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Phosphate Transport in Mitochondria: Past Accomplishments, Present Problems, and Future Challenges

Gloria C. Ferreira[1] and Peter L. Pedersen[2]

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The requirement of inorganic phosphate (P_i) for oxidative phosphorylation in eukaryotic cells is fulfilled through specific P_i transport systems. The mitochondrial proton/phosphate symporter (P_ic) is a membrane-embedded protein which translocates P_i from the cytosol into the mitochondrial matrix. P_ic is responsible for the very rapid transport of most of the P_i used in ATP synthesis. During the past five years there have been advances on several fronts. Genomic and cDNA clones for yeast, bovine, rat, and human P_ic have been isolated and sequenced. Functional expression of yeast P_ic in yeast strains deficient in P_i transport and expression in *Escherichia coli* of a chimera protein involving P_ic and ATP synthase α subunit have been accomplished. P_ic, in contrast to other members of the family of transporters involved in energy metabolism, was demonstrated to have a presequence, which optimizes the import of the precursor protein into mitochondria. Six transmembrane segments appear to be a structural feature shared between P_ic and other mitochondrial anion carriers, and recent-site directed mutagenesis studies implicate structure–functional relationships to bacteriorhodopsin. These recent advances on P_ic will be assessed in light of a more global interpretation of transport mechanism across the inner mitochondrial membrane.

KEY WORDS: Inorganic phosphate; carrier protein; mitochondria; transport.

INTRODUCTION

Phosphates are ubiquitous in biological systems. Inorganic phosphate (P_i) is essential for cell growth and division, since it is required for ATP production and is the central structure of genetic material. P_i or pyrophosphates are also essential in many anabolic and catabolic processes. In addition, most coenzymes are esters of phosphoric acid or pyrophosphoric acid (Westheimer, 1987). This ubiquity of P_i in biological systems reflects its own chemistry. P_i, by being an ionized species, can be kept easily within cells, and phosphates, by being stable and negatively charged, offer enough reactivity to be used in biological reactions (Westheimer, 1987). Availability of P_i for cellular processes in eukaryotic cells is dependent on the transport of P_i by specific carrier proteins across the plasma membrane and the membranes of the various organelles. In this review we will focus on structural and functional aspects of the mitochondrial P_i carrier (P_ic) and their relationship to the molecular mechanism of P_i transport across the inner mitochondrial membrane.

The supply of P_i required for oxidative phosphorylation in eukaryotic cells is maintained with two transport systems, which are responsible for the translocation of P_i across the inner mitochondrial membrane. The major transport system, P_ic, catalyzes a P_i/H^+ symport (or P_i/OH^- antiport) (reviewed in Wohlrab, 1986; Wehrle and Pedersen, 1989). The other system, the P_i/dicarboxylate carrier, catalyzes an electroneutral P_i:dicarboxylate or $P_i : P_i$ exchange (reviewed in Coty and Pedersen, 1975a; Kaplan and Pedersen, 1985; Kramer and Palmieri, 1992). P_ic deli-

[1] Department of Biochemistry and Molecular Biology, College of Medicine; Institute for Biomolecular Science; and The H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612.
[2] Laboratory for Molecular and Cellular Bioenergetics, Department of Biological Chemistry, The John Hopkins University of Medicine, Baltimore, Maryland 21205.

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vers P_i to the mitochondrial ATP synthase complex, the site of synthesis of most ATP required by cells. Since newly synthesized ATP is continuously being hydrolyzed, P_ic has to provide the ATP synthase complex with P_i according to the cell energy requirements. Remarkably, the V_{max} of the P_ic in intact mitochondria is approximately $3,000\,\text{nmol} \times \text{min}^{-1}\,\text{mg}^{-1}$ at 22°C, a rate much higher than that of most mitochondrial reactions (Ligeti *at al.*, 1985).

