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Kinetics and thermodynamics of ethanol oxidation catalyzed by genetic variants of the alcohol dehydrogenase from *Drosophila melanogaster* and *D. simulans*

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Four naturally occurring variants of the alcohol dehydrogenase enzyme (ADH; EC 1.1.1.1) from *Drosophila melanogaster* and *D. simulans*, with different primary structures, have been subjected to kinetic studies of ethanol oxidation at five temperatures. Two amino acid replacements in the N-terminal region which distinguish the ADH of *D. simulans* from the three ADH allozymes of *D. melanogaster* generate a significantly different activation enthalpy and entropy, and Gibbs free energy change. The one or two amino acid replacements in the C-terminal region between the ADH allozymes of *D. melanogaster* do not have such clear-cut effects. All four ADH variants show highly negative activation entropies. Sarcosine oxidation by the ADH-71k variant of *D. melanogaster* has an activation energy barrier similar to that of ethanol oxidation. Three amino acid differences between the ADH of *D. simulans* and the ADH-F variant of *D. melanogaster* influence the k_{cat} and $k_{\text{cat}}/K_{\text{m}}^{\text{eth}}$ constant by a maximum factor of about 2 and 2.5, respectively, over the whole temperature range. Product inhibition patterns suggest a 'rapid equilibrium random' mechanism of ethanol oxidation by the ADH-71k, and the ADH of *D. simulans*.

Introduction

During the 'era of electrophoresis', a tremendous amount of genetic variation was found to be present within and between populations of species (for a review, see Refs. 1 and 2). Once this had been established, evolutionary biologists were still

left with the question of whether this protein polymorphism has functional significance. A number of more traditional fields of research such as enzymology and comparative biochemistry have proven to be important in an in-depth study of the potential functional differences between allozymes (enzymes encoded from alleles of the same locus) and protein homologues [3].

The present study concerns the naturally occurring polymorphism at the alcohol dehydrogenase locus of the fruit fly. *Drosophila melanogaster* (*Adh*, chromosome II – 50.1), and of the common *Adh* gene of its sibling species, *D. simulans*. Natural and laboratory populations of *D. melanogaster* contain three alleles, viz. *Adh*^F, *Adh*^S, and *Adh*^{71k},

Abbreviations: ADH, alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1); MTT, thiazolyl blue; PMS, phenazine methosulphate.

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TABLE I
DIFFERENCES IN PRIMARY STRUCTURE OF ADH VARIANTS

The total ADH protein comprises 255 amino acid residues. Residues 1–32 are coded for by exon 1, residues 33–167 by exon 2, and 168–255 by exon 3 [8].

Variant	Amino acid position				Ref.
	NH ₂ COOH				
	1	82	192	214	
<i>simulans</i> -ADH	Ala	Lys	Lys	Pro	6
<i>melanogaster</i> ADH-S	Ser	Gln	Lys	Pro	7,8
<i>melanogaster</i> ADH-F	Ser	Gln	Thr	Pro	7,8
<i>melanogaster</i> ADH-71k	Ser	Gln	Thr	Ser	- ^a

^a Involved CCC (*Adh^F*) into TCC (*Adh^{71k}*) transition in codon 214 (D. de Boer, X. Andriessse, G. Thörig and P. Weisbeek, unpublished results).

whereas *D. simulans* generally is monomorphic [4,5]. From nucleotide and amino acid sequence data, the primary structure of the alcohol dehydrogenase proteins (ADH, EC 1.1.1.1) is known. It turned out that they differ in up to four amino acids (Table I). To probe differences in properties between these four genetic variants, various approaches have been used. Population genetical studies have revealed differences in fitness (reproduction and/or survival) under dietary ethanol conditions between individuals carrying different *Adh* genes [4,9–12]. Such data suggest functional differences in biochemical properties between the ADH variants. In vitro activities of fly extracts toward ethanol show a rank order of ADH-71k > ADH-F > ADH-S > *simulans*-ADH [4,11,13,14]. This rank order has been substantiated from in vivo studies in third-instar larvae [12]. The question remained whether activity differences originate from differences in protein quantity in vivo and/or in kinetic-catalytic properties [12,15]. Previous kinetic studies on purified ADHs from *Drosophila* at a fixed temperature under optimum pH conditions have demonstrated differences in kinetic properties [11,14,16,17]. However, the enzyme proteins function at a presumed physiological pH of about 7 [5], whereas *Drosophila* experiences a temperature range of 15–35 °C [18]. Hence, kinetic studies should be performed under those experimental conditions. Moreover, if enzyme

kinetic studies are performed in a suitable range of temperature, the activation parameters of the reaction can also be explored. A proper evaluation of these parameters requires a knowledge of the kinetics mechanism involved [19]. Therefore, it is useful to measure the substrate-isotope effect and to conduct product inhibition studies [20,21]. In the present report, the exploration of enzyme kinetics and thermodynamics of ethanol oxidation by the ADH-variants from *Drosophila* are described.

