Drosophila melanogaster alcohol dehydrogenase: product-inhibition studies

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The *Drosophila melanogaster* alleloenzymes Adh^S and Adh^F have been studied with respect to product inhibition by using the two substrate couples propan-2-ol/acetone and ethanol/acetaldehyde together with the coenzyme couple NAD^+/NADH. With both substrate couples the reaction was consistent with an ordered Bi Bi mechanism. The substrates added to the enzyme in a compulsory order, with coenzyme as the leading substrate, to give two interconverting ternary complexes. The second ternary complex broke down with release of products in an obligatory order, with the aldehyde/ketone leaving first. Both the acetaldehyde and acetone products formed binary complexes with the enzyme that affected NAD^+ binding. However, only an enzyme–acetone complex seemed to affect NADH binding and hence the reverse reaction. The inhibitory pattern with acetaldehyde as product was also affected by the formation of a ternary enzyme–NAD^+–acetaldehyde complex, which broke down to acetic acid and NADH. The product-inhibition pattern shown in the present work is different from that published for *Drosophila* Adh previously and this discrepancy can not be explained by the use of different variants of *Drosophila* Adh.

INTRODUCTION

The enzyme alcohol dehydrogenase (Adh) has been isolated and characterized from many different fruitfly (*Drosophila*) species (for review, see [1–3]) and catalyses the following reactions:

$$NAD^+ + \text{alcohol} \leftrightarrow \text{aldehyde/ketone} + NADH + H^+ \quad (1)$$

$$NAD^+ + \text{acetaldehyde} \rightarrow \text{acetic acid} + NADH + H^+ \quad (2)$$

In addition to oxidizing primary and secondary alcohols to the respective carbonyl products (eqn. 1), the enzyme is also able to further oxidize the formed aldehyde to its corresponding acid (eqn. 2) [4,5]. The Adh has been assumed to take part in the detoxification of alcohols/aldehydes and ketones, as well as in fatty acid and pheromone metabolism [1]. The Adh from fruitflies differ from the well-characterized Adhs from horse liver (vertebrate) and yeast in many respects. The former belong to the 'short-chain' dehydrogenase family [6,7], with a subunit M_r of approx. 27400 [1–3] and they lack a metal ion in their active site and are not metalloenzymes [1–3]. In contrast with yeast and liver Adh, all *Drosophila* Adhs studies so far show a preference for secondary alcohols compared with primary alcohols [1–3]. They also show an opposite stereospecificity with respect to hydride transfer from coenzyme as well as from primary alcohols, i.e., the pro(S) hydrogen atom is transferred in both cases [8,9] and (1S)-[$^3H_1$]ethanol is produced in the reduction of acetaldehyde by [4-(S)-$^3H_1$]NADH [10]. Site-directed mutagenesis showed that tyrosine-152 and lysine-156, in contrast with the two cysteines, were essential for activity [11–13]. Binding of alcohol and alcohol competitive inhibitors to *Drosophila* Adh was dependent on a residue in the active site, which showed a pK value of about 7.6 in the binary enzyme–NAD^+ (EO) complex [14]. This indicated that tyrosine-152 could be the residue that interacts with the hydroxy group in the alcohol and hence have the same function as Zn^{2+} in horse liver Adh [15].

Studies with alternate substrates and dead-end inhibitors indicated that the oxidation of primary as well as secondary alcohols follows a compulsory ordered pathway, with coenzymes as the leading substrate (Scheme 1) [3]. The principal difference in the oxidation of primary and secondary alcohols resides in different rate-limiting steps. For most secondary alcohols, dissociation of NADH from the binary ER product complex was rate-limiting, while for ethanol and primary alcohols the velocity was limited by a slow hydride-transfer step, i.e., interconversion of the ternary complexes [3]. In contrast with the observations supporting a compulsory ordered pathway are product-inhibition studies of *Drosophila* Adh, which indicated a rapid-equilibrium random ordered mechanism (Scheme 2) without ternary dead-end complexes [16,17]. Similar discrepancies in the interpretation of the reaction mechanism has been seen in studies with sorbitol dehydrogenase, where product-inhibition studies indicated a rapid equilibrium random ordered mechanism, while alternative substrate studies and dead-end inhibitors showed a compulsory ordered pathway ([18] and references cited therein).

In the present study we have investigated the product-inhibition patterns for the oxidation of both ethanol and propan-2-ol with the *Drosophila melanogaster* alleloenzymes Adh^S and Adh^F. Our aim was to verify whether there was a discrepancy between the previously established mechanism from alternate-substrate and dead-end-inhibitor studies and that from product-inhibition patterns. Here we show that there is no inconsistency between the different methods, and that both alleloenzymes follow the same mechanism. Hence a single or a few amino acid substitutions in the enzyme are not enough to change the enzymic reaction mechanism.

EXPERIMENTAL

Reagents

Grade III NAD^+ and NADH were from Sigma. Ethanol (96%) was from A/S Vinmonopolet and propan-2-ol (99.7%; pro

Abbreviations used: Adh, alcohol dehydrogenase; Adh^S, slow alcohol dehydrogenase (EC 1.1.1.1) from *Drosophila melanogaster*; E, enzyme; O, NAD^+; R, NADH; S, alcohol; P, acetaldehyde/acetone; EO, enzyme–NAD^+ complex; ER, enzyme–NADH complex; EP, enzyme–carbonyl complex; EOS, enzyme–NAD^+–alcohol complex; ERP, enzyme–NADH–carbonyl complex; EOP, enzyme–NAD^+–carbonyl complex.

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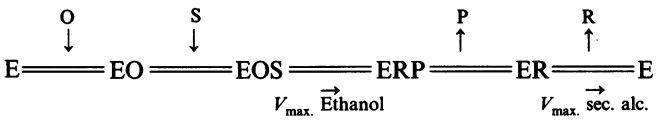


Scheme 1 Compulsory ordered ternary complex mechanism describing the kinetics of *Drosophila* Adh catalysis

This mechanism was deduced from alternate-substrate and dead-end-inhibitor studies (3,14,24–27] and covered both primary and secondary alcohols (sec alc.). It was only the rate-determining step ($V_{max.}$ or $1/\phi_0$) that distinguished the oxidation of ethanol and secondary alcohols. This reaction mechanism can be described by eqn. (3), where e is the concentration of enzyme active sites, O is NAD^+, S is the alcohol and the ϕ symbols are those described by Dalziel [19]. The presence of the product R (NADH) gives eqn. (4), and the product P (acetone or acetaldehyde) gives eqn. (5) [20,21]. The relation between the Dalziel parameters (ϕ) and the Cleland notations (K and V) is shown in Table 1.

