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THE EFFECTS OF ASSAY TEMPERATURE ON THE COMPLEX KINETICS OF ACETALDEHYDE OXIDATION BY ALDEHYDE DEHYDROGENASE FROM HUMAN ERYTHROCYTES

GARY T. M. HENEHAN and KEITH F. TIPTON*
Department of Biochemistry, Trinity College, Dublin 2, Ireland

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Abstract—Several studies have shown preparations of the cytosolic aldehyde dehydrogenase (EC 1.2.1.3) from sheep and human liver and from human erythrocytes to exhibit complex kinetic behaviour in which the dependence of the initial velocity on the concentration of acetaldehyde gives rise to downwardly curving double-reciprocal plots. This behaviour has often been analysed in terms of a sharp discontinuity in the double-reciprocal plots and its possible implications for the oxidation of acetaldehyde and other pharmacologically important aldehydes has been a subject of speculation. In the present work, it is shown that the purified, apparently homogeneous, enzyme from human erythrocytes exhibits such complex kinetic behaviour when initial rates are determined at 25°, although the double-reciprocal plots describe a smooth curve with no sharp discontinuity. However, when the assays were performed at 37° there was no significant deviation from Michaelis–Menten kinetics over a wide range of acetaldehyde concentrations (0.2–30 mM). At higher concentrations of acetaldehyde inhibition occurred which was competitive with respect to NAD^+. These results, which indicate that the complex kinetic behaviour of aldehyde dehydrogenase is not important at physiological temperature, are interpreted in terms of the mechanisms that have been advanced to explain the phenomena.

Pure preparations of cytosolic aldehyde dehydrogenase (EC 1.2.1.3) from sheep and human liver and from human erythrocytes have been shown to exhibit complex kinetics, when the effects of aldehyde concentration on initial velocity are studied. This gives rise to biphasic double-reciprocal plots with certain aliphatic aldehyde substrates [1–4]. At high concentrations of the varied aldehyde substrate an apparently sharp increase in the slope of the double-reciprocal plot has been reported. Often two K_m values for aldehyde substrates, obtained by extrapolation of the apparently linear portions of the biphasic plot to their intersection point on the reciprocal substrate concentration axis, are quoted. However, even if this behaviour were a result of the presence of two enzymes, or two independent active sites with different K_m values and maximum velocities, such a procedure would not given an accurate measure of the kinetic parameters of the two contributing components (see Ref. 5). Although some workers have reported the occurrence of this phenomenon, often called substrate activation, with the human erythrocyte enzyme [4, 6], others have not apparently observed it [7, 8] and it has been suggested that the biphasic kinetics may be related to the assay pH [3]. The human erythrocyte enzyme has also been shown to exhibit this apparent substrate activation with physiologically occurring aromatic aldehydes such as 3,4-dihydroxy-phenylacetaldehyde and 5-hydroxyindole-acetaldehyde [6].

In view of the potential importance of aldehyde dehydrogenase in the oxidation of ethanol-derived acetaldehyde as well as its role in the biotransformation of endogenously formed and ingested aldehydes, the physiological relevance of this substrate activation merits further study. The present work demonstrates that the assay temperature influences the kinetics of acetaldehyde oxidation by the enzyme from human erythrocytes and that substrate activation is not apparent at the physiological temperature of 37°. A preliminary account of part of this work has been published [9].

MATERIALS AND METHODS

Buffer salts, 2-mercaptoethanol, EDTA and acetaldehyde were obtained from BDH (Poole, U.K.). NAD^+ was from the Boehringer Corporation (Mannheim, F.R.G.). Acetaldehyde was distilled in an all-glass apparatus, diluted 1:10 (v/v) with distilled deionized water and stored frozen at −20° until use. Solutions of acetaldehyde were standardized enzymically [10] using commercially available yeast aldehyde dehydrogenase (Sigma Chemical Co., Poole, U.K.). Aldehyde dehydrogenase from human erythrocytes was purified to homogeneity as described essentially by Inoue *et al.* [4] except that the final gel-filtration step was found to be unnecessary and was omitted. In addition, all buffers subsequent to the DEAE-Sephadex chromatography step contained 10% glycerol to stabilize the enzyme. Such storage did not affect the kinetic behaviour significantly. 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°. The purified enzyme was found to be

* To whom correspondence should be addressed.

