Isolation and Partial Characterization of the Glutamate/Aspartate Transporter from Pea Leaf Mitochondria Using a Specific Monoclonal Antibody

Jeevalatha Vivekananda and David J. Oliver*
Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843

ABSTRACT

A library of monoclonal antibodies directed against the proteins of the inner mitochondrial membrane was screened for antibodies that could bind to the glutamate/aspartate transporter of pea mitochondria and thereby inhibit its activity. One antibody, 2C7, had the property of inhibiting glutamate and aspartate-dependent oxaloacetate metabolism by pea mitochondria without affecting the metabolism of other substrates. The antibody specifically recognized a 21,000 dalton protein, which was tentatively identified as the glutamate/aspartate transporter. The antibody was used to follow the extraction of this protein by Triton X-114 and cardiolipin and the partial purification of the protein by centrifugation and chromatography on hydroxylapatite. The partially purified preparation was reconstituted into azolectin vesicles and shown to catalyze glutamate/glutamate and glutamate/aspartate exchange in an apparently nonelectrogenic manner. The antibody was shown to specifically bind to the glutamate/aspartate exchanger by its ability to inhibit this reconstituted exchange reaction.

The translocation of substrates across the mitochondrial membranes plays an important role in regulating a number of metabolic pathways. The inner mitochondrial membrane, although impermeable to even small molecules, contains several carrier-mediated transport systems which facilitate the exchange of metabolites between the cytosol and the mitochondrial matrix (9, 18, 31). The transport proteins found in plant mitochondria are similar to the carriers found in animal systems with some specific differences. For example, plant mitochondria exchange glutamate for dicarboxylic acid ions (3, 5) and for aspartate in a nonelectrogenic reaction (13), whereas liver mitochondria contain an electrogenic glutamate/aspartate antiport as well as a glutamate/hydroxyl ion exchanger (1, 5, 20). Plant mitochondria also contain a specific high-affinity, high capacity carrier protein for oxaloacetate (4, 7, 24). This carrier apparently exchanges OAA2 for malate and, combined with excess malate dehydrogenase activity in the cytosol and matrix, provides a mechanism for connecting NADH pools in these two compartments. A similar carrier has been found in the chloroplast (10) which allows redox equilibration between all three metabolic compartments (6). While this carrier has a defined role in photosynthesis, its presence in nongreen tissues is still enigmatic (24, 25).

Several of the transport proteins have been isolated from animal mitochondria and reconstituted into artificial phospholipid vesicles. These include the monocarboxylate (22, 23), dicarboxylate (14, 26, 28) and tricarboxylate (27) carriers, the adenylate transporter (19), the α-ketoglutarate transporter (2, 12), the phosphate transporter (15, 30), and the glutamate carrier (1, 8, 17). The dicarboxylate transporter has also been isolated from plant mitochondria (28). The study of these transport proteins has been greatly aided by the relative ease with which they are separated from most other inner membrane proteins. This is accomplished by passing the crude detergent-solubilized fraction over a hydroxylapatite column. The transporters are so deeply imbedded within the detergent micelle that they do not bind to hydroxylapatite, thus affording a substantial purification.

Despite this advantage, few if any of these proteins have been purified to homogeneity, and only the phosphate and adenylate transporters have been unequivocally identified. The identification of the adenylate carrier was aided by the high-specificity, high-affinity interaction of carboxyactractractylidade with this carrier (19). Unfortunately, few chemicals are known which can be used to specifically label transport protein. We have recently reported a technique which allows the production of monoclonal antibodies which will specifically label these proteins. We used this technique to identify and isolate the dicarboxylate transporter from pea mitochondria (28). This technology has allowed us to identify a second transporter from both green and etiolated pea mitochondria. In this paper we report the generation of a monoclonal antibody that recognizes a single 21 kD polypeptide that was identified as the glutamate/aspartate transporter. This transporter was partially purified and shown to catalyze the antibody-sensitive transport of glutamate and aspartate in phospholipid vesicles.

MATERIALS AND METHODS

Plant Materials

Pea plants (Pisum sativum) were grown in commercial soil in a greenhouse for 2 to 3 weeks, and the etiolated seedlings were grown in sterile soil in the dark for 7 days at room temperature. Mitochondria were isolated from both green and etio-

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1 This work was supported by Grant DMB84-14866 from the Metabolic Biology Program of the National Science Foundation and is Publication 8956 of the Idaho Agricultural Experiment Station.

2 Abbreviation: OAA, oxaloacetate.
lated plants by grinding and differential centrifugation (29). The mitochondrial-enriched fraction was resuspended and subjected to controlled osmotic shock in 50 mM sorbitol (28). This osmotic shock removed much of the outer mitochondrial membrane allowing the antibodies access to the outer surface of the inner mitochondrial membrane, the site of the targeted transport proteins. We have shown (28) that shaking mitochondria at 50 mM sorbitol did not damage the oxidative reactions of the mitochondrial matrix. The mitochondria were centrifuged and resuspended in 0.3 M sorbitol, 20 mM Mops, 2 mM EDTA, and 0.1% BSA (pH 7.2) for further use.

