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Choline kinase II is present only in nodules that synthesize stable peribacteroid membranes

(nodulin-like protein/*Rhizobium japonicum*/*Glycine max*/symbiosis)

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ABSTRACT **Host-cell cytoplasm from soybean plants infected with the peribacteroid membrane (PBM)-building *Rhizobium japonicum* strain 61-A-101 (effective, N_2-fixing) had much higher choline kinase activity than cytoplasm from either uninfected tissue or tissue infected with the non-PBM-building (ineffective, non-N_2-fixing) strain 61-A-24. Ion-exchange chromatography showed that both types of nodule and root tissue possessed constitutive choline kinase I activity that had a K_m for choline of ≈150 μM. The nodules of the effective symbiosis had another activity, choline kinase II (K_m = 81 μM). Nondenaturing and NaDodSO$_4$ electrophoresis revealed no multimeric subunit structure of the two enzyme forms but did show the molecular sizes for choline kinase I, 58–59 kDa, and choline kinase II, 60 kDa. Choline kinase I and II had pI values of 8.1 and 8.5, respectively, and two-dimensional gel electrophoresis of whole cytoplasm from control and infected tissue showed a spot corresponding to choline kinase II only in the case of the effective symbiosis, whereas both tissue types had spots corresponding to choline kinase I. Choline kinase II is presumed to be encoded by the plant as neither free-living nor symbiotic (bacteroid) forms of the prokaryote showed any choline kinase activity.**

Following the successful invasion of *Glycine max* cells by symbiotic strains of *Rhizobium japonicum*, the plant cell is transformed and becomes filled with prokaryotic cells (1). During the transformation process several genes are turned on (2, 3), resulting in the synthesis of nodule-specific proteins or "nodulins"; the best characterized of which is leghemoglobin (4). As effective (N_2-fixing) symbiotic bacteria (bacteroids) multiply and fill the eukaryotic cell, a new membrane type, the peribacteroid membrane (PBM), which encloses the individual bacteroids, is built (5). With some ineffective (non-N_2-fixing) symbiotic bacterial strains, however, the plant builds either a partial PBM or no PBM (6). While it appears that the PBM in the initial stages of plant cell infection is derived from parts of the plant plasma membrane, there is electron microscopic (7, 8) and biochemical (9–11) evidence that the subsequent massive proliferation of the membrane is derived directly from the endoplasmic reticulum and Golgi. Major components of the PBM include glycoconjugates (11) and phospholipids (12). Our studies have shown that phosphatidylcholine is synthesized in plant endoplasmic reticulum by the CDP-choline pathway from a cytoplasmic choline kinase and endoplasmic reticulum phosphorylcholinecytidyltransferase and choline phosphotransferase. Phosphatidylcholine thus formed is then transferred from the endoplasmic reticulum, through the Golgi to the growing PBM. In this communication we demonstrate that this choline kinase activity in PBM-building symbioses is made up of two enzymes.

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MATERIALS AND METHODS

Growth and Preparation of Tissue. The titer-dependent infection of *Glycine max* var. Maple Arrow seedlings with *Rhizobium japonicum* strains 61-A-101 (effective, N_2-fixing, PBM-building), 61-A-24 (ineffective, non-N_2-fixing, builds short-lived PBM), and RH-31-Marburg (ineffective, non-N_2-fixing, early fusion of PBM, giving large vacuoles; see ref. 6) was performed exactly as described (9). Twenty-one days after infection nodules were picked, washed, and used immediately. Bacteroids were isolated (12). Cytoplasmic fractions were obtained for ion-exchange chromatography by homogenization in ice-cold buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, and 1 mM reduced glutathione and for isoelectric focusing as above, except that the homogenization medium contained 0.01% Nonidet P-40. Cytoplasmic fractions were obtained from such homogenates by taking the supernatant after centrifugation at 100,000 × *g* for 30 min at 2°C. The free-living *Rhizobium japonicum* strain 61-A-101 and the *Agrobacterium tumefaciens* strain C 6-6 were grown in medium 20 E (5), harvested by centrifugation, washed twice with fresh sterile medium, then resuspended in the homogenization buffer described above to a concentration of 2×10^8 cells per ml and broken by passage through a French pressure cell at 1000 psi (1 psi = 6.895 kPa). The supernatant from a subsequent centrifugation at 100,000 × *g* for 30 min at 2°C was designated cytoplasmic fraction.