Materials and Methods

Chemicals

Ethanol (99.5%) was purchased from Baker Chemicals, Deventer, The Netherlands; NAD (grade I) and NADH (grade I) from Boehringer, Mannheim, F.R.G.; and hexadeutero-(*d*₆)-ethanol from BDH Chemicals Ltd. Poole, U.K. For other reagents, see Refs. 14 and 22.

Stocks and rearing of flies

Stocks of *D. melanogaster* were homozygous for the *Adh^F* and *Adh^{71k}* allele, respectively, and one was homozygous for the *Adh^S* allele (Groningen population). The first two stocks were present in our laboratory [12], whereas the latter was kindly provided by Prof. W. van Delden (University of Groningen). The stock of *D. simulans* originated from Malaga (Spain) and is monomorphic for the common *Adh* gene of this species [12]. Flies were maintained on the standard cornmeal, glucose, agar, dead-yeast medium supplemented with 0.8% (v/v) propionic acid to suppress growth of fungi [13].

Protein purification

ADH protein was purified according to the affinity procedure given by Eisses et al. [14]. In brief, about 5 g freshly frozen adult flies (age 5–10 days) were hand-homogenized in a mortar by means of a pestle in 50 ml 20 mM Tris-HCl buffer, 5 mM MgCl₂, 0.4 mM EDTA, pH 6.4 (buffer A). The homogenate was centrifuged at 16000 × *g* for 30 min at 4 °C. After passing through glass-wool, the supernatant was applied to a Blue Sepharose CL-6B column. Overnight elution with buffer A removed unbound proteins.

Then, ADH was eluted by a 5 mM NAD solution in buffer A (pH re-adjusted). Fractions containing ADH activity were pooled and rapidly concentrated to about 3 ml using an Amicon PM10 Diaflow membrane. NAD was removed by finally running the aliquots over a Sephadex PD10 mini-column. Small aliquots of protein were stored at -30°C and used within 2 weeks of storage. All purification steps were conducted at 4°C . The purification procedure was always completed within 24 h. The purity of the protein was checked by means of nondenaturing polyacrylamide gel electrophoresis [14], and total protein staining (Coomassie Blue G-250) and specific ADH activity staining [24] afterwards. The quantity of protein in aliquots was determined by means of the Coomassie Blue G-250 dye-binding assay using bovine serum albumin as standard [14]. As an additional check of the absolute ADH protein quantities, radial immunodiffusion was applied using rabbit antisera raised against each separate ADH protein variant (see Ref. 12 for further details). The antibodies against the four ADH antigens possessed very strong cross-reactivity and immunological identity revealed by the absence of spurs between precipitin lines [12].

Enzyme kinetics

For the kinetic experiments, a microprocessor-controlled Gilford Response UV/VIS spectrophotometer provided with a kinetics program and equipped with a Haake N3 thermostat was used.

Primary Lineweaver-Burk plots were obtained at varying NAD concentrations (0.5–4-times the K_m^{NAD}) and various fixed concentrations of ethanol (0.5–5-times the K_m^{eth}) at 25°C in a 50 mM phosphate buffer pH 7.4. Product inhibition by NADH was performed at a subsaturating concentration of NAD or ethanol and varying concentrations of ethanol and NAD, respectively. All measurements were made in duplicate. The kinetic parameters were derived from replots according to Fromm [20]. The regression coefficients of the replots were always greater than 0.99. The inhibition constant for NADH was determined from replots of slopes versus NADH concentration [20].

Kinetic studies at a range of temperatures were conducted in a solution which was 50 mM in sodium phosphate buffer, 1 mM NAD and which

