Steady-state kinetic analysis of aldehyde dehydrogenase from human erythrocytes

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The steady-state kinetics of purified cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3) from human erythrocytes have been studied at 37 °C. Previous studies of the enzyme from several mammalian sources, which used a lower assay temperature, have been difficult to interpret because of the substrate activation by acetaldehyde which led to complex kinetic behaviour. At 37 °C the initial-rate data do not depart significantly from Michaelis–Menten kinetics. Studies of the variation of initial rates as a function of the concentrations of both substrates and studies of the inhibition by NADH were consistent with a sequential mechanism being followed. High-substrate inhibition by acetaldehyde was competitive with respect to NAD⁺. The enzyme was not inhibited by the product acetate and thus the results of these studies, although consistent with an ordered mechanism in which NAD⁺ was the first substrate to bind, were inconclusive. That such a mechanism was followed was confirmed by determination of the initial-rate behaviour in the presence of acetaldehyde and glycolaldehyde as alternative substrates. When the reciprocal of the initial rate of NADH formation was plotted against the acetaldehyde concentration at a series of fixed ratios between that substrate and glycolaldehyde, a linear ‘mixed inhibition’ pattern was obtained, confirming the mechanism to be ordered with NAD⁺ being the leading substrate and with kinetically significant ternary complex-formation.

INTRODUCTION

Kinetic characterization of the cytosolic aldehyde dehydrogenase (EC 1.2.1.3) from several mammalian species has been described (Duncan & Tipton, 1971; Eckfeldt & Yonetani, 1976; MacGibbon *et al.*, 1977; Vallari & Pietruszko, 1981; Hart & Dickinson, 1982). These studies were complicated by the phenomenon of ‘substrate activation’ exhibited even by homogeneous preparations of the enzyme (Duncan & Tipton, 1971; MacGibbon *et al.*, 1977; Hart & Dickinson, 1982; Helander & Tottmar, 1986). This manifests as non-linear double-reciprocal plots of initial velocity against aldehyde concentration. This complication has led to the kinetic mechanism being studied only at relatively low concentrations of the aldehyde substrate (typically less than 100 μ M) where the double-reciprocal plots were apparently linear. Such an analysis was based on the interpretation of the double-reciprocal plots as being composed of two linear segments with a relatively sharp break between them. However, we have shown that the double-reciprocal dependence of initial rate on substrate concentration describes a smooth curve (Henehan & Tipton, 1991).

The underlying mechanisms that give rise to substrate activation have been the subject of some controversy. Blackwell *et al.* (1983) have explained this phenomenon in terms of two binding sites for the aldehyde substrate, whereas Hart & Dickinson (1982) have provided evidence for more rapid dissociation of NADH from the enzyme at high aldehyde concentrations (for review, see Duncan, 1985). Neither of these models would predict a double-reciprocal plot composed of linear sections linked by a sharp discontinuity; indeed it is difficult to envisage any mechanism that would give such behaviour (see Cornish-Bowden, 1988).

Human erythrocyte aldehyde dehydrogenase appears to be identical with the aldehyde dehydrogenase found in the cytosol of human liver (Agarwal *et al.*, 1984; Harrington *et al.*, 1987). We have shown that the complex kinetics are dependent on the temperature used for the initial-rate measurements (Henehan &

Tipton, 1991). At 25 °C the curvature of the double-reciprocal plots was marked, whereas at the more physiological temperature of 37 °C no significant deviation from Michaelis–Menten kinetics could be detected over a wide range of acetaldehyde concentrations, until high-substrate inhibition became apparent at concentrations above about 30 mM. Clearly the conclusions from any attempted steady-state analysis that assumes a restricted section of a curved double-reciprocal plot to be linear must be regarded as being suspect. The observation of this temperature effect prompted re-investigation of the kinetic mechanism at 37 °C where complications of non-linear double-reciprocal plots were absent.

MATERIALS AND METHODS

Buffer salts, 2-mercaptoethanol, EDTA and acetaldehyde were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and NAD⁺ and NADH were from the Boehringer-Mannheim G.m.b.H., Mannheim, Germany. Acetaldehyde was distilled in an all-glass apparatus, diluted 1:10 (v/v) with distilled deionized water and stored frozen at –20 °C until use. Solutions of acetaldehyde were standardized enzymically (Racker, 1949) using commercially available yeast aldehyde dehydrogenase (Sigma Chemical Co., Poole, Dorset, U.K.). Glycolaldehyde (Sigma), which was used in the mixed-substrate experiments, exists as a dimeric species when freshly made up in solution. Depolymerization was accomplished by allowing filtered solutions to stand at room temperature for at least 24 h before use (see Dawson *et al.*, 1986).

