A comparison of nitrophenyl esters and lactones as substrates of cytosolic aldehyde dehydrogenase

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1. *p*-Nitrophenyl (PNP) acetate and propionate show a burst of *p*-nitrophenoxide release when their hydrolysis is catalysed by sheep liver cytosolic aldehyde dehydrogenase. This is not seen in the presence of NAD⁺ or NADH, implying a change in rate-determining step. 2. 6-Nitrodihydrocoumarin (6-NDC) shows no burst of absorbance in the visible region. We propose that the p*K*_a of the transient 'reporter group' produced during the hydrolysis of this lactone is high (approx. 10) and that the incipient covalently linked *p*-nitrophenoxide moiety is protonated immediately on formation. The small burst seen in the hydrolysis of 5-nitro-2-coumaranone (5-NC) suggests that the p*K*_a of its reporter group is about 8.5. 3. NADH markedly enhances the

steady-state rate with the lactones. 5-NC shows a large rapid burst of colour development in the presence of NADH; this implies that NADH decreases the p*K*_a of the reporter group to 7–7.5. 4. In the presence of NAD⁺, 5-NC and 6-NDC give an unusual 'negative burst' in the stopped-flow traces. We propose that, under these circumstances, acylation of the enzyme is extremely fast and that the first event seen in the stopped-flow traces is protonation of the reporter group. NAD⁺ also greatly increases the steady-state rate. 5. With the lactones in the presence of NADH, the *k*_{cat} value (nearly 6 s⁻¹), a measure of the deacylation rate, is compatible with the single-site model for dehydrogenase and esterase activities.

INTRODUCTION

For more than 20 years there has been interest in the ability of mammalian aldehyde dehydrogenase to catalyse the hydrolysis of reactive esters such as *p*-nitrophenyl (PNP) acetate, as originally observed by Feldman and Weiner [1]. During this time, a lively debate has been carried out in the literature as to whether this esterase activity occurs at the same active site as the enzyme's 'natural' dehydrogenase activity and involves attack by the same putative enzymic nucleophile, or whether the two activities are mediated at quite different sites on the enzyme (see [2–7], and references therein). Today it seems that the simpler picture of a common active site is at last becoming firmly established; some of the evidence for this is as follows. The enzyme has been shown to possess a uniquely reactive cysteine residue (Cys-302) that can be labelled by a chromophoric aldehyde substrate [8] and by ester substrate analogues [8,9]. Of several cysteine residues that variously occur in all the forms of aldehyde dehydrogenase so far sequenced (over 20), only Cys-302 is conserved [10]. Most tellingly, mutation of Cys-302 to alanine completely abolishes both esterase and dehydrogenase activity [11]. Furthermore, aldehyde dehydrogenase can catalyse the formation of traces of acetaldehyde from PNP acetate and NADH [5], which can only be reasonably explained if the esterase and dehydrogenase activities share a common acyl-enzyme intermediate. Thus we believe that studying the esterase action of aldehyde dehydrogenase does indeed have the potential of helping to elucidate how the enzyme works as a dehydrogenase.

In previous work we have made use of analogues of PNP acetate that contain a carbamate in place of a simple ester functional group. Such compounds react slowly with sheep liver cytosolic aldehyde dehydrogenase under mild conditions to provide an acyl enzyme that is completely inert to hydrolysis. For instance, PNP di[¹⁴C]methylcarbamate was one of the

reagents that led to the identification of Cys-302 as the enzyme's active-site nucleophile [9]. The cyclic analogue of this compound, 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB), behaves similarly, but furnishes the enzyme with a stable covalently linked *p*-nitrophenol 'reporter group' (as defined by Burr and Koshland [12]) that has allowed interesting conclusions about the environment of the active site to be drawn [13]. At pH values around 7 or 8, the nature of this site is such as to favour strongly the un-ionized form of the reporter group in the absence of NAD⁺, but binding of the latter causes the reporter group to ionize immediately.

In this paper, for the first time, cyclic esters (i.e. lactones) have been investigated as substrates of aldehyde dehydrogenase. 6-Nitrodihydrocoumarin (6-NDC) is the cyclic analogue of PNP propionate and is also a close structural analogue of our cyclic carbamate reagent, DMNB. It was therefore expected to provide a transient reporter group (in the short period between acylation and deacylation of the enzyme) that would be amenable to study by stopped-flow spectrophotometry. Our aim was to monitor this reporter group in the presence and absence of NAD⁺ and NADH and compare the experimental results with what we would predict from our previous experiments with DMNB [13]. We have also investigated the unsaturated lactone, 6-nitrocoumarin, and the cyclic analogue of PNP acetate, 5-nitro-2-coumaranone (5-NC).

Abbreviations used: DMNB, 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one; 6-NDC, 6-nitrodihydrocoumarin; 5-NC, 5-nitro-2-coumaranone; PNP, *p*-nitrophenyl.

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EXPERIMENTAL

Materials

Cytosolic aldehyde dehydrogenase from sheep liver was purified as before [13]. The method includes polyethylene glycol precipitation, ion-exchange chromatography and affinity chromatography [14] and is known to give pure enzyme uncontaminated by the mitochondrial form of aldehyde dehydrogenase. The nitrolactones used here were made by nitration of the commercially available precursors with a standard nitration method as used in the synthesis of DMNB [15]. 5-NC and 6-NDC had a melting point of 187–189 and 134–136 °C respectively; Tobias et al. [16] give 189–189.5 and 130–131 °C. The identities of 5-NC and 6-NDC were confirmed by NMR spectrometry. 6-Nitrocoumarin had a melting point of 195–196 °C. Desai et al. [17] give a value of only 185–186 °C; the identity of our material was confirmed by NMR and mass spectrometry ($M^+ = 191$). PNP esters were commercially available.

