BINDING OF VARIOUS LIGANDS TO PEA ALCOHOL DEHYDROGENASE

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The inhibition was studied of alcohol dehydrogenase (ADH) isolated from pea seeds by lower fatty acids and their SH-derivatives, by the alkyl derivatives of berberine, and by chloride anions. The results obtained show that the character of the binding site of ADH for ethanol is hydrophobic and that this site is not identical with the binding site for acetaldehyde. Chloride anions interact with pea ADH at two different sites at least: at the binding site for the pyrophosphate group of NAD and at the binding site for ethanol.

The present state of our concepts of the structure of horse liver alcohol dehydrogenase (EC 1.1.1.1) including its interaction with various ligands[1,2] is fairly good. Fatty acids are bound to the binding site of horse liver ADH for ethanol and their inhibitory effect increases with the increasing length of the carbon chain[3-5]. This fact provides evidence of the marked hydrophobic character of the binding site for ethanol, as demonstrated also by X-ray diffraction analysis[1,2]. Another proof in favor of the hydrophobic character of the binding site for ethanol is the binding of various berberine derivatives; their hydrophobic molecule is also bound to the binding site for ethanol[6]. Numerous analogs of NAD, such as ATP, ADP, AMP (ref.[7-9]), are bound to the binding site for the coenzyme. It has been demonstrated that the part of the molecule of NAD essential for its binding to the enzyme is its adenosine moiety[10]. The interaction between various small anions (bromides, chlorides) and several binding sites for anions on the ADH molecule has also been observed (ref.[2,11-13]). Far less attention has been devoted to plant ADH's. Certain elements of the structure of animal ADH's, such as the presence of sulfhydryl groups and zinc ions in the active center are obviously also retained by the structure of plant alcohol dehydrogenases[14-17].

EXPERIMENTAL

Pea (*Pisum arvense* L. cv. *Raman Elita*) served as a source of vegetal material. ADH was prepared from pea seeds which had been allowed to swell 24 h; plant tissue fibers were homogenized and extracted with 0·1M phosphate buffer at pH 8·5. The part of material precipitated at 40—60%

saturation was used in subsequent experiments; this material was desalted on Sephadex G-25. The proteins were fractionated on a column of DEAE-cellulose, an elution gradient of 0·025 to 0·5M Tris-acetate buffer at pH 6·4 containing 10 mM L-cysteine was used[16,17]. The activity was determined by a modification of the method of Racker[16,18] in the reaction mixture (total volume 1 ml) at 20°C.

Inhibitory constants K_i were determined kinetically from Lineweaver-Burke plots[19] or according to Dixon[20]. Constants $K_{0.5}$ equal an inhibitor concentration which brings about a 50% inhibition under standard conditions.

RESULTS AND DISCUSSION

Inhibition by Fatty Acids and their Derivatives

Pea ADH is inhibited by propionic and butyric acid. The effect of fatty acids on the enzyme is different in the presence of ethanol and different in the presence of acetaldehyde. The character of the inhibition by both fatty acids in the presence of ethanol is strictly competitive and can be expressed by defined inhibitory constants (Table I). Inhibition in the presence of acetaldehyde is uncompetitive since the fatty acids (*I*) do not compete with acetaldehyde and do not form an ADH–NADH–I complex yet obviously react with the ADH–NAD product only, thus giving rise to an ADH–NAD–I complex. Similarly to liver ADH, it is the increasing hydrophobic character of the carbon backbone of fatty acids[4] which is responsible for the increasing inhibitory power in the series propionic acid – butyric acid. The negative charge of the fatty acid anion is responsible for the stabilizing effect in the ternary ADH–NAD–I complex, in analogy to the postulated formation of zinc alcoholate during enzymic oxidation of ethanol by yeast ADH (ref.[21]).

TABLE I

Inhibitory Constants Characterizing Inhibition by Fatty Acids and their SH-derivatives of Ethanol Oxidation by Pea ADH

Experimental conditions: 0·2M phosphate buffer, pH 8·5; [NAD] = 0·5 mM; [ethanol] = 10–100 mM; [inhibitor] = 0–20 mM.

Inhibitor	K_i, mM
Propionic acid	42
Butyric acid	25
2-Mercaptopropionic acid	20
3-Mercaptopropionic acid	20
Mercaptobutyric acid	5

TABLE II

Inhibitory Constants Characterizing Inhibition by Berberine Derivatives and Chloroprothixene of Ethanol Oxidation by Pea ADH

Experimental conditions: 0·1M phosphate buffer, pH 7·5; [NAD] = 50 μM; [ethanol] = 100 mM; [inhibitor] = 0–600 μM.

Inhibitor	$K_{0.5}$, μM
Berberine	500
13-Methylberberine	70
13-Ethylberberine	~30
Chloroprothixene	75

Two conclusions follow from the interaction of fatty acids with pea ADH. First, the binding site for ethanol has probably a rather strongly hydrophobic character. Second, the binding site for ethanol is not identical with the binding site for acetaldehyde and only on this assumption the competition with ethanol and the uncompetitive behaviour toward acetaldehyde can be explained. The nonequivalence of the binding sites for ethanol and acetaldehyde can be accounted for by a conformational change of the protein backbone caused, *e.g.* by some amino acid residue acting as an acid-base catalyst of the redox reaction catalyzed by ADH (ref.[21]).

