1972, p. 126; MacGibbon *et al.*, 1977*c*).

RESULTS AND DISCUSSION

Determination of decay constants for NADH displacement at pH 7.0

Since our published values for the decay constants for NADH-displacement experiments at pH 7.6 (MacGibbon *et al.*, 1977*c*) have been questioned (Dickinson, 1985; Dickinson & Haywood, 1986) on the basis of similar experiments at pH 7.0, we began by redetermining the values at pH 7.0. The decrease in fluorescence observed when a solution of aldehyde dehydrogenase in pH 7.0 phosphate buffer ($I = 0.1$ M) at 25 °C was mixed with a solution of NAD^+ in the same buffer is shown in Fig. 1. The data were clearly biphasic and were fitted to two exponentials by the iterative non-linear least-squares procedure described above. The amplitude of the fast process constituted 43% of the total fluorescence change, and the values for the decay constants were 1.64 ± 0.06 s^{-1} and 0.25 ± 0.004 s^{-1}. These values differ from those reported previously (2.3 s^{-1} and 0.4 s^{-1}, at pH 7.0) by Dickinson (1985).

In order to demonstrate the correctness of our values, the decay constants and the amplitudes obtained from our fit of the data to eqn. (1) by the non-linear least-squares method were used to reconstruct the biphasic fluorescence-decay curve on the computer by using eqn. (1). The reconstructed curve, when superimposed on the experimental data, gave an excellent fit over more than 90% of the total displacement reaction (Fig. 1). As a second check on the internal consistency of the fitting procedure, the non-linear least-squares curve-fitting program was then used to fit this same computer-generated fluorescence-decay curve after random noise had been added to simulate the experimental data. The values obtained for the decay constants by this procedure ($\lambda_F = 1.62 \pm 0.06$ s^{-1}; $\lambda_S = 0.25 \pm 0.005$ s^{-1}) were identical with the decay constants calculated from the experimental data.

If the fluorescence at infinite time (Y_f) is known, as was the case for the computer-simulated fluorescence-decay curve, the same values of the decay constants can be

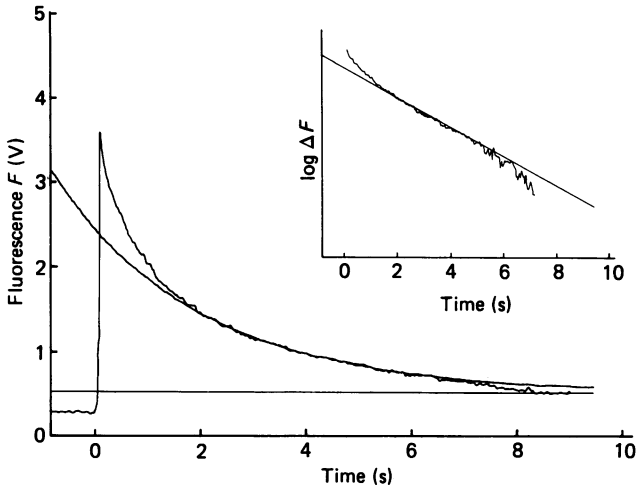

Fig. 2. NADH-displacement data collected over 10 s

Aldehyde dehydrogenase (9.0 μM in active sites) and NADH (40 μM) from one syringe were rapidly mixed with NAD^+ (2 mM) from the second syringe in pH 7.0 phosphate buffer ($I = 0.1$ M) at 25 °C. The sweep time was 10 s and the voltage setting was 5 V full scale. A solution of NADH (52 μM) gave a fluorescence of 6.25 V. The data were fitted by using the logarithm difference procedure described in the Experimental section. The infinity line and regenerated curve for the slow exponential are shown. The insert clearly shows the biphasic nature of the logarithm of the fluorescence difference as a function of time.

obtained by using the logarithm difference procedure described in the Experimental section. For the experimental data (Fig. 1), the pre-trigger fluorescence value (which corresponds to the final fluorescence of the previous reaction, which is still present in the light-path of the stopped-flow spectrophotometer) can be used as the infinity value. The derived decay constants (1.62 ± 0.04 s^{-1} and 0.25 ± 0.004 s^{-1}) were indeed identical with the values obtained by the non-linear curve-fitting procedure, validating this choice of infinity value.

