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Studies on the mechanism of sheep liver cytosolic aldehyde dehydrogenase

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1. The dissociation of the aldehyde dehydrogenase·NADH complex was studied by displacement with NAD^+. The association reaction of enzyme and NADH was also studied. These processes are biphasic, as shown by McGibbon, Buckley & Blackwell [(1977) *Biochem. J.* **165**, 455–462], but the details of the dissociation reaction are significantly different from those given by those authors. 2. Spectral and kinetic experiments provide evidence for the formation of abortive complexes of the type enzyme·NADH·aldehyde. 3. Kinetic studies at different wavelengths with *trans*-cinnamaldehyde as substrate provide evidence for the formation of an enzyme·NADH·cinnamoyl complex. Hydrolysis of the thioester relieves a severe quenching effect on the fluorescence of enzyme-bound NADH.

Previous studies have indicated that cytosolic aldehyde dehydrogenase operates by a compulsory-order mechanism in which NAD^+ is the first substrate to bind and NADH is the last product to leave (McGibbon *et al.*, 1977*a*; Hart & Dickinson, 1982). Substrate activation by high propionaldehyde concentrations has been explained as being due to the participation of an alternative pathway of substrate addition (McGibbon *et al.*, 1977*a*), but Hart & Dickinson (1982) argued in favour of a pathway involving an abortive enzyme·NADH·aldehyde complex with an alternative route of product release. It is generally assumed that the mechanism of aldehyde dehydrogenase involves the formation of a thioester intermediate, as is the case with glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976).

There have been difficulties in fitting some of the above ideas with existing data. Thus the slowest step in NADH dissociation, with $k = 0.22\,s^{-1}$ (McGibbon *et al.*, 1977*b*), was too slow to explain the non-activated and activated $V_{max.}$ values for propionaldehyde oxidation of $0.4\,s^{-1}$ and $1.4\,s^{-1}$ respectively (Hart & Dickinson, 1982). No evidence has yet been brought forward to show that abortive complexes of the type enzyme·NADH·aldehyde can exist. The present paper presents new information on these important points. Rapid-scanning single-turnover experiments, particularly with *trans*-4-(*NN*-dimethylamino)cinnamaldehyde and 4-(*NN*-dimethylamino)benzaldehyde, have provided direct evidence for the formation of a thioester intermediate in catalysis (Buckley & Dunn, 1982). Now further evidence is provided on this point and related matters from stopped-flow experiments involving saturating concentrations of *trans*-cinnamaldehyde.

Experimental

Materials

NADH (grade I) and NAD^+ (grade II) were from Boehringer Corp., London W.5, U.K. NAD^+ was in some instances further purified before use by chromatography on DEAE-cellulose by the method of Dalziel & Dickinson (1966). *trans*-Cinnamaldehyde was obtained as a 'gold label' product from Aldrich Chemical Co., Gillingham, Dorset, U.K. Other chemicals were analytical-reagent grade, where available, obtained from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Propionaldehyde was redistilled before use.

Cytosolic aldehyde dehydrogenase, free from the corresponding mitochondrial enzyme, was prepared as described by Dickinson *et al.* (1981), and was assayed by the method of Hart & Dickinson (1977). Protein concentrations were calculated by using $A^{1\%}_{1cm} = 11.3$ at 280nm (Dickinson & Hart, 1982). Enzyme solutions were normally prepared for use by dialysis against 50mM-sodium phosphate buffer, pH7.0, containing 0.3mM-EDTA and 100μM-dithiothreitol.

Methods

Substrate and coenzyme concentrations. Solutions (approx. 13mM) of *trans*-cinnamaldehyde were

prepared by mixing aldehyde and water for about ½h followed by removal of excess aldehyde by centrifugation. *trans*-Cinnamaldehyde and propionaldehyde concentrations in stock solutions were determined enzymically with aldehyde dehydrogenase and NAD^+. NAD^+ and NADH were assayed enzymically with yeast alcohol dehydrogenase by the methods of Dalziel (1962, 1963). For all these determinations $\varepsilon = 6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ for NADH at 340nm (Horecker & Kornberg, 1948).