Ion-Exchange Chromatography. Cytoplasmic protein fraction (2 mg) was applied to a 15 × 1 cm column of DEAE-Sepharose connected to a continuous-flow UV monitor and fraction collector and eluted in homogenization buffer. At the peak of unbound protein (which contained no choline kinase activity) the fraction collector was turned on and fractions of 40 drops (≈2 ml) were collected. After the elution of all unbound protein a concentration gradient of 100 ml of KCl ranging from 0.05 M to 0.45 M was applied. Collected fractions were immediately used for choline kinase assay or were concentrated by dialysis against Sephadex G-10 powder overnight at 4°C prior to electrophoresis.

Electrophoretic Methods. Gels containing between 5% and 10% acrylamide but without NaDodSO$_4$ were polymerized in tubes and electrophoresed in the native electrophoresis system described by Davis (13). Immediately after electrophoresis these gels were either (*i*) stained with Coomassie brilliant blue G250 or (*ii*) cut into 0.5-mm segments and assayed for choline kinase. Gels processed as in (*i*) above could be treated subsequently with detergent and reductant, embedded crosswise into slab gels containing NaDodSO$_4$ and 12% acrylamide and electrophoresed (14). Conversely, those gel bands containing choline kinase activity could be cut out of duplicate G250-stained gels, treated with detergent and

Abbreviation: PBM, peribacteroid membrane.

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reductant (14), and electrophoresed individually on 15% acrylamide gels containing $NaDodSO_4$ (11). Isoelectric focusing was performed using Servalyte Pre-Coats pH 3–10 (Serva, Heidelberg) with methods and marker proteins recommended by the manufacturer. Immediately after focusing the gels were either cut into 0.5-mm segments and assayed for choline kinase or fixed and stained as above. Such stained strips could be treated as above for separation in a second dimension, $NaDodSO_4$-containing 12% acrylamide slab gel (14). All gels containing proteins electrophoresed a second time were stained with Coomassie brilliant blue R250.

Enzymic and Other Methods. Choline kinase was assayed exactly as described (12), protein was measured as described (9), and K^+ concentration was measured by ion-specific electrode.

Materials. [^{14}C]Choline (2.146 GBq/mmol) was from Amersham Buchler (Braunschweig, F.R.G.). DEAE-Sepharose and Sephadex G-10 were from Pharmacia (Uppsala, Sweden), and molecular size marker proteins from Boehringer Mannheim. All other chemicals were from either Sigma (München, F.R.G.) or Serva (Heidelberg).

RESULTS AND DISCUSSION

Plant cytoplasmic fractions either from nodules formed as a result of symbioses with *Rhizobium japonicum* strains 61-A-101 or 61-A-24 or from uninfected root tissue contained one choline kinase activity that eluted from the DEAE-Sepharose column with 0.18 M KCl. The cytoplasmic fraction from the PBM-building 61-A-101-containing symbiosis showed a second choline kinase activity that eluted with 0.3 M KCl (Fig. 1). (*i*) The K_m values for choline kinase I peaks were similar whereas the K_m value for choline kinase II was significantly lower (Table 1). Furthermore, the K_m value for the activity in the whole cytoplasm from those tissue types possessing only one choline kinase was almost identical to the value for the gradient-eluted peak, indicating one enzyme. The apparent K_m value for whole cytoplasm from the 61-A-101 symbiosis, however, was between the values for the two column-purified enzymes (Table 1), indicating a mixed-enzyme population. (*ii*) After isoelectric focusing of host effective nodule cytoplasm, two choline kinases were clearly discernible, but only the enzyme with the more basic pI value appeared in control plant root cytoplasm (Fig. 2), indicating that two charge isomers of the choline kinase exist in the PBM-building symbiosis.