Immunization of Mice and Hybridoma Production

The mitochondria were further purified on self-generating Percoll gradients (29). The purified mitochondria were washed to remove Percoll, suspended in 80% acetone to solubilize lipids, washed in PBS to remove soluble proteins, and homogenized into Freund's complete adjuvant. BALB/c mice were injected and hybridomas prepared (28). Hybridoma cultures producing monoclonal antibodies against the glutamate/aspartate transporter were selected from the resulting library by their ability to produce antibodies capable of inhibiting the transport-dependent metabolism of glutamate and aspartate coupled to the metabolism of OAA.

Glutamate-Dependent Metabolism of [4-14C]OAA

Transport-dependent substrate metabolism was measured following a 1 h incubation of the osmotically shocked mitochondria with supernatants from either control or experimental hybridoma cultures. The mitochondria were then distributed to media containing 10 mM glutamate, aspartate, malate, citrate, α-ketoglutarate, succinate, glycine, or pyruvate. Following a 2 min preincubation, [4-14C]OAA (prepared as in ref. 11) was added to the mitochondria and substrate-dependent OAA metabolism was allowed to proceed for 1 min. The reaction was stopped by acidification and the unreacted [4-14C]OAA selectively decarboxylated by heating with Cu2+ (7, 11, 28). The residual radioactivity was stable products formed from the transamination or reduction of OAA in the presence of the different substrates. The inhibition of substrate transport caused by binding of the monoclonal antibody to the transport protein resulted in a decrease in substrate flux into the matrix with a concomitant decrease in OAA metabolism.

Solubilization and Partial Purification of the Glutamate/Aspartate Transporter and Measurement of Glutamate Transport in Phospholipid Vesicles

The transporter-enriched fraction was isolated from etiolated pea mitochondria (28). The washed mitochondrial membranes were dissolved in 3% Triton X-114 containing 2 mg/mL of cardiolipin. Following centrifugation, the soluble portion was passed over a hydroxylapatite column to remove most of the proteins that are not substrate transporters.

This fraction, which contains many of substrate carriers, was reconstituted into azolectin vesicles (14, 28). The vesicles were preloaded with the desired substrate and excess external substrate was removed on a Dowex AG 1-X column.

The exchange of [14C]glutamate for the preloaded substrate was performed as described earlier (14, 28). The transport reaction was initiated with 1 mM [U-14C]glutamate and terminated at the indicated time by the addition of 10 mM N-ethylmaleimide. The proteoliposomes were separated from the unincorporated radioactivity on a spun Sephadex G-50 column and the amount of radioactivity transported in exchange for the preloaded substrate was determined by liquid scintillation counting (14, 28). In order to study the inhibitory effect of the monoclonal antibodies, the reconstituted proteoliposomes were incubated with the experimental or control hybridoma supernatants for 1 h on ice immediately preceding the uptake measurements.

RESULTS AND DISCUSSION

Monoclonal antibodies were produced against the full range of epitopes in the inner mitochondrial membrane. The monoclonal procedure produced about 750 hybridoma cultures each one of which recognized a single specific epitope. Analysis of the hybridoma library showed that over 95% of the cultures produced antibodies and that approximately 85% of the cultures contained antibodies that reacted with extracted inner mitochondrial membranes.

Inhibition of Glutamate and Aspartate Metabolism

The mitochondria were able to metabolize [4-14C]OAA at rapid rates provided that they were supplied with any of a number of readily metabolized substrates (28). These substrates were either oxidized or transaminated within the mitochondrial matrix. Following the oxidation of the substrates, the NADH produced could be used to reduce OAA to malate in a reaction catalyzed by malate dehydrogenase. Alternatively the substrate could serve as an amino donor for the transamination of OAA to aspartate. Aspartate could transaminated [14C]OAA to form [14C]aspartate and unlabelled OAA. Following either reaction the radioactive products formed from the [4-14C]OAA would no longer be decarboxylated by the copper treatment (7, 11, 28).

The supernatants from the individual hybridoma cultures were screened for their ability to bind to the transporters involved in the various reaction pathways and thereby block OAA metabolism. One group of antibodies screened, exemplified by antibody 2C7, was unique in that it inhibited glutamate and aspartate-dependent OAA metabolism without affecting metabolism linked to other substrate. The results for glutamate, aspartate, and glycine are shown in Table I. In addition, the metabolism of malate, succinate, α-ketoglutarate, citrate, and pyruvate were not inhibited by pretreating the mitochondria with this monoclonal antibody. Under these conditions the amount of inhibition of glutamate and aspartate metabolism was about 35%. This incomplete inhibition may result from the fact that the osmotic shock does not remove all of the outer mitochondrial membrane which protects some of the target transporter from the monoclonal antibody (28).