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[S]} + \frac{\phi_{12}}{[O][S]} \quad (3)$$

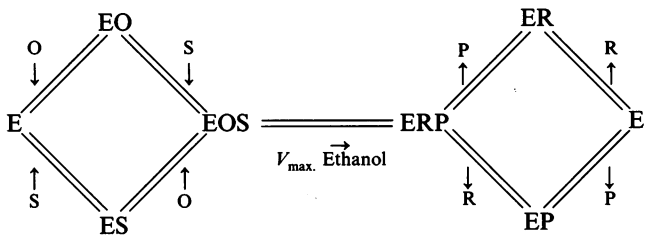
$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[O]}\left(1 + \frac{[R]}{\phi'_{12}/\phi'_2}\right) + \frac{\phi_2}{[S]} + \frac{\phi_{12}}{[O][S]}\left(1 + \frac{[R]}{\phi'_{12}/\phi'_2}\right) \quad (4)$$

$$\frac{e}{v_0} = \phi_0\left(1 + \frac{[P]}{K_{ip}}\right) + \frac{\phi_1}{[O]} + \frac{1}{[S]}\left(\phi_2 + \frac{\phi_{12}}{[O]}\right)\left(1 + \frac{[P]}{\phi'_{12}/\phi'_1}\right) \quad (5)$$

Table 1 Relation between Dalziel parameters (ϕ) and Cleland parameters (K and V) [21]

The rate constants (k_i) describing K_{ip} are those in Scheme 4.

$\phi_0 = 1/V_f \quad \phi_1 = K_O/V_f \quad \phi_2 = K_S/V_f \quad \phi_{12} = K_{iO}K_S/V_f$
$\phi'_0 = 1/V_f \quad \phi'_1 = K_R/V_f \quad \phi'_2 = K_P/V_f \quad \phi'_{12} = K_{iR}K_P/V_f$
$K_{ip} = (k_7 + k_9)/k_8 \quad \phi'_{12}/\phi'_1 = K_{iR}K_P/K_R$



Scheme 2 Rapid-equilibrium random-ordered reaction mechanism determined with *Drosophila melanogaster* Adh^{F71K}, Adh^F and *D. simulans* Adh for the oxidation of ethanol, based on the product-inhibition pattern [16,17]

The mechanism can also be described by eqn. (3) (Scheme 1), and the products, R and P, will only affect the ϕ_{12} term by $1 + \{[R]/(\phi'_{12}/\phi'_2)\}$ and $1 + \{[P]/(\phi'_{12}/\phi'_1)\}$ respectively [21]. Heinstra et al. [17] found that the slopes (varied NAD^+ or ethanol) gave parabolic plots in the presence of acetaldehyde (P). To explain this requires the additional formation of a dead-end EP complex.

analysis grade) was from Merck. Acetone (pro analysis grade) was from May & Baker and anhydrous acetaldehyde (puriss p.a. grade) was from Fluka.

Enzyme

Drosophila melanogaster Adh^S and Adh^F were purified as described previously [22]. Freeze-dried samples of the two enzymes were dissolved in 0.1 M phosphate buffer of pH 7.0 and 7.5 respectively, and dialysed against two changes of the same buffer at 4 °C. Denaturated protein was removed by centrifugation for 20 min at 25000 *g*.

Rate assay

To determine the amount of enzyme, i.e. the enzyme active-site concentration in the assay cuvette, the previously described rate assay for *Drosophila* Adh was used [23]. The enzyme concentration is expressed as the amount of subunits in nM. This is twice the amount of enzyme molecules, as the enzyme is a dimer. The assay solution consisted of 0.5 mM NAD^+ and 100 mM ethanol in a total volume of 1 ml 0.1 M glycine/NaOH buffer pH 9.5.

Kinetic measurements

Initial-rate measurements at 340 nm (absorption by the NADH produced) were carried out spectrophotometrically in either 0.1 M glycine/NaOH or 50 mM pyrophosphate buffer, pH 9.5, at 23.5 °C. A Perkin–Elmer Lambda 15 spectrophotometer coupled to a computer was used along with the PECSS program (Perkin–Elmer). Pyrophosphate buffer was used in experiments with acetaldehyde and acetone, as glycine may form Schiff bases with these two compounds and hence interfere with the kinetic measurements. The reason for choosing pH 9.5 is the favourable equilibrium [14], which permits a reliable determination of the initial velocity in the presence of product. All data that fitted eqn. (3) in Scheme 1 were analysed with linear regression, using the computer program Statgraphics (Statistical Graphics Corp.).

RESULTS

NADH as a product inhibitor (Figure 1)

NAD^+ varied

With the Adh^S alleloenzyme, NADH gave linear competitive inhibition against varied NAD^+ and a constant concentration of either ethanol (500 mM and 5.7 mM) or propan-2-ol (5 mM) (Figures 1a–1c). It is noteworthy that identical K_i values were obtained independently of the alcohol or the fixed concentration of alcohol as shown in Table 2.

