The role of the metal ion in the mechanism of the K^+-activated aldehyde dehydrogenase of *Saccharomyces cerevisiae*

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The effect of K^+ on assays of the enzyme was studied and it appears that the activation occurs slowly by a two-step process. Kinetic measurements suggest that the enzyme-catalysed reaction can proceed slowly (0.4 %) in the complete absence of K^+. The enzyme exhibits a K^+-activated esterase activity, which is further activated by NAD^+ or NADH. Stopped-flow studies indicated that the principal effect of K^+ on the dehydrogenase reaction is to accelerate a step (possibly acyl-enzyme hydrolysis) associated with a fluorescence and small absorbance transient that occurs after hydride transfer and before NADH dissociation from the terminal E–NADH complex. The variation of activity of the enzyme with pH was studied. An enzyme group with pK_a approx. 7.1 apparently promotes enzyme activity when in its alkaline form.

INTRODUCTION

Recent work with sheep liver cytosolic aldehyde dehydrogenase has shown that an internal step in the mechanism is significantly accelerated (approx. 3-fold) by Mg^{2+} (Dickinson & Haywood, 1986). The step so influenced appears to be hydrolysis of the acyl-enzyme species, and this process occurs more rapidly with

$$\underset{\underset{Mg^{2+}}{|}}{\overset{\overset{NADH}{|}}{E-acyl}} \quad \text{than with} \quad \overset{\overset{NADH}{|}}{E-acyl}. \quad \text{The accelerating effect of}$$

Mg^{2+} here is in marked contrast with its inhibiting effect on the overall reaction rate. The rate-limiting step is NADH dissociation, and this occurs much more slowly

$$\text{from} \quad \underset{\underset{Mg^{2+}}{|}}{\overset{\overset{NADH}{|}}{E}} \quad \text{than from} \quad \overset{\overset{NADH}{|}}{E} \quad \text{(Dickinson \& Hart, 1982;}$$

Bennett *et al.*, 1983).

The K^+-activated aldehyde dehydrogenase of yeast is a much more effective catalyst than the liver enzyme referred to above. Comparison of the specific activities of the purified enzymes under roughly similar conditions indicates that the yeast enzyme is some 100–200-fold faster in the oxidation of acetaldehyde. This is a remarkable degree of 'improvement', which really reflects the fact that the liver enzyme is rather ineffective as a catalyst ($V_{max.} = 0.4 \text{ s}^{-1}$ with non-activating aldehyde concentrations; Hart & Dickinson, 1982). It is very curious that this rate is so low when the substrate aldehydes are thought of as toxic, when the rate is actually limited by NADH dissociation (i.e. not by aldehyde oxidation) and when this is itself likely to be dramatically lowered by Mg^{2+}, which is present at high intracellular concentrations. It is, however, interesting and perhaps pertinent to note that yeast alcohol dehydrogenase also exhibits a $V_{max.}$ some 150-fold greater than that of its liver counterpart (Dickinson & Monger, 1973).

Since Mg^{2+} moderates the activity of the liver cytosolic

aldehyde dehydrogenase and since the K^+-activated enzyme is so active, one is prompted to enquire into the role of the K^+. It is important to know whether this ion is absolutely essential for activity or whether it merely facilitates certain step(s) within the mechanism that would otherwise occur only slowly. These points are the subject matter of the present paper.

EXPERIMENTAL

Materials

NAD^+ (grade II) was from Boehringer Corp., London W.5, U.K. 4-Nitrophenyl acetate was obtained from Sigma Chemical Co., Poole, Dorset, U.K., and was dissolved in acetone before addition to enzyme assays. Other chemicals were of analytical grade, where available, obtained from Fisons Chemicals, Loughborough, Leics., U.K. Acetaldehyde was redistilled before use.

Aldehyde dehydrogenase was prepared from fresh commercial yeast obtained from NG&SF, British Fermentation Products, Hull, U.K.

Methods

Solutions were usually made with glass-distilled water, but for experiments requiring low concentrations of K^+ the glass-distilled water was first passed through an Elgastat deionizer (Elga Products, Lane End, Bucks., U.K.). For these experiments glassware was cleaned by immersion in 10 % HNO_3 and rinsed in deionized water. Commercial NAD^+ was found to contain significant amounts of K^+. For some experiments, therefore, more highly purified NAD^+ was required, and this was obtained by chromatography on a DEAE-cellulose column at pH 7.5 with a concentration gradient of Tris/HCl buffer, deionized water being used for all reagents. Acetaldehyde concentrations were obtained by spectrophotometric assay at pH 7.0 with aldehyde dehydrogenase by using a value of $\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for NADH.

The procedure used for preparing enzyme was essentially that described by Bostian & Betts (1978a), starting with an acetone-dried powder and with phenylmethanesulphonyl fluoride as a proteinase inhibitor. The procedure was taken as far as fraction IV without

modification. Thereafter chromatography on a DEAE-cellulose column replaced bulk adsorption by this material. The affinity-chromatography step on NAD^+–Sepharose was omitted, but gel filtration on a large column of Sephacryl S-300 was introduced followed by chromatography on hydroxyapatite at pH 7.0 with a potassium phosphate gradient. The final product (approx. 30 mg from 300 g of acetone-dried powder) had a specific activity in the range 36–42 units/mg in the assay system of Bostian & Betts (1978a). (These authors gave a value of 34 units/mg for the purified enzyme.) The enzyme exhibited only one active species on poly-acrylamide-gel electrophoresis, and thus showed no evidence of partial proteolysis, which can be a serious problem with this enzyme (Clark & Jakoby, 1970a,b). The enzyme was routinely stored in potassium phosphate buffers, pH 7.0, containing 1 mM-EDTA and 1 mM-dithiothreitol, and was kept in a liquid-N_2 container. Under these conditions the preparations were completely stable for at least 2 months. When K^+-free samples of enzyme were required for catalytic experiments, 0.2 ml portions were passed down a TSK G3000-SW gel-filtration column in an LKB fast-protein-chromatography apparatus and with elution by a K^+-free buffer. When substantial amounts of enzyme were required, as for example in stopped-flow experiments, dialysis was used.