EARLY INVESTIGATIONS ON MITOCHONDRIAL P_ic: IDENTIFICATION, PURIFICATION, AND CHARACTERIZATION

Sulfhydryl specific reagents played a major role in the identification of the mitochondrial P_ic (Fonyo and Bessman, 1968; Chappell, 1968; Tyler, 1968). These reagents are, in general, inhibitors of transport systems. However, there are reagent specificities which permit singling out the P_ic from the other mitochondrial carriers. Both the P_ic and the P_i/dicarboxylate carrier are sensitive to mercurials, but only P_ic is inhibited by *N*-ethylmaleimide (NEM) (Tyler, 1968; Palmieri *et al.*, 1970; Coty and Pedersen, 1975a). However, the effect of NEM is apparently restricted to higher eukaryotes (see below). Likewise, the ATP/ADP carrier is inhibited with NEM under certain conditions, but not with mercurials (Aquila *et al.*, 1982, Klingenberg, 1989a). The specific inhibition of P_ic with NEM was successfully used to identify the rat liver mitochondrial P_ic (Coty and Pedersen, 1975b). These authors identified a 32-KDa protein, which was selectively labeled with [^{3}H]NEM in rat liver mitochondria, and shown to be associated with the transport of P_i in the mitochondria.

The identification of mitochondrial P_ic was followed by the development of solubilization, purification, and reconstitution methods for P_ic from beef heart, rat liver, blowfly flight muscle, and yeast mitochondria (Wohlrab, 1980; Wehrle and Pedersen, 1983; Wohlrab *et al.*, 1984; Bisaccia and Palmieri, 1984; Guerin *et al.*, 1990). The different types of methods used for successful solubilization of mitochondrial carriers, and their purification and reconstitution into liposomes, have been extensively reviewed in the past few years (Wohlrab 1986; Wehrle and Pedersen, 1989; Kramer and Palmieri, 1989). The general purification scheme involves solubilization with a nonionic detergent, chromatography on hydroxylapatite, followed, in some cases, by SH-affinity chromatography (Kolbe *et al.*, 1984; Kaplan *et al.*, 1986; Guerin *et al.*, 1990). In all the developed purification schemes, lipids, in particular cardiolipin, must be added during solubilization in order to improve the protein stability in the presence of detergents. Further, the solubilization with the nonionic detergent Triton X-100 reduces the affinity of P_ic and several anion carriers for hydroxylapatite, permitting their separation from other mitochondrial proteins.

Wohlrab *et al.* (1984), in an attempt to purify the beef heart mitochondrial P_ic, treated the P_ic-containing hydroxylapatite eluate with SDS and urea and rechromatographed it on hydroxylapatite. This coupled SDS–urea treatment was proposed to replace Triton and lipids bound to P_ic and/or unfold the protein, exposing functional groups, which then would interact more strongly with the hydroxylapatite matrix. The resulting purified beef heart mitochondrial P_ic protein consisted of two protein bands (α and β) of molecular masses close to 34 KDa (Kolbe *et al.*, 1984). P_ic from porcine heart was also purified as a homogeneous protein of 34 KDa (Bisaccia and Palmieri, 1984). Kaplan *et al.* (1986, 1989) purified the rat liver mitochondrial P_ic to near homogeneity by introducing novel aspects to the existing purifications schemes. The P_i transport system was extracted with Triton-114 and purified in the presence of cardiolipin by sequential chromatography on hydroxylapatite, DEAE-Sepharose CL-6B, and Affi-gel 501. Depending on the capacity of the affinity resin, i.e., the mercury content of the Affi-gel resin, and the elution conditions, i.e., the mercaptoethanol concentration in the elution buffer, the rat liver P_ic appeared either as a single 33-KDa protein or as two proteins of 33 and 35 KDa on SDS-PAGE. However, upon alkylation with NEM the two latter protein bands co-migrated at 35 KDa. All of the above successfully purified P_ic systems have been reconstituted into liposomes in an active form, confirming their identity and role in transport of P_i across the inner mitochondrial membrane (Bisaccia and Palmieri, 1984; Kolbe *et al.*, 1984; Kaplan *et al.*, 1986). Finally, it is perhaps important to note the difficulty in bringing this problem to this critical stage. Thus, following the identification of the P_ic as a 32-KDa protein in 1975 by Coty and Pedersen, it took three different laboratories almost a decade to obtain stable, purified, reconstitutively active protein. Over "30 people years effort" were expended to complete this important task.

