Biochem. J. (1986) 238, 75–82 (Printed in Great Britain)

Studies on the mechanism of sheep liver cytosolic aldehyde dehydrogenase

The effect of pH on the aldehyde binding reactions and a re-examination of the problem of the site of proton release in the mechanism

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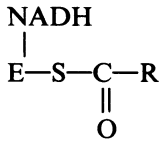
Initial-rate measurements and stopped-flow spectrophotometric experiments over a wide range of pH implicate an enzyme group of $pK_a \sim 6.6$ affecting the aldehyde binding reactions. It is possible, though not proved, that the group involved is the cysteine residue involved in catalysis. Stopped-flow fluorescence studies show that a group of $pK_a > 8.5$ facilitates hydrolysis of the NADH-containing acyl-enzyme species. The identity of this group is quite unknown. Studies with 4-nitrobenzaldehyde show that this substrate gives marked substrate inhibition at quite low ($< 20\ \mu M$) concentrations. The mechanism of catalysis seems to be the same as for propionaldehyde oxidation. It is argued that proton release occurs with both substrates on hydrolysis of the NADH-containing acyl-enzyme and not before hydride transfer, as has been previously suggested [Bennett, Buckley & Blackwell (1982) Biochemistry **21**, 4407–4413].

INTRODUCTION

Recent studies by stopped-flow methods have shown that the first NADH-containing species to be formed in the oxidation of aldehydes with cytosolic aldehyde dehydrogenase exhibits little or no fluorescence characteristic of enzyme-bound NADH (Hart & Dickinson, 1982; Dickinson, 1985). This species can be identified with:



The second NADH-containing species to form exhibits a large enhanced fluorescence characteristic of enzyme-bound NADH and this

complex develops as hydrolysis of the first species proceeds. It seemed worthwhile to examine the pH-dependencies of the reactions leading to the formation and breakdown of these species. The observation of pK_a values might be expected to help identify enzyme groups important in the mechanism of catalysis. The present paper provides some data and conclusions on these points.

Bennett *et al.* (1982) deduced, on the basis of experiments with 250 μM-4-nitrobenzaldehyde as substrate, that proton release in the enzyme mechanism occurred before hydride transfer and was probably associated with a conformational change occurring immediately after aldehyde binding. The conclusion is difficult to reconcile with my previous work (Dickinson, 1985). This suggested that, for propionaldehyde, acyl-enzyme hydrolysis, and presumably release of the acid product, occurs synchronously with the development of the fluorescent E–NADH species, i.e. one step after hydride transfer. Further work in this laboratory has now shown that the oxidation of 4-nitrobenzaldehyde with cytosolic aldehyde dehydrogenase exhibits some rather unusual properties. Marked substrate inhibition occurs even at quite low ($< 20\ \mu M$) concentrations. It seemed that this feature might explain the discrepancies apparently existing in the results with propionaldehyde and 4-nitrobenzaldehyde. One hoped that both aldehydes could be shown to be oxidized by the same mechanism. This, too, is the subject of the present work.

EXPERIMENTAL

Materials

NAD^+ (grade II) was from Boehringer Corp., London W.5, U.K. 4-Nitrobenzaldehyde was from Sigma Chemical Co., Poole, Dorset, U.K. Other chemicals were analytical-reagent grade, where available, obtained from Fisons Chemicals, Loughborough, Leics., U.K. Acetaldehyde, propionaldehyde and butyraldehyde were redistilled before use.

Cytosolic aldehyde dehydrogenase 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 an $A^{1\%}_{280}$ value of 11.3 (Hart & Dickinson, 1983). Enzyme solutions were normally prepared for use by dialysis against 50 mM-sodium phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 100 μM-dithiothreitol.

Methods

Buffer solutions for conventional assays contained 300 μM-EDTA. Initial-rate measurements were performed in a filter fluorimeter of the type described by Dalziel (1962). Aldehyde concentrations were determined spectrophotometrically at pH 7.0 with aldehyde dehydrogenase using a ϵ_{340} value of $6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$ for NADH (Horecker & Kornberg, 1948). The M_r of the enzyme was taken to be 212000 (MacGibbon *et al.*, 1979).

Stopped-flow experiments

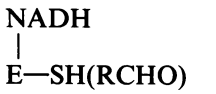
The apparatus used was that described by Hart & Dickinson (1982). The effective cell path length for the spectrophotometric experiments was 2.2 cm. The proton-burst experiments were monitored spectrophotometrically at 560 nm under the conditions described by Bennett *et al.* (1982). Thus solutions contained 10–20 μM-Phenol Red, 0.1 M-Na_2SO_4 and 0.1 M-KNO_3 in 0.5 mM-sodium phosphate buffer, pH 7.6. The enzyme was usually pre-mixed with NAD^+ and was rapidly mixed with a solution containing aldehyde and NAD^+. The solutions were adjusted to pH 7.6 with either dilute HCl or KOH immediately before use. Preliminary experiments confirmed the finding of Bennett *et al.* (1982) that the presence of Phenol Red (15 μM), Na_2SO_4 and KNO_3 does not affect the rate of aldehyde dehydrogenase assays. Further, recovery of unused enzyme solutions from the stopped-flow apparatus at the end of the experiment showed that the enzyme was perfectly stable under the conditions used for the proton-burst experiments.

