Mitochondrial Proton/Phosphate Transporter

AN ANTIBODY DIRECTED AGAINST THE COOH TERMINUS AND PROTEOLYTIC CLEAVAGE EXPERIMENTS PROVIDES NEW INSIGHTS ABOUT ITS MEMBRANE TOPOLOGY

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Gloria C. Ferreira, Raymond D. Pratt, and Peter L. Pedersen
From the Laboratory for Molecular and Cellular Bioenergetics, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Molecular cloning and sequencing of a full-length cDNA encoding the rat liver mitochondrial phosphate transporter (H⁺/P⁻ symporter) has revealed its primary structure (Ferreira, G. C., Pratt, R. D., and Pedersen, P. L. (1989) J. Biol. Chem. 264, 15628-15633). To date, no experimental data pertinent to the membrane topology of this transporter are available. For this reason, four different peptides which represent different regions of the H⁺/P⁻ symporter were synthesized and used to raise polyclonal antibodies. Each of the antipeptide antibodies exhibits immunoreactivity with its synthetic peptide antigen, but only antiserum against a COOH-terminal peptide reacts with the native transporter, suggesting that the other peptides are either conformally restricted or located in the interior of the protein.

Competitive radioimmunoassays, using intact "mitoplasts" (outer membrane-free mitochondria) and inverted inner membrane vesicles, show that the COOH-terminal antibodies bind only to the cytoplasmic surface of the inner membrane, indicating that the COOH terminus of the protein is normally exposed to the mitochondrial intermembrane space. In support of this conclusion, tryptic digestion of mitoplasts but not of the inside-out vesicles, cleaves the antigenic site for the COOH-terminal antibodies.

In other experiments, it was shown that N-ethylmaleimide, a sulfhydryl alkylating agent known to inhibit the mitochondrial phosphate transporter, markedly reduces the accessibility of the COOH terminus to trypsin. These studies provide the first direct experimental data relevant to the membrane topology of the mitochondrial H⁺/P⁻, symporter. In addition, they support the view that alkylation of a reactive cysteine residue induces a significant conformational change in the transporter.

The mitochondrial proton/phosphate symporter (Pc) is a membrane-embedded protein which translocates phosphate from the cytoplasm into the mitochondrial matrix, where it is used to synthesize ATP. The rat liver mitochondrial Pc has been purified to homogeneity and functionally reconstituted into liposomes (1). Its primary structure has been determined by sequencing a full-length cDNA clone encoding the protein (2). The deduced protein has 312 amino acids and a molecular mass of 34,740 daltons.

Hydropathy profiles of the rat liver Pc predict six hydrophobic α-helical segments connected by hydrophilic loops, and a short hydrophilic carboxyl terminus (2-4). Of the 8 cysteine residues in the protein, only Cys-41 has been shown to be reactive with N-ethylmaleimide (NEM) (5). This "Cys-essential" residue has been shown also to be readily accessible from the cytosolic side of the inner mitochondrial membrane (6). Significantly, alkylation of the transporter by NEM inhibits transport activity and induces a large mobility shift in SDS-PAGE (1, 5).

With the exception of the above information, very little is known about the topology of the mitochondrial Pc within the inner membrane. Therefore, those regions of the protein which are exposed to the cytosol or to the mitochondrial matrix remain to be identified. For this reason, we have examined the accessibility of the Pc to both antibodies directed against specific peptide regions and to the protease trypsin. These studies, which are described in detail below, were carried out with both intact rat liver "mitoplasts" (outer membrane-free mitochondria) and with inverted (inside-out) inner membrane vesicles.