More recently Guerin *et al.* (1990) purified the mitochondrial P_ic in a reconstitutively active form

from *Saccharomyces cerevisiae* and *Candida parapsilosis*. The P_ic from *S. cerevisiae* appeared as a single band of 35 KDa on SDS-PAGE. In contrast to the mammalian P_ic, the *S. cerevisiae* P_ic is insensitive to NEM, although its transport activity is inhibited with mersalyl. Significantly, Kolbe and Wohlrab (1985) had previously assigned Cys-42 as the only NEM-reactive sulfhydryl group on the beef heart P_ic. These authors sequenced an N-terminal formic acid fragment of [^{3}H-NEM]-labeled and carboxymethylated bovine mitochondrial P_ic, and found that the only NEM-reactive cysteine, Cys-42, was located between two basic amino acids, Lys-41 and Arg-43. Given the reactivity of the Cys-42 and the total lack of transport activity when P_ic was treated with NEM, Cys-42 was suggested to play an essential role in the transport of P_i in the mitochondria (Kolbe and Wohlrab, 1985; Ligeti and Fonyo, 1987). In yeast P_ic, the NEM-reactive Cys-42 of the beef heart protein is replaced by a threonine residue. This substitution probably leads to the NEM insensitivity of the yeast P_ic and led Guerin *et al.* (1990) to propose that Cys-42 of the P_ic protein had no essential catalytic role in the mechanism of mitochondrial P_i transport. Moreover, the P_ic proteins from *S. cerevisiae* and beef heart mitochondria show significant differences structurally, as demonstrated by the non-cross-reactivity of anti-beef heart P_ic sera with the *S. cerevisiae* P_ic protein and by the strikingly different CNBr peptide maps of the yeast and beef heart proteins (Guerin *et al.*, 1990). Much of the controversy centered around the NEM-reactive Cys-42 has been resolved and is discussed in the section on functional studies.

STRUCTURAL STUDIES: CLONING, SEQUENCING, PRIMARY AND SECONDARY STRUCTURE

In the late 1980's it became clear that the understanding of the mechanism of P_i transport at the molecular level would require elucidation of the structure of the mitochondrial carrier protein. With this in mind, several investigators initiated research programs to isolate cDNA or genomic clones of the different P_ics, so that expression systems could be developed and larger amounts of P_ic protein could be made available. In addition, changes of amino acids in P_ic could be possible, by mutagenesis at the DNA level, and proposed molecular mechanisms for P_ic transport could then be tested in relation to the structure of the carrier. Using a mixture of oligonucleotides, which were synthesized based on the amino acid sequence from the N-terminal and internal sequences of the bovine heart P_ic, Runswick *et al.* (1987) isolated a cDNA encoding the precursor form of the carrier protein. The protein predicted from the nucleotide sequence indicated that the mature bovine heart P_ic is preceded by a peptide of 49 amino acids. As with other mitochondrial proteins, this presequence has a net positive charge and was proposed to direct the P_ic precursor to the mitochondria (Runswick *et al.*, 1987). In 1989, Ferreira, Pratt, and Pedersen isolated a full-length cDNA clone of the rat liver P_ic. Similar to the bovine heart carrier, an import sequence of 44 amino acids precedes the mature 312 amino acid-long rat liver P_Ic. The beef heart and rat liver P_ic are highly homologous, with only 32 amino acid differences existing between the two proteins. More recently, Palmieri *et al.* (1990) have cloned and sequenced the human heart transporter, which is more than 90% homologous to the bovine heart and rat liver proteins. Amino acid sequence comparisons of the mammalian P_ic with P_i-binding proteins and other H^+-linked symporters did not reveal significant sequence homology (Ferreira *et al.*, 1989), making the assignment of putative amino acid residues, involved either in the binding of P_i or H^+, difficult.

The gene for the *S. cerevisiae* P_ic was cloned from a yeast recombinant library by Phelps *et al.* (1991). In contrast to the mammalian P_ic proteins, no N-terminal import sequence is present in the yeast protein. Potential CAAT-box and TATA-box promoter elements were located at positions −456 and −362, respectively (Phelps *et al.*, 1991). A striking feature is the tripartite P_ic gene structure (Fig. 1). In fact, the mitochondrial anion carrier superfamily, which comprises the P_ic, ADP/ATP carrier, oxoglutarate carrier, and one of the two citrate carriers, is characterized by triplication of a given motif at the primary structure level (Runswick *et al.*, 1987; Klingenberg, 1990; Runswick *et al.*, 1990, Kaplan and Mayor, 1993). These proteins have three internal repeats of approximately 100 amino acids in length, which are highly conserved. The mitochondrial anion carriers are thought to be derived from a common ancestor by gene duplication of an internal repeat of 100 amino acids (Aquila *et al.*, 1987).

