THE CHARACTER OF THE BINDING SITES FOR ETHANOL AND ACETALDEHYDE IN PEA ALCOHOL DEHYDROGENASE

Sylva LEBLOVÁ and Noemi ČEŘOVSKÁ

Department of Biochemistry,
Charles University, 128 40 Prague 2

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The inhibition measurements carried out with pea alcohol dehydrogenase (PADH) in the presence of acids, acid amides, and dimethyl sulfoxide show that the binding sites for ethanol and acetaldehyde are not identical. The binding site for ethanol lies in the hydrophobic domain of the PADH molecule as follows from the inhibitory action of acids: with the increasing hydrophobicity of the organic acid the inhibitory power also increases. The inhibitory effect of amides indicates that the binding site for acetaldehyde is most likely localized in the part of the enzyme molecule which is not hydrophobic. The difference in the binding sites for ethanol and acetaldehyde has been postulated before on the basis of the measurements with chloride ions, which inhibit the two substrates to different degrees, and on the basis of differences in pH-optima for ethanol oxidation and acetaldehyde reduction.

Pea alcohol dehydrogenase (EC 1.1.1.1) is a relatively stable enzyme[1] whose properties have been partly studied from the viewpoint of substrate specificity[2,3] and mechanism of action[4]. Our recent studies have been focused on the elucidation of the character of the binding sites for the two substrates and coenzymes. The character of the binding sites for the coenzymes has been discussed before[5] and some of the properties of the binding sites for ethanol and acetaldehyde are outlined in this study in which acids and their amides were used as inhibitors.

EXPERIMENTAL

Vegetal material: The plant used to start with was pea (*Pisum arvense* L., cv. Raman-Elita). The seeds were allowed to germinate 48 h on filter paper in closed Petri dishes, 15 cm in diameter. Ten ml of deionized water was used per 5 g of seeds.

Chemicals: NAD-Imuna, Šarišské Michalany, NADH-Boehringer, Mannheim, FRG, Tris-Merck A.G., Darmstadt, FRG. The remaining chemicals were of analytical purity and were supplied by Lachema, Brno.

The isolation of the enzyme was carried out as described in the preceding study[6,7].

The assay of enzymatic activity was carried out by the method of Racker[8]. The enzyme solution (0·1 ml) was pipetted into the reaction mixture containing 0·1 ml of 1M ethanol, 0·33 ml of 0·5M sodium phosphate buffer, pH 8·5, 0·5 ml of 10 mM-NAD, and 0·42 ml of H_2O. The measurements were carried out at the pH-optimum of the reaction given. One enzyme unit is defined as an absorbance change of 0·001 under the above conditions.

Determination of inhibition constants: If the inhibition is reversible the enzyme reacts with the inhibitor according to the equation $E + I \rightleftarrows EI$. The inhibitory constant regarded as the dissociation constant of the complex EI was determined by the method of Dixon[9]: The reciprocal initial rate of the reaction inhibited was plotted *versus* the varying inhibitor concentration at constant concentration of ethanol and NAD or acetaldehyde and NADH. Since we determined the type of inhibition with respect to these two substrates the experiment was always carried out at two different concentrations of these substrates and the intersection of the lines thus obtained indicated the K_i-value.

RESULTS AND DISCUSSION

It has been demonstrated that liver alcohol dehydrogenase (LADH) is inhibited by fatty acids[10]. The inhibitory effect of the first four members of the organic acid series, *i.e.* of formic, acetic, propionic, and butyric acid, on PADH has been examined. As shown in Fig. 1, the fatty acids studied are competitive inhibitors with respect to ethanol and noncompetitive inhibitors with respect to acetaldehyde. The following conclusions can be deduced from our results (Fig. 1 and Table I): The behavior of the acid toward the oxidation and reduction substrates (ethanol and acetaldehyde) is different. The decrease of the K_i-values as a function of the length of the carbon backbone indicates that, similarly to LADH (ref.[10]) the increasing hydrophobicity of the carbon chain of the acids is responsible for the inhibitory power. The negative charge of the acid anion is obviously responsible for the stabilizing effect on the binary enzyme-acid(EI) complex or the ternary E–NAD–acid (E–NAD–I) complex; the increase of the inhibition with the increasing hydrophobicity of the acid chain points to the conclusion that the binding site for ethanol is probably also considerably hydrophobic. If we examine the inhibitory effect of the acids on acetaldehyde reduction we observe noncompetitive inhibition suggesting that the ternary E–NAD–I complex is probably not formed and that the acids react most likely with the product (E–NAD) forming the complex E–NAD–I; this is actually the essence of