Table 1. Enzyme substrate affinity for choline kinase from various sources

Tissue	K_m values, μM		
	Cytoplasmic fraction	Peak 1	Peak 2
61-A-101-infected nodules	97	152	81
61-A-24-infected nodules	150	148	—
Control roots	152	156	—

After ion-exchange chromatography, choline kinase in the two peaks from the effective symbiosis was concentrated and subjected to native polyacrylamide gel electrophoresis in gels of various acrylamide concentration. Such gels were assayed for choline kinase activity. Each peak from the ion-exchange column gave one activity band after electrophoresis. The bands comigrated with a minor protein band (Fig. 3*a*), whose native molecular size, calculated by using the differential sieving effect of the various acrylamide concentrations (15), appeared to be 58 kDa for choline kinase I and 60 kDa for choline kinase II (Fig. 3*b*). To find out if the native protein was composed of subunits and to determine the molecular size more accurately, the following two strategies were used. The first was a two-dimensional technique, where proteins were electrophoresed on a native gel and then on a $NaDodSO_4$-containing gel. The second was to cut out the choline kinase bands from a non-denaturing gel and electro-

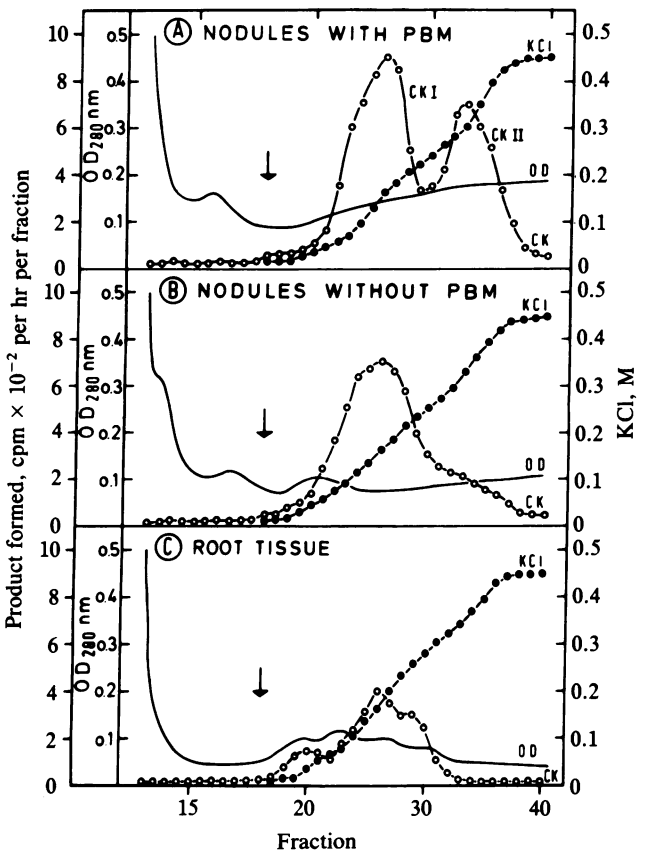

FIG. 1. DEAE-Sepharose elution profiles of proteins from cytoplasmic fractions from plant cells. (*A*) *Rhizobium japonicum* 61-A-101-infected nodules. (*B*) *Rhizobium japonicum* 61-A-24-infected nodules. (*C*) Control root tissue. Unbroken line, protein concentration (absorption at 280 nm); closed circles, KCl concentration gradient; open circles, choline kinase activity.

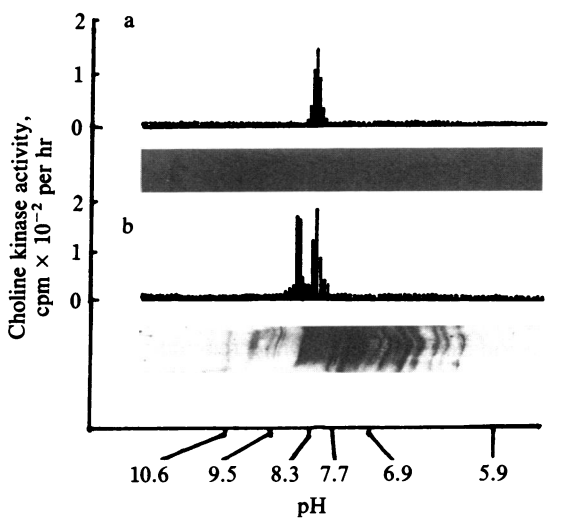

FIG. 2. Isoelectric focusing gels of cytoplasm from control root tissue (*a*) and *Rhizobium japonicum* 61-A-101-infected nodule tissue (*b*). Duplicate gels were cut into segments and assayed for CK activity (shown in histogram form).

