Alternative system of succinate oxidation in glyoxysomes of higher plants

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Abstract Succinate oxidation in scutella of germinating seeds of wheat and maize was investigated. Besides oxidation via succinate dehydrogenase (SDH; EC 1.3.99.1), an alternative path of succinate oxidation insensitive to SDH inhibitors – malonate and thenoyltrifluoroacetone (TTFA) – was revealed. Using isopicnic sucrose gradient it was shown that this path is localized in glyoxysomal membranes. Glyoxysomal succinate oxidase (GSO) converts succinate directly into malate with the production of hydrogen peroxide identified using auxilary enzymes malate dehydrogenase and peroxidase. GSO is most active during the intensive operation of the glyoxylate cycle (3–5 days of germination). Quinacrine, the inhibitor of flavine-containing oxidases, strongly suppressed the activity of GSO. K_m for succinate is 18 mM for GSO from maize scutellum. It is concluded that in scutella of cereal seeds the glyoxysomal succinate oxidation non-linked with ATP synthesis operates.

Key words: Glyoxysomal succinate oxidase; Succinate dehydrogenase; *Zea mays*

1. Introduction

In oil-storing tissues of germinating seeds, the glyoxylate cycle intensively utilizes acetyl CoA generated in β-oxidation of fatty acids resulting in succinate production. It is always proposed that the latter is transported from glyoxysomes to mitochondria where it is converted by the reactions of the TCA cycle, and further turns to gluconeogenesis. It was shown that in castor bean endosperm the branch of the TCA cycle from succinate to oxaloacetate is strongly intensified, and oxidation of other substrates is realized via the cyanide-resistant oxidase [1]. In soybean cotyledons succinate itself is oxidized via the cyanide resistant path which escapes it from adenylate control [2]. In scutellum of maize and of other cereals, the glyoxylate cycle may be not tightly connected with gluconeogenesis, so as carbohydrates are supplied from endosperm [3]. The main function of scutellum may be the acidification of a milieu for the activation of hydrolases. It was proposed earlier that in this case succinate may be oxidized directly in glyoxysomes [4], but concrete pathway was not determined.

In the present paper we characterize the glyoxysomal oxidation of succinate and investigate the effect of inhibitors of electron transport and metabolism on this process.

2. Materials and methods

Glyoxysomes and mitochondria from scutella of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) etiolated seedlings of age 2–7 days were isolated using differential and isopicnic centrifugation in sucrose gradient as described elsewhere [5]. The purity of organelles was controlled by the activity of marker enzymes catalase (for glyoxysomes) [6] and fumarase [7] and succinate dehydrogenase (for mitochondria) [8]. Organelles were washed twice in 50 mM Tris-HCl buffer, pH 7.8, containing 0.4 M sucrose, 10 mM KCl and 1 mM EDTA. Cross-contamination was less than 2% for glyoxysomes and 7% for mitochondria. Organelles were broken using osmotic shock in the same buffer without sucrose. In certain variants it contained detergents (0.05% digitonin, 0.01% Tween-80 or 0.01% Triton X-100).

The activity of GSO was measured spectrophotometrically by the production of malate or hydrogen peroxide in 50 mM Tris-HCl buffer, pH 7.8, containing 20 mM succinate. Malate was detected by the increase of NADH at 340 nm after the addition of malate dehydrogenase (EC 1.1.1.37; 5 U/ml) and 0.6 mM NAD (final concentrations). The formation of H_2O_2 was measured at 435 nm by the addition of peroxidase (EC 1.11.1.6; 10 U/ml) and 0.5 mM *o*-dianisidine [9].

Succinate dehydrogenase activity was measured using artificial electron acceptors dichlorophenolindophenol (DCPIP) and phenazine methosulphate (PMS) at 600 nm [8] in 50 mM Tris-HCl buffer, pH 7.8, containing 1–20 mM succinate, 1 mM KCN, 0.1 mM PMS, 0.005 mM DCPIP.