Aldehyde dehydrogenase was purified to homogeneity as previously described by Henehan & Tipton (1991). The purified enzyme was stored in the presence of 30 mM-sodium phosphate buffer, pH 7.2, containing 0.1 % (v/v) 2-mercaptoethanol, 1 mM-EDTA and 60 % (v/v) glycerol at –20 °C. Before kinetic studies the enzyme was dialysed for 14 h at 4 °C against 500 vol., with two changes, of the above buffer mixture without glycerol.

Assays were performed at 37 °C in 50 mM-sodium phosphate

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buffer, pH 7.2, containing 0.1% 2-mercaptoethanol, 1 mM-EDTA, enzyme and substrates. The reaction was started by the addition of enzyme. Initial steady-state rates were determined from the linear portions of reaction progress curves spectrophotometrically, by following the increase in absorbance at 340 nm, or fluorimetrically, by monitoring the increase in fluorescence at 460 nm at an excitation wavelength of 340 nm. Protein concentration was determined by the method of Bradford (1976). Kinetic data were analysed by non-linear regression by the procedure of Wilkinson (1961) as modified by Cleland (1967). All kinetic parameters were calculated in this way and double-reciprocal plots, drawn by eye, are presented for illustrative purposes only.

RESULTS

A linear relationship between initial velocity and enzyme concentration was obtained in the range 0–50 μg of enzyme per assay and all assays were performed using enzyme concentrations within this range.

In common with all mammalian aldehyde dehydrogenases studied thus far, the erythrocyte enzyme was found not to be inhibited by the acid product of the reaction. Inclusion of acetate at concentrations up to 3 mM in the assay mixture had no effect on the oxidation of acetaldehyde by the enzyme. The K_m and maximum velocity values for glycolaldehyde were determined in the presence of 500 μM -NAD⁺ to be $0.59 \pm 0.06 \mu\text{M}$ and $0.88 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ (mean values $\pm$ S.E.M.) respectively.

Initial-velocity studies

Initial velocities were determined when the concentration of acetaldehyde was varied in the presence of several fixed NAD⁺ concentrations (Fig. 1a). A linear intersecting pattern of lines was obtained and replots of the slopes and intercepts of these lines against the reciprocal of the NAD⁺ concentration were linear (Figs. 1b and 1c). Similar results were obtained when NAD⁺ was the varied substrate (not shown). Kinetic constants determined from these data are summarized in Table 1.

Product inhibition

Inhibition patterns could be determined for NADH only, since the acid product was not inhibitory. NADH was found to be a linear mixed inhibitor with respect to acetaldehyde at a non-saturating concentration of NAD⁺ and this inhibition was alleviated at saturating concentrations of coenzyme (Fig. 2). NADH was found to be a linear competitive inhibitor with respect to NAD⁺ at both saturating and non-saturating concentrations of acetaldehyde (Fig. 3). The inhibition constants determined from these data are summarized in Table 2.

High-substrate inhibition

Double-reciprocal plots of various acetaldehyde concentrations at fixed concentrations of NAD⁺ showed substrate inhibition to occur at concentrations above about 30 mM. Reaction progress curves were linear for the initial-rate periods, indicating that there was no significant time-dependent inhibition of the enzyme over these periods at the high concentrations of acetaldehyde used. The form of this inhibition with respect to NAD⁺ was determined at a series of fixed inhibitory acetaldehyde concentrations and various concentrations of NAD⁺ (Fig. 4). High-substrate inhibition by acetaldehyde was found to be competitive with respect to NAD⁺. A replot of the slopes of the competitive plot against the inhibitor concentrations was used to determine the K_i for this inhibition (see Table 2).

Alternative substrates

Alternative substrate procedures can be used to distinguish between enzyme reaction mechanisms (see Huang, 1979). The alternative substrate is assumed to undergo the same catalytic events as the substrate but gives rise to an alternative reaction pathway. Experimentally the substrate concentration is varied in the presence of a fixed ratio of alternative substrates and the initial rate of formation of the common product (NADH) is determined. The results are plotted in double-reciprocal form and this process is repeated for several values of β , where $\beta =$

Fig. 1. Variation of initial velocity with acetaldehyde concentration determined at a series of fixed concentrations of NAD⁺

(a) The concentrations of NAD⁺ were held constant at the following values: 5 μM ($\circ$); 10 μM ($\bullet$); 16.66 μM ($\square$); 25 μM ($\blacksquare$); 50 μM ($\triangle$) and 100 μM ($\blacktriangle$). The data are presented as double-reciprocal plots. Experimental conditions are given in the text. Replots of (b) the slopes (dimensions: $\text{min} \cdot \text{mg}$ of protein per l) and (c) intercepts (dimensions: $\text{min} \cdot \text{mg}$ of protein per μmol) of the data from (a) against the reciprocal of the concentration of NAD⁺.

