Aldehyde dehydrogenase catalyses acetaldehyde formation from 4-nitrophenyl acetate and NADH

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Incubation of sheep liver cytoplasmic aldehyde dehydrogenase with the substrate 4-nitrophenyl [^{14}C]acetate in the presence of NADH leads to the formation of ^{14}C -labelled acetaldehyde. This observation strongly supports the idea that the esterase and dehydrogenase activities of the enzyme occur at the same site and involve the intermediacy of a common acyl-enzyme.

INTRODUCTION

Mammalian aldehyde dehydrogenases catalyse the oxidation by NAD^+ of a wide range of aldehydes, and also have the ability to catalyse the hydrolysis of 4-nitrophenyl acetate. Some workers have drawn the natural conclusion that the dehydrogenase and esterase activities of the enzyme take place at the same active site and occur via a common acyl-enzyme intermediate, probably involving a catalytically essential cysteine residue (Sidhu & Blair, 1975; Takahashi & Weiner, 1981). Studies of the esterase activity might therefore have the potential to provide valuable information about the mechanism of the enzyme's dehydrogenase action. However, Blackwell, Buckley and co-workers (MacGibbon *et al.*, 1978; Blackwell *et al.*, 1983a,b; Deady *et al.*, 1985) have presented a large body of kinetic results concerning the dehydrogenase and esterase reactions of sheep liver cytoplasmic aldehyde dehydrogenase that they interpret as showing distinct and separate sites for the two enzymic activities. Their arguments have been vigorously criticized by Duncan (1985) and by Dickinson & Haywood (1986), and at least one of their observations (the nature of the inhibition of the esterase activity by chloral hydrate in the presence of NAD^+ ; Blackwell *et al.*, 1983a) disagrees with the results reported by other workers (Sidhu & Blair, 1975; Kitson, 1986).

The most telling and unequivocal support for a common active site would be the reduction by NADH of the presumed acetyl-enzyme formed in the esterase reaction (thereby giving NAD^+ and acetaldehyde), since it would be highly unlikely that enzyme-bound NADH could deliver a hydride ion to a site different from the one that provides a hydride ion for NAD^+ in the dehydrogenase reaction. That this reduction does indeed occur was reported by Duncan (1979) for rabbit liver aldehyde dehydrogenase, but his technique for detecting the formation of low concentrations of NAD^+ was cumbersome (involving the addition of 5 M-HCl to destroy NADH selectively, extraction with 1,2-dichloroethane to remove 4-nitrophenol, and determination of NAD^+ by alkali-induced fluorescence). Control values of NAD^+ of up to 25% of those from the complete reaction mixture were found. Moreover, the formation of acetaldehyde was not directly demonstrated but only inferred from the greater production of NAD^+ when yeast alcohol dehydrogenase was included in the reaction mixture. In experiments with cytoplasmic aldehyde dehydrogenase

from sheep liver, Blackwell *et al.* (1983a) found that no acetaldehyde was formed within the limits of detection (approx. 1%, based on the initial concentration of NADH) of their experimental method (Stowell *et al.*, 1978).

In view of these conflicting results and the importance of the continuing lively debate in the literature over whether aldehyde dehydrogenase has one kind of active site or two, we considered it crucial that, if acetaldehyde is produced from the enzyme/NADH/ester system, then its identification should be firmly established. Accordingly, the technique we chose to detect acetaldehyde (by allowing the ^{14}C -labelled material to distil spontaneously into a semicarbazide trap) was simple, sensitive, direct and, in contrast with Duncan's (1979) method, completely 'non-invasive' (to use a medical analogy).

EXPERIMENTAL

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified as previously described (Dickinson *et al.*, 1981). 4-Nitrophenyl acetate labelled with ^{14}C in the carbonyl group was prepared from [^{14}C]acetic anhydride essentially by the method of Fife (1965). The product was judged to be identical with an authentic sample of unlabelled material on the basis of t.l.c. and by monitoring the extent of production of 4-nitrophenoxide in enzyme-catalysed hydrolysis. It had a specific radioactivity of 9.39×10^{10} d.p.m./mol. Radioactivity was determined as described previously (Kitson, 1978), and values were corrected for the background level.

