Human class I alcohol dehydrogenases catalyze the oxidation of glycols in the metabolism of norepinephrine

(4-hydroxy-3-methoxyphenyl glycol/4-hydroxy-3-methoxymandelic acid/ethanol)

GÖRAN MÄRDH, CRAIG A. LUEHR, AND BERT L. VALLEE

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Contributed by Bert L. Vallee, April 12, 1985

ABSTRACT Investigations of the function of human liver alcohol dehydrogenase (ADH) in norepinephrine metabolism have revealed that class I ADH catalyzes the oxidation of the intermediary alcohols 4-hydroxy-3-methoxyphenyl glycol (HMPG) and 3,4-dihydroxyphenyl glycol (DHPG) in vivo. The \( k_{cat}/K_m \) values for the individual homogeneous class I isoforms are generally in the range from 2.0 to 10 \( \text{mM}^{-1}\text{min}^{-1} \), slightly lower than those obtained for ethanol oxidation, 16-66 \( \text{mM}^{-1}\text{min}^{-1} \), but considerably higher than those obtained for ethylene glycol oxidation, 0.23-1.5 \( \text{mM}^{-1}\text{min}^{-1} \). Importantly, HMPG and DHPG are not substrates for the class II or class III ADHs. 4-Methylpyrazole and 1,10-phenanthroline inhibit the class I ADH-catalyzed oxidation of HMPG, DHPG, and ethanol with inhibition constants of 75-90 nM and 19-22 \( \mu \text{M} \), respectively, indicating that these substrates interact at the same catalytic site of ADH. Moreover, ethanol inhibits the oxidation of DHPG. The competition of ethanol with HMPG for ADH provides a basis for the in vivo changes observed in norepinephrine metabolism after acute ethanol intake. Any assessment of norepinephrine function through the study of metabolites in peripheral body fluid must include monitoring the oxidation of HMPG by ADH.

The remarkable polymorphism of human liver alcohol dehydrogenase (ADH) has given new perspectives to both its structure and function as well as to its catalytic and metabolic roles. The detection, isolation, and definitive characterization of the individual forms is attributable to the introduction of affinity chromatography based on pyrazole derivatives (1, 2). The subsequent identification of both \( \alpha \)-ADH (3) and \( \beta \)-ADH (4) has altered the views on the genetics of ADH and its expression. The "three gene loci" hypothesis, the perception of the field for a decade, has given way to one in which a minimum of five loci account for the three classes of dimeric ADH encompassing up to eight phenotypic variants (5-7). Virtually all of the resultant homo- and heterodimeric isozymes have now been isolated and purified and they are being characterized both functionally (8-10) and structurally (5, 11). Consequently, in addition to their amino acid sequences (12, 25), it has become possible to detail their substrate specificities, inhibition and catalytic constants, and, ultimately, metabolic roles.

Previous investigations along such lines have established marked differences among the three classes; they vary in substrate specificities, inhibition by pyrazole derivatives, and antigenicity (7). Moreover, as noted, almost uniformly, ethanol is far from unique or even superior as a substrate for all the ADH isozymes (7); for class III ADH in particular, ethanol is an exceptionally poor substrate (10). Such observations have raised considerable doubt that the oxidation of ethanol and/or reduction of acetaldehyde are the primary and sole metabolic functions of all human alcohol dehydrogenase isozymes. Hence, we have initiated an intensive search for biological products of intermediary metabolism that might be specific substrates for particular isozymes or isozyme classes.

This and reports to follow examine the potential participation of the three classes of ADH isozymes in the oxidationreduction of intermediates in catecholamine metabolism. In norepinephrine catalysis, the major metabolite 4-hydroxy-3-methoxymandelic acid (VMA, HMMA) forms in vivo by oxidation of 4-hydroxy-3-methoxyphenyl glycol (HMPG, MHPG, MOPEG) (13). Importantly, when \( ^{14} \text{C} \)-labeled HMPG is incubated in vitro the liver proves to be the major site of its oxidation (unpublished work), and in the rat this process is inhibited by ethanol in vivo (14). The present data suggest that one or more of the human ADH class I isozymes account(s) for the oxidation of HMPG.