The hydropathy profiles of the mammalian and yeast P_ic predict the presence of six transmembrane segments. The P_ic polypeptide chain possibly spans the inner mitochondrial membrane (Runswick *et al.*,

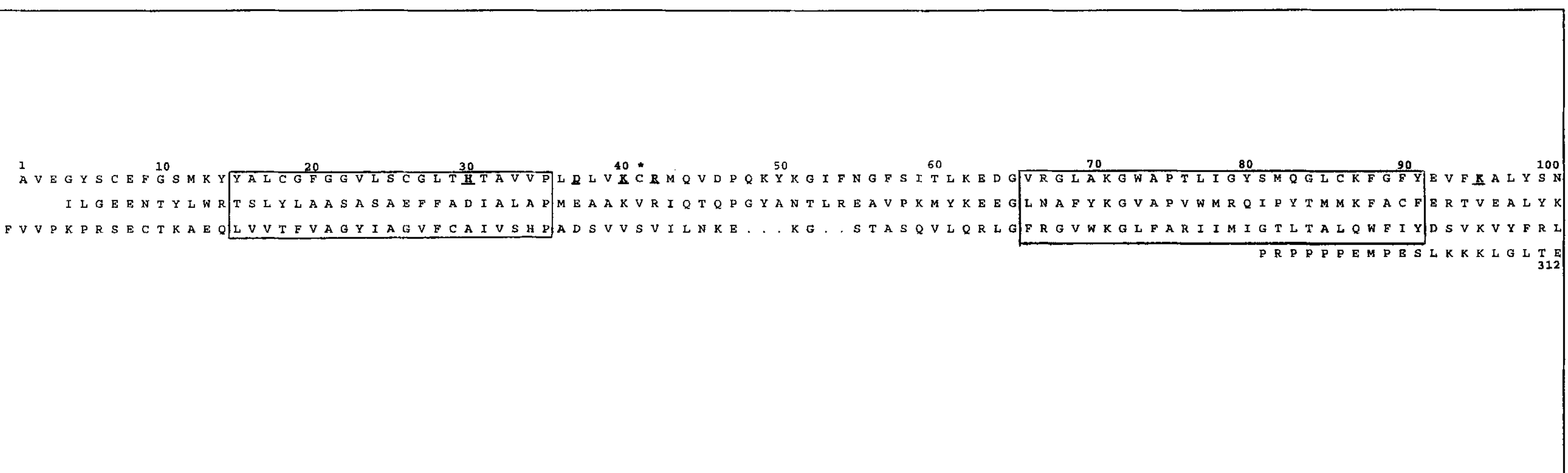

Fig. 1. Primary structure of the rat liver P_ic. Data are from Ferreira *et al.* (1989). Each of the three rows represent an ~ 100 amino acid internal repeat in the P_ic protein, and each of the three rows of amino acids within a "boxed" region represents a predicted transmembrane α-helix. This gives a total of six predicted transmembrane spanning helices in the P_ic. Conserved amino acid residues, which may be important for function, are in bold type and underlined, and the NEM-reactive cysteine is indicated by an asterisk.

1987; Ferreira *et al.*, 1989; Phelps *et al.*, 1991). While the six transmembrane α-helical structural feature is shared among most of the members of the anion carrier family, Dalbon *et al.* (1988), upon photolabeling the ADP/ATP carrier, proposed the presence of only five-membrane spanning segments in this carrier protein. Both the ADP/ATP carrier and the uncoupler binding protein appear to be functional as dimers (Klingenberg, 1989b), whereas the functional oligomeric form of P_ic in its native membrane environment remains to be established with certainty. Curiously, bacterial carriers (e.g., *E. coli* lactose permease, xylose/H^+ symporter, and melibiose carrier) are much more hydrophobic than the mitochondrial carriers and have twelve transmembrane segments instead of six (Maloney, 1990; Henderson *et al.*, 1992). Clearly, at the primary and secondary structural level, bacterial and mitochondrial anion carriers are quite distinct. Nevertheless, Griffith *et al.* (1992), after an exhaustive comparison of the primary sequences of many bacterial transport proteins with different substrate specificities, proposed that these bacterial carriers belong to the same family of transporter proteins. The underlying hypothesis is that bacterial carriers have similar three-dimensional structures and molecular mechanisms of transport, and that small structural differences account for the recognition of different substrates. Previously, Maloney (1990) proposed that prokaryotic and eukaryotic carriers have similar structural motifs, and postulated that some carriers function as monomers whereas others function as dimers. The bacterial carriers would correspond to monomeric proteins of twelve-transmembrane helical domains, while the mitochondrial carriers would correspond to dimers of six-transmembrane helical elements. In all cases this architecture for the transport systems assumes that all membrane carriers are built from a common structural element.

