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SEPARATION AND PROPERTIES OF THE NAD-LINKED AND NADP-LINKED ISOZYMES OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE IN *EUGLENA GRACILIS* z

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Summary

Euglena gracilis z contained two succinic semialdehyde dehydrogenases (EC 1.2.1.16), one requiring NAD and the other NADP, and these isozymes were separated from each other and partially purified. The NAD-linked isozyme was relatively stable on storage at 5°C whereas the NADP-linked one was extremely unstable unless 30% glycerol or ethyleneglycol was added. The optimum pH was 8.7 and optimum temperature 35—45°C for both isozymes. They were inhibited by Zn^{2+} and activated, particularly the NAD-linked enzyme, by K^+. Sulfhydryl reagents activated both isozymes. The K_m values for succinic semialdehyde were $1.66 \cdot 10^{-4}$ M with the NAD-linked isozyme and $1.06 \cdot 10^{-3}$ M with the NADP-linked one. The NADP-linked isozyme was induced by glutamate while the NAD-linked one was not. Probable roles of these isozymes in the physiology of *Euglena gracilis* are discussed.

Introduction

Euglena gracilis has been known to grow on glutamic acid as the sole carbon (and nitrogen) source [1], but the presence of the γ-aminobutyric acid shunt [2] or by-pass pathway [3] which involves the enzymes of glutamate decarboxylase (EC 4.1.1.15), γ-aminobutyric acid transaminase (EC 2.6.1.19) and succinic semialdehyde dehydrogenase (EC 1.2.1.16) and provides an alternative route linking glutamic acid to the tricarboxylic acid cycle, has not been reported. In the course of studying this metabolic route in *E. gracilis* z, which is of interest from the point of view of comparative glutamate metabolism, we have found that this organism contains two isozymes of succinic semialdehyde dehydrogenase with different co-factor specificities, one depending on NAD

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and the other on NADP. The present paper reports partial purification and some properties of these isozymes from *E. gracilis* z, and discusses the probable physiological roles of them.

Materials and Methods

Organism and culturing. E. gracilis z was maintained in a peptone medium. A bleached strain was derived by using streptomycin according to McCalla [4], and this strain was used throughout experiments except in induction studies. The bleached cells were cultured in the Koren and Hutner medium [5] at 25° C.

Assays. The activity of succinic semialdehyde dehydrogenase was assayed in 2 ml of reaction mixture containing 100 mM of potassium phosphate buffer (pH 8.3) for the NAD-linked enzyme or 50 mM of Tris · HCl buffer (pH 8.7) for the NADP-linked one, 1 mM of succinic semialdehyde, 0.1 mM of NAD or NADP, 1 mM of β-mercaptoethanol and enzyme. The mixture minus the enzyme was preincubated at 35° C for 3 min and the reaction was started by adding the enzyme. Formation of NADH or NADPH was followed by measuring increase of the absorbance at 340 nm with a Hitachi 124 spectrophotometer. One unit of succinic semialdehyde dehydrogenase was defined as the amount of enzyme catalyzing reduction of 1 μmol of pyridine nucleotide per min under the above assaying conditions.

The activity of succinate dehydrogenase was assayed in 2 ml of reaction mixture containing 100 mM of potassium phosphate buffer (pH 7.4), 30 mM of sodium succinate, 1 mM of KCN and 500 μg of cytochrome *c* (Sigma, type III). The reaction was started by the addition of enzyme and the absorbance at 550 nm was monitored with the spectrophotometer.

Succinic semialdehyde was determined by colorimetry after reaction with *o*-aminobenzaldehyde [6]. Protein was determined by the method of Lowry et al [7].

Chemicals. Succinic semialdehyde was prepared according to Jakoby [8] and malonic semialdehyde to Robinson and Coon [9]. Other chemicals were obtained from ordinary commercial sources.

Results and Discussion

Purification of two succinic semialdehyde dehydrogenase isozymes from E. gracilis. In a preliminary experiment the NAD-linked and NADP-linked succinic semialdehyde dehydrogenases were found to be separated from each other by column chromatography on Celite by eluting with a buffer containing a gradient of $(NH_4)_2SO_4$. The former enzyme was eluted at about 15% saturation of $(NH_4)_2SO_4$ while the latter around 40% saturation. Purification of each isozyme was carried out by including this separating procedure.

