The action of cytoplasmic aldehyde dehydrogenase on methyl *p*-nitrophenyl carbonate and *p*-nitrophenyl dimethylcarbamate

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Cytoplasmic aldehyde dehydrogenase catalyses the hydrolysis of methyl *p*-nitrophenyl (PNP) carbonate at an appreciable rate that is markedly stimulated by NAD⁺ or NADH. The nucleotides accelerate the rate-limiting hydrolysis of the acyl-enzyme intermediate while slowing the observed burst of *p*-nitrophenoxide production. With PNP dimethylcarbamate the enzyme catalyses the slow release of approx. 1 molecule of *p*-nitrophenoxide per tetrameric enzyme molecule; during the reaction the enzyme becomes effectively inactivated, as the rate of hydrolysis of the acyl-enzyme is virtually zero. The presence of NAD⁺, NADH, propionaldehyde, chloral hydrate, diethylstilboestrol or disulfiram slows the reaction of enzyme with PNP dimethylcarbamate. The reaction appears to be dependent on a group of p*K*_a 7.6, possibly a cysteine residue. The effect of PNP dimethylcarbamate on the dehydrogenase activity of the enzyme is consistent with there being a single type of active site for the enzyme's dehydrogenase and esterase activities. Steric and electronic factors that govern reaction of the enzyme with PNP substrates are discussed.

INTRODUCTION

Some very interesting work has been done to compare and contrast the action of aldehyde dehydrogenase towards its natural aldehyde substrates and towards *p*-nitrophenyl (PNP) esters, although there is not yet full agreement on the broad conclusions to be drawn from this work concerning the enzyme's active site or sites (see Blackwell *et al.*, 1983; Duncan, 1985). In the preceding paper (Kitson, 1989) a study of the kinetics of enzyme-catalysed hydrolysis of PNP pivalate (trimethylacetate) was reported; attack on this substrate is limited by steric hindrance due to the highly branched acyl group. Another way in which the activity of PNP substrates could be moderated is by the incorporation of a heteroatom such as oxygen or nitrogen adjacent to the carbonyl group. It was the purpose of the present work to investigate examples of such compounds (namely methyl PNP carbonate and PNP dimethylcarbamate) as possible substrates of aldehyde dehydrogenase in the hope that further illumination on the catalytic properties of the enzyme would be provided.

EXPERIMENTAL

Materials

Cytoplasmic aldehyde dehydrogenase was prepared and assayed as indicated in the preceding paper (Kitson, 1989). Methyl PNP carbonate was prepared by the method of Clark & Chan (1978) except that toluene was used in place of benzene. The product was recrystallized three times from methanol to give pale pinkish-buff crystals, m.p. (uncorr.) 109–110 °C [Pocker & Guilbert (1974) give m.p. 111–112 °C]. PNP dimethylcarbamate was prepared either by the method of Bender *et al.* (1966) (by using *p*-nitrophenol and dimethylcarbamoyl chloride in pyridine) or by a method based on that of Hegarty & Frost (1973) (by using equimolar amounts of *p*-nitro-

phenyl chloroformate and dried dimethylammonium chloride and 2 equivalents of triethylamine in dry chloroform). Both methods gave large flat almost colourless crystals (after recrystallization twice from ethanol), m.p. (uncorr.) 104–106 °C [Bender *et al.* (1966) give m.p. 107.5–108 °C]. The identity of the product was confirmed by m.s. [molecular ion *m/z* 210, base peak *m/z* 72 (Me₂NCO)].

Methods

Enzyme assay (as an esterase). This was performed at 25 °C in 50 mM-sodium phosphate or 50 mM-Tris/HCl buffer, pH 7.4, or (for the pH-activity profile experiment) 200 mM-sodium phosphate buffer. The production of *p*-nitrophenoxide was monitored at 400 nm in a Zeiss PMQ II or a Unicam SP.1800 spectrophotometer. Assay volume was usually 3 ml, but occasionally was 1 ml (so as to economize with the enzyme when a high concentration was used). Other conditions were as before (Kitson, 1989). Measured rates were corrected for the spontaneous hydrolysis of the substrate where this was significant.

Enzyme assay (as a dehydrogenase). This was performed as indicated in the preceding paper (Kitson, 1989).

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. 1. Before mixing, syringe 1 contained enzyme and in some cases NAD⁺ or NADH in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA. Syringe 2 contained methyl PNP carbonate (the stock solution of

which was in acetone) and where appropriate NAD^+ or NADH in the same buffer as in syringe 1. Immediately after mixing the concentrations were: enzyme, $9.15 \mu\text{M}$; substrate, $200 \mu\text{M}$; acetone 1.5% (v/v); NAD^+ , 0 or $400 \mu\text{M}$; NADH , 0 or $100 \mu\text{M}$.