Stopped-flow experiments

These were performed with a Hi-Tech Scientific instrument at 25 °C. One syringe contained enzyme in either 50 or 70 mM sodium phosphate buffer, pH 7.4, with or without NAD⁺ or NADH (0.1 mM); the other syringe contained a mixture of 4.5 ml of 50 or 70 mM sodium phosphate buffer, pH 7.4, and 0.5 ml of substrate in acetone, again with or without nucleotide (0.1 mM). Enzyme, substrate and acetone concentrations (after mixing) were generally 7.2–7.7 μ M, 0.25 mM and 5% (v/v) respectively. Although in principle the substrate concentration should be as high as possible to ensure saturation of the enzyme, we wished to avoid any higher acetone concentration; this limited the substrate solubility. Moreover the lactones we used as substrates undergo rapid spontaneous hydrolysis, necessitating speed in performing the experiments before the background absorbance reaches an unacceptable level; this problem would be made worse by using higher concentrations. The non-cyclic esters do not present this difficulty, but we used the same concentrations as of the lactones, to allow meaningful comparisons to be made. Another factor for consideration here is that very high concentrations of PNP acetate give detectable substrate inhibition [18]. Non-cyclic ester hydrolysis was monitored at 400 nm; lactone hydrolysis was usually monitored at 420 or 440 nm as stated in the Figure legends. The products from hydrolysis of the lactones absorb maximally at about 415 nm, but we suspected from our work with DMNB [13] that the reporter groups might absorb at a longer wavelength when bound to the enzyme. Stopped-flow traces were computer-fitted as appropriate either to a straight line or to an exponential burst followed by a straight line, using software supplied by Hi-Tech Scientific. In the latter case, the program automatically gave the best estimates of burst amplitude, burst rate constant, and steady-state rate.

Conventional spectrophotometric experiments

These were performed with a Varian Cary 1 spectrophotometer at 25 °C. The assay mixture consisted of 3.0 ml of 50 mM sodium phosphate buffer, pH 7.4, containing enzyme (approx. 0.27 μ M) and substrate (added as 20 μ l of an acetone solution). The reaction was monitored at 400 nm for PNP esters and 415 nm for lactones. Rates were corrected for spontaneous hydrolysis of substrate, which was particularly necessary with the lactones. In the presence of NAD⁺, reactions with 5-NC were monitored for only 30 s, as substrate depletion became evident after this.

RESULTS AND DISCUSSION

Studies with PNP acetate and propionate

We first investigated the hydrolysis of non-cyclic esters for purposes of comparison with the results described below, obtained with the corresponding lactones. Figure 1 shows that PNP acetate and propionate (in the absence of cofactor) exhibit a classic burst of production of *p*-nitrophenoxide in the pre-steady-state phase of the reaction, implying that hydrolysis of the rapidly formed acyl-enzyme is the rate-determining step. The size of the burst is slightly bigger for the acetate than for the propionate (by a factor of 1.2) and the rate constant is also larger (23 compared with 15 s⁻¹). These results agree well with the previous work that one of us (T. M. K.) carried out in Dr. F. M. Dickinson's laboratory [19], which also showed the acetate to have a burst 1.2 times larger than the propionate, with rate constants of 21 and 18 s⁻¹ respectively. The latter work showed that, for a series of PNP esters, the burst magnitude and the rate constant decrease as the steric bulk of the acyl group increases, culminating in the observation of no burst at all with PNP pivalate (trimethylacetate). For this substrate, acylation of the enzyme is evidently the rate-determining step of the reaction.

Other workers have reported bursts in the esterase action of sheep liver cytosolic aldehyde dehydrogenase. Dickinson and Haywood [7] give 30 s⁻¹ for the burst rate constant for PNP propionate (1 mM) and a burst magnitude of 0.7 mol of *p*-nitrophenol per mol of enzyme tetramers. With PNP acetate, Blackwell et al. [2] reported a rate constant of 12 s⁻¹ and a burst magnitude of between 18 and 30 % of the dehydrogenase active site concentration, which in turn is quoted as being generally between 1 and 2 active sites per tetramer [20]. In other words, these workers detected a burst of between 0.18 and 0.6 mol of product per tetramer, depending on the specific activity of the

Figure 1 Stopped-flow traces of aldehyde dehydrogenase-catalysed hydrolysis of PNP acetate and propionate

The experiments were performed as described in the Experimental section at pH 7.4 and 25 °C with 0.25 mM substrate and an enzyme concentration of 7.7 μ M in (a) and (b) and 7.2 μ M in (c) and (d). In some cases, 0.1 mM NAD⁺ or NADH was also present, as shown. (a and c), PNP acetate with or without NAD⁺ and NADH respectively; (b and d), PNP propionate with or without NAD⁺ and NADH respectively.