contained ethanol at varying concentrations (range of 0.5–6-times the K_m^{eth}) at pH 7.4 in the range of $15\text{--}35^{\circ}\text{C}$ with 5° increments. After thermal equilibration of the mixture, an enzyme aliquot was added to initiate the reaction. The total volume amounted to 0.55 ml. Rates of enzymic NADH production were determined from the increase in absorption in the UV/VIS spectrum at 340 nm. According to the tests of Selwyn [25], no significant enzyme denaturation occurred at the assay temperatures. Initial rates were proportional to the quantity of protein used. Omission of either NAD or ethanol gave no background activity. Initial rates were calculated using a linear regression program loaded by the manufacturer and using an absorption coefficient of $6.3 \cdot 10^3$ l/mol per cm. The kinetic parameters, maximum velocity (V) and the apparent Michaelis constant for ethanol (K_m^{eth}), were calculated using various linear transformations of the Michaelis-Menten equation, viz. those according to Lineweaver-Burk, Hanes, Eadie-Hofstee, and Eisenthal-Cornish Bowden [20,21]. All procedures gave similar answers. The turnover number, $k_{\text{cat}} = V/[E_0]/2$, was calculated starting from the fact that *Drosophila*-ADH (M_r 54 800) is a homodimeric protein with two identical subunits, each containing one substrate and coenzyme binding site [26]; and assuming that no interaction between the subunits occurs. All these kinetic experiments were performed in triplicate. The kinetic parameters were derived from weighted least-squares linear regression analysis.

Initial rates of sarcosine oxidation were measured at 550 nm in a temperature range of $20\text{--}35^{\circ}\text{C}$ with 5° increments. The assay mixture consisted of 100 mM sarcosine, 1.5 mM NAD, 1.5 mM MTT and 1 μM PMS in a 50 mM sodium phosphate buffer, final pH 8.0. Sarcosine oxidation was only measured with purified ADH-71k enzyme. The other ADH variants showed no activity towards this substrate (see also Ref. 27).

Thermodynamics

The activation parameters, ΔG^{\ddagger} , ΔH^{\ddagger} , and ΔS^{\ddagger} , were calculated using the equations:

$$\ln k_{\text{cat}} = \ln(kT/h) - \Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R \quad (1)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (2)$$

(see Refs. 19, 20 and 28). ΔH^\ddagger and ΔS^\ddagger were obtained from plots of $\ln(k_{\text{cat}}/T)$ versus $1/T$. Arrhenius activation energies, E_a , were calculated from plots of $\ln k_{\text{cat}}$ versus $1/T$. Statistical evaluation was according to Student's *t*-test. Physical units and symbols are according to the recommendations in Ref. 29.

Results

Enzyme purification

The mild and rapid purification which we applied resulted in high purity of the three ADH allozymes (ADH-F, ADH-S, and ADH-71k) from *D. melanogaster* (>99%). For all four ADH variants, two ADH isozymes were separated, i.e.

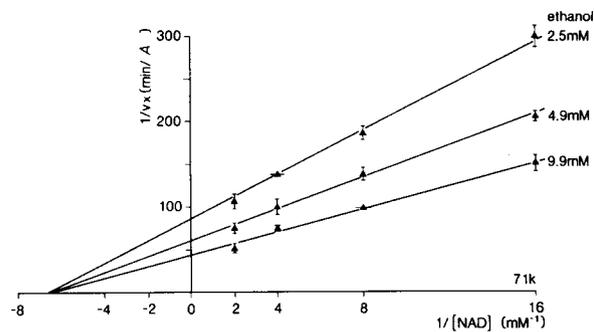


Fig. 1. Lineweaver-Burk plot from ethanol oxidation catalyzed by the ADH-71k allozyme of *D. melanogaster*. Bars indicate standard deviations.

the ADH-5 and ADH-3 isozyme. The contribution of the ADH-3 isozyme, known to be associated with one NAD-ketone adduct [24,30], was always less than 10% of the total ADH protein. The purification samples of the ADH from *D. simulans* contained an amount (approximately 15% of the total protein) of an electrophoretically distinct protein (without alcohol oxidizing activity). Therefore, radial immunodiffusion was applied to determine the actual quantities of ADH protein ($[E_0]$) used in the kinetic studies.

Enzyme kinetics

Steady-state. Primary Lineweaver-Burk plots obtained with the ADH-71k enzyme show one intersection point on the *x*-axis (Fig. 1). This means that the apparent Michaelis constants for ethanol and NAD are identical to their respective dissociation constants, whereas a sequential type of catalytic mechanism is suggested (see also Ref. 14). From replots, the K_m^{eth} was computed to be $4.5 (\pm 0.3)$ mM, and for the K_m^{NAD} , $150 (\pm 10)$ μM . Very similar values of their respective dissociation constants were computed too. Primary Lineweaver-Burk plots obtained with the ADH enzyme of *D. simulans* show an intersection point slightly above the *x*-axis (Fig. 2). Statistically, this point is non-significantly different from zero. This means that the apparent Michaelis constants for both substrates are slightly lower than their respective dissociation constants; however, a

