Studies of the esterase activity of cytosolic aldehyde dehydrogenase with resorufin acetate as substrate

Trevor M. KITSON and Kathryn E. KITSON

Departments of Chemistry and Biochemistry, Massey University, Private Bag 11222, Palmerston North, New Zealand

Resorufin acetate is a very good substrate for sheep liver cytosolic aldehyde dehydrogenase, both from the point of view of practical spectrophotometry and in terms of information provided about the nature of the catalysis shown by this enzyme. *p*-Nitrophenyl (PNP) acetate competes against resorufin acetate for the enzyme's active site (although relatively weakly as the latter substrate has the lower Michaelis constant), but acetaldehyde (in the presence of NAD^+) inhibits the hydrolysis of resorufin acetate only at very high aldehyde concentration. In the absence of cofactor, the rate-limiting step in the hydrolysis of resorufin acetate and of PNP acetate is hydrolysis of the common acetyl-enzyme, as shown by the observation of bursts of chromophoric product and very similar values of k_{cat} . In the presence of NAD^+ or NADH, however, the deacylation step with resorufin acetate is greatly

accelerated until acylation seems to become rate-limiting, because no burst is seen under these conditions. Millimolar concentrations of Mg^{2+} activate the hydrolysis of resorufin acetate both in the presence and absence of cofactors. With both Mg^{2+} and cofactor the k_{cat} for hydrolysis of resorufin acetate is 30–35 s^{-1} ; this is three orders of magnitude higher than the k_{cat} for aldehyde oxidation in the presence of Mg^{2+} , showing that the enzyme's potential catalytic efficiency is very much hampered by the slowness with which NADH dissociates from its binding site. The pH profile for the hydrolysis of resorufin acetate in the presence of NAD^+ or NADH fits well to a theoretical ionization curve of $\text{p}K_{\text{a}}$ approx. 8.2; it is suggested that this might belong to the enzyme's putative catalytic residue (Cys-302).

INTRODUCTION

Over the past 25 years there has been much interest in the esterase activity of aldehyde dehydrogenase, largely because of the continuing disagreement on whether or not the twin activities of the enzyme occur at the same active site [1–7]. In that time, only *p*-nitrophenyl (PNP) esters have been used as substrates for the esterase action, until our recent work with resorufin acetate. Although this compound was suggested to have potential as an esterase substrate quite some time ago [8], it seems to have been neglected. We have found that it is a useful substrate for chymotrypsin [9] and a particularly good one for sheep liver cytosolic aldehyde dehydrogenase [10], its main practical advantage over PNP esters being the very much higher absorption coefficient of the resorufin anion (the product of hydrolysis) compared with *p*-nitrophenoxide (Scheme 1). Our preliminary studies [10] have already suggested that (in the absence of cofactors) the hydrolysis of resorufin acetate and that of PNP acetate catalysed by aldehyde dehydrogenase are governed by the rate-limiting hydrolysis of the common acetyl-enzyme intermediate. However, the two substrates differ very strikingly when their hydrolysis is studied in the presence of NAD^+ or NADH; the reaction with resorufin acetate is greatly accelerated, whereas there is only a modest effect with PNP acetate.

In the present work we have extended our investigation of the effect of nucleotides on the hydrolysis of resorufin acetate by investigating the pre-steady-state phase of the reaction. We have also studied the effects of Mg^{2+} and of changes in pH on the steady-state rate of reaction. The results give some very interesting insights into the nature of the catalysis performed by aldehyde dehydrogenase.

EXPERIMENTAL

Materials

Cytosolic aldehyde dehydrogenase was purified from sheep liver and its concentration was measured as before [11]. Dithio-dithreitol, used to protect the enzyme against oxidation, was removed by dialysis against 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA, before experiments were carried out. Resorufin acetate was synthesized as previously described [9,12].

Conventional spectrophotometric experiments

These were performed with a Varian Cary 1 spectrophotometer at 25 °C. The volume of the assay mixture was 3.0 ml and the buffer was 50 mM sodium phosphate, pH 7.4, unless otherwise stated. Experiments with Mg^{2+} used 50 mM Tris/HCl buffer, pH 7.4. Experiments at other pH values used buffers made by mixing either *x* ml of 0.1 M citric acid, (20–*x*) ml of 0.2 M Na_2HPO_4 and 10 ml of water, or 10 ml of 0.1 M Tris, *x* ml of 0.1 M HCl and (20–*x*) ml of water. Dehydrogenase assays used 1 mM NAD^+ and 1 mM acetaldehyde. Esterase assays used the concentration of substrate noted, added as a small volume (e.g. 20 μl) in ethanol or acetone. The inactivator disulfiram was added similarly; an equivalent volume of organic solvent was added to the control assays. The hydrolysis of resorufin acetate was monitored at 571 nm and that of PNP acetate at 399 nm. All esterase data were corrected for the rate of spontaneous hydrolysis of substrate in the absence of enzyme. In the pH profile experiments, the observed rates of increase in A_{571} were corrected to what they would have been if the resorufin product had been

Scheme 1 Structures of resorufin acetate (left panel) and resorufin (anionic form) (right panel)

completely ionized, based on a pK_a value of 5.8 [10]. All assays were done in duplicate or triplicate, always with very satisfactory agreement.