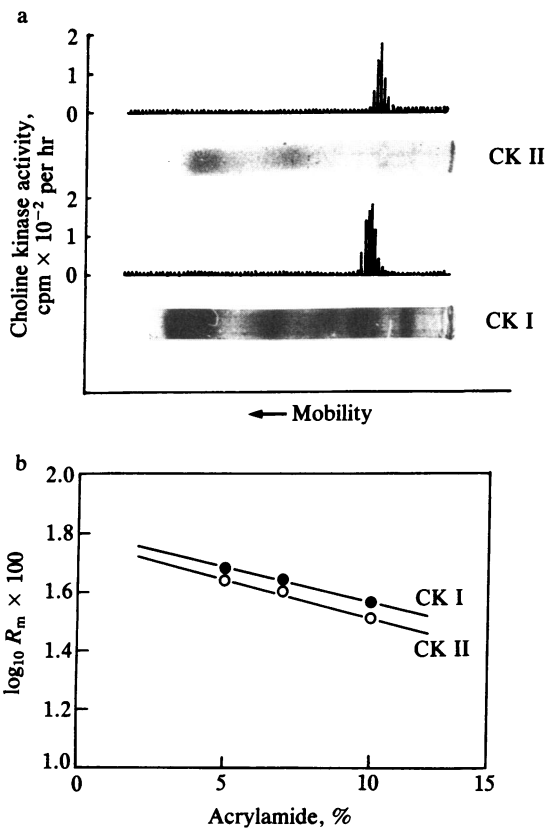

FIG. 3. (*a*) 10% acrylamide nondenaturing polyacrylamide gels of peak choline kinase I (CK I) and choline kinase II (CK II) fractions from ion-exchange chromatography of *Rhizobium japonicum* 61-A-101-infected nodule cytoplasm. Duplicate gels were cut into sections and assayed for activity (shown in histogram form). R_m is the mobility as a fraction of that of the dye front. (*b*) Effect of acrylamide concentration on the mobility of choline kinase I (●) and choline kinase II (○) peak activity fractions in nondenaturing polyacrylamide gels.

phorese each on $NaDodSO_4$ gels. The results of the first strategy for choline kinase I are shown in Fig. 4. Correlation between mobility in the first gel and actual molecular size was linear throughout the whole gel, with no irregularities to suggest multiple subunit composition. Similar correlations were obtained by using gels from the choline kinase II fraction in the first dimension (data not shown). The second strategy enabled us to estimate the molecular size of the two choline kinase proteins without interference from other proteins. Fig. 5 shows that the molecular sizes of the single bands obtained on $NaDodSO_4$ gels are in reasonable agreement with the calculated values from native gels, namely, 58 kDa for choline kinase I and 60 kDa for choline kinase II.

To determine whether choline kinase II is in fact a constitutive protein of soybean that is only activated in PBM-building symbioses, two-dimensional gels of cytoplasm