Enzyme active-site concentrations were calculated by assuming $A^{1\%}_{1\,cm} = 10.0$ and taking an M_r of 240000 with four NAD^+-binding sites and therefore four active sites/molecule (Bostian & Betts, 1978a).

Initial-rate measurements of dehydrogenase activity were made fluorimetrically in a filter fluorimeter based on the design of Dalziel (1962). The esterase activity was monitored by spectrophotometry by using an absorption coefficient for the p-nitrophenoxide ion of 18300 $M^{-1} \cdot cm^{-1}$ at 400 nm (Kézdy & Bender, 1962) and a pK_a of 7.1 for p-nitrophenol (Behme & Cordes, 1967).

Stopped-flow experiments were performed in the apparatus described by Hart & Dickinson (1982). The enzyme was usually pre-mixed with NAD^+ and stored in one syringe, with the NAD^+/acetaldehyde mixture in the other syringe. All solutions contained 1 mM-EDTA and 1 mM-dithiothreitol. When required, KCl was added to the syringe contents.

K^+ concentrations were determined by atomic absorption spectrophometry with a Baird Atomic A3400 instrument. Calibration was with KCl.

RESULTS AND DISCUSSION

Preliminary experiments in Tris/HCl buffer at pH 8.0 established that the enzyme preparation requires K^+ for full activity. Rb^+ and NH_4^+ can substitute for K^+, but not Li^+, Na^+, Mg^{2+}, Ca^{2+} or Cs^+. These results are in agreement with the report by Black (1955). In our experiments the rates with K^+, Rb^+ and NH_4^+, presented as the chlorides at a concentration of 100 mM, were in the proportions of 100, 92 and 57. As the radii of these ions are very similar (approx. 0.14 nm) and are substantially different from those of the other ions tested, the specificity, particularly in relation to the univalent cations, is understandable.

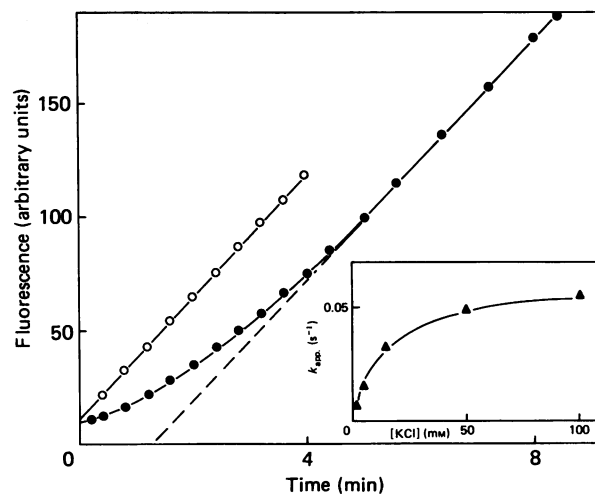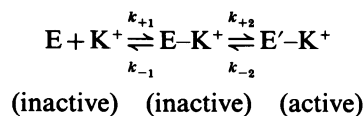The behaviour of the enzyme in assays was dependent upon its treatment before assay. This is shown in Fig. 1.

Fig. 1. Assays of aldehyde dehydrogenase

The fluorescence record shown follows the addition of enzyme treated by gel filtration to remove K^+ (●) or untreated (○) to assay mixtures containing 500 μM-NAD^+, 1 mM-acetaldehyde, 0.1 M-KCl and 1 mM-dithiothreitol in 65 mM-Tris/HCl buffer, pH 7.5, at 25 °C. The enzyme concentration used was 9 nM. The inset shows how the apparent first-order rate constant ($k_{app.}$) obtained by analysis of the lag phase in accordance with Dalziel et al. (1978) varies with the KCl concentration.

If the K^+ used for protection in storage was removed by rapid gel filtration before a normal assay, a pronounced lag phase was observed in the progress curve of the reaction. The final rate achieved was about the same as for untreated enzyme. Preincubation of the treated enzyme for 2 min in the assay, with all components present except NAD^+, followed by addition of the NAD^+ to start the reaction completely abolished the lag phase. No other preincubation conditions succeeded in this. Simple dilution of stock enzyme to the extent achieved by the gel-filtration procedure did not produce lag phases in assays either.