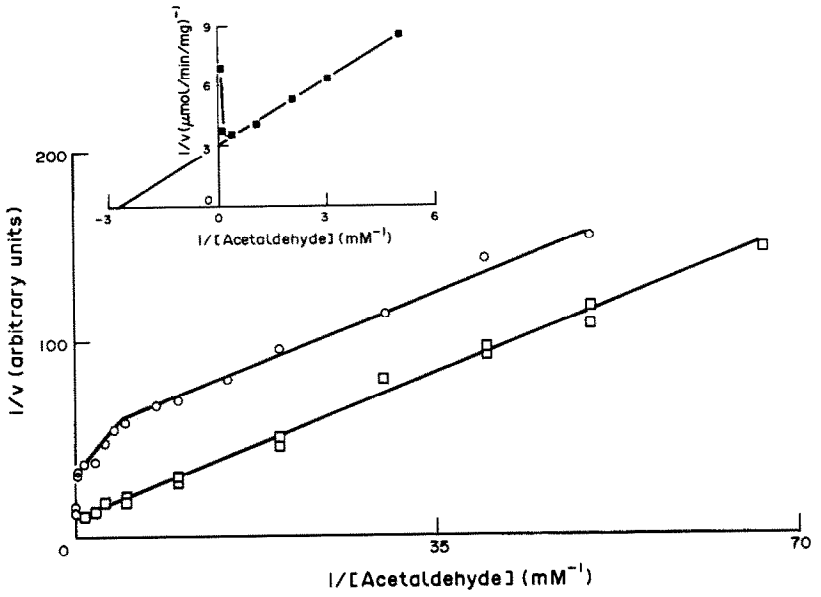

Fig. 1. Dependence of initial velocity of the reaction catalysed by human erythrocyte aldehyde dehydrogenase on the concentration of acetaldehyde. Assays were performed at 25° (○), with the acetaldehyde concentration varying within the range 0.02 to 120 mM, and at 37° (□), with the acetaldehyde concentration varying in the range 0.015 to 100 mM. Other details are given in the text. The inset shows a plot of the inhibition by high concentrations of acetaldehyde at 37°.

homogeneous by the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate followed by staining with Coomassie brilliant blue.

Before kinetic studies the enzyme was dialysed for 14 hr at 4° against 500 volumes, with two changes, of the above buffer mixture without glycerol. Enzyme assays were performed in 50 mM sodium phosphate buffer, pH 7.2, containing 0.1% 2-mercaptoethanol and 1 mM EDTA. The specific activities of preparations of the enzyme under these conditions, and at 37° in the presence of 3 mM acetaldehyde and 500 μM NAD^+, varied between 0.3 and 0.73 μmol/min/mg. Protein concentration was determined by the method of Bradford [11]. For kinetic studies, with varied acetaldehyde concentrations, the NAD^+ concentration was 500 μM and when NAD^+ was varied the concentration of acetaldehyde was 3 mM. Assays were started by the addition of acetaldehyde and the increase in absorbance at 340 nm was followed with time.

RESULTS AND DISCUSSION

The most commonly used assay temperature in published studies of aldehyde dehydrogenase is 25° (e.g. Refs 1–3 and 6), although some workers have used 22° [4] and in a few cases the physiological temperature of 37° has been used [8, 12].