EXPERIMENTAL PROCEDURES

Materials

All the t-butoxycarbonyl-protected amino acids, the peptidylglycine α-amidating monoxygenase reagents, and the reagents for the peptide assembly were from Applied Biosystems, Inc. [3H] Protein A was purchased from ICN Biochemicals. Immulon 2 wells (Removawell) were obtained from Dynatech Laboratories, Inc., Alexandria, VA. Nitrocellulose (BA 85, 0.45 μm) was from Schleicher & Schuell. Acrylamide gel reagents and Affi-Gel 10 were purchased from Bio-Rad. The biocinchonic acid protein assay reagents were from Pierce Chemical Co. Trypsin and trypsin inhibitor were products of Boehringer Mannheim. N-(γ-Maleimidomethyl)oxysuccinimide was obtained from Behring Diagnostics. N-ethylmaleimide was purchased from Sigma. All other chemicals were of the highest quality available. Centricon-30 microconcentrators were obtained from Amicon.

Methods

Peptide Synthesis—Peptides (Table I) were synthesized on an Applied Biosystems Model 430A peptide synthesizer by the solid phase method developed by Merrifield (7). Peptide assembly was initiated with the appropriate peptidylglycine α-amidating monoxygenase reagents and the t-butoxycarbonyl-protected amino acids using a synthetic amidhvde activation procedure (8). A cysteine residue

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was added to the COOH terminus of the NH₂-terminal peptide and to the NH₂ terminus of the COOH-terminal peptide to form the coupling to the carrier protein. The synthetic protected peptide resins were deprotected and cleaved according to the method described in Ref. 8. The crude peptides were subjected to purification by high performance liquid chromatography on a semipreparative C₁₈ column as described in Ref. 8. The peptides were at least 95% pure.

Preparation of Antibodies—COOH- and NH₂-terminal peptides were used to raise antisera or antibodies for 2-3 h at room temperature or overnight at a dilution of 1:200 and 1:500, respectively. The blots were incubated with the antisera and purified antibodies at a dilution of 1:20-100 peptide. Two rabbits were immunized with the conjugated N-peptide, two with the conjugated C-peptide, two with the free conjugated Cys-peptide, three with the free Cys-peptide, and two with the free P₁₃₄₋₁₆₉ peptide. Preimmune sera were drawn before the first injection. Antisera titer determinations were performed every 3–4 weeks for a period of approximately 1 year. Additional injections of the antigen were made 2 and 10 weeks after the first injection.

Affinity Purification of Anti-C-peptide Antibodies—Antibodies directed to the C-peptide were purified by affinity chromatography according to the manufacturer's instructions and as described in Ref. 11. Basically, the C-peptide (5 mg) was coupled to Affi-Gel 10 (2 ml) in 50 mM Tris-HCl, pH 7.4, at 4 °C for 16 h. The coupling reaction was terminated by the addition of Tris-HCl, pH 8.0, and equilibrated in a 10 ml column with 10 mM phosphate-buffered saline (PBS), pH 7.4. The antisera was added to the column, washed with PBS followed by PBS, 2 mM NaCl. The antibodies were eluted with 3.5 mM NaSCN in PBS, pH 6.6, dialyzed against PBS, pH 7.4, concentrated in a Centricon-30 microconcentrator, and stored at -70 °C.

Mitoplasts and Inner Membrane Vesicle Preparation—Intact, outer membrane free, mitochondrial vesicles (mitoplasts), and purified “inverted” inner membrane vesicles were prepared from rat liver mitochondria exactly as described by Pedersen et al. (12). The mitoplasts are intact as judged by electron microscopy, acceptor control ratios, and enzyme marker analysis.

Standard Radioimmunoassay (RIA). An antigen solution (100 μl containing 10 μg/ml free synthetic peptides, 2 mg/ml mitoplasts, or 1 mg/ml inner membrane vesicles) were incubated in microtiter wells at 4 °C for 2-12 h (13). After washing with PBS (50 mM Tris-HCl, pH 8.0, and 150 mM NaCl), 0.1% BSA (or 0.1% gelatin), the free sites on the wells were blocked with PBS, 1% BSA or with PBS, 1% gelatin (in the cases where the antigens were coupled to BSA), for 1 h at room temperature. The antibodies diluted in PBS, 0.1% BSA or with 0.1% gelatin were then incubated for 1 h at room temperature. The wells were then washed with PBS, 0.1% BSA (or 0.1% gelatin) and the antibodies were detected with [¹²⁵I] Protein A (10⁶ cpm/ml). Finally, the wells were washed with PBS, separated, and the radioactivity determined in a γ-counter.