The lag phases seen for the treated enzyme were not dependent on the concentration of enzyme used in the assay, but they were dependent on the K^+ concentration used. The higher the concentration of K^+ the shorter was the observed lag phase. Analysis of the results by the method of Dalziel et al. (1978) showed that the process displays apparent first-order kinetics. The apparent first-order rate constant was not, however, linearly related to the K^+ concentration, but reached a finite maximum at high concentrations of K^+ (Fig. 1 inset). The results showed that the activation by K^+ of the enzyme is a rather slow process, and they indicate that a mechanism such as

$$E + K^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{-}K^+ \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'\text{-}K^+$$

(inactive)   (inactive)   (active)

may be involved. According to Gutfreund (1972), with rapid equilibration of the first step the apparent rate constant for the appearance of active enzyme is given by the expression:

$$k_{app.} = k_{-2} + \frac{k_{+2}}{1 + \dfrac{k_{-1}}{k_{+1}[K^+]}}$$

The intramolecular step, perhaps a conformational change, would determine the value of $k_{app.}$ at high concentrations of K^+.

Lineweaver–Burk plots of the steady-state rates obtained with enzyme stored in K^+ or enzyme depleted of K^+ gave very similar K_m values for K^+ at pH 7.5 at 25 °C (5.0 ± 0.25 mM for enzyme stored in K^+ and 5.5 ± 0.25 mM for enzyme depleted of K^+) with acetaldehyde as substrate. Bostian & Betts (1978b) report a K_m value for K^+ of 7 mM with glycolaldehyde at pH 8.0. There is, incidentally, no difficulty in making accurate rate measurements with the K^+-depleted enzyme. The lag period is long (approx. 15 min) with low K^+ (1 mM), but progress curves are strictly linear for at least a further 15 min at the completion of the lag with enzyme concentrations of the order of 9 nM, as here. By using scrupulously cleaned glassware, chromatographically purified NAD^+ and deionized water it was possible to produce reaction conditions with as little as 1.7 μM-K^+ present. Assays using these conditions or supplemented with 100 mM-K^+ provided the results given in Table 1. At pH 7.5 the rate observed with 1.7 μM-K^+ was some 12-fold greater than was expected on the basis of the K_m value for K^+ of 5.0 mM reported above. This result suggests that the activity of the enzyme is not absolutely dependent on K^+, but that some residual activity remains (approx. 0.4%) when K^+ is completely absent. Results found on working at pH 7.0 and pH 5.5 confirmed this. The rates obtained with 100 mM-K^+ show a very much greater pH-dependence than those obtained with 1.7 μM-K^+. If the residual activity at pH 7.5 was due to the K^+-activated enzyme it should show the same pH-dependence as that activity. Finally, the K_m values determined for acetaldehyde at pH 7.0 at 25 °C with high and low K^+ concentrations support the view that a low level of dehydrogenase activity is sustained in the absence of K^+. Thus the K^+ enhances the rate of certains steps, but is not fundamental to the mechanism.

The non-activated $V_{max.}$ at pH 7.0 for the dehydrogenase activity is about $0.10\ s^{-1}$. This is rather similar to the value obtained for the sheep liver cytosolic enzyme ($V_{max.} = 0.4\ s^{-1}$; Dickinson & Hart, 1982) and is in contrast with the very large difference in rates when the yeast enzyme is activated by K^+. It seems, however, that

the similarity of the non-activated rates is coincidental, because the liver enzyme $V_{max.}$ is determined by NADH dissociation whereas for the K^+-activated yeast enzyme a different step (see below) is slow.

The fluorescence emission spectrum of the enzyme in 50 mM-Bistris buffer, pH 7.5, was completely unchanged [$\lambda_{max.}$ (uncorrected) 340 nm] on the introduction of 100 mM-KCl. This suggests that there are no gross structural changes arising from combination with K^+. On the other hand fluorescence titrations of the enzyme with NADH were infuenced by K^+, as shown in Fig. 2. The additional enhancement of the NADH fluorescence

in the presence of K^+ shows the formation of an $\underset{|}{\overset{NADH}{\underset{|}{E}}}$
$$K^+$$

complex in addition to an $\overset{NADH}{\underset{|}{E}}$ complex. For both titrations there is no obvious end point, and they do not provide estimates of the number of NADH-binding sites or of the dissociation constants for NADH. However, there is no real indication that the presence of saturating concentrations of K^+ tighten the binding of NADH. This might be because the binding of K^+ and NADH occur independently and not sequentially. Certainly the stability studies by Bostian & Betts (1978b) indicate an interaction between free enzyme and K^+. Fluorescence titrations of the enzyme with nicotinamide-1,N^6-etheno-adenine dinucleotide with excitation at 310 nm and

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Table 1. Effect of K^+ concentration on the kinetics of acetaldehyde oxidation by NAD^+ at 25 °C catalysed by aldehyde dehydrogenase

Experiments were conducted in 50 mM-Bistris buffer containing 1 mM-dithiothreitol. The K^+ concentrations were determined by atomic absorption spectrophotometry, and the NAD^+ was purified chromatographically with the use of metal-ion-free buffers. The initial velocity at pH 7.0 with 100 mM-K^+ is arbitrarily set at 100. The given initial velocities (v) were obtained with 50 μM-acetaldehyde and 500 μM-NAD^+. The K_m values for acetaldehyde were obtained from data collected at pH 7.0 at 25 °C with 500 μM-NAD^+ and acetaldehyde concentrations in the range 2–50 μM.

