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Isolation of Π-alcohol dehydrogenase of human liver: Is it a determinant of alcoholism?

(ethanol metabolism/multiple molecular forms/affinity chromatography)

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ABSTRACT **Human liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1), homogeneous by physicochemical criteria, has been available in quantity only recently [Lange, L. G. & Vallee, B. L. (1976) *Biochemistry* 15, 4681–4686]. Until now, the biochemical basis of human alcohol metabolism had to be extrapolated from the properties and behavior of enzymes from other species, primarily horses and yeast. The biological determinants of human alcoholism have remained obscure, although recent evidence indicates a genetic predisposition, requiring delineation. A functionally distinct form of human liver alcohol dehydrogenase (ADH), which we have designated Π-ADH, is provocative since, thus far, it seems to be unique to human beings. It has a high K_m for ethanol and is remarkably insensitive (apparent K_I, 500 μM) to pyrazole and its derivatives, which are usually potent ADH inhibitors (K_I, 1 μM), a property that is the basis for the isolation of Π-ADH. The affinity resin 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole-Sepharose binds all other known forms of ADH but not Π-ADH, thereby separating it selectively by affinity chromatography. In turn, this has led to the establishment of its identity with that enzyme form which was previously known as the anodic band and characterized by a high K_m for ethanol (20 mM at pH 7.5). The remarkable insensitivity of Π-ADH to pyrazole inhibition has also permitted quantitation of its role in hepatic ethanol oxidation. At 5 mM ethanol, a saturating concentration for virtually all other forms of ADH, Π-ADH contributes less than 15% to total ethanol oxidation. However, at intoxicating concentrations, e.g., 60 mM, it can account for as much as 40% of the total ethanol oxidation rate of liver, indicating a seemingly unique role for this enzyme form in ethanol elimination. Thus far, we have found the amount of Π-ADH varies from liver to liver of individuals and is considerably more labile than the other molecular forms, phenomena whose inter- or independence requires further study. The isolation of human Π-ADH advances efforts to recognize and understand biochemical mechanisms that may be biological determinants of alcoholism and alcohol-related disease states, now generally approached and managed largely as psychosocial disorders.**

The biochemical features of alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase; EC 1.1.1.1.) have been studied in detail in the yeast and the horse enzymes (1), but the physicochemically homogeneous human enzyme has been available for study only recently (2). It is evident that this enzyme must play a critical role in the normal metabolism of alcohol and the pathological consequence of this intoxicant. There is universal agreement that the chemical properties of ethanol—and/or its products of degradation—must relate directly to the deleterious effects of ethanol consumption, which are manifested primarily as behavioral, psychological, and socioeconomic disturbances. It seems therefore paradoxical that understanding of the pharmacological, addictive, and pathological effects of ethanol on humans is generally sought in psychosocial rather than biochemical terms. Yet, the complexity of the problem and lack of fundamental knowledge have left few but such pragmatic alternatives, since biological determinants have remained elusive and obscure, much as they have been postulated (3). A more biologically oriented view has gained considerable support in recent years from increasingly convincing evidence indicating a genetic predisposition in some individuals for the consumption of alcohol (4) and for alcoholism (5, 6). On this basis, the delineation of the genetic variability of the enzymes of ethanol metabolism has become an important objective of alcoholism research. However, little progress in the understanding of the biochemistry potentially pertinent to these deviations has been apparent.

In this context, alcohol dehydrogenase (ADH) is of particular interest, since it is the principal enzyme responsible for the elimination of ethanol (1). Human livers contain multiple molecular forms of this enzyme (7–11), the number and amounts of which vary, seemingly dependent upon the genetic background (10, 12) and the state of health of the donors (13, 14). Therefore, elucidation of the properties of each of the major enzyme forms and the assessment of their contribution to total hepatic ethanol oxidation rates are fundamental to our understanding of normal alcohol metabolism and its pathologic derangements. Moreover, a new molecular form of the enzyme has recently been discovered. Initially identified by electrophoresis on starch gels as the "anodic band" (14, 15), this enzyme form was recently purified and shown to exhibit kinetic properties that are strikingly different from those of the other molecular forms (16). Importantly, its K_m for ethanol is as much as 100 times that of the others, suggesting that it may serve a unique role in the elimination of ethanol.

