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PURIFICATION AND PROPERTIES OF NADP-DEPENDENT GLUTAMATE DEHYDROGENASE FROM YEAST NUCLEAR FRACTIONS

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Summary

1. NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) extracted from nuclear fractions of *Saccharomyces cerevisiae* was partially purified. The final purification achieved was over 100-fold over the initial extract.

2. Cellulose acetate electrophoresis shows that the preparation is close to homogeneity and that the enzyme is slightly more anionic than cytoplasmic glutamate dehydrogenase.

3. The response of the nuclear activity to variation of pH, of inorganic phosphate and other electrolyte concentration and of the concentration of the reaction substrates has been investigated. Several differences were detected in comparison with cytoplasmic glutamate dehydrogenase.

Introduction

In a previous communication [1], the existence of a NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) in nuclear fractions of *Saccharomyces cerevisiae* has been reported. The distribution of the nuclear and cytoplasmic enzymes has been studied in wild type as well as in mutant strains.

Although in higher organisms glutamate dehydrogenase was commonly believed to be exclusively localized in mitochondria, several investigators have recently found it associated with purified nuclear fractions [2—8]. The ox liver nuclear enzyme, recently crystallized [9], was shown to differ from mitochondrial glutamate dehydrogenase, extracted from the same source, in catalytic, structural and immunological properties [10,11].

This investigation has been extended to yeast cells, in which a cytoplasmic NADP-dependent glutamate dehydrogenase has previously been described and characterized [12—14]. Data are now presented on the purification and some catalytic properties of yeast nuclear glutamate dehydrogenase. A comparison of the electrophoretic mobility of the two yeast enzymes with ox liver glutamate

dehydrogenase has also been performed. The results show that the two yeast enzymes differ in several aspects.

Experimental

Cultures. The yeast strain used for the preparation of the enzyme was a spontaneous *rho*$^-$ mutant from *Saccharomyces cerevisiae* 1511 α pts strain. The *rho*$^-$ mutant strain was chosen for its high level of enzymatic activity [1].

Cells were grown in YEP medium consisting of 1% Difco yeast extract, 2% Difco peptone and 2% glucose. Cultures were grown at 23°C with vigorous shaking.

Preparation of protoplasts and isolation of nuclei. Early stationary phase cells were collected by centrifugation and washed twice with cold distilled water. Protoplasts were prepared according to a modification [1] of the procedure of Kováč [15]. Nuclei were isolated and purified essentially following the method of Rozijn and Tonino [16], except that after centrifugation of the homogenate at 3000 × *g*, the sucrose gradient was substituted by directly suspending the nuclei in 2 M sucrose containing 8% polyvinylpyrrolidone (mol. wt. 40 000), 0.5 mM $MgCl_2$ and 20 mM potassium phosphate buffer, pH 6.5, and centrifuging at 105 000 × *g* for 1 h. By phase contrast microscope observation, the purified nuclei appeared morphologically intact and free of cytoplasmic contaminants.

Assay. The enzyme activity (reverse reaction) was measured spectrophotometrically at 30°C by recording in a 1-ml volume, the decrease in absorbance of NADPH at 340 nm; the forward reaction was measured by recording the fluorescence due to the formation of NADPH, in a 1.2 ml volume. Activity is expressed as μmol of cofactor oxidized (or reduced) per min, using a molar extinction coefficient of $6.22 \cdot 10^3$ [17], 1 μmol representing one activity unit.

Protein was determined by the method of Lowry et al. [18].

Cellulose acetate electrophoresis. Electrophoresis was carried out at 300 V on cellulose acetate strips for 25 min at room temperature, in 40 mM Veronal buffer pH 8.5 [19]. The strips were stained for protein with 0.2% Ponceau S in 3% trichloracetic acid and destained with 5% acetic acid, and for activity according to Talal et al. [20], except that NAD^+ was replaced by $NADP^+$.

Materials. Glusulase was from Endo Lab., NADPH and $NADP^+$ from Calbiochem, α-ketoglutarate from Pierce Chemicals, sodium glutamate from Sigma, cellulose acetate strips from Gelman and the anionic exchanger DE 52 from Whatman. All other reagents were of the highest purity commercially available.

