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INTERACTION OF NAD WITH RAPE ALCOHOL DEHYDROGENASE

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Key Word Index—*Brassica napus*; Cruciferae; rape; alcohol dehydrogenase; coenzyme; inhibitor; interaction constant.

Abstract—Rape alcohol dehydrogenase is competitively inhibited with respect to NAD by nicotinamide, as well as by compounds containing adenine (adenine, adenosine, AMP, ADP, ATP). Adenine and adenosine are bound more firmly to the enzyme than nicotinamide. The two types of compound, as component parts of the NAD coenzyme, are bound to different sites on the enzyme. Adenine and adenosine compete for the adenine nucleotide bonding site, but they do not compete for the *o*-phenanthroline bonding site. Nicotinamide competes with *o*-phenanthroline for the binding site at which the metal is apparently present.

INTRODUCTION

Plant alcohol dehydrogenase (ADH) (EC 1.1.1.1), like the animal and yeast enzyme, is a NAD-dependent metalloprotein enzyme. It follows from inhibition studies with *o*-phenanthroline as a chelating agent that the coenzyme of the plant ADH is probably bound through the metal component, as the *o*-phenanthroline inhibition is competitive with respect to the coenzyme [1]. The present paper is an attempt to contribute to the understanding of the catalysis mechanism concerning the NAD bond to rape ADH, on the basis of further experiments with the simultaneous effect of two inhibitors.

RESULTS AND DISCUSSION

Inhibition studies

It was found earlier [2] that nucleotides ATP, ADP and AMP, as partial analogues of NAD, inhibit rape ADH. We found that rape ADH is also inhibited by adenosine and adenine. Substances like ATP, ADP and AMP inhibit rape ADH competitively with respect to NAD. It can be seen from these inhibitions that the adenosine part of the NAD molecule takes part in the interaction with the enzyme protein surface. Adenosine is bound more firmly than adenine, as follows from the inhibition constant values for these inhibitors (Table 1). The bonding is thus apparently affected by the presence and the arrangement of the furanose ring in ribose.

To ascertain whether adenosine and adenine are bound to the sites for the adenosine parts of nucleotides, which are parts of the NAD molecule, kinetic measurements were carried out always in the presence of two inhibitors competitive with the coenzyme. The measurements indicate that adenosine and adenine are bound to the same site as AMP, since the interaction constant values are infinity for pairs adenosine–AMP and adenine–AMP. As ATP and ADP compete with AMP for the same binding site [1], the measurements with ATP and ADP were not carried out.

Although bonding of nucleotides ATP, ADP and AMP is strongly pH-dependent, the bonding of adenosine and adenine does not depend on pH (Table 1). It can be concluded from these findings that phosphate groups exert a strong influence on the coenzyme bonding.

In the measurement of the enzyme activity in Tris–acetate and phosphate buffers with a phosphate concentration of up to 0.5 M, the two values were virtually identical. However, the measurements indicated that phosphate groups are important for the coenzyme bonding and their interaction with the enzyme is apparently strongly dependent on the presence of other coenzyme components, their mutual bonding and arrangement in the coenzyme molecule.

Rape ADH is also inhibited by nicotinamide, which is a part of the coenzyme molecule and this part is important for the enzymic reaction as it mediates the hydride transfer. Nicotinamide is strictly competitive with NAD and NADH. The inhibition with respect to ethanol is of mixed type (competitive–noncompetitive) and that with respect to acetaldehyde is purely noncompetitive (Fig. 1, Table 2). In view of the competition with respect to the coenzyme it can be stated that nicotinamide interacts with the coenzyme bonding site.

Table 1. Inhibition constants for adenine, adenosine and nucleotides in dependence on pH

Inhibitor	K_i(mM)		
	pH 7.5	pH 8.5	pH 10.4
Adenine	5	5	5
Adenosine	4	4	3.95
AMP*	0.08	0.072	0.045
ADP*	0.4	0.3	0.25
ATP*	1.4	0.65	0.5

*Ref. [2] Assay conditions: 0.1 M NaPi buffer (pH 8.5); 0.86 and 1.43 mM NAD; 100 mM EtOH; 0–4 mM adenine and adenosine in a total vol. of 1 ml.

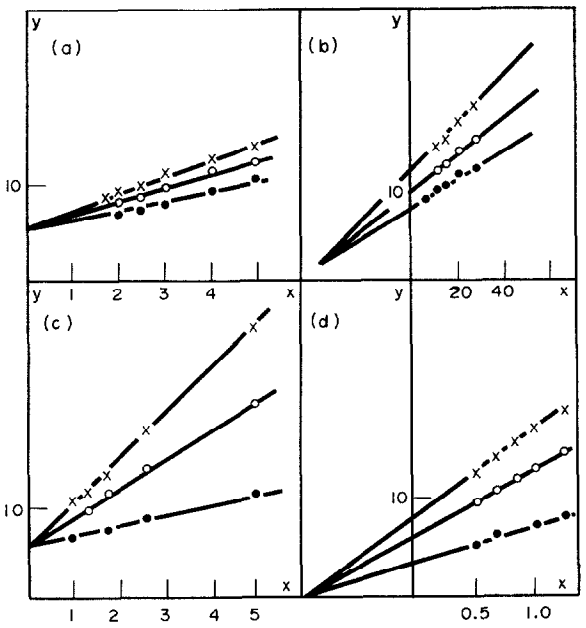

Fig. 1. Double-reciprocal plot showing effect of nicotinamide on activity of rape ADH. Abscissa. A 1/NAD (mM) B 1/ethanol (mM) C 1/NADH (mM) D 1/acetaldehyde (mM) i.e. reciprocal concentrations of basic substrates Ordinate for each graph $1/\Delta A_{366}$/min i.e reciprocal changes in A at 366 nm during 1 min. —○—○— without nicotinamide, —●—●— 50 mM nicotinamide, —×—×— 100 mM nicotinamide.

