Kinetics of *p*-nitrophenyl pivalate hydrolysis catalysed by cytoplasmic aldehyde dehydrogenase

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The effects of modifiers (NAD⁺, NADH, propionaldehyde, chloral hydrate, diethylstilboestrol and *p*-nitrobenzaldehyde) on the hydrolysis of *p*-nitrophenyl (PNP) pivalate (PNP trimethylacetate) catalysed by cytoplasmic aldehyde dehydrogenase are reported. In each case a different inhibition pattern is obtained to that observed when the substrate is PNP acetate; for example, propionaldehyde and chloral hydrate competitively inhibit the hydrolysis of PNP acetate, but are mixed inhibitors with PNP pivalate. The kinetic results can be rationalized in terms of different rate-determining steps: acylation of the enzyme in the case of the pivalate but acyl-enzyme hydrolysis for the acetate. This is confirmed by stopped-flow studies, in which a burst of *p*-nitrophenoxide is observed when the substrate is PNP acetate, but not when it is the pivalate. PNP pivalate inhibits the dehydrogenase activity of the enzyme competitively with the aldehyde substrate; this is most simply explained if the esterase and dehydrogenase reactions occur at a common enzymic site.

INTRODUCTION

Aldehyde dehydrogenase catalyses the oxidation of a wide variety of aliphatic and aromatic aldehydes by NAD⁺. Since 1972 it has been known that the enzyme also catalyses the hydrolysis of *p*-nitrophenyl (PNP) acetate (Feldman & Weiner, 1972). One group of workers has argued that the two types of activity, namely dehydrogenase and esterase, occur at different active sites on the enzyme (Blackwell *et al.*, 1983; Deady *et al.*, 1985). Several other workers are of the opinion that a common active site is involved and that the same acetylated form of the enzyme participates in the oxidation of acetaldehyde and in the hydrolysis of PNP acetate (Sidhu & Blair, 1975; Takahashi & Weiner, 1981; Duncan, 1985; Loomes & Kitson, 1986).

Little work has been done on the action of aldehyde dehydrogenase on esters other than PNP acetate. It was thought that PNP pivalate (PNP trimethylacetate), in which reaction at the ester group is subject to pronounced steric hindrance, might warrant investigation, as had indeed proved to be the case for other hydrolytic enzymes such as chymotrypsin (Bender *et al.*, 1967; Milstein & Fife, 1969). Accordingly the present paper reports the results of detailed studies designed to compare and contrast the actions of sheep liver cytoplasmic aldehyde dehydrogenase on PNP pivalate and on PNP acetate.

EXPERIMENTAL

Materials

Cytoplasmic aldehyde dehydrogenase, free from the corresponding mitochondrial enzyme, was prepared from sheep liver as described by Dickinson *et al.* (1981), and was assayed by the method of Hart & Dickinson (1977). Protein concentrations were calculated by using $A_{1\text{cm}}^{1\%} = 11.3$ at 280 nm (Dickinson & Hart, 1982). Enzyme solutions were normally prepared for use by

dialysis against 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA.

PNP isobutyrate was prepared by the method of Fife (1965), but with triethylamine in place of pyridine. The flaky white crystals had m.p. 35–36 °C [Kreisky (1956) gives m.p. 36.5–37 °C]. All other chemicals were obtained from the usual commercial sources.

Methods

Enzyme assay (as an esterase). This was performed at 25 °C in 50 mM-sodium phosphate buffer, pH 7.4, by monitoring the production of *p*-nitrophenoxide at 400 nm in a Zeiss PMQ II spectrophotometer. The final volume of the assay solution was 3 ml, and the substrate and certain modifiers (such as *p*-nitrobenzaldehyde and diethylstilboestrol) were added as small volumes (10–25 μ l) of ethanol solutions. Where appropriate, equal volumes of ethanol were incorporated in the control assays. Other modifiers such as NAD⁺, NADH, propionaldehyde and chloral hydrate were added as small volumes of solutions in the buffer above. Where necessary, measured rates were corrected for spontaneous ester hydrolysis; this was negligible for PNP pivalate.

