Reversal of Part of the Aldehyde Dehydrogenase Reaction Pathway during the Hydrolysis of an Ester

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An aldehyde dehydrogenase from rabbit liver, a homogeneous protein on three distinct polyacrylamide-gel systems, has an associated 4-nitrophenyl esterase activity. At pH 7.0 in the presence of 80 μ M-NADH and 800 μ M-4-nitrophenyl acetate the enzyme produces NAD⁺ and a stoichiometric amount of an aldehyde, as well as hydrolysing the ester. On this and other evidence it is proposed that ester hydrolysis occurs at the usual active site of the enzyme.

Several workers have shown that highly purified aldehyde dehydrogenases (EC 1.2.1.3) from the livers of several species have an associated 4-nitrophenyl esterase activity (Feldman & Weiner, 1972; Sidhu & Blair, 1975; Eckfeldt & Yonetani, 1976; Duncan, 1977; MacGibbon *et al.*, 1978). It has generally been assumed that the aldehyde dehydrogenase and the 4-nitrophenyl esterase reactions are catalysed by the same protein at the same active site, but recently some doubt has been cast on this assumption. Almost complete inhibition of the dehydrogenase activity of aldehyde dehydrogenase from the cytoplasm of sheep liver by disulfiram (tetraethylthiuram disulphide) results in only slight inhibition of the associated esterase (Kitson, 1978), and both chloral hydrate and NAD⁺ differentially inhibit the dehydrogenase and esterase activities of the enzyme(s) from the same source (MacGibbon *et al.*, 1978). In addition, differential induction of aldehyde dehydrogenase and 4-nitrophenyl esterase by phenobarbital in the cytoplasm of the liver of the rat suggests that the two activities may be controlled by distinct genetic factors (Nousiainen *et al.*, 1978).

There are theoretical grounds for the expectation that some acetaldehyde will be produced during the hydrolysis of 4-nitrophenyl acetate by aldehyde dehydrogenase in the presence of NADH (Duncan, 1977), but this requires that the two reactions be catalysed at the same active site. Hart & Dickinson (1978) have shown that purified aldehyde dehydrogenase from both the mitochondria and cytoplasm of sheep livers will, when acetylated with acetic anhydride in the presence of NADH, catalyse the oxidation of the NADH and the simultaneous production of an aldehyde. The present paper demonstrates that NAD⁺ and an aldehyde are

produced during the hydrolysis of 4-nitrophenyl acetate in the presence of NADH under appropriate conditions, and so provides further evidence that the enzyme has an active site capable of catalysing at least two apparently distinct reactions.

Materials

Aldehyde dehydrogenase from rabbit liver was purified to homogeneity as described elsewhere (Duncan, 1977). NAD⁺ (lithium salt), NADH (grade 1) and freeze-dried yeast alcohol dehydrogenase (EC 1.1.1.1) were obtained from Boehringer Corp. (London) Ltd. 4-Nitrophenyl acetate and iodoacetamide were from Sigma (London) Chemical Co.

Methods and Results

Routine assays for aldehyde dehydrogenase were conducted spectrophotometrically at 340 nm by following the rate of production of NADH from 1 mM-NAD⁺ with 1 mM-propionaldehyde (freshly distilled). 4-Nitrophenyl esterase was also assayed spectrophotometrically, by measurement of the rate of increase of A_{400} caused by the production of 4-nitrophenol from 500 μ M- or 800 μ M-4-nitrophenyl acetate. Both activities were assayed at 30°C. Two pH values were used on a routine basis in this study: pH 7.0 in 50 mM-sodium/potassium phosphate and pH 9.0 in 100 mM-sodium pyrophosphate. Results are given as means $\pm$ S.E.M., with the numbers of observations in parentheses.

A millimolar absorption coefficient of 18.3 cm⁻¹ was assumed for 4-nitrophenol at pH 9.0 (Kezdy & Bender, 1962), but the value for use at pH 7.0 was not ascertainable from the literature. Use of a method similar to that of Kezdy & Bender (1962) showed that 4-nitrophenol has an apparent pK of 6.99 $\pm$ 0.01 (5) at 30°C in 50 mM-phosphate, pH 7.0, and an

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Fig. 1. Enzyme inhibition by iodoacetamide

Enzyme was incubated with iodoacetamide and remaining activity assayed as described in the text. ▲, 4-Nitrophenyl esterase activity; ●, aldehyde dehydrogenase activity; ■, aldehyde dehydrogenase activity in the presence of 20 μ M-diethylstilboestrol (see Duncan, 1977).

absorption of 9.2 ± 0.1 (5) litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$ under these conditions.

