THE BONDING OF 8-ANILINO-1-NAPHTHALENE SULFONATE TO RAPE (*BRASSICA NAPUS*) ALCOHOL DEHYDROGENASE

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Received 31 May 1979

1. Introduction

Alcohol dehydrogenase (ADH) (EC 1.1.1.1.) is an enzyme which is widely distributed in both the animal and the plant world [1]. ADH of higher plants plays an important role in the energetic metabolism of the higher plant seeds. During germination of seeds plants undergo a short period of natural anaerobiosis in which degradation of carbohydrates is the main source of energy. Most seed carbohydrates are metabolised by alcoholic fermentation, producing ethanol. ADH takes part in processes mentioned above [2].

While the liver ADH and the yeast ADH have been well investigated [1] much less is known about ADH of the plant origin.

We have found that rape ADH has a quite broad substrate specificity with respect to alcohols and aldehydes. This enzyme which is an SH-enzyme has a metal ion in its molecule [3]. The bonding of the coenzyme (NAD) to the enzyme protein molecule was also studied [4–6]. The kinetics of redox reaction catalysed by rape ADH obey the bi-bi ordered mechanism [7].

Further information about the rape ADH is given and the possible similarities between this enzyme and alcohol dehydrogenases from other sources are discussed.

2. Materials and methods

2.1. *Plant material and chemicals*

Rape seeds (*Brassica napus* L., var. Třebíčská) were used for the enzyme isolation. NAD^+ from Koch-Light, 8-anilino-1-naphthalene sulfonate Mg salt (ANS) and acrylamide from Serva, Tris from Merck and other chemicals from Lachema Brno, all of p.a. purity, were used.

2.2. *Isolation of the enzyme*

The rape seeds germinating 24 h (500 g) were homogenised and ADH was extracted with 0.1 M Na phosphate buffer pH 8.5 (1000 ml). After centrifugation (20 min, 15 000 × *g*, 4°C) the extract was precipitated with ammonium sulphate. The active fraction saturated by 35–60% ammonium sulphate was dialysed against 25 mM Tris–acetate buffer, pH 6.4, containing 10 mM mercaptoethanol (24 h) and further purified by chromatography on DEAE-cellulose DE-32 column (3.4 × 75 cm) (Tris–acetate buffer, pH 6.4, containing 10 mM mercaptoethanol; a linear gradient 25–600 mM Tris (2000 ml) was used). The fractions with the highest specific activity of ADH were collected and concentrated (ultrafiltration, lyophilisation) and then applied to a Sephadex G-100 column (4 × 70 cm). The elution was by the addition of 0.15 M Tris–acetate buffer, pH 6.4, containing 10 mM mercaptoethanol. The active fractions were further purified by gel filtration on the same Sephadex column repeatedly. The whole isolation process was carried out at 4°C.

2.3. *Disc electrophoresis on polyacrylamide gel*

Disc electrophoresis was carried out by the method of Slustr [8] in 7.5% polyacrylamide gel at pH 8.9. The proteins were detected by amidoblack 10 B in 7% acetic acid. The enzyme activity was detected by incubation of the gels in a medium containing 1.5 ml tetrazolium blue (1 mg/ml), 0.15 ml phenazine methosulfate (1 mg/ml), 5 mg NAD which was

Table 1
Isolation of rape alcohol dehydrogenase

	Activity (units)	Protein (mg)	Spec. activity (units/mg)	Degree of purification
Crude extract	138.83	15 000	0.009	1
35–60% $(NH_4)_2SO_4$ fraction	133.69	5264	0.025	2.74
Desalted fraction	92.55	4200	0.022	2.4
Fraction after DEAE-cellulose	90.50	168	0.538	58.2
Fraction after G-100 (1)	72.03	42	1.715	188.4
Fraction after G-100 (2)	12.35	2.9	4.258	467.2

added as the solid substance, 2 ml 0.2 M glycine–NaOH buffer, pH 9.5, and 0.2 ml 96% ethanol. The gels were incubated 30 min in above medium.

2.4. *Determination of the enzyme activity*

The ADH activity was measured as the increase of the absorbance at 366 nm as described in our previous paper [3]. The activity unit (table 1) is that amount of enzyme which catalyses reduction of 1 μmol NAD^+ per minute under the above conditions [3].

2.5. *Determination of the protein content*

The protein content was determined by the Lowry method [9] using serum albumin as a standard.

2.6. *Titration of the enzyme by ANS*

The dissociation constant of the binary complex ADH–ANS (K_{EB}) is defined as:

$$K_{EB} = \frac{([E] - [EB]) \cdot ([B] - [EB])}{[EB]} \qquad (1)$$

where [E], [B], [EB] are total concentrations of the enzyme, ANS and the ADH–ANS complex, respectively [10,11]. From the titration of the enzyme by ANS the dissociation constant of the ADH–ANS complex, the concentration of the active sites (bonding sites) of the enzyme and the turnover number can be obtained [12].

3. Results and discussion

3.1. *Isolation of rape ADH*

The preparation of rape ADH which has 467 times higher specific activity than the crude extract was obtained by the above enzyme isolation method

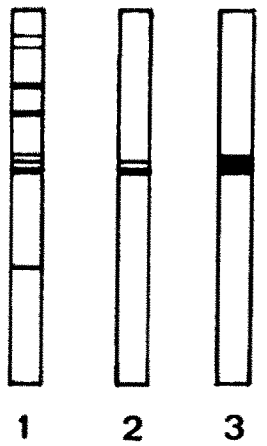

Fig.1. Disc electrophoresis on polyacrylamide gel. (1) Pattern on disc electrophoresis for sample rape ADH isolated by DEAE-cellulose column chromatography only stained with amidoblack 10B. (2) Pattern on disc electrophoresis for sample rape ADH isolated by the whole isolation process stained with amidoblack 10B. (3) Pattern on disc electrophoresis for sample as 2 stained with medium for the enzyme activity.