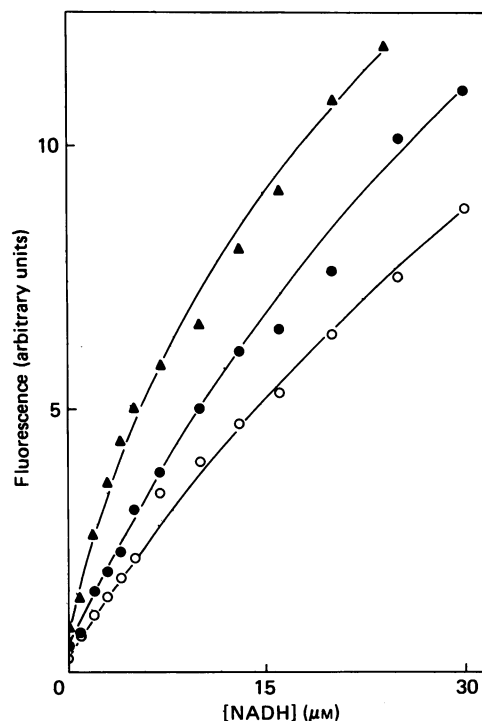| Concn. of K^+ (mM)	v (pH 7.0)	v (pH 5.5)	$K_m^{acetaldehyde}$ (μM)
100	100	0.97	5.6 ± 0.5
0.0017	0.4	0.06	21 ± 2

Fig. 2. Fluorescence titrations of aldehyde dehydrogenase with NADH

The experiments were conducted in 50 mM-Bistris buffer, pH 7.0, containing 1 mM-dithiothreitol at 25 °C. NADH was added to buffer alone (○), buffer containing 1.3 μM-enzyme (●) and buffer containing 1.3 μM-enzyme and 100 mM-KCl (▲).

emission at 410 nm showed a slight enhancement of fluorescence with no KCl present, and this, if anything, was diminished in the presence of 100 mM-KCl. These results are more difficult to interpret. It may be noted, however, that the fluorescent NAD^+ analogue is an effective coenzyme in this system, giving a rate some 70 % of that with NAD^+ when substituted in the normal assay.

The enzyme exhibited a substantial K^+-activated esterase activity towards p-nitrophenyl acetate, and some results obtained at pH 7.0 are shown in Fig. 3. Inclusion of NAD^+ or NADH caused marked activation, as with horse liver cytosolic (F1) enzyme (Eckfeldt & Yonetani, 1976). When 8 μM-NADH was present the K_m increased from 0.05 to 0.125 mM and V_{max} from 6.7 to 16.0 s^{-1}. Little esterase activity occurred in the absence of added K^+, but because the substrate hydrolyses in the absence of enzyme it was difficult to determine accurately what the residual activity was.

The occurrence of an esterase activity is not unexpected. Such activity is also seen with liver cytosolic and mitochondrial aldehyde dehydrogenases and with glyceraldehyde-3-phosphate dehydrogenase. The esterase and dehydrogenase activities occur at the same site on these enzymes, and the mechanism suggested for liver cytosolic aldehyde dehydrogenase is shown in Scheme 1. This mechanism is based on that established for glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976), but it also rests on extensive studies of the cytosolic enzyme by various groups (MacGibbon et al., 1977; Hart & Dickinson, 1982; Buckley & Dunn, 1982;
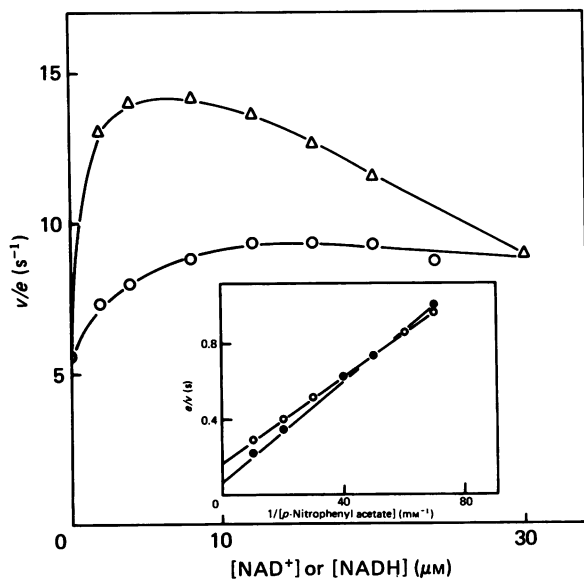

Fig. 3. Effect of NAD^+ and NADH on the specific initial rate of the esterase activity of aldehyde dehydrogenase

Spectrophotometric assays were conducted at 400 nm in 50 mM-Bistris buffer, pH 7.0, containing 1 mM-dithiothreitol at 25 °C. Assay mixtures contained 0.1 M-KCl, 0.33 mM-p-nitrophenyl acetate and the NAD^+ (O) or NADH ($\triangle$) concentrations shown. The enzyme concentration used in the experiments was 15 nM. The inset shows Lineweaver–Burk plots obtained on varying the p-nitrophenyl acetate concentration with 8 μM-NADH present ($\bullet$) or with no NADH present (O).
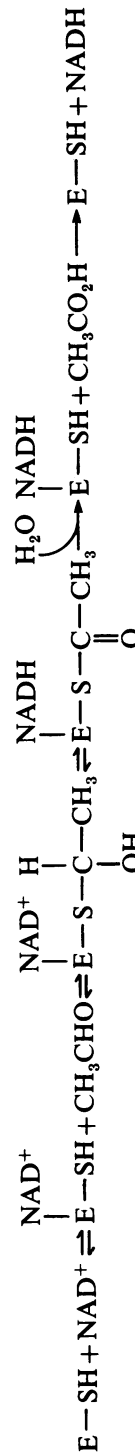
Scheme 1. Mechanism of aldehyde dehydrogenases

It is presumed that K^+ is bound to all the various enzyme species when the yeast enzyme is under consideration and the activator is present. It is also assumed that the esterase and dehydrogenase activities utilize the same thiol group as appears to be the case for the sheep liver cytosolic enzyme (Dickinson & Haywood, 1986). The species would, therefore, be formed in p-nitrophenyl acetate hydrolysis in the presence of NADH and in the acetaldehyde dehydrogenase reaction.