Stopped-flow experiments. The apparatus used was that described in an earlier paper (Hart & Dickinson, 1982). For these experiments all solutions contained 0.3mM-EDTA. The performance of the apparatus was tested on the displacement of NADH from the liver alcohol dehydrogenase·NADH binary complex by NAD^+. Alcohol dehydrogenase (45 μM) was pre-mixed with 45 μM-NADH in 50mM-sodium phosphate buffer, pH7.0. This mixture was then pushed against 9.0mM-NAD^+ in the same buffer, Observations were made spectrophotometrically at 350nm. The observed displacement reaction was strictly first-order throughout, with $k = 3.13 s^{-1}$, as expected from the results reported by Shore & Gutfreund (1970). The fluorescence attachment was tested with the same reaction but with 50mM-NaCl included. For this experiment the enzyme and NADH concentrations were 7 μM, the reaction observed was again strictly first-order throughout, with $k = 9.3 s^{-1}$. Shore & Gutfreund (1970) obtained values in the range 8.5–9.5s^{-1} for the reaction in the presence of 50mM-NaCl.

Fluorescence measurements. These were performed in a Farrand mark I spectrofluorimeter. The instrument was standardized with a 6 μM solution of NADH.

Results and discussion

Dissociation and association of NADH

Fig. 1 shows the decrease in fluorescence of a mixture of enzyme and NADH at pH7.0 at 25°C on mixing with a large excess of NAD^+. At the NAD^+ concentration used here the kinetics of the displacement reaction are unchanged by further increase. Analysis of the results shows that the displacement occurs in a biphasic manner, with rate constants of $2.3 s^{-1}$ and $0.4 s^{-1}$. Repeat

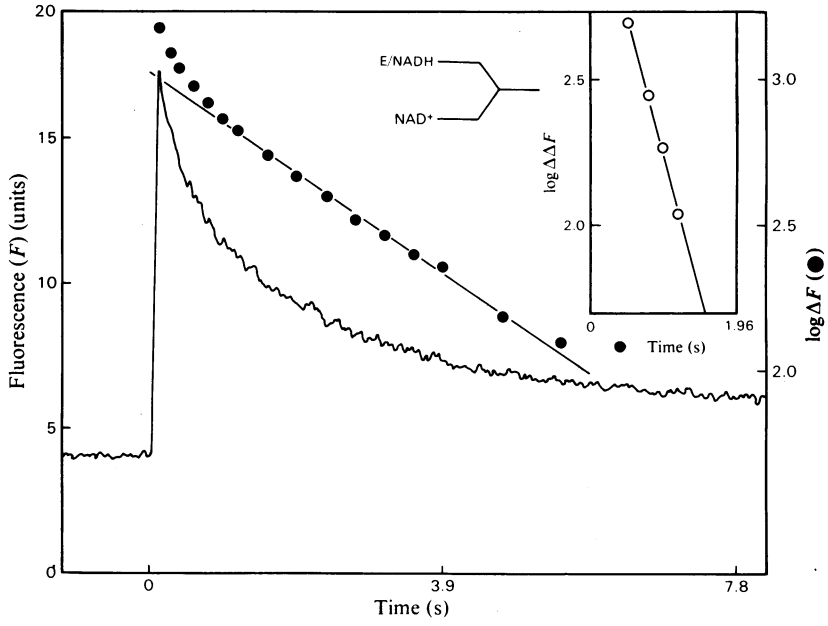

Fig. 1. *Dissociation of NADH from the aldehyde dehydrogenase–NADH complex*

A solution containing enzyme and NADH was mixed with an equal volume of solution containing NAD^+ in the stopped-flow apparatus. The concentrations and conditions after mixing were 2 μM-enzyme, 8 μM-NADH and 8mM-NAD^+ in 50mM-phosphate buffer, pH7.0, at 25°C. The Figure shows the decrease in fluorescence as the dissociation of NADH proceeds, and analysis of the data. The inset relates to the early part of the reaction record. Ten fluorescence units (F) ≡ 0.62V and a solution of 6 μM-NADH gave a signal of 0.8V. The dissociation constants of the E·NADH and E·NAD^+ complexes under these conditions are 3 μM and 7 μM respectively (Hart & Dickinson, 1983).

experiments gave estimates in the range $3.5 \pm 1.4 s^{-1}$ and $0.4 \pm 0.004 s^{-1}$. The relative amplitudes of the fast and the slow processes were about 35% and 65% respectively. These results are similar to those at pH 7.6 published by McGibbon *et al.* (1977*b*), but there are important differences. Thus the earlier work reports rate constants of $1.0 s^{-1}$ and $0.22 s^{-1}$ with amplitudes of 60% and 40% respectively. When the experiment of Fig. 1 was repeated at pH 7.6, the results were essentially unchanged. Calibration of the stopped-flow apparatus (see the Experimental section) showed that it was performing satisfactorily.