The choice of infinity value is, however, crucial. Dickinson (1985) collected data for the same displacement reaction over only 8 s and, as can be seen from our experimental displacement data collected for only 9 s (Fig. 2), the fluorescence is still decreasing at these times. We used the log difference procedure by fitting an (incorrect) infinity line through the last 100 data points (8.1–9.0 s) of the decay curve (Fig. 2) and obtained values for the decay constants (1.92 ± 0.07 s^{-1} and 0.36 ± 0.004 s^{-1}) similar to those reported by Dickinson (1985). Fitting of the simulated data (obtained as described above with $\lambda_F = 1.64$ s^{-1} and $\lambda_S = 0.25$ s^{-1}) by using an infinity line between 8.1 and 9 s also gave high (2.17 ± 0.05 s^{-1} and 0.38 ± 0.008 s^{-1}) values. The magnitude of the slow decay constant so obtained (which corresponds to a half-life of 1.8 s) is clearly inconsistent with the fact that the fluorescence was still decaying markedly after 9 s (Fig. 2).

The differences between our results and those of Dickinson (1985) appear therefore to arise from his choice of an infinity value from data collected over too short a time interval. A warning is given that, when fitting exponential processes by logarithmic plots, it is essential to obtain a valid and reproducible final value (Y_f) by collecting the data over a sufficient number of

$$*E \cdot NADH \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} E \cdot NADH \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} E + NADH$$

Scheme 2.

half-lives. This is particularly important for biphasic processes, such as observed in the NADH-displacement experiments, where sufficient data must be collected for the slow phase. If this is not done, then invalid rate constants will be obtained. When data from such experiments are fitted by non-linear least squares, this problem does not arise.

We proposed (MacGibbon *et al.*, 1977*c*) that the biphasic nature of the NADH displacement is due to an obligatory conformational change before NADH can be released from the enzyme (Scheme 2).

Alternatively a two-site model, involving displacement of NADH from two different types of binding sites on the enzyme, with different dissociation constants and different rate constants, could also explain the biphasic displacement of NADH. Both models predict that the same decay constants should be observed whether excess enzyme is present or not. However, if there were in fact two different high-affinity NADH sites on the enzyme, these sites should be occupied to different extents when excess enzyme is present; the amplitudes of the two processes should be significantly different from those when NADH is in excess. We carried out a displacement experiment at pH 7.6 using an excess of enzyme (7.0 μM) over NADH (2.6 μM), and observed a biphasic decrease in NADH fluorescence with decay constants and relative amplitudes similar to those reported previously (MacGibbon *et al.*, 1977*c*). This observation confirms that the displacement of NADH from aldehyde dehydrogenase must occur in two steps, as shown in Scheme 2.

Evidence that the *E·NADH complex lies on the catalytic pathway for aldehyde dehydrogenase at pH 7.6

Although the displacement experiments provide evidence for an isomerization of binary E·NADH complexes, they do not show that this isomerization lies on the catalytic pathway in the steady state. However, the second step in Scheme 2, which involves the release of the NADH from the enzyme, must lie on the catalytic pathway, since it has been shown that the NADH is released after the acid product (MacGibbon *et al.*, 1977*a*).

We carried out a single-turnover experiment, in which an excess of enzyme and NAD^+ were mixed in the stopped-flow spectrophotometer with only sufficient propionaldehyde for one passage through the catalytic pathway. The results are shown in Fig. 3. There was an initial fast increase in NADH fluorescence as NADH was formed rapidly, but, in the presence of the remaining NAD^+, the NADH was subsequently displaced from the enzyme. A decrease in fluorescence was observed because free NADH has 5.6-fold lower fluorescence than NADH bound to the enzyme (MacGibbon *et al.*, 1977*c*). The data were fitted to two exponentials by using the non-linear least-squares fitting program. The curve of best fit is shown through the data in Fig. 3 and gave a rate constant for the initial fast increase in fluorescence of 1.23 ± 0.04 s^{-1}, and a decay constant for the slow decrease in fluorescence of 0.305 ± 0.008 s^{-1}.

The rate of NADH formation is partially limited at the low concentrations of propionaldehyde used in the

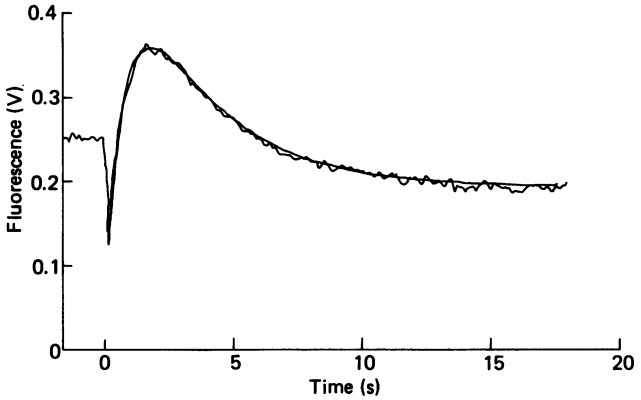

Fig. 3. Single-turnover experiment for the oxidation of propionaldehyde at pH 7.6