Dickinson, 1985, 1986). In the dehydrogenase reaction the aldehyde substrate reacts with an active-site thiol group, forming a thiohemiacetal, with hydride transfer and acyl-enzyme formation following. The same active-site thiol group is involved in a nucleophilic attack on the ester substrate with production of the acyl-enzyme species. In both cases acyl-enzyme hydrolysis releases the acid product before the catalytic cycle begins again.

Interpretation of the yeast aldehyde dehydrogenase data is given below in terms of Scheme 1. The occurrence of the esterase activity is not the only evidence to support it. The NAD$^+$-binding and kinetic studies by Bostian & Betts (1978a,b) and those shown in Fig. 2 support a sequential mechanism for the dehydrogenase, with NAD$^+$ as the leading substrate and NADH dissociation as the final step. Circumstantial evidence indicates an essential thiol group at the active centre. The enzyme requires a thiol compound present in assays (2-mercaptoethanol or dithiothreitol) to exhibit full activity. Enzyme freed from the protective agent by gel filtration loses activity rapidly (50 % loss at 0 °C at pH 7.0 in 2 h). Incubation of the thiol-free enzyme with a 10-fold molar excess of disulfiram (tetraethylthioperoxydicarbonic diamide) or methyl methanethiosulphonate at room temperature causes almost total loss of activity within 3 min. Severe inhibition of the liver cytosolic aldehyde dehydrogenases by disulfiram is well known.

In considering the mechanisms of the two enzyme-catalysed reactions it is of importance to know what role is played by K$^+$. As indicated above, there is evidence of the binding of K$^+$ to free enzyme and to the enzyme–coenzyme species. Further, Fig. 1 suggests that the combination with K$^+$ is much too slow to account for the overall rate if K$^+$ was presumed to dissociate in each catalytic cycle. It seems most likely then that the K$^+$ remains bound. But what steps are activated? The common step for both reactions in Scheme 1 is acyl-enzyme hydrolysis, and this seems a likely candidate. The $V_{max.}$ values for p-nitrophenyl acetate hydrolysis (16 s^{-1} in the presence of 8 μM-NADH) and acetaldehyde oxidation (20 s^{-1}) are very similar at pH 7.0 at 25 °C. As the same acyl group is involved, one is prompted to suggest that both reactions are rate-limited by hydrolysis

$$\text{of the } \overset{\overset{\displaystyle \text{NADH}}{\displaystyle |}}{\underset{\underset{\displaystyle \text{K}^+}{\displaystyle |}}{\text{E--acetyl}}} \text{ species with K}^+ \text{ promoting this}$$

reaction. If this is true, then a parallel exists with sheep liver cytosolic aldehyde dehydrogenase, where Mg^{2+}, but not K$^+$, promotes acyl-enzyme hydrolysis (Dickinson & Haywood, 1986). Of course, if other kinetically significant species are involved in the dissociation sequence from

$$\overset{\overset{\displaystyle \text{NADH}}{\displaystyle |}}{\underset{\underset{\displaystyle \text{K}^+}{\displaystyle |}}{\text{E--acyl}}} \text{ than are indicated in Scheme 1, there would}$$

be other common steps for dehydrogenase and NADH-activated esterase reactions and other potential rate-limiting steps. This is considered further below.

Studies of the pre-steady state of the dehydrogenase activity were conducted in the stopped-flow apparatus with enzyme that had been dialysed for a 45 min period

to remove excess K$^+$ and that was subsequently supplemented with KCl if required. We did not attempt to remove all K$^+$, only to decrease it to a low concentration (approx. 0.5 mM). The enzyme is unstable in the absence of K$^+$ (Bostian & Betts, 1978b), and operations with the stopped-flow apparatus can take appreciable time to accomplish. By using the spectrophotometric monitoring system, saturating (1 mM) acetaldehyde and 13 mM-KCl, two pre-steady state transients in A_{340} were obtained (Fig. 4). The amplitudes of the phases were equivalent to 1.3 and 0.65 mol of NADH/mol of enzyme with apparent first-order rate constants of 110 and 30 s^{-1}. It was difficult to get accurate values for the small transient. When KCl was omitted the second transient appeared to be absent, the steady-state rate was much lowered, but the initial transient was more or less unchanged, although
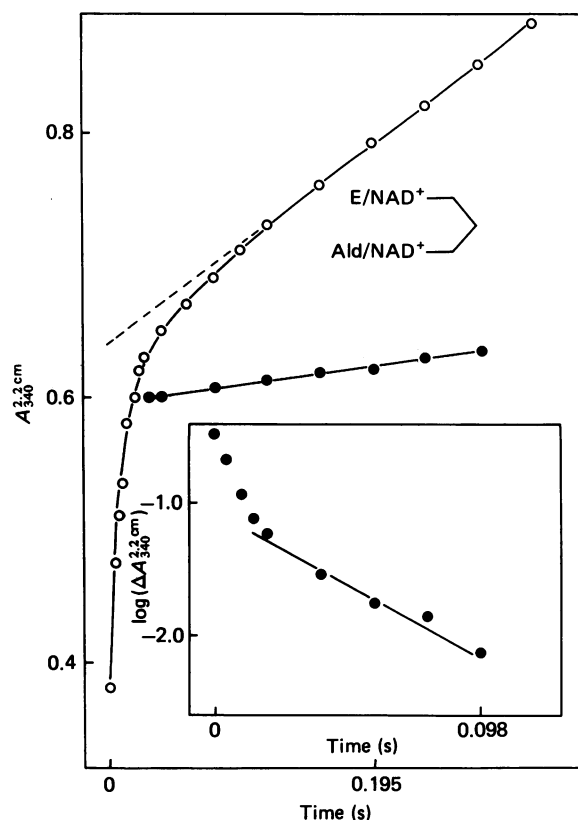