Reaction mixtures contained the following in a volume of 4.5 ml (concentrations being those after mixing): enzyme (0.80 μM), 4-nitrophenyl [^{14}C]acetate (1.1 mM), NADH (83 μM) and sodium phosphate buffer, pH 8.0 (35 mM). Each reaction mixture was incubated at 30 °C for 120 min (by which time the hydrolysis was complete) in the outer compartment of a Conway diffusion unit. The inner compartment contained 1.0 ml of semicarbazide (0.10 M) and potassium acetate (0.18 M) solution. The diffusion units were sealed with a greased glass plate. Control mixtures were as follows: (1) without NADH, (2) without enzyme, (3) with enzyme inactivated by disulfiram (13 μM), and (4) with addition of enzyme and NADH (and semicarbazide solution to the other compartment) only after the 4-nitrophenyl [^{14}C]acetate had undergone complete spontaneous hydrolysis, as judged spectrophotometrically. After incubation, dupli-

ate 0.1 ml samples of the semicarbazide solutions were assayed for radioactivity. The remaining semicarbazide solution from the three complete reaction mixtures was then pooled and freeze-dried, as was that from three of each type of control mixture. The dried material was extracted with ethanol (4 ml), the extract was concentrated, and the residue was applied to a plastic-backed sheet of silica gel (Merck Kieselgel 60 F₂₅₄, 3.5 cm × 10 cm) for t.l.c. The eluent was ethanol/ethyl acetate (1:1, v/v). The chromatogram was cut into transverse sections; the silica gel from each piece was scraped into a scintillation bottle and the radioactivity was determined. On another occasion, the silica from the chromatogram section corresponding to the R_F of authentic acetaldehyde semicarbazone was extracted with ethanol and after evaporation the residue was examined by mass spectrometry.

RESULTS AND DISCUSSION

Table 1 shows the results of a typical set of experiments. In all cases the trapped radioactivity from the complete reaction mixtures was consistently greater than that from any of the various controls. Repetition of the whole series of experiments always led to the same conclusion, although there was some minor variation in the absolute amounts of radioactivity observed. We considered various possibilities for the radioactivity found in the control samples: (1) a volatile radioactive impurity in the substrate, (2) acetic acid, the hydrolysis product (although diffusion of this is likely to be small at pH 8.0), and (3) diffusion of 4-nitrophenyl [¹⁴C]acetate itself (which would then become hydrolysed in the semicarbazide solution). Either or both of the last two possibilities is likely to contribute, as t.l.c. of the control samples showed a peak corresponding to the R_F (0.1–0.4)

Table 1. Radioactivity trapped in semicarbazide solution after incubation of 4-nitrophenyl [¹⁴C]acetate under various reaction conditions

For full experimental details see the text. Each value given refers to an individual reaction mixture.

Reaction mixture	Radioactivity (d.p.m.)
(I) Complete (enzyme/NADH/ester)	490, 449, 481
(II) Control 1 (enzyme/ester)	71, 90, 108
(III) Control 2 (NADH/ester)	166, 276, 180
(IV) Control 3 (inactivated enzyme/NADH/ester)	152, 229, 179
(V) Control 4 (pre-hydrolysed ester/enzyme/NADH)	69, 73, 65

of potassium acetate (see Fig. 1). The variation of the size of this peak may reflect the different extents of ester hydrolysis in the various control mixtures; the greater the hydrolysis, the less scope for diffusion of the ester. In support of this we observed that where the acetate peak in Fig. 1 was large the freeze-dried semicarbazide sample was noticeably yellow (this presumably being due to 4-nitrophenoxide).

The most important and striking observation in Fig. 1 is that of a large peak corresponding to the R_F (0.6–0.7) of authentic acetaldehyde semicarbazone from the complete reaction mixtures. This contrasts with the complete absence of such a peak in any of the controls; clearly, acetaldehyde is formed from the ester only in the presence of active enzyme and NADH. Duplication of the whole experiment on a separate occasion gave the same result. As a final confirmation that the radioactivity

Fig. 1. T.l.c. profiles of the trapped radioactivity from the reaction mixtures (I)–(V) mentioned in Table 1

The R_F of acetaldehyde semicarbazone is 0.6–0.7.