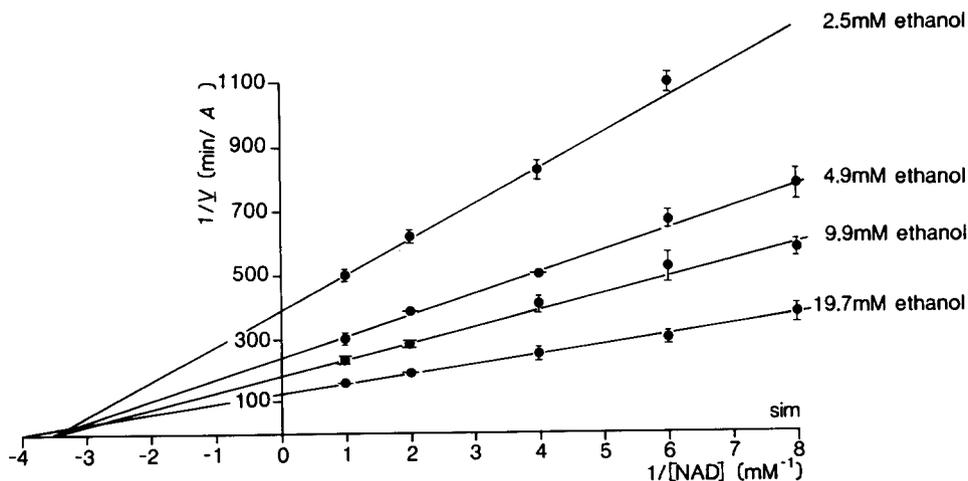


Fig. 2. Lineweaver-Burk plot from ethanol oxidation catalyzed by the ADH of *D. simulans*. Bars indicate standard deviations.

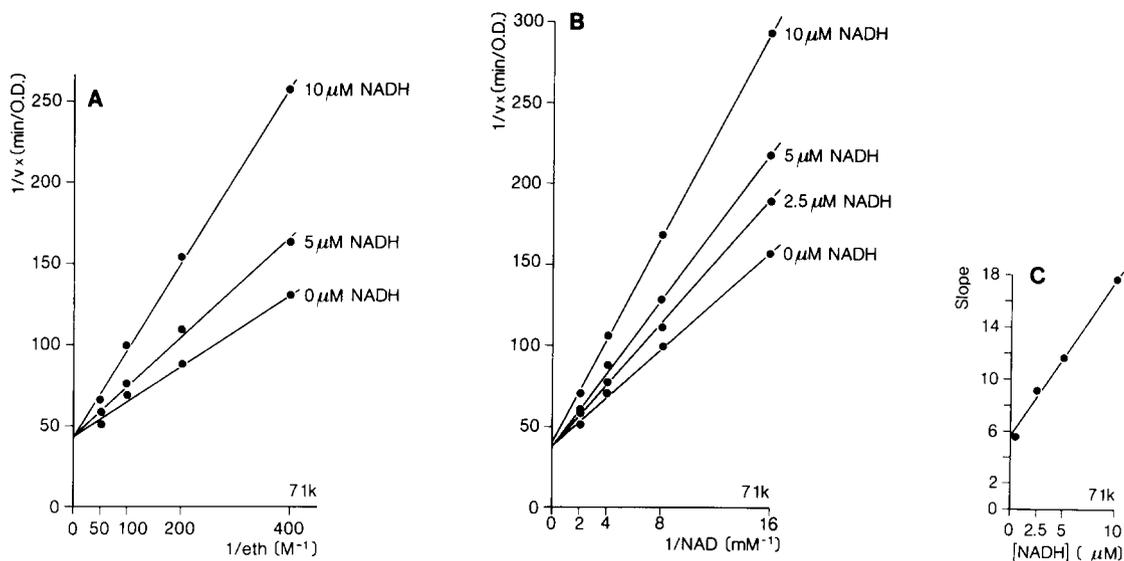


Fig. 3. NADH product inhibition of the ADH-71k enzyme. (A) Activities were determined at a subsaturated NAD concentration of 200 μM , variable ethanol and NADH at pH 7.4. (B) Activities were determined at a subsaturated ethanol concentration of 10 mM, variable NAD and NADH at pH 7.4. (C) Replot of slopes versus NADH concentration from data shown in (B).

sequential type of catalytic mechanism remains valid [20]. From replots, the K_m^{eth} was computed to be $6.1 (\pm 0.5)$ mM, and for the $1/K_m^{\text{NAD}}$, 280 (± 30) μM . The dissociation constants were 7 (± 0.3) mM and 340 (± 30) μM , respectively.