Figure 1 shows double-reciprocal plots of the dependence of initial velocity on substrate concentration determined over a wide range of acetaldehyde concentrations using purified erythrocyte aldehyde dehydrogenase. The double-reciprocal plots were determined at 25° and at 37° in the presence of a saturating concentration of NAD^+ (500 μM). Because of the wide range of substrate concentrations used, the plots show features not recognized previously with this enzyme. At an assay temperature of 37°, the double-reciprocal plot was apparently linear over the concentration range 0.2 to 30 mM but at substrate concentrations greater than 30 mM, high-substrate inhibition was observed (Fig. 1, inset). There was no significant deviation from hyperbolic behaviour when the data in the non-inhibitory region were directly analysed by non-linear regression. At 25° the double-reciprocal plot showed a more complex profile (Fig. 1), which might be interpreted in terms of four separate regions: (i) an apparently linear portion between 0.02 and 0.125 mM acetaldehyde; (ii) a second apparently linear portion, of increased slope, between 0.2 and 2.8 mM acetaldehyde; (iii) at concentrations of acetaldehyde greater than 2.8 mM the double-reciprocal plot curves downward towards the x-axis; (iv) at acetaldehyde concentrations greater than 30 mM substrate inhibition was observed. However, closer inspection of the data in the region below 30 mM acetaldehyde revealed that this region formed a smooth curve.

Apparent kinetic constants determined at both temperatures are shown in Table 1. Two K_m and maximum velocity values were determined from the behaviour at 25° by fitting the initial rate data in the region where there was no apparent high-substrate inhibition to an equation describing the sum of two rectangular hyperbolas [13]. Theoretical curves constructed using the kinetic parameters determined in this way were in close agreement with the experimentally determined initial-rate curves (Fig.

Table 1. Kinetic parameters determined for human erythrocyte aldehyde dehydrogenase

Temperature (°C)	Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)
37	Acetaldehyde	348 ± 29	0.337 ± 0.009
37	NAD^+	11.2 ± 2.3	0.287 ± 0.03
25	Acetaldehyde	178 (181)	0.27 (0.32)
		8.3 (54)	0.052 (0.22)

The biphasic double-reciprocal plots obtained at 25° were analysed in terms of the sum of two rectangular hyberbolas by the procedure of Spears *et al.* [13] to give two K_m and maximum velocity values. The values shown in parenthesis are the values obtained by attempting to fit the two apparently linear portions of the curve, in the ranges 0.02 to 0.125 mM and 0.2 to 2.8 mM, to straight lines (as shown in Fig. 1) by linear regression. The values for the behaviour at 37° were determined from the data, where no high-substrate inhibition was evident by fitting to a rectangular hyperbola by nonlinear regression. The concentrations of the fixed substrates and other assay conditions are given in the text.