Procedures for purifying the NAD-linked isozyme are summarized in Table I. The supernatant of a homogenate (French press) of 800 g wet cells of *E. gracilis* was centrifuged at 6500 × *g* and then at 105 000 × *g*, and the supernatant submitted to chromatography on DEAE-cellulose by eluting with 5 mM potassium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and

TABLE I

PURIFICATION OF NAD-LINKED SUCCINIC SEMIALDEHYDE DEHYDROGENASE FROM *E. GRACILIS*

One unit of the enzyme is defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD per min.

Fraction	Total protein (mg)	Enzyme activity		Yield (%)	Purification (fold)
		Total (unit)	Specific (unit/mg protein)		
1. Crude extract	58 320	1184.0	0.02	100	1.0
2. Ultracentrifugation	32 320	1131.2	0.04	95.5	1.7
3. DEAE-cellulose	5 076	532.0	0.10	44.9	5.2
4. $(NH_4)_2SO_4$ fractionation	2 465	408.0	0.17	34.5	8.2
5. DEAE-cellulose	192	298.0	1.56	25.1	76.8
6. $(NH_4)_2SO_4$ fractionation	130	275.0	2.11	23.2	104.2
7. Sephadex G-150	47	153.2	3.25	12.9	159.9

1 mM EDTA. The active fractions were brought to 35% saturation of $(NH_4)_2SO_4$, and the precipitate, dissolved in the same buffer and desalted, was again chromatographed on DEAE-cellulose by eluting with 20 mM potassium phosphate buffer. The active fractions were brought to 70% saturation of $(NH_4)_2SO_4$ and the precipitate chromatographed on a Celite column with a linear concentration gradient of $(NH_4)_2SO_4$ from 35 to 0% saturation. The active fractions were concentrated and the precipitate submitted to Sephadex G-150 chromatography.

The enzyme preparation was thus purified 160-fold over the crude extract. Gel filtration with Sephadex G-150 by the method of Whitaker [10] together with several proteins with known molecular weights indicated that this isozyme had a molecular weight of 150 000.

The NADP-linked isozyme of *Euglena* succinic semialdehyde dehydrogenase was purified about 50-fold from 100 g of wet cells simply by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography as shown in Table II. To the supernatant of centrifuged homogenate of disrupted cells was added

TABLE II

PURIFICATION OF NADP-LINKED SUCCINIC SEMIALDEHYDE DEHYDROGENASE FROM *E. GRACILIS*

One unit of the enzyme activity is defined as the amount of enzyme catalyzing the reduction of 1 μmol of NADP per min.

Fraction	Total protein (mg)	Enzyme activity		Yield (%)	Purification (fold)
		Total (unit)	Specific (unit/mg protein)		
1. Crude extract	3530	602.7	0.17	100	1.0
2. $(NH_4)_2SO_4$ fractionation	750	409.5	0.55	67.9	3.2
3. DEAE-cellulose	8	63.5	7.94	10.5	46.7

$(NH_4)_2SO_4$ to about 25% saturation. The precipitate was removed, and to the supernatant was added further amount of $(NH_4)_2SO_4$ to make finally about 50% saturation. The precipitate was collected by centrifugation and dissolved in 5 mM potassium phosphate buffer containing β-mercaptoethanol, EDTA and 30% glycerol, and after desalting chromatographed on DEAE-cellulose by eluting with 20 mM phosphate buffer in the presence of 30% glycerol. The enzyme preparation had a specific activity as high as 7.94 units per mg of protein.

Stabilities of the isozymes. When the crude extract containing both isozymes of *E. gracilis* was stored at 5°C, the NAD-enzyme was so stable that it remained about 85% of the activity after 1 day, and 60% even after 3 days, whereas the NADP-enzyme lost about 90% of the activity after 1 day and it was completely inactive after 2 days. The NADP-linked succinic semialdehyde dehydrogenase was not stabilized by the addition of excess of succinic semialdehyde, NADP, a variety of metal ions, sulfhydryl reagents or proteins, but it was markedly stabilized by 30% glycerol (Fig. 1) or 30% ethyleneglycol. Similar effect of protecting enzyme activity of glycerol has been known with many enzymes, for example pyruvate kinase from *Mucor rouxii* [11].

The NADP-linked succinic semialdehyde dehydrogenase from other organisms appear to be more stable; the enzyme from *Pseudomonas fluorescens* [12], for example, lost only 10% of the activity after 2 weeks at 2°C.

