BIOORGANIC CHEMISTRY 17, 268–274 (1989)

On the Oxidation of Aldehydes by Alcohol Dehydrogenase of *Drosophila melanogaster*: Evidence for the *gem*-diol as the Reacting Substrate

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Received August 5, 1988

Purified alcohol dehydrogenase from *Drosophila melanogaster* is able to catalyze the oxidation of aldehydes in addition to primary and secondary alcohols. Acetaldehyde, methoxy-acetaldehyde, chloro-acetaldehyde, and trichloro-acetaldehyde (chloral hydrate) are used here to show that the *gem*-diol structure is the reacting form of the aldehydes. The reaction rates depend primarily on the degree of hydration of the aldehydes and to a lesser extent on the resulting electronic state of the hydrated molecules. These results resemble the chemical oxidation of aldehydes. The V/K_m values for the oxidation of acetaldehyde hydrate and chloro-acetaldehyde hydrate are very similar to that for ethanol. The very high oxidation rates of secondary alcohols compared to primary alcohols by *Drosophila* ADH are explained as the result of the structural resemblance to hydrated aldehydes and a more favorable electronic state. This behavior is in contrast to mammalian and yeast ADH, which have a low affinity for secondary alcohols and aldehydes. The oxidation of aldehydes by ADH apparently enables *D. melanogaster* to live in alcoholic environments.

INTRODUCTION

Alcohol dehydrogenases (ADH; EC 1.1.1.1) from various sources have been examined for many years. ADH and its variant forms in *Drosophila* species have been investigated because of their special value in understanding evolutionary mechanisms, e.g., selection and adaptation (*1*). High correlations exist between the tolerance to the presence of ethanol in the living environments of *D. melanogaster* and the specific enzyme activities of the various ADH allozymes (*1*).

Alcohol dehydrogenases of *Drosophila* species (DADH)[2] differ in a number of properties from yeast ADH (YADH) and horse liver ADH (HLADH). The latter are zinc containing enzymes with much larger molecular weights per subunit (*2, 3*). Striking differences were found in substrate specificities. Secondary alcohols are oxidized by HLADH at a very low rate, with a rate limiting step in the ternary complex, while primary alcohols are oxidized in a Theorell-Chance type mecha-

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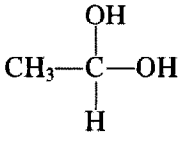
[2] Abbreviations used: DADH, *Drosophila* alcohol dehydrogenase; HLADH, horse liver alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase.

0045-2068/89 $3.00

nism (*4*). The opposite is true for DADH. DADH oxidizes primary alcohols slowly, with a rate limiting ternary complex, and secondary alcohols very fast in a Theorell-Chance type mechanism (*5*). There is little sequence homology between the zinc and non-zinc containing enzymes (*6*).

The question remains about the physiological significance of the high affinity for secondary alcohols by DADH, because of the activity inhibiting properties of these substrates: their oxidation products (ketones) form inhibitory complexes with ADH and the coenzyme NAD(H) (*7–9*). This problem is studied in this paper by referring to and exploring a recently described property of DADH, i.e., an NAD^+-dependent acetaldehyde oxidation by purified DADH in addition to its ethanol oxidation (*10, 11*). This observation has been confirmed by others (*12, 13*). The same kind of reaction was reported earlier with HLADH, but at that time its physiological significance and the particular oxidizable form of the aldehyde were questioned (*14–16*). The *gem*-diol or aldiol, i.e., the hydrated aldehyde, was suggested to be the reacting molecule. A hydrated aldehyde (Structure [1]) resembles a secondary alcohol in its molecular structure:



[1]

In acqueous solutions at neutral pH approximately 55% of acetaldehyde is in the hydrated form. Electron withdrawing substituents increase this percentage up to almost 100%, as in chloral hydrate (*14, 16, 17*). If substrates like chloral hydrate can be oxidized by purified DADH, further knowledge might be obtained about the mechanism of aldehyde oxidation.