Competition RIA—Antiserum diluted 1:300 in TBS, 0.1% gelatin was incubated with increasing concentrations of peptide in gelatin-blocked microtiter wells for 1 h at room temperature (14). The unreacted antibodies were then transferred to microtiter wells coated with mitoplasts or inner membrane vesicles and the standard RIA was performed.

Intact mitoplasts have fewer molecules of Pₐ/mg of protein than intact membrane vesicles. In order to normalize the amount of antigen in two preparations, equal aliquots of mitoplasts and inner membrane vesicles, at the same protein concentration were subjected to SDS-polyacrylamide gel electrophoresis. This step was followed by immunoblotting with the Pₐ antisera (as described below). After autoradiography, strips of nitrocellulose containing the [³⁵S]protein were excised and counted in a γ-counter.

Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis was carried out by the procedure of Laemmli (15), using 15% acrylamide and 1.5-mm gels. Electrophoretic transfer to nitrocellulose filters was according to Towbin et al. (16) in a Bio-Rad Trans Blot cell with 20% (v/v) methanol, 192 mM Tris, and 0.1% SDS. Antisera and purified antibodies were used at a dilution of 1:200 and 1:500, respectively. The blots were incubated with the antisera or antibodies for 2-3 h at room temperature or overnight at 4 °C. The binding of the antibodies to the Pₐ and its proteolytic fragments on the nitrocellulose filters was detected with ¹²⁵I-Protein A (approximately 3 × 10⁶ cpm/ml) in TBS, 0.1% TSA (or 0.1% gelatin), at room temperature for 1 h. The filters were extensively washed in TBS, air dried, and autoradiographed at ~70 °C.

Tryptic Digestions—Mitoplasts (45 μl, 29 mg/ml) or inner membrane vesicles (45 μl, 29 mg/ml) were suspended in 35 μl of 250 mM KP, pH 7.4, 5 mM EDTA, and 10 μl of 100 mM CaCl₂. The reaction was performed at room temperature and initiated by the addition of 10 μl of trypsin (1 mg/ml), and terminated by the addition of 10 μl of trypsin inhibitor (5 mg/ml). The reaction mixture was centrifuged twice at 48,000 rpm for 30 min. The final pellet was suspended in H medium (0.21 mM mannitol, 0.07 mM sucrose, 0.006 M Hepes, pH 7.4). Mitoplasts and inner membrane vesicles were incubated with 5 mM NEM for 5 min on ice.

Protein Determination—Protein concentrations were determined by the biureinic acid assay using bovine serum albumin as the standard.

RESULTS

Hydropathy Analysis of the Mitochondrial Phosphate Transporter Pₐ—Fig. 1 illustrates the hydropathy profile of the rat liver mitochondrial Pₐ and two possible working models reported for the arrangement of the transporter in the membrane (2, 4). The model presented in Fig. 1A is based on the finding that the highly reactive NEM-sensitive cysteine (Cys-41) is accessible to alkylation from the cytoplasmic side of the membrane (6), and that an even number of membrane-spanning segments would then predict a mitochondrial matrix location for both the NH₂ and COOH termini. In the model presented in Fig. 1B, a 12 amino acid β-strand is predicted to transverse the membrane between the two first α-helices.
This prediction, while satisfying the requirement that Cys-41 must be accessible from the cytoplasmic surface, necessitates that the P, cross the membrane seven times. Consequently, NH₂ and COOH termini would be on different sides of the membrane (4).