Optimum pH and temperature. Both NAD-linked and NADP-linked isozymes of *Euglena* succinic semialdehyde dehydrogenase had an optimum pH at 8.7 (Fig. 2). The NADP-linked enzyme was inhibited by potassium phosphate buffer. Optimum temperature for the reaction of both enzymes, as determined by assaying the initial reaction rates at different temperatures, was 35—45°C.

Co-factor specificities. Each isozyme of the *Euglena* succinic semialdehyde dehydrogenase required a specifically linked pyridine nucleotide. NADP did not

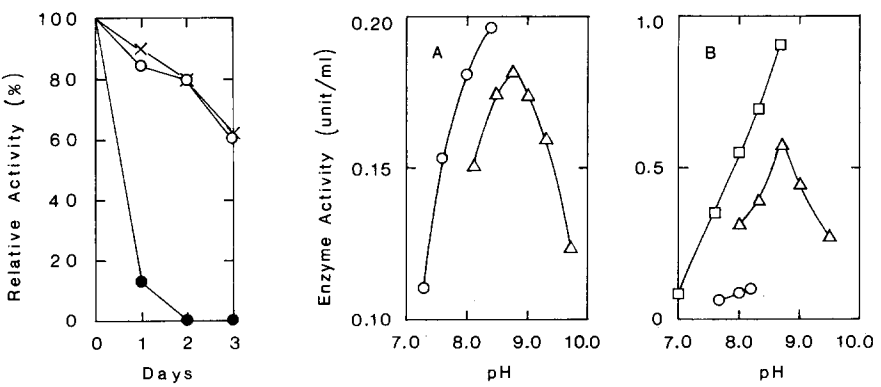

Fig. 1. Stabilities of *Euglena* succinic semialdehyde dehydrogenase isozymes at 5°C. A crude enzyme preparation in 20 mM potassium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM EDTA was allowed to stand at 5°C and assayed after intervals for the NAD-linked isozyme (○———○), and the NADP-linked isozyme in the absence (●———●) and presence (X———X) of 30% glycerol.

Fig. 2. Effect of pH on the enzyme activity. The reaction mixture contained 1 mM of succinic semialdehyde, 0.1 mM of NAD or NADP, buffer solution and 0.1 ml of the succinic semialdehyde dehydrogenase isozyme. A, NAD-linked isozyme; B, NADP-linked isozyme. ○———○, 0.1 M potassium phosphate buffer; □———□, 50 mM Tris · HCl buffer; △———△, 0.1 M glycine/KCl/KOH buffer.

replace NAD in the reaction of the NAD-linked isozyme and NAD did not take the part of NADP in that of the NADP-linked one.

Succinic semialdehyde dehydrogenases have been studied previously in some microorganisms, a plant [13] and animals. While brains [14,15], *P. fluorescens* [12] and *Rhodopseudomonas spheroides* [16] contain only one species, or no isozyme, of succinic semialdehyde dehydrogenase, other *Pseudomonas* species studied [17—19] contain two isozymes but with rather complicated co-factor specificities. Two isozymes of succinic semialdehyde dehydrogenase with strict co-factor specificity have been found in *Escherichia coli* [20] and *Saccharomyces cerevisiae* [21]. The *Euglena* succinic semialdehyde dehydrogenase belongs to the last category.

Apparent K_m values. The apparent K_m values for succinic semialdehyde were $1.66 \cdot 10^{-4}$ M for the NAD-linked isozyme and $1.06 \cdot 10^{-3}$ M for the NADP-linked one in the presence of 0.2 mM of co-factors. Succinic semialdehyde showed substrate inhibition at higher than 2 mM on the NAD-linked enzyme and at higher than 1 mM on the NADP-linked one. The apparent K_m values for the co-factors were $0.71 \cdot 10^{-4}$ M for the NAD-enzyme and $0.38 \cdot 10^{-4}$ M for the NADP-enzyme.