The magnitude of the proton burst in stopped-flow experiments was obtained by comparing the steady-state rates seen at 340 nm and 560 nm in the stopped-flow apparatus after the completion of the burst phases. The same solutions were used for both measurements. The stoichiometry of the aldehyde dehydrogenase reaction requires the release of 2 mol of H^+/mol of NADH produced. Thus comparing the steady-state records at 340 nm and 560 nm provides a yardstick by which to measure the magnitude of the proton burst in the pre-steady state. It may be noted that the pH changes seen in these experiments were very small because of the presence of significant concentrations (0.5 mM) of phosphate buffer. Only a small fraction (5–10%) of the A_{560} was titrated over the time course of the experiments.

RESULTS AND DISCUSSION

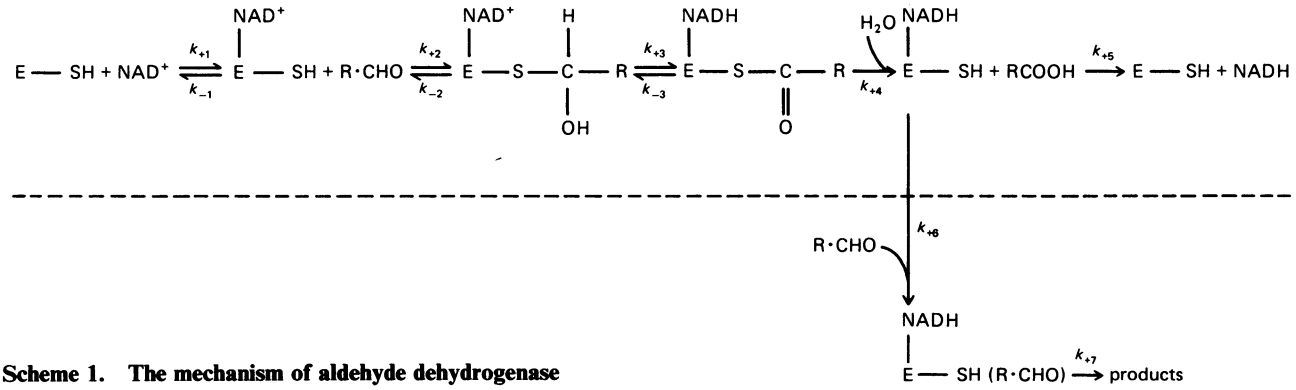
pH effects on the aldehyde binding reactions

Detailed studies by various workers have led to the view that the basic mechanism of cytosolic aldehyde dehydrogenase is that shown in Scheme 1. The compulsory nature of the basic mechanism was established by kinetic studies (MacGibbon *et al.*, 1977*b*; Hart & Dickinson, 1982). The participation of an enzyme thiol group and the formation of an acyl-enzyme species was deduced by analogy with the mechanism of glyceraldehyde 3-phosphate dehydrogenase and from the sensitivity of the enzyme to thiol reagents, particularly disulfiram (tetraethylthioperoxydicarbonic diamide). It was confirmed by spectrophotometric observation of thioester intermediates formed in the oxidation of various derivatives of cinnamaldehyde (Buckley & Dunn, 1982; Dickinson, 1985). The alternative pathway of product release shown in Scheme 1 involving the abortive



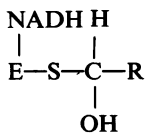
complex is thought to occur only at high substrate concentrations. With propionaldehyde this alternative pathway leads to substrate activation (Hart & Dickinson, 1982), but with 4-nitrobenzaldehyde it leads to substrate inhibition (see below). The activation or inhibition arises because the rate of product release from the abortive complex is faster or slower than it is from the normal terminal complex, E–NADH. Thus, for propionaldehyde, $k_{+7} > k_{+5}$, and for 4-nitrobenzaldehyde, $k_{+7} < k_{+5}$.

Fig. 1 shows the spectrophotometric and fluorescence records after mixing of an enzyme/NAD^+ mixture with propionaldehyde/NAD^+ in the stopped-flow apparatus. Fig. 1 confirms previous findings (Hart & Dickinson, 1982) that the transient in absorbance due to NADH formation precedes the transient due to NADH fluorescence. The steady-state rate is achieved at the end of the fluorescence transient. It may appear superficially that the absorbance record shows an earlier entry into the steady state. This, however, is not so. Careful inspection shows a reproducible 'kink' in the absorbance trace on the same time scale as the fluorescence transient. MacGibbon *et al.* (1977*a*) did not observe any significant



Scheme 1. The mechanism of aldehyde dehydrogenase

The alternative pathway below the broken line is thought to occur only at high substrate concentrations. The structure of the abortive complex may be



Certainly high propionaldehyde concentrations protect the enzyme towards disulfiram in assays (Kitson, 1985). The details of product release from the abortive complex are unknown. For 4-nitrobenzaldehyde $k_{+7} < k_{+5}$; the reverse is true for propionaldehyde.