Aldehyde dehydrogenase (18 μM) and NAD^+ (2.7 mM) were mixed with propionaldehyde (4.9 μM) in 25 mM-phosphate buffer, pH 7.6, at 25 °C. The sweep time was 20.6 s and the voltage setting was 0.5 V (9.78 μM-NADH gave 1 V). The data were fitted by the iterative procedure described in the Experimental section, and the reconstructed curve is superimposed on the experimental data.

single-turnover experiment by the rate of binding of propionaldehyde to the enzyme. The rate constant for the burst at this concentration of propionaldehyde is similar to that obtained by MacGibbon *et al.* (1977*b*) in their studies of the dependence of the burst rate constant on propionaldehyde concentration. The magnitude of the slow decay constant is similar to the slow decay constant observed in the NADH-displacement experiments. This suggests that the two-step displacement reaction shown in Scheme 2 does lie on the catalytic pathway.

Further evidence was obtained from simulation of the single-turnover experiment by using Scheme 1 with the Continuous System Modelling package on the Prime computer. The following rate constants are assigned the same values as those used by Bennett *et al.* (1982): $k_{+1} = 0.2\ \mu M^{-1}\cdot s^{-1}$, $k_{+2} = 1.0\ \mu M^{-1}\cdot s^{-1}$, $k_{-1} = 50.0\ s^{-1}$, $k_{+3} = 12.0\ s^{-1}$, $k_{-3} = 0.1\ s^{-1}$. Since the hydride transfer step is fast, k_{+4} and k_{-4} were chosen as 100 s^{-1} and 50 s^{-1} respectively. Although the decay constants, λ_F and λ_S, are complex functions of all of the rate constants in Scheme 2, it is possible to extract the individual rate constants from the data (MacGibbon *et al.*, 1977*c*). At pH 7.6 the values of the rate constants which gave the best fit of Scheme 2 to the kinetic data were: $k_{+6} + 0.2\ s^{-1}$, $k_{-6} = 0.05\ s^{-1}$, $k_{+7} = 0.8\ s^{-1}$ and $k_{-7} = 0.5\ \mu M^{-1}\cdot s^{-1}$ (MacGibbon *et al.*, 1977*c*); these values were used in the computer simulations. The initial concentrations of enzyme, NAD^+ and propionaldehyde used in the simulations were the same as those used in the single-turnover experiment (Fig. 3), and k_{+5} was varied to attempt to fit this model to the experimental data.

The experimental data could only be reproduced if k_{+5} was at least 5 s^{-1}, as for smaller values of this constant the slow decay constant for the single-turnover experiment was always significantly less than the measured value of 0.305 s^{-1}. Furthermore, simulations of burst experiments using this model with values of k_{+5} less than about 5 s^{-1} showed pronounced lags after the burst in NADH production before the steady-state production of NADH was established. As shown in Fig. 4, no such lags were observed in the burst experiments at pH 7.6 and at a concentration of propionaldehyde which is saturating for the burst (higher concentrations are needed for saturation in the burst than in the steady state). The data could be fitted to an exponential burst followed by a linear steady state.

Although the single-turnover and burst experiments could be accurately simulated by using Scheme 1 and values of k_{+5} of at least 5 s^{-1}, the possibility still remains that the *E·NADH complex is an inactive form of the enzyme which lies as a shunt to the normal catalytic pathway. The rate of the slow decrease in fluorescence which follows the burst in the single-turnover experiment would be controlled by some step other than isomerization, presumably deacylation (k_{+5}). This possibility was modelled by suitably modifying Scheme 1 so that the *E·NADH complex lay off the catalytic pathway and was in equilibrium (by the step with rate constants k_{+6} and k_{-6}) with E·NADH, which does lie on the catalytic pathway. The value for k_{+5} was again varied, keeping all the other rate constants at their previous values. The changes in fluorescence observed during the single-turnover experiment could be reproduced by this model if k_{+5} was decreased to around 0.3 s^{-1}.

When the burst experiments were simulated by using this model with $k_{+5} = 0.3\ s^{-1}$, the burst in NADH was followed by a slow decrease in the steady-state rate of production of NADH, rather than the linear steady state observed experimentally (Fig. 4). This model is therefore inconsistent with the observed behaviour in the burst experiment, and the two-step release of NADH from the enzyme must lie on the catalytic pathway shown in Scheme 1.