Given that the metabolism of both glutamate and aspartate were inhibited while the metabolism of none of the other substrates was altered, we have tentatively identified mono-
Table I. Effect of Monoclonal Antibody 2C7 on Substrate-Dependent Oxaloacetate Metabolism by Osmotically Shocked Mitochondria from Pea Leaves

Osmotically shocked mitochondria were incubated with the supernatant from hybridoma culture 2C7 for 1 h. The mitochondria were then distributed into microtitre dishes (12.5 μg of mitochondrial protein per well) with 100 μL of reaction medium containing 10 mM glutamate, aspartate, or glycine. In the glutamate reactions, 10 mM thiamine pyrophosphate, 1 mM ADP, and 1 mM coenzyme A were included. Following a 2 min preincubation, 0.6 mM [4-14C]OAA was added. After 1 min the reaction was stopped and the reacted [14C]OAA measured as described in (28). The controls were treated with hybridoma growth medium.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conditions</th>
<th>Rate of OAA Metabolism</th>
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</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>Control</td>
<td>919</td>
</tr>
<tr>
<td></td>
<td>+2C7</td>
<td>580 (63%)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Control</td>
<td>662</td>
</tr>
<tr>
<td></td>
<td>+2C7</td>
<td>433 (65%)</td>
</tr>
<tr>
<td>Glycine</td>
<td>Control</td>
<td>559</td>
</tr>
<tr>
<td></td>
<td>+2C7</td>
<td>640 (114%)</td>
</tr>
</tbody>
</table>

clonal antibody 2C7 as specifically binding to and preventing the function of the glutamate/aspartate transporter in these mitochondria.

SDS-PAGE and Western Blotting

Percoll-purified pea mitochondria were dissolved in SDS sample buffer and separated by SDS-PAGE. The separated proteins were then electrophobotted to a nitrocellulose membrane and, after blocking with 3% gelatin in TBS (10 mM Tris-HCl, 150 mM NaCl [pH 7.0]), the membrane was probed with the monoclonal antibody, 2C7. The bound antibody was visualized using an alkaline phosphatase conjugated indicator antibody (28). The monoclonal antibody reacted with a single band from plant mitochondria (Fig. 1, lane 3). This protein, which is identified as a subunit of the glutamate/aspartate transporter, had an apparent mol wt of 21 kD.

Purification of the Glutamate/Aspartate Transporter

Once we had established an assay system for the glutamate/aspartate transporter based on SDS-PAGE, electrophobouting to nitrocellulose, and immunodetection with antibody 2C7, we proceeded with the isolation and characterization of this protein. The method adopted for the purification of reconstitutively active glutamate/aspartate transporter was a two-step process based on our successful earlier attempts to isolate and reconstitute a number of plant mitochondrial transporters (14, 28). The washed mitoplasts from total mitochondria were extracted with the nonionic detergent Triton X-114 in the presence of cardiolipin. Following a ultracentrifugation step to remove undissolved membranes, the solubilized proteins were subjected to column chromatography using hydroxylapatite. This technique has been applied to the purification of several other transport proteins (2, 12, 14, 15, 23, 27, 28). The crude Triton extract and the pass from the hydroxylapatite column were subjected to SDS-PAGE and Western blotting using antibody 2C7. Figure 1 shows that the antibody identified the same 21 kD protein in these two preparations as identified from total mitochondria (Fig. 1). The glutamate/aspartate transporter from pea mitochondria behaves like the other mitochondrial transporters studied in that it is extracted into Triton X-114 containing cardiolipin and does not bind to hydroxylapatite.

The total proteins present in the washed mitochondria (lane 1), the crude Triton X-114 extract (lane 2), and the hydroxylapatite purified fraction (lane 3) were analyzed by SDS-PAGE followed by silver staining (Fig. 2). The extensive purification achieved by the hydroxylapatite chromatography is evident. Prior to this column a considerable number of bands are visible; whereas in the subsequent steps there are only a few bands mostly present between 21.5 and 45 kD. The same type of polypeptide pattern was reported in our earlier investigations (28) and also observed in other laboratories (12, 14, 16). Although there is a diffuse protein band in the region where the glutamate/aspartate transporter was localized by Western blotting, we cannot identify this protein as the glutamate/aspartate transporter. In fact, we have shown that this band contains the dicarboxylate transporter (28) and the monocarboxylate carrier (our unpublished data) in addition to the glutamate/aspartate transporter.