(table 1). This isolation process is more effective than that described in our previous paper [3]. This rape ADH preparation was fully active for some weeks; in the lyophilised state even for several months. Rape ADH obtained by above isolation process was homogeneous with respect to the molecular weight. This follows from its behaviour on Sephadex G-200 column. The gels on which isolated rape ADH was applied are shown in fig.1. When coloured by amidoblack two close lying strips of proteins were seen while only one strip was seen when coloured by medium for the enzyme activity. The gel with the strips of protein, which had been obtained by DEAE-cellulose column isolation process only has been shown in the same figure.

It can be emphasized that the isolation of alcohol dehydrogenases of plant origin is no simple matter. Hitherto the homogenous plant ADH has been obtained by the purification of the tea (*Thea sinensis*) [13] and peanut (*Arachis hypogaea*) ADH only [14].

3.2. *The turnover number and the active sites of rape ADH*

The method of determination of the plant ADH active site concentration has not been known till now. From this viewpoint some methods used in the case of horse-liver ADH were also examined with the rape ADH. Unfortunately, the methods of titration by NADH in the presence of isobutyramide or acetamide titration by pyrazole or imidazole [15] failed. The explanation of the above fact is rather complicated. It can be supposed that the non-fluorescent binary enzyme–NADH complex is present. As follows from this explanation the further methods based on fluorescence of the enzyme–NADH binary complex were also unsuccessful. The only suitable method in our case was titration method based on fluorescence of binary ADH–ANS complex. It was found that ANS interacts with rape ADH and the fluorescent rape ADH–ANS complex is present. The interaction ANS with liver ADH and the ANS bonding site to this enzyme were also studied [16]. It is known that ANS is bound to the coenzyme bonding site of liver ADH [16]. ANS can be substituted from the rape ADH–ANS complex by NADH, NAD and *o*-phenanthroline. As follows from the fact that these substances are bound to the coenzyme binding site of rape ADH [4], the ANS binding site on rape ADH molecule could

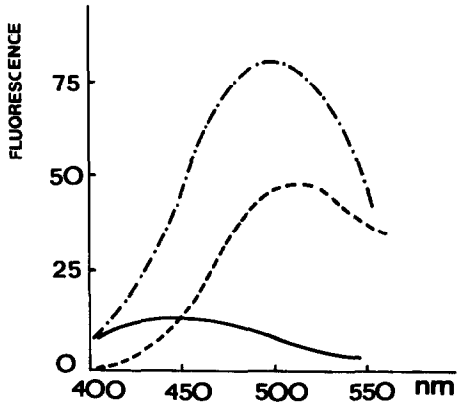

Fig.2. The fluorescent spectra of rape ADH (——, 4 μN), ANS (- - - -, 13.3 μN) and the binary ADH–ANS complex (- · - · -, 4 μN). (0.1 M sodium phosphate, pH 7.5, excitation 365 nm.)

be present in the coenzyme binding site of the enzyme or its nearest vicinity.

The fluorescent spectra of rape ADH, ANS and the ADH–ANS binary complex are shown in fig.2. A sharp shift and enhancement of the fluorescent peak of the ADH–ANS complex compared with the fluorescent peak of free ANS can be seen. The titration of ADH by ANS is shown in fig.3. The dissociation constant of binary ADH–ANS complex the value of which is 4.7 μM (pH 7.5) can be determined from above titration [12]. The turnover number in direction of ethanol oxidation can be also determined from the titration. The value of the turnover number is 11 active sites^{-1} .min^{-1} under our conditions (pH 8.5, T = 20°C, saturation by substrates). The value of the

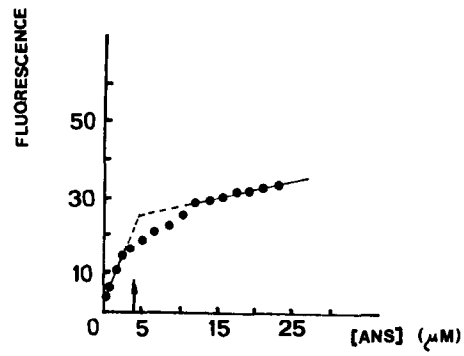

Fig.3. The fluorescent titration of rape ADH by ANS. (0.1 M sodium phosphate, pH 7.5; 4 μN ADH; 0–20 μN ANS; excitation 365 nm; emission 520 nm.)

turnover number of ADH from horse or human liver and from yeast vary from 11–60 000 active sites^{-1} .min^{-1} [17].

The number of active sites (binding sites) per one rape ADH molecule can be also determined by above titration. The rape ADH preparation used in method based on titration of the enzyme by ANS was 2.04 μM. The ANS concentration which equals the equivalence point (4 μM) can be determined from the titration curve (fig.3).

It can be concluded from results given that two ANS molecules are bond to one rape ADH molecule. As follows from the finding that ANS is probably bound to the rape ADH coenzyme binding site it can be also stated that two coenzyme molecules are bound to one rape ADH molecule, too, and the number of coenzyme binding sites per molecule of rape ADH equals two.

In a comparison of rape ADH with ADH from other sources it can be stated that two coenzyme binding sites per molecule of the enzyme were found in horse liver ADH molecule, too [15]. However four coenzyme binding sites were found in the yeast ADH molecule [19]. From the viewpoint of number of coenzyme binding sites the rape ADH is similar to the liver ADH.

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