Table 1 Results of stopped-flow experiments with a range of PNP acetate and NAD⁺ concentrations

The concentrations of substrate and cofactor given below are those after mixing in the stopped-flow spectrophotometer. In all cases the buffer was 50 mM sodium phosphate, pH 7.4, and the concentrations of enzyme and acetone were 5.4 μ M and 5 % respectively. The burst size is given relative to that seen at each substrate concentration in the absence of NAD⁺. At 0.3 and 1.0 mM NAD⁺, the spectrophotometer trace was slightly irregular over the first 0.15 s, but this could not be described as a burst and could not be fitted as such by the computer. The burst rate constant is given directly in s⁻¹. The steady-state rate after the burst is given relative to that seen at each substrate concentration in the absence of NAD⁺. Each value in this Table represents the mean $\pm$ S.D. for several closely agreeing results.

[NAD ⁺] (mM) ...	[PNP acetate] (mM) 0.25	0.50	1.0
(a) Relative burst size			
0	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	1.00 $\pm$ 0.02
0.02	0.63 $\pm$ 0.02	0.74 $\pm$ 0.06	0.74 $\pm$ 0.05
0.03	0.55 $\pm$ 0.03	0.64 $\pm$ 0.01	0.63 $\pm$ 0.03
0.05	0.49 $\pm$ 0.01	0.46 $\pm$ 0.01	0.57 $\pm$ 0.06
0.1	0.26 $\pm$ 0.01	0.30 $\pm$ 0.01	0.37 $\pm$ 0.04
0.3	0	0	0
1.0	0	0	0
(b) Burst rate constant (s ⁻¹)			
0	24.3 $\pm$ 0.4	32.2 $\pm$ 0.6	36.3 $\pm$ 0.7
0.02	25.6 $\pm$ 1.4	30.9 $\pm$ 2.1	38.0 $\pm$ 1.9
0.03	24.2 $\pm$ 2.1	32.5 $\pm$ 0.8	37.8 $\pm$ 2.3
0.05	25.9 $\pm$ 1.8	30.2 $\pm$ 1.0	33.0 $\pm$ 2.1
0.1	27.4 $\pm$ 0.9	31.6 $\pm$ 2.7	35.8 $\pm$ 1.3
(c) Relative steady-state rate			
0	1.00 $\pm$ 0.03	1.00 $\pm$ 0.01	1.00 $\pm$ 0.03
0.02	0.96 $\pm$ 0.01	1.28 $\pm$ 0.03	1.37 $\pm$ 0.03
0.03	1.34 $\pm$ 0.04	1.43 $\pm$ 0.01	1.54 $\pm$ 0.03
0.05	1.42 $\pm$ 0.03	1.56 $\pm$ 0.02	1.68 $\pm$ 0.04
0.1	1.60 $\pm$ 0.01	1.69 $\pm$ 0.02	1.93 $\pm$ 0.03
0.3	1.38 $\pm$ 0.03	1.90 $\pm$ 0.06	2.36 $\pm$ 0.03
1.0	0.95 $\pm$ 0.01	1.47 $\pm$ 0.05	2.18 $\pm$ 0.03

enzyme. In the present study, our burst with PNP acetate represents 1.1 mol of product per tetramer. The linear phase of the reaction after the burst gives a k_{cat} value of 0.55 s⁻¹, which is somewhat greater than the 0.2 s⁻¹ reported by Blackwell et al. [2]. The higher values here may be a reflection of recent improvements to the method of enzyme isolation [13]. (Incidentally, we have observed that the affinity chromatography step [14] removes a minor impurity that is not an aldehyde dehydrogenase but does have very high activity towards PNP acetate.)

Figure 1 also shows the results of otherwise identical experiments carried out in the presence of either 0.1 mM NAD⁺ or 0.1 mM NADH. When the traces are drawn in this way (on the same scale as those obtained without nucleotide), it is clear that the burst has largely disappeared. Expanding the scale allows the burst still to be measurable in the case of the acetate; curve-fitting shows that its magnitude in the presence of 0.1 mM NAD⁺ and 0.1 mM NADH is approx. 17 % and 10 % respectively of the burst seen in the absence of cofactor.

Blackwell et al. [2], in experiments done under conditions fairly similar to ours, found that NAD⁺ had no effect on either the rate constant or the amplitude of the burst that they found with PNP acetate. Dickinson and Haywood [7] reported that NADH has some effect on the burst seen with PNP propionate, reducing it from 0.7 to 0.5 mol per tetramer, but with little alteration in the rate constant. Because of the conflicting reports on the effect of nucleotides on the burst size, we decided to perform a more comprehensive series of stopped-flow experi-

ments over a range of PNP acetate and NAD⁺ concentrations; the results are presented in Table 1. On this occasion (with a different enzyme preparation) the amplitude of the residual burst seen with 0.25 mM substrate in the presence of 0.1 mM NAD⁺ was a little higher (26 %) than that reported above (17 %), but there is a clear unequivocal trend for the burst to decline effectively to zero as the NAD⁺ concentration rises, and this is true for all three substrate concentrations studied.

We performed a few experiments with an acetone concentration of 1.25 % instead of the normal 5 % that was used in obtaining the data in Table 1 and Figures 1, 3 and 4. Under these conditions, the burst amplitude with 0.25 mM PNP acetate and no NAD⁺, for example, was 83 % of that found at the higher acetone concentration, and the corresponding value in the presence of 0.1 mM NAD⁺ was 111 %. Thus the results depend to some extent on acetone concentration, but not enough to change the conclusions we draw below.