Isolation of E-X-CO-NMe₂ (X represents the essential nucleophilic atom at the enzyme's active site). A small volume (0.5–2 ml) of enzyme (approx. $40\text{--}100 \mu\text{M}$) in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA was mixed with a small volume (25–100 μl) of an ethanol solution of PNP dimethylcarbamate to give a concentration of 0.4 or 0.5 mM, and left at room temperature overnight. (On some occasions a preliminary incubation of approx. 1 h at 30°C was used before the overnight stand at room temperature.) The protein was then isolated from excess reagent and product (*p*-nitrophenoxide) by gel filtration on a small column of Bio-Gel P-6 that had been equilibrated either with 50 mM-sodium phosphate buffer, pH 7.4, or (for experiments with Mg^{2+}) with 50 mM-Tris/HCl buffer, pH 7.4. The modified form of the enzyme was then assayed for residual activity, which typically was approx. 11% of a control treated in the same way except for the omission of PNP dimethylcarbamate. Doubtless further decrease in the activity would have been achieved by longer but less convenient reaction times.

RESULTS AND DISCUSSION

Action of aldehyde dehydrogenase on methyl PNP carbonate

Table 1 shows that methyl PNP carbonate is indeed a substrate for sheep liver cytoplasmic aldehyde dehydrogenase, albeit a poor one compared with PNP acetate; the relative rates of reaction under the conditions used are 1 and 170 respectively. The low rate of enzyme-catalysed hydrolysis of the carbonate, however, is markedly stimulated by the presence of NAD^+ or NADH , relatively much more so than reported for PNP

Table 1. Action of aldehyde dehydrogenase on methyl PNP carbonate

The enzyme-catalysed hydrolysis of methyl PNP carbonate was assayed under various conditions as described in the Experimental section. Some results with PNP acetate are included for purposes of comparison. The enzyme concentration was $0.33 \mu\text{M}$.

Substrate	Modifier	Rate	
		(A_{400}/min)	(relative to control)
Methyl PNP carbonate	None	0.00156	(1)
Methyl PNP carbonate	1 mM- NAD^+	0.0200	12.8
Methyl PNP carbonate	100 μM - NADH	0.0412	26.4
Methyl PNP carbonate	0.3 μM -Disulfiram	0.000754	0.48
PNP acetate	None	0.269	(1)
PNP acetate	0.3 μM -Disulfiram	0.102	0.38

Fig. 1. Stopped-flow studies of the hydrolysis of methyl PNP carbonate catalysed by aldehyde dehydrogenase

Experiments were conducted as described in the Experimental section with an enzyme concentration of $9.15 \mu\text{M}$ and a substrate concentration of $200 \mu\text{M}$. The symbol $\circ$ represents the increase in A_{400} observed when enzyme reacts with substrate in the absence of nucleotide. Symbols $\bullet$ and $\square$ show the results of otherwise identical experiments in the presence of $400 \mu\text{M}$ - NAD^+ and $100 \mu\text{M}$ - NADH respectively.

acetate (MacGibbon *et al.*, 1978). Pre-modification by a stoichiometric amount of disulfiram lowers the activity of the enzyme towards both substrates to a broadly similar degree.

Fig. 1 and Table 2 show the results of stopped-flow studies with methyl PNP carbonate, carried out to identify the rate-determining step of the enzyme-catalysed hydrolysis. Although the K_m was not determined, it is probably safe to assume that $200 \mu\text{M}$ -substrate is saturating in the absence of nucleotides (by comparison with the known very low K_m values for PNP acetate and PNP propionate). Under these conditions a burst of *p*-nitrophenoxide is clearly demonstrable; this appears to be biphasic and represents 0.89 molecule of *p*-nitrophenoxide produced per tetrameric enzyme molecule [in

Table 2. Characteristics of the burst observed in the enzyme-catalysed hydrolysis of methyl PNP carbonate

Stopped-flow experiments were performed as described in the Experimental section. The results obtained are shown in Fig. 1. Analysis of the results as first-order processes gave the data presented in this Table.