When mixtures of enzyme and NADH (4 μM-enzyme, 16 μM-NADH) were mixed with equal volumes of buffer at pH 7.0 at 25°C, a biphasic decay of fluorescence was observed as some of the NADH dissociated. The measured rate constants were $1.6 s^{-1}$ and $0.03 s^{-1}$. In a similar experiment at pH 7.6 McGibbon *et al.* (1977*b*) obtained values of $3 s^{-1}$ and $0.83 s^{-1}$. When 4 μM-enzyme was mixed with 16 μM-NADH at pH 7.0 at 25°C the fluorescence record showed a biphasic increase in signal strength, with rate constants of $5.0 \pm 0.5 s^{-1}$ and $1.1 \pm 0.1 s^{-1}$ and amplitudes of about 45% and 55%. This result confirms the findings obtained by McGibbon *et al.* (1977*b*) for this reaction at pH 7.6.

The results so far recorded confirm the biphasic nature of the reactions of NADH with the enzyme, but the details of the dissociation reaction differ significantly from those reported by McGibbon *et al.* (1977*b*). More recently it has been found that preparations of cytoplasmic aldehyde dehydrogenase exhibit two active bands with very similar pI values on isoelectric focusing (Agnew *et al.*, 1981). We have obtained a similar result (Hart & Dickinson, 1983). It may be that the different results recorded are at least partly caused by different amounts of the two isoenzymes being present. At the moment there appears to be no prospect of separating the isoenzymes and testing this possibility.

McGibbon *et al.* (1977*b*) interpreted their results on NADH binding in terms of the two-step model:

$$E^* \cdot NADH \overset{k_{+1}}{\rightleftharpoons} E \cdot NADH \rightleftharpoons E + NADH$$

Their detailed kinetics of the association reaction established this scheme rather than the alternative with two species of enzyme having different rates of combination. If the same model is adopted here, then the slow step is the isomerization of the $E^* \cdot NADH$ complex with $k_{+1} = 0.4 s^{-1}$. Steady-state kinetics with propionaldehyde and butyraldehyde as substrates have shown that with low aldehyde concentrations linear primary and secondary reciprocal plots are obtained (Hart & Dickinson, 1982). The results are consistent with a compulsory-order mechanism in which NAD^+ binds before aldehyde and NADH is the last product to leave. The $V_{max.}$ obtained from these results was $0.4 s^{-1}$. Thus the rate-limiting step of the overall reaction can be equated with the slow isomerization of the E·NADH complex described above. The corresponding value of $0.22 s^{-1}$ obtained by McGibbon *et al.* (1977*b*) for the isomerization reaction is, of course, too slow to fit in with the $V_{max.}$ value given. That the rate-limiting step does occur after NADH formation was established in transient experiments with NAD^+ and propionaldehyde, when a burst of NADH occurred before the steady state was established (McGibbon *et al.*, 1977*c*; Hart & Dickinson, 1982).

Abortive complexes

With high propionaldehyde concentrations (above 50 μM) activation is observed in kinetic studies (McGibbon *et al.*, 1977*a*) and the $V_{max.}$ achieved is $1.4 s^{-1}$ (Hart & Dickinson, 1982). One must ask what the rate-limiting step is under the new conditions. McGibbon *et al.* (1977*b*) suggested that activation occurred because of the increased role of an alternative pathway of substrate addition; the mechanism became random-order. We argued againt this (Hart & Dickinson, 1982) and suggested as an alternative that activation occurs because of the formation of an abortive complex from which NADH dissociates more readily than from E·NADH. Further argument against the random mechanism theory for activation is that a slow step in NADH dissociation of $0.4 s^{-1}$ could not explain an activated $V_{max.}$ of $1.4 s^{-1}$ however substrate addition occurred. Although the abortive-complex hypothesis can accommodate the results, there has so far been no independent evidence that such complexes exist for this enzyme. Fig. 2 now presents such evidence. The changes in emission spectrum of enzyme-bound NADH are reproducible, and they indicate that two complexes of the type enzyme·NADH·aldehyde might form. It is possible that the decreased fluorescence at high aldehyde concentrations is due to an increase in the dissociation constant for NADH rather than to the formation of a complex of lower quantum yield. However, the fact that lower aldehyde concentrations increase the fluorescence (and shift the spectrum somewhat) means that the decrease in fluorescence must involve a different complex.