TABLE I

Inhibition constants K_i of acids for ethanol oxidation. Experimental conditions: 0·1M Na-phosphate buffer, pH 8·5; NAD 0·5 mM; ethanol 50—120 mM; inhibitor 0—20 mM

Acid	K_i, mM
Formic	62
Acetic	54
Propionic	42
Butyric	25

noncompetitive inhibition. The fact that the acid anions cannot compete with acetaldehyde is probably caused by the negative charge they bear.

LADH is also inhibited by acid amides[10]. We found that the acid amides have an inhibitory effect also on PADH. The effect of formamide, acetamide, and butyramide on PADH has been tested. Like with the acids the behavior of the amides toward acetaldehyde and ethanol is different; the amides, however, unlike the acids compete for the binding site with acetaldehyde; they show a noncompetitive inhibition toward ethanol (Fig. 2).

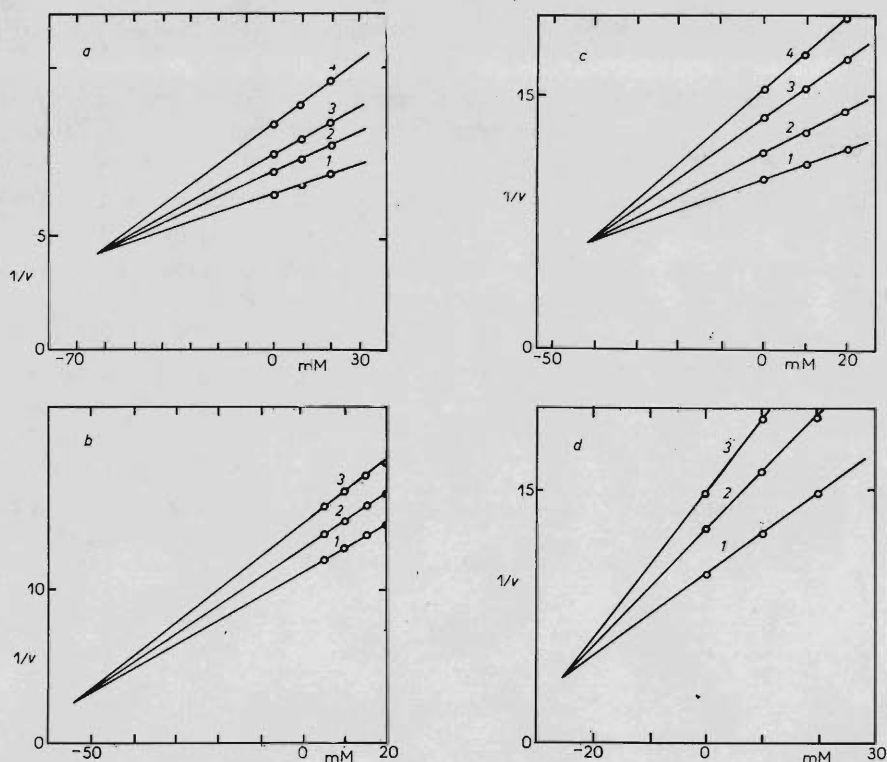

FIG. 1

Reciprocal initial reaction rate as a function of acid concentration. *a* I formic acid 0—20 mM; ethanol (mM): *1* 120, *2* 100, *3* 80, *4* 60. *b* I acetic acid 5—20 mM; ethanol (mM): *1* 100, *2* 70, *3* 50. *c* I propionic acid 0—20 mM; ethanol (mM): *1* 100, *2* 100, *3* 80, *4* 50. *d* I butyric acid 0—20 mM; ethanol (mM): *1* 120, *2* 100, *3* 50. 0·1M phosphate buffer, pH 8·5; 0·5 mM NAD; I = inhibitor