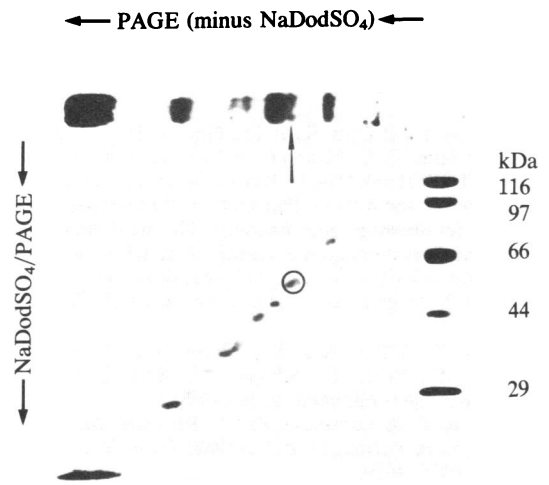

FIG. 4. Two-dimensional PAGE of proteins from choline kinase I ion-exchange chromatography fractions from *Rhizobium japonicum* 61-A-101-infected nodule cytoplasm subjected, in the first dimension, to nondenaturing PAGE in 7% acrylamide and, in the second dimension, $NaDodSO_4$/PAGE (12% acrylamide). Arrow, choline kinase activity in duplicate gels from the first dimension; circle, final position of choline kinase I.

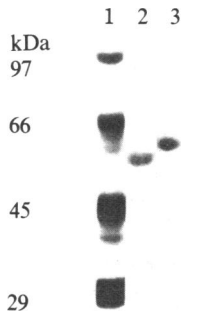

FIG. 5. $NaDodSO_4$/PAGE of peak activity segments of nondenaturing polyacrylamide gels containing choline kinase I (lane 2) and choline kinase II (lane 3) ion-exchange chromatography peak fractions of *Rhizobium japonicum* 61-A-101-infected nodule cytoplasm. Molecular size markers are in lane 1.

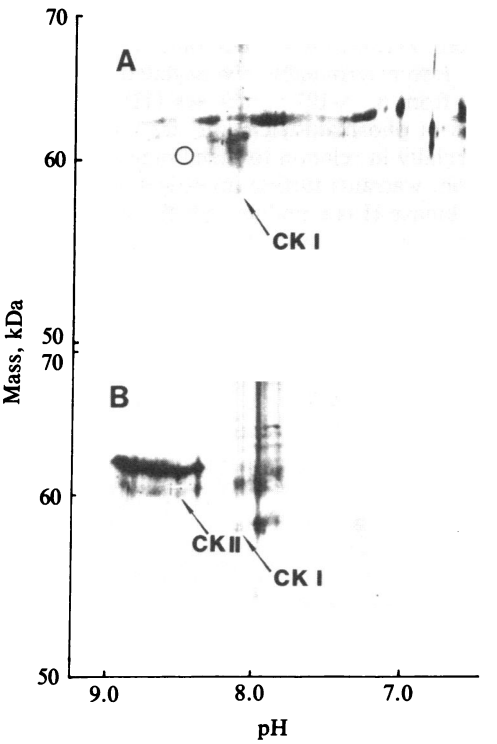

FIG. 6. Two-dimensional isoelectric focusing × $NaDodSO_4$/PAGE analysis of control root cytoplasm (*A*) and *Rhizobium japonicum* 61-A-101-infected nodule cytoplasm (*B*) showing the expected positions of choline kinase I (CK I) and choline kinase II (CK II) (arrows) and a clear space in *A* where choline kinase II would be expected (circle). Protein (80 μg) was initially applied in the first dimension.

Table 2. Stimulation of some membrane-building enzyme activities in soybean nodules

Strain	Choline kinase (EC 2.7.1.32)	Choline-phospho-transferase* (EC 2.7.8.2)	GDP-DMP mannosyl-transferase† (EC 2.4.1.83)	UDP-ASGF galactosyl-transferase‡ (EC 2.4.1.38)	UDP-ASGF *N*-acetyl-galactosamine transferase‡
61-A-101	350	200	300	780	1600
RH 31-Marburg	80	120	280	800	800
61-A-24	50	90	180	550	570

Tissue, infected with various strains of *Rhizobium japonicum*, was assayed for various enzymes. Figures given are percent stimulation over root tissue. ASGF, asialoagalactofetuin; DMP, dolicholmonophosphate.
*Data calculated from ref. 12.
†Data summarized from ref. 9.
‡Data taken from ref. 11.

from the effective symbiosis were compared to gels of control root cell cytoplasm. Fig. 6*b* shows that while the gel of the host cell cytoplasm between pH 7.8 and 8.8 and 70 and 50 kDa has many proteins, there were two spots that corresponded to the predicted positions of choline kinase I and choline kinase II (arrows). Fig. 6*a* shows the same area in the gel made from control root tissue. Among the many proteins present one lies where choline kinase I should lie (arrow), but there is a clear area where choline kinase II should be (circle).