Modifier	Burst size		Burst rate constant (s^{-1})	Relative steady-state rate
	(A_{400} unit)	(relative to control)		
None	0.248	(1)	2.0, 0.81	1
400 μM - NAD^+	0.193	0.78	0.68	3.3
100 μM - NADH	0.069	0.28	0.86	6.6

Fig. 2. Inhibition of enzyme-catalysed hydrolysis of PNP acetate by methyl PNP carbonate

Enzyme was pre-mixed with methyl PNP carbonate and after 1 min various concentrations of PNP acetate were added. The linear rate achieved after a further 0.5–1 min was recorded. Enzyme concentration was $0.4 \mu\text{M}$ and methyl PNP carbonate concentrations were $0 \mu\text{M}$ ($\circ$), $5 \mu\text{M}$ ($\bullet$) and $10 \mu\text{M}$ ($\square$).

good agreement with values obtained before with PNP esters (Dickinson & Haywood, 1986)]. Thus, like PNP acetate and PNP propionate, but unlike PNP pivalate (Kitson, 1989), deacylation is rate-limiting for methyl PNP carbonate. The presence of NAD^+ or NADH is known to increase the size of K_m for PNP esters (Kitson, 1986) and thus under these conditions $200 \mu\text{M}$ substrate is perhaps no longer saturating. Thus the experimental observation that NAD^+ or NADH appears to eliminate the fast phase of the burst must be treated with caution. However, a decrease in the acylation rate caused by NAD^+ or NADH has been observed with PNP pivalate (Kitson, 1989) and PNP dimethylcarbamate (see below), and this lends credence to the conclusion here that the nucleotides also slow the acylation step with methyl PNP carbonate (while, as is obvious from Fig. 1, simultaneously speeding up the deacylation rate).

It is interesting to contrast the steric effect of the bulky acyl group in PNP pivalate (Kitson, 1989) and the electronic effect of the methoxycarbonyl group in methyl PNP carbonate on the acylation and deacylation steps of the enzyme-catalysed reaction. The steric effect markedly lowers the rate of acylation (approx. 100-fold compared with PNP acetate) but must have a much smaller effect on the rate of acyl-enzyme hydrolysis (otherwise deacylation would be rate-limiting for the pivalate as it is for the acetate). On the other hand the electronic effect lowers the rate of acylation only approx. 10-fold [from 21.2 s^{-1} with PNP acetate to 2.0 s^{-1} with methyl PNP carbonate determined under identical conditions; Kitson (1989) and Table 2], but decreases the steady-state rate (deacylation) by approx. 170-fold (see Table 1). Evidently, the two types of reaction here, nucleophilic attack and general base catalysis, are differentially affected by steric and electronic factors.

Fig. 2 shows the very potent effect of methyl PNP carbonate on the enzyme-catalysed hydrolysis of PNP

acetate. Enzyme was mixed first with the carbonate and secondly with PNP acetate; in each assay the initial increase in A_{400} was very slow but increased over 0.5–1 min to a final linear rate (which is that recorded in the Figure). The explanation of these results is as follows. Initially, before the addition of PNP acetate, much of the enzyme would be in the form E-X-CO-OMe ; as this is slowly hydrolysed, methyl PNP carbonate and PNP acetate compete for the enzyme that is liberated until a steady state is attained. Each enzyme molecule that happens to be acylated by the carbonate is unavailable for a long period of time compared with the time of turnover with PNP acetate; effectively therefore the carbonate has the same effect on the kinetics as an extremely tightly binding competitive inhibitor. The apparent K_i value is too low to determine accurately ($< 1 \mu\text{M}$). [At low PNP acetate concentration the contribution to the increase in A_{400} from hydrolysis of methyl PNP carbonate will no longer be negligible (as it is at high PNP acetate concentration), and this is presumably why the Lineweaver–Burk plots curve downward in this region.]

Action of aldehyde dehydrogenase on PNP dimethylcarbamate

Under the usual assay conditions, with $100 \mu\text{M}$ -PNP dimethylcarbamate and $0.8 \mu\text{M}$ -enzyme, there is no measureable production of *p*-nitrophenoxide over a period of 2 h. However, if after various times $50 \mu\text{M}$ -PNP acetate is then added to the reaction mixtures in order to assay the enzyme activity, it becomes evident that the enzyme is suffering a gradual inactivation. (Approx. 98% of activity is abolished within 1.5–2 h under these conditions.) This suggests that the enzyme is acylated to give a stable and inactive E-X-CO-NMe_2 species. To confirm this idea, experiments with much higher enzyme concentrations were performed (in which a more substantial increase in A_{400} would be expected) and the results are given in Fig. 3.