Characterization of Peptide-specific Antibodies—Antibodies were raised against synthetic peptides representing four different regions of the P,: the COOH-terminal region (E302–E312), the NH₂-terminal region (Al–S11), the reactive cysteine region (V34–E62), and the central region (E134–G169). These antibodies were then assayed by RIA (see “Methods”). Fig. 2A shows that antibodies to the COOH-terminal peptide were elicited in rabbits by the adequate conjugate. An antisera produced by immunization of rabbits with purified P, also reacted with the COOH-terminal peptide, indicating that the COOH terminus is immunogenic in the intact protein. Both the COOH-terminal and the P₁₃₄₋₁₆₉ antisera recognized the P, in assays involving immunoblots (Fig. 2B, Lanes 2 and 5). Antibodies directed against the NH₂-terminal and Cys peptides did not react with the P, in the immunoblot detection assay (Fig. 2B, Lanes 3 and 4).

In data not presented here, the NH₂-terminal peptide-BSA conjugate was shown to elicit peptide-specific antibodies, but the anti-P, serum does not contain antibodies that recognized this peptide. Likewise, the Cys-peptide antisera reacts with its own peptide antigen but the antitransporter antibodies do not react with the Cys-peptide. Finally, a high anti-P₁₃₄₋₁₆₉ peptide antibody titer was produced in the serum of rabbits immunized with this peptide. In this case, antibodies present in the anti-P, serum do react with the P₁₃₄₋₁₆₉ peptide.

Competition RIA: Location of the P, Carboxyl Terminus—In order to establish on which side of the membrane the COOH terminus is exposed, competitive RIA experiments were performed using mitoplasts and inverted inner membrane vesicles. Prior to carrying out these experiments, it was essential to establish the P, content in mitoplasts and inner membrane vesicles so that the amounts of each used in RIA experiments would contain the same amount of antigen. This measurement was made by quantitative immunoblotting, which demonstrated that inner membrane vesicles contain twice the number of P, molecules as do the mitoplasts on a per mg of protein basis (data not presented).

As shown in Fig. 3, intact mitoplasts exhibit a high binding capacity for the COOH-terminal peptide-specific antisera, whereas inner membrane vesicles, whose membrane orientation is inverted, have very little capacity for antibody binding. Preincubation of the COOH-terminal peptide-specific antisera with the synthetic peptide removed in a titratable fashion the binding of the antibodies to the mitoplasts (Fig. 3). These data indicate that the COOH terminus of the P, is accessible to COOH-terminal-specific antibodies only in mitoplasts. Thus, it can be concluded that the COOH terminus is located on the cytoplasmic side of the membrane.

Topology of Tryptic Sites—As an independent criterion for establishing the inner membrane topology of the mitochondrial P, mitoplasts and inverted inner membrane vesicles were subjected to trypsin proteolysis. After SDS-PAGE, proteolytic patterns were then analyzed by immunoblotting using the anti-COOH-terminal peptide antibody as a probe.

Treatment of the mitoplasts and inner membrane vesicles with trypsin for 5–60 min led to complete loss of reactivity of the COOH-terminal peptide antibody against the P, protein in mitoplasts (Fig. 4, Lanes 2–5), whereas the reactivity was maintained in inner membrane vesicles (Fig. 4, Lanes 7–10). These results would be expected only if the tryptic sites were present near the carboxyl-terminus and located on the cytosolic side of the membrane.

Effect of NEM on the Accessibility of the P, COOH-terminal to Trypsin—NEM alkylation of the P, has been shown to induce a large mobility shift in SDS-PAGE (1). To determine whether this “conformational” change affects the accessibility of tryptic sites, mitoplasts and inner membrane vesicles were pretreated with NEM and then reacted with trypsin. The reaction of the NEM-treated mitoplasts with trypsin was markedly inhibited (Fig. 5, Lanes 4 and 6). Moreover, no proteolysis was induced by NEM treatment of inner membrane vesicles (Fig. 5, Lanes 8, 10, and 12). These findings support the suggestion that NEM induces a significant change in the conformation of the mitochondrial P, (1). (It should be noted that in prior studies we have demonstrated that NEM does react with the P, in both mitoplasts and inverted inner membrane vesicles (18, 19.).