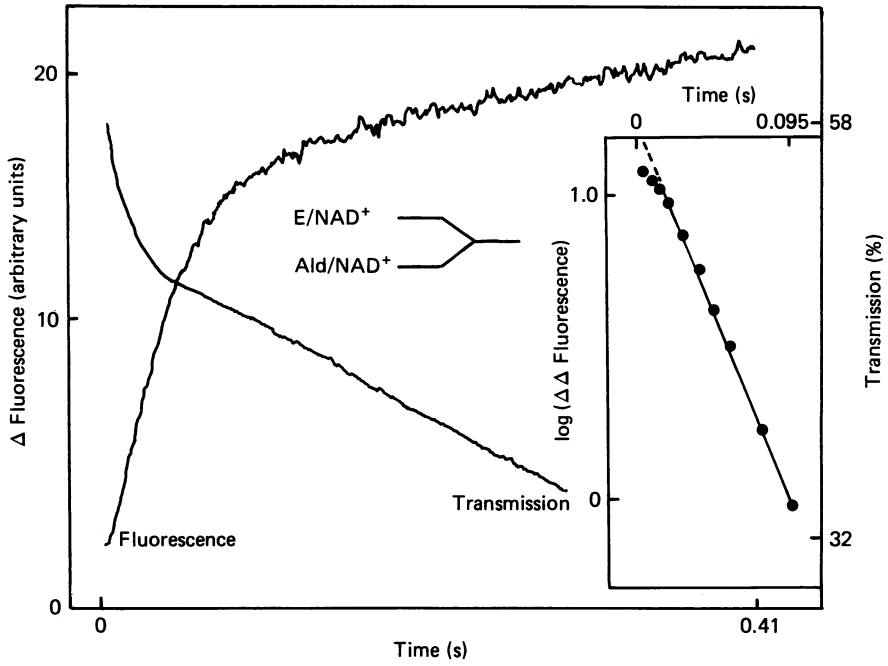
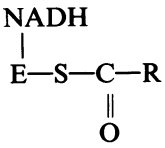


Fig. 1. Stopped-flow studies of the propionaldehyde dehydrogenase reaction

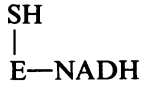
Experiments were conducted at pH 7.0 in 50 mM-sodium phosphate buffer at 25 °C. Solutions of enzyme (E) and NAD^+ were mixed rapidly with solutions containing NAD^+ and propionaldehyde (Ald). These pre-mixing arrangements are indicated. The traces show the decrease in transmission at 340 nm and the increase in fluorescence at 430 nm after mixing. In the spectrophotometric experiments the final conditions were enzyme, 8 μM; NAD^+, 400 μM; and propionaldehyde, 50 μM. For the fluorimetric experiments, 1 μM-enzyme was used with the same reagent concentrations (10 fluorescence units = 0.79 V, and 6 μM-NADH gave a signal of 0.8 V). The inset shows a first-order plot of the fluorescence transient obtained by taking the difference between the observed reading at any time and the reading obtained by extrapolating back to that time from the steady-state phase of the reaction. The first-order rate constant obtained was 3.2 s^{-1}.

difference between the time courses of spectrophotometric and fluorescence transients. Presumably they happened to use a substrate concentration at which the apparent rate constants for the two transients were very similar (see below).

The explanation of the results in Fig. 1 is that the first NADH-containing species to form:

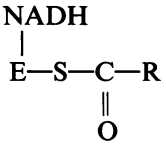


is non-fluorescent, whereas the second,



is. Detailed evidence has been presented in support of these ideas (Dickinson, 1985; Dickinson & Haywood, 1986).

Fig. 2 shows how the first-order rate constants for the spectrophotometric and fluorescence transients respond to changes in the propionaldehyde concentration at pH 7.0. The two behave quite differently, with the absorbance burst showing strong dependence on the propionaldehyde concentration and the fluorescence burst showing little. The latter observation forms part of the evidence that the step is associated with hydrolysis of the



complex. The amplitudes of the two transients are essentially constant over the concentration ranges used. The absorbance experiments, together with an ϵ_{340} value of 5.5×10^3 $M^{-1} \cdot cm^{-1}$ for enzyme-bound NADH (MacGibbon *et al.*, 1979), indicate the formation of about 0.7 mol of NADH/mol of enzyme in the initial burst.

Fig. 3 shows how the transients of Fig. 1 respond to changes in pH over the range 5.2–9.6. For the fluorescence measurements the values of the first-order rate constants ($k_1^{fluor.}$) are plotted. These were obtained by extrapolation of a plot of $1/k_{app.}^{fluor.}$ versus 1/[propionaldehyde] to infinite aldehyde concentration. For the spectrophotometric experiments the second-order rate constants ($k_2^{abs.}$) derived from the slopes of the equivalents of Fig. 2 are plotted. The characteristics of the experiments over the pH range used were as indicated in connection with Fig. 2, but with some minor variation. At pH 5.5 the plot of the spectrophotometric data equivalent to Fig. 2 was concave towards the abscissa,

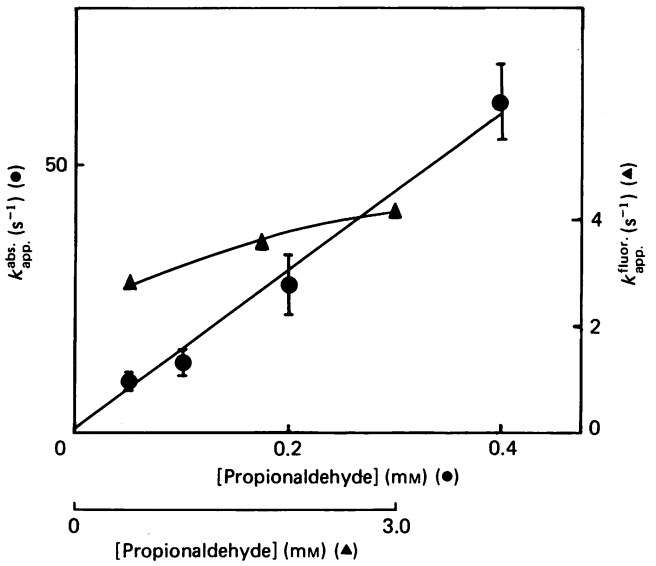


Fig. 2. Dependence of spectrophotometric and fluorimetric transients on the propionaldehyde concentration

The experiments were conducted as for Fig. 1, except that the propionaldehyde concentrations were varied as indicated. The apparent first-order rate constants for the transients in absorbance (●) and fluorescence (▲) were determined by the same procedure as for the inset to Fig. 1. The vertical bars indicate the spread of values obtained in quadruplicate estimates of the rate constants for the spectrophotometric experiments. The fluorescence measurements were much easier to make, and duplicate experiments usually agreed to within 5%.