With the Adh^F alleloenzyme, NADH was again a competitive

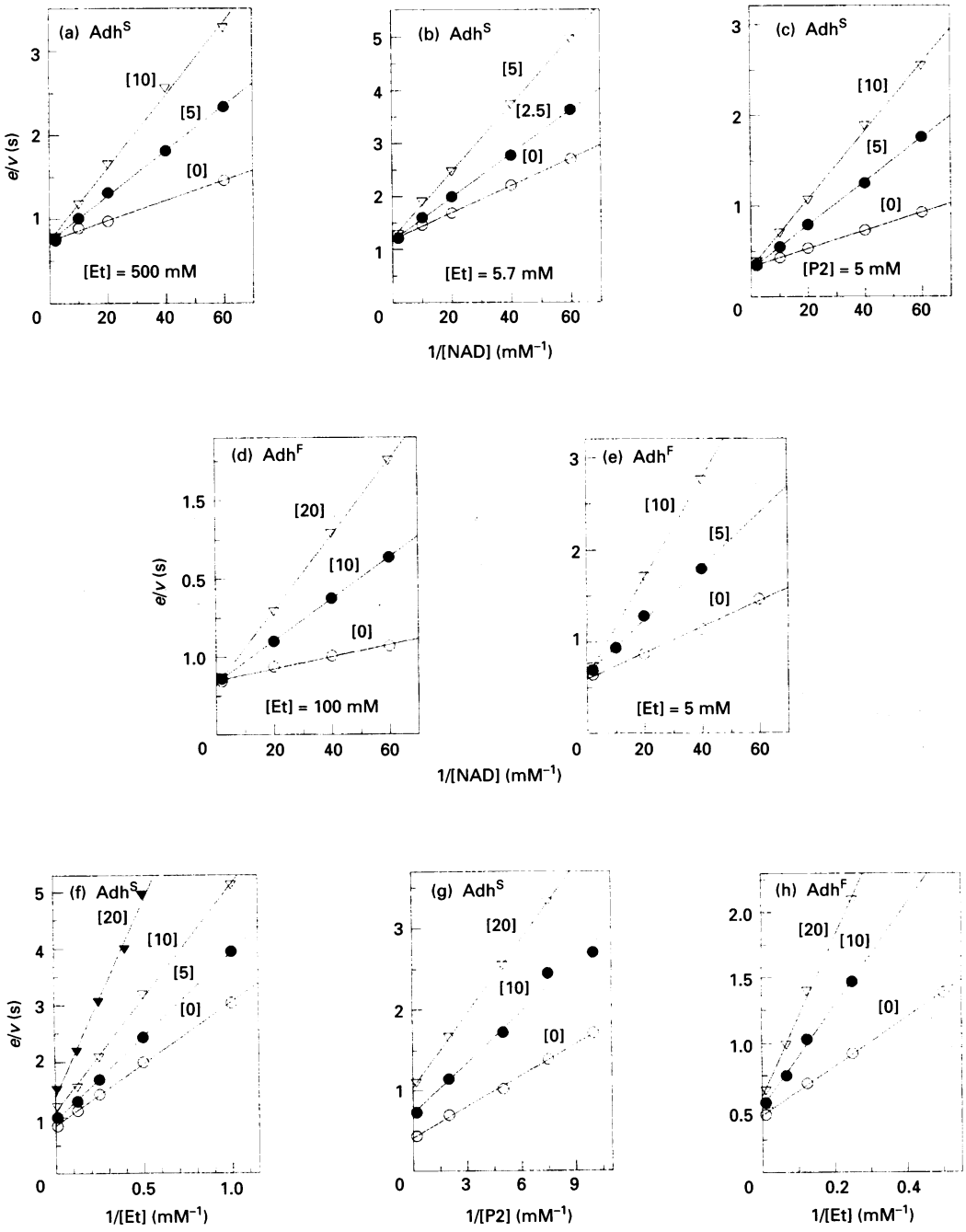


Figure 1 NADH as a product inhibitor of the Adh^S (a–c, f and g) and the Adh^F (d, e and h) alleloenzymes

(**a–e**) Varied NAD$^+$ and a constant concentration of either ethanol (Et) or propan-2-ol (P2). (**f–h**) Varied alcohol at a constant concentration of 100 μM NAD$^+$. The NADH concentrations in μM are shown in square brackets.

inhibitor against varied NAD$^+$ and a constant ethanol concentration (Figures 1d and 1e). Using a constant ethanol concentration of either 5 mM or 100 mM resulted in virtually identical K_i values of 4.2 μM and 4.1 μM respectively.

Alcohol varied

With Adh^S, NADH showed a linear non-competitive inhibition against either varied ethanol (Figure 1f) or propan-2-ol (Figure 1g) at a constant concentration of 100 μM NAD$^+$. Replots of slopes and intercepts versus inhibitor concentration were linear in both cases.

With Adh^F, NADH was again a non-competitive inhibitor against varied ethanol, at a constant concentration of 100 μM NAD$^+$ (Figure 1h) and gave linear replots.

Acetaldehyde as a product inhibitor (Figure 2)

NAD$^+$ varied

With varied coenzyme and a constant concentration of 5.7 mM

Table 2 Kinetic coefficients obtained from product inhibition with the Adh^s alleloenzyme

Sources: [a]From slopes with varied NAD^+ and 5 mM propan-2-ol (Figure 1c); [b]from slopes and intercepts with varied alcohol and 0.1 mM NAD^+ (Figures 1f and g); [c]from intercepts with varied alcohol and 0.5 mM NAD^+ (Figures 2h and 3h); [d]from slopes with varied propan-2-ol and 0.5 mM NAD^+ (Figure 3i); [e1]from slopes with varied NAD^+ and 5 mM propan-2-ol (Figure 3f); [e2]from slopes with varied NAD^+ and 0.5 mM propan-2-ol (Figure 3c); [f]from slopes with varied NAD^+ and 500 mM ethanol (Figure 1a); [g]from slopes with varied NAD^+ and 5.7 mM ethanol (Figure 1b); [h]from intercepts with varied NAD^+ and 500 mM ethanol (Figure 2e); [i]from the linear part of the slopes with varied ethanol and 0.5 mM NAD^+ in Figure 2g (re-plot not shown); [j]based on previously determined ϕ's for the acetaldehyde/NADH reaction [14].

