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Kinetic Studies on the Esterase Activity of Cytoplasmic Sheep Liver Aldehyde Dehydrogenase

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The hydrolysis of 4-nitrophenyl acetate catalysed by cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3) from sheep liver was studied by steady-state and transient kinetic techniques. NAD^+ and NADH stimulated the steady-state rate of ester hydrolysis at concentrations expected on the basis of their Michaelis constants from the dehydrogenase reaction. At higher concentrations of the coenzymes, both NAD^+ and NADH inhibited the reaction competitively with respect to 4-nitrophenyl acetate, with inhibition constants of 104 and 197 μM respectively. Propionaldehyde and chloral hydrate are competitive inhibitors of the esterase reaction. A burst in the production of 4-nitrophenoxide ion was observed, with a rate constant of $12 \pm 2\,s^{-1}$ and a burst amplitude that was 30% of that expected on the basis of the known NADH-binding site concentration. The rate-limiting step for the esterase reaction occurs after the formation of 4-nitrophenoxide ion. Arguments are presented for the existence of distinct ester- and aldehyde-binding sites.

A number of tissue aldehyde dehydrogenases (EC 1.2.1.3) have been shown to catalyse the hydrolysis of 4-nitrophenyl esters (Mathew *et al.*, 1967; Feldman & Weiner, 1972; Sidhu & Blair, 1975*a*; Eckfeldt & Yonetani, 1976; Duncan, 1977) and it has been proposed (Feldman & Weiner, 1972) that the oxidation of aldehydes and hydrolysis of 4-nitrophenyl esters proceed via a common acyl-enzyme intermediate. According to this hypothesis, after the formation of this acyl-enzyme intermediate the two reactions follow essentially identical pathways. A comparison of the two reactions could therefore be expected to lead to the elucidation of further steps in the mechanism of aldehyde oxidation. By using the enzyme isolated from horse liver, Weiner *et al.* (1976) found no evidence for a pre-steady-state phase in the production of 4-nitrophenoxide ion, and concluded that for this enzyme the rate-limiting step of the esterase reaction occurs before the formation of the presumed acyl-enzyme intermediate.

We have reported the results of steady-state and transient kinetic studies on the oxidation of aldehydes catalysed by cytoplasmic aldehyde dehydrogenase isolated from sheep liver (MacGibbon *et al.*, 1977*a*,*b*,*c*). To explore further the similarities and differences between the sheep liver enzyme and other aldehyde dehydrogenases, as well as with the hope of obtaining further insights into the mechanism of action of the enzyme, we decided to study the esterase activity of this enzyme, using 4-nitrophenyl acetate as a substrate.

Experimental

Materials

NADH (grade III) and NAD^+ (grade III) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), propionaldehyde was obtained from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), chloral hydrate was from B.D.H. (Poole, Dorset, U.K.) and 4-nitrophenyl acetate was purchased from Aldrich Chemical Co., (Milwaukee, WI, U.S.A.). The enzyme was purified essentially as described previously (Crow *et al.*, 1974) to the same specific activity and dialysed against 25mM-sodium phosphate buffer (pH 7.6) immediately before use to remove 2-mercaptoethanol.

Methods

The enzyme active-site concentration was determined by a fluorimetric assay for the dehydrogenase reaction at pH 7.6 (MacGibbon *et al.*, 1977*a*). The rate of hydrolysis of 4-nitrophenyl acetate was determined spectrophotometrically at 25°C by following the increase in A_{400} corresponding to the production of the 4-nitrophenoxide ion. A molar absorption coefficient of 18.3×10^3 litre·mol^{-1}·cm^{-1} at 400nm for 4-nitrophenoxide ion (Kezdy & Bender, 1962) and a pK_a of 7.1 for 4-nitrophenol (Behme & Cordes, 1967) were used to calculate rates in terms of μmol of ester hydrolysed/s. Because of its poor solubility in water, 4-nitrophenyl acetate was dissolved in acetone and diluted to the required

concentration in the assay mixture. Acetone has a small but significant inhibitory effect at concentrations above 60mM (inhibition largely uncompetitive, with inhibition constant about 1.8M); thus the inhibitory effect was minimized by maintaining the final concentration of acetone in all assay mixtures constant at 0.1% (v/v) (13mM). Stock solutions remained transparent during the course of the esterase experiments. Reaction mixtures contained 25mM-NaH_2PO_4 buffer, pH7.6, enzyme (about 0.3μM) and a range of ester and modifier concentrations as specified in Figure legends.

