Subcellular Localization of Quinate:Oxidoreductase from *Phaseolus mungo* L. Sprouts

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Quinate:oxidoreductase (QORase, EC 1.1.1.24) was isolated and purified from etiolated mung bean (*Phaseolus mungo* L.) sprouts and a monospecific antiserum was raised in rabbit to the homogeneous protein. Highly intact etioplasts were isolated from the same plant material. The stroma of the purified etioplasts was enzymatically characterized. Contamination by cytosol, mitochondria and vacuole was estimated from activities of marker enzymes. QORase activity was localized in the stroma (about 91% for both NAD^+ and $NADP^+$ as a cofactor). Western blotting and immunoprinting of the stroma proteins revealed a single band that migrated identically with the purified QORase. The results suggest that the QORase is localized predominantly, if not exclusively, in the etioplast stroma. The physiological role of the enzyme is discussed.

Introduction

Quinic acid is widely distributed among higher and lower plants (Karrer, 1958; Minamikawa and Yoshida, 1972; Boudet, 1973; Yoshida *et al.,* 1975). Therefore, its physiological function and metabolism are of interest. At the turn of the 1960's Weinstein *et al.* (1959, 1961) demonstrated that QA could be synthesized from ^{14}C-labeled CO_2 and that the radioactive label was then transferred to phenylalanine and tyrosine. This was reported also by other researchers (Leuschner and Schultz, 1991 a) by importing ^{14}C-labeled quinic acid into the isolated leucoplasts. The first enzyme found in higher plant material, which introduces quinic acid into the main trunk of the SKA pathway, was quinate dehydrogenase or quinate:oxidoreductase (EC 1.1.1.24) from mung bean cell suspension (Gamborg, 1966, 1967). Since then the enzyme has been isolated from four other plant materials: corn seedlings (Graziana *et al.,* 1980), carrot cell culture (Refeno *et al.,* 1982), tobacco callus (Beaudoin and Thorpe, 1983, 1984) and conifer needles (Osipov and Shein, 1987). Two of them were reported to be purified to homogeneity (Refeno *et al.,* 1982; Kang and Scheibe, 1993). However, the characteristics of these enzymes appeared to be very divergent. The quinate:NAD^+ oxidoreductase from carrot cell culture was reported to be regulated through phosphorylation/dephosphorylation and the enzyme can be activated by Ca^{2+}/calmodulin (Refeno *et al.,* 1982; Graziana *et al.,* 1983, 1984; Ranjeva, 1983). These phenomena were not yet observed with QORase from mung bean sprouts (Kang and Scheibe, 1993). Besides, most reported QORases from plant materials are NAD(H) specific, while only two among them were reported to be able to use both NAD(H) and NADP(H) as a cofactor (Osipov and Shein, 1987; Kang and Scheibe, 1993). Nevertheless, two other enzymes were reported to introduce QA into the main trunk: (1) a bifunctional enzyme QORase/DHQase converting QA to DHS (Graziana *et al.,* 1980) and (2) a QA hydrolyase transferring QA directly to SKA (Graziana and Boudet, 1983). From these results it is not yet possible to deduce the physiological roles of QORase and the other enzymes, respectively.

In this paper we demonstrate that the QORase from etiolated mung bean sprouts is located

Abbreviations: AAA, aromatic amino acid(s); ADPGlcPPase, ADP glucose pyrophosphorylase; BSA, bovine serum albumin; DHS, dehydroshikimic acid; DQA, dehydroquinic acid; QA, quinic acid; QORase, quinate:oxidoreductase; SKA, shikimic acid; UDPGlcPPase, UDP glucose pyrophosphorylase.

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mainly in the plastid stroma of cells. This is the first report of the subcellular localization of a QORase. This work can be a basis for further studies to elucidate the role of QORase and QA metabolism in higher plants.

Materials and Methods

Chemicals and plant material

Goat anti-(rabbit IgG) IgG horse-radish peroxidase (HRP)-conjugate was from BioRad, Munich, Germany. SDS-PAGE molecular mass standards and dialysis tubing were purchased from Sigma, Deisenhofen, Germany. Percoll, Sepharose G-25 medium, Phenylsepharose CL-4 B, Blue Sepharose CL-6 B, Superose 12 were from Pharmacia, Freiburg, Germany. DEAE cellulose was from Serva, Heidelberg, Germany. Etiolated mung bean sprouts were bought from a local supermarket.

Enzyme assay

Because of problems with the enzyme assay in crude plant extracts described earlier (Kang and Scheibe, 1993), extracts were prepared as follows: after homogenization and centrifugation at 20,000×*g* for 20 min the proteins of the supernatant were precipitated with 100% saturated ammonium sulfate. After centrifugation at 10,000×*g* for 15 min the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, and desalted with a Sepharose G-25 column equilibrated with the same buffer.