It is also very clear from Table 1 that at NAD⁺ concentrations of 0.1 mM and below, there is no significant change in the rate constant associated with the observed burst. This result agrees with the previous reports [2,7]; perhaps if these workers had investigated more than a single set of concentrations they might also have observed the correlation between burst size and NAD⁺ concentration.

We interpret the results shown in Table 1 and Figure 1 as follows, referring to the scheme below (where P is *p*-nitrophenoxide and EQ is the acyl enzyme):

In the absence of cofactor, the deacylation step is rate-limiting (i.e. $k_{+3} < k_{+2}$) and the size of the burst is given by [21]:

$$\pi = e_0 / (1 + k_{+3} / k_{+2})^2$$

(π approximates to the enzyme concentration, e_0 , only if k_{+2} is very much greater than k_{+3}). However, when the enzyme is saturated by NAD⁺ (which is evidently at a concentration between 0.1 and 0.3 mM) we propose that k_{+2} is much smaller than k_{+3} , so that π becomes effectively zero, as experimentally observed. In other words, acylation is now rate-limiting and it is the rate of this that is reflected in the observed steady-state rate. At intermediate NAD⁺ concentrations, we have (at the start of the stopped-flow experiments) a mixed population of E and E.NAD⁺, and thus we see a proportionally smaller burst (but with the same rate constant) because only the E species, not E.NAD⁺, gives rise to a burst of *p*-nitrophenoxide production. During the experiment (again at intermediate NAD⁺ concentrations) we also have a mixture of EQ and EQ.NAD⁺. Hydrolysis of the latter is evidently faster than that of EQ itself, accounting for the observed increase in steady-state rate (see Table 1). In short, the results are explained by proposing that NAD⁺ increases k_{+3} while reducing k_{+2} , resulting in a change of rate-determining step and loss of the burst.

The expression given above for π only holds if the enzyme is saturated by substrate. This is certainly true in the absence of cofactors, but their presence increases K_m such that the substrate concentration used for most of the experiments here (0.25 mM) is not truly saturating, and this will tend to result in a smaller burst. However, it certainly cannot explain the complete loss of the transient; from previously obtained K_m values for PNP acetate in the presence of 0.1 mM NAD⁺ or NADH [18] we calculate that the enzyme would be 80–90 % saturated in our stopped-flow experiments. Furthermore an NAD⁺ concentration

of 0.3 mM or higher completely eliminates the burst even with a PNP acetate concentration as high as 1 mM (Table 1). Therefore we maintain that only the suggestion of a change in rate-determining step, as discussed above, can properly explain the results.

The observation of no effect of NAD^+ on the burst with PNP acetate was one piece of evidence used by Blackwell et al. [2] to support their hypothesis that esterase and dehydrogenase activities occur at different sites. If, as it now seems (see the Introduction section, and as strongly argued by Dickinson and Haywood [7]), the two activities share the same site, then it could be easier to believe that NAD^+ should have some effect on the burst (as we see in Figure 1) rather than none. Intuitively, one might have thought that the enzyme's cofactors would promote its attack on esters rather than retard it as we are suggesting here; in the dehydrogenase reaction, attack on the aldehyde occurs only after prior binding of NAD^+ [20]. However, there is a precedent for NAD^+ and NADH slowing down the acylation rate with ester substrates and analogues. For instance, the cofactors are inhibitors of the enzyme-catalysed hydrolysis of PNP pivalate [19], for which the steady-state rate is a reflection of the acylation rate, as mentioned above. The cofactors do not simply inhibit the binding of PNP pivalate; at infinite substrate concentration, NAD^+ and NADH still markedly reduce the rate. Similarly with PNP dimethylcarbamate and DMNB, which undergo only the acylation step, NAD^+ and NADH act in an inhibitory way [13,22]. A similar observation with glyceraldehyde 3-phosphate dehydrogenase has also been reported [23]: NAD^+ inhibits acylation of the enzyme by PNP acetate, but if NAD^+ is added after formation of the acyl-enzyme it promotes the rapid transfer of the acetyl group to phosphate ion.

The effect of cofactors on the steady-state rate of hydrolysis of PNP acetate has been reported to be activatory at low concentrations and inhibitory at high concentrations [24]. We agree but find the results are, perhaps not surprisingly, highly dependent on the relative concentrations of enzyme, substrate and cofactor. In the present study, NAD^+ and NADH affected the steady-state rate observed in the stopped-flow experiments with PNP acetate as shown in Table 1. In conventional spectrophotometric experiments (with a much lower enzyme concentration), the effect of a range of NAD^+ concentrations on the rate of hydrolysis of 0.5 mM PNP acetate is shown in Figure 2. Low concentrations (approx. 10 μM) give a modest activation; higher concentrations are inhibitory. These results are consistent with the explanation put forward above involving a change in rate-determining step. Low concentrations of NAD^+ speed up the initially rate-determining deacylation rate, but as the enzyme becomes more saturated by NAD^+ , the inhibitory effect on the acylation step becomes dominant and this process then takes over the rate-limiting role. Previous work with methyl PNP carbonate is of interest here [22]. For this substrate also, NAD^+ and NADH seem to slow down the acylation rate while simultaneously accelerating the deacylation rate. As the burst rate is one-tenth as fast as with PNP acetate, whereas the rate-limiting deacylation rate is only 1/170 as fast, there is much greater scope with methyl PNP carbonate than with PNP acetate for nucleotides to enhance the steady-state rate by a large factor before any inhibitory effect on the burst rate becomes evident.