In certain symbioses in which peribacteroid membranes are made in large quantities, enzymes involved in membrane biogenesis are stimulated (Table 2). The effective symbiont stimulates all the enzymes researched, from the cytoplasm through the endoplasmic reticulum to the distal face of the Golgi. This contrasts with the 61-A-24 symbiosis, in which the degree of enzyme stimulation is much smaller. In the RH-31 symbiosis peribacteroid membranes with a higher stability are formed, and enzyme stimulation is correspondingly higher, although not as high as in the wild type. Terminal glycosylation and choline kinase are notable exceptions here. Peribacteroid membranes from RH-31 symbioses have fewer terminally glycosylated glycoconjugates than those from 61-A-101 symbioses (11), but the relative proportions of phosphatidylcholine are unknown, and this point, especially in relation to membrane stability, fluidity, and function, warrants further investigation.

Choline kinase II is a nodule-specific membrane-building enzyme that is expressed as a result of transformation. This begs the question: Is the new form produced by the bacteria that are then enclosed by a PBM or is it produced by the plant host? It is known that when effective nodules cut in half are given [^{14}C]choline chloride for periods up to 3 hr no radioactivity appears in the isolated bacteroids (12). The actual lipid composition of the symbiotic bacteria's cytoplasmic membrane is unknown. Most bacteria do not contain any phosphatidylcholine (16); notable exceptions are certain gram-negative species, including *Agrobacterium* (17). It is, however, known that *Agrobacterium tumefaciens* makes its phosphatidylcholine not by the choline kinase-containing choline CDP phosphate pathway but by stepwise methylation from phosphatidylethanolamine (18). Thus, it would seem improbable that *Rhizobium* possesses choline kinase. To be certain, however, cytoplasmic fractions of mid-logarithmic and lag phase *Agrobacterium tumefaciens* C 6-6, *Rhizobium japonicum* 61-A-101, nodules infected with *Rhizobium japonicum* 61-A-101 bacteroids, and ion-exchange chromatography choline kinase II fraction (also passed through a French pressure cell) were compared for choline kinase activity. Fig. 7 shows that the eukaryote-derived protein mixture had a much higher activity than any of the prokaryotic extracts. We, therefore, assume choline kinase II, like choline kinase I, is plant derived. Whether choline kinase II is a new gene product or is posttranslationally modified from choline kinase I is not yet known. Nodulins detected after two-dimensional electrophoresis at the correct location for choline kinase II from soybean have not yet been described (3). However, Bisseling *et al.* (2) have located a nodulin, nodulin 12, at the correct molecular mass for choline kinase from peas.

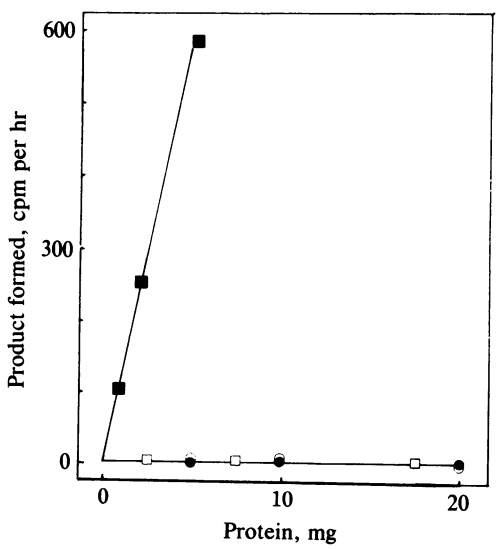

FIG. 7. Choline kinase activity in French pressed choline kinase II from ion-exchange chromatography of *Rhizobium japonicum* 61-A-101-infected nodule cytoplasm (■), bacteroid cytoplasm (□), free-living *Rhizobium japonicum* 61-A-101 cytoplasm (○), or free-living *Agrobacterium tumefaciens* C 6-6 cytoplasm (●).

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