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The Phosphate Transporter from Pea Mitochondria (Isolation and Characterization in Proteolipid Vesicles)

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The phosphate transporter from mitochondria will exchange matrix phosphate for cytosolic phosphate and facilitate either phosphate/proton symport or phosphate/hydroxyl ion antiport. The phosphate transported into the matrix by this carrier is either p-hydroxymercuribenzoate, pyridoxal 5-phosphate, and dansyl dicarboxylate transporter, permitting entry of malate and succinate phosphate/proton symport or phosphate/hydroxyl ion antiport.

The phosphate transporter facilitates phosphate transport across the inner mitochondrial membrane. It catalyzes the thermodynamically indistinguishable phosphate/proton symport or phosphate/hydroxyl ion antiport as well as the exchange of matrix and cytosolic phosphate against each other (Wiskich, 1974, 1977; LaNoue and Schoolwerth, 1979; Kaplan and Pedersen, 1983; Day and Wiskich, 1984; Wohlrab, 1986). The involvement of this transport reaction with the pH gradient across the mitochondrial membrane allows this carrier to connect the proton gradient generated by the mitochondrial electron transport chain to the accumulation of high levels of phosphate in the matrix. Physiologically, this transporter serves two purposes. The phosphate in the matrix serves as a substrate for oxidative phosphorylation and the synthesis of ATP. This ATP can then exchange with cytosolic ADP on the adenylate carrier. The phosphate transport can also exchange back out of the mitochondria against the uptake of cytosolic dicarboxylic acids (malate and succinate) on the dicarboxylate transporter (Kaplan and Pedersen, 1985; Vivekananda et al., 1988). Thus, the phosphate transporter connects the electrochemical gradient generated by electron transport to the uptake of dicarboxylates and eventually α-ketoglutarate and citrate (LaNoue and Schoolwerth, 1979).

The phosphate transporter has been isolated, purified, and studied in detail from beef heart and rat liver (Wehrle and Pedersen, 1983; Kolbe et al., 1984; Kaplan et al., 1986; Wohlrab, 1986). The gene for the protein has been cloned from rat (Ferreira et al., 1989), yeast (Phelps et al., 1991), and cow (Runswick et al., 1987). The mature protein predicted by these sequences contains 311 to 313 amino acids and has a mol wt of about 33,000. Sequence and structural similarities have been noted between the phosphate transporter, the adenylate transporter, and brown fat uncoupling protein (Aquila et al., 1987; Runswick et al., 1987).

In this paper we describe the isolation and partial purification of the phosphate transporter from pea mitochondria.

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DEPC, diethyl pyrocarbonate; NEM, N-ethylmaleimide.
This preparation was reconstituted into phospholipid vesicles and the exchange of internal phosphate for external \(^{32}\)Pi was characterized.

**MATERIALS AND METHODS**

**Preparation of Phospholiposomes**

\(^{32}\)Pi was from New England Nuclear. Dowex AG1-X8, azolectin, cardiolipin, Percoll, and other standard reagents were from Sigma. Hydroxylapatite was from Bio-Rad, and p-hydroxyphenylglyoxal was from the Pierce Chemical Co.

Pea (*Pisum sativum* L. cv Alaska) seeds imbibed overnight in running water and were grown in vermiculite in the dark. Etiolated seedlings were harvested after 10 d, and mitochondria were isolated by grinding and differential centrifugation and further purified on a self-generating Percoll gradient (Walker et al., 1982a). The mitochondria were resuspended in 20 mM Mops, pH 7.2, the matrix was released by two freeze-thaw cycles (–20°C), and the membranes were pelleted at 20,000g for 30 min. The mitochondrial membranes (about 10 mg protein/mL) were solubilized in 3% Triton X-114 in 10 mM KPi, 20 mM KCl, 1 mM EDTA, pH 7.2, containing 6 mg cardiolipin mL\(^{-1}\), and the transporter-enriched fraction was prepared by hydroxylapatite chromatography as described earlier (Kaplan and Pedersen, 1985; Kaplan et al., 1986; Vivekananda et al., 1988; McIntosh and Oliver, 1992). In those experiments where the effect of phosphate concentration was studied the transporter fraction had to be prepared in 0.1 mM phosphate. To accomplish this the mitochondria were solubilized with 3% Triton X-114 in 20 mM Hepes, 0.1 mM KPi, 20 mM KCl, 1 mM EDTA, pH 7.2, containing 6 mg/mL cardiolipin. The hydroxylapatite column was eluted with the same solution containing 3% Triton X-114 and 3 mg cardiolipin mL\(^{-1}\).