FUNCTIONAL STUDIES: EXPRESSION AND SITE-DIRECTED MUTAGENESIS

Phelps and Wohlrab (1991) have demonstrated the expression of the yeast P_ic at functional levels in yeast which were deficient in endogenous P_ic. In addition, the authors mutated the yeast Thr-43 to a Cys and analyzed the transport activity of this mutated P_ic once it was alkylated with NEM. The yeast P_ic has only 38% sequence similarity with the beef heart carrier protein and, in contrast to the mammalian P_ic as described above, shows no NEM sensitivity. By mutating the yeast P_ic Thr-43 residue to Cys, the authors produced a P_i transport system, (P_ic-T43C), with a Cys residue flanked by two basic amino acids (Lys-42 and Arg-44), resembling the bovine heart P_ic. Given the adjacent basic residues, the reactivity of the new Cys residue was greatly increased, and the yeast P_ic-T43C acquired NEM-sensitivity. The transport activity of P_ic-T43C was less than 10% of the wild-type P_ic activity, which was decreased even further upon reaction with NEM. In light of these results, it has been proposed that the yeast Thr-43 (and likewise Cys-42 and Cys-41 in the bovine and rat P_ic, respectively) has a location close to the path for P_i transport (Phelps and Wohlrab, 1991). By having this strategic location, Cys-42 (in the bovine heart P_ic) or Cys-41 (in the rat liver P_ic), may play a crucial role in the P_i transport mechanism, as suggested earlier (Ligeti and Fonyo, 1987).

The results from the studies of Phelps and Wohlrab (1991) also fulfilled a second purpose. Murakami *et al.* (1990) have recently isolated a yeast genomic clone with a sequence identical to that of P_ic, which the authors proposed to encode a mitochondrial import receptor. The functional expression of the yeast P_ic gene in a yeast strain deficient in the endogenous P_i transport system, and subsequent reconstitution of the expressed P_ic protein, unequivocally demonstrated that the yeast gene encoded P_ic. Neither the possibility of a dual functional protein (i.e., as a carrier protein and as a mitochondrial import receptor) nor the possibility of P_ic cross-reactivity with the anti-idiotypic antibodies used by Murakami *et al.* (1990) have been ruled out at this time.

Ferreira and Pedersen (1992) have demonstrated that the rat liver mitochondrial P_ic can be expressed in *E. coli*, as long as the P_ic-encoding DNA fragment is truncated at the 3′ end and fused to a DNA fragment encoding a water-soluble protein, as the ATP synthase α subunit. In fact, the level of expression increased with the number of nucleotides deleted from the 3′ end of the P_ic cDNA and, consequently, the number of amino acids deleted from the C-terminus of the P_ic protein. The minimal truncation necessary for the expression of the fusion protein was of 64 amino acids from the C-terminus for the P_ic. This truncation corresponded to the removal of one of the six predicted membrane-spanning segments. However, the overexpression of the full-length P_ic in *E. coli*, as an active transporter, remains to be accomplished, and more broadly, the molecular requirements for

the expression of genes encoding eukaryotic membrane proteins, in particular P_ic, in *E. coli*, remain to be established. The overexpression of P_ic would facilitate the structural and functional studies on P_ic, which are essential to dissect the transport mechanism at the molecular level.

Finally, it should be noted that in a potentially exciting new development Wohlrab and Phelps (1993) report briefly that Glu 126, Glu 137, and an unidentified histidine of the yeast P_ic may be essential for function. In addition, they indicate that these amino acids may be in positions in the P_ic identical to those, respectively, of Asp 85, Asp 96, and Lys 216 of bacteriorhodopsin, which are believed to be involved in proton translocation. This is the first report that a member of the anion carrier family may be structurally and functionally related to bacteriorhodopsin.

