Localization of Nitric-oxide Synthase in Plant Peroxisomes*

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The presence of nitric-oxide synthase (NOS) in peroxisomes from leaves of pea plants (Pisum sativum L.) was studied. Plant organelles were purified by differential and sucrose density gradient centrifugation. In purified intact peroxisomes a Ca²⁺-dependent NOS activity of 5.61 nmol of L-[³H]citrulline mg⁻¹ protein min⁻¹ was measured while no activity was detected in mitochondria. The peroxisomal NOS activity was clearly inhibited (60–90%) by different well characterized inhibitors of mammalian NO synthases. The immunoblot analysis of peroxisomes with a polyclonal antibody against the C terminus region of murine iNOS revealed an immunoreactive protein of 130 kDa. Electron microscopy immunogold-labeling confirmed the subcellular localization of NOS in the matrix of peroxisomes as well as in chloroplasts. The presence of NOS in peroxisomes suggests that these oxidative organelles are a cellular source of nitric oxide (NO) and implies new roles for peroxisomes in the cellular signal transduction mechanisms.

Nitric oxide (NO) is a widespread intra- and intercellular messenger which is involved in a wide range of functions such as the regulation of vascular tone, neuronal signaling, and immune response to infection (1). As a consequence of the physiological importance of the free radical NO, numerous studies have been focused on the enzyme responsible for its endogenous production. Nitric-oxide synthase (NOS; EC 1.14.13.39) catalyzes the NADPH-dependent conversion of L-arginine to NO and implies new roles for peroxisomes in the cellular signal transduction mechanisms.

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The abbreviations used are: NO, nitric oxide; d-NNAME, N⁶-nitro-L-arginine methyl ester; t-NNAME, N⁶-nitro-L-arginine methyl ester; L-NMMA, N⁶-monomethyl-L-arginine; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; perNOS, peroxisomal NOS; O₂⁻, superoxide radical; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

In plants, several recent studies have shown that NO could function as a signal in disease resistance (5–8). However, NOS-like activity has only been detected in plant extracts during the interaction of Rhyzobium-legume (9) and fungi plants (10), and in soybean cell suspensions induced with an elicitor from Pseudomonas syringae (8). Very recently, the presence of NOS in the cytosol and nucleus of maize cells has been reported (11). But no information is available concerning the occurrence of NOS in other plant cell organelle.

Peroxisomes are single-membrane-bound organelles that may have a role in the cellular metabolism of activated oxygen species (22). In this work, we report the presence of NOS in peroxisomes (perNOS) of pea leaves using four complementary approaches: (i) distribution of NOS activity in cell organelle fractions purified by sucrose density gradient centrifugation, (ii) sensitivity of NOS activity to well characterized NOS inhibitors, (iii) cross-reactivity of peroxisomes with Western blot antibodies against iNOS, and (iv) electron microscopy immunocytochemical analysis of intact plant tissue.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Pea (Pisum sativum L., cv. Lincoln) seeds, obtained from Royal Sluis (Enkhuizen, Holland), were surface-sterilized with 3% (v/v) commercial bleaching solution for 3 min, and then were washed with distilled water, and germinated in vermiculite for 15 days. Healthy and vigorous seedlings were selected and grown in the greenhouse in nutrient solutions under optimum conditions (23) for 15 days.

Purification of Peroxisomes—All operations were performed at 0 to
4 °C. Peroxisomes were purified from pea leaves by differential and sucrose density gradient centrifugation (35–60%, w/w) as described by López-Huertas et al. (24). Purified peroxisomes were practically free of chloroplasts and mitochondria, as confirmed by measuring the activities of the appropriate marker enzymes (24, 25).