In the absence of other modifiers, it is obvious that aldehyde dehydrogenase does indeed catalyse a release of *p*-nitrophenoxide from PNP dimethylcarbamate. After approx. 24 h the increase in A_{400} represents 1.1 molecules of *p*-nitrophenoxide per tetrameric enzyme molecule. Expressing the data in Fig. 3(a) as a first-order plot gives a fairly good straight line with $k = 0.38 \text{ h}^{-1}$, and from Fig. 3(b), where substrate concentration is more likely to be saturating, $k = 0.43 \text{ h}^{-1}$. This burst of *p*-nitrophenoxide (if ‘burst’ be the right word for such a leisurely process) is slowed by the presence of species that are known or thought to bind to the enzyme’s active site (i.e. propionaldehyde, chloral hydrate and diethylstilboestrol), as shown in Figs. 3(c) and 3(d). Pre-modification of the enzyme by disulfiram largely abolishes its ability to react with PNP dimethylcarbamate (as is the case, of course, with other substrates; Kitson, 1982). The presence of NAD^+ or NADH has a potent inhibitory effect (Fig. 3a); a similar nucleotide-induced slowing of acylation has previously been seen with methyl PNP carbonate (see Fig. 1) and PNP pivalate (Kitson, 1989). Little or no effect on the reaction is produced by Mg^{2+} alone, but Mg^{2+} and NADH together appear to inhibit the reaction totally (Fig. 3b).

It is interesting to compare the first-order rate constants for acylation of aldehyde dehydrogenase by PNP acetate, methyl PNP carbonate and PNP

Fig. 3. Reaction of aldehyde dehydrogenase with PNP dimethylcarbamate

Enzyme was mixed with PNP dimethylcarbamate at 25 °C in 50 mM-sodium phosphate buffer, pH 7.4 [or 50 mM-Tris/HCl buffer, pH 7.4, in (b)] and the increase in A_{400} was recorded for a long period of time. In each set of three traces, trace 1 was obtained in the absence of other modifiers. In (a), trace 2 was with 1 mM-NAD⁺ and trace 3 with 0.5 mM-NADH. In (b), trace 2 was with 20 mM-Mg²⁺ and trace 3 with 20 mM-Mg²⁺ and 100 μ M-NADH. In (c), trace 2 was with 50 μ M-diethylstilboestrol and trace 3 with 5 mM-propionaldehyde. In (d), trace 2 was with 1 mM-chloral hydrate and trace 3 with enzyme pre-modified by 40 μ M-disulfiram. Enzyme concentration was 19 μ M, 15.4 μ M, 14.7 μ M and 19 μ M in (a), (b), (c) and (d) respectively. PNP dimethylcarbamate concentration was 0.2 mM in (a), (c) and (d), and 0.5 mM in (b).

dimethylcarbamate; they are 21.2 s⁻¹, 2.0 s⁻¹ and 0.43 h⁻¹ respectively (or, relatively, 180 000:17 000:1). Steric effects are likely to be unimportant here. The rate constant for PNP isobutyrate (which is virtually identical in size with PNP dimethylcarbamate) is decreased only by a factor of 2-fold compared with PNP acetate (Kitson, 1989). Therefore the difference of several orders of magnitude in acylation rates with the carbonate and the carbamate must be a reflection of the way nucleophilic attack of the enzyme on the substrate's carbonyl group is disfavoured much more by an adjacent nitrogen atom than by an oxygen atom, in line with their respective electron-donating abilities.

Enzyme and PNP dimethylcarbamate were mixed and left at room temperature overnight; the modified enzyme (E-X-CO-NMe₂) was subsequently isolated by gel filtration (see the Experimental section). It was then left to incubate under a variety of conditions and the activity was periodically monitored; the results are shown in Fig. 4. In the absence of NADH there is a very slight trend towards increased activity in phosphate buffer and a similarly slight trend towards decreased activity in Tris buffer (suggesting the enzyme may be intrinsically less stable in the latter), but the changes are probably insignificant. In the presence of NADH, however, there is a more definite trend towards some small recovery of activity with time; this is not unexpected in view of the accelerating effect of NADH on the rate of deacylation when the substrate is methyl PNP carbonate (see above) or PNP propionate (Dickinson & Haywood, 1986). No

significant effect is caused by Mg²⁺. The only broad conclusions from this experiment are that in the presence of NADH hydrolysis of E-X-CO-NMe₂ is extremely slow and that in its absence it is negligible.