For inhibition of succinate oxidizing enzymes we used 1–20 mM malonate, 5–8 mM TTFA or 0.5 mM quinacrine. The quenching of superoxide-free radicals was carried out with Nitrotetrazolium blue according to [10].

Electrophoretic detection of succinate oxidizing enzyme was conducted according to Davis [11] in 7.5% polyacrylamide gel. After separation gels were incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM succinate, 1 mg/ml Nitrotetrazolium blue, 1 mg/ml PMS and 40 U/ml malate dehydrogenase (for GSO). Malonate (up to 100 mM) was used for the inhibition of succinate oxidase activity.

Metabolism of succinate by isolated glyoxysomes and mitochondria was investigated using [2,3-^{14}C]succinate. Organelles isolated from 5 g of scutella of 4-day-old maize seedlings were put into 2 ml of 50 mM Tris-HCl buffer containing 15 mM succinate-Na (radioactivity 10 kBq/ml) and incubated for 10 min. $^{14}CO_2$ was absorbed by monoethanolamine (0.2 ml in the side arm of Warburg flask). Chromatographic separation of the products of succinate metabolism was carried out as described earlier [12]. Radioactivity was measured by liquid scintillation counter SBS-2 (Russian Federation).

3. Results

During the incubation of isolated glyoxysomes from maize scutella with [2,3-^{14}C]succinate, the latter was metabolized mostly in malate (Table 1). The radioactivity of other organic acids and CO_2 was insignificant. In ruptured glyoxysomes the accumulation of malate was more intensive (by 30%), and radioactivity of other metabolites was even lower. In ruptured mitochondria the main part of radioactivity from succinate was detected in fumarate. In malate only traces of radioactivity could be observed.

Using auxilary enzyme malate dehydrogenase, we also showed that the product of the glyoxysomal succinate converting enzyme is malate. Using peroxidase, the stoichiometric production (one mole per one mole of converted succinate and produced malate) of H_2O_2 was detected. The activity of GSO

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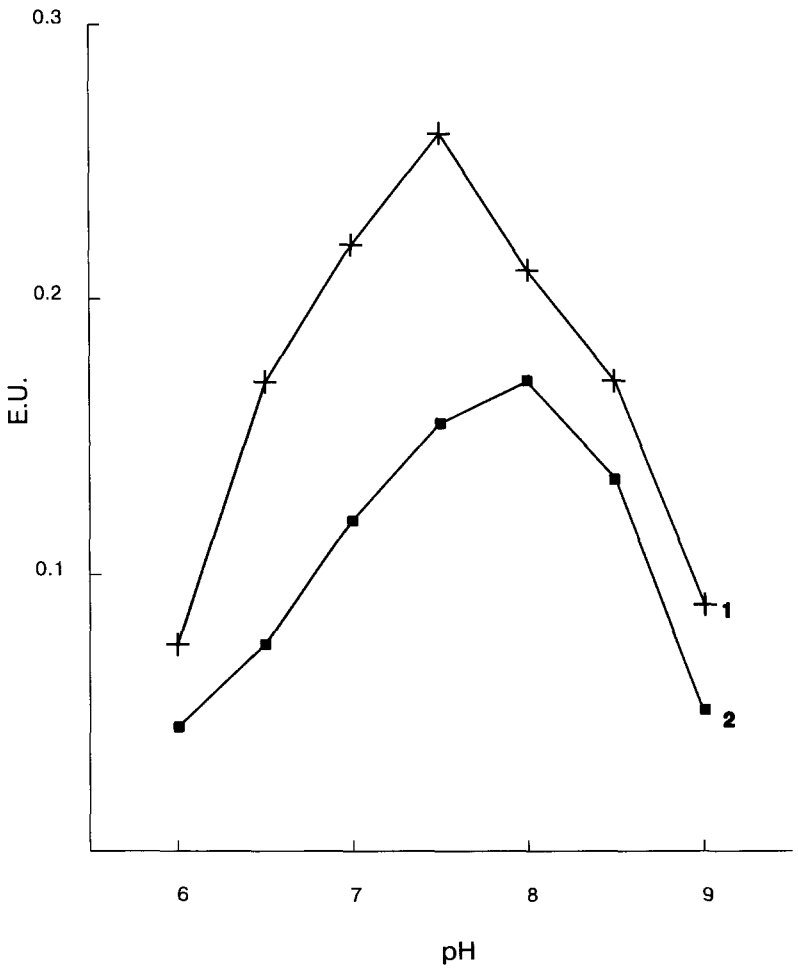

