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EFFECT OF ADENINE NUCLEOTIDES ON NAD-DEPENDENT ISOCITRATE DEHYDROGENASES IN RHIZOBIA AND BACTEROIDS OF LEGUME ROOT NODULES

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Summary

ATP, ADP, AMP and cyclic AMP inhibit NAD-dependent isocitrate dehydrogenase (L_s-isocitrate : NAD^+ oxidoreductase, EC 1.1.1.41) from rhizobia but have no effect on the enzyme from corresponding bacteroids. This was observed using three rhizobial strains two of which are effective, and one ineffective, with *Lotus pedunculatus*. Using partially purified enzyme from each of the three rhizobial strains it was found that the adenine nucleotides inhibit the enzyme by competing with NAD^+, not with the isocitrate.

The rate of reaction catalysed by the enzyme (expressed as activity per mg protein) in cell-free extracts of each of the effective rhizobial strains was about three times that of the reaction in extracts of the corresponding bacteroids. No correlation was found between effectiveness and NAD-dependent isocitrate dehydrogenase activity in the rhizobial cells.

Introduction

In the legume symbiotic system rhizobia, free-living soil bacteria, enter the host-plant root cells where they develop into the nitrogen-fixing bacteroids. During this transformation many biochemical changes take place in the bacterial cells and in the tissues of the host plant (for a review see Bergersen [1]). In the course of a study of the effect of nucleotides on nitrogen fixation by legume root nodules it was found that ATP, ADP, AMP and cyclic AMP inhibited NAD-dependent isocitrate dehydrogenase (L_s-isocitrate : NAD oxidoreductase, EC 1.1.1.41) but had no effect on the enzyme from the corresponding bacteroids. Here we report these results and describe experiments carried out to study the nature of inhibition of the rhizobial enzyme by these nucleotides.

Materials and Methods

Bacterial cells

Three rhizobial strains were selected for their degree of effectiveness on *Lotus pedunculatus* Cav., the host plant used in this work. These strains are 2169 (CC814S) highly effective, 1303 (CC829) moderately effective, and 3153 (NZP 2238) ineffective with *L. pedunculatus*. Rhizobial cells were harvested during the log phase from broth cultures [2].

Bacteroids were isolated by differential centrifugation [3,4] of homogenates of nodules from plants grown from surface-sterilized seed and heavily inoculated with the appropriate rhizobia strain.

Preparation of cell-free extracts

This was done by ultrasonic irradiation (3 min at 2—4 °C) or shaking with ballotini beads in the presence of octan-2-ol (0.2 ml per 50 ml) as an antifoaming agent. The latter was carried out for 3 30-s periods at full speed in a Braun-type 853030 shaker at 2—4 °C.

Preparation of calcium phosphate gel

Calcium phosphate gel (6.4% dry weight) was prepared by the method described by Kunitz [5].

Determination of isocitrate dehydrogenase activity

The rate of reaction was determined by measuring the reduction of NAD^+ at 340 nm (Unicam SP 800). The reaction mixture (3 ml) contained 75 μmol Tris · HCl (pH 7.4), 11 μmol $MgCl_2$, 2 μmol NAD^+ and 2 μmol sodium isocitrate. When crude extracts were used the enzyme assay was conducted under anaerobic conditions to eliminate the effect of an O_2-dependent NADH oxidase present in these extracts.

Determination of NADH oxidase activity

The rate of reaction was determined by measuring the oxidation of NADH at 340 nm. The reaction mixture (3 ml) contained 75 μmol Tris · HCl (pH 7.4), 11 μmol $MgCl_2$ and 2 μmol NADH.

Protein determination

This was done using Lowry's method [6].

Results and Discussion

NAD-dependent isocitrate dehydrogenase in rhizobia and bacteroids

Table I shows the rates of reactions catalysed by the enzyme in crude cell-free extracts of each of the three rhizobial strains alongside those of the extracts of the bacteroids formed by the effective strains. It will be noted that the rate of reaction (expressed per mg protein) in the extract of a rhizobial strain is about three times that of the extract of the corresponding bacteroids. There is no correlation between the rate of reaction catalysed by isocitrate dehydrogenase and the effectiveness of rhizobia with the host plant.