Enzyme assay (as a dehydrogenase). This was performed fluorimetrically as described by Hart & Dickinson (1977) at 25 °C in 50 mM-sodium phosphate buffer, pH 7.4, with 1 mM-NAD⁺ and various concentrations of propionaldehyde or D-glyceraldehyde as substrate. The final volume of the assay solution was 4 ml. PNP pivalate was added as 25 μ l of an ethanol solution; an equivalent volume of ethanol was added to control assays.

Stopped-flow studies. The apparatus and its associated data-storage facilities were as described by Hart & Dickinson (1982). The effective cell path-length was 2.2 cm. Experiments were performed at 25 °C. The

transmittance values obtained at 400 nm were transformed into the absorbance values shown in Fig. 6. Before mixing, syringe 1 contained $14.3 \mu\text{M}$ -enzyme in 10 ml of 50 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA. Syringe 2 contained 9.7 ml of the same buffer and 0.3 ml of an acetone solution of PNP acetate or propionate, or 9 ml of the same buffer and 1 ml of an acetone solution of the less soluble PNP pivalate or isobutyrate. In all cases the substrate concentration in the syringe was $400 \mu\text{M}$.

RESULTS

Steady-state studies with PNP pivalate as substrate

The maximum velocity of the enzyme-catalysed hydrolysis of PNP pivalate was found to be approx. 0.35 that for the acetate under identical conditions, and the K_m values are approx. $40 \mu\text{M}$ and $2\text{--}3 \mu\text{M}$ for the pivalate and acetate respectively.

With PNP acetate as substrate it has previously been found that NAD^+ and NADH at certain concentrations have a marked activatory effect on the enzyme-catalysed reaction (MacGibbon *et al.*, 1978). In contrast, Fig. 1 shows that with PNP pivalate NADH is an inhibitor of the linear mixed type. A similar result was obtained with NAD^+ . The K_i values are $6.5 \mu\text{M}$ and $23 \mu\text{M}$ respectively, which agree well with the dissociation constants for NADH and NAD^+ reported by Hart & Dickinson (1983).

Aldehyde substrates and analogues (such as propionaldehyde and chloral hydrate) have previously been reported to be competitive inhibitors of the esterase reaction in the absence of nucleotides (MacGibbon *et al.*, 1978), and this was confirmed in the present work. With PNP pivalate a completely different pattern of inhibition

Fig. 1. Inhibition of enzyme-catalysed hydrolysis of PNP pivalate by NADH

Enzyme concentration was $0.55 \mu\text{M}$ and NADH concentrations were $0 \mu\text{M}$ (○), $5 \mu\text{M}$ (●), $10 \mu\text{M}$ (□) and $20 \mu\text{M}$ (■). The inset is a replot of the slopes against inhibitor concentration.

Fig. 2. Inhibition of enzyme-catalysed hydrolysis of PNP pivalate by propionaldehyde

Enzyme concentration was $0.92 \mu\text{M}$ and propionaldehyde concentrations were 0 mM (○), 0.5 mM (●), 1 mM (□), 2 mM (■) and 3 mM (Δ). The inset is a replot of the slopes against inhibitor concentration.

Fig. 3. Inhibition of enzyme-catalysed hydrolysis of PNP pivalate by chloral hydrate

Enzyme concentration was $0.55 \mu\text{M}$ and chloral hydrate concentrations were $0 \mu\text{M}$ (○), $20 \mu\text{M}$ (●), $40 \mu\text{M}$ (□) and $60 \mu\text{M}$ (■). The inset is a replot of the slopes against inhibitor concentration.

Fig. 4. Inhibition of enzyme-catalysed hydrolysis of PNP pivalate by diethylstilboestrol

Enzyme concentration was $0.92 \mu\text{M}$ and diethylstilboestrol concentrations were $0 \mu\text{M}$ (○), $3.33 \mu\text{M}$ (●), $6.67 \mu\text{M}$ (□), $13.3 \mu\text{M}$ (■) and $20 \mu\text{M}$ (Δ). The inset is a replot of the slopes against inhibitor concentration.