As reported in this communication, yet another distinctive property of this enzyme form is its remarkable insensitivity to inhibition by pyrazole compounds, which are potent inhibitors of all types of mammalian alcohol dehydrogenases thus far studied. We have designated this new form of pyrazole-insensitive human liver alcohol dehydrogenase Π-ADH. The low affinity of Π-ADH for pyrazole compounds not only accounts for its ease of separation from the other ADH forms by affinity chromatography on 4-[3-(*N*-6-aminocaproyl)aminopropyl]-pyrazole-Sepharose (16), but also allows the elucidation of its functional role: at concentrations of ethanol that produce moderate to severe intoxication, Π-ADH can account for as much as 40% of the rate of ethanol oxidation in certain human livers.

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Abbreviations: ADH, alcohol dehydrogenase; CapGapp-Sepharose, 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole-Sepharose.

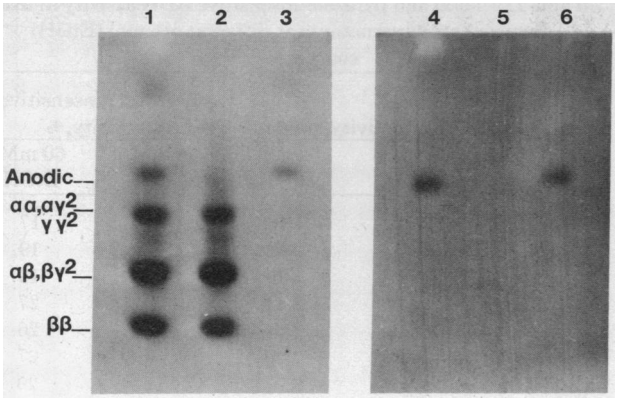

FIG. 1. Identification of the human liver ADH molecular forms in a homogenate supernatant and the enzyme fractions separated by CapGapp-Sepharose. Starch gel electrophoresis of the homogenate supernatant (samples 1 and 4) and the enzyme fraction that bound (samples 2 and 5) and did not bind (samples 3 and 6) to the affinity resin was performed at pH 7.7. Gels were stained at pH 8.5 with 100 mM ethanol in the absence (samples 1–3) or presence (samples 4–6) of 2 mM 4-methylpyrazole.

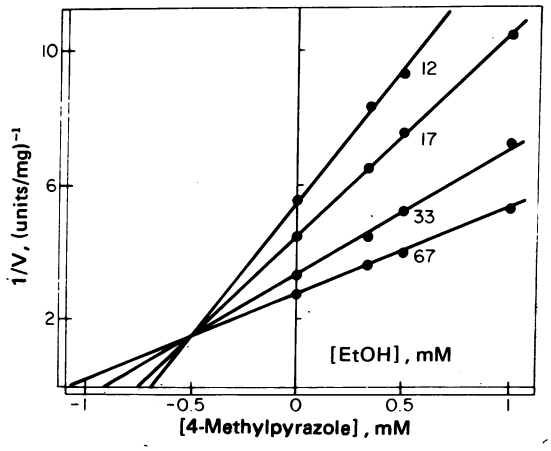

FIG. 2. Inhibition of II-ADH by 4-methylpyrazole. The purified enzyme form was assayed for ethanol oxidation with 2.4 mM NAD^+ in 0.1 M sodium phosphate, pH 7.5, in the absence or presence of 4-methylpyrazole. Data were analyzed by plotting $1/V$ against 4-methylpyrazole concentration, a Dixon plot.

METHODS

Human liver specimens were obtained at post-mortem examination from apparently healthy individuals who had succumbed to sudden death. Informed consent was obtained from the next-of-kin with assurance to protect the anonymity of the donor. Only those specimens exhibiting pH-rate profiles for ethanol oxidation at present considered "typical" (17), i.e., pH optimum at 10.5, were used. Liver samples, 5 g, were homogenized in 5 ml of 50 mM sodium phosphate, pH 7.5, and centrifuged for 60 min at 100,000 × *g* and 4°. Endogenous NAD^+-reducing activity in the absence of ethanol was removed by gel filtration of the homogenate supernatant on Sephadex G-25 (40–120 μm) in 5 mM sodium phosphate, pH 7.5. In contrast to all other forms of ADH, II-ADH does not bind to 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole-Sepharose (CapGapp-Sepharose) in the presence of 1.2 mM NAD^+ (18), and appears, with other contaminating proteins, in the breakthrough fraction. II-ADH, or the anodic enzyme form, was then purified to homogeneity by chromatography on agarose-AMP (14). The other ADH's bound to CapGapp-Sepharose could be eluted, free of all contaminating proteins, by 0.5 M ethanol.