Product inhibition. In the reaction catalyzed by the ADH-71k variant, NADH behaves as a linear competitive product inhibitor at both changing and fixed substrate and coenzyme concentrations (Fig. 3A and B). From Fig. 3C, the inhibition constant for NADH was deduced to be 4.8 μM . The inhibition by NADH could be prevented by using 50 mM ethanol and 1 mM NAD, respectively (data not shown). NADH was shown to be a linear competitive product inhibitor at a subsaturating concentration of ethanol and variable NAD in the reactions catalyzed by *simulans*-ADH [31]. Moreover, at a subsaturating concentration of NAD, and variable ethanol, NADH also shows linear competitive product inhibition (Fig. 4A and B). The K_1^{NADH} was computed to be 1.1 μM , a value identical to that reported previously with varying NAD concentrations [31].

Temperature. Figs. 5a–c depict the kinetic parameters as determined in a range of temperature for the four ADH variants. At each temperature, the rank order of the catalytic rate constants

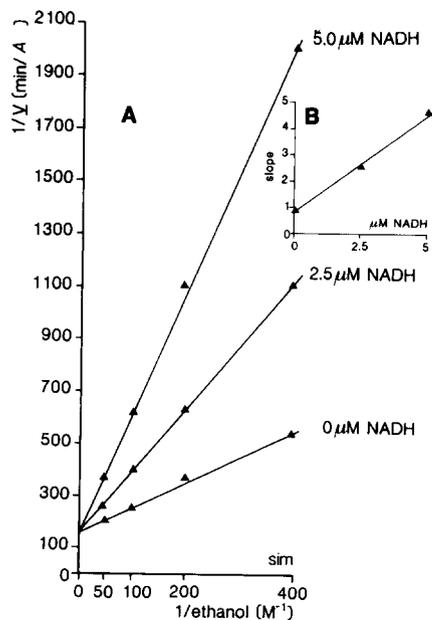


Fig. 4. NADH product inhibition of the *simulans*-ADH. (A) Activities were determined at a subsaturated NAD concentration of 400 μM , variable ethanol and NADH at pH 7.4. (B) Replot of slopes versus NADH concentration from data shown in (A).