BIOGENESIS AND IMPORT INTO MITOCHONDRIA

In contrast to the ADP/ATP carrier and uncoupler binding protein, the mammalian P_ic does have a presequence (Runswick *et al.*, 1987; Ferreira *et al.*, 1989). All three proteins are synthesized in the cytosol and imported into the mitochondria. In the case of the ADP/ATP carrier and uncoupler protein, the mitochondrial targeting information resides within the encoding region of the protein (Adrian *et al.*, 1986; Liu *et al.*, 1990; Shore *et al.*, 1992). Deletion mutagenesis studies indicated that the information required to direct these proteins to the inner mitchondrial membrane is centered in different regions of the proteins. In the ADP/ATP carrier, this information resides within the C-terminal 20 amino acids (Smagula and Douglas, 1988) and in the uncoupler protein within the N-terminal third of the protein (Liu *et al.*, 1988; Pfanner and Neupert, 1990b). ADP/ATP carrier and uncoupler protein have been proposed to be imported into the mitochondria through a nonconservative, or stop-transfer sorting, pathway (Pfanner and Neupert, 1990a; Glick *et al.*, 1992; Shore *et al.*, 1992). In the stop-transfer sorting pathway, the ADP/ATP carrier and the uncoupler protein are inserted directly into the inner mitochondrial membrane without first passing through the matrix and then being redirected to export (Shore *et al.*, 1992; Glick *et al.*, 1992; Pfanner and Neupert, 1990a).

Mammalian P_ic precursors are synthesized with presequences of 44 (rat) and 49 (bovine and human) amino acid residues (Runswick *et al.*, 1987; Ferreira *et al.*, 1989; Dolce *et al.*, 1991). Since the P_ic belongs to the family of carrier proteins comprising the ADP/ATP carrier and uncoupler binding protein, the role of its presequence has been of interest, particularly since the yeast P_ic precursor is synthesized without a presequence (Phelps *et al.*, 1991). Pratt *et al.* (1991) demonstrated that the rat liver mitochondrial P_ic precursor, synthesized *in vitro*, was properly imported into the mitochondria and processed to yield the mature carrier protein. The import process required a membrane potential and external ATP. The requirement for ATP is specific, since GTP does not replace the role of ATP in the import process, as demonstrated by Zara *et al.* (1991).

The import of P_ic seems to occur at membrane contact sites between outer and inner mitochondrial membranes, as suggested by Zara *et al.* (1991). The deletion of 35 amino acids at the N-terminus of the P_ic presequence yielded a mutant protein with low mitochondrial import capacity (Pratt *et al.*, 1991). These investigators proposed that P_ic, in contrast to ADP/ATP carrier and the uncoupler binding protein, requires a presequence for optimal import into mitochondria (Pratt *et al.*, 1991). More recently, Zara *et al.* (1992) removed the entire P_ic presequence and obtained a protein, which was imported into mitochondria at approximately 50% of the precursor rate. In addition, the fusion of the P_ic presequence to dihydrofolate reductase conferred only 4 to 5% mitochondrial import capacity to the fusion protein, suggesting that although the P_ic presequence contains specific targeting information, the presequence by itself has "a weak transport-directing ability" (Zara *et al.*, 1992). These investigators proposed that P_ic, as the ADP/ATP carrier and uncoupler protein, contains sufficient targeting information, for import and functional assembly, in the mature region of the protein. Nevertheless, the P_ic presequence as shown by Pratt *et al.* (1991) does markedly enhance the translocation of P_ic into the inner mitochondrial membrane. It should be emphasized that these are *in vitro* studies, and it remains to be established whether the presequence is essential for P_ic import into mitochondria within mammalian cells.

MEMBRANE TOPOLOGY: A MODEL FOR THE TRANSMEMBRANE ARRANGEMENT OF P_ic IN RELATION TO ITS FUNCTION

In the absence of information on the tertiary structure of the mitochondrial P_ic, the use of specific antibodies has been a powerful tool in determining the

orientation and the topography of P_ic in the inner mitochondrial membrane (Palmieri *et al.*, 1990; Ferreira *et al.*, 1990; Capobianco *et al.*, 1991). From the hydropathy plots for the bovine heart and rat liver mitochondrial P_ic, secondary structural models for the arrangement of the protein in the membrane have been predicted (Aquila *et al.*, 1987; Runswick *et al.*, 1987, Ferreira *et al.*, 1989). In fact, the models proposed thus far are variants of a major secondary structural model of P_ic, which predicts the presence of six transmembrane α-helices connected by hydrophilic loops (Runswick *et al.*, 1987). In earlier studies, Aquila *et al.* (1987) suggested the presence of a short amphipatic β-structure peptide, which spanned the membrane between the transmembrane α-helices 1 and 2. This model placed N- and C-termini on opposite sides of the mitochondrial membrane.