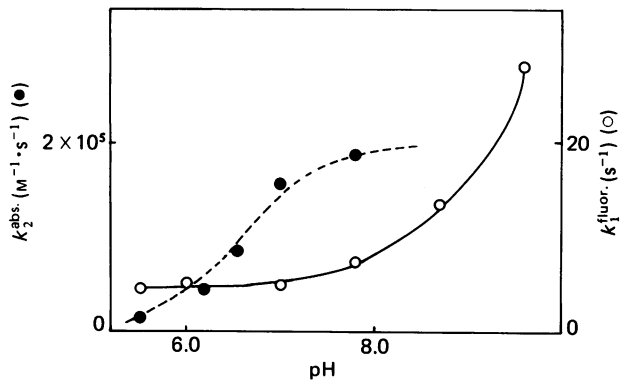
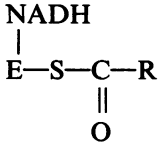


Fig. 3. pH-dependence of spectrophotometric and fluorescence transients seen in stopped-flow experiments with propionaldehyde

All experimental data was obtained at 25 °C. Buffers used were as follows: pH range 6–8, 50 mM-sodium phosphate; pH 8.7 and 9.6, 10 mM-sodium bicarbonate buffer containing 50 mM-Na_2HPO_4; pH 5.5, 50 mM-sodium acetate. Values for $k_1^{fluor.}$ (○), the first-order rate constant controlling the fluorescence burst, and $k_2^{abs.}$ (●) the second-order rate constant controlling the absorbance burst, were obtained as described in the text. The broken line is a theoretical curve applicable to the case where $k_2^{abs.}$ is controlled by a group of pK_a 6.6 and where $k_2^{abs.}$ varies from 0 to 200000 $M^{-1} \cdot s^{-1}$ over the full range of the titration curve.

reaching an apparent maximum value for the rate constant of 70 s^{-1} at 10 mM-propionaldehyde. The point in Fig. 3 from the pH 5.5 experiment is taken from the initial slope of this plot. The amplitudes of the fluorescence transients were larger at more alkaline pH values. Presumably the enzyme complex involved is more fluorescent under these conditions.

Fig. 3 shows that the rate of the fluorescence transient is enhanced at more alkaline pH values. This confirms the earlier work of MacGibbon *et al.* (1977*a*). It is believed (see above) that this process is associated with hydrolysis of the



complex. Unfortunately the data of Fig. 3 are not complete enough to allow an estimate of the pK_a or speculation about the nature of the enzyme group(s) controlling this process. By contrast, the spectrophotometric data of Fig. 3 suggest a pK_a of about 6.6 for a group on the enzyme associated with the aldehyde binding reaction.

We have sought more evidence on the pH-dependence of aldehyde binding by studying the steady-state kinetics of aldehyde oxidation over the pH range 4.75–9.0. The experiments were conducted by varying the aldehyde concentration at a constant and high (400 μM) concentration of NAD^+. Earlier detailed kinetic work (Hart & Dickinson, 1982) suggests that this concentration of NAD^+ would be saturating. In this case the slopes of the Lineweaver–Burk plots equal $K_{m,aldehyde}/k_{cat.}$. Fig. 4 show the variation of $k_{cat.}/K_{m,aldehyde}$ with pH for acetaldehyde, propionaldehyde and butyraldehyde. All the aldehydes show the same broad features, though the pH profile for butyraldehyde is rather flatter than for the other two. The curves are a similar shape to that for the spectrophotometric transient with propionaldehyde in Fig. 3. The initial-rate data appear to be consistent with a group of p$K_a \sim 6.6$ affecting aldehyde binding.

Comparison of the data for propionaldehyde in Fig. 4 and the data for $k_2^{abs.}$ in Fig. 3 shows that the absolute values agree well at pH 5.5 and 6.0, but differ somewhat (about 2-fold) at alkaline pH values. In general one might expect $k_{cat.}/K_{m,aldehyde}$ to be less than, or equal to, the values for the rate constant seen in the stopped-flow apparatus. It is significant here, however, that the good agreement noted is found when the measurements are easiest to make. At low pH the transients are slower and easier to monitor, and with relatively high $K_{m,aldehyde}$ values, measurement of the slopes of Lineweaver–Burk plots is not difficult. At high pH values the reverse is true. It is particularly difficult to determine the slopes of Lineweaver–Burk plots accurately with values of $K_{m,aldehyde}$ of 2 μM or less. In view of these considerations it will be assumed for now that for propionaldehyde estimates of $k_{cat.}/K_{m,aldehyde}$ and $k_2^{abs.}$ measure the same thing.