Alcohol dehydrogenase activity was assayed in 0.1 M sodium phosphate, pH 7.5, as described by Lange and Vallee (18) and expressed as μmol of NADH produced per min (1 unit). Starch gel electrophoresis was performed at pH 7.7 as described by Li and Magnes (14); protein concentrations were determined with the biuret reagent (19). NAD^+ and Sephadex G-25 were obtained from Sigma Chemical Co., St. Louis, MO, and 4-methylpyrazole from K and K Laboratories, Plainview, NY. All other chemicals were reagent grade, and distilled water was used throughout.

RESULTS

The molecular forms of ADH contained in the supernatant of a human liver homogenate and in the enzyme fractions after separation by CapGapp-Sepharose affinity chromatography are compared in Fig. 1. The starch gels after electrophoresis at pH 7.7 were stained for ethanol-oxidizing activity in the absence (samples 1–3) and presence (samples 4–6) of 4-methylpyrazole. The enzyme form with the lowest electrophoretic mobility is II-ADH, or the "anodic band" (14). The other bands of activity correspond to the molecular forms characteristic of phenotype $ADH_3 2$, by the nomenclature of Smith *et al.* (10). The fraction that binds to the affinity column (sample 2) comprises all molecular forms in the liver extract except II-ADH (sample 3).

As might be expected on this basis, 2 mM 4-methylpyrazole inhibits all enzyme forms (Fig. 1, sample 5) except II-ADH (samples 4 and 6). This differential inhibition was examined quantitatively with a purified enzyme preparation (Fig. 2). In contrast to the other enzyme forms whose K_I for pyrazole and its 4-substituted derivatives is less than 1 μM (18, 20, 21), the apparent K_I of II-ADH for 4-methylpyrazole is 500 μM. Thus, compared with all other known forms of the enzyme, II-ADH is highly insensitive to pyrazole inhibition.

This marked difference between these enzyme forms enabled quantitation of the contribution of II-ADH to total enzyme activity in the supernatant of the above liver homogenate. At 100 mM ethanol, 0.2 mM 4-methylpyrazole inhibits the activity of purified II-ADH by less than 10%, while the rest of the purified enzyme forms are inhibited more than 90%. In the absence of 4-methylpyrazole, enzyme activity in the homogenate supernatant increases progressively when measured over a range of ethanol concentration from 0.3 to 100 mM (Fig. 3). However, in the presence of 0.2 mM 4-methylpyrazole, activity is observed only when ethanol concentration exceeds 3 mM and increases thereafter in parallel with that observed in the absence of 4-methylpyrazole. Thus, II-ADH begins to contribute significantly to total activity only at concentrations of ethanol above 5 mM, in accord with the known high K_m for ethanol, 20 mM at pH 7.5, of this enzyme form (16). At ethanol concentrations approaching saturation for II-ADH, 100 mM, *it accounts for 40% of the total activity in the homogenate supernatant.*

The contribution of the *pyrazole-sensitive* forms to total activity was calculated from the difference in activity measured in the absence and presence of 4-methylpyrazole (Fig. 3). Importantly, this activity becomes constant at about 5 mM ethanol, in agreement with the low K_m for ethanol reported previously for these molecular forms (2, 11, 20).