METHODS

Normal human livers were obtained at postmortem examination, stored, processed, and handled as described (8-10). The various isozymes of human liver alcohol dehydrogenase were separated and purified to homogeneity by affinity chromatography (1) followed by ion exchange chromatography (2, 8-10). The individual isozymes of all three classes of alcohol dehydrogenase obtained were homogeneous by physicochemical criteria and were in accord with reported specific activities (8-10). The present study was performed with purified isozymes of class I (\( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), \( \alpha_4 \), \( \beta_1 \), \( \beta_2 \), \( \beta_3 \), and \( \gamma_2 \)), class II(\( \gamma \)), and class III(\( \delta \)) ADH.

Kinetics were determined by using a Gilford model 2600 spectrophotometer to monitor the formation of NADH at 340 nm. All experiments were carried out in 0.1 M sodium phosphate (pH 7.40) at 25.0°C with a saturating concentration (2.5 mM) of NAD\(^+\). The kinetic parameters were calculated from duplicate determinations of initial reaction rates at 6-10 substrate concentrations. \( K_m \) and \( k_{cat} \) values were determined from Lineweaver-Burk plots. For each isozyme two enzyme preparations were used and the results differed by <2-fold.

The product of HMPG oxidation was monitored by HPLC (Waters Associates) using a methanol/10 mM phosphate, pH 7.5, gradient, 0-50% methanol, on a Waters Radial-Pak Nova-Pak C\(_{18}\) column. Prior to HPLC analysis protein was removed by centrifugal filtration (5000 \( \times \) g) through a Centricon-10 microconcentrator (Amicon). HMPG, 3,4-dihydroxyphenyl glycol (DHPG), and NAD\(^+\) (grade III) were used as purchased from Sigma.

Abbreviations: ADH, alcohol dehydrogenase; DHPG, 3,4-dihydroxyphenyl glycol; HMPG, 4-hydroxy-3-methoxyphenyl glycol; HMMA, 4-hydroxy-3-methoxymandelic acid; OP, 1,10-phenanthroline.
RESULTS

Class I ADH isozymes readily catalyze oxidation of the norepinephrine metabolites HMPG and DHPG. Although the pH optimum for ADH is 10.5 (2), these studies were carried out at physiological pH to avoid problems due to the instability of DHPG at alkaline pH. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ values for oxidation of the two glycols in the presence of saturating concentrations of NAD$^+$ by all class I isozymes examined at pH 7.40 and 25°C are listed in Table 1. Comparative data for ethanol oxidation under the same conditions are also shown for reference.

In contrast to the class I ADH isozymes, neither class II nor class III ADH oxidizes HMPG or DHPG with detectable rates at substrate concentrations up to 8.0 mM (Table 1). These results add HMPG and DHPG to the list of substrates including methanol and the dihydroxigenins that are oxidized by class I but not classes II and III, underlining the distinctive isozyme substrate specificities.

The $k_{cat}$ values for HMPG and DHPG oxidation by the various class I isozymes show a much greater variation than those for ethanol oxidation. They range from 0.3 to 33 min$^{-1}$ for HMPG and from 1.7 to 16 min$^{-1}$ for DHPG, while for ethanol the range is only from 9.1 to 39 min$^{-1}$. In the most striking case, the values of $k_{cat}$ for HMPG oxidation by $\alpha_2i$-ADH and $\beta_1i$-ADH differ by 100-fold whereas, with ethanol, the two isozymes have essentially the same $k_{cat}$, 9.4 ± 0.4 min$^{-1}$.

The $K_m$ values also show different trends. The range of values for HMPG, 0.44–5.0 mM, overlap those for ethanol oxidation, 0.16–1.1 mM. Those for DHPG fall within the same range, 1.1–3.4 mM, but vary much less. The $k_{cat}/K_m$ values are generally in the range 2.0–10 mM$^{-1}$min$^{-1}$, slightly lower than those obtained for ethanol oxidation, 16–66 mM$^{-1}$min$^{-1}$.