Stopped-flow experiments

These were performed with a Hi-Tech Scientific instrument at 25 °C. One syringe contained enzyme in 50 mM sodium phosphate buffer, pH 7.4, with or without NAD^+ or $NADH$ (0.25 mM); the other syringe contained a mixture of 4.5 ml of 50 mM sodium phosphate buffer, pH 7.4, and 0.5 ml of substrate in acetone, again with or without nucleotide (0.25 mM). Enzyme, substrate and acetone concentrations (after mixing) were 8.32 μM , 0.1 mM and 5% (v/v) respectively. Stopped-flow traces were fitted by computer to an exponential burst followed by a straight line, using software supplied by Hi-Tech Scientific; this program automatically gives the best estimates of burst amplitude and burst rate constant. All stopped-flow runs were repeated at least 10 times with very satisfactory agreement; the results quoted are means $\pm$ S.D.

RESULTS AND DISCUSSION

Competition between resorufin acetate and other substrates or modifiers of aldehyde dehydrogenase

The maximal absorbance of the resorufin anion (571 nm) is well away from that of *p*-nitrophenoxide (399 nm) and so the enzyme-catalysed hydrolysis of resorufin acetate can be monitored in the presence of PNP acetate. With equal concentrations of substrates (25 μM) the rate of resorufin acetate hydrolysis was found to be 90% of the rate in the absence of PNP acetate; with 25 μM resorufin acetate and 0.5 mM PNP acetate, the corresponding value was 44%. Cornish-Bowden ([13], p. 84) gives the following equation for competing substrates:

$$v_1 = V^A a / [K_m^A (1 + b/K_m^B) + a]$$

where, in our case, a and b are the concentrations of resorufin acetate and PNP acetate respectively, K_m^A and K_m^B are their Michaelis constants, V^A is the maximal velocity for resorufin acetate alone, and v_1 is the rate in the presence of the other substrate. Using values of 0.15 μM [10] and 2.6 μM [14] respectively for the Michaelis constants, it is calculated that the rate should be 94% and 46% of the control rate under the conditions referred to above, in excellent agreement with the experimental data obtained here. Thus, as expected, the two ester substrates can indeed be simply imagined as competing with each other for the enzyme.

The situation is not as simple when we examine the effect of dehydrogenase substrates on the hydrolysis of resorufin acetate. For example, if the ester (25 μM) is added to enzyme (0.032 μM) already in the presence of NAD^+ (0.1 or 1 mM) and acetaldehyde (1 mM), and the rate of release of resorufin is monitored, no inhibition at all of ester hydrolysis is observed. Only at very high concentrations of acetaldehyde is the rate reduced; the rate of

hydrolysis of resorufin acetate (25 μM) in the presence of NAD^+ (0.1 mM) and the following concentrations of acetaldehyde is given as a percentage of the control rate in the absence of acetaldehyde: 1 mM (104%), 10 mM (79%), 20 mM (52%), 50 mM (21%). For a variety of reasons we think that the esterase and dehydrogenase activities of aldehyde dehydrogenase occur at the same active site [4,15,16]; we must therefore provide an explanation of why acetaldehyde at a concentration (1 mM) that would be considered fully saturating is ineffective as an inhibitor of the esterase activity. During the course of the aldehyde dehydrogenation reaction catalysed by the enzyme, the slow step is the dissociation of $NADH$ from the $E \cdot NADH$ complex. This has to happen before binding of first NAD^+ then aldehyde [17]. However, binding of resorufin acetate does not have to await the dissociation of $NADH$; the hydrolysis reaction with this substrate in the presence of $NADH$ is in fact very fast [10]. Thus the enzyme can be diverted into acting as an esterase at the $E \cdot NADH$ stage. Identical reasoning has been used to explain why a low concentration of disulfiram is a rapid inactivator of cytosolic aldehyde dehydrogenase in the presence of NAD^+ and 1 mM acetaldehyde [18]. Very high concentrations of acetaldehyde are suggested to form an abortive $E \cdot NADH \cdot$ aldehyde complex [19], and this would account for the inhibition of the esterase activity under these conditions. Similarly, very high aldehyde concentrations protect the enzyme against disulfiram [20].