Table 1. Kinetic parameters for the oxidation of acetaldehyde by aldehyde dehydrogenase from human erythrocytes

Values were determined as described in the text and as defined by eqn. (2). The values shown are means±range of two separate complete determinations.

Constant	Value	Dimensions
$V_{\max}^{NAD^+}$	0.67 ± 0.03	$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$
$K_m^{NAD^+}$	15.6 ± 1.9	μM
$K_m^{\text{Acetaldehyde}}$	321 ± 80	μM
$K_s^{NAD^+}$	12.7 ± 2.1	μM

Fig. 2. Inhibition of aldehyde dehydrogenase by NADH with respect to acetaldehyde

(a) The concentration of acetaldehyde was varied while NADH concentrations were held constant at the following values: 0 μM ($\circ$); 3.33 μM (k); 6.66 μM ($\square$); 15 μM ($\blacksquare$); 20 μM ($\triangle$) and 25 μM ($\blacktriangle$). The concentration of NAD^+ was held at 20 μM throughout (non-saturating). (b) The concentration of acetaldehyde was varied while the concentration of NAD^+ was held constant at 250 μM . Assays were carried out in the absence of NADH ($\square$) and in the presence of 6.66 μM -NADH ($\bullet$). Each point represents the mean±range of three separate determinations.

[alternative substrate]/[substrate]. The resulting pattern of lines can provide information on the kinetic mechanism followed. The kinetic parameters of glycolaldehyde were found to be suitable for its use as an alternative substrate to acetaldehyde in this way (see Huang, 1979). The resulting plots took the form of families of intersecting straight lines (Fig. 5).

Fig. 3. Inhibition of aldehyde dehydrogenase by NADH with respect to NAD^+

(a) Acetaldehyde was held at a non-saturating concentration of 300 μM and the NADH concentrations were: 0 μM ($\bullet$); 3.33 μM ($\circ$); 4.49 μM ($\blacklozenge$); 13.32 μM ($\diamond$). (b) Acetaldehyde was held at a saturating concentration of 3 mM and the NADH concentrations were: 0 μM ($\bullet$); 6.66 μM ($\square$); 10.0 μM ($\circ$); 16.66 μM ($\blacksquare$); 25 μM ($\blacktriangle$).

Table 2. Inhibition constants for purified human erythrocyte aldehyde dehydrogenase

All values quoted are means $\pm$ S.E.M. K_{is} and K_{ii} were obtained from slope and intercept replots respectively. In the cases where competitive inhibition was observed K_{is} , determined from slope replots, was equal to K_i . However, in the case where NADH showed mixed inhibition (i.e. with acetaldehyde as the varied substrate and a non-saturating concentration of NAD⁺) values for K_i and K_i' were calculated from the equations given by Dixon & Webb (1979, pp. 354–355) to be 35.4 and 10.8 μ M respectively.

Inhibitor	Variable substrate	Fixed substrate	K_{is} (μ M)	K_{ii} (μ M)
NADH	NAD ⁺	Acetaldehyde (non-saturating)	6.08 ± 0.83	∞
NADH	NAD ⁺	Acetaldehyde (saturating)	2.57 ± 0.57	∞
NADH	Acetaldehyde	NAD ⁺ (non-saturating)	26.3 ± 5.7	74.7 ± 32.7
NADH	Acetaldehyde	NAD ⁺ (saturating)	∞	∞
Acetaldehyde	NAD ⁺	—	90000 ± 3660	∞

Fig. 4. Inhibition of aldehyde dehydrogenase by high concentrations of acetaldehyde

The concentration of NAD⁺ was varied at the following fixed concentrations of acetaldehyde; 60 mM (●); 120 mM (○); 180 mM (■); 240 mM (□).

DISCUSSION

The linear intersecting patterns of the primary initial-velocity plots (see Fig. 1) suggests that a sequential mechanism is followed. A steady-state random mechanism would give rise to curved initial-rate plots although, in practice, this non-linearity might be difficult to discern (Pettersson, 1970). A double-displacement (Ping-Pong) mechanism would give parallel lines in double-reciprocal plots.