Enzyme Activities and Other Assays—Peroxisomal catalase activity was assayed as described by Aebi (26) and fumarase activity was measured by the method of Walk and Hock (27). NO synthase activity was determined by monitoring the conversion of L-[3H]arginine to L-[3H]citrulline (28). The assays for total NOS activity were performed in duplicate for each sample for 20 min at 37 °C in a reaction medium containing 40 mM Hepes buffer, pH 7.2, 0.2 mM CHAPS, 10 μM calmodulin, 1.25 mM CaCl₂, 1 mM dNADPH, 10 μM FAD, 10 μM FMN, 10 μM tetrahydrobiopterin (BH₄), variable amounts of protein samples, and a variable concentration of L-arginine supplemented with L-[3H]arginine (1.0 μCi ml⁻¹) in a total volume of 220 μl. The reaction was terminated by addition of 1.5 ml of a cation exchange resin (Dowex 50W, 8%; cross-linkage, 200–400 mesh, Na⁺ form) at 4 °C which stops the reaction by removing the arginine substrate. Then, 5 ml of water was added and the resin was allowed to settle for approximately 10 min before aspirating 3.5 ml of supernatant which was analyzed by liquid scintillation counting.

The level of L-[3H]citrulline was computed after subtracting the blank value, which represented the nonspecific radioactivity in the control was used omitting the primary antibody.

Cell (31). Ultrathin sections were poststained in 2% (v/v) uranyl acetate for 3 min and examined in a Zeiss EM 10C transmission electron microscope. Preimmune sera were used with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech) and were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech)

Antibodies—Two antibodies have been used in this study. A monoclonal antibody against the NADPH-binding region (residues 961 to 1144) of iNOS from mouse macrophage (Transduction Lab, Lexington, KY) and a polyclonal antibody against the peptide PT387 (Ac-Cys-KY) and a polyclonal antibody against the NADPH-binding region (residues 961 to 1144)) from the C terminus of the deduced amino acid sequence of murine iNOS (30).

Protein levels were determined according to Bradford (29) using bovine serum albumin as standard. The density of the gradient fractions was calculated at room temperature using an Atago refractometer.

Electrophoretic and Immunoblot Analyses—SDS-PAGE was carried out in 7.5% acrylamide slab gels. Samples were prepared in 62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, and 10 mM dithiothreitol, and were heated at 95 °C for 5 min. For immunoblot analyses, polypeptides were transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore Corp., Bedford, MA) using a Semi-Dry Transfer System (Bio-Rad) with 10 mM CAPS buffer, 10% (v/v) methanol, pH 11.0, at 1.5 mA cm⁻² for 2 h (31). For slot blot studies, proteins were also transferred onto polyvinylidene difluoride membranes using a Bio-Dot SF Apparatus (Bio-Rad) following the instructions of the manufacturer. For immunodetections, the two antibodies against iNOS were used with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech) and were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech).

Electron Microscopy and Immunocytochemistry—Pea leaf segments of approximately 1 mm² were fixed, dehydrated, and embedded in LR White resin as described by Corpas and co-workers (31). Ultrathin sections were incubated for 3 h with IgG against iNOS (30) diluted 1/250 in TBST buffer (10 mM Tris-HCl, pH 7.6, containing 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin and 1% (v/v) goat normal serum. The sections were then incubated for 1 h with goat anti-rabbit IgG conjugated to 15-nm gold particles (Bio-Rad) diluted 1/50 in TBST plus 2% (w/v) bovine serum albumin. Sections were poststained in 2% (v/v) uranyl acetate for 3 min and examined in a Zeiss EM 10C transmission electron microscope. Preimmune serum, instead of the iNOS antibody, was used as control. A second control was used omitting the primary antibody.

RESULTS

The purification of peroxisomes from pea leaves by sucrose density gradient centrifugation is shown in Fig. 1. Peroxisomes (fractions 20–25) were identified by the peak of catalase activity, used as peroxisomal marker enzyme. The peroxisomal fraction banded at an average equilibrium density of 1.24 g cm⁻³, characteristic for these intact organelles in sucrose solutions (12, 24). The absence of fumarase activity in these fractions indicated that peroxisomes were essentially free of contamination by mitochondria. The assay of NOS activity throughout the gradient fractions showed a peak of activity in the top of the sucrose gradient (fractions 1–3), corresponding to the zone of broken organelles, and another NOS peak in the peroxisomal fractions (fractions 21–25). A maximum NOS activity of 170 pmol of L-[3H]citrulline min⁻¹ mg⁻¹ protein was found in the peroxisomal fraction 23 which coincided with the maximum of catalase activity.