Kinetic coefficient	Value
Acetone/NADH	
$\phi'_{12}/\phi'_2 = K_{ER}$ (μM)	3.65[a] (1.90–2.64)[b]
K_{ip} (mM)	15.7[c]
ϕ'_{12}/ϕ'_1 (mM)	33.5[d]
K_{ip} (mM)	23[e1] 16[e2]
Acetaldehyde/NADH	
$\phi'_{12}/\phi'_2 = K_{ER}$ (μM)	3.84[f], 3.71[g] (1.35–2.06)[b]
K_{ip} (mM)	9.4[c], 9.6[h]
ϕ'_{12}/ϕ'_1 (mM)	< 2.55[i] (1.55)[j]

ethanol (close to K_m), acetaldehyde gave a linear non-competitive pattern, using Adh^s (Figure 2a). Both linear slopes and intercept effects appeared (Figures 2b and 2c). By increasing the constant ethanol to a concentration of 500 mM, i.e. approx. $150 \times K_m$ [14], a large reduction in the slope effects appeared, with nearly parallel lines (Figure 2d). Replots of both the intercepts and the slopes against the concentration of inhibitor are linear (Figures 2e and 2f), and the pattern is still linear non-competitive.

Ethanol varied

Figure 2(g) shows that acetaldehyde is a non-competitive inhibitor with respect to ethanol, using the Adh^s alleloenzyme. At the lowest concentrations of acetaldehyde (0.5 and 1.0 mM), a linear Lineweaver–Burk plot appears. However, at higher acetaldehyde concentrations double-reciprocal plots deviate from linearity and hyperbolic plots arise. The higher the acetaldehyde concentration the earlier is the deviation from linearity. The plot is linear between 4 and 500 mM of ethanol and with up to 3 mM acetaldehyde. However, this only occurs with between 8 and 500 mM of ethanol and with 4 mM of product inhibitor. Replots of these linear parts of Figure 2(g) result in linear slopes (not shown) and intercepts (Figure 2h) versus acetaldehyde concentrations.

Acetone as a product inhibitor (Figure 3)

NAD^+ varied

At a constant propan-2-ol concentration of either 0.5 mM (approx. K_m) or 5 mM ($10 \times K_m$), acetone showed non-competition inhibition against varied NAD^+, using Adh^s (Figures 3a and 3d). In both cases re-plots of intercepts and slopes versus acetone concentration were linear (Figures 3b and 3c and Figures 3e and 3f).

Propan-2-ol varied

At a constant concentration of 500 μM of NAD^+, acetone gave non-competitive inhibition against varied propan-2-ol with linear replots of slopes and intercepts versus acetone concentrations, using Adh^s (Figures 3g–3i).

DISCUSSION

Interconversion between propan-2-ol and acetone

Previous studies with different secondary alcohols indicated that a compulsory ordered pathway was followed, with NAD^+ as the leading substrate [3] (Scheme 1). The present study shows that, in the presence of either acetone or NADH, the oxidation of propan-2-ol with NAD^+ gave rise to one competitive and three non-competitive product-inhibitory patterns. It was only the two coenzymes that competed, confirming that NAD^+ binds prior to the alcohol and that NADH also binds to the free enzyme (E). As a Theorell–Chance or a rapid-equilibrium random-order mechanism with or without dead-end complexes can give rise to at most two non-competitive patterns, these can be excluded as possible mechanisms [21]. Mechanisms that are consistent with the observed pattern are the simple compulsory ordered pathway in Scheme 1 and a pathway where the 'on' phase of the reaction is compulsory ordered (E–EO–EOS), but the 'off' phase, i.e. the product-leaving phase, is random-ordered combined with the formation of a dead-end EOP complex. As NADH binds to the free enzyme (E) in both mechanisms, both will give rise to rate eqn. (4) in Scheme 1. However, the two possible mechanisms can be distinguished by the effect acetone exerts on the kinetic coefficients. In the second mechanism, where acetone binds to free enzyme (E), the ϕ_1 and ϕ_{12} terms should be multiplied with $1+\{[P]/(\phi'_{12}/\phi'_1)\}$. The formation of a dead end EOP complex would increase the ϕ_2 term by $1+([P]/K_{ip})$. In the case of varied propan-2-ol (Figures 3g–3i), $\phi_1/[O]$ and $\phi_{12}/[O]$ would contribute about equally (4%) to the intercepts and slopes, respectively. The formed dead-end EOP complex would, in addition, increase the ϕ_2 term, and hence the slope effects should be at least equal to, or larger than, the intercept effects. Figures 3(g)–3(i) shows that the opposite is the case, and hence argues against this mechanism. The intercepts obtained with varied NAD^+ (Figures 3b and 3e) also argue against this mechanism. The increase in intercept with increasing acetone concentrations should be due solely to the increase of the ϕ_2 term, as ϕ_0 is not affected. The increase in the intercepts in Figure 3(e) should hence be much larger than in Figure 3(b), as $\phi_2/[S]$ is approx. 80% of ϕ_0 in the former case and only 8% in the latter case. As shown in the two Figures, there is a slightly larger increase in the intercepts with high propan-2-ol concentration, i.e., the opposite of what would be expected if the reaction pathway followed the second mechanism.

In the compulsory ordered reaction pathway in Scheme 1, the intercept effects obtained with varied propan-2-old should give the K_{ip} value (eqn. 5), as $\phi_1/[O]$ would account for less than 4% of the ϕ_0 value. The slopes in Figure 3(g) will give the ϕ'_{12}/ϕ'_1 value (eqn. 5). Using the obtained K_{ip} and ϕ'_{12}/ϕ'_1 values (Table 2), the theoretically calculated intercept effects from varied NAD^+ fitted perfectly with the observed values (Figures 3b and 3e). However, the slope effects could not be completely accounted for in the above proposed mechanism (Figures 3c and 3f). With varied NAD^+ and a fixed concentration of 5 mM propan-2-ol, the $\phi_{12}/[S]$ value would be approx. 8% of the ϕ_1 value (6 μM), and hence only minor slope effects should occur (Figure 3f). The observed slope effect indicates that acetone has had an effect on the ϕ_1 term, suggesting that acetone also binds to the free enzyme (E). This indicates that acetone forms a dead-end binary EP complex, which largely prevents coenzyme binding (Scheme 3). In this case, the ϕ_1 and ϕ_{12} coefficients in eqn. (5) need to be multiplied by $1+([P]/K_{ip})$, and hence acetone affects all the