Fig. 3 presents results on the kinetics of abortive-complex formation. When the enzyme·NADH complex was mixed with 2 mM-propionaldehyde there seemed to be a slight increase in fluorescence. At all higher aldehyde concentrations the fluorescence decreased. No comparable changes were observed when enzyme or NADH

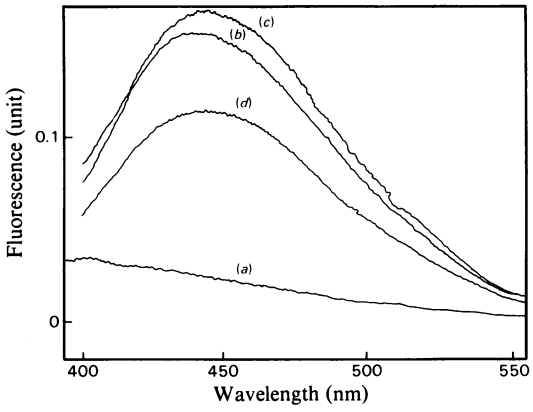

Fig. 2. *Fluorescence-emission spectra of solutions of enzyme, in the presence and in the absence of NADH and propionaldehyde*

The solutions were made up in 50mM-phosphate buffer, pH7.0, as follows: (*a*) 2.8μM-enzyme; (*b*) 2.8μM-enzyme+11.2μM-NADH; (*c*) 2.8μM-enzyme+11.2μM-NADH+13mM-propionaldehyde; (*d*) 2.8μM-enzyme+11.2μM-NADH+110mM-propionaldehyde. Excitation was at 350nm, and 10nm slits were used. A solution of 6μM-NADH gave a fluorescence of 0.04 unit at 470nm.

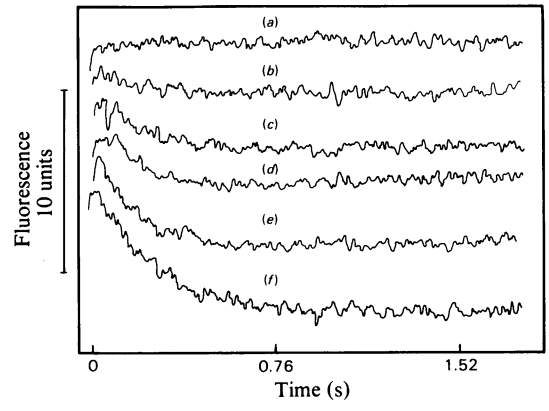

Fig. 3. *Fluorescence changes following the addition of propionaldehyde to solutions containing enzyme and NADH*

Solutions containing enzyme and NADH were mixed with equal volumes of solutions containing NADH and propionaldehyde in the stopped-flow apparatus. The final concentrations and conditions after mixing were 2μM-enzyme, 4μM-NADH and propionaldehyde at (*a*) 2mM, (*b*) 4.3mM, (*c*) 12.4mM, (*d*) 33mM, (*e*) 66mM and (*f*) 130mM in 50mM-phosphate buffer, pH7.0, at 25°C. Ten fluorescence units ≡ 0.62V, and a solution of 6μM-NADH gave a signal of 0.8V.

was mixed with 30mM-propionaldehyde. Analysis of the curves in Fig. 3 indicates a first-order process described by a rate constant of $6s^{-1}$ independent of the propionaldehyde concentration in the range 4–130mM. If the decrease in fluorescence at high propionaldehyde concentrations results from increased dissociation of NADH, then a specific rate of $6s^{-1}$ is a measure of this process and is rapid enough to fit in with the activated $V_{max.}$ noted above. At this stage the details of the activation process are still unclear, however. Activation occurs with propionaldehyde in the range 50–10000μM (Hart & Dickinson, 1982). Abortive complexes do form in this range, but they are seen much better at still higher aldehyde concentrations (Fig. 3).