Steady-state treatment of Scheme 1 yields the following relationship (Dalziel, 1957):

$$k_{cat.}/K_{m,aldehyde} = \frac{k_{+2}k_{+3}k_{+4}}{k_{-2}k_{-3}+k_2k_{+4}+k_{+3}k_{+4}}$$

At pH 7.0 $k_{+4} = 3.2\ s^{-1}$ (Fig. 1) and $k_{-3} \approx 0.5\ s^{-1}$. The lat-

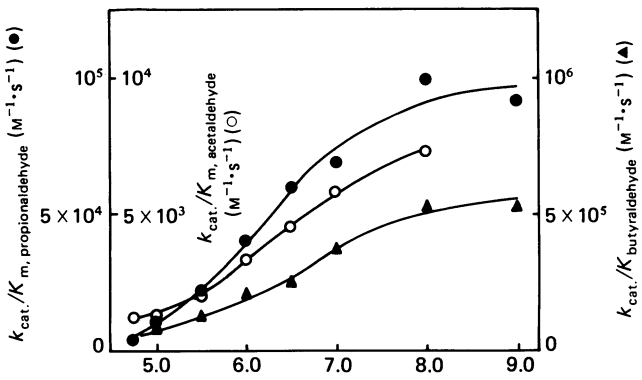


Fig. 4. Dependence of $k_{cat.}/K_{m,aldehyde}$ for various aldehyde substrates

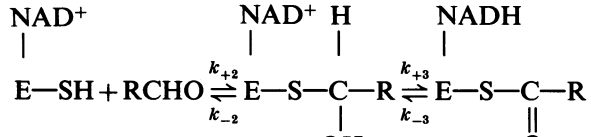
The initial-rate experiments were performed at 25 °C with an NAD^+ concentration of 400 μM. For the pH range 4.75–6.0, 10 mM-sodium acetate buffers containing 50 mM-NaH_2PO_4 were used; for the pH range 6–8, 50 mM-sodium phosphate buffers were used, and for pH 9.0, 10 mM-sodium bicarbonate buffer containing 50 mM-Na_2HPO_4 was used. Values of $k_{cat.}/K_{m,aldehyde}$ were obtained from Lineweaver–Burk plots as described in the text.

ter information is deduced from the fact that the NADH oxidation reactions using acetic or butyric anhydrides as acylating agents give $V_{max.}$ values of about 0.5 s^{-1} and exhibit large primary isotope effects when [4A-^{2}H]NADH replaces NADH (Hart & Dickinson, 1978). Thus $k_{-2}k_{+4} \gg k_{-2}k_{-3}$. So, if $k_{+3} \gg k_{-2}$, $k_{cat.}/K_{m,aldehyde} = k_{+2}$, and if $k_{+3} \ll k_{-2}$:

$$k_{cat.}/K_{m,aldehyde} = \frac{k_{+2}k_{+3}}{k_{-2}} = \frac{k_{+3}}{K_{aldehyde}}$$

$K_{aldehyde}$ is the dissociation constant for aldehyde from the reactant ternary complex.

In interpreting the spectrophotometric transients, the following simplification of Scheme 1 is appropriate:



The spectrophotometric change is associated with the second of the two steps. As only one transient is observed and no lag phase occurs, a reasonable assumption is that the first step equilibrates rapidly. According to Gutfreund (1972) this leads to the following relationship:

$$k_{obs.} = k_{-3} + \frac{k_{+3}}{1 + \frac{K_{aldehyde}}{[aldehyde]}}$$

where $K_{aldehyde} = k_{-2}/k_{+2}$ as before, and $k_{obs.}$ is the observed first-order rate constant. The plot of $k_{obs.}$ versus [aldehyde] will be concave towards the abscissa and will reach a plateau value ($k_{obs.} = k_{-3} + k_{+3}$) at high aldehyde concentrations. The slope of the plot at low aldehyde concentrations will be $k_{+3}/K_{aldehyde}$. If an alternative and probably less likely scenario is adopted where, at all aldehyde concentrations used, k_{+2} [propionaldehyde] $\ll k_{+3}$ and the equilibria lie predominantly to the right, the observed transients will be effectively determined by k_{+2} [propionaldehyde]. The plot of $k_{obs.}$ versus aldehyde concentration will then be linear with slope equal to k_{+2}.

We have then the possibilities that:

$$k_{cat.}/K_{m,aldehyde} = k_2^{abs.} = k_{+3}/K_{aldehyde}$$

or

$$k_{cat.}/K_{m,aldehyde} = k_2^{abs.} = k_{+2}$$

The analysis suggests that the pK_a value of ~ 6.6 observed in Fig. 3 and supported by the data of Fig. 4 is associated with a single amino acid residue that controls k_{+2}, k_{+3} or $K_{aldehyde}$. It is possible, though, that the observed pK_a value arises from a combination of effects on these parameters. It is tempting to assign the pK_a value to k_{+2} and thus to the cysteine residue involved in the mechanism. This could be an oversimplification, however. It might be that the group in question influences the reactions involved rather than functioning directly in the reaction mechanism.