The NOS activity of peroxisomes, measured as production of L-[3H]citrulline, showed a linear increase with the amount of peroxisomal protein (data not shown). The specific activity of the peroxisomal NOS was reduced more than 70% in the absence of calcium (Table I) and was strictly dependent on NADPH.

The effect on the peroxisomal NOS activity of seven well characterized inhibitors of the three known mammalian NO synthases is shown in Table II. An inhibition of the NOS activity by 59–100% was obtained with the inhibitors L-NAME, L-thiocitrulline, 7-nitroindazole, diphenylhydantoin, and aminoguanidine. When the NOS assays were performed using a 100 μM L-Arg concentration, a reversion of about 30% of the inhibitory effect of 7-nitroindazole, L-NAME, and L-thiocitrulline, diphenylhydantoin, and aminoguanidine. The NOS activity was not sensitive to L-NAME, an enantiomer of L-NAME, which is used as a negative control.

The analysis by slot blot of both pea leaf crude extracts and pea leaf peroxisomal fractions with a monoclonal antibody against mouse macrophage NOS (iNOS) gave a positive reac-
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TABLE I

Effect of calcium on the NOS activity of pea leaf peroxisomes

Reaction mixtures containing peroxisomal fractions were incubated in the presence and absence of Ca^{2+} (1.25 mM), EGTA (0.5 mM), or NADPH (1 mM) for 20 min. Then the NOS activity was assayed using a concentration of 1 mM Arg during 20 min. Results are mean ± S.E. of three samples from three different sucrose density gradients.

| Samples                        | NOS specific activity (nmol of L-[3H]citrulline mg^{-1} protein min^{-1}) |
|-------------------------------|-------------------------------------------------------------------------|
| Peroxisomes                    |                                                                          |
| + Ca^{2+} + NADPH              | 5.61 ± 0.54                                                             |
| − Ca^{2+} + EGTA               | 1.49 ± 0.12                                                             |
| Peroxisomes + Ca^{2+} − NADPH  | 0                                                                        |

TABLE II

Effect of different inhibitors on the NOS activity of pea leaf peroxisomes

Reaction mixtures were incubated in the presence of several inhibitors of NOS at a 1 mM final concentration for 20 min. Then, 20 μM L-arginine was added and the mixture was incubated for another 20 min. The NOS activity obtained for the control reaction was 80.5 pmol of L-[3H]citrulline mg^{-1} protein min^{-1}. Data are mean values of three samples from three different sucrose density gradients.

| Inhibitor (1 mM)      | Inhibitory action | Inhibition |
|-----------------------|-------------------|------------|
| Aminoguanidine        | Irreversible inhibitor of iNOS | 100% |
| L-NMMA                | Non-selective inhibitor | 88% |
| L-NAME                | Non-selective inhibitor | 90% |
| Thiocitrulline        | Inhibitor of nNOS | > 80% |
| 7-Nitroindazole       | Competitive inhibitor of nNOS | > 59% |
| Diphenyliodonium      | Non-selective inhibitor | 60% |
| L-N5-(I-)Imenoethyl)-ornithine | Inhibitor of eNOS | 59% |
| β-NMMA                | Enantiomer of L-NAME | 2% |

FIG. 2. Immunoblot analysis of peroxisomal fractions with NOS antibodies. A, slot blots probed with a monoclonal antibody against murine macrophage iNOS (Transduction Lab) (dilution 1/100). B, SDS-PAGE and Western blot probed with a polyclonal antibody against murine iNOS. Lane 1, molecular mass markers; 2, crude extracts of lipopolysaccharide-pretreated rat liver (30 μg of protein); 3, murine macrophage control lysate (Transduction Lab) (30 μg of protein); 4, pea peroxisomal fraction (50 μg of protein). A dilution of 1/2,500 was used for lanes 2 and 3, and 1/500 for lane 4.

FIG. 3. Effect of an antibody against iNOS on the perNOS activity. Peroxisomal fractions were incubated at 25 °C for 2 h with different dilutions of the antibody against murine iNOS. The NOS activity of samples was assayed using 1 mM Arg concentration.