The results obtained permit us to conclude that the amides form a ternary complex E–NADH–I in the case of PADH. On this assumption only the noncompetitive behavior of the amides toward ethanol can be explained. The amides probably cannot form the E–NAD–I complex yet they form a complex with the reaction product, *i.e.* E–NADH–I. Whereas the inhibitory power of the acids increases with their increasing hydrophobicity, the situation with the amides is less clear-cut. The K_i-values characterizing the first and second member of the homologous series correspond to the situation observed with the inhibition by the acids; the inhibitory power of butyramide, however, is by one order weaker than that of acetamide (Table II). Since there exists a certain structural analogy between acids and ethanol on the one hand and acetaldehyde and acid amides on the other[11], as evidenced by the strict competition observed with PADH, it can be expected that the corresponding alcohol and acid on the one hand or the aldehyde and the acid amide on the other will analogically be bound to PADH (Table III). It is known that the K_m-value of alcohols decreases with the increasing number of carbon atoms in their chain yet the binding

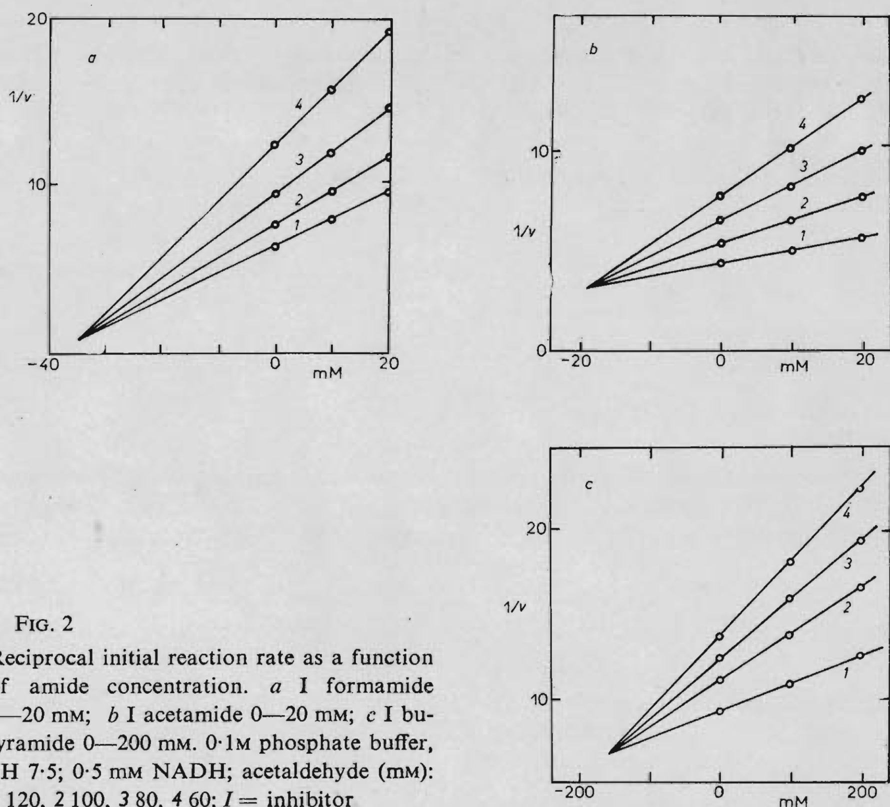

FIG. 2

Reciprocal initial reaction rate as a function of amide concentration. *a* I formamide 0—20 mM; *b* I acetamide 0—20 mM; *c* I butyramide 0—200 mM. 0·1M phosphate buffer, pH 7·5; 0·5 mM NADH; acetaldehyde (mM): *1* 120, *2* 100, *3* 80, *4* 60; *I* = inhibitor

of the acids is the strongest the higher the number of carbons in the chain. The K_m-value of the aldehydes examined[5] is not affected by the length of the chain yet the inhibitory effect of butyramide abruply decreases compared with the lower homologs. It may be therefore postulated that the length of the chain of substrates and their analogs is not the only factor affecting their binding to PADH.

If we compare the results of our inhibition measurements with the results of similar experiments carried out with LADH we can observe that the strength of the ternary complexes of LADH with fatty acids and their amides increases with the increasing length of their carbon chain (up to C_{15}). The high affinity of the long chains of acids and amides for the formation of ternary E–NAD/H/–I complexes in the case of LADH is in good agreement with the increasing rate of conversion of alcohols and aldehydes in dependence on the increasing number of carbons of the substrate. Similar measurements with yeast alcohol dehydrogenase (YADH) have been carried out with acetamide[12] only and a comparison is therefore impossible.