$k_{cat.}$ for the oxidation of propionaldehyde at pH 7.6

At concentrations of propionaldehyde that are sufficient to saturate only the active site, the $k_{cat.}$ calculated from Scheme 1 by using the rate constants specified above is about 0.18 s^{-1} per active site, which is greater than the value of 0.082 s^{-1} per active site which

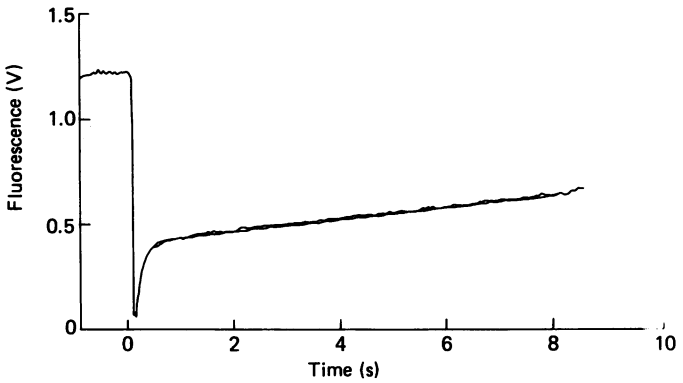

Fig. 4. Burst in NADH fluorescence at saturating concentrations of propionaldehyde at pH 7.6

Enzyme (2.4 μM) and NAD^+ (2.7 mM) were mixed with propionaldehyde (820 μM) in 25 mM-phosphate buffer, pH 7.6, at 25 °C. The concentration of propionaldehyde was sufficient to saturate the active site, but not high enough to cause activation of the steady-state rate. The data were fitted as described in the Experimental section, and the regenerated fluorescence curve is superimposed on the experimental data. The sweep time was 10 s and the voltage setting was 2 V (9.78 μM-NADH gave 1 V).

we reported previously (MacGibbon *et al.*, 1977*a*). The $k_{cat.}$ value of 0.082 s^{-1} per active site would, for Scheme 1 and the rate-constant values given above, require a k_{+5} value of about 0.15 s^{-1}; this value is inconsistent with the fact that no lag phase is seen after the burst in NADH production, as discussed above.

In order to resolve this conflict, and to address the questions raised by Dickinson (1985) about the correct value of $k_{cat.}$, we repeated our measurements of $k_{cat.}$ at pH 7.6. A stock solution of aldehyde dehydrogenase with a tetramer concentration of 1 μM was prepared by pre-dilution of a 50 μM solution in 25 mM-phosphate buffer, pH 7.6. Dehydrogenase assays were carried out at a low concentration of propionaldehyde (171 μM), with different amounts of enzyme added to the cuvette. The buffer, NAD^+ and enzyme were thermally equilibrated at 25 °C before addition of the appropriate propionaldehyde solution. Similar assays were carried out at higher enzyme concentrations by omitting pre-dilution of the enzyme.

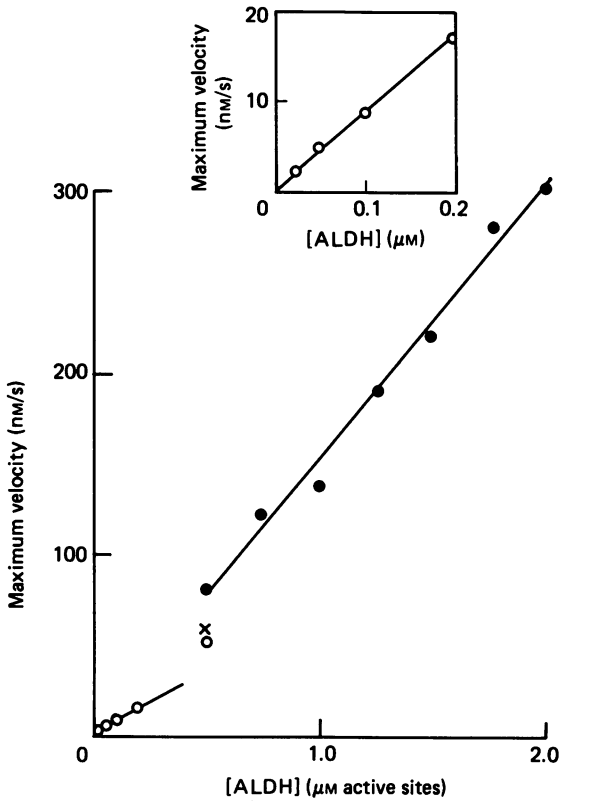

Fig. 5. Plot of $V_{max.}$ versus [E] at low concentrations of propionaldehyde at pH 7.6