Substrate specificity. With malonic semialdehyde as the substrate, the NAD-linked isozyme exhibited 3.8% of the activity observed with succinic semialdehyde while the NADP-linked one showed 8.7% of the activity. Glyoxylic acid, formic acid, formaldehyde, acetaldehyde, glyoxal, furfural and acrolein did not serve as the substrates for either isozyme. The results indicate that the *Euglena* succinic semialdehyde dehydrogenases have rather narrow substrate specificity in comparison to the enzymes from other organisms. Malonic semialdehyde served as a substrate for the NAD-linked and NADP-linked succinic semialdehyde dehydrogenases of *Pseudomonas aeruginosa* [17] in 5—15 and 30%, respectively, of the activities on succinic semialdehyde. Glyoxylic acid which has been reported to serve as a substrate for succinic semialdehyde dehydrogenases of many other organisms was rather a competitive inhibitor of succinic semialdehyde with the *Euglena* enzymes. The K_i values were $2.50 \cdot 10^{-4}$ M with the NAD-enzyme and $2.64 \cdot 10^{-3}$ M with the NADP-enzyme in the presence of 10^{-4} M co-factors.

Effects of metal ions. K^+ in 5 mM augmented the activity of the NAD-linked isozyme by 84% but that of the NADP-linked one only by 10%. K^+ appeared to contribute to the stabilization of the NAD-enzyme, since in its absence the enzyme was unstable on heating. Other monovalent cations had no significant effects on both isozymes. Divalent ions examined (Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+}) worked inhibitive; the effect was highest with Zn^{2+} particularly on the NADP-linked isozyme. The effects of metal ions on the *Euglena* succinic semialdehyde dehydrogenase are considerably different from those reported on the succinic semialdehyde dehydrogenase of *P. aeruginosa* [17] which is activated by Mg^{2+}, Mn^{2+} and Ca^{2+}.

Effects of sulfhydryl reagents and sulfhydryl inhibitors. Both isozymes of the *Euglena* succinic semialdehyde dehydrogenase were activated by β-mercaptoethanol and dithiothreitol; the effect of the latter was stronger, particularly on the NADP-linked isozyme (Table III). Such sulfhydryl inhibitors as *p*-chloromercuribenzoate, 5,5′-dithio-bis(2-nitrobenzoic acid) and *N*-ethylmale-

TABLE III

EFFECTS OF SULFHYDRYL REAGENTS AND INHIBITORS ON SUCCINIC SEMIALDEHYDE DEHYDROGENASE ISOZYMES FROM *E. GRACILIS*

Reaction mixtures minus succinic semialdehyde were preincubated for 3 min together with the sulfhydryl reagents or inhibitors and the reactions were started by adding succinic semialdehyde.

Addition		Relative activity (%)	
Compound	Concentration (M)	NAD-linked succinic semialdehyde dehydrogenase	NADP-linked succinic semialdehyde dehydrogenase
None		100	100
β-Mercaptoethanol	$1 \cdot 10^{-3}$	106.8	105.8
Dithiothreitol	$1 \cdot 10^{-3}$	117.6	178.4
p-Chloromercuribenzoic acid	$1 \cdot 10^{-5}$	0	0
5,5'-Dithio-bis(2-nitrobenzoic acid)	$1 \cdot 10^{-5}$	66.6	29.4
N-Ethylmaleimide	$1 \cdot 10^{-5}$	82.3	68.6
	$1 \cdot 10^{-4}$	0	0

imide strongly inhibited both enzymes. The results suggest that the sulfhydryl group plays an important role in exhibiting the activity in both isozymes like in the succinic semialdehyde dehydrogenases from other organisms.

Induction of the succinic semialdehyde dehydrogenase isozymes. Induction of the isozymes by glutamate was studied by adding glutamate after autotrophic culture of the original green strain and after culture of the bleached strain on glucose as the sole carbon source, and by determining the activities of the NAD-linked and NADP-linked isozymes of succinic semialdehyde dehydrogenase as well as succinate dehydrogenase, the marker enzyme of mitochondria, after intervals. The results are shown in Fig. 3.

It is evident from the data that after both types of preculture the addition of glutamate increases the activities of succinate dehydrogenase and of the NADP-linked succinic semialdehyde dehydrogenase but does not change the activity of the NAD-linked isozyme. When cycloheximide (15 μg/ml of culture) was added together with glutamate, the increase of the activity of the former two enzymes was not observed indicating that the increase of the enzyme activity by glutamate is due to induction but not to activation.