The kinetic parameters for oxidation of HMPG, a glycol, were also determined at pH 10.0 for some of the class I isozymes (Table 2), and the results are compared with those for the simplest glycol, ethylene glycol (8). The $K_m$ values for HMPG are one to two orders of magnitude lower than those for ethylene glycol whereas the corresponding $k_{cat}$ values differ by only a factor of 3–6, with the exception of $\alpha_b$, whose $k_{cat}$ for HMPG is lower by a factor of 50. For $\alpha_1i$ and $\beta_1i$, the $k_{cat}/K_m$ values for HMPG are an order of magnitude higher than those for ethylene glycol, while for $\alpha_b$ and $\beta_1i$, the values for both substrates are comparable. Overall, as is apparent from the relevant $k_{cat}/K_m$ values, the catalytic preference for HMPG of the isozymes spans a 70-fold range, much greater than that for ethylene glycol, which spans only an 8-fold range.

The oxidation product of HMPG by class I ADH isozymes was identified by HPLC. A single peak formed linearly throughout a 2-hour incubation of $\alpha_2i$-ADH with HMPG (10 mM HMPG/20 mM NAD$^+/0.1$ M sodium phosphate, pH 7.4, 37°C). It elutes with a retention time identical to that of authentic dimeric 4-hydroxy-3-methoxymandeldehyde (HMMAL), which was synthesized and purified for reference and identified by NMR and mass spectrometry.

The inhibition of HMPG oxidation by ethanol was monitored directly by HPLC determination of the amount of aldehyde (HMMAL) formed as a result of the action of $\alpha_2i$-ADH at various ethanol concentrations. HMMAL production decreases with increasing ethanol concentration, thus demonstrating competition (Fig. 1). At 5 mM ethanol, 50% inhibition of HMPG oxidation is noted in this system.

1,10-Phenanthroline (OP) is known to inhibit the ethanol oxidase activity of horse liver ADH by binding reversibly to the E-Zn complex forming an E-Zn-OP ternary complex (15). Similarly, the human isozymes are also reversibly inhibited by OP. The $K_i$ value for OP inhibition of ethanol oxidation by $\beta_1i$-ADH is 22 µM. OP inhibits the oxidation of HMPG and DHPG with $K_i$ values of 19 and 21 µM, respectively (Fig. 2). These values are virtually identical to that obtained for ethanol, indicating that the active-site zinc atom is essential for catalysis of these two intermediates in norepinephrine metabolism.

4-Methylpyrazole is another reversible active-site inhibitor that is highly specific for class I ADHs. Dixon plots show that this compound inhibits $\beta_1i$-ADH catalysis of HMPG and ethanol with the virtually identical $K_i$ values of 75 and 90 nM, respectively (Fig. 3). Thus, the results of both OP and 4-methylpyrazole inhibition of HMPG, DMPG, and ethanol oxidation show that all of these substrates interact at the same catalytic site of ADH.

DISCUSSION

During the last 20 years studies of human ADH have resulted in the recognition of a large number of isozymes (16) that differ in amino acid composition, charge, primary sequence, substrate specificity, and immunogenicity (5, 11, 12, 25). In addition, their distribution is organ specific (17). Given a particular organ such as the liver, the isozyme distribution is typical for an individual and—in the case of some of the isozymes—for population groups. The characteristics of the isozymes serve to define three isozyme classes. These apparently arise from at least five genetic loci that express multiple polypeptides that, in turn, combine to form the various dimeric isozymes. Ethanol and acetalddehyde have conventionally served as the substrates to assay oxidation and reduction by ADH, which has been thought to be primarily an ethanol dehydrogenase. However, the large