Buffer (pH 7.6, 25 mM-phosphate), NAD^+ (1 mM) and various amounts of enzyme (○) prediluted 1:50 in 25 mM-phosphate buffer, pH 7.6, were thermally equilibrated at 25 °C before addition of propionaldehyde (171 μM). The steady-state production of NADH was monitored by absorbance on an Aminco DW-2A spectrophotometer. For undiluted enzyme (●) the propionaldehyde was added within 30 s of the enzyme sample. The point X was obtained by pre-diluting the enzyme 1:50 with an NAD^+ (1 mM)/buffer solution. The enzyme concentration ([ALDH], in μM active sites) was calculated by assuming $k_{cat.}$ = 0.082 s^{-1} per active site (MacGibbon *et al.*, 1977*a*) for the pre-diluted samples from the insert, which shows the data points corresponding to dilutions originally used by MacGibbon *et al.* (1977*a*).

The $V_{max.}$ values for lower enzyme concentrations (with pre-dilution) did not show the same dependence on enzyme concentration as those at higher enzyme concentrations (Fig. 5). Although a linear plot was obtained in each case, the pre-diluted samples gave a slope (corresponding to the $k_{cat.}$ value) approximately half that obtained for the higher concentrations. The lower concentrations correspond to the enzyme concentrations used by MacGibbon *et al.* (1977*a*) in their original determination of $k_{cat.}$ (0.082 s^{-1} per active site) by fluorescence measurements. From the slope of the plot at low concentrations of enzyme (Fig. 5), an active-site concentration of 160 μM was obtained for this enzyme sample. This corresponds to three active sites per tetramer, which ranks among the most active preparations obtained. A change of slope was also observed at 17 mM-propionaldehyde, where substrate activation occurs, and a similar active-site concentration was obtained by using the appropriate value of $k_{cat.}$.

The $k_{cat.}$ value for the enzyme samples which were not prediluted was 0.16 s^{-1} per active site at low propionaldehyde concentrations (171 μM). This is in good agreement with the value determined above by simulating Scheme 1 under these conditions.

The reason for the difference between pre-diluted and stock enzyme samples is not known, but it is clearly related to time. When rates were measured for assays which contained the same total amount of enzyme delivered from the pre-diluted enzyme (which had stood for some time) on the one hand and the stock enzyme on the other, higher rates (Fig. 5, 0.5 μM) were obtained for the stock enzyme sample. When the enzyme sample was pre-diluted in a solution of NAD^+ (1 mM) in 25 mM buffer, pH 7.6, the rate obtained was consistent with those for samples pre-diluted in buffer alone (Fig. 5). Thus NAD^+ did not protect against the dilution-time effect. There was no significant decline in the steady-state rate over 100 s for the assays carried out with stock enzyme for either low or high concentrations of propionaldehyde. This indicates that either the time scale for the dilution effect is much longer than 100 s, or propionaldehyde, in the presence of NAD^+, stabilizes the enzyme against the effects of dilution.

The most obvious explanation for the values obtained on prediluted enzyme samples is that the enzyme is inactivated by approx. 50% by this treatment. Since this dilution/time phenomenon was not previously recognized, the reported $k_{cat.}$ values obtained by dividing the maximum velocities determined on pre-diluted enzyme samples by the active-site concentration determined at higher enzyme concentrations (1–2 μM active sites) by using NADH titration (MacGibbon *et al.*, 1979) are too low. If enzyme concentrations are determined by fluorescence (or sensitive absorbance) measurements by using the $k_{cat.}$ values reported by MacGibbon *et al.* (1977*a*) for prediluted enzyme samples, the correct active-site concentration will be obtained. However, use of these values in assays employing more concentrated enzyme samples which are not extensively prediluted (Bennett *et al.*, 1982; Motion, 1986, for example) will give active-site concentrations which are twice their correct values. Further work is clearly required to understand this phenomenon completely, but it is important to recognize its existence and use the $k_{cat.}$ value appropriate to the experiment under consideration.