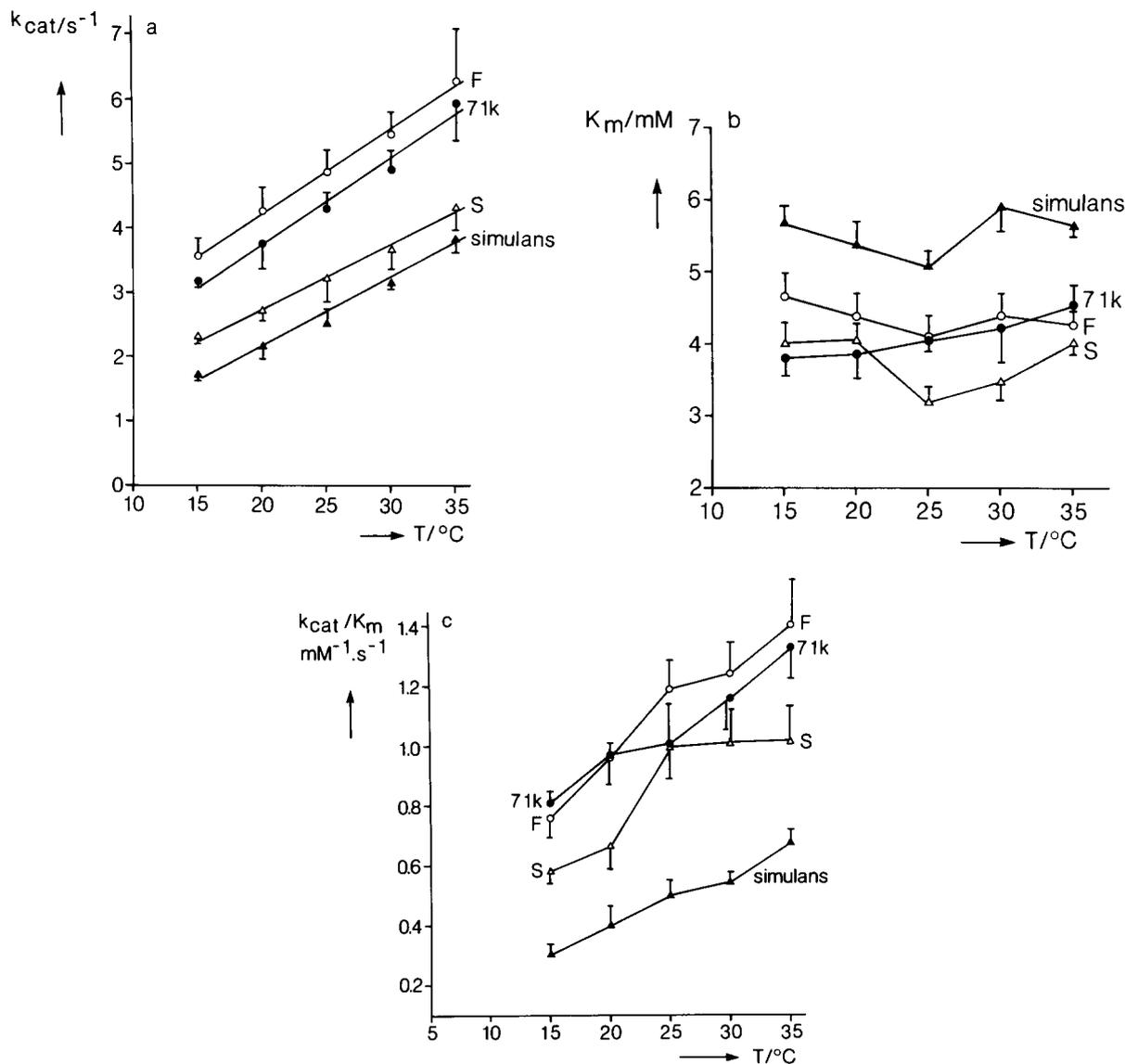


Fig. 5. Kinetic parameters obtained from ethanol oxidation catalyzed by four genetic variants of *Drosophila*-ADH at pH 7.4 within the indicated temperature range. Bars indicate standard deviations.

is $k_{cat}(\text{ADH-F}) > k_{cat}(\text{ADH-71k}) > k_{cat}(\text{ADH-S}) > k_{cat}(\text{simulans-ADH})$. The k_{cat} values of all variants increase with increasing temperature. On the other hand, their K_m^{eth} values are relatively insensitive towards changes in temperature. At each temperature, the K_m^{eth} of *simulans*-ADH is significantly higher than the K_m^{eth} of the ADH-S from *D. melanogaster*. In contrast, the ADH allozymes of *D. melanogaster* show similar K_m^{eth} values; however, at lower temperatures the ADH-S

and ADH-71k are more similar, whereas ADH-F and ADH-71k are at higher temperatures. The relative differences in the K_m^{eth} values between the ADH variants of *Drosophila* are in general agreement with previous data in the literature obtained at a fixed temperature (23 or 30°C) and at optimum pH values (8.6–9.5) [11,14,16,17]. The k_{cat}/K_m^{eth} values of *simulans*-ADH are lower over the whole temperature range when compared to the respective values of the ADH allozymes from

TABLE II
PRIMARY ISOTOPE EFFECTS

Obtained from kinetic experiments at 25 °C; concentration of NAD was 1 mM. Substrate concentrations of ethanol (H) or d_6 -ethanol (D) varied between 0.5- and 6-times the K_m . For details, see text.

Protein variant	k_{cat}^H/k_{cat}^D	K_m^H/K_m^D	$(k_{cat}/K_m)^{H/D}$
ADH-F	1.76 ± 0.20	0.83 ± 0.01	2.12 ± 0.21
ADH-71k	2.11 ± 0.36	1.09 ± 0.08	1.94 ± 0.21
ADH-S	1.86 ± 0.23	0.65 ± 0.16	2.86 ± 0.47
<i>simulans</i> -ADH	2.03 ± 0.23	0.64 ± 0.16	3.17 ± 0.58

D. melanogaster (Fig. 5c, four out the five values are significantly lower than those of ADH-S). The k_{cat}/K_m^{eth} values of ADH-S lie between those of ADH-71k and *simulans*-ADH. Differences between ADH-71k and ADH-F are smaller. The k_{cat}/K_m values give the lower limit of the second-order rate constant for binding of the respective substrate [45]. These values for *Drosophila*-ADH are about 10^3 lower compared to other dehydrogenases [45], suggesting a low interaction of ethanol with the ADH-NAD complex.

Isotope effect. Primary isotope effects were calculated from separate kinetic studies in which d_6 -ethanol and ethanol were used as substrates. The results for the four ADH variants are given in Table II. All ADHs show an isotope effect on k_{cat} of approximately two. The K_m of ADH-71k decreases slightly on deuteration, whereas the other variants show an increase of the K_m by a factor of

about 1.5. Significant isotope effects on the k_{cat}/K_m constants can be observed too.