The failure of the acid product to inhibit the enzyme means that product inhibition studies are inadequate to define the mechanism. Although the inhibition patterns observed with NADH are inconsistent with an ordered mechanism where acetaldehyde is the first substrate to bind to the enzyme, they do

not rule out such a mechanism where NAD⁺ is the first substrate to bind or a Theorell–Chance mechanism in which either substrate is first to bind. These results also tend to rule out a random mechanism under equilibrium conditions in which NADH could bind only to the free enzyme, although a more likely variant of this mechanism where an abortive enzyme–NADH–aldehyde complex can be formed would give similar patterns to those obtained.

A compulsory-order mechanism would predict that the inhibition constant for NADH as a competitive inhibitor with respect to NAD⁺ would be independent of the concentration of acetaldehyde, whereas in a random mechanism this value might be expected to be different if NADH were to bind to the free enzyme or the enzyme–NAD⁺ complex. However, as shown in Fig. 1 and Table 1, the apparent K_m value for either substrate is little affected by the concentration of the other. Furthermore, the differences in the K_i values shown in Table 2 are too small to allow any useful conclusions to be drawn. In the only previous study of the enzyme from a human source, Vallari & Pietruszko (1981) found that the apparent K_i value for competitive inhibition by NADH remained constant when the acetaldehyde concentration was varied within the low concentration range (up to 290 μ M) but increased at higher acetaldehyde concentrations (1.6 and 9 mM). They interpreted this behaviour as being the result of a ‘degree of randomness’ becoming apparent in the mechanism at high (mM range) concentrations of the latter substrate. However, the data obtained in that work cannot be interpreted in such a simple way since the enzyme exhibited complex kinetics with respect to acetaldehyde under the experimental conditions used. Vallari & Pietruszko (1981) interpreted the behaviour in terms of biphasic double-reciprocal plots exhibiting two K_m values for acetaldehyde. In the remainder of their kinetic studies the acetaldehyde concentrations were restricted to the low concentration range (up to 300 μ M) where they detected no apparent deviation from linearity in the double-reciprocal plots. As mentioned earlier, such apparent linearity is misleading (see Henehan & Tipton, 1991). Furthermore, in the studies of NADH inhibition the extension of the acetaldehyde concentration range to the millimolar range where the non-linearity of the double-reciprocal plots became pronounced would be complicated by the operation of the ‘substrate activation pathway’ which Hart & Dickinson (1982) have shown to result from acetaldehyde binding to the E–NADH complex.

The high-substrate inhibition observed with acetaldehyde was found to be competitive with respect to NAD⁺. If acetaldehyde were the second substrate to bind in an ordered mechanism, competitive high-substrate inhibition would be expected if it were to form an abortive binary complex with the free enzyme, thereby preventing the binding of NAD⁺ (Dalziel, 1957). For such inhibition to be competitive, acetaldehyde must bind to the same enzyme form as NAD⁺ according to Scheme 1, where A and B are NAD⁺ and acetaldehyde respectively and P and Q are NADH and acetate respectively. This mechanism is expected to give rise to an initial-rate equation of the form (see Dixon & Webb, 1979, pp. 133–137):

$$v = \frac{V_{\max}}{1 + \frac{K_m^A}{[A]} \left(1 + \frac{[B]}{K_i}\right) + \frac{K_m^B}{[B]} + \frac{K_s^A K_m^B}{[A][B]} \left(1 + \frac{[B]}{K_i}\right)} \quad (1)$$

Scheme 1.

Fig. 5. Alternative substrate effects on the initial velocity of NADH production

Acetaldehyde and glycolaldehyde were used as the alternative substrates. Activities are plotted against the concentration of acetaldehyde in double-reciprocal form and at the following values of β : 0.0 ($\diamond$); 0.25 ($\bullet$); 1 ($\circ$); 5 ($\blacksquare$); and 10 ($\square$), where $\beta = [\text{glycolaldehyde}]/[\text{acetaldehyde}]$. The NAD^+ concentration was $25 \mu\text{M}$ $5000 \mu\text{M}$ (a) or (b).