Azolectin was prepared as described earlier (McIntosh and Oliver, 1992) and sonicated at room temperature under NZ and Oliver, 1989) transporters. It is generally assumed, but unproven, that these bands contain all the substrate transporters from plant mitochondrial membranes. Several other bands are present on the gels at about 50, 30, and <20 kD. In animal systems, the major contributors to these bands are the adenylate and phosphate transporters (Kolbe et al., 1984). It is generally assumed, but unproven, that these bands contain all the substrate transporters from plant mitochondrial membranes. Several other bands are present on the gels at about 50, 30, and <20 kD. In animal systems, the major contributors to these bands are the adenylate and phosphate transporters (Kolbe et al., 1984).

**Assays**

The proteoliposomes were preincubated with inhibitors as noted. Inhibitors were dissolved in water or ethanol at 100X. The ethanol had no effect on the assay. The exchange reactions were initiated by adding 10 \(\mu\)L (5 \(\mu\)Ci) of \(^{32}\)Pi to the 330 \(\mu\)L of proteoliposomes and, after the desired time at 22°C, a 160-\(\mu\)L aliquot of the reaction was pipetted into 340 \(\mu\)L of ice-cold DEPC (final concentration 32 mM) to stop the exchange reaction. Following the exchange reaction the samples were passed over Dowex AG1-X8 to remove unincorporated \(^{32}\)Pi (McIntosh and Oliver, 1992), and internalized \(^{32}\)Pi was determined by liquid scintillation counting of Cerenkov radiation without scintillation cocktail at 87% efficiency. All results were corrected for the amount of phosphate exchanged in liposomes that were incubated with 32 mM DEPC for 3 min before \(^{32}\)Pi addition. The phosphate exchange rates thereby represented are for DEPC-sensitive exchange.

**RESULTS**

The substrate transporters are highly hydrophobic molecules that are about 90% buried in the membrane. As a result, there are very few hydrophobic residues at the surface of the protein and these proteins interact poorly with hydroxylapatite. When Triton X-114-solubilized membrane proteins are passed over a hydroxylapatite column, the vast majority of the proteins stick to the resin while a small number of proteins, including the substrate transporters, pass through the column. When solubilized pea mitochondria membranes are passed over hydroxylapatite, about 99.8% of the proteins bind with no apparent loss in the phosphate transport activity. The hydroxylapatite-purified fraction, when reconstituted into azolectin liposomes preloaded with either 0.1 or 10.0 mM phosphate, showed a 459- to 523-fold increase in the specific activity compared with the crude Triton X-114 extract (Table I). This fraction was used for all of the following experiments.

The protein composition of the crude Triton X-114 extract and the hydroxylapatite pass fraction are shown in Figure 1. The major protein bands have apparent molecular masses of about 33 and 35 kD. In animal systems, the major contributors to these bands are the adenylate and phosphate transporters (Kolbe et al., 1984). It is generally assumed, but unproven, that these bands contain all the substrate transporters from plant mitochondrial membranes. Several other bands are present on the gels at about 50, 30, and <20 kD and have not been identified. The protein extracts prepared in low phosphate were very similar to those prepared in 10 mM phosphate. In plant systems this preparation has been shown to contain the monocarboxylate (Vivekananda and Oliver, 1990), dicarboxylate (Vivekananda et al., 1988), tricarboxylate (McIntosh and Oliver, 1992), \(\alpha\)-ketoglutarate (Genchi et al., 1991), and glutamate/aspartate (Vivekananda and Oliver, 1989) transporters.