When disulfiram (10 μM) is added to enzyme (0.32 μM) and then after 1 min the remaining activity is assayed by adding resorufin acetate (25 μM), it is found that approx. 96% inactivation has occurred. In contrast, if disulfiram is added to enzyme already in the presence of the ester, only a slow gradual loss of activity is observed; after 5 min, for example, the enzyme retains approx. 35% of the control activity. A similar protection of the enzyme against disulfiram has been seen before with PNP acetate [18]. Evidently with saturating levels of ester substrate, the enzyme is almost always in the form of either the ES complex or the acyl-enzyme and hence is protected against modification of the active site by disulfiram, whereas in the dehydrogenase mode the enzyme is vulnerable to modification when in the $E \cdot NADH$ form (unless the aldehyde concentration is very high) as mentioned above.

Effect of NAD^+ on the hydrolysis of resorufin acetate catalysed by aldehyde dehydrogenase

We have previously reported [10] that 0.1 mM NAD^+ or $NADH$ greatly affects the rate of enzyme-catalysed hydrolysis of resorufin acetate, much more so than it does that of PNP acetate. In Figure 1 we show the effect of a wide range of NAD^+ concentrations. Even a very low concentration has an appreciable activatory effect (e.g. 1 μM causes a 2.5-fold enhancement); the activation rises to a maximum under these conditions of approx. 23-fold at 0.5 mM NAD^+ . Plotting some of the data from Figure 1(a) in double-reciprocal form gives a smooth curve (Figure 1b) consistent with the mechanism for hyperbolic activation discussed by Cornish-Bowden ([13], p. 88). At higher concentrations of NAD^+ (1 mM and above) the rate of hydrolysis of resorufin acetate begins to fall (Figure 1a).

In previous work [16] we explained the effect of NAD^+ on the hydrolysis of PNP acetate by aldehyde dehydrogenase in terms of a change in rate-determining step. Low concentrations of NAD^+ enhance the initially rate-limiting deacylation step (i.e. the hydrolysis of the acyl-enzyme intermediate). However, increasing NAD^+ concentration also slows the acylation step; this soon takes over the rate-limiting role, and at higher NAD^+ concentrations the steady-state rate of hydrolysis of PNP acetate is

Figure 1 Effect of NAD^+ concentration on the steady-state rate of hydrolysis of resorufin acetate catalysed by aldehyde dehydrogenase

The experiments were performed at pH 7.4 and 25 °C with a substrate concentration of 25 μM and an enzyme concentration of 0.0161 μM . In (a), data are plotted as a multiple of the rate in the absence of cofactor. In (b), some of the data are plotted in double-reciprocal form. In this figure and Figures 6–8, the data are the means of determinations performed in duplicate; in general the agreement was such that the spread of the results was similar in size to the symbol used on the graph and so error bars are not included.

significantly less than in the absence of NAD^+ . In the present work, if a similar change in rate-determining step occurs with resorufin acetate, then clearly it happens at a higher NAD^+ concentration; the maximum in the curve in Figure 1(a) occurs at approx. 0.5 mM rather than at approx. 10 μM as we found with PNP acetate [16]. In other words, the accelerating effect that NAD^+ has on acyl-enzyme hydrolysis is directly observable with resorufin acetate to a much higher NAD^+ concentration (before it becomes hidden by the acylation step taking over as the limiting factor) than with PNP acetate. With the lactone substrate 5-nitro-2-coumaranone, NAD^+ activates over the full concentration range examined, and it was concluded that for this substrate deacylation remains the rate-limiting step throughout [16].

Stopped-flow studies with resorufin acetate and PNP acetate

Figure 2 (upper panel) shows a typical burst of *p*-nitrophenoxide production in the hydrolysis of PNP acetate (0.1 mM) catalysed by cytosolic aldehyde dehydrogenase (8.32 μM). The curve fits extremely well to a single exponential followed by a linear steady-state rate. The burst rate constant is $13.9 \pm 0.2 \text{ s}^{-1}$. When this value and others obtained at higher substrate concentrations [16] are plotted in double-reciprocal fashion, a limiting value at infinite concentration of $46.1 \pm 1.3 \text{ s}^{-1}$ can be derived (see Figure 2, lower panel). (In the present work we limited the concentration of PNP acetate to 0.1 mM so that it would be exactly the same as that used with the less soluble resorufin acetate.) The dependence of burst-rate constant on PNP concentration is consistently reproducible and conflicts with an early report [22] that

Figure 2 Stopped-flow trace of aldehyde dehydrogenase-catalysed hydrolysis of PNP acetate