TABLE I

RATE OF REACTION CATALYSED BY NAD-DEPENDENT ISOCITRATE DEHYDROGENASE FROM CELL-FREE EXTRACTS OF RHIZOBIA AND CORRESPONDING BACTEROIDS

Reaction mixture was as described in the text and the assay was conducted under anaerobic conditions to eliminate the effect of the O_2-dependent NADH oxidase.

Rhizobial strain	Rate of reaction (nmol NADH/min per mg protein)	
	Rhizobia	Bacteroids
CC814S	11.3 ± 0.8 (5)	3.2 ± 0.6 (5)
CC829	14.3 ± 0.6 (5)	3.1 ± 0.3 (5)
NZP2238 *	12.8 ± 0.7 (5)	—

* NZP2238 is an ineffective strain which does not form bacteroids with *L. pedunculatus*.

Effect of adenine nucleotides on rhizobial and bacteroid isocitrate dehydrogenases

The results given in Table II show that adenine nucleotides inhibited the enzyme in crude extracts of rhizobial strain CC829 but had no effect on the enzyme from the corresponding bacteroids. Similar results were also obtained when the other effective strain CC814S was used. The effect of adenine nucleotides on the rhizobial enzyme was observed with all three strains used in the present work.

Many biochemical changes occur in rhizobial cells in the course of transformation into nitrogen-fixing bacteroids, e.g. the accumulation of poly-β-hydroxybutyrate instead of polysaccharides as energy reserves [7,8] and the development of high activity of β-hydroxybutyrate dehydrogenase [9]. The results presented here show that changes also occur in the mechanisms by which certain enzymic reactions are controlled, viz. the absence of inhibitory effects of adenine nucleotides on the bacteroid NAD-dependent isocitrate dehydrogenase. This may be related to the demand by the bacteroids for NADH

TABLE II

EFFECT OF ADENINE NUCLEOTIDES ON THE RATE OF REACTION CATALYSED BY NAD-DEPENDENT ISOCITRATE DEHYDROGENASE FROM RHIZOBIA AND CORRESPONDING BACTEROIDS

Reaction mixture (3 ml) contained 75 μmol Tris·HCl (pH 7.4), 11 μmol $MgCl_2$, 1.5 μmol NAD^+, 2 μmol sodium isocitrate and 3 μmol nucleotide. The assay was conducted using crude extracts and under anaerobic conditions to eliminate the effect of the NADH oxidase (see Table III).

Additions	Rate of reaction (nmol NADH/min per mg protein)	
	Rhizobia (CC829)	Bacteroids
None	14.0	3.0
ADP	12.5	2.9
AMP	11.0	3.0
cAMP	10.2	2.9
ATP	9.4	2.9

TABLE III

NAD-DEPENDENT ISOCITRATE DEHYDROGENASE AND NADH OXIDASE IN FRACTIONS OBTAINED DURING PARTIAL PURIFICATION OF THE ISOCITRATE DEHYDROGENASE IN EXTRACTS OF RHIZOBIAL STRAIN CC829

Isocitrate dehydrogenase assay was conducted anaerobically using a reaction mixture as described in the text. NADH oxidase assay mixture (3 ml) contained 75 μmol Tris·HCl (pH 7.4), 11 μmol $MgCl_2$ and 2 μmol NADH.

Fraction	Specific activity (nmol NAD reduced or oxidised/min per mg protein)	
	Isocitrate dehydrogenase	NADH oxidase
Crude extract	14	4.2
$(NH_4)_2SO_4$	39	0.5
Phosphate gel	82	0.2
DEAE	334	0.1

(as an electron source) and for large amounts of ATP, for the process of nitrogen fixation [10—12].

Nature of the inhibitory action of adenine nucleotides on isocitrate dehydrogenase

A partially purified NAD-dependent isocitrate dehydrogenase prepared from rhizobial cell-free extract was used in these studies. The cell-free extract (40 ml) was fractionated with $(NH_4)_2SO_4$ and the fraction precipitated between 60 and 75% saturation was dissolved in 10 ml 0.01 M Tris · HCl buffer (pH 7.4), treated with 10 ml calcium phosphate gel, and then centrifuged. The supernatant, which contained most of the enzyme activity, was subjected to DEAE chromatography in a 20 cm × 1.2 cm column and the enzyme eluted