When proteoliposomes were prepared with the hydroxylapatite-purified fraction and internal and external phosphate concentrations of 10.0 mM, phosphate/phosphate exchange occurred for at least 40 min at room temperature (Fig. 2). No true initial rate of uptake could be established. The exchange rate for the 1st min was substantially faster than the rate measured during the subsequent time periods. Uptake, however, was fairly constant between 2 and 20 min, suggesting that after an initial burst, uptake occurred at a more uniform rate. In this investigation 32 mM DEPC was used as an inhibitor-stop reagent. The inhibition by DEPC is not instantaneous (data not presented) and, therefore, time resolution below 1 min is difficult with this system. The relatively slow time course of phosphate/phosphate exchange with the
Table I. Purification of the phosphate transporter on hydroxylapatite

The membranes from Percoll-purified pea mitochondria were solubilized in Triton X-114 and either reconstituted directly into phospholipid vesicles or passed over hydroxylapatite before forming proteoliposomes. The vesicles were preloaded with either 0.1 or 10.0 mM KPi. Phosphate/phosphate exchange proceeded for 2 min before the reactions were stopped with 32 mM DEPC and the external phosphate was removed by Dowex AG1-X8 chromatography. Assays were done in duplicate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Yield</th>
<th>Specific Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0 mM phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton extract</td>
<td>92</td>
<td>130</td>
<td></td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite fraction</td>
<td>0.181</td>
<td>134</td>
<td>103</td>
<td>743</td>
<td>523</td>
</tr>
<tr>
<td>0.1 mM phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton extract</td>
<td>103</td>
<td>29.9</td>
<td>83</td>
<td>0.29</td>
<td>459</td>
</tr>
<tr>
<td>Hydroxylapatite fraction</td>
<td>0.187</td>
<td>24.9</td>
<td></td>
<td>133</td>
<td></td>
</tr>
</tbody>
</table>

Phosphate transporter from peas is very similar to that observed with the equivalent transporter from bovine heart mitochondria (Genchi et al., 1988).

There are two possible mechanisms for phosphate/phosphate exchange in mitochondria; it can be facilitated by either the phosphate or the dicarboxylate transporters. The dicarboxylate transporter is sensitive to n-butylmalonate (LaNoue and Schoolwerth, 1979; Wohlrab 1986; Vivekananda et al., 1988). When the phosphate/phosphate exchange reaction with the reconstituted transporter in proteoliposomes was run in the presence of 20 mM n-butylmalonate, the exchange rate was inhibited less than 10% (Fig. 2). This suggests that over 90% of the phosphate transport is being carried on the phosphate transporter with only a minor contribution by the dicarboxylate transporter.

The rate of phosphate transport was dependent on the internal (Table I) and external (Fig. 3) phosphate concentration. The method of preparing these liposomes results in an initial ratio of internal to external phosphate near unity. To measure the effect of external phosphate concentration on the exchange rate, proteoliposomes were prepared with the hydroxylapatite-purified membrane fractions and 0.1 mM phosphate. The phosphate uptake rates shown in Figure 3 are for proteoliposomes with an initial internal phosphate concentration of 0.1 mM and an external phosphate concentration of 0.1 to 5.0 mM, as indicated. The rate of phosphate transport was determined by measuring the uptake of radioactive phosphate into the liposomes

Figure 1. SDS-PAGE analysis of the crude Triton X-114-solubilized mitochondrial membranes and the fraction recovered from hydroxylapatite chromatography. The proteins were separated on a 15% SDS-polyacrylamide gel and stained with silver. Lanes A and C, BioRad high molecular mass markers; lane B, 6 μg of Triton X-114-solubilized membrane proteins; lane D, 0.14 μg of hydroxylapatite-purified protein in 10 mM phosphate buffer; lane E, 0.14 μg of hydroxylapatite-purified protein in 0.1 mM phosphate buffer; lane F, 1.4 μg of acetone-precipitated hydroxylapatite-purified protein prepared in 10 mM phosphate buffer; lane G, 1.4 μg of acetone-precipitated hydroxylapatite-purified protein prepared in 0.1 mM phosphate buffer.

Figure 2. Time course for phosphate/phosphate exchange by the hydroxylapatite-purified fraction reconstituted into phospholipid vesicles. The reaction was initiated by addition of the isotope to proteolipid vesicles prepared with 1 μg of protein and stopped at the time indicated by the addition of 32 mM DEPC. The internal and external phosphate concentrations were 10 mM. n-Butylmalonate was present at 20 mM in those samples indicated. Assays were done in duplicate, and the data presented are the averages of two determinations.
uptake showed kinetics with an apparent $K_{0.5}$ of 1.6 mM and a $V_{max}$ of 209 nmol (mg protein)$^{-1}$ min$^{-1}$. Increasing the internal phosphate concentration to 10 mM would be expected to increase the measured $V_{max}$ to approximately 1200 nmol (mg protein)$^{-1}$ min$^{-1}$ if the increase is proportional to that noted in Table I.