The experiments were performed as described in the Experimental section, at pH 7.4 and 25 °C with 0.1 mM substrate and an enzyme concentration of 8.32 μM . Upper panel: a typical stopped-flow trace generated from the 'raw data', showing the excellent signal-to-noise ratio. This and six repeats under the same conditions gave an average burst rate constant of $13.9 \pm 0.2 \text{ s}^{-1}$ and a burst amplitude of 0.101 ± 0.001 absorbance units. Lower panel: the burst rate constant from the upper panel together with data similarly obtained at other substrate concentrations (0.25 mM, $24.3 \pm 0.4 \text{ s}^{-1}$; 0.50 mM, $32.2 \pm 0.6 \text{ s}^{-1}$; 1.0 mM, $36.3 \pm 0.7 \text{ s}^{-1}$ [16]) are plotted in double-reciprocal form. The reproducibility of the data is such that error bars would be smaller than the symbol used on the graph.

the rate constant ($12 \pm 2 \text{ s}^{-1}$) is independent of ester concentration (over the range 24–337 μM , although it is unclear whether these concentrations refer to the substrate syringe of the stopped-flow spectrophotometer or to the reaction chamber after mixing). A possible factor that might contribute to the disagreement here is that the earlier report [22] used data analysis methodology (photographing an oscilloscope screen, tracing the photograph on to paper, and analysing the curve graphically) that is far more onerous and prone to error than the automatic computer system used in the present work.

We investigated resorufin acetate under exactly the same conditions as used with PNP acetate, and a typical result is shown in Figure 3. There is a rapid burst of resorufin production, for which the rate constant is $153 \pm 4 \text{ s}^{-1}$. This is about 11 times that found with the same concentration of PNP acetate and is considerably faster even than the limiting rate with the latter substrate at infinite concentration. The faster burst with resorufin acetate might be a reflection of resorufin's being a better leaving group than *p*-nitrophenoxide; the $\text{p}K_a$ values of the two phenols are 5.8 [10] and 7.1 [23] respectively.

The amplitude of the burst of *p*-nitrophenoxide seen in Figure 2 (upper panel) represents 0.99 molecule of product released per

Figure 3 Stopped-flow trace of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate in the absence of cofactor

The experiments were performed as described in the Experimental section, at pH 7.4 and 25 °C with 0.1 mM substrate and an enzyme concentration of 8.32 μ M. This and 10 repeats under the same conditions gave an average burst rate constant of 153 ± 4 s⁻¹ and a burst amplitude of 0.293 ± 0.011 absorbance units.

Figure 4 Stopped-flow trace of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate in the presence of NAD⁺

The experiments were performed as described in the Experimental section, at pH 7.4 and 25 °C with 0.1 mM substrate, 0.25 mM NAD⁺ and an enzyme concentration of 8.32 μ M. A typical trace is shown; in seven repeats the linear slope (after the lag) was 3.82 ± 0.09 absorbance units/s, showing a high degree of reproducibility.

tetrameric enzyme molecule, similar to our previously found value of 1.1 [16]. The burst of resorufin seen in Figure 3, although larger in terms of absorbance units, is equivalent to 0.52 molecule of product per tetramer. These relatively small values (compared with the theoretical maximum of 4 for an enzyme made up of four identical subunits) are typical of results previously found with aldehyde dehydrogenase [1,6,24–26].

The occurrence of a burst with resorufin acetate shows that hydrolysis of the acetyl-enzyme intermediate is the rate-determining step of the reaction. Because breakdown of the identical intermediate also governs the rate of enzyme-catalysed hydrolysis of PNP acetate, the values of k_{cat} for the two substrates should be identical; experiment shows this to be so, as reported before [10] (see also below).

As mentioned above, we have suggested that the presence of NAD⁺ or NADH changes the rate-determining step in the enzyme-catalysed hydrolysis of PNP acetate from deacylation to

Figure 5 Stopped-flow trace of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate in the presence of NADH

The experiments were performed as described in the Experimental section, at pH 7.4 and 25 °C with 0.1 mM substrate, 0.25 mM NADH and an enzyme concentration of 8.32 μ M. A typical trace is shown; seven repeats were all practically indistinguishable.

acylation. Although in some dispute with results of other workers [1,6], we consistently find that a sufficient concentration of NAD⁺ or NADH (say, 0.1–0.3 mM) abolishes the burst of *p*-nitrophenoxide release that is otherwise seen [16], consistent with the suggestion above. Figures 4 and 5 show the results of our similar experiments with resorufin acetate. The conditions were exactly the same as for Figure 3 except for the presence of 0.25 mM nucleotide in both syringes of the stopped-flow spectrophotometer. Figure 4 shows that in the presence of NAD⁺ there is no sign of a burst of resorufin; on the contrary, there is a slight lag before establishment of the linear steady-state rate (which under these conditions is approx. 10 times the rate in the absence of NAD⁺). (At present we have no explanation for this lag, except to speculate that the E·NAD⁺ complex perhaps has to undergo a conformational change before the hydrolysis of resorufin acetate can be catalysed to maximum effect.)