QORase activity was determined following the method of Kang and Scheibe (1993), ADPGlcPPase was measured as described by Sowokinos (1976), UDPGlcPPase was assayed as given in Bergmeyer (1974), citrate synthase and α-mannosidase were determined as described in Stitt *et al.* (1989). All enzyme assays were performed at 25 °C.

Isolation of plastids

Etioplasts were isolated from 2 kg sprouts according to the method of Neuhaus *et al.* (1993), using 35% Percoll each for the isopycnic (30 min) and the rate zonal (25 min) centrifugation step, respectively. The final etioplast pellet was resuspended in a medium consisting of 0.3 M sorbitol, 15 mM Hepes-KOH, pH 7.2, 1 mM EDTA and 2 mM $MgCl_2$. After removal of an aliquot for protein determination, BSA was added to 0.1% final concentration.

Protein determination

Protein determination was performed according to Bradford (1976) using BSA as a standard.

Contamination of the isolated plastid stroma and estimation of the QORase localization

The stroma of the isolated plastids was released by ultrasonication with three strokes each for 3 sec and membrane fragments were removed by centrifugation. The following marker enzymes were used (Neuhaus *et al.*, 1993): ADPGlcPPase for plastid stroma, UDPGlcPPase for cytosol, citrate synthase for mitochondria and α-mannosidase for vacuoles. Enzyme activities were measured in the crude extracts (CE) and in the stroma preparation. The percentage of the total activity localized in the plastidic fraction was calculated according to the following equation:

$$\left(\frac{\text{ADPGlcPPase (CE)}}{\text{enzyme (CE)}}\right) \Big/ \left(\frac{\text{ADPGlcPPase (stroma)}}{\text{enzyme (stroma)}}\right) \cdot 100$$

Enzyme purification

All purification steps were carried out at 4 °C. The enzyme purification was conducted according to the method of Kang and Scheibe (1993) with the following modifications: Crude extract was prepared from 5 kg mung bean sprouts homogenized in one volume of buffer A containing 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA and 28 mM 2-mercaptoethanol, with a Waring blender. The homogenate was then centrifuged at 20,000×*g* for 20 min. For the enzyme assay, the protein in the supernatant was precipitated with 100% saturated ammonium sulfate and resuspended in buffer B containing 50 mM Tris-HCl, pH 8.0, and desalted through a Sepharose G-25 column equilibrated with the same buffer.

For further purification the 30–60% ammonium sulfate fraction was resuspended in buffer B (about 500 ml) and was dialyzed for 36 h against 15 l of buffer B with three changes. Then the sample was applied to the columns of DEAE cellulose (400 ml bed volume), Phenylsepharose CL-4 B (100 ml bed volume), Blue Sepharose CL-6 B (60 ml bed volume) and Superose 12. The

DEAE cellulose was eluted using a HighLoad system (Pharmacia, Sweden). All the other chromatography steps were carried out with an FPLC system (Pharmacia, Sweden).

Antibody production

Purified QORase (about 100 μg) was dialyzed against 100 ml of 20 mM phosphate buffer, pH 7.4, adjusted with KOH. The dialyzate was then concentrated by ultrafiltration with a Centricon 30 tube (Amicon, Witten, Germany) to about 500 μl and emulsified with complete Freund's adjuvant (Sigma, Deisenhofen, Germany) to a final volume of 1 ml. The antigen was injected subcutaneously into a 3 kg rabbit. Booster injections (70–90 μg) of the same protein emulsified in incomplete Freund's adjuvant were given subcutaneously in the 4th, 7th and 11th week. 10 days after the final injection, blood was collected from the ear vein. After incubation at room temperature overnight, the clotted blood cells were removed by centrifugation at 1500×*g* for 10 min. The crude antiserum was divided into 100 μl aliquots, and stored at −80 °C.

Electrophoresis and Western blotting

SDS-PAGE was performed with the BioRad (Munich) 2D mini-gel apparatus with 12% polyacrylamide. The proteins were transferred to nitrocellulose by semi-dry electroblotting at 1 mA/cm^2 for 2 h. After blotting, the gel was stained with Coomassie Brilliant Blue R-250, while the nitrocellulose filter was first blocked with 3% gelatine in Tris-buffered saline and then incubated with the antiserum (1:5000 dilution). For detection, the filter was incubated in goat anti-(rabbit IgG) IgG-horse-radish peroxidase conjugate, BioRad (Munich) (1:2500 dilution). The nitrocellulose membrane was developed with 4-chloro-naphthol and H_2O_2. The color reaction was carried out at 37 °C for about 2–3 min.