Results

Purification

The purified nuclear fraction was sonically disrupted at 0°C for 3 min in the presence of 0.1 M potassium phosphate buffer, pH 7.0. Following centrifugation for 1 h at 105 000 × *g*, the activity was recovered in the supernatant. Solid ammonium sulphate was then slowly added up to 40% saturation; the suspension was centrifuged, the precipitate discarded and more ammonium sulphate was added to the suspension to bring the final concentration to 60%

saturation. After centrifugation, the sediment was suspended in a small volume of 50 mM potassium phosphate buffer, pH 7.0, and heated in a water bath for 3 min at 55°C. The precipitate was removed by centrifugation; the supernatant was extensively dialyzed against 5 mM phosphate buffer, pH 6.8, and run through a column of DE 52 (0.5 × 6 cm) equilibrated with the same buffer. Elution was carried out by means of a phosphate buffer concentration gradient made of two linear portions. Fig. 1 shows the elution profile of the column. The activity was associated to the protein peak emerging at 140 mM buffer. The fractions with the highest specific activity were pooled and concentrated by precipitation with ammonium sulphate at 60% saturation. Table I summarizes the purification outlined above. All experiments to be described were carried out with the enzyme solution resulting from the last purification step.

Cellulose acetate electrophoresis

Following the last precipitation step, the enzyme solution, after dialysis against 0.1 M potassium phosphate buffer, pH 7.0, was run on two parallel strips of cellulose acetate (see Experimental). The staining for protein revealed that the preparation was only slightly contaminated (10—15%); the major band was shown to be enzymatically active and to possess a slightly higher anodic mobility than the cytoplasmic enzyme purified from the same yeast culture (Fig. 2). The figure also shows that in comparison with the mitochondrial ox liver glutamate dehydrogenase, both enzymes from yeast appear to be proteins with a more acidic character.

Response of activity to pH variations

Fig. 3 represents the pH activity curves for the forward and reverse reactions; the latter showed optimal activity at pH 6.7, whereas the forward reaction did not reach an optimum up to pH 9.0. Under identical assay conditions, the optimal activity for cytoplasmic glutamate dehydrogenase was shown to be at pH 6.9 and 7.7 respectively; an optimum of pH 8.6 was also reported for the latter enzyme in 0.1 M Tris · HCl [14].

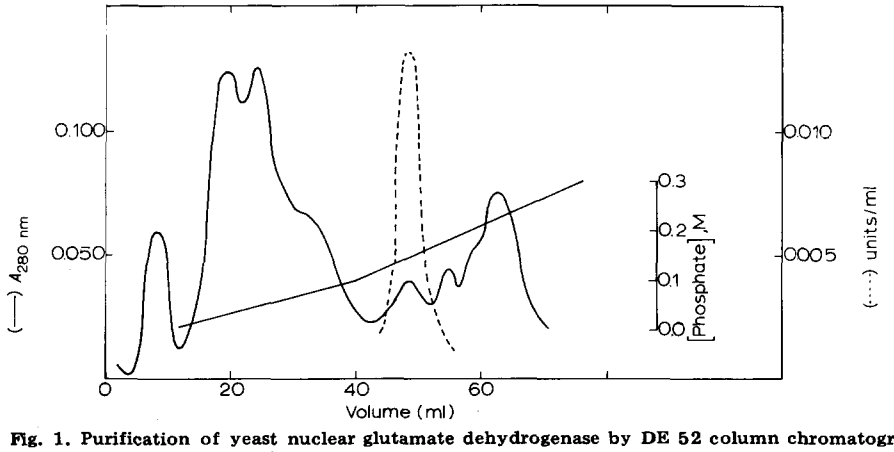

Fig. 1. Purification of yeast nuclear glutamate dehydrogenase by DE 52 column chromatography. Forward reaction (1 mM $NADP^+$, 50 mM glutamate, 100 mM potassium phosphate buffer, pH 8.0).

TABLE I

PURIFICATION OF YEAST NUCLEAR GLUTAMATE DEHYDROGENASE

The activity was measured in the reverse direction (0.1 M potassium phosphate buffer, pH 7.0; 100 mM NH_4Cl, 10 mM α-ketoglutarate, 0.1 mM NADPH). The starting material was 10 g (wet weight) of yeast cells.

Fraction	Units/ml	Vol. (ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield	Purification (-fold)
Supernatant after disruption of nuclei	0.046	27	2.0	0.023	100	1.0
$(NH_4)_2SO_4$ precipitate (40—60% sat.)	0.464	2.1	3.6	0.129	78	5.6
Supernatant after heating at 55°C	0.464	2.0	1.9	0.244	75	10.6
DE 52 eluate (after concentration)	2.032	0.2	0.8	2.540	33	110.4

Effect of inorganic phosphate and other electrolytes

The nuclear glutamate dehydrogenase activity was strongly enhanced by inorganic phosphate when the reaction was assayed in the forward direction; however, a strong inhibition was demonstrated at phosphate concentrations