Antibodies raised against a C-terminus peptide of P_ic, in conjunction with proteolytic studies using intact and inside-out mitochondrial vesicles, demonstrated that the P_ic C-terminus is located on the cytosolic side of the inner mitochondrial membrane (Ferreira *et al.*, 1990). Further, treatment of the intact mitochondrial vesicles with NEM reduced the accessibility of the P_ic C-terminus to trypsin. Since NEM had been previously shown to induce a mobility shift of the P_ic in SDS-PAGE (Kaplan *et al.*, 1986), and the highly NEM-reactive cysteine (Cys-41) of the rat liver P_ic had been previously shown to be accessible from the cytosolic side of the inner mitochondrial membrane (Houstek and Pedersen, 1985), the reduced accessibility of the C-terminus to the protease was interpreted as a consequence of a change in P_ic conformation, which was transmitted to the C-terminus

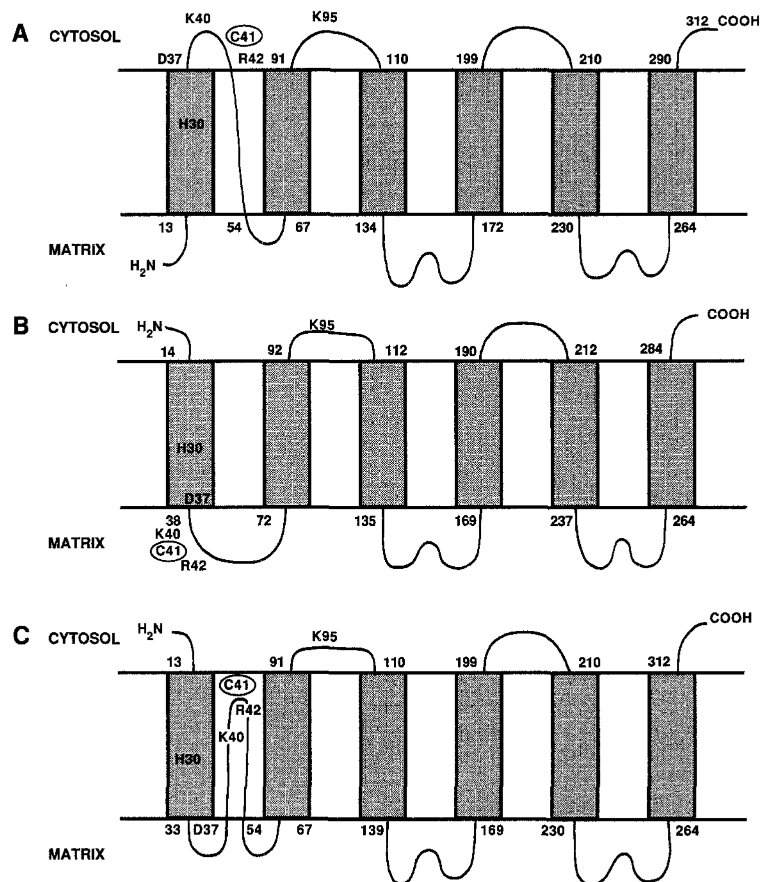

Fig. 2. Secondary structure models of P_ic showing the amino acids which may be involved in P_i and/or H^+ translocation. (A) From Ferreira *et al.* (1990). (B) From Capobianco *et al.* (1991). (C) From the combination of some of the features of the models in (A) and (B) [Similar to the secondary structure model for the uncoupler binding protein proposed by Klingenberg (1990)].

of the protein. This conformational change was induced by NEM (Ferreira *et al.*, 1990). Taking together the information on the location of the P_ic C-terminus and the accessibility of NEM-reactive cysteine residues from the cytosolic side of the membrane, Ferreira *et al.* (1990) proposed a model for the P_ic arrangement in the mitochondrial membrane, in which N- and C-termini were on opposite sides of the membrane (Fig. 2A).