Fig. 1. pH dependence of activities of succinate dehydrogenase (1) and glyoxysomal succinate oxidase (2) from maize scutellum.

in the glyoxysomal fraction of maize was 0.315 mmol of NAD formed in one minute during oxidation of malate produced by GSO per 1 mg of total protein. This corresponds to 12-fold purification compared to the coarse fraction of cell organelles (mitochondria and glyoxysomes) and about 40-fold purification compared to the tissue homogenate of maize scutellum.

The inhibitor of SDH malonate (from 5 to 20 mM) had no detectable influence on GSO activity. TTFA (5–8 mM) which inhibits succinate:ubiquinone reductase was also uneffective against GSO. Quinacrine, the inhibitor of flavine-containing enzymes, strongly suppressed GSO activity: in its presence in 0.5 mM concentration the GSO activity was not detected.

Investigation of some catalytic properties of GSO showed that K_m (succinate) is 18 ± 4 mM. K_m SDH (succinate) is 0.8 mM. pH optimum of GSO is 8.0 (Fig. 1). pH optimum of SDH is 7.5.

Electrophoretic investigations of isolated mitochondria and glyoxysomes from maize scutella revealed the activity of succinate oxidizing enzymes in both organelles. This activity was detected via electron transfer to Nitrotetrazolium blue in the presence of phenazine methosulphate. Malate dehydrogenase was necessary for revealing of succinate oxidative activity in glyoxysomes. Four isoforms of SDH highly sensitive to malonate inhibition were detected in mitochondria. In glyoxysomes one form of succinate oxidizing enzyme which was insensitive to malonate even in 100 mM concentration was clearly identified. Its R_f is 0.55 whereas R_fs of mitochondrial SDH isozymes are 0.08, 0.16, 0.37 and 0.50.

The pattern of change of GSO and SDH activities during germination of maize seedlings is presented on Fig. 2. The activity was maximal for both enzymes at the 4–5th days of germination, i.e. at the period of the most intensive operation of the glyoxylate cycle. The SDH activity was about twice higher than that of GSO.

GSO activity was detected also in scutella of wheat seedlings, but it was absent in soybean and sunflower cotyledons and

Table 1
Incorporation of radioactive label to organic acids after incubation of organelles with [2,3-^{14}C]succinate

Organic acid	Intact glyoxysomes	Broken glyoxysomes	Intact mitochondria
Succinate	18,320	19,900	19,600
Malate	3,100	4,700	130
Fumarate	70	80	4,300
Glycolate	950	800	300
Citrate	100	100	300
CO_2	80	100	100