Neither acetone (0.43M) nor ethyl acetate (1.02mM) altered the steady-state rate of production of NADH at pH7.6 (25mM-sodium phosphate buffer) as measured by changes in A_{340} in an assay mixture that contained NAD^+ (15mM), enzyme (1μM) and propionaldehyde (20mM) in a total volume of 3ml.

Stopped-flow experiments were carried out in a Durrum–Gibson D110 stopped-flow spectrophotometer as described previously (Boland & Hardman, 1973). One syringe contained the enzyme (about 4μM) and the other a solution of 4-nitrophenyl acetate solution (20–350μM) prepared by diluting a stock solution of the ester in acetonitrile with either the sodium phosphate buffer for low ester concentrations, or with water for the highest ester concentration. Acetonitrile had no inhibitory effect on the enzyme activity. The data were processed as previously described (MacGibbon *et al.*, 1977*a*,*c*).

Results

Lineweaver–Burk plots of the initial-velocity data gave a K_m value for 4-nitrophenyl acetate of 5μM and a $k_{cat.}$ value of 0.2s^{-1}. In calculating $k_{cat.}$ values it is assumed that the active-site concentration for the dehydrogenase reaction is the same as the esterase active-site concentration.

Effect of added NAD^+ and NADH

The variation of v/E (where E is enzyme concentration) as a function of NAD^+ concentration at a constant ester concentration of 118μM (saturating in the absence of NAD^+) is shown in Fig. 1. v/E increased up to concentrations of about 100μM-NAD^+ and passed through a maximum as the concentration was increased, so that at still higher NAD^+ concentrations a decrease was observed. A similar dependence on NAD^+ concentration was observed when a lower concentration of the ester was used (23μM), except that the maximum in the curve occurred at about 30μM-NAD^+.

At high NAD^+ concentrations (in the region where inhibition is occurring) double-reciprocal plots of the data show the inhibition pattern characteristic of competitive inhibition, with a K_i value of 104μM. However, these plots gave on extrapolation an

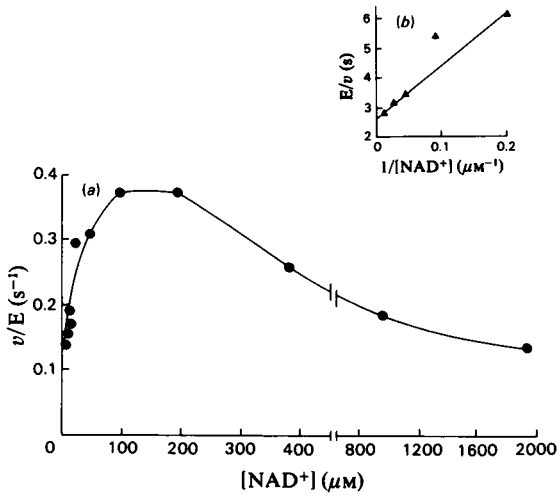


Fig. 1. *Effect of NAD^+ on initial velocity at* 118μM-*4-nitrophenyl acetate*

The initial-velocity data are plotted against the NAD^+ concentration. A reciprocal replot of the stimulation of the esterase activity is shown in the inset. The concentration giving half-maximal effect (slope/intercept) is 7μM.

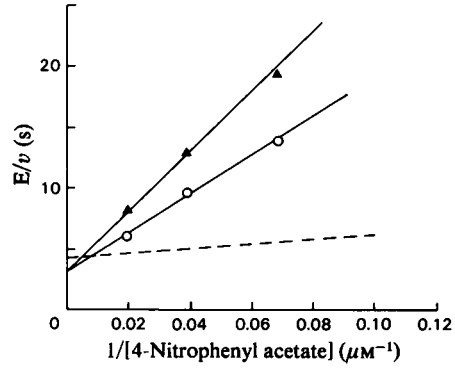


Fig. 2. *Double-reciprocal plot showing competitive inhibition by NAD^+*

The initial velocity was measured at various concentrations of 4-nitrophenyl acetate at fixed concentrations of NAD^+ (○, 478μM; ▲, 956μM) and the data were plotted as shown. The broken line indicates the plot of the slope and intercept values from a K_m determination with 4-nitrophenyl acetate in the absence of modifier (slope/intercept, 5μM; 1/intercept, 0.25s^{-1}), showing that the activating effect of NAD^+ is not removed by high concentrations of ester. The inhibition constant from the replot of slopes is 104μM.

apparent $k_{cat.}$ which was twice that obtained in the absence of NAD^+ (Fig. 2). At low concentrations of NAD^+, where activation is occurring, the concentration of NAD^+ required to give half the maximum activation effect was 7 μM when the ester concentration was 118 μM (Fig. 1) and about 5 μM when the ester concentration was 23 μM.