### Table 1. Kinetic parameters for various ADH isozymes with norepinephrine-derived glycols and ethanol

<table>
<thead>
<tr>
<th>Class</th>
<th>Isozyme</th>
<th>$k_{cat}$ (HMPG)</th>
<th>$K_m$ (HMPG)</th>
<th>$k_{cat}/K_m$ (HMPG)</th>
<th>$k_{cat}$ (DHPG)</th>
<th>$K_m$ (DHPG)</th>
<th>$k_{cat}/K_m$ (DHPG)</th>
<th>$k_{cat}$ (Ethanol)</th>
<th>$K_m$ (Ethanol)</th>
<th>$k_{cat}/K_m$ (Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$\alpha_1i$</td>
<td>7.5</td>
<td>1.1</td>
<td>6.8</td>
<td>7.6</td>
<td>3.2</td>
<td>2.4</td>
<td>10.5</td>
<td>0.32</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>$\alpha_2i$</td>
<td>33.0</td>
<td>5.0</td>
<td>6.6</td>
<td>9.9</td>
<td>2.8</td>
<td>3.5</td>
<td>9.8</td>
<td>0.16</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>$\alpha_b$</td>
<td>2.0</td>
<td>1.3</td>
<td>1.7</td>
<td>3.4</td>
<td>3.0</td>
<td>1.1</td>
<td>39.0</td>
<td>0.98</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>$\beta_1i$</td>
<td>2.9</td>
<td>0.44</td>
<td>6.6</td>
<td>16.0</td>
<td>3.3</td>
<td>4.8</td>
<td>30.0</td>
<td>1.1</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>$\beta_2i$</td>
<td>9.6</td>
<td>0.94</td>
<td>10.0</td>
<td>14.0</td>
<td>2.3</td>
<td>6.1</td>
<td>14.0</td>
<td>0.21</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>$\alpha_1$</td>
<td>2.4</td>
<td>3.6</td>
<td>6.8</td>
<td>6.9</td>
<td>3.4</td>
<td>2.0</td>
<td>11.0</td>
<td>0.40</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>$\beta_1i$</td>
<td>0.30</td>
<td>2.2</td>
<td>0.14</td>
<td>1.7</td>
<td>3.1</td>
<td>0.55</td>
<td>9.1</td>
<td>0.55</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>$\gamma_2i$</td>
<td>14.0</td>
<td>1.8</td>
<td>7.8</td>
<td>11.0</td>
<td>1.1</td>
<td>10.0</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>$\pi$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>470*</td>
<td>120*</td>
<td>3.9*</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>$\chi$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined; NA, no activity noted at ≤8.0 mM substrate and 1 µM enzyme. Units are as follows: $k_{cat}$, min$^{-1}$; $K_m$, mM; $k_{cat}/K_m$, min$^{-1}$mM$^{-1}$. Values are for pH 7.4, 25°C.

*Data (obtained at pH 10) are from ref. 7.
number of isozymes of ADH suggests that they may have different metabolic functions and quite possibly act on endogenous metabolites.

Not until the constituent isozymes of human liver ADH were resolved (3–10) did it become possible to examine their kinetic characteristics and substrate specificities. Although, in general, quantitative variations in the kinetic behavior of isozymes toward different substrates are quite common, qualitative differences are less so (18). However, the three ADH classes exhibit substantial differences in activity not only toward ethanol but also toward methanol and digitoxigenins, the pharmacologically active constituents of digitoxin (7–10, 19). Moreover, ethanol competes with these toxic agents for the active site of the isozymes. Nevertheless, until now we had not observed absolute differences between the isozymes in their substrate specificities for endogenous intermediary alcohols and aldehydes, substances whose metabolism would be affected adversely by concomitant oxidoreduction of ethanol/acetaldehyde.

We here report observations pertinent to this problem, which demonstrate that the class I ADH isozymes, but not those of classes II or III, catalyze the oxidation of HMPG and DHPG, the glycol intermediates of norepinephrine and epinephrine metabolism. The present in vitro experiments cannot differentiate between the involvement of any one or all of the class I ADH isozymes in the in vivo oxidation of HMPG and DHPG. Further work is needed to localize the tissue specificity (4) of particular ADH isozymes to differentiate between those that are crucial for in vivo oxidation of these glycol intermediates but the present results extend the broad specificity of ADH to include metabolically derived alcohols.

Table 2. Kinetic parameters for various ADH isozymes with HMPG and ethylene glycol

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>HMPG</th>
<th>Ethylene glycol*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>$\alpha_\gamma$</td>
<td>23.0</td>
<td>1.3</td>
</tr>
<tr>
<td>$\beta_\gamma$</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>$\beta_\gamma$</td>
<td>16.0</td>
<td>1.8</td>
</tr>
<tr>
<td>$\beta_\gamma$</td>
<td>8.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Measurements were made at pH 10. Units are as follows: $k_{cat}$, min$^{-1}$; $K_m$, mM; $k_{cat}/K_m$, mM$^{-1}$ min$^{-1}$.

*Data are from ref. 8.