Mechanism for the oxidation of propionaldehyde at pH 7.6

The near-identity of the experimental and simulated $k_{cat.}$ values (based on Scheme 1 and the rate constants given above) strongly supports the suggestion that the rate of the E·NADH isomerization step completely controls the steady-state rate of oxidation at pH 7.6. According to Scheme 1, $k_{cat.}$ must always be less than λ_s because of the reversibility of the slow isomerization step. This is clearly the situation for the present data at pH 7.6, since λ_s (0.25 s^{-1}) is significantly greater than the $k_{cat.}$ value (0.16 s^{-1} per active site) at low (100 μM) concentrations of propionaldehyde.

The analysis of the displacement and steady-state data given by Dickinson (1985) must be questioned, even though he reached conclusions about the mechanism of this reaction similar to those reported in the present paper. The slow decay constant for NADH dissociation from the enzyme at pH 7.0 has been shown in the present work to be 0.25 s^{-1} (as at pH 7.6), rather than 0.4 s^{-1} as reported by Dickinson (1985), and hence is less than the $k_{cat.}$ value inadvertently referred to by Dickinson (1985) as $V_{max.}$, with a value of 0.4 s^{-1}. Such a high value for $k_{cat.}$ is clearly inconsistent with Scheme 1, and most likely arises because the $k_{cat.}$ value is reported on the basis of the tetramer (or protein) concentration. (If the number of active sites were two per tetramer, $k_{cat.}$ would become 0.2 s^{-1} and the inconsistency would disappear.) The rate of isomerization of the binary E·NADH complexes is therefore probably the rate-limiting step in the steady-state phase of the reaction at both pH values.

It has been argued that the rate-limiting step in the pre-steady-state phase of the reaction (Bennett *et al.*, 1982) is a conformational change in the ternary enzyme·NAD^+·aldehyde complex at saturating concentrations of aldehyde. This conclusion is based on a large number of experimental results, which must all be taken into consideration when the overall reaction mechanism is being discussed. In particular, any mechanism describing the pre-steady-state phase of the reaction must account for: the hyperbolic concentration-dependence of the burst rate constants on aldehyde concentration (MacGibbon *et al.*, 1977*b*; Eckfeldt & Yonetani, 1976; Bennett *et al.*, 1982); the lack of an isotope effect on the burst rate constant for the production of NADH monitored by absorbance (Eckfeldt & Yonetani, 1976) and fluorescence (MacGibbon *et al.*, 1977*b*) at saturating aldehyde concentrations; the lack of an isotope effect on the proton burst (Bennett *et al.*, 1982); the uncoupling of the proton and NADH-fluorescence bursts for 4-nitrobenzaldehyde (Bennett *et al.*, 1982), for which hydride transfer is probably rate-limiting (MacGibbon *et al.*, 1977*b*); and the identity of the rate constants for the NADH burst in absorbance, protein fluorescence and nucleotide fluorescence for propionaldehyde (MacGibbon *et al.*, 1977*b*) and the absorbance and nucleotide-fluorescence bursts for acetaldehyde (Eckfeldt & Yonetani, 1976; MacGibbon *et al.*, 1977*b*), all at saturating aldehyde concentrations. The mechanism shown in Scheme 1 successfully accommodates all of these results. However, Dickinson & Haywood (1986) have suggested that the observable transient in NADH fluorescence represents acyl-enzyme hydrolysis for propionaldehyde, even though such a suggestion is untenable in the light of the data reported above. Furthermore the results of a pH study of the burst (Motion, 1986) at saturating concentrations of propionaldehyde are quite inconsistent with the proposal, since at pH 5.0, when acyl-enzyme hydrolysis is slow and rate-limiting in the steady state, a fluorescence burst is still observed. The processes controlling the burst must therefore occur before acyl-enzyme hydrolysis, as indicated by the data leading to Scheme 1.

The reasons for the differences in the absorbance and fluorescence bursts and the order of mixing effects reported by Hart & Dickinson (1982) and Dickinson & Haywood (1986) are not clear, but they cannot easily be accounted for by Scheme 1. It is noteworthy, however, that these data were all obtained under non-saturating conditions with respect to propionaldehyde. The propionaldehyde concentration required for half-saturation of the burst is 50 μM (MacGibbon *et al.*, 1977*b*); therefore burst data reported at 40–50 μM concentrations of propionaldehyde (Dickinson, 1985; Dickinson & Haywood, 1986) cannot be considered maximum burst rate constants.

Thus, in summary this model (Scheme 1) explains a wide range of kinetic data for a variety of experiments at concentrations of propionaldehyde which bind only at the active site, and can be regarded as a valid representation of the reaction mechanism for the catalytic pathway of aldehyde dehydrogenase.

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