6-Nitrocoumarin is not a substrate

We report that 6-nitrocoumarin shows no substrate activity at all with cytosolic aldehyde dehydrogenase. If 0.1 mM 6-nitrocoumarin is added to an enzyme-catalysed assay with 0.1 mM PNP acetate as substrate, the rate is 86% of the rate

Figure 2 Effect of NAD^+ concentration on the steady-state rate of ester and lactone hydrolysis catalysed by aldehyde dehydrogenase

The experiments were performed at pH 7.4 and 25 °C with a substrate concentration of 0.5 mM and an enzyme concentration of approx. 0.27 μM . Data were corrected for the rate of spontaneous hydrolysis and are plotted as a fraction or multiple of the rate in the absence of NAD^+ (shown as a broken line). In (a) and (b) the concentration of NAD^+ is plotted as the $\log_{10}$ of the value of the concentration in μM . (a) PNP acetate; (b) 5-NC; (c) some of the data for 5-NC are plotted again in double-reciprocal form.

without 6-nitrocoumarin. With 0.1 mM PNP propionate, the value is 76%. A delay of 30 min before adding PNP propionate to the mixture of enzyme and 6-nitrocoumarin gives a similar result (77%). These data show that 6-nitrocoumarin acts as an inhibitor of the esterase activity but not as a progressive inactivator of the enzyme. With PNP pivalate (which has a more convenient K_m for inhibition studies), 6-nitrocoumarin was confirmed to be a competitive inhibitor, with K_i approx. 0.6 mM. The inertness of the compound as a substrate or inactivator is explained by the stability of its electronic structure; there is apparently a significant benzopyrylium aromatic character to the compound as the following resonance forms show:

Its lack of reactivity is also reflected in the negligible rate of spontaneous hydrolysis compared with the other PNP lactones and esters studied here.

Figure 3 Stopped-flow traces of aldehyde dehydrogenase-catalysed hydrolysis of 5-NC and 6-NDC, with and without NADH

The experiments were performed as described in the Experimental section at pH 7.4 and 25 °C with 0.25 mM substrate and an enzyme concentration of 7.2 μM . (a and c) 5-NC in the absence and presence of 0.1 mM NADH respectively; (b and d) 6-NDC in the absence and presence of 0.1 mM NADH respectively.

5-NC and 6-NDC as substrates of aldehyde dehydrogenase

In the absence of nucleotides, 5-NC and 6-NDC are moderately good substrates for cytosolic aldehyde dehydrogenase. The K_m for 5-NC is 71 μM and its $V_{\max}$ is 0.73 times that of PNP acetate ($K_m = 2.6 \mu\text{M}$); the K_m for 6-NDC is 53 μM and its $V_{\max}$ is 0.80 times that of its non-cyclic analogue, PNP propionate ($K_m = 8.0 \mu\text{M}$).

Figure 3 shows the results of stopped-flow experiments carried out under the same conditions as with the non-cyclic esters (Figure 1). There is a burst in production of product from 5-NC ($k = 39 \text{ s}^{-1}$) but it is small, less than 10% of the size observed with PNP acetate. No burst is seen with 6-NDC. The lack of a burst with 6-NDC could imply that acylation is rate-limiting (as it is with the sterically hindered PNP pivalate), but there is reason to expect from their structure that lactones are more open to nucleophilic attack than the corresponding esters (as reflected in their greater rate of spontaneous hydrolysis). Moreover the observation of a burst, albeit a small one, with 5-NC argues that acylation with the lactones (as with PNP acetate and propionate) is a fast process. Therefore we propose the following alternative explanation for the lack of an observable burst with 6-NDC.

Our work with DMNB [13] showed that (in the absence of cofactor) the pK_a of the enzyme-linked reporter group is close to 10; i.e. at pH 7.4 the *p*-nitrophenol group is totally un-ionized. (Free *p*-nitrophenol in aqueous solution has a pK_a of 7.1.) We assume that the pK_a of the similar group transiently linked to the enzyme during the hydrolysis of 6-NDC will also be perturbed to a high value (because DMNB and 6-NDC are of such similar structures). This transient reporter group is presumably liberated initially in its ionized form (on breakdown of the tetrahedral intermediate formed by attack of the enzyme on the lactone's carbonyl group). The fact that no burst of absorbance is seen in

Scheme 1

For 6-NDC, $x = 2$; for 5-NC, $x = 1$.

the visible region of the spectrum (Figure 3b) implies that the *p*-nitrophenoxide moiety is then immediately protonated; that is, step B is faster than step A in Scheme 1. The reporter group then remains in this preferred un-ionized form until the rate-limiting deacylation step liberates it from the enzyme.

The transient reporter group produced from 5-NC is similar to that from 6-NDC, but has a linker arm that is one methylene group shorter. We cannot therefore assume that its pK_a when linked to aldehyde dehydrogenase will necessarily be as high as we suspect for 6-NDC (and as it is known to be for DMNB). If the pK_a were in the region of 8.5, then the equilibrium represented as step B in Scheme 1 would lie predominantly, but not completely, to the right at pH 7.4, and this would then neatly explain why a small burst of visible absorbance occurs.