Proton release in the enzyme mechanism and studies with 4-nitrobenzaldehyde

Bennett *et al.* (1982) performed stopped-flow studies with cytosolic aldehyde dehydrogenase at pH 7.6 with Phenol Red as indicator. With propionaldehyde as substrate they observed a burst of protons equivalent to 1 mol/mol of enzyme, with an observed first-order rate constant of 8.1 s^{-1}. These experiments were repeated here under almost identical conditions and with similar results. With 10 μM-enzyme, 50 μM-propionaldehyde and 400 μM-NAD^+, a burst of 13.5 μM-protons was observed at 560 nm with a first-order rate constant of 4.5 s^{-1}. The results were virtually the same when 500 μM-propionaldehyde was used. When the reactions were monitored at 340 nm using the same solutions, the bursts were 0.5 mol of NADH/mol of enzyme, and first-order rate constants were 13.5^{-1} and 60 s^{-1}, essentially as seen at pH 7.0 in Figs. 1 and 2. Comparison of the results with Phenol Red and Fig. 3 indicates a reasonable correlation between the rate constant for the fluorescence transient (6 s^{-1} at pH 7.6) and that for the proton burst (4.5 s^{-1}). If the fluorescence transient is associated with acyl-enzyme hydrolysis and release of the acid product as suggested (Dickinson, 1985) this agreement is expected. One may note here that the lag phases seen in the early stages of the proton-burst experiments (Bennett *et al.*, 1982) and observed here also correlate well with the lags seen in the early stages of development of the fluorescence transient (Hart & Dickinson, 1982; see also Fig. 1).

The interpretation just given is at variance with the conclusions of Bennett *et al.* (1982). They showed that, with 250 μM-4-nitrobenzaldehyde as substrate, proton bursts of the same amplitude and on a similar time scale as for propionaldehyde occurred, but no spectrophotometric or fluorescence transients like those of Fig. 1 could be demonstrated. The proton-burst experiments have been confirmed in the present work. With the same conditions as those used by Bennett *et al.* (1982), and with 20 μM- and 250 μM-4-nitrobenzaldehyde, bursts of 1.3 and 1.4 mol of H^+/mol of enzyme were obtained with apparent first-order rate constants of 2.8 and 3.0 s^{-1}. Bennett *et al.* (1982) concluded on the basis of their experiments that proton release must precede hydride transfer (and so could not correlate with acyl-enzyme

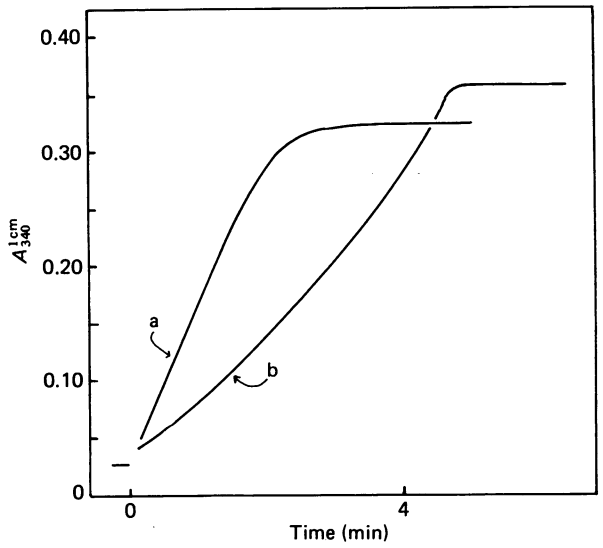


Fig. 5. Spectrophotometric assay of propionaldehyde and 4-nitrobenzaldehyde with aldehyde dehydrogenase

Experiments were conducted in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C. Assay mixtures contained 400 mM-NAD^+ and either (a) 48 μM-propionaldehyde or (b) 54 μM-4-nitrobenzaldehyde. The enzyme concentration was 0.3 μM.

hydrolysis). They suggested that proton release is associated with a conformational change in the enzyme occurring immediately after aldehyde binding to the

$$\begin{array}{l} NAD^+ \\ | \\ E\text{—}SH \end{array}$$

complex. The following experiments argue against this conclusion and support the proposal made above that proton release occurs as acyl-enzyme hydrolysis proceeds.

Fig. 5 shows the results of spectrophotometric assays of propionaldehyde and 4-nitrobenzaldehyde with aldehyde dehydrogenase. The behaviour of the 4-nitrobenzaldehyde assay is unusual. The reaction rate accelerates throughout the assay and is still accelerating as the substrate is suddenly exhausted. Clearly the K_m for 4-nitrobenzaldehyde is very low, and much lower than for propionaldehyde, but also 4-nitrobenzaldehyde oxidation is subject to substrate inhibition at low (< 20 μM) concentrations.

Substrate activation by high (> 50 μM) concentrations of propionaldehyde has been explained by abortive complex-formation (Hart & Dickinson, 1982). Formation of the

$$\begin{array}{l} NADH \\ | \\ E\text{—}SH\ (propionaldehyde) \end{array}$$

complex has since been observed (Dickinson, 1985). The explanation of the activation is that this complex releases products more quickly than does the normal rate-limiting complex,

$$\begin{array}{l} NADH \\ | \\ E\text{—}SH \end{array}$$

(see Scheme 1). Suppose that an analogous

$$\begin{array}{l} NADH \\ | \\ E\text{—}SH(4\text{-nitrobenzaldehyde}) \end{array}$$

complex can form very rapidly and that it has a very small dissociation constant, has a severely quenched fluorescence and releases product more slowly than the

$$\begin{array}{l} NADH \\ | \\ E\text{—}SH \end{array}$$

complex. Then, fluorescence transients would not be seen in stopped-flow experiments using high 4-nitrobenzaldehyde concentrations, and the substrate inhibition would be easily explained. The suggestion that the abortive complex might have a severely quenched fluorescence is reasonable when one considers that the acyl-enzyme species

$$\begin{array}{l} NADH \\ | \\ E\text{—}S\text{—}C\text{—}R \\ \quad\quad \| \\ \quad\quad O \end{array}$$

also has little or no NADH fluorescence.