Experiments Conducted with Antisera Against Other P, Peptides—As emphasized earlier, the antisera raised against the P₁₃₄₋₁₆₉ peptide showed good reactivity with the denatured P, (Fig. 2B, Lane 5). However, in experiments not presented...
Polyacrylamide gel, electrotransferred onto nitrocellulose, and immunodetected with COOH-terminal peptide antibodies. Lane 1, mitoplasts (200 μg); Lanes 2-5, mitoplasts (100 μg) treated with trypsin for 0, 15, 30, and 60 min, respectively. Lane 6, inverted inner membrane vesicles (50 μg) treated with trypsin for 0, 15, 30, and 60 min, respectively. Lane 7,9, and 10, inverted inner membrane vesicles digested with trypsin for 0, 15, and 60 min, respectively. Lanes 11 and 12, NEM-treated mitoplasts digested with trypsin for 0, 15, and 60 min, respectively. Lanes 1, 3, and 5, mitoplasts digested with trypsin for 0, 15, and 60 min, respectively; Lanes 2, 4, and 6, NEM-treated mitoplasts digested with trypsin for 0, 15, and 60 min, respectively. Lanes 7, 9, and 10, inverted inner membrane vesicles digested with trypsin for 0, 15, and 60 min, respectively; Lanes 8, 10, and 12, NEM-treated inner membrane vesicles digested with trypsin for 0, 15, and 60 min, respectively.

FIG. 4. Immunoblot analysis of the tryptic cleavage of the P1c in mitoplasts and in inverted inner membrane vesicles. Trypsin treatment was for 0–60 min, exactly as described under “Methods.” The proteolytic products were separated on a 15% SDS-polyacrylamide gel, electrotransferred onto nitrocellulose, and immunodetected with COOH-terminal peptide antibodies. Lane 1, mitoplasts (200 μg); Lanes 2-5, mitoplasts (100 μg) treated with trypsin for 0, 15, 30, and 60 min, respectively. Lane 6, inverted inner membrane vesicles (100 μg); Lanes 7–10, inverted inner membrane vesicles (50 μg) treated with trypsin for 0, 15, 30, and 60 min, respectively.

DISCUSSION

In the absence of information about tertiary structure, the use of specific antibodies has become a powerful tool in determining membrane protein topography (20, 21). In this study, antibodies to four different peptides of the P1c/H+ symporter were raised (Table I, Fig. 2), and used to probe the orientation of the corresponding P1c epitopes in rat liver right-side and inside-out mitochondrial vesicles.

There was a distinct correlation between the antigenicity of the peptides and their hydrophilicity (Table I). Antibodies to the synthetic COOH-terminal peptide (E302–E312) reacted most strongly with the transporter, and competition RIA demonstrated that the carboxyl terminus is exposed at the cytoplasmic surface of the inner mitochondrial membrane (Fig. 3). These results were supported by experiments in which the accessibility of the COOH terminus to trypsin cleavage was tested (Fig. 4). When mitoplasts were treated with trypsin and the proteolytic pattern was analyzed by incubating the protein blot with the C-terminal antibody, the reactivity against the P1c protein was lost, thus establishing that the carboxyl terminus has a cytosolic orientation.

The highly reactive cysteine (Cys-41) of the rat liver P1c, previously shown to be readily accessible from the cytoplasmic side of the inner mitochondrial membrane (6), can be alkylated with NEM resulting in inhibition of P1c transport (5). NEM also induces a significant mobility shift of the P1c in SDS-PAGE (1), a “conformational alteration” that must be induced by reaction of NEM with Cys-41, another Cys residue, or both. In this study, this change in conformation was investigated further to establish whether it is transmitted to the COOH-terminal region of the transporter. Significantly, the COOH-terminal region of the P1c in NEM-treated mitoplasts is much less susceptible to cleavage by trypsin than the same region in control (untreated) mitoplasts (Fig. 5). As there are no Cys residues in the COOH-terminal peptide (E302–E312), these results indicate that NEM alkylation of 1 or more Cys residues upstream from the COOH terminus induces a conformational change that is transmitted to this region. The nearest Cys residue (C227) is 75 amino acids upstream from E302. In response to this conformational change, the COOH-terminal region may become less accessible to protease either by “dipping” into the inner membrane or by interacting with neighboring cytosolic regions of the transporter.