The purified enzyme was inhibited with iodoacetamide by incubating approx. 2 μ M-enzyme with 1 mM-iodoacetamide in 20 mM-triethanolamine hydrochloride/NaOH, pH 8.6, 0°C, for an appropriate time (as shown in Fig. 1), followed by dilution of the inhibition solution 200-fold into the routine assay system for determination of the dehydrogenase or esterase. This dilution served to terminate the inhibition of the enzyme, and the subsequent rate of production of NADH or 4-nitrophenol was constant for several minutes. Fig. 1 shows the time course of inhibition of the esterase, dehydrogenase and steroid-sensitive dehydrogenase by iodoacetamide at pH 8.6.

When the purified enzyme was incubated at about 16 μ g/ml with 4-nitrophenyl acetate (800 μ M) and NADH (80 μ M), it was not possible to show any oxidation of NADH by spectrophotometric means at either pH 9.0 (Duncan, 1977) or pH 7.0 because of the high and changing background absorbance as 4-nitrophenoxide was produced. However, if a sample (250 μ l) from a solution of the above composition after incubation at pH 7.0, 30°C, was treated with 10 μ l of 5 M-HCl and left for 15 min at room temperature [to stop the enzymic reaction, protonate 4-nitrophenoxide, and destroy NADH selectively (Lowry & Passonneau, 1972)], then thoroughly extracted with 1 ml of water-saturated ethylene dichloride, it was possible to demonstrate the presence of enzymically produced NAD $^{+}$ in the resulting

aqueous phase by the alkali-induced-fluorescence technique of Lowry & Passonneau (1972). Authentic NAD $^{+}$ added to an incubation solution which was then treated as described produced 93% of the fluorescence of an appropriate quantity of NAD $^{+}$ added to the 6 M-NaOH used in the induction of fluorescence, and the fluorescence was proportional to the concentration of NAD $^{+}$ over the range of interest (up to at least 3 nmol in 250 μ l of the incubation mixture). The fluorescence absorption and emission spectra of the enzymically produced NAD $^{+}$ were identical with those of authentic NAD $^{+}$. The following controls were always performed: (i) enzyme, (ii) NADH and (iii) 4-nitrophenyl acetate, added after the 5 M-HCl. In each of these controls the fluorescence ascribable to NAD $^{+}$ was always constant and low [less than 25% of that of the NAD $^{+}$ in the complete sample after a 40 min (routine) incubation] and could be traced to small amounts of NAD $^{+}$ in the NADH. The concentration of NAD $^{+}$ produced by the enzyme with increasing length of incubation was as shown in Fig. 2 along with the amount of 4-nitrophenol produced during the same time. The amount of NAD $^{+}$ produced after a constant time was proportional to the concentration of enzyme up to at least 45 μ g/ml.

If NAD $^{+}$ is produced from NADH during the hydrolysis of 4-nitrophenyl acetate by aldehyde dehydrogenase, then acetaldehyde should also be produced in amounts stoichiometric with the

Fig. 2. Time course of NAD $^{+}$ production during ester hydrolysis

Enzyme was incubated with 80 μ M-NADH and 800 μ M-4-nitrophenyl acetate. NAD $^{+}$ and 4-nitrophenol were then measured as described in the text. ■, NAD $^{+}$; ●, 4-nitrophenol; ▲, % of hydrolytic cycles leading to NAD $^{+}$ production.

amounts of NAD^+ produced. That this is the case was demonstrated by incubating 4-nitrophenyl acetate ($800\mu\text{M}$), NADH ($80\mu\text{M}$) and enzyme (about $4\mu\text{g}/250\mu\text{l}$) for 40 min along with the above controls, then adding $12.5\mu\text{g}$ of yeast alcohol dehydrogenase to half of the set of incubation tubes and continuing the incubation for a further 10 min. After this time the enzymic reactions were stopped with 5M-HCl , and NAD^+ was then assayed as described above. In the absence of yeast alcohol dehydrogenase, 1.23 ± 0.02 (5) nmol of NAD^+ was produced during the incubation. When yeast alcohol dehydrogenase was added towards the end of the incubation, the NAD^+ produced was 2.34 ± 0.07 (5) nmol. Under these conditions the amount of NAD^+ produced in the

hemiacetal at the dehydrogenase active site, as proposed by Feldman & Weiner (1972), then an enzyme-NADH-thiohemiacetal must presumably be formed during ester hydrolysis in the presence of NADH. Such an intermediate would have circumvented the energetic barrier to reversal of the usual aldehyde dehydrogenase reaction, and so at least some of this intermediate might rearrange and dissociate to produce NAD^+ and acetaldehyde (Duncan, 1977). The present work shows that this is the case: NAD^+ is produced during the hydrolysis of 4-nitrophenyl acetate in the presence of NADH, and an aldehyde is produced in amounts stoichiometric with the NAD^+ , as would be expected from the equations representing this reaction:

presence of alcohol dehydrogenase was not significantly different from twice the amount produced in the absence of alcohol dehydrogenase ($P > 0.2$). When alcohol dehydrogenase was present throughout the incubation, not merely during the final 10 min, more than twice as much NAD^+ ($P < 0.005$) was produced as in the absence of the alcohol dehydrogenase [1.27 ± 0.04 (4) nmol to 2.93 ± 0.08 (4) nmol produced].