Thermodynamics. The thermodynamic parameters as derived from the kinetic constants are given in Table III. The ΔH^\ddagger (*simulans*-ADH) turns out to be significantly higher than the ΔH^\ddagger values of the ADH allozymes from *D. melanogaster*. Slight variations in the ΔH^\ddagger values between the latter ADH variants are found. The ΔS^\ddagger (*simulans*-ADH) is significantly less negative than the values of ADH-S and ADH-F. The corresponding ΔS^\ddagger and ΔH^\ddagger values seem to have a compensating effect on their ΔG^\ddagger values, a phenomenon also observed for other enzymes [19,28]. The ΔG^\ddagger values decrease in the order *simulans*-ADH > ADH-S > ADH-71k > ADH-F. We further differentiated the ΔG^\ddagger values into ΔG^\ddagger_T (Gibbs free energy change from free enzyme into substrate-bound enzyme in the transition state) and into ΔG_S (Gibbs free energy change from free enzyme into substrate-bound enzyme) [32]. The ΔG^\ddagger_T values showed no significant differences between the ADH variants. The ΔG_S values of *simulans*-ADH are always significantly different from those of the ADH-S variant at each temperature (Table III; data at other temperatures not shown).

An Arrhenius plot of $\ln V$ versus $1/T$ for sarcosine oxidation mediated by the ADH-71k allozyme revealed an activation energy barrier of 26.2 ± 0.2 kJ · mol⁻¹. This barrier lies between values of ethanol oxidation by ADH-S and *simulans*-ADH (Table III).

TABLE III
THERMODYNAMIC PARAMETERS OF ETHANOL OXIDATION

The parameters were deduced from the relationship between k_{cat} and temperature (see Materials and Methods).

Protein variant	E_a (kJ · mol ⁻¹)	ΔH^\ddagger (kJ · mol ⁻¹)	ΔS^\ddagger J · mol ⁻¹ · K ⁻¹	ΔG^\ddagger (kJ · mol ⁻¹) (T)	ΔG_S (kJ · mol ⁻¹) (T)
ADH-F	20.9 ± 1.4	18.6 ± 1.0	-169.9 ± 10.0	69.1 ± 0.2 (25 °)	-3.5 ± 0.2 (25 °)
ADH-71k	23.1 ± 1.1	20.5 ± 0.9	-163.7 ± 13.4	69.4 ± 0.2 (25 °)	-3.5 ± 0.2 (25 °)
ADH-S	24.0 ± 0.9	21.3 ± 0.9	-164.1 ± 6.7	70.1 ± 0.4 (25 °)	-2.8 ± 0.2 (25 °) *
<i>simulans</i> -ADH	29.9 ± 0.4 *	27.8 ± 0.6 *	-143.6 ± 2.9 **	70.7 ± 0.2 (25 °)	-4.0 ± 0.2 (25 °) ***

* Significantly different from the other ADH variants (P < 0.05).

** Significantly different from ADH-F and ADH-S (P < 0.05).

*** Significantly different from ADH-S (P < 0.05).

Discussion

Mechanism

The ADH of *D. melanogaster* has been shown to oxidize not only ethanol, but its product, acetaldehyde as well [12,14,22,30,33–35]. Preliminary studies indicated that during initial periods of ethanol oxidation, the ratio of product formation of acetaldehyde/acetate is about 8:1 [35]. Therefore, the kinetic parameters reported would mainly refer to the first-half reaction.

NADH is found to be a competitive product inhibitor in the reactions catalyzed by both ADH-71k and *simulans*-ADH. At low concentrations, acetaldehyde also shows competitive inhibition on both enzyme variants [31]. At higher concentrations of acetaldehyde ($> 100 \mu\text{M}$), inhibition with stimulation of the maximum velocity was found, suggesting the formation of dead-end ternary complexes [31]. Nevertheless, inhibition of both produces can be prevented by saturation of either substrate. These patterns of product inhibition at physiological reactant conditions suggest a ‘rapid equilibrium random’ mechanism of ethanol oxidation at pH 7.4 by the two *Drosophila*-ADH variants comprising the largest difference in primary structure (Table I) [21]. The isotope effects on the k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ values suggest a mechanism in which hydrogen-transfer is a significant rate-limiting step. This catalytic mechanism of *Drosophila*-ADH is the opposite of that of yeast and horse-liver ADH, both of which display an ‘ordered’ mechanism of ethanol oxidation at pH 7.2 [36]. The ‘rapid equilibrium’ mechanism allows us to make a thermodynamic simplification. Then the activation parameters would refer to the k_{cat} rate constant of the hydrogen-transfer step [19,32].

