Evidence that the cytoplasmic aldehyde dehydrogenase-catalysed oxidation of aldehydes involves a different active-site group from that which catalyses the hydrolysis of 4-nitrophenyl acetate

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Acylation of the aldehyde dehydrogenase·NADH complex by acetic anhydride leads to the production of acetaldehyde and NAD⁺. By monitoring changes in nucleotide fluorescence, the rate constant for acylation of the active site of the *enzyme·NADH complex was found to be $11 \pm 3 \text{ s}^{-1}$. The rate of acylation by acetic anhydride at the group that binds aldehydes on the oxidative pathway is clearly rapid enough to maintain significant steady-state concentrations of the required active-site-acylated *enzyme·NADH intermediate despite the rapid hydrolysis of this *enzyme·acyl·NADH intermediate ($5\text{--}10 \text{ s}^{-1}$) [Blackwell, Motion, MacGibbon, Hardman & Buckley (1987) *Biochem. J.* **242**, 803–808]. Hence reversal of the normal oxidative pathway can occur. However, although acylation of the aldehyde dehydrogenase·NADH complex by 4-nitrophenyl acetate also occurs rapidly with a rate constant of $10.9 \pm 0.6 \text{ s}^{-1}$, even under the most extreme trapping conditions only very small amounts of acetaldehyde are detected [Loomes & Kitson (1986) *Biochem. J.* **235**, 617–619]. Furthermore enzyme-catalysed hydrolysis of 4-nitrophenyl acetate is limited by the rate of deacylation of a group on the enzyme (0.4 s^{-1}), which is an order of magnitude less than deacylation of the group at the active site ($5\text{--}10 \text{ s}^{-1}$). It is concluded that the enzyme-catalysed 4-nitrophenyl ester hydrolysis involves a group on the enzyme that is different from the active-site group that binds aldehydes on the normal oxidative pathway.

INTRODUCTION

The aldehyde dehydrogenase-catalysed hydrolysis of 4-nitrophenyl esters has been widely studied (Feldman & Weiner, 1972; Sidhu & Blair, 1975*a,b*; Eckfeldt & Yonetani, 1976; Duncan, 1977, 1979; MacGibbon *et al.*, 1978; Takahashi *et al.*, 1981; Blackwell *et al.*, 1983; Dickinson & Haywood, 1986; Loomes & Kitson, 1986). The interest in the ester hydrolysis reaction stems from the fact that an acyl-enzyme intermediate is formed along the normal catalytic pathway (Buckley & Dunn, 1985) during aldehyde oxidation. If the same intermediate is formed during 4-nitrophenyl acetate hydrolysis, then not only might kinetic studies of this reaction be expected to provide mechanistic information relating to acyl-enzyme hydrolysis, but also acylation in the presence of NADH should lead to a reversal of the oxidative acylation pathway. Acylation of enzyme·NADH mixtures with acetic anhydride or butyric anhydride results not only in a clearly observable decrease in NADH

absorbance at 340 nm (Hart & Dickinson, 1978) but also in the production of significant amounts of acetaldehyde (Motion, 1986). However, only very small amounts of acetaldehyde have been found from reaction of enzyme·NADH mixtures with 4-nitrophenyl acetate (Duncan, 1979; Loomes & Kitson, 1986).

For a reverse reaction to occur the acylating agent must acylate the active-site group on the conformationally rearranged binary *E·NADH complex that binds aldehydes on the oxidative pathway (Blackwell *et al.*, 1987). Scheme 1 shows this process for acylation by either acetic anhydride or 4-nitrophenyl acetate. Following formation of the *E·NADH·acyl intermediate either a hydride ion can be transferred to the carbonyl group, ultimately producing free enzyme, NAD⁺ and acetaldehyde (the process shown with rate constant k_r in Scheme 1), or deacylation by water produces acetic acid and re-forms *E·NADH (rate constant k_d). The acylating agent may also undergo spontaneous hydrolysis (rate constant k_h).

Scheme 1.

X = OCOCH₃ or OC₆H₄NO₂.

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Clearly if the rate of acylation (the step with rate constant k_a) is much less than the total rate of hydrolysis (the $k_d + k_h$ steps) and the rate of deacylation is also much greater than hydride transfer and subsequent steps ($k_d \gg k_r$) the reverse reaction will not be able to proceed to any great extent before the acylating agent is depleted. This has been the explanation suggested for the small amount of acetaldehyde actually observed with 4-nitrophenyl acetate on the assumption that its total rate of hydrolysis (via the k_d and k_h steps) is much faster than reduction of the $*E \cdot NADH \cdot acyl$ species by NADH (Loomes & Kitson, 1986).