It is apparent now that the abortive complex appearing at the higher aldehyde concentrations has contributed to transient experiments with 30mM-propionaldehyde (Hart & Dickinson, 1982). At that time it was observed that bursts of NADH formation observed by fluorescence were apparently of smaller amplitude with 30mM substrate than with 50μM. It was also seen that following the initial burst of fluorescence ($k = 12s^{-1}$) there was a small decrease in fluorescence before the steady state was established (see Fig. 8 in Hart & Dickinson, 1982). If the process observed in Fig. 3 begins to occur as the enzyme·NADH complex is being generated it is easy to see how the amplitude of the increase in fluorescence would be significantly diminished and why a subsequent slower decrease in fluorescence might be seen before the steady state is established.

NAD^+–trans-cinnamaldehyde reactions

It has become accepted on the basis of largely circumstantial evidence that the aldehyde dehydrogenase mechanism involves a thioester intermediate. A difficulty does remain, though, in that disulfiram inhibition, which is thought to involve the active thiol group, is not complete (1–2% activity remains) even with a large excess of reagent (Dickinson *et al.*, 1981). More recently, however, direct evidence has been obtained in single-turnover experiments with a variety of aldehydes, but particularly with *trans*-4-(*NN*-dimethylamino)cinnamaldehyde and 4-(*NN*-dimethylamino)benzaldehyde (Buckley & Dunn, 1982). Pepsin forms a thioester, on reaction with *trans*-cinnamoylimidazole, that exhibits a characteristic absorption band at 326nm ($\varepsilon = 22500 M^{-1}\cdot cm^{-1}$; Bender & Kedzy, 1965). *trans*-Cinnamaldehyde might be expected to yield a similar intermediate with aldehyde dehydrogenase, and indeed the results reported by Buckley & Dunn (1982) indicate that it does. It has been thought important here to confirm this finding

with our enzyme preparations and to try to estimate the rates of formation and hydrolysis of the intermediate. This would facilitate an interpretation of the rather complex picture of transients that has been observed in the spectrophotometric and fluorimetric records of pre-steady-state experiments with other aldehydes as substrates (Hart & Dickinson, 1982).

Fig. 4 shows a Lineweaver–Burk plot of data obtained in the oxidation of *trans*-cinnamaldehyde. This aldehyde has some absorbance at 340nm, and so the usable substrate range is limited. It is clear that the K_m is very low (less than 1 μM) and that the $V_{max.}$ is very similar to the non-activated $V_{max.}$ for propionaldehyde. Results for the latter aldehyde are presented for comparison. It is presumed that activation does not occur with cinnamaldehyde, but we cannot really know.

On mixing a mixture of NAD^+ and enzyme with *trans*-cinnamaldehyde at pH7.0 at 25°C, an initial transient increase in absorbance occurred at 340nm (Fig. 5). $\Delta A_{340}^{2cm} = 0.13$ and the burst rate constant was $42 s^{-1}$. When the same experiment was monitored at different wavelengths the following bursts were noted: $\Delta A_{325}^{2cm} = 0.14$, $\Delta A_{332}^{2cm} = 0.135$ and $\Delta A_{365}^{2cm} = 0.036$, with rate constants of $40 \pm 5 s^{-1}$. When the *trans*-cinnamaldehyde concentration was halved (11 μM final) the burst size at 340nm was essentially unchanged, but the rate constant was $23 s^{-1}$. The rate of this process is clearly dependent on the aldehyde concentration. When the experiment of Fig. 5 was repeated with propionaldehyde as substrate (50 μM final), a burst of $\Delta A_{340}^{2cm} = 0.053$ was observed, with a rate constant of $10.5 s^{-1}$. The latter confirms the previous findings by Hart & Dickinson (1982).

The results with propionaldehyde show a burst of NADH production equivalent to approx. 0.8mol/mol of enzyme active sites. The much larger bursts observed with *trans*-cinnamaldehyde could be due to a large hyperchromic effect on the spectrum of bound NADH, but this seems unlikely. In fact, the binary enzyme·NADH complex shows hypochromic effects with respect to free NADH at the shorter wavelengths (McGibbon *et al.*, 1979). This has been confirmed in the present work. This spectrum appears to be unaltered by including large propionaldehyde concentrations in the mixture, which would promote the formation of the abortive complex (see above). A more-likely explanation of the results is that a second absorbing species is formed concomitantly with the NADH and that this contributes to the observed changes. This is in accord with the findings obtained by Buckley & Dunn (1982) with *trans*-4-(*NN*-dimethylamino)cinnamaldehyde as substrate. The changes at 325nm and 332nm being apparently larger than at 340nm suggests that the other species absorbs more strongly at the shorter wavelengths, as might be expected for the supposed thioester intermediate. This species would contribute very little at 365nm, and this too seems to be the case.