MATERIALS AND METHODS

The thermostable alcohol dehydrogenase allozyme ADH^{71k} from a *D. melanogaster* strain, homozygous for Adh^{71k}, was purified as described elsewhere (*11*). The Blue Sepharose CL-6B column was regenerated before use with the buffer system mentioned there, but instead of urea, 2 M NaCl was used. The enzyme solution was essentially pure as measured with Phastgel isoelectric focusing and a silver staining. All solutions and buffers were made up in triple glass-distilled water, and the aldehyde solutions in freshly degassed water as well. The liquid aldehydes were g.c. pure. Acetaldehyde was purchased from Merck, chloroacetaldehyde from Fluka, methoxy-acetaldehyde from TCI (Tokyo Kasei Organic Chemicals), and solid chloral hydrate was analytical grade from the Norsk Medisinsk Depot.

Kinetic measurements were performed in 3 ml 0.1 M glycine–NaOH buffer, pH 9.5, and 0.5 mM NAD^+ (grade III-C, Sigma) at 23.5°C. After 1 min equilibration of the solution with added enzyme, the reaction was started by substrate addition. The enzyme samples were freshly thawed twice a day. There was no significant loss in enzyme activity during the period of experiments, when measured with

TABLE 1

Hydration of Aldehydes and Relative Oxidation Rates by *Drosophila* Alcohol Dehydrogenase ADH^{71k}

Hydration	% Hydrate	Activity[c]
$CH_3CHO + H_2O \rightleftarrows CH_3CH(OH)_2$	53[a]	27%
$CH_3OCH_2CHO + H_2O \rightleftarrows CH_3OCH_2CH(OH)_2$	85[b]	36%
$CH_2ClCHO + H_2O \rightleftarrows CH_2ClCH(OH)_2$	97.4[a]	66%
$CCl_3CHO + H_2O \rightleftarrows CCl_3CH(OH)_2$	99.99[a]	11%

Note. Percentages of hydration of acetaldehyde, methoxy-, chloro-, and trichloro-acetaldehyde at 25°C, based on equilibrium constants for the hydration reaction. The activities of *Drosophila* alcohol dehydrogenase with these substrates are relative to the activity with ethanol (100%) at an equimolar concentration.

[a] Data from Ref. (*17*) and references therein.

[b] Extrapolated from Fig. 1.

[c] At 1 mM concentrations, relative to 1 mM ethanol.

ethanol as a substrate. The initial rate measurements of NADH formation at 340 nm were performed in duplicate with a variation of 0.5% at higher and moderate concentrations to 3.5% at lower substrate concentrations. Background activities without substrate were negligible. A Pye-Unicam SP6-550 spectrophotometer, coupled to a Kontron 1100 W + W recorder and a MGW LAUDA thermostat, was used. The Lineweaver–Burk plots were calculated using the linear regression program of a Texas Instruments TI-59.

Due to the spectrophotometer and the temperature used the experiments with aldehydes had to be performed outside a fume-cupboard, which forced the experimenter to wear a gas mask with carbon filter.

RESULTS

At 1 mM substrate concentrations the initial rates of the reactions with free aldehydes or aldehyde hydrates are lower than with ethanol. Table 1 gives the values for the allozyme ADH^{71k}. It shows that even chloral hydrate can be oxidized by DADH, although at a relatively low rate, whereas chloro-acetaldehyde (or rather its hydrate) is oxidized at a high rate, and methoxy-acetaldehyde and acetaldehyde (or its hydrates) at moderate rates. At a constant coenzyme concentration of 0.5 mM NAD^+ the initial rates were measured for a wide range of concentrations (0.02–15 mM) of acetaldehyde and chloro-acetaldehyde. The Lineweaver–Burk plots over the whole concentration range were very nearly linear ($r > 0.998$). Both the apparent K_m values and the relative V values were lower than those with ethanol. When the apparent K_m and V are recalculated for the actual aldehyde hydrate concentrations, very different values are obtained in the case of acetaldehyde (Table 2, in parentheses). In the case of chloro-acetaldehyde these data do not differ because of the very low concentration of free aldehyde (Table

TABLE 2

Oxidation of Aldehydes by *Drosophila* Alcohol Dehydrogenase ADH^{71k}

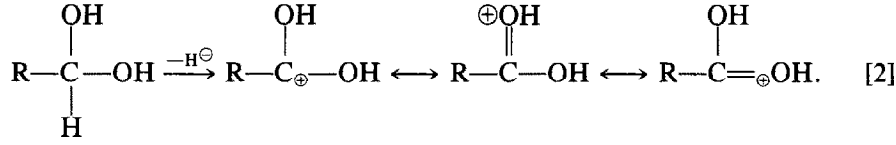
Substrate	1 mM	K_m (mM)	V (%)	V/K_m
Ethanol	100	3.2	100	31
Acetaldehyde	27 (37)	1.0 (0.65)	18 (19)	18 (29)
Chloro-acetaldehyde	66 (66)	0.60 (0.59)	24 (24)	40 (40)
Methoxy-acetaldehyde	36	0.62	n.d.	n.d.