It was found previously [1] that rape ADH contains a metallic component in its molecule. In view of the inhibition by *o*-phenanthroline which is a strong chelating agent and the strict competitive character of the inhibition with respect to coenzyme, it can be assumed that the metal component is localized at the coenzyme bonding site. The values of the interaction constants for inhibitor pairs of *o*-phenanthroline with nucleotides ATP, ADP, and AMP [1] indicate that the metal component does not interact with the adenosinediphosphoribosyl part of the coenzyme, through which the coenzyme is bound to the enzyme protein molecule. Therefore, we tried to find out with which part of the coenzyme the metal component reacts. Because the types of nicotinamide inhibition with respect to basic substrates are identical with the types of *o*-phenanthroline inhibition (competitive with respect to NAD and NADH, mixed with respect to ethanol and noncompetitive with respect to acetaldehyde [1]) the kinetic measurements were performed in the presence of *o*-phenanthroline and nicotinamide, i.e. two inhibitors competitive with the coenzyme. We found that the bonding site for nicotinamide is identical to that for *o*-phenanthroline, as follows from the interaction constant value for this inhibitor pair in a complex with the enzyme which equals infinity. Thus it can be assumed that the nicotinamide part of the coenzyme interacts with the metal part of the enzyme molecule. The higher values of the inhibition constants (Table 2) indicate that this interaction is apparently weaker than that of the adenosinediphosphoribosyl part of the coenzyme with the enzyme. To verify the binding sites, further kinetic measurements were carried out in the presence of nicotinamide and ATP. It was found that the two ligands are bound to different binding sites, independently of one another, as follows from the value of their interaction constant, i.e. unity. Therefore, nicotinamide does not interact with the enzyme protein molecule at the nucleotide bonding site, i.e. with the adenosinediphosphoribosyl binding site of the enzyme.

Table 2. Inhibition by nicotinamide

Substrate	Inhibition type	K_i (mM)
NAD	competitive	32
EtOH	mixed	30
NADH	competitive	54
Acetaldehyde	noncompetitive	60

Assay condition: 0.1 M NaPi buffer (pH 8.5) for EtOH and pH 7.5 for acetaldehyde; 0.286–0.86 mM NAD; 0.286–0.86 mM NADH; 10–100 mM EtOH; 1–10 mM acetaldehyde; 0–100 mM nicotinamide in a total vol. of 1 ml.

EXPERIMENTAL

Rape seeds (*Brassica napus* L., var. Třebíčská) were germinated in a closed Perti dish (diam. 15 cm) on filter paper. 10 ml of deionized H_2O was used for 5 g of seed. Enzyme was prepared each day by the method of ref. [3]. Adenosine, nicotinamide, NAD, ATP and AMP were obtained from Koch-Light, NADH from Boehringer and other chemicals from Lachema, Brno.

Enzyme activity was determined as in ref. [3]. The following values were obtained in a statistical evaluation of the determination of alcohol dehydrogenase by the above method: $x = 413.3$; $s = 5.6$; $v\% = 1.4$.

Enzyme isolation was described previously [3]. The enzyme prepn obtained had a sp. act. of 9420 units/mg protein.

Inhibition constant K_i is considered as the dissociation constant of the enzyme–inhibitor complex and was determined by the method of ref. [4]

Kinetic measurements in the presence of two inhibitors competitive with the coenzyme If two competitive inhibitors I_1 and I_2 react with enzyme E, complexes EI_1, EI_2 and EI_1I_2 may be formed. Interaction constant α provides information about mutual interaction between inhibitors I_1 and I_2 in the complex with the enzyme, the inhibitors I_1 and I_2 interact with the same site on the enzyme, the interaction constant is infinity and complex EI_1I_2 is not formed. If inhibitors I_1 and I_2 interact with different sites on the enzyme, $\infty > \alpha > 0$ and the EI_1I_2 complex is formed. The inhibitors I_1 and I_2 can mutually interact in this complex, to a greater or lesser degree. If $\alpha < 1$, the inhibitors positively affect one another, if $\alpha < 1$, repulsion occurs between the inhibitors. The interaction constants were obtained graphically, according to ref. [5]. The measurements were carried out in a medium with a vol. of 1 ml, containing 100 mM EtOH, 0.86 mM NAD, 0.1 MNaPi buffer of pH 8.5 and two different inhibitors, the concn of one being constant and that of the other being varied. The temp. was 20°.

The kinetic constants were calculated from 6 or 9 parallel measurements. The values of inhibition and interaction constants calculated from different experiments did not differ.

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