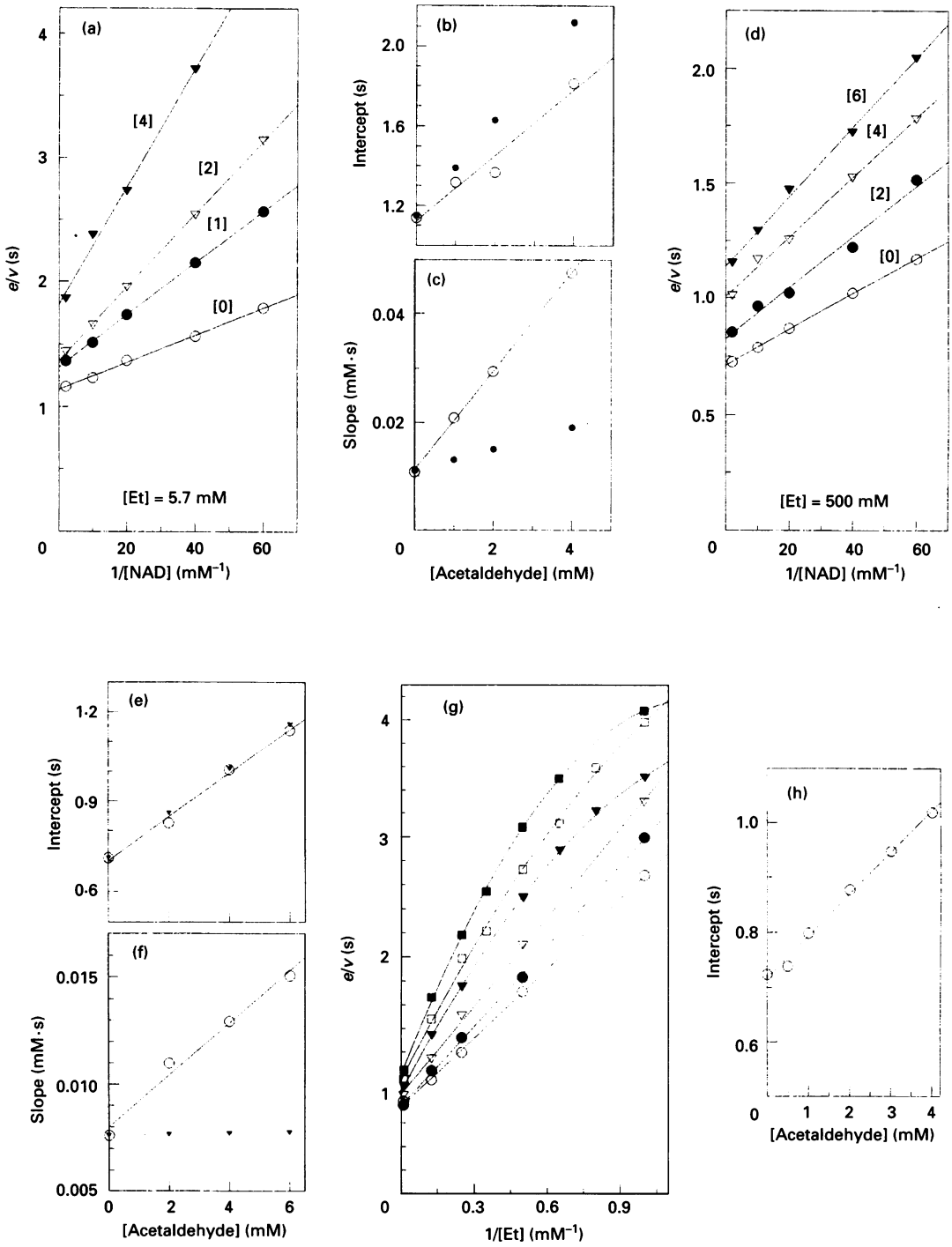


Figure 2 Acetaldehyde as a product inhibitor of the Adh^S alleloenzyme

Primary plots with varied NAD^+ and a constant ethanol concentration of 5.7 mM (**a**) and 500 mM (**d**), with the acetaldehyde concentrations in mM shown in square brackets. Replots of intercepts (**b**, **e**) and slopes (**c**, **f**) from (**a**) and (**d**) respectively versus acetaldehyde concentration (○). The theoretical intercepts and slopes (●, ▼) are calculated for an ordered reaction mechanism as described in Scheme 1 with the kinetic constants in Table 2 and [14]. The following coefficients were used for the calculations; $\phi_0 = 0.72$ s; $\phi_2 = 2.5$ mM·s; $\phi_1 = 6.0$ μM·s; $\phi_{12} = 0.029$ mM^2·s; $K_{ip} = 9.5$ mM and $\phi'_{12}/\phi'_1 = 2.55$ mM. In (**f**) a ϕ_1 value of 7.5 μM·s gave the best result. (**g**) Primary plot with varied ethanol (Et) and a constant concentration of 500 μM NAD^+. [Acetaldehyde]: ○, Control; ●, 0.5 mM; ▽, 1.0 mM; ▼, 2 mM; □, 3 mM; ■, 4 mM. (**h**) Replots of intercepts versus acetaldehyde concentrations, which were obtained from the linear part of the plots in (**g**).

kinetic coefficients. The suggested reaction pathway in Scheme 3 gives a good prediction of the inhibitory patterns shown in Figures 3c and 3f.

Previously we have shown that acetone is a very poor substrate, with much larger ϕ'_0 and ϕ'_1 values than for acetaldehyde [14]. If acetone forms an abortive EP complex, both the ϕ'_1 and ϕ'_{12} terms in the reverse reaction should be multiplied by $1 + ([P]/K_{ip})$ [21] and hence account for the large ϕ'_1 coefficient for acetone