Note. Reaction rates at 1 mM substrate concentrations, relative to ethanol (= 100%), V, and apparent Michaelis constants for ethanol, and aldehyde oxidation by DADH at a range of concentrations at 23.5°C. In parentheses the apparent constants as calculated for the actual aldehyde hydrate concentrations.

1). The K_m value for acetaldehyde oxidation by ADH^{71k} is of the same order of magnitude as the value obtained by Moxon *et al.* (*12*) for the ADH^F allozyme.

DISCUSSION

Although the oxidation of aldehydes by alcohol dehydrogenase seems to be a surprising function of this enzyme, it can be explained by chemical properties of aldehydes, which depend on the electronic nature of the substituents (*18*), expressed by Taft's polar substituent constant σ^* (*19*); i.e., the equilibrium of the hydration reaction of the carbonyl group can be affected by electron donating or electron withdrawing substituents, as for example by alkyl groups or halogen atoms, respectively. Figure 1 shows the Taft plot for $\log K_{(\text{Hydration})}$ versus σ^* (*19*). K is the equilibrium constant of the hydration reaction at 25°C (*17*). Because of the linear relationship (correlation $r > 0.998$, with slope $\rho^* = 1.69$), the $K_{(\text{Hydration})}$ value for methoxy-acetaldehyde can be calculated. The chemical oxidations of alcohols and fully hydrated aldehydes resemble each other, which favors the hypothesis of identical reaction mechanisms (*18*). The hydride transfer to the oxidators (Formula [2]) is considered as the rate determining step in the chemical oxidation of aldehydes (*18*). The two hydroxyl groups stabilize the intermediate carbonium ion formed in this step. Therefore, hydride transfer is facilitated:



[2]

The order of reactivity of various aldehydes corresponds in principle to the degree of hydration (*15, 18*).

In the biological situation, with hydride transfer to NAD^+, a similar order of

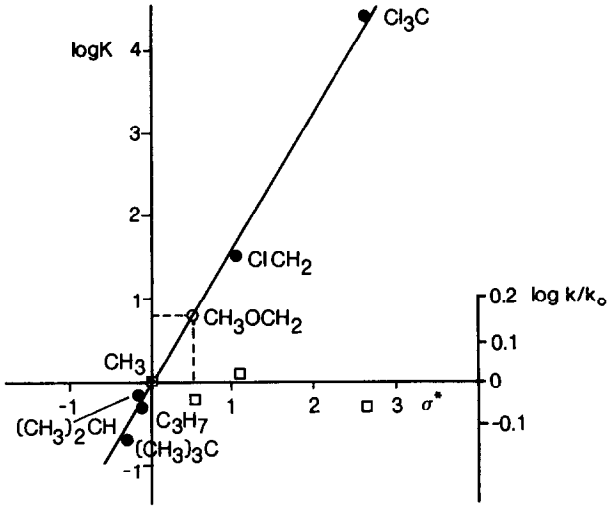


FIG. 1. Taft plot. Effects of polar and alkyl substituents of the carbonyl group, as expressed by σ^* (*19*), on the equilibrium constant K of the hydration reaction of aldehydes (*17*) (●), and on the relative reaction rates k/k_o (□). The rates were calculated with respect to the concentration of hydrated aldehyde, assuming that only the hydrated form is reactive. k_o is the calculated reaction rate with 1 mM acetaldehyde hydrate. The slope of log K versus σ^* is $\rho^* = 1.69$.

reactivity seems to occur (Table 1). The almost completely hydrated chloral hydrate can be dehydrogenated by DADH (Table 1) and HLADH (*16*), but the reaction rate is lower than that expected. This can be explained by the presence of the three Cl atoms. On the one hand an increase of the concentration of the hydrated aldehyde increases the reaction rate k, which points to the involvement of the *gem*-diol structure. On the other hand electron withdrawing substituents destabilize the intermediate carbonium ion. The cleavage of the C–H bond is less facilitated in that case (*18*). Roček (*18*) measured this relationship with three almost completely hydrated aldehydes, mono-, di-, and trichloro-acetaldehyde by oxidation with transition metals. A negative ρ^* was obtained, which means a lower reaction rate with increasing polarity of the substituents.