Reconstitution of the Glutamate/Aspartate Transporter into Proteoliposomes

The crude Triton X-114 extract and the partially purified hydroxylapatite fraction were reconstituted into azolectin vesicles. Both preparations were capable of exchanging preloaded unlabeled glutamate for [U-14C]glutamate in the medium (Fig. 3). The exchange rate catalyzed by the partially purified fraction was approximately 75 times faster than the rate catalyzed by the crude fraction. This increase in specific
activity reflects the substantial purification afforded by the hydroxylapatite column (Fig. 2).

In the reconstituted system, [14C]glutamate in the medium could exchange for either glutamate or aspartate preloaded into the liposomes (Fig. 4). Glutamate uptake, however, could not be driven by a pH gradient established by preloading the liposomes with buffers to create an artificial pH gradient. This may suggest that glutamate/OH- counter exchange is not important in plant mitochondria or simply that this carrier is not present in the hydroxylapatite fraction.

In order to conclusively determine that the monoclonal antibody, 2C7, was specifically binding to and inactivating the glutamate/aspartate transporter, the proteolipid vesicles containing the hydroxylapatite fraction were incubated with the monoclonal antibody 2C7 for 1 h before the exchange reaction was measured. Incubation with the control hybridoma supernatant resulted in a 23% inhibition in the exchange rate exhibited by the proteoliposomes. Once this correction has been made, incubation with the antibody 2C7 inhibited the rate of glutamate-glutamate exchange by 80% (Table II).

Unlike the inhibition of glutamate or aspartate-dependent OAA metabolism, the exchange reaction is not dependent on any other proteins than the glutamate/aspartate carrier. This inhibition provides conclusive evidence that the antibody 2C7 reacts specifically with the glutamate/aspartate transporter.

**CONCLUSIONS**

The production of antibodies by hybridoma technology offers the ability to produce highly specific monoclonal antibodies without first purifying the antigen. This is of particular value in studying membrane proteins where it is often not possible to purify the antigen to homogeneity. By screening for antibodies that inhibit transport function, it is possible to obtain monoclonal antibodies that are specific for the desired transporter, even though it is, at this point, impossible to obtain the purified transport protein. While this technique has proven its specific applicability in studying plant mitochondrial substrate transporters, it is easy to envision how it could be used to study other low abundance membrane proteins.

The subunit of the glutamate/aspartate transporter as identified by the monoclonal antibody 2C7 has an apparent molecular mass of 21 kD. Further work will be needed to determine if the functional transporter is comprised of additional polypeptide chains. The size of the transporter subunit identified is similar to, although distinctly different from, the dicarboxylate transporter from pea mitochondria (26 kD). The other mitochondria transporters either conclusively or tentatively identified from mammalian sources are in the same size range. The dicarboxylate transporter is believed to be between 27 and 37 kD (15), the \( \alpha \)-ketoglutarate transporter
The vesicles were formed from azolectin and the hydroxylapatite eluate and were preloaded with 6 mM glutamate. The liposomes were then incubated with either control supernatant or the supernatant from hybridoma culture 2C7. After 1 h the rate of NEM-sensitive glutamate exchange was measured.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rate of [14C]Glutamate Uptake (nmol (mg protein)^{-1} (min)^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>133</td>
</tr>
<tr>
<td>+ Control supernatant</td>
<td>102</td>
</tr>
<tr>
<td>+ Antibody 2C7</td>
<td>20</td>
</tr>
</tbody>
</table>

Table II. Effect of Monoclonal Antibody 2C7 on Glutamate Transport in Reconstituted Proteolipid Vesicles

is reported to be 31 KD (2, 12), and phosphate transporter preparations contain proteins of 30 and 35 KD (15, 30).

Glutamate transport in mammalian mitochondria proceeds by two mechanisms, an electroneutral glutamate/OH^- exchange reaction (20) and an electrogenic glutamate (+H^+)/ aspartate exchange. The electrochemical gradient imposed by mitochondrial electron transport makes this latter exchange reaction nearly unidirectional with glutamate taken into the mitochondria and aspartate exported to the cytosol. The situation in plant mitochondria is different. Day and Wiskich (3) have provided evidence for a glutamate/dicarboxylate exchange. More recently Journet et al. (13) presented evidence that plant mitochondria can rapidly exchange external aspartate for internal glutamate (see also ref. 21). This reaction, which cannot occur in energized mammalian mitochondria, is electroneutral and therefore readily reversible. This exchanger apparently was the one detected by monoclonal antibody 2C7. Our reconstituted proteoliposome system provided no evidence for an electrogenic exchange. The possibility of glutamate/dicarboxylate exchange was not tested.

LITERATURE CITED

8. Gaugenon DC, Juliard JH (1979) Isolation of a glutamate carrier system from pig heart mitochondria and incorporation into liposomes. Methods Enzymol 56: 419-430