The percentage of the contribution of II-ADH to total ADH activity was estimated in ten liver specimens measured at substrate concentrations approaching saturation for this and for the other enzyme forms, 60 and 5 mM, respectively (Table 1). At 5 mM, the average contribution of II-ADH to total activity was 7%. In three of the samples, virtually no II-ADH activity was found at this substrate concentration. At 60 mM

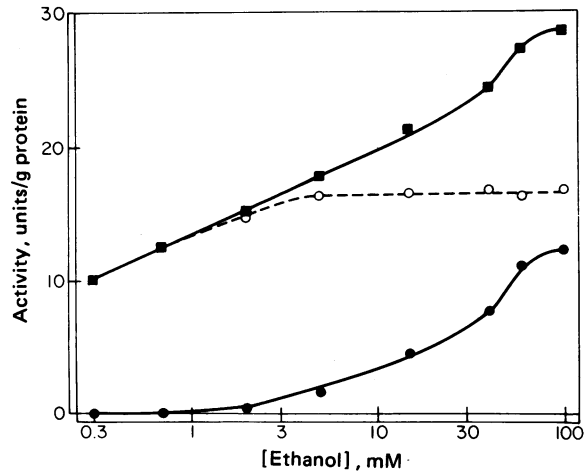

FIG. 3. Pyrazole-sensitive and -insensitive ADH activities in a liver homogenate. Alcohol dehydrogenase activity in the homogenate supernatant was determined at 0.3–100 mM ethanol with 2.4 mM NAD^+ in 0.1 M sodium phosphate, pH 7.5. Pyrazole-insensitive activity was determined in the presence of 0.2 mM 4-methylpyrazole. The difference between total and pyrazole-insensitive activity is calculated to be the pyrazole-sensitive activity (dashed line). ■, Without 4-methylpyrazole; ●, with 4-methylpyrazole.

ethanol, however, the contribution of II-ADH ranged from 17 to 39%, with an average of 27%. Although, in accord with previous observations (13, 14), total ADH activity varied significantly in the individual specimens, the sample size is not large enough to establish any correlations.

DISCUSSION

In the past, information regarding the molecular properties of human liver alcohol dehydrogenase has been incomplete as reviewed recently (22). This seems attributable in large measure to the lack of effective methods for its purification capable of yielding homogeneous enzyme in the amounts mandatory for definitive characterization. The situation has now altered decisively owing to the development of an effective affinity chromatographic procedure specific for the purification of alcohol dehydrogenases and using an immobilized derivative of pyrazole as the affinity ligand (18). In this manner, most of the molecular forms of human liver ADH can be isolated collectively and purified in high yield, allowing the characterization of their physicochemical properties (2).

The utilization of this affinity resin has further proven the existence of the "anodic enzyme form" through its physical isolation (16) and established the presence of two functionally distinct forms of alcohol dehydrogenase in human livers. One is that characterized by low K_m for ethanol (1 mM or less at pH 7.5) and low K_I for pyrazole and its 4-substituted derivatives (less than 1 μM) and comprises the molecular forms studied by most investigators in the past. The other is the molecular form previously identified as the anodic band (14, 15) and now designated II-ADH, which exhibits new and different catalytic properties. Both its K_m (15–30 mM at pH 7.5) for ethanol (14) and apparent K_I (500 M) for pyrazole and its derivatives are high (Fig. 2). The separation of II-ADH by affinity chromatography was based on this difference in K_I for pyrazole compounds, since the formation of the ternary complexes, enzyme·NAD^+·pyrazole and enzyme·NAD^+·ethanol, is the basis for specific binding to and elution from the affinity resin, respectively (18). However, II-ADH and the other ADH forms are identical in molecular weight, subunit composition, and zinc content (16).

Table 1. Total and pyrazole-insensitive ADH activity in liver homogenate supernatants at different ethanol (EtOH) concentrations

Sample	Total activity, units/g* 5 mM EtOH	Total activity, units/g* 60 mM EtOH	Pyrazole-insensitive activity, % 5 mM EtOH	Pyrazole-insensitive activity, % 60 mM EtOH
1	1.31	1.46	0	17
2	0.63	0.68	0	19
3	1.30	1.70	0	19
4	1.63	2.14	8	27
5	2.25	2.94	9	26
6	1.83	2.20	10	27
7	1.41	2.03	11	29
8	1.63	1.98	12	30
9	1.77	2.39	11	36
10	1.36	1.93	13	39
Mean	1.51	1.94	7	27

* g of liver wet weight.