Fig. 4. Stopped-flow studies of acetaldehyde oxidation catalysed by aldehyde dehydrogenase monitored by spectrophotometry

The records show the time course of absorbance at 340 nm following the mixing of enzyme (8 μM) with NAD$^+$ (500 μM) and acetaldehyde (1 mM) in the presence of 13 mM-KCl (○) or with no KCl added (●). The pre-mixing conditions are indicated. The experiment was conducted at 25 °C in 50 mM-Bistris buffer, pH 6.6, containing 1 mM-dithiothreitol and 1 mM-EDTA. The initial $A_{340}^{2.2 \text{ cm}}$ of the reaction mixtures based on the absorbances of the parent solutions was expected to be 0.37. The inset shows the first-order plot of the transient obtained by taking the difference between the observed readings at any time and the reading obtained at the same time by extrapolating back from the steady-state region. The apparent first-order rate constant for the slow phase of the reaction is 30 s^{-1} and that for the fast phase 110 s^{-1}, obtained from a secondary plot.

data were not collected in this region. When the reaction was monitored by fluorescence (Fig. 5) in the presence of KCl only one transient was obtained ($k_{app.}$ = 37 s^{-1}) on a similar time scale to the slower absorbance transient. When the fluorescence experiment was performed in the absence of added KCl no fluorescence transient was seen and the steady-state rate was much lowered. Further, when KCl was added only to the NAD$^+$/aldehyde syringe before mixing there was no fluorescence transient. Thus the effects brought about by KCl are established slowly. This was already clear from the results of Fig. 1.

The pre-steady-state experiments just described can be interpreted in terms of Scheme 1. It seems that the hydride-transfer step equilibrates rapidly and that the

NADH
|
E–S–C–CH$_3$ species exhibits absorbance at 340 nm,
| ‖
K$^+$ O

NADH
|
but little fluorescence. As the generation of E–SH
|
K$^+$

proceeds by hydrolysis the fluorescence characteristic of

NADH
|
NADH develops, and as more E–S–C–CH$_3$ forms to
| ‖
K$^+$ O

maintain the equilibrium of the hydride-transfer step a second absorbance transient is seen on the same time scale as that seen in the fluorescence mode. It is not unusual that the product of the hydride-transfer step should exhibit little fluorescence. This has been seen also with liver aldehyde dehydrogenase (Hart & Dickinson, 1982), glutamate dehydrogenase (di Franco & Iwatsubo, 1972), lactate dehydrogenase (Whitaker et al., 1974) and yeast alcohol dehydrogenase (Dickinson & Dickenson, 1978).

The stopped-flow experiments show that the rate-limiting step for the dehydrogenase reaction occurs after hydride transfer. One might suggest that the slow step is dissociation of the terminal E–NADH complex, as is the case for the sheep liver cytosolic enzyme (Dickinson, 1985). However, displacement of NADH by NAD$^+$ monitored by fluorescence (Fig. 6) shows that the specific rate of NADH dissociation is greater than 100 s^{-1}, which is substantially greater than the steady-state rate of acetaldehyde oxidation (20 s^{-1}). The rate-limiting step must then occur between hydride transfer and coenzyme dissociation.

It appears that the effect of K$^+$ is to increase dramatically the rate of the step associated with the fluorescence transient of Fig. 5. The steady-state rate is greatly increased too, but whether this is because this step is actively promoted or simply because the enzyme species participating in it is greatly increased in concentration by the increased rate of the previous step is not known.

What, then, is the step activated by K$^+$? Comparison with the effects of Mg^{2+} on sheep liver cytosolic aldehyde
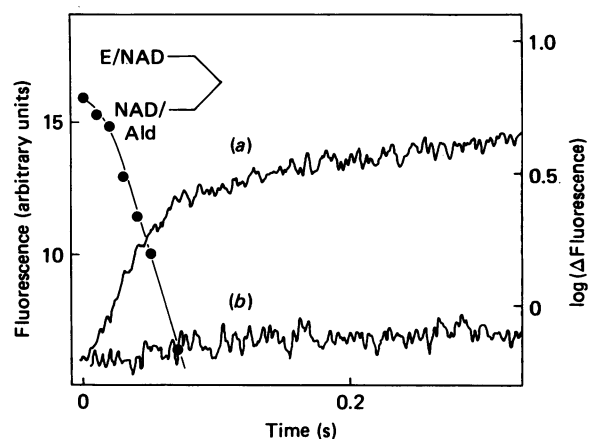

Fig. 5. Stopped-flow studies of acetaldehyde oxidation catalysed by aldehyde dehydrogenase monitored by fluorescence