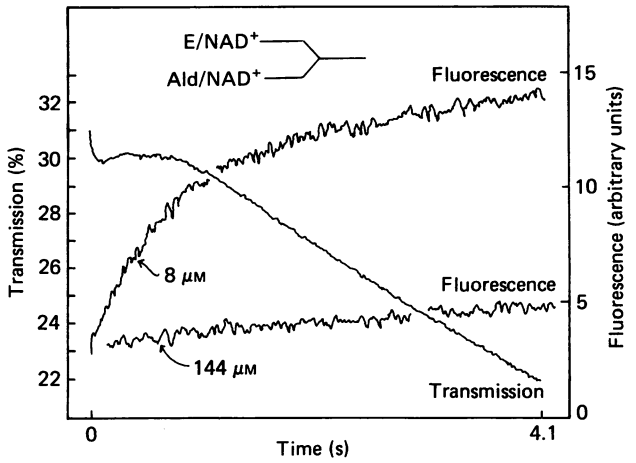
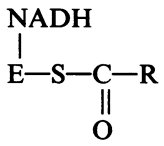


Fig. 6. Stopped-flow studies of the 4-nitrobenzaldehyde dehydrogenase reaction

Experiments were conducted at pH 7.6 and at 25 °C. Solutions of enzyme (E) and NAD^+ were mixed rapidly with solutions containing NAD^+ and 4-nitrobenzaldehyde (Ald). The pre-mixing arrangements are indicated. The traces show the decrease in transmission at 340 nm and the increase in fluorescence at 430 nm after mixing. In the spectrophotometric experiment the final conditions were: enzyme, 10 μM; NAD^+, 400 μM; and 4-nitrobenzaldehyde, 20 μM. This experiment was performed with solutions also used for pH-burst experiments (see the text). Solutions, therefore, contained 10 μM-Phenol Red, 0.5 mM-sodium phosphate buffer, pH 7.6, 0.1 M-KNO_3 and 0.1 M-Na_2SO_4. The apparent first-order rate constant of 54 s^{-1} for the initial transient (see the text) was determined from similar experiments, but with a more suitable time scale and with greater amplification. The record given here is chosen to facilitate comparison with the fluorescence record. For the fluorimetric experiments 1 μM-enzyme was used with 400 μM-NAD^+ and either 8.0 or 144 μM-4-nitrobenzaldehyde as indicated (10 fluorescence units = 0.8 V, and 6 μM-NADH gave a signal of 0.8 V).

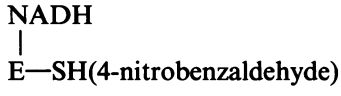
Fig. 6 shows the spectrophotometric and fluorescence records after mixing enzyme/NAD^+ mixtures with 4-nitrobenzaldehyde/NAD^+ mixture at pH 7.6, 25 °C. When 20 μM-substrate is used, there is a rapid transient in A_{340}. The amplitude of the burst corresponds to 0.13 mol of NADH/mol of enzyme, with an apparent first-order rate constant for the process of 54 s^{-1}. The fluorescence trace for similar conditions shows a clear transient with a first-order rate constant of 2 s^{-1}. When the 4-nitrobenzaldehyde concentration was increased to 144 μM, the transient in fluorescence was completely abolished. No transient in absorbance, either, was detectable, under these conditions. These results with high substrate concentrations confirm the findings of Bennett *et al.* (1982).

The results in Fig. 6 with 20 μM-4-nitrobenzaldehyde are very similar to those for 50 μM-propionaldehyde in Fig. 1. The only differences are in the amplitudes of the transients, particularly that in absorbance, which is about five times smaller with the aromatic substrate, and the more widely separated rates of the two transients with 4-nitrobenzaldehyde. It may be concluded that the basic mechanism of oxidation of the two aldehydes is the same, and is as shown in Scheme 1. As with propionaldehyde, the first-order rate constant for proton production (3 s^{-1}) correlates reasonably well with that for the fluorescence transient (2 s^{-1}). Again it is reasonable (see above) to conclude that proton release at the level of about 1 mol of H^+/mol of enzyme occurs on hydrolysis of the



complex.

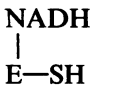
The lack of fluorescence transient with 250 μM-4-nitrobenzaldehyde occurs apparently because of the severely quenched fluorescence of the



complex. No significant concentrations of the terminal enzyme–coenzyme complex accumulate, presumably because of the extremely rapid formation of the abortive complex. That such a complex can form is readily demonstrated. Addition of 5–50 μM-4-nitrobenzaldehyde to a mixture of 2.7 μM-enzyme and 11.7 μM-NADH causes increasing and marked quenching of the emission peak (430 nm) characteristic of bound NADH. There is no indication that the 430 nm peak is shifted to longer wavelengths on the binding of 4-nitrobenzaldehyde, which would suggest an increased disposition to dissociate NADH.

These arguments explain the substrate inhibition by high 4-nitrobenzaldehyde concentrations and the lack of fluorescence transient. They do not explain the apparent absence of an absorbance transient under these conditions. It seems likely that this is due to a technical matter. As we have seen with 20 μM-4-nitrobenzaldehyde (Fig. 6), the absorbance transient is small. Fig. 2 suggests that increasing substrate concentration to 144 μM will dramatically increase the rate constant of the process from what is seen with 20 μM-substrate. ($k_{obs.}$ = 54 s^{-1}). One might expect that the transient would be completed within the mixing time of the stopped-flow apparatus (~ 2 ms), and no burst would be seen on the record. Observing a small 'dead-time' transient occurring against a high background absorbance created by the presence of Phenol Red and high concentrations of NAD^+ would be very difficult.