We have now measured the rates of acylation of the enzyme (the step with rate constant k_a in Scheme 1) for acetic anhydride and 4-nitrophenyl acetate in order to test the above assumptions. By consideration of the model shown in Scheme 1 we conclude that most ester hydrolysis is catalysed by a different group on the enzyme from that which binds aldehydes on the oxidative pathway.

EXPERIMENTAL

Cytoplasmic aldehyde dehydrogenase was purified from sheep liver as described previously (MacGibbon *et al.*, 1979). Acetic anhydride from May and Baker (Dagenham, Essex, U.K.) was distilled before use, and 4-nitrophenyl acetate from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) was recrystallized from acetone. NAD^+ and NADH were grade III products from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and propionaldehyde was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.) and was distilled under N_2 before use. Dehydrogenase assays were carried out as described by Blackwell *et al.* (1983), and active-site concentrations were calculated from standard assays with 20 mM-propionaldehyde and 1 mM- NAD^+ at pH 7.6 for which k_{cat} is $0.56 s^{-1}$ per active site (Blackwell *et al.*, 1987). Stock solutions of 4-nitrophenyl acetate were made up in acetonitrile or acetone and diluted to the appropriate concentration so that the organic solvent composition did not exceed 1–2% (MacGibbon *et al.*, 1978). Stopped-flow experiments were performed and analysed as described by Bennett *et al.* (1982), Motion *et al.* (1984) and Blackwell *et al.* (1987). The acetaldehyde concentration was determined according to the procedure of Stowell *et al.* (1978).

RESULTS AND DISCUSSION

When enzyme, NADH and acetic anhydride were mixed an extensive decrease in the absorbance at 340 nm was observed, as described by Hart & Dickinson (1978). Simultaneous monitoring of the acetaldehyde and NADH concentrations (Fig. 1) clearly showed that acetaldehyde was both produced and subsequently utilized at the same rate as the accompanying changes in the NADH concentration as expected from Scheme 1. The fact that 80% of the initial NADH was oxidized in the presence of acetic anhydride (at an almost constant rate) shows that there is no intrinsic barrier to the reverse reaction once the active-site group that binds aldehydes on the $*E \cdot NADH$ species has been acylated. The concentration of acetic anhydride used in these experiments maintains the steady-state concentration of the acylated intermediate in the face of competing hydro-

Fig. 1. Acetaldehyde and NADH concentrations during reverse reaction with acetic anhydride

The solution initially contained aldehyde dehydrogenase ($1.7 \mu M$), NADH ($51 \mu M$) and acetic anhydride ($17 mM$) in $0.2 M$ -phosphate buffer, pH 7.0. NADH concentrations ($\bullet$) were monitored by the absorbance at 340 nm, and acetaldehyde concentrations ($\circ$) were determined by withdrawing samples (2 ml), denaturing the enzyme with 3% (v/v) $HClO_4$ and analysing for acetaldehyde by using the method of Stonewell *et al.* (1978). The solution volume was 40 ml.

lysis (the k_d and k_h steps in Scheme 1) for at least 4–5 min (see Fig. 1). Measurements of the initial rate of the reverse reaction as a function of enzyme concentration at saturating concentrations of NADH ($60 \mu M$) and acetic anhydride ($17 mM$) gave a k_{cat} value of $0.2 s^{-1}$ (Motion, 1986). Thus the reverse reaction proceeds at almost the same overall rate as the enzyme-catalysed oxidation of acetaldehyde and propionaldehyde (Blackwell *et al.*, 1987).

Eventually insufficient anhydride and NADH are left to overcome the irreversible utilization of the NAD^+ and acetaldehyde (which have been generated) via the normal dehydrogenase pathway. A slow increase in the concentration of NADH (and concomitant decrease in the concentration of acetaldehyde) therefore occurred (Fig. 1), with about one-third of the acetaldehyde ($12 \mu M$) still remaining after 60 min. Although the enzyme retained some activity after the reverse reaction, further addition of acetic anhydride produced only a small amount of reverse reaction, showing that other sites on the enzyme were also being modified.

When acetic anhydride ($3.2 mM$) and NADH ($15 \mu M$) were rapidly mixed with enzyme ($10 \mu M$) and NADH ($24 \mu M$) in a stopped-flow spectrophotometer, there was an exponential decrease in the nucleotide fluorescence with a rate constant of $11 \pm 3 s^{-1}$ and an amplitude equal to about 30% of the enzyme active-site concentration (approximately equal to the equilibrium concentration of $*E \cdot NADH$; MacGibbon *et al.*, 1977). Increasing the acetic anhydride or NADH concentration above these values had no effect on the rate constant or amplitude of the burst. The transient decrease was followed by a steady-state decrease until a minimum value was reached, after which the nucleotide fluorescence began to increase again exactly as observed in the absorbance traces (Fig. 1). There are three steps on the pathway for the reverse