The rapid protonation of the *p*-nitrophenoxide reporter group we have proposed above may involve an enzymic amino acid side chain rather than a water molecule (especially as the active site may well be hydrophobic in character [13]). It has been suggested [25] that the anionic form of Glu-268 (which is totally conserved and can be labelled with an active-site-directed modifier) acts to remove the proton from Cys-302, enhancing the latter's nucleophilicity. If this occurs during enzyme-catalysed lactone hydrolysis, Glu-268 may then be in an ideal position to donate the proton back to the incipient *p*-nitrophenoxide group.

Effect of NADH on hydrolysis of 5-NC and 6-NDC

Superficially, the presence of 0.1 mM NADH seems to have little effect in the stopped-flow experiments shown in Figure 3; for example, the shapes of Figures 3(a) and 3(c) obtained with 5-NC are very similar. However, note the different absorbance scales; both the size of the burst and the subsequent steady-state rate are markedly increased by NADH, by factors of 13 and 19 respectively. The burst is now approximately 90% as big as is observed with PNP acetate (in the absence of cofactor) and its rate constant is 59 s^{-1} , increased from 39 s^{-1} in the absence of NADH. With 6-NDC, the steady-state rate is also much enhanced (12-fold) and, although the early part of the trace is not linear, no real burst is observed. (The large steady-state rates mean that substrates soon become depleted under these conditions, but not in the time frame of Figure 3.)

With DMNB, the pK_a of the reporter group is decreased somewhat in the presence of NADH (from approx. 10 to 9.2) [13]. If something similar happens with 6-NDC, then at pH 7.4 the transient reporter group will still prefer to be very largely but not entirely in its un-ionized form. Assuming, as before, that the *p*-nitrophenoxide ion can be rapidly protonated, this would account for the absence of a proper burst but only the suggestion of an early rapid process in the trace (Figure 3d). In Figure 3(c), the situation is more clearcut; the results are consistent with NADH's lowering the pK_a of the reporter group derived from 5-

Figure 4 Stopped-flow traces of aldehyde dehydrogenase-catalysed hydrolysis of 5-NC and 6-NDC in the presence of NAD^+

The experiments were performed as described in the Experimental section at 25 °C with 0.25 mM substrate, 0.1 mM NAD^+ and an enzyme concentration of 7.7 μM in (a) and (b), and 7.2 μM in (c) to (f). (a and b) 5-NC and 6-NDC respectively at pH 7.4; (c and d) 5-NC and 6-NDC respectively at pH 7.4, monitored over a much shorter time scale; (e and f) 5-NC and 6-NDC respectively at pH 5.6.

NC to approx. 7–7.5, thus giving a burst similar in size to those obtained with the non-cyclic esters.

Effect of NAD^+ on hydrolysis of 5-NC and 6-NDC

In the presence of NAD^+ , the pK_a of the DMNB-derived reporter group attached to aldehyde dehydrogenase is markedly lowered [13], such that at pH 7.4 it exists virtually completely as the anion. By analogy, we predicted that the transient reporter group from 6-NDC would similarly be ionized; i.e. in Scheme 1 there would be no tendency for reaction B to occur. Thus a burst of colour development would be seen, unless the presence of NAD^+ were to slow down the acylation rate (rendering it rate-determining) as it evidently does with the non-cyclic esters, in which case a linear stopped-flow trace would be expected. Figure 4b shows that neither of these possibilities actually occurs. Instead there is a large initial decline in absorbance, a 'negative burst', similar in size to the burst observed with PNP esters in the absence of NAD^+ . A qualitatively similar picture is seen with the five-membered lactone (Figure 4a). The rate constants for the negative burst processes are approx. 13 and 12 s^{-1} for 5-NC and 6-NDC respectively, and the subsequent steady-state rates are

Figure 5 Steady-state rate and 'negative burst' size obtained in the aldehyde dehydrogenase-catalysed hydrolysis of 6-NDC in the presence of NAD^+ as a function of wavelength

The experiments were performed as described in the Experimental section at 25 °C and pH 7.4 with 0.25 mM substrate, 0.1 mM NAD^+ and an enzyme concentration of 7.2 μM . The absorbance was monitored over a range of wavelengths as shown. Symbols: ○, steady-state rate, taken from the slope of the linear part of traces such as that shown in Figure 4b; ●, size of the 'negative burst', measured by extrapolating the linear part of the trace to zero time and subtracting the absorbance from the observed initial absorbance in the experimental stopped-flow trace.

again greatly enhanced over the corresponding rates without NAD^+ , by 17-fold and 8-fold respectively.

A series of identical stopped-flow experiments was performed with 6-NDC in the presence of 0.1 mM NAD^+ , monitoring the absorbance change at different wavelengths. The results are shown in Figure 5. The steady-state rate (which does not actually alter) seems maximal at 415 nm; this is as expected because the steady-state rate reflects the appearance of the free product, 3-(2-hydroxy-5-nitrophenyl)propanoate, and this has a λ_{max} of 415 nm (in the phenoxide form). In contrast, the magnitude of the negative burst (measured by extending the linear part of the trace back to zero time) seems largest at about 435 nm. This is very close to the λ_{max} of the DMNB-derived reporter group in the presence of NAD^+ [13]. Thus we are led to conclude that the ionized form of the acyl-enzyme is already present at the beginning of the stopped-flow traces of Figures 4(a) and 4(b), and the negative burst is a consequence of the protonation of this species. In other words, referring to Scheme 1, in the presence of NAD^+ the rate of reaction A is too fast to observe even by stopped-flow spectrophotometry, and the rate of the subsequent step B is slower but still fast enough to ensure a build-up of the (colourless) un-ionized acyl-enzyme before the rate-limiting deacylation step.