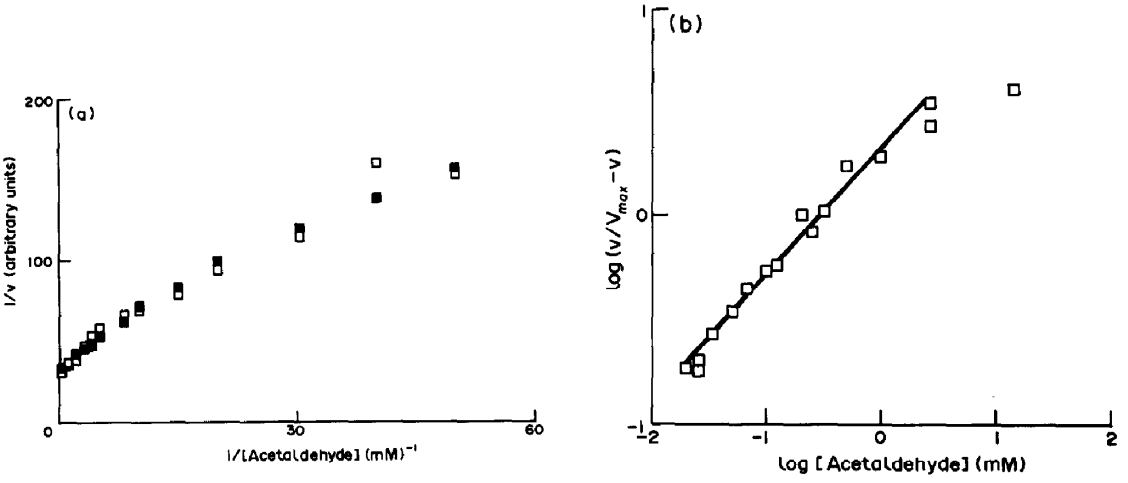

Fig. 2. Kinetic analysis of the behaviour of human erythrocyte aldehyde dehydrogenase at 25°. (a) The data from Fig. 1, in the region where high-substrate inhibition was not apparent, were analysed in terms of the presence of two independent activities towards acetaldehyde, with different K_m and maximum velocity values, by the procedures of Spears *et al.* [13]. The calculated kinetic parameters were used to determine the predicted variation of reciprocal velocity with the reciprocal of the acetaldehyde concentration (■), and this is compared with the experimentally observed values (□). (b) The same data expressed as a Hill plot.

2a). The lines drawn to the 25° curve in Fig. 1 correspond to the commonly used procedure of drawing tangents to the apparently linear portions of the curve. The kinetic parameters obtained in this way are also shown in Table 2 and illustrate the large discrepancy between the values obtained in this way and those determined by curve-fitting. The use of a wider range of acetaldehyde concentrations in the present study than used previously indicates that it is not justifiable to assume that the curve is composed of two linear regions with a relatively sharp discontinuity between them. Indeed, it is not easy to accommodate sharp discontinuities in initial-rate behaviour in terms of simple enzyme kinetic theory (see Ref. 14).

Although the analysis used here would be valid for systems containing two enzymes or independent active sites with different kinetic parameters towards acetaldehyde, the values determined would have no real meaning if the nonlinear double-reciprocal plot resulted from negative cooperativity or from complexities in the steady-state mechanism followed [15]. A Hill plot of the initial-rate data at 25° (Fig. 2b), indicates that they may be equally well interpreted in terms of negative cooperativity. A Hill constant of 0.48 ± 0.06 was determined from that plot.

These results indicate that substrate activation is not significant at physiological temperatures and pH values. However, it must be noted that one group did not observe substrate activation at 25° [7], suggesting that this phenomenon may be influenced also by other factors such as cofactor concentration. Kitson and Crow [16] have reported the activation

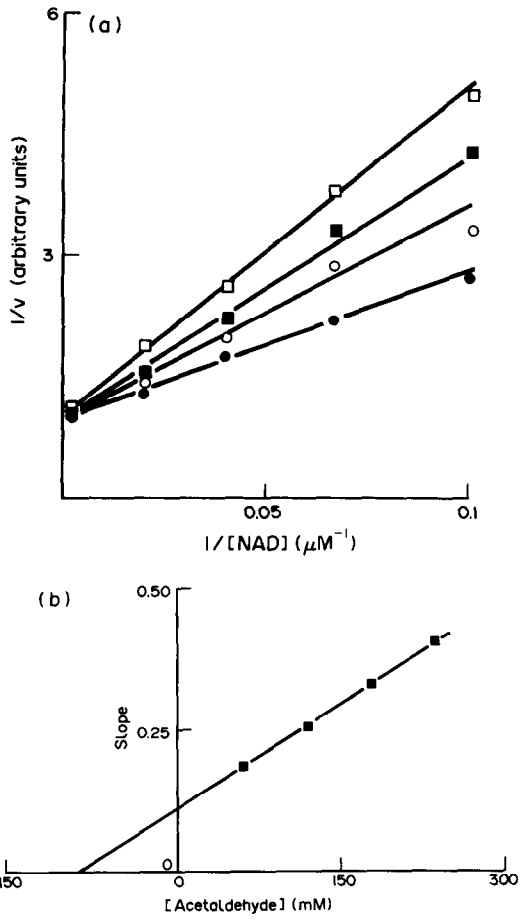

Fig. 3. Inhibition of human erythrocyte aldehyde dehydrogenase by high concentrations of acetaldehyde. Assays were performed at 37°. (a) Inhibition, presented as double-reciprocal plots with respect to NAD^+. The concentrations of acetaldehyde used were: 60 mM (●); 120 mM (○); 180 mM (■) and 240 mM (□). (b) Plots of the slopes of the lines shown in (a) against the concentration of acetaldehyde.