In two species of *Pseudomonas* [18,19], the NADP-linked succinic semialdehyde dehydrogenase has been reported to be constitutive while the NAD-linked one inducible. In *E. coli* [20] both isozymes are inducible, but the NAD-linked enzyme is induced more extensively (200-fold) than the NADP-linked one (10-fold). The pattern of induction in the *Euglena* succinic semialdehyde dehydrogenase isozymes differs from these data. It is noted, however, that the NADP-linked isozyme of succinic semialdehyde dehydrogenase of *E. gracilis* may also be constitutive since the total activity of this enzyme was higher than that of the NAD-linked isozyme even in the absence of glutamate, and the increase of the enzyme activity by glutamate induction was only 2-fold.

Probable physiological roles of the isozymes. The NADP-linked isozyme of *Euglena* succinic semialdehyde dehydrogenase has an apparent K_m for succinic semialdehyde about six times larger than the NAD-linked isozyme, or the latter

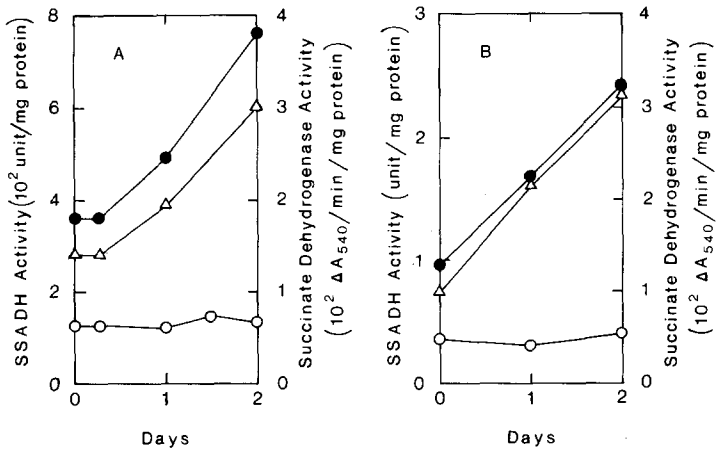

Fig. 3. Inducibilities of the *Euglena* succinic semialdehyde dehydrogenase isozymes and succinate dehydrogenase by glutamate. (A) Green strain of *E. gracilis* was cultured autotrophically in a modified Koren and Hutner medium containing no carbon sources at all under lighting of 2000 lux, and into the culture of the stationary phase (3 weeks after inoculation) 4 g/l of monosodium glutamate was added. (B) Bleached strain of *E. gracilis* was cultured in another modified Koren and Hutner medium in which all carbon sources were replaced by 12 g/l of glucose, and into the culture of the stationary phase (8 days after inoculation) 4 g/l of glutamate was added. In both cases the activities of the three enzymes were assayed after intervals up to 2 days. ○———○, NAD-linked isozyme of succinic semialdehyde dehydrogenase (SSADH); ●———●, NADP-linked isozyme of succinic semialdehyde dehydrogenase (SSADH); △———△, succinate dehydrogenase.

has a higher affinity toward the substrate, and the NADP-linked enzyme is induced by glutamate whereas the NAD-linked one is not. These results suggest that the NAD-linked isozyme mainly operates under ordinary cellular conditions of low succinic semialdehyde concentrations, and as glutamate is accumulated the NADP-linked succinic semialdehyde dehydrogenase is inductively synthesized to meet the augmented flow of substrate through the γ-aminobutyric acid shunt which bypasses the glutamate.

TABLE IV

COMPARISON OF MAJOR PROPERTIES OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE ISOZYMES FROM *E. GRACILIS*.

	NAD-isozyme	NADP-isozyme
Co-factor specificity	NAD	NADP
Activity remaining after 24 h at 5°C	85%	10% (90%) *
Optimum pH	8.7	8.7
Heat stability (up to)	30°C	60°C *
Optimum temperature,		
in sodium phosphate buffer	20°C	35—45°C
in potassium phosphate buffer	35—45°C	35—45°C
K_m for succinic semialdehyde	1.66×10^{-4} M	1.06×10^{-3} M
K_m for co-factor	0.71×10^{-4} M	0.38×10^{-4} M
Effect of K^+ (activation by 5 mM)	84%	10%
Effect of Zn^{2+} (inhibition by 1 mM)	43%	100%
Effect of SH reagents	Activated	Activated
Induction by glutamate	None	Induced

* In the presence of 30% glycerol.

Comparison of properties of the Euglena succinic semialdehyde dehydrogenase isozymes. Table IV lists the properties of the two isozymes of succinic semialdehyde dehydrogenase from *E. gracilis.* The data include those not described in the text.

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