The two transport reactions of the phosphate transporter, phosphate/phosphate and phosphate/hydroxyl exchange, are demonstrated in Table II. When the external phosphate concentration was 100 times greater than the internal concentration, the rate of $^{32}$Pi uptake was more than doubled when the uncoupler CCCP was included in the reaction medium. In the absence of the uncoupler phosphate/hydroxyl ion antiport (or phosphate/proton symport) would result in the formation of a pH gradient; collapsing this gradient with CCCP increased uptake. When high concentrations of phosphate are present on both sides of the membrane, however, phosphate/phosphate exchange predominated and CCCP addition had no effect.

Fast, effective transport inhibitors are important for these types of studies. As noted below, NEM was not effective in these studies. A search of a range of potential inhibitors showed that DEPC had the properties needed. Inhibition by DEPC was concentration dependent, with 32 mM needed for 95% inhibition with a 3-min incubation period (Fig. 4). DEPC reacts with His residues and has been shown to inhibit the phosphate transporter from rat liver mitochondria with about the same concentration requirements (Kaplan et al., 1986). Unless otherwise indicated, all of the data presented are from DEPC-sensitive phosphate/phosphate exchange.

The Arg-directed reagent $p$-hydroxyphenylglyoxal was also a potent inhibitor of phosphate/phosphate exchange. The inhibition was concentration dependent, with 20 mM inhibiting exchange over 90% during a 3-min preincubation (Fig. 5).

The traditional inhibitor for the phosphate transporter in mammalian mitochondria is the sulfhydryl reagent NEM (Wehrle and Pedersen, 1983; Kolbe and Wohlrab, 1985; Kaplan et al., 1986). In fact, the high reactivity of NEM with Cys$^44$ in the beef protein was used to specifically label and isolate the protein (Kolbe et al., 1984). The phosphate transporter from pea mitochondria, however, is surprisingly insensitive to NEM. Preincubation of proteoliposomes with 15 mM NEM for up to 40 min did not inhibit phosphate/phosphate exchange (data not presented).

In addition to sensitivity to DEPC and $p$-hydroxyphenylglyoxal, the phosphate transporter was inhibited by the sulfhydryl reagent $p$-hydroxymercurobenzoate and the Lys-directed compounds pyridoxal 5-phosphate and dansyl chloride (Table III). Carboxyatractylsode, $\alpha$-cyano-4-hydroxycinnamate, and $n$-butylmalonate, inhibitors specific for the

<table>
<thead>
<tr>
<th>Phosphate Uptake</th>
<th>Phosphate Uptake</th>
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<tbody>
<tr>
<td>Concentration</td>
<td>Concentration</td>
</tr>
<tr>
<td>Phosphate</td>
<td>Phosphate</td>
</tr>
<tr>
<td>$\mu$M</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Internal</td>
<td>External</td>
</tr>
<tr>
<td>0.1</td>
<td>10.0</td>
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<tr>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Figure 3. The effect of external phosphate concentration on the rate of phosphate/phosphate exchange. The phospholipid vesicles were reconstituted with hydroxylapatite fraction (1 $\mu$g assay) prepared with 0.1 mM potassium phosphate. The reaction was initiated by adding $^{32}$Pi and sufficient unlabeled KPi to obtain the external concentration indicated. The reaction was stopped with DEPC after 1 min, and the samples were immediately processed over Dowex AG1-X8. Assays were done in duplicate.

Figure 4. The effect of DEPC on the phosphate/phosphate exchange reaction. Proteoliposomes were prepared with the hydroxylapatite fraction (1 $\mu$g protein assay) and preloaded with 10 mM KPi. The vesicles were preincubated with the indicated concentration of DEPC for 3 min before the reaction was initiated and stopped 5 min later with DEPC. Assays were done in duplicate and data are the averages of two determinations.
adenylate, monocarboxylate, and dicarboxylate transporters, respectively, did not inhibit the phosphate transporter. Sulfate, pyrophosphate, and nitrate, possible phosphate analogs, did not inhibit transport when presented in a 5-fold excess over the phosphate concentration (50 versus 10 mM phosphate).