At pH 9.0 NADH is a partially competitive inhibitor (Dixon & Webb, 1964) of the esterase activity of rabbit liver aldehyde dehydrogenase (Duncan, 1977), but at pH 7.0 NADH stimulates the hydrolysis of 4-nitrophenyl acetate [160 ± 4.3 (5) $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ increased ($P < 0.001$) to 339 ± 7 (5) $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the presence of $25\mu\text{M-NADH}$].

Discussion

Fig. 1 shows that for the present preparation of aldehyde dehydrogenase a well-known enzyme inhibitor, iodoacetamide, produces similar inhibition of the aldehyde dehydrogenase, steroid-sensitive aldehyde dehydrogenase and esterase activities of the preparation. Such inhibition is consistent with all these reactions being catalysed by the active sites of the same enzyme, but is apparently distinct from the inhibition of a preparation of cytosolic aldehyde dehydrogenase from sheep liver, whose esterase activity is more resistant to inhibition by disulfiram than is the dehydrogenase activity (Kitson, 1978).

Stimulation of the esterase activity by NADH at pH 7.0 or the partially competitive inhibition at pH 9.0 by the same ligand (Duncan, 1977) both imply that the enzyme-NADH adduct is active in ester hydrolysis. If ester hydrolysis occurs via a thio-

with acetaldehyde being trapped according to:

The proportion of the NADH-enzyme-(acetyl) that breaks down with liberation of acetaldehyde is small. Fig. 2 shows that 10 nmol of NAD^+ /ml is formed concurrently with 819 nmol of 4-nitrophenol/ml after 135 min, so about 1.2% of the ternary complex yields NAD^+ plus acetaldehyde. This proportion is rather greater at earlier stages of the reaction (2.1% at 10 min), as shown by the broken line in Fig. 2, and it is probable that some of the acetaldehyde and NAD^+ react in the normal way with the dehydrogenase, resulting in the formation of acetate and re-formation of NADH leading to an apparent decrease of the NAD^+ produced. In agreement with this is the observation that when alcohol dehydrogenase is present throughout the incubation significantly more NAD^+ is formed than would be expected from the incubation without alcohol dehydrogenase, because re-reduction of the NAD^+ is prevented. When alcohol dehydrogenase is added to trap acetaldehyde at the end of the incubation, the true stoichiometry of the production of acetaldehyde and NAD^+ is revealed although the total amount of acetaldehyde formed during the hydrolysis is under-estimated.

The present results are most easily explicable if aldehyde dehydrogenase has the capacity to hydrolyse certain esters at its normal active site. In favour of this are co-purification of the activities through ion-exchange and affinity chromatography, co-inhibition of all the enzymic activities by iodoacetamide, interaction of diethylstilboestrol and progesterone with both dehydrogenase and esterase activities, and reversal of part of the dehydrogenase reaction during ester hydrolysis under appropriate conditions. Also, as reported by Hart & Dickinson (1978), acetic

anhydride (which is presumed to acetylate the enzyme to give an intermediate similar to that postulated in ester hydrolysis) may be partially reduced to an aldehyde in the presence of NADH.

If the dehydrogenase and esterase activities are catalysed at the same enzymic active site, the observations of Kitson (1978), MacGibbon *et al.* (1978) and Nousiainen *et al.* (1978) should be explicable in terms of the present hypothesis. The data of Nousiainen *et al.* (1978) do not rule out the existence of aldehyde dehydrogenase and 4-nitrophenyl esterase activities on the same protein; an equally valid explanation is that there is more than one type of esterase, with most of the activity not being contributed by the phenobarbital-inducible aldehyde dehydrogenase. The data of Kitson (1978), showing sheep liver aldehyde dehydrogenase and esterase being differentially inhibited by disulfiram, may have to be reassessed in view of the report by Dickinson & Berrieman (1979) of the difficulty of separation of cytosolic and mitochondrial aldehyde dehydrogenases in that species, and the resistance of the mitochondrial enzyme to inhibition by disulfiram. If most of the esterase activity of the enzyme preparation studied by Kitson (1978) is provided by contaminating mitochondrial disulfiram-resistant aldehyde dehydrogenase the apparent differential inhibition of the two activities simply reflects the different dehydrogenase/esterase activity ratios of the two isoenzymes. The differential inhibition by NAD⁺ and by chloral hydrate of the two activities in a similar enzyme preparation (MacGibbon *et al.*, 1978) may perhaps be resolved on similar

grounds. The evidence at present is that at least one isoenzyme of aldehyde dehydrogenase has the capacity to hydrolyse certain esters at its normal active site.

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