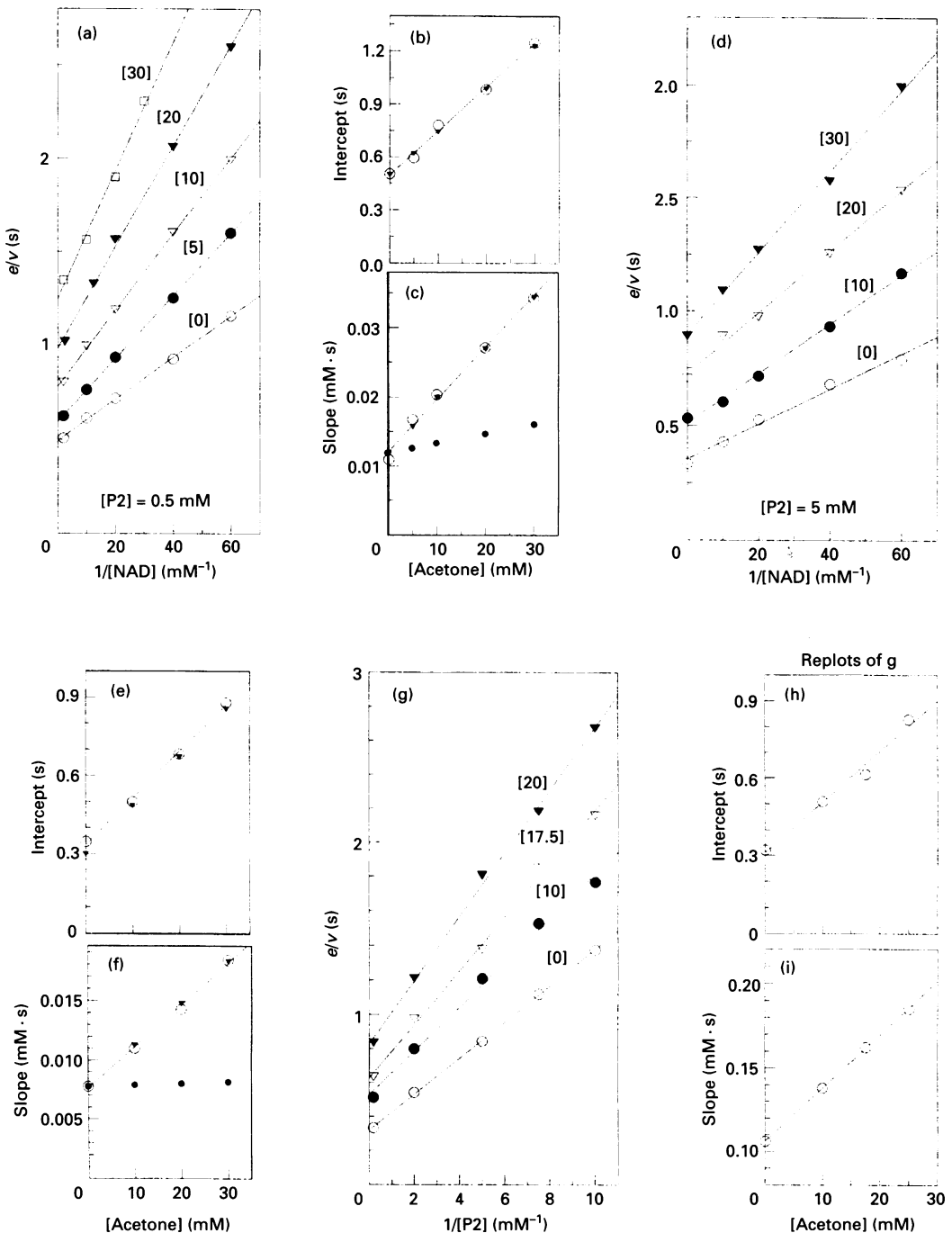
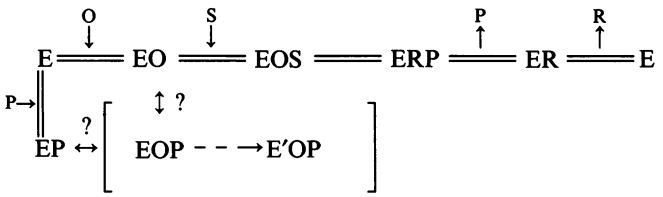


Figure 3 Acetone as a product inhibitor of the Adh^S alleloenzyme

Primary plots with varied NAD^+ and a constant propan-2-ol (P2) concentration of 0.5 mM (**a**) and 5.0 mM (**d**), with the acetone concentrations in mM shown in square brackets. Replots of intercepts (**b**, **e**) and slopes (**c**, **f**) from (**a**) and (**d**) respectively versus acetone concentration (○). The theoretical intercepts (▼) and slopes (●) are calculated for an ordered reaction mechanism as described in Scheme 1 with the kinetic constants in Table 2 and [14]. The following kinetic coefficients were used for the calculations; $\phi_0 = 0.28$ s; $\phi_2 = 0.11$ mM·s; $\phi_1 = 7.4$ μM·s; $\phi_{12} = 0.0023$ mM^2·s; $K_{ip} = 15.7$ mM and $\phi'_{12}/\phi'_1 = 33.5$ mM. The theoretical slopes (▼) were obtained as described above, but with the addition of a dead-end EI complex with a K_{ip} of 16 mM (**c**) and 23 mM (**f**). (**g**) Primary plots with varied propan-2-ol and a constant concentration of 500 μM NAD^+, with the acetone concentrations in mM shown in square brackets. (**h**, **i**) Replots of intercepts (**h**) and slopes (**i**) versus acetone concentrations.

compared with acetaldehyde [14]. By taking this factor into account when calculating K_{ER} from ϕ'_{12}/ϕ'_2, the value fits with that from the acetaldehyde reaction [14]. The reason that we did not observe substrate inhibition may be due to the low acetone concentrations used in these experiments.

Our previous studies with either dead-end inhibitors or alternative substrates also indicated that the products left in a compulsory ordered pathway, with the dissociation of NADH from the binary product ER complex as the last and rate-determining step (Scheme 1). With the *D. melanogaster* Adh^S



Scheme 3 Reaction mechanism extended for the oxidation of propan-2-ol with *D. melanogaster* Adh^S in the presence of product, P

The binary EP complex formed will in addition to the reverse reaction affect both the ϕ_1 and ϕ_{12} terms in eqn. (5) (Scheme 1) by the factor $(1 + [P]/K_{ip})$. The square brackets represent the previously shown isoenzyme conversion of Adh-5 into Adh-1 [28], where the almost inactive Adh-1 variant (E'OP) contains one NAD^+ and one acetone molecule covalently bound per active site [28]. The reversible EOP complex was loose ($K_{EO,P} = 1.7$ M) [28], which explains why it was not observed in the present product-inhibition study. The question marks denote the lack of knowledge concerning the reaction pathway for the conversion process.