Transients occurring over longer time periods after the mixing of an enzyme·NAD^+ mixture with *trans*-cinnamaldehyde are shown in Fig. 6.

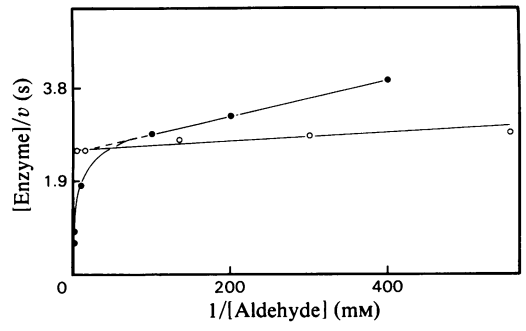

Fig. 4. *Kinetics of aldehyde oxidation by aldehyde dehydrogenase*

Experiments were conducted at 25°C in 50mM-phosphate buffer, pH7.0, with an NAD^+ concentration of 250 μM, and with *trans*-cinnamaldehyde (○) or propionaldehyde (●) as substrate.

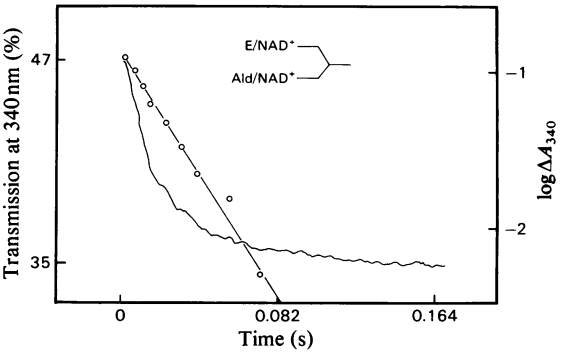

Fig. 5. *Stopped-flow experiments with trans-cinnamaldehyde as substrate*

The reaction occurred on mixing a solution containing enzyme and NAD^+ with an equal volume of one containing NAD^+ and *trans*-cinnamaldehyde. The concentrations and conditions after mixing were 5 μM-enzyme, 200 μM-NAD^+ and 20 μM-*trans*-cinnamaldehyde in 50mM-phosphate buffer, pH7.0, at 25°C. The reaction was followed spectrophotometrically at 340nm in a 2cm-path-length cell.