The existence of II-ADH was not realized in the past, presumably owing to its considerable lability both *in vivo* and *in vitro* (14, 16), compared with the pyrazole-sensitive molecular forms. Our studies have consistently shown that the amount of II-ADH activity present in liver specimens obtained at postmortem examination from otherwise healthy individuals who had succumbed due to accidental injury varies from instance to instance (Table 1). It is clearly too early to state to what extent possible inherent biologic variation may have been modified by post-mortem change. While only one such enzyme form has been observed consistently, liver specimens have occasionally exhibited at least one other activity-staining band on starch gel electrophoresis which is pyrazole-insensitive, especially at pH 8.6. This leaves open the potential existence of yet other pyrazole-insensitive forms.

The identification of II-ADH as a functionally distinct liver alcohol dehydrogenase may bear upon the understanding of the physiology of human alcohol metabolism. It has been estimated that hepatic oxidation accounts for more than 75% of the ethanol elimination *in vivo* (23). Although several lines of evidence indicate that ADH is the principal enzyme responsible for the oxidation of ethanol in liver, quantitative assessments of its contribution to total hepatic ethanol-oxidizing capacity have largely been based on studies in experimental animals (22). The failure of pyrazole compounds to fully inhibit ethanol oxidation (24, 25) has been thought to favor the existence of alternate pathways of oxidation, e.g., the microsomal ethanol-oxidizing system (26) and/or catalase (27). Since the present study demonstrates that II-ADH can account for a substantial fraction of the ethanol oxidized, such alternate pathways or their lack in humans cannot be inferred exclusively from the effects of this compound.

The belief is held widely that the rate of ethanol elimination in man becomes maximal when ethanol concentration in blood exceeds approximately 5 mM (20, 28). Below this level, the elimination rate of ethanol is consistent with Michaelis–Menten kinetics (29) and, hence, the saturation of an enzyme system characterized by low K_m for ethanol, perhaps the pyrazole-sensitive forms of liver ADH. However, the rates of ethanol oxidation by the supernatant fractions of liver homogenates do not reach a maximum in the range of ethanol concentration expected for an enzyme exhibiting low K_m for ethanol. In fact, at 60 mM ethanol, total activity can be as much as 40% higher than that measured with 5 mM ethanol as substrate (Fig. 3 and

Table 1). This increase in activity, seemingly almost entirely due to the action of II-ADH (Fig. 3), would suggest that, *in vivo*, ethanol elimination rates should increase substantially when blood ethanol concentrations are increased to intoxicating levels.

It may be surmised that failure of previous studies to discern such effects *in vivo* is the consequence of the relatively low loading doses of ethanol customarily used, i.e., maximal blood ethanol concentrations of less than 20 mM; under these conditions, the contribution by II-ADH would still be relatively small (Fig. 3). However, a preliminary report indicates that alcohol elimination rates are 14–21% higher when measured at blood alcohol concentrations above 16 mM than when they are below this concentration (30). If substantiated, these findings would provide at the very least a partial explanation for clinical observations (31, 32) that chronically intoxicated individuals can consume considerably larger quantities of ethanol than those estimated from ethanol elimination rates measured with low loading doses of ethanol. Clearly, further investigations into this important aspect of the physiology of ethanol elimination are needed.

Because ADH is the principal enzyme responsible for ethanol oxidation in humans, its actions and properties are fundamentally related also to the pathologic effects of ethanol and to alcoholism itself. Alcoholism, defined as a pathologic state of behavioral abnormalities associated with physical and/or psychological dependence on alcohol, is a condition unique to human beings. It has been difficult to unravel the etiology and underlying mechanisms of this condition owing to the complex interrelationship among behavioral, psychosocial, and biologic determinants. However, predisposing biologic factors undoubtedly exist, as exemplified by the studies of alcoholism in the adopted children of alcoholics (6). Moreover, both ethanol-metabolizing capacity (33) and the molecular heterogeneity of liver ADH (10) appear to be under genetic control.

These considerations raise the provocative question of whether the presence or absence of II-ADH, or any of the other molecular forms already identified or other as yet unidentified forms, may prove to be biochemical links to alcoholism. Whether chronic alcohol ingestion, which induces an increase in alcohol metabolic rate *in vivo* (34, 35), alters the relative distribution and amounts of II-ADH and other enzyme forms is a particularly pertinent question. The elucidation of these interrelationships provides a realistic experimental basis from which to advance the understanding of the biological determinants of and biochemical mechanisms in alcoholism and alcohol-related diseases.

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