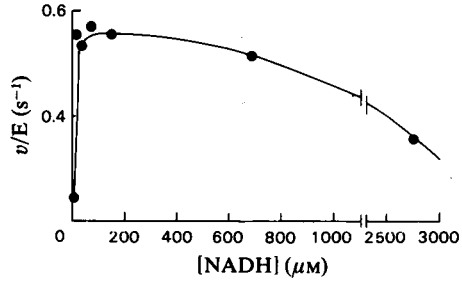


Fig. 3. *Effect of NADH on initial velocity at* 91 μM-4-*nitrophenyl acetate*

The initial velocity was measured at different concentrations of NADH and plotted as shown. The concentration giving half-maximal effect can be estimated as about 2 μM by plotting the initial points on a 10-fold expansion of the concentration scale. The enzyme concentration was 0.56 μM.

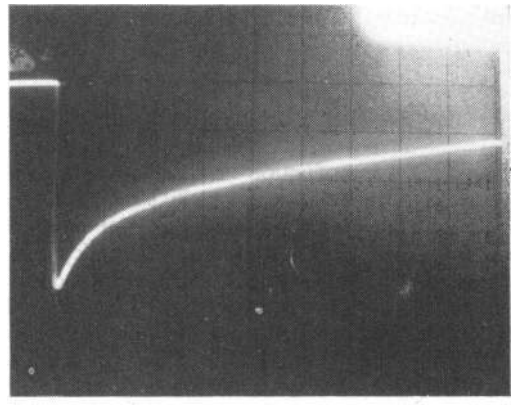


Fig. 4. *Burst in the production of* 4-*nitrophenoxide ion during the hydrolysis of* 4-*nitrophenyl acetate catalysed by cytoplasmic aldehyde dehydrogenase*

The reaction mixture consisted of enzyme (4 μM) from one syringe and 4-nitrophenyl acetate (337 μM) from another in 25 mM-sodium phosphate buffer, pH 7.6 at 25°C. The reaction was monitored by the A_{400}. The horizontal scale is 0.1 s/division and the vertical scale is 0.1 V/division. The burst size (0.6 μM) is 30% of the active-site concentration and the burst rate constant is 12.9 s^{-1}.

Table 1. *Kinetic constants for hydrolysis of* 4-*nitrophenyl acetate at pH* 7.6

Initial-velocity data were collected by using a range of 4-nitrophenyl acetate concentrations (usually 5–100 μM), and fixed concentrations of modifier were present in the determination of inhibition constants. Inhibition constants were determined from a replot of slopes against modifier concentration by using a value of 25 $\mu M \cdot s^{-1}$ for the intercept in the absence of modifier, and the $k_{cat.}$ values were calculated from the extrapolated maximum velocity. The enzyme concentration was about 0.3 μM as a routine. Activation constants were determined as described in the text.

Reaction	Modifier	$k_{cat.}$ (s^{-1})	K_m (μM)	K_i (μM)	Activation constant (μM)
Ester hydrolysis	None	0.2	5	—	—
	NAD^+ (478, 956 μM)	0.34	63*	104	7
	NADH (369, 1846 μM)	0.36	—	197	2
	Propionaldehyde (9.4, 18.8 mM)	0.17	—	4200	—
	Chloral hydrate (1.14, 2.28 mM)	0.23	—	287	—
Aldehyde oxidation		0.25	(20 mM-propionaldehyde)		

* Value obtained with 930 μM-NAD^+

The effect of added NADH on the initial velocity of the esterase reaction at concentrations (90.9 μM) of ester that were saturating in the absence of coenzyme was qualitatively similar to that observed when adding NAD^+ (Fig. 3), with the maximum in the curve occurring at 100 μM-NADH. At higher concentrations of NADH the inhibition was again competitive, with a K_i value of 197 μM, and an apparent value of $k_{cat.}$ which was about twice that observed in the absence of NADH. The concentration of NADH which gave half the maximal activation effect was about 2 μM (Fig. 3).

Inhibition by propionaldehyde and chloral hydrate

Propionaldehyde competitively inhibited ester hydrolysis in the absence of coenzymes, with a slope inhibition constant, determined for replots of slopes, of 4.2 mM and a $k_{cat.}$ value (0.17 s^{-1}) which was identical within experimental error with that determined in the absence of modifier (Table 1). Chloral hydrate, which competitively inhibits the dehydrogenase reaction with respect to propionaldehyde (MacGibbon *et al.*, 1977*a*), was also a competitive inhibitor with respect to 4-nitrophenyl acetate in the esterase reaction with a slope inhibition constant of 287 μM (Table 1) and a $k_{cat.}$ value of 0.23 s^{-1}, again

identical with $k_{cat.}$ values determined in the absence of a modifying agent.