**DISCUSSION**

The phosphate/phosphate exchange reaction showed many characteristics that were similar to those identified for the yeast and mammalian phosphate transporter. The \( K_{0.5} \) for the pea protein was 1.6 mM. This is very similar to the values of 1.5 to 2.5 mM for mammalian transporters (Wohlrab, 1986). Using a rapid filtration assay that allows time resolution of 150 ms (DuPont and Moutin, 1987), Douce and Neuburger (1990) reported a \( K_{0.5} \) of 0.6 mM and a \( V_{\text{max}} \) of 5.6 \( \mu \)mol (mg protein\(^{-1} \) min\(^{-1} \) with isolated potato mitochondria. Rebeille et al. (1984) measured phosphate concentrations in sycamore cells of 5 to 6 mM (3 mM when grown in phosphate-free medium). Under any of these conditions, the phosphate transporter is nearly saturated with phosphate and, given the \( V_{\text{max}} \) measured, would not pose a limitation on Pi transport and respiration. The protein profiles on SDS-PAGE for the hydroxylapatite fraction from pea mitochondria are also similar to those seen with yeast and rat liver. The tentative identification of the transporters as bands at \( 33 \) to \( 35 \) kD agrees with the masses of the phosphate transport protein purified more thoroughly from other sources (Kolbe et al., 1984; Wohlrab, 1986). The phosphate transporter from peas, as with all other transporters identified to date, is sensitive to the mercurial sulphydryl reagents like \( p \)-hydroxymercuribenzoate.

![Figure 5](image_url)

**Figure 5.** The effect of \( p \)-hydroxyphenylglyoxal on phosphate/phosphate exchange. Proteoliposomes were prepared with the hydroxylapatite fraction (1 mg/assay) and preloaded with 10 mM KPi. The exchange reactions were initiated after a 3-min incubation with the concentration of \( p \)-hydroxyphenylglyoxal and terminated after 5 min by the addition 32 mM DEPC containing the same concentration of \( p \)-hydroxyphenylglyoxal. Assays were done in duplicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM</td>
<td>15</td>
<td>-7</td>
</tr>
<tr>
<td>( p )-Hydroxymercuribenzoate</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Dansyl chloride</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Pyridoxal 5-phosphate</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>( N )-Acetylimidazole</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>( \beta )-Mercaptoethanol</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>DEPC</td>
<td>16</td>
<td>73</td>
</tr>
<tr>
<td>DEPC</td>
<td>32</td>
<td>93</td>
</tr>
<tr>
<td>( p )-Hydroxyphenylglyoxal</td>
<td>20</td>
<td>94</td>
</tr>
<tr>
<td>( n )-Butylmalonate</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>( \alpha )-Cyan 4-hydroxycinnamate</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Carboxyatractyloside</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>PEP</td>
<td>50</td>
<td>-7</td>
</tr>
<tr>
<td>PPI</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Sulfate</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>Nitrate</td>
<td>50</td>
<td>-4</td>
</tr>
</tbody>
</table>

There are some differences. Like the yeast transporter, the pea mitochondrial membrane transporter is not inhibited by NEM. The beef and rat liver transporters have a reactive Cys residues that results in NEM sensitivity (Phelps et al., 1991). The yeast phosphate transporter lacks this residue and, as a result, is not inhibited by NEM. Since the pea mitochondrial phosphate transporter is not inhibited by NEM, it seems reasonable to predict that it is more similar to the yeast protein and lacks this reactive Cys residue.

Chemical modifiers directed against the basic amino acid residues \( \text{Arg} (p \text{-hydroxyphenylglyoxal}), \text{His} (\text{DEPC}), \) and \( \text{Lys} (\text{dansyl chloride and pyridoxal 5-phosphate}) \) suggest that a number of positively charged amino acid side chains are essential for transport function. These residues are likely to be involved in binding the phosphate anion to the carrier protein.

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**LITERATURE CITED**


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**Table III. Inhibition of phosphate/phosphate exchange in proteoliposomes by chemical modifiers and substrate analogs**

The proteoliposomes were preincubated with the inhibitor for 5 min and the \(^{32}\)Pi uptake reaction was for 4 min before the reaction was stopped with 32 mM DEPC. All assays were done in duplicate and the data presented are the average of two or three replications.
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