Although the other three antipeptide antibodies raised in these studies (i.e. the NH2-terminal peptide antibody, the Cys-peptide antibody, and the P134–169 antibody) showed high immunoreactivity against their respective “parent” peptides, they were unreactive with the P1c in its native inner membrane environment. As it is known that the reactivity of an antipeptide antibody to a native protein will only occur if the conformation of the peptide in the native protein is identical to the conformation recognized by the antibody, or if the protein segment is sufficiently flexible to adopt the conformation that would allow the binding of the antipeptide antibody (21–23), these results are not surprising. Rather, they suggest that, within the native P1c in the inner membrane, these three peptide regions are either conformationally restricted or located within the membrane. Failure of the NH2-terminal and Cys-peptide antibodies to react with the P1c in SDS gels does not negate this suggestion, as SDS itself can be used to purify an active P1c (24).

The results presented here are most consistent with the model presented in Fig. 1C which depicts the COOH-terminal region of the P1c on the cytoplasmic side of the inner membrane. However, additional experiments will be necessary to confirm or refine this model, to identify the cysteine residue involved in initiating the long-range conformational change transmitted to the COOH-terminal region, and to elucidate
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Membrane Topology of the Proton/Phosphate Symporter

TABLE I

Properties of antibodies to synthetic segments of the P: transporter

Average hydropathy values were calculated from the average hydropathy values of individual amino acids summarized by Kyte and Doolittle (17); the average value for P: is 0.13. Immunoreactivity was determined by RIA exactly as described under "Experimental Procedures" (also see Fig. 2 for experimental data).

<table>
<thead>
<tr>
<th>Peptide segment</th>
<th>Sequence</th>
<th>Average hydropathy</th>
<th>Immunoreactivity against</th>
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</thead>
<tbody>
<tr>
<td>1-11</td>
<td>AVVGYSCEFGS</td>
<td>+0.05</td>
<td>+</td>
</tr>
<tr>
<td>34-62</td>
<td>VPLDLVKRCMVDPQKYDGPSITLKE</td>
<td>−0.26</td>
<td>+</td>
</tr>
<tr>
<td>134-169</td>
<td>EAAKVR1QTQPGYANTLREAVPMKMYKEEGLNAFYKG</td>
<td>−0.78</td>
<td>+</td>
</tr>
<tr>
<td>302-312</td>
<td>ES100GL1TR</td>
<td>−0.84</td>
<td>+</td>
</tr>
</tbody>
</table>

the relationship of this change to the transport functions of the phosphate carrier.

Acknowledgments—We wish to thank Joanne Hullihen for her expert technical assistance in preparing both mitoplasts and purified inner membrane vesicles. We also gratefully acknowledge Starlene Murray for processing the manuscript for publication.

Note Added in Proof—During the revision of this article, Palmieri et al. (Palmieri, F., Bisaccia, F., Capobianco, L., Iacobazzi, V., Indiveri, C., and Zara, V. (1990) Biochim. Biophys. Acta 1018, 147-150) proposed that Arg-140 of the Pi transporter has a mitochondrial matrix location. This interpretation is also consistent with the model presented in Fig. 1C.

REFERENCES


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Mitochondrial proton/phosphate transporter. An antibody directed against the COOH terminus and proteolytic cleavage experiments provides new insights about its membrane topology.

G C Ferreira, R D Pratt and P L Pedersen


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