alleloenzyme, a constant $V_{max.}$ of 3.4 s^{-1} was obtained for different secondary alcohols such as propan-2-ol [14,23], (*R*)-(−)-butan-2-ol, (*R*)-(−)-octan-2-ol [24] and cyclohexanol [25]. This is approx. 2.4 times the $V_{max.}$ value with ethanol. Substrate activation was observed at high concentrations of propan-2-ol, which was explained by the formation of a ternary ER–alcohol complex dissociating faster than the binary ER complex [24]. The ethanol competitive inhibitor imidazole shows competitive inhibition with stimulation against varied propan-2-ol or varied NAD^+ and fixed propan-2-ol [26]. The stimulatory pattern is explained by the formation of a ternary ER–imidazole complex in the 'off' phase of the reaction that dissociates faster than the binary ER complex [26]. Thus it can be concluded that there is no inconsistency in the deduced reaction mechanism, whether it is based on product inhibition, dead-end inhibition or alternative substrates.

Interconversion between ethanol and acetaldehyde

Previously Heinstra et al. [16,17] found that NADH as a product inhibitor was competitive against both NAD^+ and ethanol. This was also the case with acetaldehyde as an inhibitor. The four competitive patterns indicated that these enzymes followed a rapid-equilibrium random-ordered mechanism without ternary dead-end complexes. This was in contrast with our previous studies with different *Drosophila* Adhs which indicated an ordered reaction mechanism [14,23–27]. Ethanol differed from the secondary alcohols only by its rate-limiting step, as the interconversion of the ternary complexes, i.e., the hydride-transfer step was rate-limiting (Scheme 1). The present product-inhibitory study with the *D. melanogaster* Adh^S alleloenzyme shows one competitive and three non-competitive patterns. It was only the two coenzymes that competed, and hence NAD^+ binds prior to ethanol and NADH must bind to the free enzyme (E). This pattern excludes a rapid-equilibrium random-ordered mechanism with or without ternary dead-end complexes as a likely reaction pathway for this enzyme. The observed pattern is consistent with the two patterns discussed for the interconversion of propan-2-ol to acetone. However, hyperbolic plots were obtained with acetaldehyde as an inhibitor of varied ethanol (Figure 2g). This pattern can be explained by the previously described alternate reaction pathway (eqn. 2), where acetaldehyde in the presence of NAD^+ is oxidized to acetic acid [4,5]. The alternate mechanism alters the ϕ_2 term, as shown in eqns. (6) and (7) in Scheme 5 and the mechanism behind the alternative reaction pathway will determine how the different ϕ coefficients for ethanol oxidation should be modified in addition to that from the reverse reaction. However, in spite of the complications that arise from the alternative reaction pathway in the presence of acetaldehyde (Scheme 4), the two possible product leaving patterns in the 'off' phase of the ethanol oxidation can be distinguished.

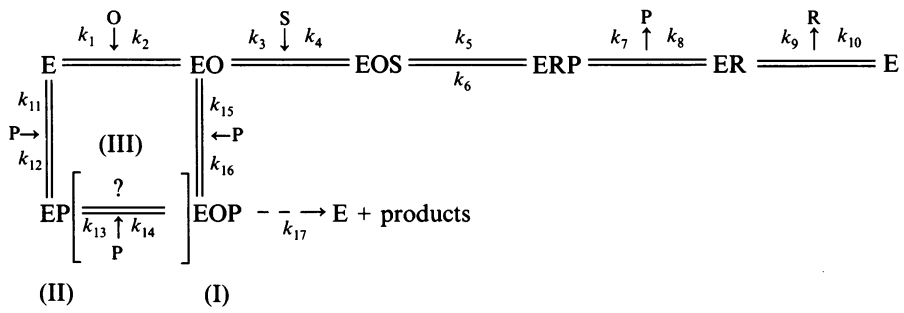
The intercept effects obtained with acetaldehyde as inhibitor against varied ethanol and a constant high NAD^+ concentration (Figure 2h) should be due to an effect on ϕ_0 alone, as $\phi_1/[O]$ is only 1.6% of ϕ_0. The ethanol concentration is infinite, and the branched pathway (independent of its mechanism) should not have an effect on the ϕ_0 term. In the 'off' phase of the ethanol oxidation, only an ordered pattern with acetaldehyde leaving from the ternary ERP complex affects the ϕ_0 term (eqn. 5; Scheme 1). This results in a K_{ip} value of 9.4 mM (Table 2). The intercept effects obtained with varied NAD^+ and 500 mM ethanol (Figure 2d) should also be due to an effect exclusively on ϕ_0, as the $\phi_2/[S]$ term is less than 0.7% of ϕ_0. As [S] > 100 times [P], the ϕ_0 term should not be affected by the branched pathway. The two curves (Figures 2e and 2h) give an almost identical inhibition (Table 2), with K_{ip} values of 9.4 and 9.6 mM respectively. Thus the intercept effects must have been produced by the reverse reaction, and hence the mechanism is consistent with an ordered leaving pattern for the products, as shown in Schemes 1 and 4. If the two products, NADH and acetaldehyde, had left randomly, acetaldehyde would have been competitive with NAD^+ at high ethanol concentrations.

The intercept effects obtained with varied NAD^+ and a low ethanol concentration (Figure 2a) should be due to the effect of acetaldehyde on both ϕ_0 and $\phi_2/[S]$. As shown in Figure 2b, the increase in the intercepts are lower than when calculated from the reverse reaction (eqn. 5; Scheme 1). This can be explained by the effect of the branched alternative mechanism on the ϕ_2 term (eqns. 6 and 7; Scheme 4). The linear slope effect with increasing acetaldehyde against varied NAD^+ and a high ethanol concentration shows that the acetaldehyde product affects the ϕ_1 term. During the conditions used, $\phi_{12}/[S]$ is less than 1% of ϕ_1 and, as acetaldehyde in an ordered reverse reaction ('off' phase) does not affect the ϕ_1 term, acetaldehyde must react with the free enzyme and form a binary EP complex. This would be consistent with an ordered alternative reaction pathway where acetaldehyde binds to the EO complex and, in addition, forms a dead-end binary EP complex. However, in a random alternative reaction pathway, the ϕ_1 term should be multiplied with $[(\alpha_6 + \alpha_7 P)/(\alpha_6 + \alpha_8 P + \alpha_9 P^2)]$ (eqn. 7). As the slope increases, the numerator term must dominate over the denominator term, as would be expected for a preferred ordered pathway, with the majority of the reaction channelled through the E–EO–EOP pathway. However, the slope effects with either a constant high or low ethanol concentration (Figs 2c and 2f) do not distinguish between these alternatives.