Fig. 5. Inhibition of enzyme-catalysed hydrolysis of PNP acetate by *p*-nitrobenzaldehyde

Enzyme concentration was $0.28 \mu\text{M}$ and *p*-nitrobenzaldehyde concentrations were $0 \mu\text{M}$ (○), $25 \mu\text{M}$ (●) and $50 \mu\text{M}$ (□).

is obtained, as shown in Figs. 2 and 3. Propionaldehyde and chloral hydrate are linear mixed inhibitors with K_i values of 1.3 mM and $20 \mu\text{M}$ respectively.

The modifier diethylstilboestrol was shown to inhibit the enzyme-catalysed hydrolysis of PNP acetate in a rather complex way (Kitson, 1986). The observed pattern was of the partial mixed variety, but the Lineweaver–

Fig. 6. Stopped-flow studies of the hydrolysis of PNP esters catalysed by cytoplasmic aldehyde dehydrogenase

Enzyme concentration was $7.2 \mu\text{M}$ and substrate concentration was $200 \mu\text{M}$. Traces 1, 2, 3 and 4 refer to PNP acetate, PNP propionate, PNP isobutyrate and PNP pivalate respectively. Other experimental details are given in the Experimental section. The traces have been drawn separated along the horizontal axis for clarity; in each case the time of observation was 1 s.

Burk plots curved down markedly at high substrate concentration. Again, with the pivalate as substrate, a different result is obtained; Fig. 4 shows that in this case diethylstilboestrol is a simple linear competitive inhibitor with K_i equal to $8.3 \mu\text{M}$.

Like diethylstilboestrol, the dehydrogenase substrate *p*-nitrobenzaldehyde is a linear competitive inhibitor (with K_i $50 \mu\text{M}$) of the enzyme-catalysed hydrolysis of PNP pivalate (results not shown), a result that is in contrast with the behaviour of propionaldehyde (Fig. 2). Once again, PNP acetate gives a different result. Fig. 5 shows that here the inhibition is certainly not competitive, but is reminiscent of the pattern obtained with this substrate and diethylstilboestrol (Kitson, 1986).

Stopped-flow esterase studies

Fig. 6 and Table 1 show typical results of experiments designed to show if a burst of *p*-nitrophenoxide production occurs in the aldehyde dehydrogenase-catalysed hydrolysis of a series of PNP esters. The initial ester concentration ($200 \mu\text{M}$) in these experiments is at least 5 times the K_m values of the esters, so the enzyme should be over 80% saturated at the start of each reaction. It is confirmed that the acetate and propionate show significant bursts, as previously reported (Blackwell *et al.*, 1983; Dickinson & Haywood, 1986); the size of the burst with PNP acetate, for example, represents 0.7 molecule of *p*-nitrophenoxide released per tetrameric enzyme molecule, a similar result to that obtained by Dickinson & Haywood (1986). PNP isobutyrate shows a smaller and slower burst, and clearly PNP pivalate produces no burst of *p*-nitrophenoxide at all. The experiment was repeated at least nine times with the latter substrate and in no case was a burst observed.

Table 1. Stopped-flow studies of the hydrolysis of PNP esters catalysed by cytoplasmic aldehyde dehydrogenase

Analysis of the curves shown in Fig. 6 gave the following data.

Substrate	Burst size <i>A</i>	Burst rate constant (s ⁻¹)	Relative steady-state rate
PNP acetate	0.152	21.2	(1)
PNP propionate	0.123	17.7	1.65
PNP isobutyrate	0.054	10.4	1.19
PNP pivalate	0	(0.23*)	0.21

* This value is that calculated for k_{cat} , by dividing the observed rate of production of *p*-nitrophenoxide (1.62 $\mu\text{M/s}$) by the enzyme concentration (7.2 μM).

Fig. 7. Inhibition of enzyme-catalysed dehydrogenation of D-glyceraldehyde by PNP pivalate

Enzyme concentration was 0.55 μM and PNP pivalate concentrations were 0 μM (○) and 75 μM (●).