The ultrastructural immunolocalization of NOS with the antibody against murine iNOS showed that gold particles were localized inside two cell compartments, peroxisomes and chloroplasts, while no labeling was observed inside mitochondria (Fig. 4, panels B and C). No significant labeling was observed in the controls (Fig. 4, panel A).

DISCUSSION

In higher plants, recent data have shown that exogenous nitric oxide has an important role as messenger in resistance against diseases produced by plant pathogens (6, 8, 32), and several groups have detected NOS activity in plant extracts (8–11). Considering that plant peroxisomes have a role in the metabolism of reactive oxygen species (22) and are also a source of H_{2}O_{2}, which may function as a second messenger (33–35), the potential presence of NOS in peroxisomes was examined.

The first approach was to study the distribution of NOS activity throughout the sucrose density gradient fractions. Two peaks of L-[3H]citrulline production were detected in the gradient fractions. One peak was in the top of the gradient, and corresponded to the broken organelles, and another peak of NOS activity was observed in the peroxisomal fractions. The peroxisomal NOS activity, measured as L-citrulline production, was protein- and calcium-dependent (Table I).

As part of the initial characterization of the NOS activity of leaf peroxisomes, the effect of seven archetype NOS inhibitors, including specific and unspecific inhibitors of different types of NOS isoforms was assayed. The results indicated a clear inhibition of the perNOS activity of 59–100%, being the L-aminoguanidine the most effective inhibitor. In extracts of maize seedlings a 31% inhibition of NOS activity by 3 mM L-aminoguanidine plus 3 mM L-NAME was reported (11), and in extracts of lupin roots a 50% inhibition of NOS activity by 1 mM L-NMMA was described (9). However, the specific activity reported by these authors is much lower (0.2 and 0.7 pmol of L-citrulline mg^{-1} protein min^{-1}, respectively) than that obtained in pea leaf peroxisomes (5.6 nmol of L-citrulline mg^{-1} protein · min^{-1}). Likewise, in this work the production of citrulline was specifically due to a NOS because an specific antibody against iNOS inhibited this reaction by more than 80% (Fig. 3).

The NOS activities described in crude extracts of roots of Lupinus albus (9), soybean cell suspensions (8), and maize seedlings (11) were calcium-dependent, moderately sensitive to...
The electron micrographs are representative of thin sections of pea leaves. The electron microscopy immunolocalization of NOS demonstrated the presence of the enzyme in the matrix of peroxisomes and also in chloroplasts. However, no immunogold labeling of NOS was detected in mitochondria. This contrasts with results obtained in mammalian tissues where a NOS was found in mitochondria isolated from rat liver (4) which was characterized as a new isoform (mtNOS) (43). The mtNOS from rat liver is similar to the inducible form (iNOS) but is constitutively expressed and bound to the mitochondrial membrane (4).

The presence of NOS in peroxisomes suggests an interaction of this enzyme with other components of the metabolism of activated oxygen species of leaf peroxisomes. An important point is that the NADP-dehydrogenases, recently found in the peroxisomal matrix (18), could provide the necessary NADPH for the perNOS reaction. But on the basis of the recent results of Sakuma and co-workers (44), a potential function of NO inside peroxisomes can be proposed. NO could react with O2· radicals generated in the peroxisomal matrix by xanthine oxidase (19) to form the powerful oxidant peroxynitrite which, according to Sakuma et al. (44) could regulate the conversion of xanthine dehydrogenase into the superoxide-generating xanthine oxidase. On the other hand, NO could also diffuse through the peroxisomal membrane, reacting with O2· produced in the cytosolic side of the membrane by a small electron transport chain (22), thus generating the oxidant peroxynitrite in the cytosol. Likewise, the NO diffused into the cytosol could also function as a cellular transduction signal, as it has been described for H2O2 (33–35).

In summary, the biochemical, immunological, and immunocytochemical data reported in this work provide evidence that peroxisomes contain nitric-oxide synthase activity (perNOS) which is calcium-dependent, constitutively expressed, and immunorelated with the mammalian iNOS. The occurrence in peroxisomes of a NOS suggests that these organelles are a cellular source of NO and implies new roles for peroxisomes in the cellular signal transduction mechanisms.

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