TABLE II

Inhibition constants K_i of acid amides for acetaldehyde reduction. Experimental conditions: 0·1M Na-phosphate buffer, pH 7·5; NADH 0·5 mM; acetaldehyde 60—120 mM; formamide, acetamide 0—20 mM; butyramide 0—200 mM

Inhibitor	K_i, mM
Formamide	35
Acetamide	20
Butyramide	160

TABLE III

Inhibition constants K_i and type of inhibition of PADH by dimethyl sulfoxide. Experimental conditions: 0·1M Na-phosphate buffer, pH 7·5 and 8·5; NADH 0·2—0·6 mM; acetaldehyde 8—12 mM; ethanol 10—100 mM; NAD 9·78 mM; dimethyl sulfoxide 0—200 mM

Substrate	K_i, mM
Ethanol, pH 8·5	46[b]
Acetaldehyde, pH 7·5	37[a]
Acetaldehyde, pH 8·5	3·4[a]
NADH, pH 8·5	20[a]

Type of inhibition: [a] competitive; [b] noncompetitive.

Inhibitory Effect of Dimethyl Sulfoxide

The hypotheses assuming differences in the binding sites for ethanol and acetaldehyde are evidenced also by the results of the inhibition measurements carried out with dimethyl sulfoxide. This compound is an inhibitor of LADH and is therefore used in alcoholism therapy. We found that PADH is also inhibited by dimethyl sulfoxide. The type of inhibition again varies with ethanol and acetaldehyde. The inhibition of ethanol is noncompetitive whereas the inhibition of acetaldehyde is strictly competitive; the inhibition of LADH by dimethyl sulfoxide is of a similar type (ref.[6]). The inhibition constant characterizing the inhibition of LADH by dimethyl sulfoxide with respect to ethanol and acetaldehyde differs by one order (10^{-2} M with respect to ethanol and 10^{-3} M with respect to acetaldehyde); this difference has not been explained so far. If we measure the inhibition constants K_i with PADH for the two directions of the reaction under identical pH (8·5), the inhibition constant with respect to acetaldehyde is by one order lower than with respect to ethanol; the situation is therefore analogous to that observed with LADH. If, however, the measurement of the inhibition is carried out at a pH close to the pH-optimum for the given reaction the K_i-values are of the same order. Since dimethyl sulfoxide inhibits PADH even at concentrations of the order of 10^{-3} M yet YADH at a relatively high concentration (10^{-1} M) only, there seems to exist an analogy between PADH and LADH rather than between PADH and YADH. The competitive character of the inhibition by dimethyl sulfoxide with respect to acetaldehyde and the partial structural resemblance of dimethyl sulfoxide with acetaldehyde permit us to assume that the inhibitor could bind to the binding site for acetaldehyde as in the case of LADH. It has been hypothesized that the site binding in LADH the carbonyl contains a zinc atom; dimethyl sulfoxide, known to form complexes with zinc salts, then interacts with this atom through an oxygen atom. The similarities in the behavior of PADH and LADH permit us to conclude that the substrate may react with the metal component of the enzyme and thus occupy one of its free valences. Since, however, dimethyl sulfoxide competes also with the coenzyme of PADH, it should be taken into consideration that it may bind also to the coenzyme binding site and hence indirectly that the coenzyme might react with the metal component of the enzyme. The competition of dimethyl sulfoxide with the coenzyme of LADH has not been observed and the inhibitor therefore acts noncompetitively[6].

The results given above provide additional evidence in favor of the conclusion that the binding sites for ethanol and acetaldehyde are different. The difference in the binding sites for the two substrates were suggested by the finding of different pH-optima for ethanol oxidation (pH 8·7) and acetaldehyde reduction (pH 7, ref.[1]). Likewise, the results obtained with chloride anions acting as competitive inhibitors with respect to ethanol and noncompetitive with respect to acetaldehyde indicate that the binding sites are not equivalent[4]. We do not account for this nonequivalence

necessarily by the existence of two different sites for the binding of each substrate but rather by a minor conformational change in the closest neighborhood of the binding site, a change caused by some amino acid residue acting as an acid-base catalyst. This conformational change may result in a far-reaching effect on the affinity for a certain substrate.

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