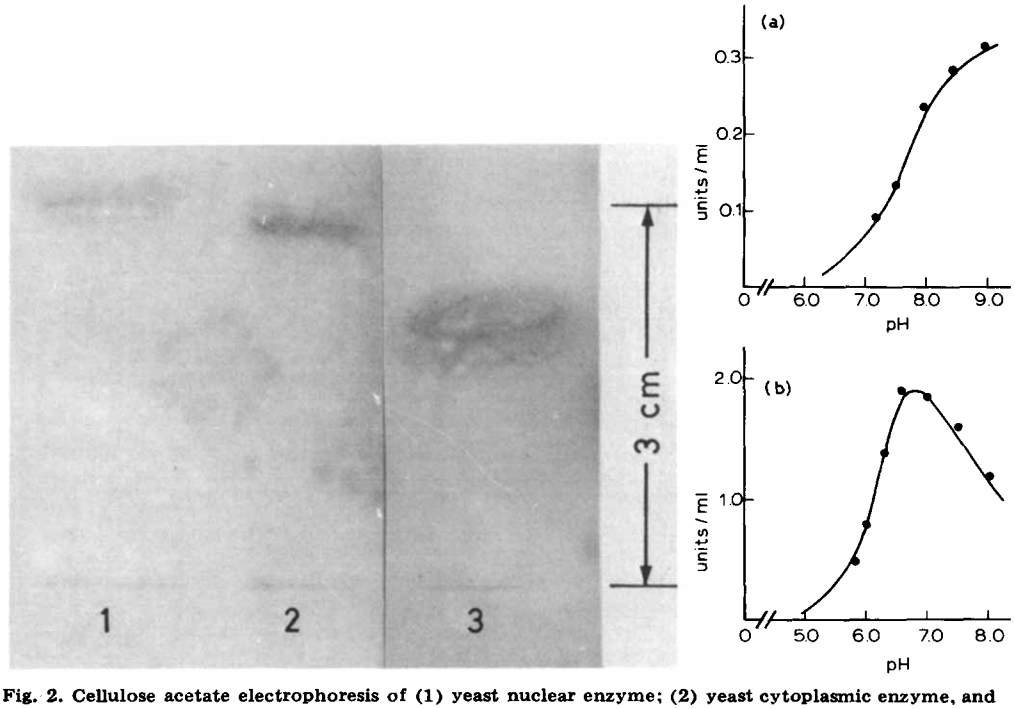

Fig. 2. Cellulose acetate electrophoresis of (1) yeast nuclear enzyme; (2) yeast cytoplasmic enzyme, and (3) ox liver mitochondrial enzyme. Staining for enzymatic activity.

Fig. 3. pH activity curves of yeast nuclear glutamate dehydrogenase. Forward reaction (1 mM $NADP^+$, 100 mM glutamate), curve a; reverse reaction (0.1 mM NADPH, 30 mM α-ketoglutarate, 100 mM NH_4Cl), curve b. 100 mM potassium phosphate buffer.

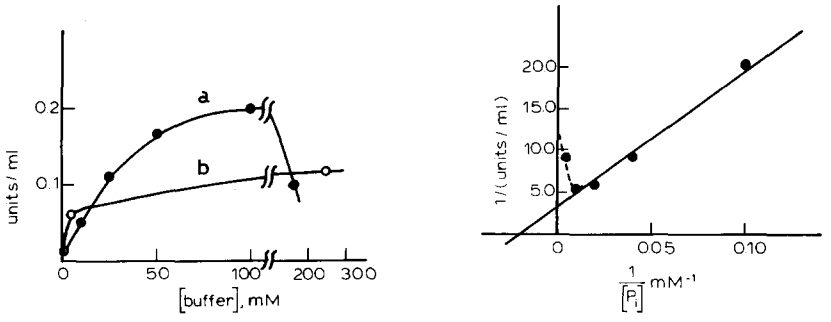

Fig. 4. Effect of inorganic phosphate (curve a) and Tris · HCl (curve b) on yeast nuclear glutamate dehydrogenase activity (forward reaction; pH 7.7, 1 mM $NADP^+$, 100 mM glutamate).

Fig. 5. Double reciprocal plot, as a function of inorganic phosphate concentration (forward reaction; assay conditions, as in Fig. 4).

higher than 0.1 M (Fig. 4, curve a). From the double reciprocal plot (Fig. 5) an "apparent dissociation constant" of 50 mM was calculated. Yeast cytoplasmic glutamate dehydrogenase was also shown to be activated by phosphate, but no inhibition at higher concentrations was reported; moreover, the "apparent dissociation constant" of the cytoplasmic enzyme was 10 mM [14].