![Fig. 1](image)

**Fig. 1.** Competition of ethanol with HMPG for $\alpha_\gamma_\gamma$-ADH. Relative amounts of HMMAL produced within 20 min of incubation are plotted vs. initial ethanol concentration in 1 mM HMPG/20 mM NAD$^+$/2.0 $\mu$M $\alpha_\gamma_\gamma$-ADH/0–33.3 mM ethanol/0.1 M phosphate, pH 7.40, at 37°C. HMMAL was determined by HPLC at 340 nm.

![Fig. 2](image)

**Fig. 2.** Instantaneous inhibition of $\beta_\gamma_\gamma$-ADH oxidation of DHPG and HMPG by OP. Substrates were assayed at 2.0 (•), 4.0 (○), and 8.0 (△) mM in mixtures of 2.5 mM NAD/0.18 mM enzyme in 0.1 M phosphate, pH 7.40, at 25°C. $K_i$ values for inhibition of HMPG and DHPG oxidation are 19 and 21 μM, respectively.

These might be oxidized either at the same or possibly at different active sites of the ADH isozyme molecules. To answer this question we have examined (i) the oxidation of HMPG in the presence of ethanol; (ii) the kinetic effects of 1,10-phenanthroline, known to interact with the active site zinc atoms of all ADH isozymes, resulting in instantaneous inhibition; and (iii) the effect of 4-methylpyrazole, which inhibits the class I isozymes but not classes II and III isozymes (1). The results show that HMPG oxidation is inhibited by the alternative substrate, ethanol (Fig. 1), and that the oxidation of both ethanol and the metabolic substrates is inhibited in the same manner by either 4-methylpyrazole or OP (Figs. 2 and 3). This supports the deduction that ethanol competes with the catecholamine alcohols for the same site of the enzyme.

The present results are consistent with the facts that VMA (13) is formed as a result of HMPG oxidation in men (14, 20, 21), that this process is inhibited by ethanol in vivo (14), and that the liver is the major and dominant source of such oxidation (unpublished work). Hepatic involvement is further substantiated by the virtual absence of class I ($\alpha$, $\beta$, $\gamma$) isozymes from the central nervous system, which virtually contains only...
class III (x) ADH (unpublished work); as would then be expected, the amount of VMA in brain is minute (22).

The overall effects of ethanol on the metabolism of norepinephrine are well known (23, 24). Small amounts of ethanol increase the excretion of HMPG and decrease that of VMA. These ethanol-induced alterations have been attributed to changes in the overall tissue NAD+/NADH ratios resulting from ethanol oxidation and to competition with acetaldehyde for aldehyde dehydrogenase in the formation of acids. Most VMA is formed by oxidation of HMPG (13), not directly from deaminated and methylated norepinephrine and epinephrine. Based on the evidence presented here, the most likely reason for the observed changes in the patterns of the catecholamine metabolites subsequent to the intake of ethanol would be a direct effect on the HMPG oxidizing enzyme. Since our results indicate that HMPG and ethanol compete for ADH (Fig. 1) and that the kcat/Km for HMPG is slightly lower than that for ethanol, administration of ethanol would impair HMPG oxidation and thereby decrease VMA formation. Consistent with this view, a study in the Sprague-Dawley rat, which oxidizes HMPG less effectively than the human, revealed a 66% inhibition of [3H]HMPG oxidation 1 hour after an intraperitoneal injection of 1.5 g of ethanol/kg of body weight (14).

Even though the metabolism of DHPG has not been explored in man, the present data lead to the expectation that DHPG might also be oxidized by ADH isozymes in vivo and that this would be presumed to be the analogous major pathway for the formation of 3,4-dihydroxyxymandelic acid.

In conclusion, class I ADH oxidizes intermediary alcohols in norepinephrine metabolism with high efficiency, presumably accounting for the perturbations of related metabolite patterns after acute ethanol intake. Hence, the levels of HMPG and VMA in body fluid levels cannot be used as probes for functional norepinephrine activity without taking into account the metabolic consequences of ethanol intake, ADH action, and the pharmacological consequences of the products.

We are grateful to Christine Russian, Diane Gminski, and Dr. Charles Ditlow for their help and to Annelie Sjögren for excellent technical assistance. This work was supported by a grant from the Samuel Bronfman Foundation Inc. with funds provided by Joseph E. Seagram and Sons, Inc.