Palmieri *et al.* (1990), using endoproteinases and antibodies against the N- and C-termini of P_ic, proposed that the P_ic region containing residues Arg-140 and Arg-152 has a mitochondrial matrix location. Subsequently, Capobianco *et al.* (1991), using results obtained with freeze-thawed mitochondria and N- and C-termini antibodies, proposed that both termini of the P_ic are exposed to the cytosolic side of the inner mitochondrial membrane and that the loop containing Arg-140 and Arg-152 protrudes into the matrix space (Fig. 2B). Both models (Fig. 2A and 2B) predict the same transmembrane arrangement for the 75% C-terminal region of the P_ic protein, which corresponds to five transmembrane α-helices, a matrix loop covering Arg-140 and Arg-152, and the C-terminus on the cytosolic side of the inner mitochondrial membrane. The models differ, however, in the prediction of the transmembrane arrangement of the 25% N-terminal region of P_ic. The placement of Cys-41 on the matrix side of the membrane (Fig. 2B) is in apparent conflict with previous results of Houstek and Pedersen (1985), which demonstrate that eosin maleimide does not translocate across the membrane but, similar to NEM, inhibits P_i transport. At the present time, it is difficult to resolve these two possible interpretations. The orientational integrity of the mitochondrial membranes used by Palmieri *et al.* (1990) remains uncertain, since frozen mitochondrial membranes were used rather than freshly purified inner mitochondrial membrane vesicles. Likewise, it is necessary to identify the cysteine residue involved in the conformational change transmitted to the C-terminal region, upon maleimide alkylation.

A third and unifying possibility for this region of P_ic would place the N-terminus on the cytosolic side of the inner mitochondrial membrane, and the stretch covering the amino acid residues 37–54 would penetrate the membrane, exposing Cys-41 close to the surface of the cytosolic side of the inner mitochondrial membrane (Fig. 2C). [A similar model has been proposed for the folding of the uncoupler binding protein through the mitochondrial inner membrane (Klingenberg, 1990).] The arrangement of 25% of the P_ic protein (from amino acid 1–78) in the inner mitochondrial membrane awaits definition, and further studies must be carried out.

In order to understand the mechanism of phosphate transport across the mitochondrial membrane, it is necessary to determine the P_ic structure, specifically the atomic features responsible for phosphate selectivity and binding. Recently Luecke and Quiocho (1990) demonstrated that the high specificity of a bacterial P_ic-binding protein was determined by hydrogen bonds. P_i is held in the interior of the membrane by 12 strong hydrogen bonds with protein groups [five NHs of the main chain, two NHs of an arginine (Arg-135) side chain, four OHs of two serines and two threonines, and one oxygen of an aspartate (Asp-137) side chain]. These investigators proposed that "although Arg-135 participates in P_i binding, salt-linking with the Asp-137 facilitates transport by reducing the charge-coupling interaction between the guanidinium and P_i" (Luecke and Quiocho, 1990). A similar mechanism for P_i binding and translocation may be common to the mammalian mitochondrial P_ic. Swindells *et al.* (1993), using the coordinates for three "P_i-binding" proteins [i.e., phosphocarrier protein, acylphosphatase, and ribonucleoproteins A and C] and a flexible structural alignment program, identified a fold consisting of three antiparallel β-strands and two α-helices and covering positively charged residues with a functional role. This finding led the authors to propose that the fold corresponds to a "stable phosphate-binding motif."

CONCLUSION

During the last five years significant advancements have positioned investigators to address now the question of the mechanism of P_i transport at the molecular level (Table I). Genomic and cDNA clones for mammalian and yeast P_ic have been isolated, sequenced, and in some cases, functionally expressed. The role of the P_ic presequence in import, and the assembly of the protein in mitochondria have been evaluated in comparison with the import processes of the ADP/ATP carrier and uncoupler binding protein. Models for the folding of P_ic in the inner mitochondrial membrane have been proposed, but they remain to be resolved. Now the questions are poised at a different level. The P_ic amino acid resi-

Table I. Progress on Mitochondrial Phosphate Transport Systems (H^+/P_i) Studied to Date

	Purification	Functional reconstitution	Cloning and cDNA sequencing	Topology data	Functional expression	Functional overexpression	Crystallization	3-Dimensional information
Rat liver	+	+	+	+	NR[a]	NR	NR	NR
Bovine heart	+	+	+	+	NR	NR	NR	NR
Yeast	+	+	+	NR	+	NR	NR	NR
Human	NR	NR	+	NR	NR	NR	NR	NR

[a] NR = not reported.

dues involved in binding of P_i and the amino acid residues, which dictate the specificity of the carrier protein, remain to be established. While this field has advanced relatively rapidly over the past few years, it appears that now structural answers are necessary for the interpretation of the P_ic mechanism of transport. Thus, the very small field dealing with phosphate transport across biological membranes, like all other areas of membrane transport, will not significantly advance unless three-dimensional structural information becomes available. Certainly, the real challenges of the future for such membrane-related problems will be to overexpress functionally the proteins, and obtain either three- or two-dimensional crystals of sufficient quality to permit three-dimensional structures to be obtained at high resolution.

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