of sheep liver cytosolic aldehyde dehydrogenase by diethylstilboestrol to be temperature-dependent. Since this activator modifies a thiol group on the enzyme, it may be that this group is also involved in the processes that give rise to the temperature-dependent change in the kinetics of aldehyde oxidation.

Dickinson [17] reported high-substrate inhibition of the sheep liver cytosolic enzyme, but only at low, non-saturating concentrations of NAD^+ (33 μM). At 37° the inhibition by high concentrations of acetaldehyde observed in the present work was found to be competitive with respect to NAD^+ (Fig. 3a). The replot of the slopes against the acetaldehyde concentration was linear and a K_i value of 90 mM was estimated (Fig. 3b).

Aldehyde dehydrogenase preparations from several sources have been found to follow an ordered reaction pathway, in which the binding of NAD^+ to the enzyme is followed by aldehyde binding, leading to the formation of a ternary complex (see Ref. 18). If such a pathway were followed by the human erythrocyte enzyme at 37°, the pattern of high-substrate inhibition by acetaldehyde would be consistent with the formation of an abortive enzyme–aldehyde binary complex. If acetaldehyde can bind to the free enzyme at both 25° and 37°, this may explain the observation that high acetaldehyde concentrations (10 mM) protect the enzyme against inhibition by disulfiram, whereas lower concentrations (1 mM) do not [17, 19].

Hart and Dickinson [2] have suggested that the activation and inhibition characteristics observed with the sheep liver aldehyde dehydrogenase are similar to those seen with horse liver alcohol dehydrogenase in the oxidation of cyclohexanol. These workers have explained the substrate activation seen at 25° with the sheep liver enzyme by proposing that an enzyme–NADH–aldehyde complex can form at high concentrations of aldehyde, and that dissociation of the reduced cofactor from this complex is more rapid than from the enzyme–NADH binary complex (see Fig. 4). Direct experimental evidence for the existence of an enzyme–NADH–aldehyde complex has been provided [17].

According to this model, at low NAD^+ concentrations the enzyme would be present largely as free enzyme and enzyme–aldehyde complex; the latter being formed either as a result of the breakdown of the enzyme–NADH–aldehyde complex or by direct binding of aldehyde to the free enzyme. In a recent extension of this model, Hill and Dickinson [20] have proposed that the enzyme–aldehyde complex may react with NAD^+ to form the productive ternary complex. With such a mechanism inhibition by high concentrations of acetaldehyde might result if NAD^+ bound to this complex more slowly than to the free enzyme. Although such a mechanism might be consistent with the behaviour of the sheep liver enzyme at 25°, the ability of the enzyme–aldehyde binary complex to bind NAD^+ would result in only partial high-substrate inhibition by acetaldehyde, which is not apparently the case in the present studies at 37°. Thus, the results presented here would be consistent with the complex between enzyme and acetaldehyde being, effectively, a dead-end. The effects of increased temperature on the kinetics of aldehyde dehydrogenase are most simply explained by proposing that at 37° a step in the reaction prior to release of reduced coenzyme becomes rate-limiting. Blackwell *et al.* [21] have shown that a slow, rate-determining, conformational change occurs in the enzyme–NADH complex before coenzyme release, and, thus, the increase in temperature might increase the rate of this process.