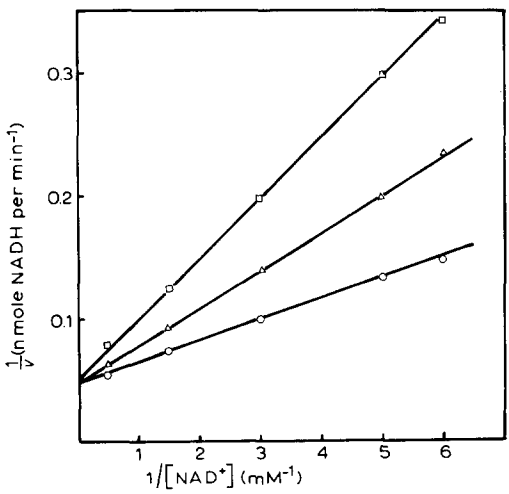

Fig. 1. Double-reciprocal plots of initial reaction velocity versus NAD^+ concentration in the absence and in the presence of two fixed ATP concentrations. ○———○, reaction without ATP; △———△, reaction with 0.5 mM ATP; □———□, reaction with 1 mM ATP. Reaction mixture (3 ml) contained 75 μmol Tris · HCl (pH 7.4), 11 μmol $MgCl_2$ and 2 μmol sodium isocitrate.

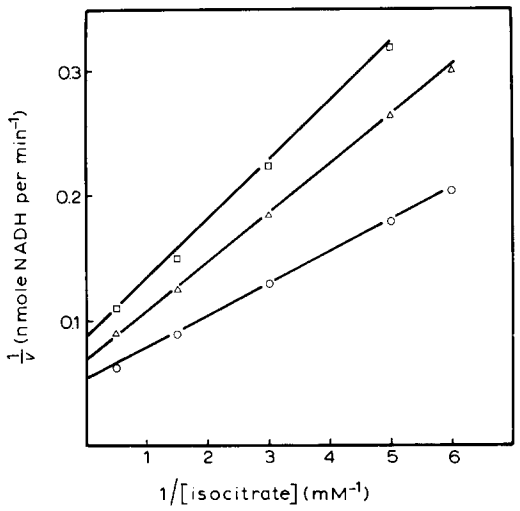

Fig. 2. Double-reciprocal plots of initial reaction velocity versus isocitrate concentration in the absence and in the presence of two fixed ATP concentrations. ○———○, reaction without ATP; △———△, reaction with 0.5 mM ATP; □———□, reaction with 1 mM ATP. Reaction mixture (3 ml) contained 75 μmol Tris · HCl (pH 7.4), 11 μmol $MgCl_2$ and 1.5 μmol NAD^+.

with 0.01 M Tris · HCl (pH 7.4) containing 22 mM KCl. The specific activity of the final preparation was about 24 times that of the crude extract and contained less than 3% of the activity of an oxygen-dependent NADH oxidase present in the crude extract (Table III).

The effect of isocitrate or NAD^+ concentration on the reaction velocity when the partially purified rhizobial enzyme was used followed Michaelis-Menten kinetics, unlike that of the yeast enzyme which followed sigmoid kinetics [13]. AMP which activates the yeast enzyme [13] and ADP which activates the myocardial enzyme [14] inhibit the rhizobial isocitrate dehydrogenase. The inhibitory effect of AMP and ADP on NAD-dependent isocitrate dehydrogenase has been reported in only a few microorganisms, for example *Pseudomonas fluorescens* and *Thiobacillus thiroxidans* [15]. AMP as well as the other adenine nucleotides inhibit the rhizobial enzyme by competing with NAD^+ for the site of binding of the enzyme. This was demonstrated by results presented in Fig. 1. When the reciprocal of initial velocity was plotted against the reciprocal of NAD^+ concentration in the presence of different concentrations of ATP straight lines with the same intercept were obtained (Fig. 1). Similar results were obtained when ADP, AMP or cyclic AMP was used. These nucleotides do not appear to compete with isocitrate for the site of binding on the enzyme (Fig. 2). The inhibition of adenine nucleotides observed here is not attributable to the effect on available Mg^{2+} as increasing Mg^{2+} concentration (up to 6 μmol/ml) does not cause any decrease in the rate of inhibition by any of these nucleotides.

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