In the presence of NADH we find that the stopped-flow trace of resorufin production seems to tail off smoothly over the first 0.2 s, as shown in Figure 5. Part of this curvature might be spurious; under these conditions the rate of reaction is so fast that very high A_{571} values are soon reached and it is possible that the linearity range of the detection system is exceeded. Furthermore the concentration of substrate (which is initially 0.1 mM) is significantly decreased over the time period; a ΔA_{571} of 1.4 represents the formation of about 20 μ M resorufin. No doubt the experimental conditions could have been altered to avoid these effects, but we wanted to compare the results in the presence and absence of NADH without introducing the complication of altering any other variables such as enzyme or substrate concentration. Careful comparison of Figures 3 and 5 shows that although the reaction is remarkably fast in the presence of NADH it is nevertheless slower than the burst phase of the reaction in the absence of NADH.

We therefore conclude that the qualitative effect of the nucleotides on the enzyme-catalysed hydrolysis of resorufin acetate is the same as with PNP acetate, that is to slow the acylation rate while enhancing the rate of acyl-enzyme hydrolysis, thus eliminating the burst. The acylation rate is intrinsically faster with resorufin acetate than with PNP acetate (as shown by the burst rate constants reported above), but as mentioned previously the deacylation rate is necessarily the same for both

Table 1 Effect of Mg^{2+} in the presence and absence of cofactors on the rate of aldehyde dehydrogenase-catalysed hydrolysis of esters

The rates of enzyme-catalysed hydrolysis of PNP acetate or resorufin acetate at pH 7.4 and 25 °C in the absence (indicated by —) or presence (indicated by +) of NAD^+ (0.1 mM) or NADH (0.1 mM) and the concentration of Mg^{2+} shown are given as means $\pm$ S.D. Also shown are the activation factors relative to the rate in the absence of both cofactor and Mg^{2+} (set at unity). With PNP acetate the substrate concentration was 0.5 mM and the enzyme concentration was 0.255 μM ; with resorufin acetate the substrate concentration was 75 μM and the enzyme concentration was 0.0127 μM .

Substrate	$[\text{Mg}^{2+}]$ (mM)	NAD^+	NADH	Rate (absorbance units/min)	Activation (fold)
<i>p</i> -Nitrophenyl acetate	0	—	—	0.0450 ± 0.0016	1.0
	1	—	—	0.0491 ± 0.0008	1.1
	10	—	—	0.0545 ± 0.0030	1.2
	0	+	—	0.0499 ± 0.0004	1.1
	0	—	+	0.0558 ± 0.0002	1.2
	1	+	—	0.0460 ± 0.0004	1.0
	10	+	—	0.0543 ± 0.0050	1.2
	1	—	+	0.0572 ± 0.0009	1.3
	10	—	+	0.0640 ± 0.0030	1.4
	0	—	—	0.0146 ± 0.0016	1.0
Resorufin acetate	1	—	—	0.0184 ± 0.0013	1.3
	10	—	—	0.0321 ± 0.0021	2.2
	0	+	—	0.479 ± 0.032	33
	0	—	+	0.685 ± 0.021	47
	1	+	—	1.54 ± 0.08	105
	10	+	—	1.84 ± 0.04	125
	1	—	+	1.75 ± 0.03	120
	10	—	+	1.81 ± 0.03	125

these acetate substrates. It is thus possible that the different effects of cofactor on the two reaction steps with resorufin acetate could result in a situation in which neither step happens to be cleanly rate-determining. This might help to account for the stopped-flow trace in the presence of NADH which, as we have seen in Figure 5, is curved but does not display a proper burst. For PNP acetate, however, it must definitely be concluded that acylation is very much slower than deacylation in the presence of NAD^+ or NADH. We know that the rate constant for hydrolysis of the E-S-COCH₃ species under these circumstances must be at least 7 s⁻¹ (because k_{cat} for resorufin acetate has about this value in the presence of NAD^+ or NADH [10] and no individual step can be slower than the overall k_{cat}). Therefore it must be the previous step (acylation) that limits k_{cat} for PNP acetate in the presence of NADH; values obtained under a variety of conditions are 0.98 [16], 0.38 [5] or 0.3 s⁻¹ (calculated from Table 1; see below).

Effect of Mg^{2+} on enzyme-catalysed hydrolysis of resorufin acetate and PNP acetate

Mg^{2+} ions have been reported to have some interesting effects on the dehydrogenase activity of various forms of aldehyde dehydrogenase. The mitochondrial enzyme from horse liver is dissociated from tetramers to dimers by Mg^{2+} , resulting in activation [25], whereas the cytosolic sheep liver enzyme is inhibited by Mg^{2+} (without dissociation) by a slowing down of the NADH off-rate [27,28]. In contrast, cytosolic aldehyde dehydrogenase from yeast is activated by Mg^{2+} [29]. We thought it would be interesting to examine the effect of Mg^{2+} on the esterase activity.