Steady-state studies with PNP pivalate as inhibitor

The rate of enzyme-catalysed hydrolysis of PNP pivalate in the presence of 1 mM-NAD⁺ is negligible. Thus this ester can be investigated as an inhibitor of the dehydrogenase reaction without interference from the simultaneous production of two species with overlapping absorbances, NADH and *p*-nitrophenoxide (unlike the case of PNP acetate; Blackwell *et al.*, 1983). Fig. 7 shows quite clearly that PNP pivalate is a competitive inhibitor of the dehydrogenation of D-glyceraldehyde (with K_i 30 μM), and Fig. 8 shows that the ester also competitively inhibits the dehydrogenation of propionaldehyde

Fig. 8. Effect of PNP pivalate on the enzyme-catalysed dehydrogenation of propionaldehyde

Enzyme concentration was 0.069 μM and PNP pivalate concentrations were 0 μM (○) and 75 μM (●). The inset shows a scale expansion of the region where substrate concentration was 0.5–5 mM (on the same vertical scale) showing the activation caused by PNP pivalate in this region.

(with K_i 58 μM) in the low substrate concentration region, where the Lineweaver-Burk plot is linear. As is well known, high concentration of propionaldehyde activate the enzyme, and Fig. 8 shows that in this region of substrate concentration the presence of PNP pivalate causes some further degree of activation rather than inhibition.

DISCUSSION

Scheme 1 shows the general case for enzyme-catalysed hydrolysis of a PNP ester (A) in the presence of a reversible modifier (Q). From previous work it is known that, when the substrate is PNP acetate, then $k_{+2} \gg k_{+3}$ (because a burst of *p*-nitrophenoxide, X, is observed). Under these circumstances, Laidler & Bunting (1973) give:

$$k_{\text{cat.}} = k_{+3} (1 + c[Q]/K_{AQ}) / (1 + [Q]/K_{AQ})$$

If $k_{+3} \gg k_{+2}$ (and guessing for the moment that this might apply to PNP pivalate) then:

$$k_{\text{cat.}} = k_{+2} (1 + b[Q]/K_{AQ}) / (1 + [Q]/K_{AQ})$$

Consider first the case of small aldehyde modifiers (propionaldehyde and chloral). If the enzyme has an essential nucleophilic thiol group, as is usually assumed (possibly Cys-302; see Hempel *et al.*, 1985), then presumably these modifiers interact with the enzyme by reversible thiohemiacetal formation. This is impossible when the putative thiol group has been acylated by the

Scheme 1. General case for the inhibition of the enzyme-catalysed hydrolysis of a PNP ester (A) by a compound Q

Key: EA, the Michaelis complex; EA', the acyl-enzyme; X, *p*-nitrophenoxide; Y, the carboxylate product.

ester substrate (i.e. in the form EA' in Scheme 1); under these circumstances $K_{A'Q}$ is infinite and for PNP acetate the expression for $k_{\text{cat.}}$ reduces to k_{+3} . In other words, the vertical intercept on a Lineweaver-Burk plot is independent of modifier concentration, as is indeed observed experimentally. Another consequence of the supposed thiohemiacetal formation would be that $b = 0$ (i.e. the catalytic group is temporarily precluded from reacting with the ester substrate). Now, for PNP pivalate, $k_{\text{cat.}}$ reduces to $k_{+2}/(1 + [Q]/K_{AQ})$, and there is a linear dependence of the Lineweaver-Burk intercepts on $[Q]$, again as is experimentally observed (see Figs. 2 and 3). Note that here K_{AQ} is not infinitely large; that is, we are led to conclude that small aldehydes can still interact with the essential group when the active site is occupied by an ester substrate (but before acylation has occurred).