Expanding the time scale to permit the observation of the first few milliseconds of the reaction shows no sign of any very rapid initial increase in absorbance in the hydrolysis of 6-NDC (Figure 4d), but there is a suggestion with 5-NC that we are seeing the tail-end of a rise before the negative burst sets in (Figure 4c). Lowering the pH to 5.6 in an attempt to slow down the rate of the enzyme-catalysed reaction gave quite different results with the two lactones. Once again, with 6-NDC, the initial formation of the ionized form of the transient reporter group seems to be too fast to detect, but now with 5-NC there is a clear exponential increase in absorbance ($k = 33 \text{ s}^{-1}$) before the exponential decay in absorbance ($k = 6 \text{ s}^{-1}$), which is then followed by the steady-state rate (not seen on the time scale of Figure 4c).

Our conclusion here is therefore that with 5-NC (at pH 7.4) and 6-NDC (at both pH 7.4 and 5.6) the presence of NAD^+ greatly accelerates the initial attack of aldehyde dehydrogenase

on the lactones. This is interesting in view of our conclusion above that NAD^+ slows the acylation rate with PNP esters. The esters and their corresponding lactones cannot necessarily be expected to behave identically. The lactones must be 'cisoid' in configuration, whereas the non-cyclic esters are free to adopt a 'transoid' configuration [16]; similar differences in shape are shown by DMNB and its non-cyclic analogue, PNP dimethylcarbamate [26]. The lactones are intrinsically more reactive than the esters (for example, to spontaneous hydrolysis); this may be partly a steric effect and partly an electronic one in that resonance stabilization of the following kind may not be as significant when the grouping is part of a 5- or 6-membered ring:

Clearly, however, the explanation for the different effects of NAD^+ on esters and lactones is not simply a matter of non-cyclic as opposed to cyclic structure, because NAD^+ is inhibitory to the action of the enzyme on both PNP dimethylcarbamate [22] and its cyclic analogue DMNB [13]. It has long been thought that the binding of NAD^+ causes a conformational change in the enzyme (because it is needed to bind before aldehydes can do so [20]) and this may be quite subtle. It seems that the effect may be to align Cys-302 precisely for rapid nucleophilic attack on aldehyde substrates and also, fortuitously, the lactones studied here; whereas with the various other compounds referred to, the scope for attack on the carbonyl group happens to be less favourable than in the absence of NAD^+ . The situation is evidently different with the mitochondrial form of aldehyde dehydrogenase; it has been concluded that NAD^+ increases the magnitude of the rate constant for acylation by PNP acetate [27], rather than eliminating the burst as we find. (There are other major differences between class 1 and class 2 aldehyde dehydrogenases, such as the effect of disulfiram [28].)

Returning to the stopped-flow traces seen with 5-NC and 6-NDC in the presence of NAD^+ (Figures 4a and 4b), we are faced with the question of why the ionized form of the reporter group apparently becomes protonated (which is how we have explained the negative burst) when we would have predicted from our work with DMNB that the ionized form would be favourable under these conditions? A possible explanation here is that it may have been naïve to expect the reporter groups produced from 6-NDC and DMNB to behave identically simply because they involve the same number of linking atoms between the nitrophenoxide moiety and Cys-302. In 6-NDC there are several single bonds capable of free rotation, as shown below, whereas with DMNB the linking chain is much more rigid and planar, as is emphasized by drawing its alternative resonance structure:

Thus the two reporter groups may occupy significantly different microenvironments within the active site and interact differently with NAD^+ .

The effect of increasing NAD^+ concentration on the steady-state rate of enzyme-catalysed hydrolysis of 5-NC is shown in Figure 2b, a very different profile from that obtained with PNP acetate (Figure 2a). There is a much more pronounced activation with the lactone, and it is biphasic. When the reciprocal of the

Table 2 Apparent k_{cat} values for cytosolic aldehyde dehydrogenase acting on esters and lactones with and without cofactors

The steady-state portion of the appropriate stopped-flow trace (see Figures 1, 3 and 4) was used to calculate a value for the apparent k_{cat} value under various conditions. Each value represents the mean $\pm$ S.D. for several closely agreeing data.

Substrate	k_{cat} (s^{-1})		
	No cofactor	0.1 mM NAD^+	0.1 mM NADH
PNP acetate	0.55 ± 0.01	0.92 ± 0.01	0.98 ± 0.04
PNP propionate	1.5 ± 0.04	1.3 ± 0.02	1.4 ± 0.01
5-NC	0.29 ± 0.01	4.7 ± 0.08	5.7 ± 0.22
6-NDC	0.51 ± 0.01	4.2 ± 0.05	5.9 ± 0.09

rate is plotted against the reciprocal of the NAD^+ concentration up to approx. $20 \mu\text{M}$, a straight line is not obtained (Figure 2c); this is consistent with the mechanism giving rise to hyperbolic activation discussed by Cornish-Bowden [21]. The interpretation here is that enzyme-catalysed hydrolysis of 5-NC is activated by NAD^+ up to the point at which the latter is saturating. The ratio of NAD^+ to enzyme at this point is similar to the corresponding ratio in the experiments referred to in Table 1 in which the enzyme was likewise deduced to be saturated by NAD^+ (although the absolute concentrations were much greater in the stopped-flow experiments). At very high NAD^+ concentration there is further activation of the rate of hydrolysis of 5-NC (see Figure 2b); this implies that the enzyme has two different types of NAD^+ binding site. Generally the aldehyde dehydrogenase tetramer seems to operate with less than full sites reactivity [7], but perhaps a very high NAD^+ -to-enzyme ratio forces more than the usual number of coenzyme sites to become occupied.