reaction that could result in a decrease in nucleotide fluorescence. The first is quenching of nucleotide fluorescence on the acylation of the aldehyde-binding active-site group of the $E \cdot NADH$ or $NADH$ complexes (Dickinson & Haywood, 1986); the second is the hydride-transfer step, in which $NADH$ is converted into NAD^+ ; the third is displacement of the bound $NADH$ (about 30%) following binding of the anhydride or the subsequent acylation. The steady-state kinetic isotope effect reported by Hart & Dickinson (1978) indicates that hydride transfer is slow in the reverse direction, and therefore the burst rate constant must be identified with steps preceding this. Hence either the burst rate constant is determined largely by acylation of the enzyme $\cdot NADH$ complexes (k_a is 11 s^{-1}) or acylation is faster than this and the observed process is due to the loss of $NADH$ from the enzyme.

The relative contributions of the quenching of nucleotide fluorescence and a possible change in the dissociation constant for the acylated enzyme $\cdot NADH$ complexes to the rate constant and amplitude for the transient decrease in nucleotide fluorescence cannot be estimated in these experiments. However, even if displacement of the bound $NADH$ takes place on acylation by acetic anhydride it clearly cannot be extensive, since the reverse reaction does occur (Fig. 1). More importantly, when the free enzyme is acylated before rapid mixing with $NADH$ (Motion, 1986), the usual biphasic increase in nucleotide fluorescence indicative of nucleotide binding (MacGibbon *et al.*, 1977) still occurs. The fact that this increase is then followed by the same slower decrease in the nucleotide fluorescence as observed when $NADH$ is added to the enzyme before acetic anhydride (Motion, 1986) demonstrates that the same $E \cdot NADH$ and $E \cdot NADH \cdot \text{acyl}$ complexes are formed irrespective of the order of mixing. If this were not the case no reverse reaction would be seen when acetic anhydride was added first to the reaction mixture, which is contrary to experiment (Motion, 1986).

Computer calculations (Blackwell *et al.*, 1987) based on the normal dehydrogenase pathway show that k_d must be at least $5\text{--}10 \text{ s}^{-1}$ at pH 7.6, since lag phases are not seen following the burst in the production of $NADH$ during the oxidation of acetaldehyde or propionaldehyde monitored in nucleotide fluorescence. The ready reverse reaction to produce large amounts of acetaldehyde can thus be understood kinetically on the basis of Scheme 1. Even though k_d ($5\text{--}10 \text{ s}^{-1}$) is greater than the steady-state rate (0.2 s^{-1} per active site), as fast as the $E \cdot NADH$ species is formed (either by hydrolysis of $E \cdot NADH \cdot \text{acyl}$ or from $E \cdot NADH$ as in Scheme 1) it is reacylated (11 s^{-1}), so that the steady-state concentration of $E \cdot NADH \cdot \text{acyl}$ is maintained until the acetic anhydride concentration is depleted.

On the single-site model (Scheme 1) k_d and k_{cat} must have the same values for 4-nitrophenyl acetate and acetic anhydride if the active-site group of $E \cdot NADH$ that binds aldehydes is being acylated in both cases, since the post-acylation steps are identical. To account for the low concentrations of acetaldehyde detected with 4-nitrophenyl acetate if a single active-site group is involved (Loomes & Kitson, 1986), either k_a of Scheme 1 must be small or alternatively 4-nitrophenyl acetate must predominantly acylate some other site. To determine whether this was so the kinetics of acylation of aldehyde dehydrogenase by 4-nitrophenyl acetate in the presence of

Fig. 2. Acylation of binary enzyme $\cdot NADH$ complexes by 4-nitrophenyl acetate at pH 7.6

Aldehyde dehydrogenase ($10 \mu\text{M}$) and $NADH$ ($35 \mu\text{M}$) from syringe A were rapidly mixed with 4-nitrophenyl acetate ($440 \mu\text{M}$) and $NADH$ ($30 \mu\text{M}$) from syringe B in the same buffer, and the reaction was monitored in nucleotide fluorescence. The regenerated curve for the slower fluorescence decay is superimposed on the experimental data, showing the biphasic nature of the reaction. The inset shows the logarithmic differences between the fluorescence decay curve and the computer-drawn steady-state line that follows the slower transient.

$NADH$ was measured. High concentrations (MacGibbon *et al.*, 1978; Kitson, 1986) of 4-nitrophenyl acetate ($440 \mu\text{M}$) and $NADH$ ($30 \mu\text{M}$) were rapidly mixed with aldehyde dehydrogenase ($10 \mu\text{M}$) and $NADH$ ($35 \mu\text{M}$) in 25 mM buffer, pH 7.6. A rapid biphasic quenching of nucleotide fluorescence (Fig. 2) was observed. This was followed by a linear steady-state rate as the 4-nitrophenoxide ion released during the acylation and hydrolysis steps extensively quenched the nucleotide fluorescence. Analysis of the data gave two apparent first-order rate constants ($k_t = 10.9 \pm 0.6 \text{ s}^{-1}$; $k_s = 1.8 \text{ s}^{-1}$) for the transient quenching of nucleotide fluorescence (with approximately equal amplitudes) and a steady-state rate of 0.38 s^{-1} per active site for the enzyme-catalysed hydrolysis of 4-nitrophenyl acetate, which closely approximates to k_d (Zeffren & Hall, 1973).