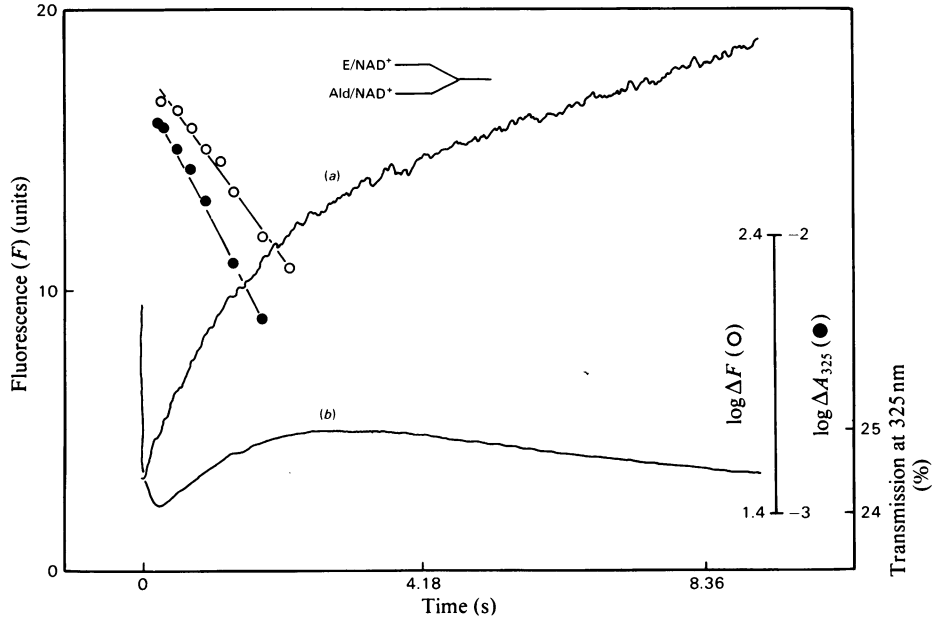

Fig. 6. *Stopped-flow experiments with trans-cinnamaldehyde as substrate*

The reactions occurred on mixing solutions containing enzyme and NAD^+ with equal volumes of solutions containing NAD^+ and *trans*-cinnamaldehyde. Experiments were conducted in 50mM-phosphate buffer, pH7.0, at 25°C, and observations were made fluorimetrically (*a*) or spectrophotometrically with a 2cm cell at 325nm (*b*). The concentrations after mixing were: (*a*) 1 μM-enzyme+200 μM-NAD^++26 μM-*trans*-cinnamaldehyde; (*b*) 5 μM-enzyme+200 μM-NAD^++20 μM-*trans*-cinnamaldehyde. For the fluorescence experiments 10 units ≡ 0.4V and 6 μM-NADH gave a signal of 0.8V.

The fluorescence record shows an initial transient appearing with a rate constant of $0.85\,s^{-1}$, and this occurs on the same time scale as the decrease in absorbance at 325nm that follows the initial burst discussed above. The decrease in absorbance at 325nm occurs with a specific rate of $1.1\,s^{-1}$. A similar decrease is also found in the record at 340nm (see Fig. 7), though it seems to occur a little more quickly at this wavelength and the amplitude is much diminished (about one-third of that at 325nm). An explanation of the results of Fig. 5 at 325nm would be that the decrease arises because of hydrolysis of the thioester intermediate (the cinnamate ion does not absorb at this wavelength; $\lambda_{max.} = 269$nm) and that hydrolysis of this species relieves a severe quenching effect on bound NADH fluorescence, which thus develops at the same time. It is to be noted that varying the *trans*-cinnamaldehyde concentration in the range 6.5–130 μM did not alter the observed rate constant for the fluorescence transient significantly. This is in accord with the proposal. With propionaldehyde as substrate it has already been reported that the fluorescence of enzyme-bound NADH develops much more slowly than does the transient development of absorbance at 340nm due to NADH appearance (Hart & Dickinson, 1982). At that time

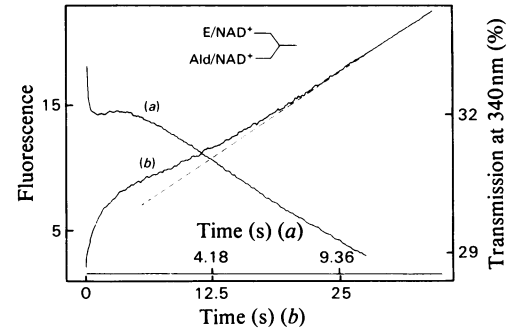

Fig. 7. *Stopped-flow experiments with trans-cinnamaldehyde as substrate*

The reactions occurred on mixing the same solutions as were used for Fig. 6. The conditions of the experiment were the same as for Fig. 6, except that the spectrophotometric measurements were made at 340nm. (*a*) shows the spectrophotometric record and (*b*) the fluorescence record. For the fluorescence experiments 10 units ≡ 0.64V and 6 μM-NADH gave a signal of 0.8V.

it was supposed that the first NADH-containing species presumably E–S–C(=O)–CH_2–CH_3·NADH,

was non-fluorescent. The results of Fig. 5 suggest that it is the rate of hydrolysis of the acyl ester that determines the rate of appearance of NADH fluorescence.

Observations at 340nm and of fluorescence over a longer time period show the approach to the steady-state rates (see Fig. 7). In both cases the steady-state rates achieved are about what one would expect from Fig. 4, but the absorbance record indicates that the steady state is achieved about 2.5s after mixing whereas the fluorescent record shows that this state is reached after about 20s. This seems a peculiar difficulty. A similar problem was encountered in stopped-flow studies with propionaldehyde as substrate (Hart & Dickinson, 1982). If the second phase of the fluorescence record is considered as a transient, the apparent first-order rate constant is about $0.1\,s^{-1}$. This is much too slow to account for the steady-state rate, and thus it cannot represent a step in the overall reaction. Perhaps the quantum yield of one of the fluorescent NADH-containing species decreases slowly to a new level because of some secondary change in the enzyme during turnover. If this change does not alter the rates of the reactions within the mechanism, or at any rate the rate-limiting step, then this change would not show itself in the absorbance record.

I am grateful to Mrs C. Gillyon for technical assistance.

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