The presence of NAD^+ also has a different effect on the K_m for the two substrates; with PNP acetate K_m is increased from 2.6 to $40 \mu\text{M}$, whereas with 5-NC it decreases from 71 to $16 \mu\text{M}$. Although Figures 2(a) and 2(b) seem different, the results are consistent with the explanations we have put forward above. Thus, in both cases, increasing NAD^+ concentration accelerates the deacylation rate, but with the lactone this effect is never swamped by a simultaneous major retardation of the acylation rate, and deacylation remains rate-limiting throughout the profile.

Turnover number of aldehyde dehydrogenase under various conditions

From the steady-state portions of the appropriate stopped-flow traces we have estimated values of k_{cat} for the four substrates studied here, with and without cofactors, and the results are shown in Table 2. (Where necessary, the absolute values of increase in observed absorbance with time were corrected to what they would be at the λ_{max} of the product and then converted into apparent V_{max} values by using experimentally determined values of the absorption coefficient for each product at pH 7.4.) The values are likely to be slight underestimates in some cases because substrate concentration was not always truly saturating. Apart from the non-cyclic esters in the presence of nucleotides (where we believe acylation is largely rate-limiting, as discussed above), the results represent the rate of hydrolysis of the acyl-enzyme, and some interesting comparisons can be made. Hydrolysis of the propionyl group is faster than of the acetyl group (by a factor of 2.7; in previous work we found 1.7 [19]), so clearly the attack of water on the acyl-enzyme is not subject to steric

hindrance. Similarly the presence of the bulky nitrophenol moiety attached to the acyl group does not slow the hydrolysis of the acyl-enzyme very much. The most striking comparison is in the turnover numbers for the lactones, where rather modest values are markedly enhanced by the presence of cofactors. When acting as a dehydrogenase, the rate-determining step for cytosolic aldehyde dehydrogenase is reported to be isomerization of the enzyme–NADH complex before dissociation of NADH [20], and so the observed low value of k_{cat} (approx. 0.2 s^{-1} [2]) tells us nothing about any of the chemical steps involved in the enzyme mechanism. Here, however, the turnover number of almost 6 s^{-1} for the lactones in the presence of NADH gives a more realistic assessment of the enzyme's catalytic ability; in this situation, unlike when it operates as a dehydrogenase, the enzyme does not have to await the dissociation of NADH before starting on the removal of the next substrate molecule.

Motion et al. [6] state that 'damaging for a simple single-site model (for esterase and dehydrogenase activities) 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 [29] for acyl-enzyme hydrolysis on the dehydrogenase pathway ($5\text{--}10 \text{ s}^{-1}$)'. According to our thinking, k_{cat} for PNP acetate in the presence of NADH represents the acylation rate rather than deacylation; with the lactones, however, where it is a measure of deacylation, the value (nearly 6 s^{-1} ; see Table 2) falls precisely in the range required to support the one-site model. Motion et al. [6] used the indirect technique of monitoring nucleotide fluorescence rather than direct observation of *p*-nitrophenoxide to investigate whether a burst occurs with PNP acetate in the presence of NADH. They came to the surprising conclusion that acetylation of the enzyme–NADH complex by acetic anhydride and acetylation by PNP acetate occur at the same rate ($k = 11 \text{ s}^{-1}$), even though acetate is a better leaving group than *p*-nitrophenoxide by more than two orders of magnitude. (The $\text{p}K_{\text{a}}$ values of acetic acid and *p*-nitrophenol are 4.8 and 7.1 respectively.) Using direct spectrophotometric observation, we find that the burst rate constant with 5-NC in the presence of NADH is 59 s^{-1} , and one intuitively feels that acetic anhydride should react even faster.

Our present results also have a bearing on the previous work that detected the production of acetaldehyde from a mixture of enzyme, NADH and PNP acetate [5]. It now seems that the formation of the acyl-enzyme in this situation is relatively slow, but the subsequent hydrolysis is fast (as evidenced by the large k_{cat} values for the lactones, where acyl-enzyme formation is not slowed); this rapid acyl-enzyme hydrolysis compared with its rate of formation may account for the fact that only a small (though definite) amount of the acyl group is reduced to acetaldehyde. It would be interesting to investigate whether the acyl-enzyme known to be formed rapidly from 5-NC in the presence of NADH (see Figure 3c) undergoes reduction to an aldehyde in any greater a proportion than in the case of PNP acetate.

Conclusion

There is no doubt that the present studies comparing the action of aldehyde dehydrogenase on PNP esters and lactones have led

to some fascinating and varied observations, with interesting implications as discussed above. The transient acyl-enzymes formed by 5-NC and 6-NDC cannot be studied by X-ray crystallography, but fortunately the stable DMNB-derived acyl-enzyme crystallizes well and structural studies are in progress [30]; in due course the enzyme's tertiary structure and the precise way in which the various reporter groups interact with residues at the active site and with NAD^+ and NADH should become known.

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