Pre-steady-state kinetics

When a solution of aldehyde dehydrogenase (about 4 μM) was mixed with a solution of 4-nitrophenyl acetate and the production of 4-nitrophenoxide ion monitored, a burst followed by the steady state was observed (Fig. 4). Within experimental error the burst rate constant and amplitude were independent of the ester concentration (Table 3) and the derived $k_{cat.}$ value was identical with that determined from the steady-state measurements in the absence of coenzyme modifiers (Table 1). Since the enzyme had not been dialysed to remove 2-mercaptoethanol for the burst experiments, a blank was carried out in which the ester and 2-mercaptoethanol were mixed. Only a very slow production of 4-nitrophenoxide ion was observed (less than 2% of the steady-state rate).

The burst rate constant was $12\pm2\,s^{-1}$ (Table 3) and the burst amplitude was about 30% of that expected on the assumption that the concentrations of dehydrogenase and esterase active sites are the same.

Table 2. *Comparison of half-maximal concentrations for activation of esterase activity by coenzymes and their dissociation constants for various aldehyde dehydrogenases*

The half-maximal concentrations for the activation by coenzymes were determined either by inspection or by double-reciprocal plots of the activation data. The inhibition constants were determined from data on the oxidation of aldehydes, assuming an ordered Bi Bi mechanism with NAD^+ as first substrate. References: (*a*) MacGibbon *et al.*(1977*a*); (*b*) Eckfeldt & Yonetani (1976); (*c*) Sidhu & Blair (1975*a*); (*d*) Sidhu & Blair (1975*b*).

Enzyme	Coenzyme	Half-maximal concentration (μM)	K_{ia} (μM)	K_i^{NADH} (μM)
Sheep liver (cytoplasmic)	NAD^+	8	8(*a*)	—
	NADH	~2	—	1.2(*a*)
Horse liver (cytoplasmic)	NAD^+	11(*b*)	14.3(*b*)	—
	NADH	6(*b*)	—	5(*b*)
Human liver	NAD^+	117(*c*)	200(*d*)	—
	NADH	35(*c*)	—	130(*d*)

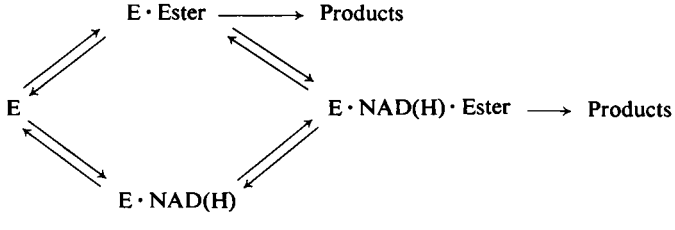
Discussion

As has been observed for other tissue aldehyde dehydrogenases (Feldman & Weiner, 1972; Sidhu & Blair, 1975*a*; Eckfeldt & Yonetani, 1976; Duncan, 1977), the sheep liver cytoplasmic enzyme exhibits significant esterase activity toward 4-nitrophenyl acetate, with a K_m value (5 μM) similar to that reported in the other studies. Since a fixed coenzyme concentration causes an increase in the apparent K_m value for 4-nitrophenyl acetate for sheep (Table 1), horse (Feldman & Weiner, 1972; Eckfeldt & Yonetani, 1976) and human (Sidhu & Blair, 1975*a*) enzymes, it seems likely that they all react with a random order of addition of coenzyme modifiers (Scheme 1), as first proposed by Sidhu & Blair (1975*a*).

Consistent with Scheme 1 is the common observation of stimulation of the esterase activity by the addition at low concentrations of either NAD^+ or NADH. In each case the concentration of coenzyme showing half the maximal activation effect has been similar to the appropriate coenzyme dissociation constant, as determined for the dehydrogenase reaction (Table 2), indicating that the stimulation is caused by NAD^+ and NADH binding in their normal coenzyme-binding sites. Since evidence for a conformational change on the binding of NADH to the sheep liver enzyme has been reported (MacGibbon *et al.*, 1977*b*), the stimulation of the esterase reaction on binding of the coenzymes may be further evidence of a ligand-induced conformational change.