Table 1 shows the effect of Mg^{2+} on the enzyme-catalysed hydrolysis of PNP acetate and resorufin acetate both in the presence and absence of NAD^+ or NADH. In the absence of nucleotide, Mg^{2+} slightly accelerates the hydrolysis of both substrates. The effect seems fairly small for PNP acetate and somewhat greater for resorufin acetate; however, with the former

substrate a 20-fold greater enzyme concentration was used in these experiments than with the latter substrate (and therefore a correspondingly smaller ratio of Mg^{2+} to enzyme), because the rate of reaction in terms of absorbance units per minute, especially in the presence of the nucleotides, is so much greater with resorufin acetate. Like us, Dickinson and Hart [27] found a small degree of activation of PNP acetate hydrolysis by Mg^{2+} (5 mM) in Tris/HCl buffer, but not in phosphate. Because we know from observations of bursts (see above) that in the absence of cofactor the hydrolysis of the acetyl-enzyme is rate-limiting, we can conclude that the rate of this process is somewhat increased by Mg^{2+} . Perhaps Mg^{2+} acts as a Lewis acid, co-ordinating to the carbonyl oxygen in the acyl-enzyme, and thereby facilitating nucleophilic attack on the carbonyl carbon atom.

We also see from Table 1 that under the conditions used here NAD^+ or NADH at 0.1 mM causes a small activation of the rate of hydrolysis of PNP acetate, but that the additional presence of Mg^{2+} has little or no further effect. We know from previous work [16] that this concentration of nucleotide largely eliminates the burst of *p*-nitrophenoxide; thus it seems that the rate of acylation of the enzyme (which is now rate-limiting) is unaffected by Mg^{2+} . Dickinson and Haywood [6] found that Mg^{2+} causes a 2-fold activation of PNP propionate hydrolysis in the presence of NADH, but their conditions differed from ours not only in the identity of the substrate but also in buffer, pH and concentrations of substrate and NADH. Our results agree with those of Blackwell et al. [1], who also reported virtually no effect of Mg^{2+} on the hydrolysis of PNP acetate in the presence of NADH.

In the presence of 0.1 mM NAD^+ or NADH the rate of enzyme-catalysed hydrolysis of resorufin acetate (75 μM) is enhanced markedly (Table 1), even more so than reported before with 25 μM substrate [10], perhaps because the higher concentration is closer to saturating conditions. (The K_m for resorufin acetate in the presence of 0.1 mM NAD^+ is 9 μM [10].) It is also strikingly clear from Table 1 that (unlike with PNP acetate) Mg^{2+} has a marked stimulatory effect (between 2.6-fold and 3.8-fold) in addition to the activation produced by the cofactors. In these

experiments the actual rates observed in the absence of cofactors can be transformed into k_{cat} values of 0.24 and 0.28 s⁻¹ for PNP acetate and resorufin acetate respectively. (The close similarity of these results supports the idea that it is the hydrolysis of the common acetyl-enzyme intermediate that is rate-limiting for both substrates under these conditions.) However, a combination of 0.1 mM NAD⁺ or NADH and 1 mM Mg²⁺ (which is a reasonable concentration, physiologically speaking [30]) raises the k_{cat} for resorufin acetate to 30–35 s⁻¹; this is far greater than any previously observed reaction catalysed by this enzyme. In the dehydrogenase action of the enzyme, Mg²⁺ lowers k_{cat} from 0.25 to 0.03 s⁻¹, an effect caused by slowing down the already rate-limiting dissociation of NADH [28]. It is intriguing that aldehyde dehydrogenase seems to be an enzyme whose catalytic efficiency may be decreased to one-thousandth of the potential value by its difficulty in ridding itself of NADH.

It has been shown that during the course of the dehydrogenase reaction catalysed by cytosolic aldehyde dehydrogenase the E-S-COCH₃·NADH intermediate is hydrolysed 3-fold faster when Mg²⁺ is present [6], although subsequently the rate of dissociation of the E·NADH complex is greatly retarded. This 3-fold effect is similar to that seen in the present work with resorufin acetate in the presence of NADH (or NAD⁺). However, the similarity might be only coincidental because we know that under these conditions a burst of resorufin production is not observed (see Figures 4 and 5), which taken at face value implies that acylation, not deacylation, is rate-limiting. Thus there would be no build-up of acyl-enzyme for Mg²⁺ to accelerate its hydrolysis. Instead, the results suggest that Mg²⁺ (in the presence of cofactor) accelerates the acylation of the enzyme by resorufin acetate, but not (as we have seen above) by PNP acetate. The acylation reactions involve the loss of different leaving groups; thus it is quite conceivable that this step of the reaction could display differences with the two substrates.