An alternative explanation for substrate activation has been proposed by Blackwell *et al.* [22] who postulated that aldehyde dehydrogenase from sheep liver cytosol has two binding sites for acetaldehyde: a high-affinity site that is occupied at low aldehyde concentrations and a low-affinity site that only becomes occupied at higher concentrations of aldehyde. The effect of temperature might be explained in terms of this model if increased temperature caused conformational changes in the

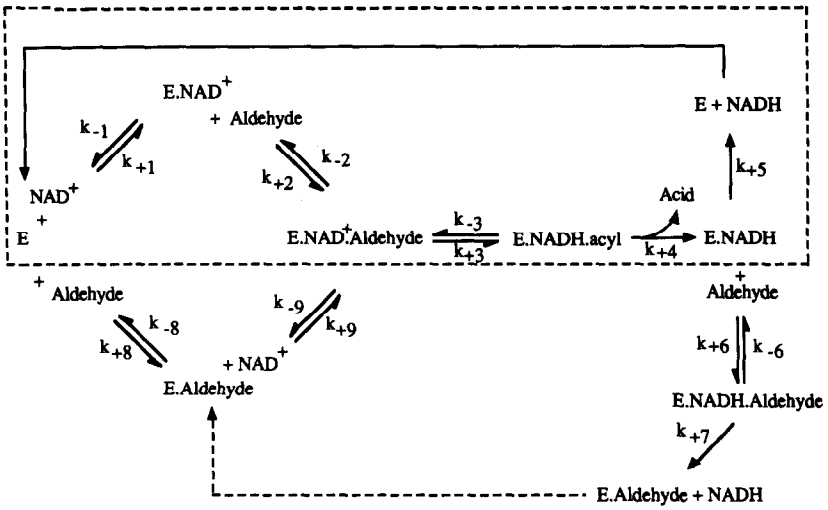

Fig. 4. The mechanism proposed by Dickinson [17] to account for the apparent activation of aldehyde dehydrogenase by high concentrations of acetaldehyde. The sequence in the boxed section is envisaged as being important only at high concentrations of acetaldehyde. The additions to this reaction pathway made by Hill and Dickinson [20] are shown by broken arrows.

enzyme to a form where all the active sites were equivalent, or where one of the two sites was no longer accessible. The necessity for proposing two sites on the enzyme has, however, been questioned by Duncan [23].

Brooks *et al.* [24] have suggested that the degree of hydration of the aldehyde substrate may provide an explanation for the biphasic kinetics. However, these workers do not explain how the degree of hydration might give rise to such kinetic behaviour. It is difficult to envisage how the presence of a substrate as a mixture of hydrated and unhydrated forms would give rise to biphasic Lineweaver–Burk plots from initial-rate measurements. If both forms of the aldehyde could be oxidized by the enzyme, or even if one form acted as a competitive inhibitor of the other, linear double reciprocal plots would still result (see Ref. 25).

These results may have some bearing on discrepancies that appear in the literature on the subcellular distribution of aldehyde dehydrogenase activities in human liver. The two major forms have often been referred to as the low and high K_m forms, the former being associated with the mitochondrion and the latter being largely cytosolic (see Ref. 9). These are estimated often by determining the enzyme activity at two arbitrarily fixed acetaldehyde concentrations, such as 15 μM and 3 mM. The first is chosen to determine the activity of the low-K_m form with minimal contribution from the other form and the latter is assumed to give the activities of both enzymes (see Ref. 9). The identity of the human erythrocyte and hepatic cytosol enzymes has been established [7]. Clearly the complex kinetic behaviour seen at 25° could be mistaken for a low-K_m enzyme, whereas this behaviour is absent at 37°. Thus, subcellular distribution studies might give different results depending on the temperature used to assay the fractions.

Because of the complex kinetics displayed at 25°, steady-state kinetic data with acetaldehyde have been difficult to interpret. However, the present work indicates that this should not be a problem at the physiological temperature of 37°. This effect of temperature indicates that great caution must be exercised when extrapolating *in vitro* experimental findings, obtained using an assay temperature of 25°, to the *in vivo* situation.

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