The records show the time course following the mixing of enzyme (0.5 μM) with NAD$^+$ (500 μM) and acetaldehyde (1 mM) in the presence of 15 mM-KCl (trace a) or with no added KCl (trace b). The pre-mixing conditions are indicated. The experiments were conducted at 25 °C in 50 mM-Bistris buffer, pH 6.6, containing 1 mM-dithiothreitol and 1 mM-EDTA. The plot (●) of the initial transient was obtained as described for Fig. 4. The apparent first-order rate constant is 37 s^{-1}.
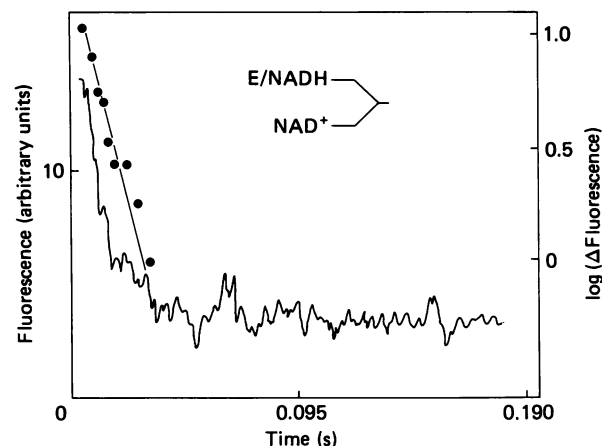

Fig. 6. Displacement of NADH from the aldehyde dehydrogenase–NADH complex

The enzyme-bound NADH was displaced by NAD$^+$ at 25 °C at pH 7.0 in 100 mM-potassium phosphate buffer containing 1 mM-dithiothreitol and 1 mM-EDTA. The pre-mixing arrangements are indicated. The composition of the reaction mixture on mixing was 2.7 μM-enzyme, 30 μM-NADH and 6 mM-NAD$^+$. The analysis (●) of the data gives an apparent first-order rate constant of 100 s^{-1}.

dehydrogenase (Dickinson & Haywood, 1986) tempts us to suggest that the effect of K$^+$ is to facilitate acyl-enzyme hydrolysis. If this is the case then the rate-limiting step occurs later and may be associated with an intramolecular change preceding NADH dissociation. If, on the other hand, acyl-enzyme hydrolysis is rate-limiting, as suggested by the similarity of $V_{max.}$ values for dehydrogenase and esterase activity in the presence of NADH, the fluorescence transient must be associated with an

intramolecular event before it. These conclusions indicate that the product release sequence is more complex than shown in Scheme 1. Thus, although K$^+$ binds to the free enzyme and to the enzyme–coenzyme complexes and the potential exists for it to modify many processes within the mechanism, it seems that the principal effect is to enhance one or maybe two steps that occur before NADH dissociation and after hydride transfer.

The pH-dependence of the K$^+$-activated activity of the enzyme is marked, as has been indicated in Table 1. The results of more detailed initial-rate studies are shown in Fig. 7. These were conducted with 500 μM-NAD$^+$ and 0.1 M-KCl with acetaldehyde concentrations kept low to avoid possible substrate inhibition effects (Bostian & Betts, 1978b). We have not attempted to study the pH-dependence of the enzyme activity in the absence of
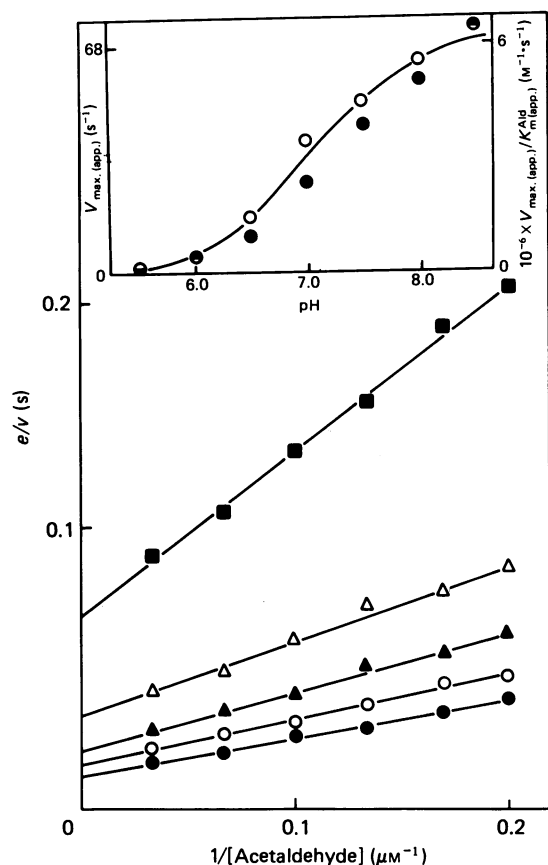

Fig. 7. Variation of the specific initial rate of acetaldehyde oxidation with the acetaldehyde concentration at different pH values catalysed by aldehyde dehydrogenase

Experiments were carried out fluorimetrically at 25 °C with 500 μM-NAD$^+$ and 0.1 M-KCl in 50 mM-Hepes buffer containing 1 mM dithiothreitol at pH 8.5, (●), 8.0 (○), 7.5 (▲), 7.0 (△) and 6.5 (■). Analogous experiments (not shown) were conducted at pH 5.5, 6.0, 6.5, 7.0 and 7.5 in 50 mM-Bistris buffers. In the overlap region very similar results were obtained with the two buffer systems. The inset shows the variation of 1/intercept ($V_{\text{max.(app.)}}$) (●) and 1/slope ($V_{\text{max.(app.)}}/K^{\text{Ald}}_{\text{m(app.)}}$) (○) with pH taken from the primary plots. For the overlap region of the buffers average values are plotted. The line shows the theoretical curve expected for a group with pK_a 7.1. The enzyme concentrations used for these experiments were variable, but in the range 5–50 nM.