In a previous study, it was shown that acetaldehyde and NADH reacted in an ordered pathway to give ethanol and NAD^+, with the coenzyme as the leading substrate. No signs of a dead-end EP complex affecting NADH binding was observed [14]. However, substrate inhibition indicated the formation of a ternary EOP complex [14], which correlates well with the observations in the present work and the alternative reaction pathway in eqn. (2).

Concluding remarks

Together with previous studies using dead-end inhibitors and



Scheme 4 Reaction mechanism extended for the oxidation of ethanol with *D. melanogaster* Adh^S in the presence of product, P

The different ϕ values for the ethanol oxidation will be modified by P in two ways. P acts as a product, as shown in the square brackets, or as an alternative substrate, shown in parentheses in eqns. (6) and (7). In a compulsory ordered alternate pathway with coenzyme as the leading substrate (I), eqn. (6) describes the effect of P on the different ϕ coefficients. If P in addition also forms a dead-end EP complex (II), both the ϕ_1 and ϕ_{12} terms should be multiplied by $(1+[P]/K_{ip})$, where $K_{ip} = k_{12}/k_{11}$, the dissociation constant of the EP complex. The effect from a random ordered alternative pathway is shown in eqn. (7). The parentheses in eqns. (6) and (7) show how the alternate pathway modifies the different ϕ values according to the structural rules of Wong and Hanes [29]. The different α_i and α'_i are products and sums of the different rate constants (k_i) and the substrates S and O.

$$\frac{e}{v_0} = \phi_0\left(\frac{\alpha'_1+\alpha'_2[P]/[S]}{\alpha'_1+\alpha'_3[P]/[S]}\right)\left(1+\frac{[P]}{K_{ip}}\right)+\frac{\phi_1}{[0]}+\left(\frac{\phi_2}{[S]}+\frac{\phi_{12}}{[S][0]}\right)\left(\frac{\alpha'_1}{\alpha'_1+\alpha'_3[P]/[S]}\right)\left(1+\frac{[P]}{\phi'_{12}/\phi'_1}\right) \quad (6)$$

$$\frac{e}{v_0} = \phi_0\left(\frac{\alpha_1+\alpha_2[P]+\alpha_3[P^2]}{\alpha_1+\alpha_4[P]+\alpha_5[P^2]}\right)\left(1+\frac{[P]}{K_{ip}}\right)+\frac{\phi_1}{[0]}\left(\frac{\alpha_6+\alpha_7[P]}{\alpha_6+\alpha_8[P]+\alpha_9[P^2]}\right)+\frac{\phi_2}{[S]}\left(\frac{\alpha_{10}+\alpha_{11}[P]}{\alpha_{10}+\alpha_{12}[P]+\alpha_{13}\dfrac{[P]}{[S]}+\alpha_7\dfrac{[P^2]}{[S]}}\right)\left(1+\frac{[P]}{\phi'_{12}/\phi'_1}\right)$$

$$+\frac{\phi_{12}}{[0][S]}\left(\frac{\alpha_6+\alpha_{14}[P]+\alpha_{15}[P^2]}{\alpha_6+\alpha_8[P]+\alpha_9[P^2]}\right)\left(1+\frac{[P]}{\phi'_{12}/\phi'_1}\right) \quad (7)$$

alternative substrates, the present product-inhibitory study shows that the *Drosophila melanogaster* alleloenzymes Adh^S and Adh^F follow a compulsory ordered reaction pathway in the interconversion of alcohols and aldehydes/ketones (Scheme 1). In contrast with this is the discrepancy between the product-inhibitory patterns in the present work and that of Heinstra et al. [16,17]. The difference in pH between the two studies should not be decisive, as the alternate substrate and dead-end inhibitory studies on the Adh^S alleloenzyme showed the same reaction mechanism over the pH range 6–10 [14]. However, it must be emphasized that the pH studies of the Adh^S enzyme [14] revealed that product-inhibition studies at pH 7 with acetaldehyde or NADH as products could be difficult to carry out, due to the unfavourable equilibrium. Heinstra et al. [16,17] used different variants of *Drosophila* Adh, which differs from the Adh^S variant (Ser-1, Gln-82, Lys-192 and Pro-214) by the following amino acid substitutions, Adh^F (Thr-192), Adh^{F71K} (Thr-192, Ser-214) and *D. simulans* Adh (Ala-1, Lys-82) [3]. As the present work shows that both Adh^S and Adh^F follow a compulsory ordered reaction mechanism, it is unlikely that a single or a few amino acid changes are enough to change the reaction mechanism. Therefore it must be assumed that all *Drosophila* Adhs follow the same reaction mechanism. It is also noticeable that the same amino acid changes did not seem to alter the substrate specificity or the differences in rate-limiting steps between secondary and primary alcohols [3], which suggests an evolutionary conservation of the active-site topology, function and reaction mechanism.

It is also noteworthy that both acetaldehyde and acetone form binary dead-end complexes with the free Adh^S enzyme (Schemes 3 and 4). Both binary complexes affect NAD^+ binding, while only the acetone complex seems to affect NADH binding. In the case of acetaldehyde, a ternary EOP complex was formed that was converted into acetic acid and NADH (Scheme 4). Although no ternary EOP complex could be observed with acetone under the conditions used, we have shown previously that this complex exists, but the determined dissociation constant $K_{EO,P}$ was 1.7 M. In contrast with acetaldehyde, acetone formed a loose reversible dead-end ternary EOP complex that was slowly converted into a tight (irreversible) dead-end E′OP complex with almost no enzyme activity [28]. Whether the different metabolic fates of acetaldehyde and acetone are due to the same interactions with the enzyme or if two different binding sites and mechanisms are involved remains to be elucidated.

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