The rate constant observed for the faster of the two transients was similar to the values observed for acylation of free enzyme (MacGibbon *et al.*, 1977) or enzyme $\cdot NAD^+$ mixtures (Bennett *et al.*, 1982; Blackwell *et al.*, 1983) and also similar to the rate constant for acylation by acetic anhydride in the present study. This strongly suggests that acylation of $E \cdot NADH$ and/or $E \cdot NADH$ is being observed. Only if loss of $NADH$ from the $E \cdot NADH$ ester or $E \cdot NADH$ ester ternary complexes occurred within the mixing time could it be argued that the decrease in nucleotide fluorescence represented acylation of the free enzyme. However, the results with acetic anhydride clearly show that a pathway to $E \cdot NADH \cdot \text{acyl}$ (and hence a reverse reaction) exists from either $E \cdot \text{acyl}$ or $E \cdot NADH \cdot \text{acyl}$ in the presence of these concentrations of $NADH$ if the same active-site group is involved. [$NADH$ clearly does bind to aldehyde

dehydrogenase in the presence of high concentrations of 4-nitrophenyl acetate, since activation of the steady-state rate of ester hydrolysis is observed (Kitson, 1986).] Hence a similar amount of acetaldehyde should be produced as with acetic anhydride as the acylating agent, even if some displacement of NADH occurs. The fact that almost no acetaldehyde is formed makes it clear that with 4-nitrophenyl acetate most acylation must be occurring at a site that is different from the dehydrogenase active-site group that binds aldehydes.

The second rapid quenching process during acylation by 4-nitrophenyl acetate was not seen in the less sensitive absorbance measurements (MacGibbon *et al.*, 1978; Blackwell *et al.*, 1983) used in the collection of those data. This second process may indicate the presence of a second transient intermediate during acylation, or alternatively the two transients could represent acylation of the two isomeric enzyme-NADH forms (see Scheme 1) at different rates. The actual identity of this process does not affect the arguments presented.

To examine the kinetics of the small amount of reverse reaction that does occur with aldehyde dehydrogenase, the enzyme (2.4 μM), NADH (50 μM) and 4-nitrophenyl acetate (320 μM) were mixed (Motion, 1986), and the small amount of acetaldehyde that was produced was monitored by the procedure described in the legend to Fig. 1. As was observed in the experiment with acetic anhydride, a maximum in acetaldehyde production was reached after 5–10 min, but with 4-nitrophenyl acetate only to a concentration of at most 1 μM (marginally above controls). Thereafter there was a slow decline in the concentrations over about 50 min. The total rate of hydrolysis of 4-nitrophenyl acetate was 5.7 $\mu\text{M}\cdot\text{min}^{-1}$, which therefore allowed ample time for the reverse reaction to proceed (50–60 min). Therefore, as required by the model shown in Scheme 1, the small amount of enzyme that is acylated by 4-nitrophenyl acetate at the active-site group that binds the aldehyde on the oxidative pathway does indeed follow the same time course as when acylated by acetic anhydride. Thus there is clearly no kinetic reason that can account for the low concentrations of acetaldehyde actually produced.

Equally damaging for a simple single-site model is the fact that the value obtained for deacylation with 4-nitrophenyl acetate in the presence of NADH (0.38 s^{-1}) is still an order of magnitude less than the value required (Blackwell *et al.*, 1987) for acyl-enzyme hydrolysis on the dehydrogenase pathway (5–10 s^{-1}), even though according to Scheme 1 the same species is being acylated in both cases. The value of 0.38 s^{-1} for its deacylation in the presence of NADH would give rise to lags following the burst during the oxidation of acetaldehyde, which, as discussed above, is contrary to experiment. This confirms that the acyl-enzyme species detected in the burst

experiments with 4-nitrophenyl acetate does not lie on the dehydrogenase pathway.

The kinetics therefore exclude the possibility that 4-nitrophenyl acetate selectively acylates the group at the active site that binds aldehydes on the oxidative pathway. An acyl-enzyme form is produced by 4-nitrophenyl acetate and observed in the stopped-flow experiments, but it clearly must involve a different group on the enzyme. It is hardly surprising that there may be other sites on the surface of a complex protein that are more readily acylated and can therefore catalyse the hydrolysis of such a labile ester as 4-nitrophenyl acetate.

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