K$^+$. The enzyme is so unstable in this condition, and with a prolonged experiment, partly necessitated by the very low rates, it seemed unlikely that reliable results could be obtained.

The inset of Fig. 7 shows the variation of 1/intercept ($V_{\text{max.(app.)}}$) and 1/slope ($V_{\text{max.(app.)}}/K^{\text{Ald}}_{\text{m(app.)}}$), and it is apparent how sensitive the K$^+$-activated reaction is to pH and how very little activity is seen at pH 5.5. If the high NAD$^+$ and K$^+$ concentrations used are saturating throughout the pH range, then the inset shows the variation of $V_{\text{max.}}$ and $V_{\text{max.}}/K^{\text{Ald}}_{\text{m}}$ with pH. The shapes of the two plots are surprisingly similar. It might have been expected that they would each reflect different steps in the mechanism. The $V_{\text{max.(app.)}}/K^{\text{Ald}}_{\text{m(app.)}}$ plot fits quite well to an ionization curve calculated on the basis of a pK_a of 7.1. The $V_{\text{max.(app.)}}$ plot suggests a pK_a slightly higher, perhaps 7.2, but in this case the fit to the theoretical curve is not quite so good. The similarity of pH behaviour of the two functions arises because $K^{\text{Ald}}_{\text{m(app.)}}$ is more or less pH-independent. The situation is in contrast with that with sheep liver cytosolic aldehyde dehydrogenase. This enzyme is also more active at alkaline pH values, but variations in $V_{\text{max.(app.)}}/K^{\text{Ald}}_{\text{m(app.)}}$ controlled by a group of pK_a 6.6 arise (Dickinson, 1986) because of variations in $K^{\text{Ald}}_{\text{m(app.)}}$, $V_{\text{max.(app.)}}$ being essentially pH-independent (less than 2-fold increase in the pH range 5.0–8.0 with butyraldehyde as substrate; F. M. Dickinson, unpublished work). For the sheep enzyme $V_{\text{max.}}$ is determined by the rate of dissociation of the terminal E–NADH complex (Dickinson, 1985), and the group of pK_a 6.6 noted above seems to control the binding of aldehyde to the E–NAD$^+$ complex (Dickinson, 1986). This is not suggested for the yeast enzyme, for the reasons outlined above. The nature of the enzyme group with pK_a approx. 7.2 that controls $V_{\text{max.}}$ and the role it fulfils remain to be elucidated.

REFERENCES

Behme, M. T. A. & Cordes, E. H. (1967) J. Biol. Chem. 242, 5500–5509

Bennett, A. F., Buckley, P. D. & Blackwell, L. F. (1983) Biochemistry 22, 776–784

Black, S. (1955) Methods Enzymol. 1, 508–511

Bostian, K. A. & Betts, G. F. (1978a) Biochem. J. 173, 773–786

Bostian, K. A. & Betts, G. F. (1978b) Biochem. J. 173, 787–798

Buckley, P. D. & Dunn, M. F. (1982) Enzymology of Carbonyl Metabolism: Aldehyde Dehydrogenase and Aldo/Ketose Reductase, pp. 23–25, Alan R. Liss, New York

Clark, J. F. & Jakoby, W. B. (1970a) J. Biol. Chem. 245, 6065–6071

Clark, J. F. & Jakoby, W. B. (1970b) J. Biol. Chem. 245, 6072–6077

Dalziel, K. (1962) Biochem. J. 84, 244–254

Dalziel, K., McFerran, N., Matthews, B. & Reynolds, C. H. (1978) Biochem. J. 171, 743–750

Dickinson, F. M. (1985) Biochem. J. 225, 159–165

Dickinson, F. M. (1986) Biochem. J. 238, 75–82

Dickinson, F. M. & Dickenson, C. J. (1978) Biochem. J. 171, 629–637

Dickinson, F. M. & Hart, G. J. (1982) Biochem. J. 205, 443–448

Dickinson, F. M. & Haywood, G. W. (1986) Biochem. J. 233, 877–883

Dickinson, F. M. & Monger, G. P. (1973) Biochem. J. 131, 261–270

di Franco, A. & Iwatsubo, M. (1972) Eur. J. Biochem. **30**, 517–532

Eckfeldt, J. H. & Yonetani, T. (1976) Arch. Biochem. Biophys. **173**, 273–281

Gutfreund, H. (1972) Enzymes: Physical Principles, p. 205, John Wiley and Sons, London and New York

Harris, J. I. & Waters, M. (1976) Enzymes 3rd Ed. **13**, 1–49

Hart, G. J. & Dickinson, F. M. (1982) Biochem. J. **203**, 617–627

Kézdy, F. J. & Bender, M. L. (1962) Biochemistry **1**, 1097–1106

MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977) Eur. J. Biochem. **77**, 93–100

Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J. & Gutfreund, H. (1974) Biochem. J. **139**, 677–697