Inhibition by Berberine Derivative and by Chloroprothixene

Berberine, an alkaloid, and its alkyl derivatives substituted at C-13 are inhibitors of pea ADH, as follows from the values of their inhibition constants (Table II). The influence of the substituent on C-13 on the inhibitory power of berberine derivatives toward pea ADH is quantitatively the same as that observed with horse liver ADH (ref.[6]). The increase of the inhibitory power from berberine to 13-ethylberberine is explained by polar and steric influences and also by a possible nonbinding interaction between the substituent on C-13 and the hydrogen on C-1, an interaction increasing the rigidity of the carbon backbone which may be important for the binding of the alkaloid to the enzyme[6].

The hydrophobic molecule of berberine derivatives binds to the binding site for the substrate and simultaneously partly affects the coenzyme binding site[6]. Hence, the interaction of berberine and its derivatives with pea ADH supports the hypothesis of the hydrophobic character of the binding site for ethanol. Similarly, chloroprothixene, a drug used in psychotherapy is an effective inhibitor of pea ADH (Table II). Its hydrophobic molecule most likely binds to the same site of horse liver ADH as the berberine derivatives[22].

Inhibition by Chloride Anions

Chloride anions interact with pea ADH. The values of the inhibitory constants of chloride anions determined with the four basic substrates are summarized in Table III. Chloride anions obviously bind at least to two different sites of pea ADH. As follows from the competitive character of chloride anions toward coenzymes, one of these sites is probably the binding site for the pyrophosphate group of coenzymes, *i.e.* of NAD and NADH. The second anionic binding site is probably identical with the binding site for ethanol, *i.e.* with the site where also fatty acid anions are bound. The hypothesis of the identity of the second anion-binding site with the binding site for ethanol is favored, besides by direct competition of chloride anions with ethanol, also by the noncompetitive behavior of chloride anions toward acetaldehyde; this phenomenon has been discussed above.

TABLE III

Inhibitory Constants Characterizing Inhibition by Chloride Anions of Oxidation and Reduction of Basic Substrates by Pea ADH

Experimental conditions: 0·1M phosphate buffer, pH 7·5; [NAD, NADH] = 100–800 μM; [ethanol] = 10–80 mM; [acetaldehyde] = 1·25–10 mM; $[Cl^-]$ = 0–100 mM.

Substrate	K_i, mM	Type of inhibition
NADH	80	competitive
NAD	100	competitive
Ethanol	120	competitive
Acetaldehyde	100	mixed (competitive – uncompetitive)

Hence, the inhibition of pea ADH by chloride anions is not only a quantitative expression of the affinity of anions for the site binding the pyrophosphate group of coenzymes but at the same time also a phenomenon which supports the hypothesis of nonequivalence of the binding sites for ethanol and acetaldehyde.

REFERENCES

1. Eklund H., Nordström B., Zeppezauer E., Söderlund G., Ohlsson I., Boiwe Z., Brändén C.-I.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *44*, 200 (1974).
2. Zeppezauer E., Jörnvall H., Ohlsson I.: Eur. J. Biochem. *58*, 95 (1975).
3. Winer A. D., Theorell H.: Acta Chem. Scand. *14*, 1729 (1960).
4. Theorell H., McKinley-McKee J. S.: Acta Chem. Scand. *15*, 1811 (1961).
5. Theorell H., McKinley-McKee J. S.: Acta Chem. Scand. *15*, 1834 (1961).
6. Pavelka S., Kovář J.: This Journal *40*, 753 (1975).
7. Yonetani T.: Acta Chem. Scand. *17*, Suppl. *1*, 96 (1963).
8. Yonetani T.: Biochem. Z. *338*, 300 (1963).
9. Yonetani T., Theorell H.: Arch. Biochem. Biophys. *106*, 243 (1964).
10. Abdallah M. A., Biellmann J.-F., Nordström B., Brändén C.-I.: Eur. J. Biochem. *50*, 475 (1975).
11. Coleman P. L., Weiner H.: Biochemistry *12*, 1702 (1973).
12. Coleman P. L., Weiner H.: Biochemistry *12*, 1705 (1973).
13. Shore J. D., Gutfreund H.: Biochemistry *9*, 4655 (1970).
14. Suzuki Y.: Phytochemistry *5*, 761 (1966).
15. Cossins E. A., Kopala L. C., Blowacki E., Spronk A. M.: Phytochemistry *7*, 1125 (1968).
16. Leblová S., Hlochová J.: This Journal *40*, 3202 (1975).
17. Leblová S., Mančal P.: Physiol. Plant. *34*, 246 (1975).
18. Racker E.: J. Biol. Chem. *184*, 313 (1950).
19. Lineweaver H., Burk D.: J. Amer. Chem. Soc. *56*, 658 (1934).
20. Dixon M.: Biochem. J. *55*, 170 (1953).
21. Klinman J. P.: J. Biol. Chem. *250*, 2569 (1975).
22. Skurský L., Kovář J., Michalský J.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *51*, 297 (1975).

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