The possibility was mentioned above that for resorufin acetate in the presence of NAD⁺ or NADH, the rates of acylation and deacylation might be of similar magnitude, rather than deacylation's being overwhelmingly faster. This scenario might account for both the lack of observation of a clear-cut burst and for activation by Mg²⁺ if this is due to speeding up the (partly rate-limiting) hydrolysis of E-S-COCH₃·NAD(H). Cornish-Bowden ([13], pp. 181–182) gives the following equations for the steady-state rate of formation of product P (i.e. resorufin) and for the amplitude of the burst (π), where k_{+2} and k_{+3} are the rate constants for acylation and deacylation respectively and e_0 is the enzyme concentration:

$$dp/dt = k_{+2}k_{+3}e_0/(k_{+2} + k_{+3})$$

and

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

From these it can be calculated that in the simple model case where k_{+2} equals k_{+3} , the amplitude of the burst would be one-quarter of the maximum that would be seen when $k_{+2} \gg k_{+3}$ (i.e. in the absence of cofactor), and the steady-state rate would double if (on the addition of Mg²⁺) k_{+3} were to increase greatly while k_{+2} remained the same. In practice, as discussed above, we see no real burst with resorufin acetate and cofactor, and an increase in steady-state rate of approx. 3-fold when Mg²⁺ is added, so the experimental results are not close enough to those predicted by the simple model (with $k_{+2} = k_{+3}$) to conclude with confidence that a situation like this is likely to exist. Adjusting the model by increasing the relative size of k_{+2} would permit a

Figure 6 pH-dependence of the rate of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate in the absence of cofactor

The rate was measured at 25 °C with a substrate concentration of 75 μ M and an enzyme concentration of 0.050 μ M. In the lower pH region (below the break in the curve) citric acid/Na₂HPO₄ buffers were used; in the upper pH region Tris/HCl buffers were used.

greater effect of Mg²⁺ on the hydrolysis of E-S-COCH₃·NAD(H) (as seen in the dehydrogenase mode [6]), but would demand that a burst would be clearly observable (contrary to experiment). In contrast, increasing k_{+3} leads us back to the situation discussed above where the lack of a burst is expected but the observed effect of Mg²⁺ must be on the acylation step rather than on the hydrolysis of the acyl-enzyme. It seems that this is the simplest rationalization of all the observations.

pH-dependence of the hydrolysis of resorufin acetate catalysed by cytosolic aldehyde dehydrogenase

The molar absorption coefficient of the resorufin ion is 69 700 M⁻¹·cm⁻¹ [this work] and that of *p*-nitrophenoxide is 18 300 M⁻¹·cm⁻¹ [32]. Thus at high pH, where both products are fully ionized, resorufin acetate is a more sensitive substrate to monitor than PNP acetate by a factor of 3.8. The advantage of using the former substrate increases as the pH falls, because the pK_a of resorufin's phenol group is 5.8 [10], whereas that of *p*-nitrophenol is 7.1 [23]. At pH 7.4, for instance, resorufin acetate is in spectrophotometric terms a better substrate than PNP acetate by a factor of 5.6, and at pH 5.8 by a factor of 40. Indeed at this lower pH, monitoring *p*-nitrophenoxide is practically impossible whereas there is no problem at all in following the hydrolysis of resorufin acetate.

In Figure 6 we see that in the absence of NAD⁺ or NADH there is a trend for the rate of hydrolysis of resorufin acetate (catalysed by cytosolic aldehyde dehydrogenase) to increase with pH. (It is known that at pH 7.4, 75 μ M substrate is fully saturating because the K_m has the very low value of approx. 0.15 μ M [10]; we assume here that we are also measuring V_{max} at the other pH values used.) At the lowest pH investigated (5.67) there is a small but reproducibly measurable activity. The break seen in the curve occurs as the buffer system changes from citric acid/Na₂HPO₄ to Tris/HCl. The rate of spontaneous hydrolysis of the substrate also increases with pH of course; the results in Figure 6 are corrected for this factor, but at the highest pH investigated (9.24) the spontaneous rate is almost 40% of the total observed rate. Thus there is likely to be considerable error

Figure 7 pH-dependence of the rate of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate in the presence of NAD^+ and NADH

The rate was measured as described for Figure 6, except that in (a) 0.1 mM NAD^+ was present and in (b) 0.1 mM NADH was present.

in the results at high pH and it is not clear whether the apparent fall in activity at pH 9.24 is real (see Figure 6).