where K_m^A and K_m^B are the Michaelis constants for NAD^+ and acetaldehyde respectively, K_i is the inhibitor constant for high-substrate inhibition, $V_{\max}$ is the maximum velocity, K_s^A is the limiting value of K_m^A as the concentration of acetaldehyde tends to zero and the square brackets denote concentrations. Such a mechanism would show competitive substrate inhibition by acetaldehyde (B) with respect to NAD^+ (A). Such high-substrate inhibition in a random-order mechanism would occur only in the unlikely case where two molecules of acetaldehyde were able to combine with the free enzyme, thereby preventing the binding of NAD^+ . Competitive high-substrate inhibition might be expected to occur in a double-displacement mechanism. However, this would not be consistent with the initial-rate data. Thus these results would favour an ordered mechanism, either involving a ternary complex or of the Theorell–Chance type with NAD^+ being the first substrate to bind. Neither mechanism would predict behaviour of this type if acetaldehyde were the first substrate to bind to the enzyme (Dalziel, 1957; Cleland, 1970).

The mechanism of high-substrate activation proposed by Hart & Dickinson (1982) would entail the interaction of acetaldehyde with the free enzyme (see also Henahan & Tipton, 1991) and, indeed, Dickinson (1985) observed high-substrate inhibition of the sheep liver cytosolic enzyme at a lower assay temperature, but only at low, non-saturating, concentrations of NAD^+ ($33 \mu\text{M}$). Hill & Dickinson (1988) have proposed that this complex may react with NAD^+ to form the productive ternary complex. However, the observation that the high-substrate inhibition was not partial would exclude such a mechanism for the human erythrocyte enzyme.

The alternative substrate method in which acetaldehyde and glycolaldehyde were held at a series of fixed ratios and the reciprocal of initial velocity of NADH formation was plotted as a function of the reciprocal acetaldehyde concentration was thus used in an attempt to distinguish between the two ordered mechanisms. The data shown in Fig. 5 gave linear patterns of intersecting lines. This intersecting ‘mixed’ pattern would be consistent with an ordered mechanism involving the formation of a ternary complex but not with a Theorell–Chance mechanism which would give a ‘competitive pattern’ (see Huang, 1979). Although these results would be consistent with either of these mechanisms if acetaldehyde were the first substrate to bind or with a random-order mechanism, such mechanisms are not consistent with the kinetic data discussed above. Thus the data presented above indicate that aldehyde dehydrogenase from human erythrocytes obeys a kinetic mechanism of the form shown in Scheme 2, where the step in which acetate is released from the ternary complex is shown to be irreversible since that product does not behave as an inhibitor of the enzyme. The step in which water binds to the enzyme is not shown. Although it is known that water is used in the hydrolysis of the acetyl-enzyme intermediate formed during the reaction (see Loomes & Kitson, 1986), there is no evidence about the step at which it binds to the enzyme, or indeed whether there is a specific site that binds the water molecule used in this process.

The lack of product inhibition by acetate necessitated the use of more elaborate methods to define the kinetic mechanism

Scheme 2.

involved than are often required with enzymes involving two substrates. Although more complicated kinetic mechanisms could be devised which would be consistent with the data obtained, and it is not possible definitely to exclude a random mechanism if the, unlikely, formation of an enzyme-(acetaldehyde)₂ ternary complex is postulated to account for the high-substrate inhibition, the compulsory mechanism in Scheme 2 is the simplest and there would be no justification for considering more complicated mechanisms unless further data were obtained that necessitated such formulations. This mechanism would give an initial-rate equation of the form.

$$v = \frac{V_{\max.}}{1 + \frac{K_m^{\text{NAD}}}{[\text{NAD}]} + \frac{K_m^{\text{Acetaldehyde}}}{[\text{Acetaldehyde}]} + \frac{K_s^{\text{NAD}} K_m^{\text{Acetaldehyde}}}{[\text{NAD}][\text{Acetaldehyde}]}} \quad (2)$$

The mechanism proposed is consistent with direct observation showing NAD⁺ to bind to the free cytoplasmic enzyme from sheep liver (Dickinson, 1985) and also with the results of some of the earlier steady-state kinetics studies with cytoplasmic aldehyde dehydrogenase preparations from other species (Duncan & Tipton, 1971, MacGibbon *et al.*, 1977; Hart & Dickinson, 1982). The interpretation of the data in those cases was, however, rendered dubious because they were based on the assumption that double-reciprocal plots that described smooth curves could be analysed in terms of Michaelis-Menten behaviour. The observation that the initial-rate behaviour of the enzyme followed Michaelis-Menten kinetics at the more physiological assay temperature of 37 °C has allowed the kinetic mechanism to be defined under conditions where possible ambiguities due to non-linear kinetics were excluded.

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