The reaction of aldehyde dehydrogenase with PNP dimethylcarbamate was studied as a function of pH and the results are shown in Fig. 5. Before the Figure was drawn, the observed readings of A_{400} at each of the five pH values were adjusted to what they would have been at pH 7.55. [The pK_a of *p*-nitrophenol is 7.1 (Behme & Cordes, 1967).] Assuming that the slower reactions at the two lowest pH values would eventually reach the same limiting value of A_{400} as was attained, within experimental error, by the three faster reactions, and assuming that 0.5 mM-PNP dimethylcarbamate is effectively saturating at each pH value, the curves were re-expressed as first-order plots and the rate constants were determined. Plotting these values against pH reveals that the rate of acylation of enzyme by PNP dimethylcarbamate appears to be controlled by a group of pK_a 7.6 (see Fig. 5). It is often assumed that the essential nucleophile in aldehyde dehydrogenase is a cysteine residue. This is by analogy with glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976) and because aldehyde dehydrogenase is susceptible to thiol-modifying reagents such as disulfiram (Kitson, 1982) and iodoacetamide (Hempel & Pietruszko, 1981). The results in Fig. 5 are not inconsistent with the proposal that X is sulphur. The typical pK_a of a cysteine residue in a protein is 8.5 (Stryer, 1988), but this can vary substantially with the local environment of the group.

Fig. 4. Investigation of possible recovery of activity from E-X-CO-NMe₂

Aldehyde dehydrogenase, pre-modified by reaction with PNP dimethylcarbamate, was isolated as described in the Experimental section. It was then left at room temperature under the following conditions, and periodically small portions were taken for assay of enzyme activity. ○, 50 mM-Sodium phosphate buffer, pH 7.4; ●, same buffer plus 0.5 mM-NADH; □, 50 mM-Tris/HCl buffer, pH 7.4; ■, same buffer plus 0.5 mM-NADH; △, same buffer plus 11 mM-Mg²⁺; ▲, same buffer plus 0.5 mM-NADH and 11 mM-Mg²⁺.

Fig. 5. Reaction of aldehyde dehydrogenase with PNP dimethylcarbamate as a function of pH

Samples (1 ml) of enzyme (16.1 μ M) in 200 mM-sodium phosphate buffer, at 25 °C, were allowed to react with PNP dimethylcarbamate (0.5 mM) while the increase in A_{400} was measured. The pH at which each numbered trace was obtained was: trace 1, 8.31; trace 2, 8.02; trace 3, 7.55; trace 4, 7.05; trace 5, 6.56. The absorbance readings were all normalized to pH 7.55 by applying a correction factor based on the measured absorbance of a standard *p*-nitrophenoxide solution at the various pH values. The first-order rate constants derived by analysis of the experimental traces are plotted against pH in the inset. The curve is that calculated for a pK_a of 7.6 and a range of k from 0 to 2.4 h⁻¹.

Fig. 6. Inhibition of the dehydrogenase activity of aldehyde dehydrogenase by PNP dimethylcarbamate

In (a) the substrate was D-glyceraldehyde and the enzyme concentration was 0.14 μ M; in (b) the substrate was propionaldehyde and the enzyme concentration was 0.069 μ M. The concentration of PNP dimethylcarbamate was 0 μ M (○) and 100 μ M (●).

Dickinson (1986) has performed initial-rate measurements and stopped-flow spectrophotometric experiments with sheep liver cytoplasmic aldehyde dehydrogenase over a wide range of pH and has found that a group of pK_a approx. 6.6 affects the aldehyde-binding reactions; he speculates that this may belong to the postulated active-site thiol group. The discrepancy between the observed pK_a values here (7.6) and in Dickinson's (1986) work (6.6) may be due to the fact that they were determined in the absence and in the presence of NAD⁺ respectively. With glyceraldehyde-3-phosphate dehydrogenase the pK_a of the essential cysteine residue, as revealed by the rate of carboxymethylation, is lowered from approx. 8 to approx. 5.5 by the binding of NAD⁺ to the enzyme (Harris & Waters, 1976).

The effect of PNP dimethylcarbamate on the dehydrogenase activity of the enzyme is shown in Fig. 6. When the substrate is either D-glyceraldehyde or propionaldehyde (in its low concentration range where the Lineweaver-Burk plot is linear), PNP dimethylcarbamate acts as a competitive inhibitor with K_i values of 84 μ M and 140 μ M respectively. Clearly PNP dimethylcarbamate can still bind to the enzyme in the presence of NAD⁺ even though its rate of acylation of the enzyme is negligible under these conditions. The results in Fig. 6 are similar to those obtained with PNP pivalate (Kitson, 1989) and provide one more piece of evidence pointing towards the identity of the enzyme's dehydrogenase and esterase active sites.

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