In the presence of NAD^+ or NADH we see, as noted before, a greatly enhanced activity; this is true over the whole pH range. The rate of spontaneous substrate hydrolysis is therefore a very much less significant factor here, even at high pH. There seems also to be no break in the curve under these conditions as the buffer system changes. Figure 7(a) shows that the experimental results in the presence of NAD^+ fit well to a theoretical ionization curve with a pK_a of 8.27 ± 0.05 , and in the presence of NADH (Figure 7b) the calculated pK_a is 8.09 ± 0.03 , with an even better fit to the data. Careful inspection of Figure 7 shows that at low pH the rate in the presence of NADH is significantly higher than in the presence of NAD^+ . This point is emphasized in Figure 8, where the activation factor produced by each nucleotide is plotted against pH. In Tris/HCl buffer (pH 7.9–8.9) both NAD^+ and NADH activate by a factor of approx. 40-fold. Over the pH range 5.7–7.5, however, the effects of the two nucleotides are markedly different; NAD^+ activates by a constant factor of approx. 20-fold, whereas the effect of NADH rises from 30-fold activation at pH 7.5 to a very impressive 85-fold at pH 5.7. In the presence of NADH the calculated k_{cat} values vary from 2.1 s^{-1} at low pH to 25 s^{-1} at high pH. (In the presence of NAD^+ at pH 7.4, a concentration of resorufin acetate of $75 \mu\text{M}$ is over eight times the K_m [10] and so we know that the measured rate approximates well to V_{max} under these particular conditions. The close fit of the data in Figure 7 to theoretical ionization curves is consistent with the assumption that we are measuring close to V_{max} throughout the pH range.)

From Figure 7 it is tempting to conclude that the rate of enzyme-catalysed hydrolysis of resorufin acetate in the presence of either NAD^+ or NADH is governed by the ionization of a

Figure 8 Activation of aldehyde dehydrogenase-catalysed hydrolysis of resorufin acetate by NAD^+ and NADH as a function of pH

From the data shown in Figures 6 and 7, the activation factor produced by NAD^+ or NADH (as a multiple of the rate in the absence of cofactor) was calculated and is plotted against pH. Symbols: $\circ$, NAD^+ ; $\bullet$, NADH.

single enzymic group, and naturally a cysteine residue would be the best candidate for a group with a pK_a of approx. 8.2. Much evidence points to Cys-302 as the catalytically essential nucleophile of aldehyde dehydrogenase ([15,16], and references therein). As discussed above, the acylation step seems to be largely if not wholly rate-determining in the presence of cofactors; it is eminently reasonable that this acylation rate should be faster when Cys-302 is in the form of -S^- rather than -SH . Note that it seems that the enzyme is definitely active to some extent when the supposed thiol group is not ionized, especially when NADH is present. It has been suggested that the proton of the thiol group of Cys-302 is removed by another enzymic group (possibly the carboxylate ion of Glu-268 [33]), rendering the former group more nucleophilic; it seems from the present work that this mechanism might apply in the lower pH region but becomes unnecessary at higher pH. If the observed pK_a of approx. 8.2 is indeed due to Cys-302, it is interesting that this is in the normal range for cysteine; apparently aldehyde dehydrogenase is unlike glyceraldehyde-3-phosphate dehydrogenase, for example, where the pK_a of the active-site thiol is perturbed to the unusually low value of 5.5 in the presence of NAD^+ [34].

We cannot fit the points shown in Figure 6 in the absence of cofactor to a simple ionization curve because of the break in the graph and because of doubt about the results in the highest pH region as discussed above. The shape of this curve is obviously similar to those in Figure 7, but we would not necessarily expect this to be so. Unlike in the presence of NAD^+ or NADH, we know that it is the deacylation of the acyl-enzyme that is rate-limiting in the absence of cofactor (see above). Under these circumstances, therefore, the rate of reaction cannot be affected by the state of ionization of Cys-302 because this residue is already in the form of E-S-COCH_3 before the slowest step occurs. Thus it might be that some other factor increases the rate of hydrolysis of the latter species as the pH is raised, resulting in a curve that is coincidentally similar to those seen in Figure 7. For instance, hydrolysis of the *S*-ester intermediate might to some extent simply depend on hydroxide ion concentration in a similar way to the rate of spontaneous hydrolysis of the ester substrate. Plotting the observed rate of spontaneous hydrolysis against pH indeed gives a curve of broadly similar shape to that shown in Figure 6 (including a similar break between the buffer

regions) but that continues to rise steeply above pH 9 (results not shown).

In previous work we examined the pH profile for the acylation of cytosolic aldehyde dehydrogenase by PNP dimethylcarbamate in the absence of cofactor, finding an apparent pK_a of 7.6 and speculating that this might belong to the postulated active-site thiol [35]. However, the ionization curve was fitted to only five data points and so the calculated pK_a was likely to be less accurate than the value of approx. 8.2 found in the present work (Figure 7).

In conclusion, although it is perhaps trivial to remark on the aesthetically delightful colour change observed when resorufin acetate is hydrolysed, it is certainly true that use of this substrate has led to some striking and important results with cytosolic aldehyde dehydrogenase. We intend to extend our work to the mitochondrial isoenzyme and we are currently examining several other derivatives of resorufin that act as covalent modifiers of aldehyde dehydrogenase.

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