Scheme 1. Proposed scheme showing the convergence on a common acyl-enzyme intermediate of the dehydrogenase and esterase pathways of aldehyde dehydrogenase

The active-site nucleophile ($-\text{X}^-$) is usually assumed to be a thiolate group.

in this peak is due to [^{14}C]acetaldehyde semicarbazone, the material on the appropriate section of a chromatogram was isolated. Mass spectrometry showed a molecular ion at 101.0585 (calculated for $\text{C}_3\text{H}_7\text{N}_3\text{O}$: 101.0589).

It could be argued that the trapped acetaldehyde arises from the hydrolysis of 4-nitrophenyl acetate at one enzyme site followed by reduction of the acetate thereby produced at a different site in a reversal of the dehydrogenase reaction. Since we were endeavouring to detect very low concentrations of acetaldehyde, this was a possibility that could not be ignored, even though the overall dehydrogenase reaction is normally considered to be irreversible (Duncan & Tipton, 1971). However, control experiment 4, in which the ester was pre-hydrolysed to [^{14}C]acetate, rules this out as an explanation of the results.

The radioactivity observed in the acetaldehyde semicarbazone peak from the complete reaction mixtures (Fig. 1) shows that at least 0.22% of the original 4-nitrophenyl acetate is diverted from hydrolysis into the reductive pathway. This is a lower limit, since presumably not all the acetaldehyde produced diffuses over into the trap. Some of it may be oxidized to acetate by the enzyme's dehydrogenase action, utilizing the NAD^+ co-product. Of course, it might have turned out that, even if both enzyme activities do occur at the same site, the hydrolysis of the acyl-enzyme could be so much faster than its reduction by NADH that no acetaldehyde could be detected. Thus in these experiments the important thing is not so much the absolute amount of acetaldehyde, but rather the simple and unequivocal demonstration that at least some of this compound is produced.

In conclusion, we have shown beyond doubt that aldehyde dehydrogenase catalyses the formation of

acetaldehyde from 4-nitrophenyl acetate and NADH. This provides the most convincing experimental foundation yet reported for the assumption that the esterase and dehydrogenase activities occur at the same site. The likely steps in a partially common pathway for the two reactions are summarized in Scheme 1.

We are grateful to Dr. D. R. K. Harding for generously providing the [^{14}C]acetic anhydride (Amersham International), to Professor R. Hodges for mass spectrometry, and to Dr. P. D. Buckley for helpful discussions.

REFERENCES

- Blackwell, L. F., Bennett, A. F. & Buckley, P. D. (1983a) *Biochemistry* **22**, 3784–3791
- Blackwell, L. F., Bennett, A. F., Crow, K. E. & Buckley, P. D. (1983b) *Pharmacol. Biochem. Behav.* **18**, Suppl. 1, 83–87
- Deady, L. W., Buckley, P. D., Bennett, A. F. & Blackwell, L. F. (1985) *Arch. Biochem. Biophys.* **243**, 586–597
- Dickinson, F. M. & Haywood, G. W. (1986) *Biochem. J.* **223**, 877–883
- Dickinson, F. M., Hart, G. J. & Kitson, T. M. (1981) *Biochem. J.* **199**, 573–579
- Duncan, R. J. S. (1979) *Biochem. J.* **183**, 459–462
- Duncan, R. J. S. (1985) *Biochem. J.* **230**, 261–267
- Duncan, R. J. S. & Tipton, K. F. (1971) *Eur. J. Biochem.* **22**, 538–543
- Fife, T. H. (1965) *J. Am. Chem. Soc.* **87**, 4597–4600
- Kitson, T. M. (1978) *Biochem. J.* **175**, 83–90
- Kitson, T. M. (1986) *Biochemistry*, in the press
- MacGibbon, A. K. H., Haylock, S. J., Buckley, P. D. & Blackwell, L. F. (1978) *Biochem. J.* **171**, 533–538
- Sidhu, R. S. & Blair, A. H. (1975) *J. Biol. Chem.* **250**, 7894–7898
- Stowell, A. R., Crow, K. E., Greenway, R. M. & Batt, R. D. (1978) *Anal. Biochem.* **84**, 384–392
- Takahashi, K. & Weiner, H. (1981) *Biochemistry* **20**, 2720–2726