The phosphate effect on cytoplasmic glutamate dehydrogenase was shown to be ion-specific [14]; the results of Fig. 4, curve b and Table II show that other electrolytes, such as Tris · HCl and KCl, were also effective in enhancing the activity of the nuclear enzyme.

Kinetics

Table III shows the apparent Michaelis constants of the reaction substrates and cofactors, in comparison with the values reported by other investigators [13] for cytoplasmic glutamate dehydrogenase. Yeast nuclear glutamate dehydrogenase, under experimental conditions identical to those used by other investigators [13,14], exhibited linear, not biphasic, double reciprocal plots of activity vs. NH_4^+ and $NADP^+$ concentrations.

TABLE II

EFFECT OF KCl ON YEAST NUCLEAR GLUTAMATE DEHYDROGENASE ACTIVITY

(Forward reaction, pH 7.7, 1 mM $NADP^+$, 100 mM glutamate). The numbers in parentheses represent concentration in mM.

Additions (mM)	Units/ml
Phosphate buffer (5)	0.04
Phosphate buffer (5) + KCl (100)	0.06
Phosphate buffer (5) + KCl (300)	0.08
Tris · HCl buffer (5)	0.05
Tris · HCl buffer (5) + KCl (300)	0.09

TABLE III

APPARENT K_m VALUES OF YEAST NUCLEAR AND CYTOPLASMIC GLUTAMATE DEHYDROGENASE

See other Tables and Figures for assay conditions.

Substrates	Yeast nuclear enzyme K_m (M)	Yeast cytoplasmic enzyme (13) K_m (M)
NADPH	$2.8 \cdot 10^{-5}$	$8.5 \cdot 10^{-5}$
$NADP^+$	$12.0 \cdot 10^{-5}$	$7.5 \cdot 10^{-5}$
Glutamate	$1.0 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$
α-Ketoglutarate	$4.0 \cdot 10^{-4}$	$13.0 \cdot 10^{-4}$
NH_4^+	$1.0 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$
		$1.0 \cdot 10^{-3}$

Discussion

The presence in higher organisms of a glutamate dehydrogenase activity associated with purified nuclear fractions obtained from liver and other tissues, has recently been reported by several investigators [2—8]. The characterization of the nuclear enzyme has produced evidence that the glutamate dehydrogenase activity in the nucleus is inherent to a protein that, although largely similar to the mitochondrial enzyme, nevertheless shows marked differences in structure, catalytic and allosteric behaviour [9] and immunological properties [10,11]. Thus, it appears that two different proteins with similar biological activity are present in the cell. Although the biological role of mitochondrial glutamate dehydrogenase is sufficiently understood, that of the nuclear enzyme is still unclear.

We have extended the investigation to another organism, yeast, and have shown the presence of a nuclear NADP-dependent activity in *Saccharomyces cerevisiae*; the activity distribution in some mutants has been recently reported [1]. As in higher organisms, roughly 10—15% of the total yeast NADP-dependent activity appears to be localized in the nuclear fraction; the remaining portion is in the soluble cytoplasmic phase. In contrast, in higher organisms, the non-nuclear activity is localized in the mitochondrial fraction.

The study of some of the properties of the nuclear enzyme, purified almost to homogeneity, in comparison with the cytoplasmic glutamate dehydrogenase already characterized [12—14], indicates that, also in yeast, the same activity is associated with two different proteins.

The pattern of electrophoretic mobility of the yeast enzymes reproduces the difference shown between the two enzymes from ox liver [9]. Other differences were detected in a number of kinetic properties. It is of interest to note that normal Michaelis-Menten kinetics were obtained with NH_4^+ and $NADP^+$, whereas activation by a high concentration of either substrate was reported with the cytoplasmic enzyme [13,14], which makes the behaviour of the latter more similar to that of the ox liver enzymes [21,22]. If, as in the case of the ox liver enzymes, such activation is indicative of a mechanism of allosteric regulation, one is led to conclude that the mechanism of action of yeast nuclear

glutamate dehydrogenase may lack some of the complexity characterizing that of the cytoplasmic enzyme.

Studies are under way to characterize the yeast nuclear enzyme further and to try to explain its biological significance, through the study of mutants with impaired nuclear or cytoplasmic activity.

As can be expected, considering the evolutionary gap, the yeast nuclear and cytoplasmic enzymes are profoundly different from the enzymes of higher organisms in structure, substrate specificity, allosteric behaviour and other characteristics. It is of interest to note that, nonetheless, yeast cells contain two different proteins having the same biological activity with different intracellular localization, reproducing the enzyme distribution of higher organisms.

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