Kinetics and population genetics

D. melanogaster and *D. simulans* are widespread cosmopolitan insects which experience a wide range of temperature climates [18]. The relatively constant values of the $K_{\text{m}}^{\text{eth}}$ for all four ADH variants are to be expected for *Drosophila* belonging to the eurytherms [37,38]. The Q_{10} values of ethanol oxidation of the ADH-S and *simulans*-ADH (average 1.0), occurring in warmer climates, on the one hand, the Q_{10} values of the ADH-F and ADH-71k variant (average 1.4), occurring

more in ‘colder’ climates, on the other, are also in line with the characteristics of eurytherms [18,37,38]. However, a different environmental factor marks the two sibling species. *D. melanogaster* can be found and breeds in habitats in which fermentations by yeast ADH may generate high concentrations of ethanol [4]. *D. simulans* does not breed in such habitats [18]. ADH plays the major role in the in vivo elimination of ethanol [12,22,23,30,33]. Therefore, ADH is thought to be a fundamental factor in the difference in habitats between the two species. This is reflected in the differentiation between both the $k_{\text{cat}}/K_{\text{m}}^{\text{eth}}$ and the k_{cat} constants of the ADH allozymes of *D. melanogaster* on the one hand, and of the ADH of *D. simulans* on the other. The $k_{\text{cat}}/K_{\text{m}}^{\text{eth}}$ specificity constant determines the reaction rate at low concentrations of substrate [39]. The k_{cat} rate constant is the sole important factor under conditions where the enzyme is saturated with substrate [5,39]. This is only relevant if the quantities of the ADH variants in larvae and flies are similar and remain so. However, several factors can influence the ADH quantity in the different *Adh* genotypes. The factors are: *cis*- and *trans*-acting regulatory elements linked and unlinked to the structural *Adh* gene [40], possible differences in ADH breakdown [11,15], dietary modulations [41], and modifiers, e.g. of body size [4]. With so many possible variables, such as temperature, genomic constitution, developmental stage, dietary conditions, and evolutionary history, and their various combinations, it seems impossible to predict the fate of *Adh* alleles in natural populations of *D. melanogaster* on the basis of kinetic properties of the ADH allozymes alone. For the evaluation of the intrinsic properties of an enzyme, the k_{cat} rate constant remains an important parameter for investigating a structure-property relationship.

Structure-property

Yeast ADH and the ADH variants of *Drosophila* are very different in primary structure except for a very few residues common to coenzyme-binding regions in dehydrogenases in general [42]. There is also a difference in activation parameters. The E_{a} and $\Delta H^{\#}$ barriers are much higher in yeast ADH than in the ADHs of *Drosophila* in the hydrogen-transfer step [43]. The

ΔS^\ddagger is positive in the former and negative in the latter. Yet, the ΔG^\ddagger value (68.6 kJ/mol per 20.0 C°) of the hydrogen-transfer step of yeast ADH is similar to those of the *Drosophila* ADHs [43].

The data in Table III show that the ΔS^\ddagger values of the ADH variants are highly negative. In general, the ΔS^\ddagger is determined by solvational and structural changes [28]. If we assume solvation effects to be equal in the ADH variants, the strong negative values of the ΔS^\ddagger suggest a compact structural orientation of the ethanol in the transition state [20]. This is particularly true for the ADH allozymes of *D. melanogaster*.

Amino acid replacements in the N-terminal region (from *simulans*-ADH to ADH-S of *D. melanogaster*, Table I) are characterized by: (i) an increase in the $k_{\text{cat}}/K_m^{\text{eth}}$ and k_{cat} constants (Fig. 5); (ii) an increase in stability of the transition state (Table III); (iii) a relatively large decrease in activation enthalpy (Table III). The same trends can be observed upon amino acid replacement in the C-terminal region (from ADH-S to ADH-F, Table I). These observations parallel kinetic and thermodynamic optimization processes in protein evolution [32], and here in micro-evolutionary change. This result is compatible with a phylogenetic reconstruction of the respective *Adh* genes based upon nucleotide sequence polymorphism [44].

The status of the ADH-71k allozyme needs additional comment. In natural populations its allele frequency is low. According to its primary structure, this allozyme would be closely related to the ADH-F allozyme (Table I). Our previous studies have shown that the ADH-71k allozyme, unlike the other ADH variants, recognizes various other alcohol-like substrates as sarcosine [27]. Only under the circumstances in which this broader substrate specificity of the ADH-71k is useful, it leads to a physiological advantage for individuals over those carrying the *Adh^F* allele [11]. This is also reflected by the activation energy barrier of the ADH-71k for sarcosine oxidation which is of the same magnitude as for ethanol oxidation.

We have predicted that the replacement of proline by serine (Table I) gives a slight change in the secondary structure of ADH-71k [14]. Such a small change is in agreement with the apparently higher affinity for *d₆*-ethanol compared to ethanol, sug-

gesting an effect in the substrate-binding region which is not evident in the other ADH variants. A further insight into the evolutionary status of the *Adh^{71k}* allele must come from molecular and further biochemical studies which are underway.

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