For the sheep liver enzyme the competitive inhibition of the esterase activity by higher concentrations of both NAD^+ and NADH indicates that a second, weaker, coenzyme-binding site exists such that binding of either NAD^+ or NADH to this site precludes the binding of 4-nitrophenyl acetate, and vice versa.

Saturating concentrations of ester therefore remove the inhibitory effect of either NAD^+ or NADH, but the magnitude of the $k_{cat.}$ values thus obtained (Table 1) shows that the normal coenzyme-binding sites are



Scheme 1. *Proposed mechanism of enzyme interactions*

Table 3. *Rate constants for the transient production of 4-nitrophenoxide ion at pH 7.6*

The reaction mixture contained aldehyde dehydrogenase (4μM) from one syringe and 4-nitrophenyl acetate from another syringe in 25mM-sodium phosphate buffer, pH 7.6, at 25°C. The burst rate constants (k_b) were calculated as previously described (Boland & Hardman, 1973). The burst size represents the amount of 4-nitrophenoxide ion produced during the burst phase.

Concn. of 4-nitrophenyl acetate (μM)	k_b (s^{-1})	Burst size (μM)	$k_{cat.}$ (s^{-1})
24.1	11.0	0.62	
96.1	11.7	0.65	0.22
192.2	12.8	0.66	
337.0	12.9	0.60	

still occupied, as expected on the basis of Scheme 1. In the dehydrogenase reaction (MacGibbon *et al.*, 1977*a*) concentrations of NAD^+ that greatly exceed the amount necessary to saturate the normal coenzyme-binding site have no inhibitory effect on the reaction. Thus, despite the fact that high concentrations of NAD^+ ($\geq 10K_{ia}$) prevent 4-nitrophenyl acetate from binding to the enzyme, no such inhibition is apparent for propionaldehyde. On the basis of these results we conclude that the binding sites for ester and aldehyde are distinct.

This conclusion is supported by the observation that although the esterase activity is competitively inhibited by high concentrations of propionaldehyde and inhibited by very high concentrations of acetone ($K_i = 1.8$M), the dehydrogenase activity is unaffected by either (or by the presence of ethyl acetate). Although chloral hydrate does competitively inhibit both the esterase and the dehydrogenase activity, the inhibition constants are much higher for the esterase reaction. (It should be noted that saturating concentrations of ester remove the inhibitory effect of both chloral hydrate and propionaldehyde to give a $k_{cat.}$ value that is the same within experimental error as that obtained in the absence of any modifier; Table 1.) Further support for the presence of separate ester- and aldehyde-binding sites on the enzyme is provided by recent findings (T. M. Kitson, personal communication) that disulfiram at stoicheiometric concentrations rapidly inactivates the dehydrogenase activity by about 90%, but at these concentrations it has little effect on the esterase activity.

On mixing 4-nitrophenyl acetate with the enzyme in the stopped-flow spectrophotometer, a burst phase was observed, followed by the usual steady-state production of 4-nitrophenoxide ion (Fig. 4). This is the first time that a burst has been reported for the hydrolysis of 4-nitrophenyl acetate catalysed by an aldehyde dehydrogenase isolated from any source. Weiner *et al.* (1976) attempted to demonstrate the presence of a burst in the esterase reaction by extrapolating back to zero time the steady-state production of 4-nitrophenoxide ion. In the present study the burst amplitude corresponds to only about 30% of the active-site concentration at all ester concentrations (Table 3). If for the horse liver enzyme the burst amplitude is also 30% of the maximum [and assuming that two sites are involved, as is claimed by Weiner *et al.* (1976) for the dehydrogenase reaction] then an absorbance change of only about 0.02 would be expected, which is not significantly different from the error involved in the extrapolation and correction procedures. Thus stopped-flow studies of the esterase reaction by using the horse liver enzyme must be carried out before the possibility of a low-amplitude burst can be excluded.

Although systematic studies on the steady-state kinetics of the esterase activity of sheep liver aldehyde dehydrogenase show this enzyme to behave in similar fashion to other tissue aldehyde dehydrogenases, our conclusion that the ester-binding site is distinct from the aldehyde-binding site does not allow us to use these results, or the results of pre-steady-state studies, to obtain fresh insights into the mechanism of the dehydrogenase reaction. However, it is clear that for the cytoplasmic sheep liver enzyme the rate-limiting step for the esterase reaction must occur after the formation of 4-nitrophenoxide ion, and since the small burst amplitude indicates the presence of other transient enzyme species, which absorb very little at 400nm, the minimum mechanism for the esterase reaction must be similar to that proposed for proteolytic enzymes (Bender & Kezdy, 1965).

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