It seems reasonable that steric requirements should forbid the simultaneous binding of substrate and bulkier molecules such as *p*-nitrobenzaldehyde and diethylstilboestrol to the enzyme's active site. This time, therefore, K_{AQ} is infinite, and for PNP pivalate $k_{\text{cat.}}$ collapses to k_{+2} , which agrees with the observed linear competitive inhibition (see Fig. 4). On the other hand, it is also reasonable to suppose that these aromatic compounds should bind to the acyl-enzyme (EA') in the position vacated by the product X (*p*-nitrophenoxide). Thus for PNP acetate there is now no simplification of the expression for $k_{\text{cat.}}$ (i.e. c and $K_{A'Q}$ have finite values) and non-linear mixed inhibition would be expected, once again as experimentally observed (see Fig. 5 and Kitson, 1986). (The curvature of the Lineweaver-Burk plots in these cases suggests that at high substrate concentration an EA'A complex may form in competition with EA'Q.)

The binding of nucleotides to aldehyde dehydrogenase

is known to affect its conformation (Bennett *et al.*, 1982). The present results suggest that the E·NAD⁺ or E·NADH complexes no longer have any appreciable ability to catalyse the hydrolysis of PNP pivalate; that is b tends to zero in Scheme 1, and $k_{\text{cat.}}$ becomes $k_{+2}/(1 + [Q]/K_{AQ})$, thus giving rise to the observed linear mixed inhibition (Fig. 1). For PNP acetate and propionate on the other hand, the presence of nucleotides reportedly has little effect on the rate of acylation of the enzyme (Blackwell *et al.*, 1983; Dickinson & Haywood, 1986), and the observed increase in steady state activity must mean that $c > 1$ in Scheme 1.

In summary, all the steady-state inhibition studies reported here can be accommodated by the initial assumption that acylation is rate-determining for PNP pivalate (i.e. $k_{+3} \gg k_{+2}$). The results shown in Fig. 6 and Table 1 give direct experimental confirmation of this suggestion. As the acyl group in the series of PNP esters becomes bulkier, the observed burst of *p*-nitrophenoxide becomes smaller and slower, culminating in no burst at all for the pivalate. The steady-state rate passes through a maximum, which is consistent with the proposed change in rate-determining step.

Fife (1965) studied the hydrolysis of a series of PNP esters catalysed nucleophilically by imidazole. He found that the relative second-order rate constants for PNP propionate, isobutyrate and pivalate were 1, 0.71 and 0.0097. The equivalent relative first-order rate constants for acylation of aldehyde dehydrogenase by these esters (calculated from the data in Table 1) are 1, 0.59 and 0.013. (The value for PNP pivalate is calculated from the steady-state rate on the basis that this represents the rate of acylation.) The very close agreement supports the view that this step of the enzyme-catalysed reaction is indeed controlled by steric hindrance to nucleophilic attack at the substrate acyl group. Intuitively one might have thought that the next step in the reaction, namely hydrolysis of the acyl-enzyme by a water molecule, presumably catalysed by some general base at the active site, would also be subject to pronounced steric hindrance when the acyl group is highly branched, resulting in deacylation still being rate-limiting for enzyme-catalysed hydrolysis of PNP pivalate and the steady-state rate being far lower than it actually is. However, Fife (1965) has shown that general catalysis of hydrolysis (of *N*-acylimidazoles) is not retarded by branching at the α -carbon atom in the acyl group (unlike the case of nucleophilic catalysis involving PNP esters; see above) but is actually slightly accelerated, and he has discussed possible reasons for this.

At high propionaldehyde concentration, PNP pivalate causes significant activation of the dehydrogenase activity, presumably by binding to the E·NADH complex and increasing the rate of dissociation of NADH (the rate-determining step) to a greater extent than does propionaldehyde itself. At low propionaldehyde concentration, and over the full range of D-glyceraldehyde concentration tested, PNP pivalate acts as a competitive inhibitor (see Figs. 7 and 8). The competition between ester and aldehyde substrates is consistent with the simple common-site model for esterase and dehydrogenase activities and augments the body of evidence in favour of such a view (see Duncan, 1985; Loomes & Kitson, 1986; Dickinson & Haywood, 1986). It is not so readily explicable in terms of the alternative two-site model (Blackwell *et al.*, 1983), in which PNP

esters bind only to a so-called P2 domain and not to the P1 domain where the dehydrogenase activity is catalysed.

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