Results

Preparative purification of the QORase

To immunize a rabbit around 100 μg of pure protein is needed for each injection. With the method described by us earlier (Kang and Scheibe, 1993) only a few μg protein can be obtained. Using the modified method as described in the Materials and Methods section about 80–100 μg protein was produced from one batch. This amount was sufficient to immunize a rabbit.

The results of the purification procedure are shown in Table I. The activities were measured with NAD^+ as the cofactor and QA as the substrate. Compared with the activity of the partially purified preparation (after 30–60% ammonium sulfate fractionation and dialysis) the enzyme was purified about 4710-fold and the final yield was 15%. The yield was better than that described earlier, while the purification was a little lower, but still in the same range as in Kang and Scheibe (1993) (4709- and 5000-fold, respectively).

Production of a specific antiserum and immunodetection of QORase

To proof the specificity of the antiserum raised against purified QORase, SDS-PAGE and Western blotting of QORase preparations was carried

Table I. Preparative purification of quinate:oxidoreductase from etiolated mung bean sprouts. The purification was carried out according to Kang and Scheibe (1993) using an FPLC system, with modifications as described in the Materials and Methods section. Activities were measured at pH 9.6 with quinic acid as substrate using NAD^+ as cofactor.

	Volume [ml]	Activity [U]	Protein [mg]	Spec. act. [U/mg]	Purification [fold]	Yield [%]
Crude extract	5000	–	11,000	–	–	–
30–60% sat. $(NH_4)_2SO_4$ fraction, dialyzed	550	10.56	3,272	0.0031	1.0	100.0
DEAE cellulose	230	6.72	1,012	0.0066	2.1	63.6
Phenylsepharose	82	4.72	188.6	0.025	8.1	44.7
Blue Sepharose	8	2.44	1.84	1.325	427.4	23.1
Superose 12	1.2	1.58	0.108	14.6	4709.7	15.0

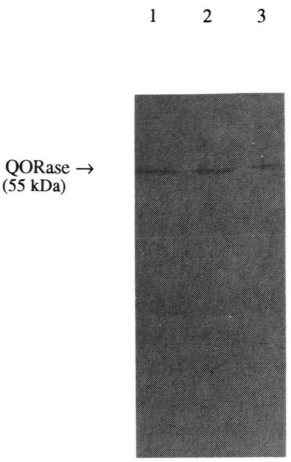

Fig. 1. Immunodetection of QORase in enzyme preparations at various purification steps using the antiserum against QORase from etiolated mung bean sprouts. Samples containing similar enzyme activities were subjected to SDS-PAGE. Lane 1, partially purified enzyme after DEAE cellulose; lane 2, after Blue Sepharose CL-6B; and lane 3, after Superose 12 gel filtration (purified enzyme). The lane with crude extract is not shown.

out. The result is shown in Fig. 1. However, it was difficult to see any band with crude extract because of the low abundance of QORase. When more protein was applied, the gel was overloaded. With the partially purified (after DEAE Sepharose) and the purified enzyme preparations identical bands at 55 kDa were obtained (Fig. 1). The molecular mass is in agreement with the earlier result (Kang and Scheibe, 1993). This result indicates that no unspecific proteolytic degradation has taken place during the purification of the enzyme.

Localization of QORase in plastids

Etioplasts were isolated from etiolated mung bean sprouts. The stroma was released by ultrasonication (3×3 sec). After removal of membrane fragments by centrifugation the stroma was used for enzyme assays. Measurement of the marker enzymes (values are the mean of three independent assays) for other cell compartments resulted in the finding that contamination of the stroma was 0.9% by cytosol using UDPGlcPPase as marker enzyme, 5.6% by mitochondria using citrate synthase as marker enzyme, and 2.1% by the vacuoles using α-mannosidase as a marker enzyme (Table II).

Table II. Contamination of the purified stroma by other compartments and percentages of QORase activities localized in the stroma preparation. Values were calculated with relative activities of the marker enzymes in stroma and crude extract according to the equation given in the Materials and Methods section. ADPGlcPPase was used as the plastidic marker enzyme and measured under V_{max} conditions according to the method of Sowokinos (1976).