A particularly convincing demonstration of the effects of large concentrations of 4-nitrobenzaldehyde on fluorescence-transient experiments is shown in Fig. 7. Experiments were conducted at 4 °C in a filter fluorimeter, and assays were initiated by adding the aldehyde substrate. At the low temperature the fluorescence burst is slow enough that the tail end of it can be seen with conventional recording equipment. Increasing concentrations of 4-nitrobenzaldehyde yield smaller bursts. At the lowest concentration used the burst is smaller, but of similar amplitude to that seen with 50 μM-propionaldehyde. When a high concentration of 4-nitrobenzaldehyde is added to an assay mixture in which the reaction has been initiated earlier with a low concentration of this substrate and which has now reached the steady state, fluorescence equivalent to that created in the initial burst is lost in the mixing time. The



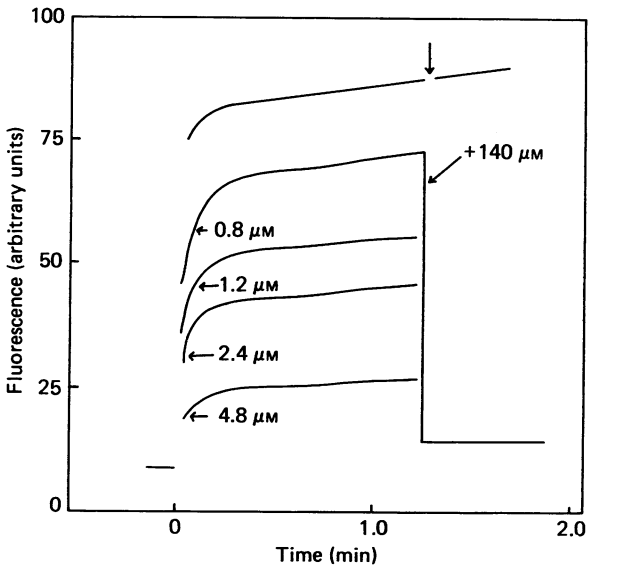


Fig. 7. Fluorescence records of reactions involving the oxidation of 4-nitrobenzaldehyde and propionaldehyde

Reactions were conducted in a filter fluorimeter at 4 °C in 50 mM-phosphate buffer, pH 7.0, and were initiated by adding the aldehyde. The top trace shows the record obtained in the oxidation of 50 μM-propionaldehyde. There is a large 'burst', followed by the steady-state phase of the reaction. At the arrow, a further 140 μM-propionaldehyde was added. The other records refer to the initial phases of the oxidations of the various concentrations of 4-nitrobenzaldehyde indicated. After about 80 s, a further 140 μM-substrate was added with immediate quenching of the fluorescence and inhibition of the reaction rate.

present is rapidly converted into the abortive complex, and, since product release from the abortive complex is slower than from the binary enzyme–coenzyme complex, substrate inhibition is established.

Finally it is interesting to return to the question of the site of proton release in the enzyme mechanism. The results of Bennett *et al.* (1982) show release of about 1 mol of H^+/mol of enzyme in the pre-steady-state phase. The results obtained here agree fairly well, with about 1.3 mol of H^+/mol of enzyme. The stoichiometry of the overall reaction of aldehyde dehydrogenase requires the release of 2 protons per cycle. Thus:

$$R\cdot CHO + NAD^+ + H_2O \rightarrow RCO_2^- + NADH + 2H^+$$

Clearly the story is incomplete. A difficulty here is the precise concentration of functioning active centres in enzyme preparations. Detailed coenzyme binding studies have indicated about 1 mol of NAD^+- or NADH-binding sites/mol of enzyme (Hart & Dickinson, 1983). If these estimates represent the concentrations of functioning active centres, the proton-burst experiments account for just over half the number of protons released. Spectrophotometric-burst experiments with propionaldehyde (Fig. 1) indicate about 0.7 mol of NADH/mol of enzyme, suggesting that the concentration of functioning sites in the enzyme preparations used here is about 0.7 of the enzyme concentration. Now clearly this is a procedure which might underestimate the functioning-site concentration. The result, with saturating substrate concentration, depends on the relative magnitudes of k_{+4}, k_{+3} and k_{-3} (Scheme 1). The data given here for pH 7.0 suggest that $k_{+4} = 3.2\ s^{-1}$, $k_{+3} > 70\ s^{-1}$ and $k_{-3} \sim 0.5\ s^{-1}$, so that the experiment might well give an accurate estimate of the active-site concentration (Dalziel, 1975). If this is accepted, then the proton-burst experiments, especially those done here, suggest that both protons are accounted for in the pre-steady-state phase. The protons are released synchronously with acyl-enzyme hydrolysis and neither is coupled directly to hydride transfer.

An alternative argument to the above is that only 1 mol of H^+/mol of enzyme is released in the proton-burst experiments and the second is released at a later stage in the mechanism. Since the time courses of the fluorescence and proton-burst experiments are the same, they enter the steady state together. As the steady-state rate is determined by NADH release from

(Hart & Dickinson, 1982; Dickinson, 1985) the second proton would be released at this stage. However, Bennett *et al.* (1982) could detect no evidence of proton release (or uptake) when NADH was displaced from this complex by excess NAD^+. This observation suggests that NADH release is not coupled to proton release, and the original argument given above is favoured.

I am grateful to Mr. G. W. Haywood for expert technical assistance.

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Received 10 March 1986; accepted 18 April 1986