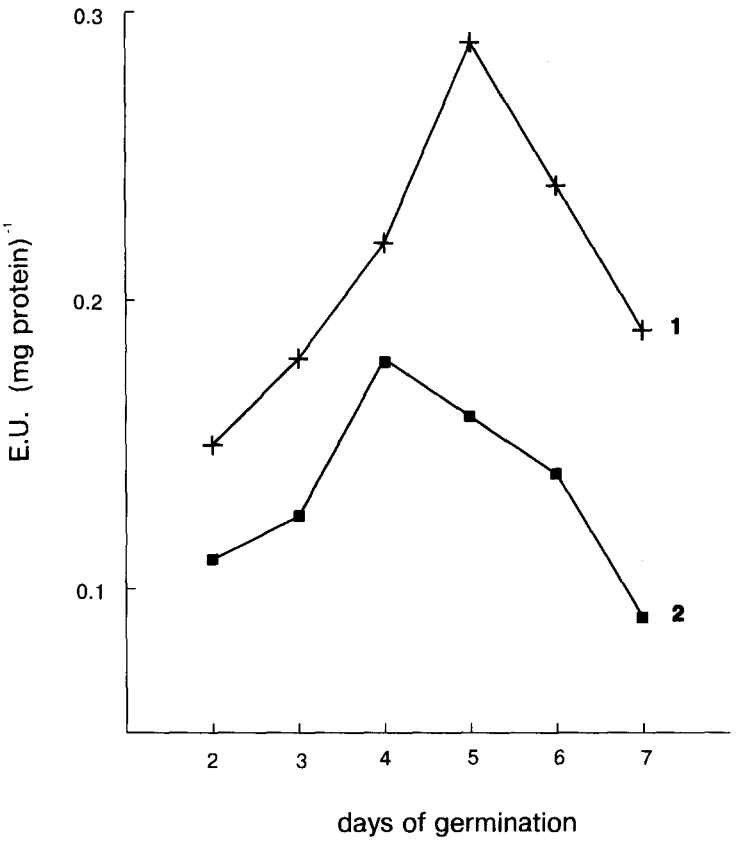

Fig. 2. Changes of activities of succinate dehydrogenase (1) and glyoxysomal succinate oxidase (2) in maize scutellum during germination.

castor bean endosperm (Table 2), whereas SDH activity in scutella of maize and wheat was about two-fold lower as compared to soybean, sunflower and castor bean.

In the presence of 5 mM succinate, the intensity of superoxide quenching by glyoxysomal fraction increased by 56%. This effect was observed only for microbodies from scutellum of maize and wheat, but not for these organelles from maize and wheat leaves, castor bean endosperm and sunflower cotyledons.

4. Discussion

The presented data shows that in glyoxysomes of cereal seedlings (but not of other fat-storing plants) an alternative enzyme which oxidizes succinate is present. It is less specific than SDH (K_m is much higher) and it converts succinate to malate with H_2O_2 production. The enzyme is flavin-containing and it is resistant to SDH inhibitors (malonate and TTFA). The data showing intensification of O_2-quenching in the presence of succinate demonstrates that superoxide radicals which are formed in glyoxysomes in high rate [13] can be involved in the mechanism of succinate oxidation in glyoxysomes. The reaction may pass via following mechanism proposed earlier by Manoilov [14] for succinate metabolism in tumors.

The main function of glyoxysomal succinate oxidation may be rapid metabolism of intensively produced succinic acid,

Table 2
Succinate dehydrogenase and glyoxysomal succinate oxidase activity in fat-storing tissues of different plants

Organ	Days of germination	Enzyme	Activity EU/g fresh weight	Specific activity EU/mg protein
Maize scutellum	4	SDH	0.286 ± 0.017	0.036 ± 0.002
		GSO	0.193 ± 0.023	0.025 ± 0.002
Wheat scutellum	5	SDH	0.200 ± 0.014	0.028 ± 0.001
		GSO	0.096 ± 0.010	0.013 ± 0.001
Castor bean endosperm	5	SDH	0.443 ± 0.031	0.047 ± 0.002
		GSO	0.000	0.000
Sunflower cotyledons	3	SDH	0.567 ± 0.043	0.106 ± 0.004
		GSO	0.000	0.000
Soybean cotyledons	7	SDH	0.318 ± 0.018	0.038 ± 0.002
		GSO	0.000	0.000

which avoids adenylate control in mitochondria. This is most important for scutellum existing in hypoxic conditions and producing mostly organic acids for hydrolysis of stored polysaccharides in endosperm and amino acids for protein synthesis [3]. The separation of succinate oxidation between glyoxysomes and mitochondria may provide effective regulation of metabolic flows, where glyoxysomal oxidation serves as an 'overflow' pathway in the conditions of high production of succinate.

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