Compartment	Marker enzyme	Activity in stroma preparation [% of total]
Cytosol	UDPGlcPPase	0.9
Mitochondria	Citrate synthase	5.6
Vacuoles	α-Mannosidase	2.1
	QORase (NAD^+)	90.6
	QORase ($NADP^+$)	91.2

For immunodetection of QORase in the plastid stroma, the purified etioplasts were lysed by ultrasonication (3×3 sec), and the membrane fragments were removed by centrifugation. About 1 mU QORase activity per slot was applied for SDS-PAGE. Western blotting and immunodetection were carried out as described in the Materials and Methods section. After blotting the SDS gel was stained with Coomassie Brilliant Blue and revealed many bands. The heavy band at 66 kDa was due to BSA (66 kDa, Sigma) present in the buffer (Fig. 2). The QORase band could not be identified among the protein bands, while immunoprinting revealed a single clear band at 55 kDa (Fig. 2). The observation that QORase could not be seen by immunoprinting with crude extract, while the crude stroma sample revealed a clear QORase band, suggested that QORase was enriched in the plastid preparation.

QORase activity was determined with NAD^+ and with $NADP^+$ as a cofactor, respectively. The data are the mean of three independent assays. Compared with the crude extract, 90.6% (NAD^+) and 91.2% ($NADP^+$) of the activities were localized in the stroma (Table II).

Discussion

About 91% QORase activity was found in the stroma preparation of the isolated mung bean sprout etioplasts (Table II). Activities with NAD^+

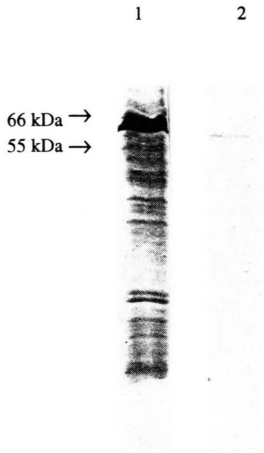

Fig. 2. SDS-PAGE and immunoprinting of QORase of the stroma of mung bean etioplasts. Stroma protein (25 μg) was subjected to SDS-PAGE. After blotting the gel was stained with Coomassie Brilliant Blue R-250 (lane 1). The heavy band is due to BSA (66 kDa, Sigma) present in the buffer medium. The blot was developed by immunoprinting using the anti-QORase serum and horse-radish peroxidase conjugate, with H_2O_2 and 4-chloro-naphthol as substrates for the color reaction. The 55 kDa band is QORase.

and with $NADP^+$ as cofactor were in the same range (90.6 and 91.2%, respectively). Immunodetection of QORase in the stroma preparation (Fig. 2) revealed a single band at 55 kDa. Compared with crude extracts, where no band could be detected upon Western blotting and immunoprinting. QORase was enriched in the stroma preparation. These results suggest that the QORase is localized in the etioplasts of mung bean sprouts. The presence of a small fraction of QORase in the cytoplasm or in other compartments can still not be completely excluded. Nevertheless, Leuschner and Schultz (1993) observed a cytosolic QORase in pea roots (personal communication). More experiments are needed to make a definite statement regarding the localization of the enzyme. However, from our data, it appears that the QORase is localized predominantly, if not exclusively, in the etioplasts.

It has been reported that plastids possess all the enzyme activities of the shikimate pathway (main trunk) (Feierabend and Brassel, 1977; Fiedler and Schultz, 1984) and were able to synthesize aromatic amino acids and prenylquinone directly from imported radioactive QA or SKA independently upon other organelles (Leuschner and Schultz, 1991 b). Recently, the localization has been verified by molecular biotechniques. The group of Amrhein has concluded from the presence of cDNA sequences for putative N-terminal transit peptides that three key enzymes of the shikimate pathway, chorismate mutase, chorismate synthase and DAHP synthase, respectively, occur as plastidic enzymes (Görlach *et al.*, 1993 a, 1993 b; Eberhard *et al.*, 1993). The location of QORase in plastids appears appropriate to its metabolic role, since it has been reported that QA could be transported across the plastid envelope and be converted into aromatic amino acids in the plastid (Leuschner and Schultz, 1991 b). Our result suggests that QORase may play a role to introduce QA into the main trunk of the SKA pathway in mung bean etioplasts, although we could not yet exclude the presence or even involvement of the other two enzymes, QORase/DHQase II and QA hydrolyase, in this part of the plastidic metabolism. These enzymes had been reported to be able to introduce QA into the main trunk of the SKA pathway in corn seedlings (Graziana *et al.*, 1980) and pea roots (Leuschner and Schultz, 1991 a) and had been localized in pea root plastids.

In conclusion, this is the first report on the subcellular localization of a QORase in a plant material. However, from the results obtained with mung bean sprouts it is premature to conclude that QORase is exclusively localized in plastids in all plants. Besides, the modified purification scheme for the enzyme described in this work might be of use to further investigate the properties of the QORase also in other tissues.

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