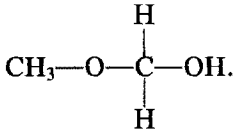
The same relationship seems to hold for the enzyme-catalyzed reactions. The log values of the relative reaction rates k/k_o, with k_o the rate with acetaldehyde, are recalculated to a 100% aldehyde hydrate concentration. In Fig. 1 the values for $\log(k/k_o)$ versus σ^* crudely represent a negative ρ^* as well, indicating a mechanism of aldehyde oxidation similar to that described for the chemical oxidation, i.e., oxidation via the *gem*-diol (*18*).

In contrast to HLADH and YADH, which exhibit low activities with secondary alcohols (*4*), DADH has a very high affinity for secondary alcohols (*5*) and a relatively good affinity for aldehydes. Whereas it looks as if secondary alcohols are natural substrates for DADH, this might be a side effect of an evolutionary process. DADH has probably evolved in the direction of an enzyme, which can dehydrogenate both primary alcohols and their related aldehydes, especially etha-

nol and acetaldehyde. Toxic intermediates, as the aldehydes are, are thus kept at low and tolerable concentrations. The efficiency of substrate oxidation at low concentrations is reflected by V/K_m (*20*). At pH 9.5 these ratios for ethanol and acetaldehyde hydrate oxidation by ADH^{71k} are almost similar (Table 2), which indicates that no acetaldehyde accumulates during ethanol oxidation. This makes it much easier to live in environments in which ethanol is often one of the major substrates (*1*).

On the other hand, ketones, the products of secondary alcohol oxidation, form inhibitory complexes with DADH and the coenzyme (*7–9*). The physiological significance of secondary alcohol oxidation by DADH is questionable, because of its suicidal character. The very fast reactions with secondary alcohols can thus be explained by their structural resemblance to hydrated aldehydes. The presence of two electron donating alkyl groups not only facilitates hydride transfer, but provides a better fit to the apparent hydrophobic reaction center as well (*5*).

The oxidative ability of DADH toward hydrated aldehydes might also imply an oxidative ability toward hemiacetals, in a manner similar to that of HLADH (*14*). This provides a clue to the remaining question of why methanol, which is not a substrate of purified DADH, has a selective interaction with two DADH allozymes, which differ in qualitative and quantitative properties (*1, 21*). The allele frequency of the gene, coding for the most active ADH^F allozyme, increases after several generations of selection on a medium containing 2.5% methanol (*21*). In such a medium methanal might be produced by microorganisms or other *Drosophila* enzymes. Formaldehyde forms a hemiacetal with methanol as fast as a hydrate with H_2O (*17*). As shown in Structure [3] the hemiacetal methoxy-methanol is a primary alcohol:



[3]

This primary alcohol is most unlikely to be a good substrate, because of the electron withdrawing effect of the methoxy group (Fig. 1; (*19*)). Its reactivity should be compared with the low reactivity of 2-chloro-ethanol ((*5, 22*), Eisses, unpublished results), and 2-methoxy-ethanol ((*22*), Eisses, unpublished results). 2-Chloro-ethanol has been described as a competitive inhibitor of the ethanol oxidation (*22*). A selective effect might be due to an inhibitory effect of methoxy-methanol. In that case inhibition has more profound effects in organisms with the lowest content of protein, including those with the ADH^S variant (*1*).

ACKNOWLEDGMENTS

The Royal Norwegian Council for Technological and Scientific Research is acknowledged for their postdoctoral fellowship. I thank Prof. J. S. McKinley-McKee for the opportunity to work at his laboratory, Øystein Brandsnes (NYCOMED, Oslo) for his help with Phastgel isoelectric focusing, Prof. W. Drenth from the Department of Organic Chemistry (University at Utrecht) for advice and

comments on the manuscript, Profs. P. Brakefield, W. Scharloo, and Dr. G. E. W. Thörig for textual improvements of the manuscript, and the Department of B & V of the University at Utrecht for preparing the figure.

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