Eur. J. Biochem. *198*, 405–410 (1991)

0014295691003511N

Biogenesis of the mitochondrial phosphate carrier

Vincenzo ZARA[1], Joachim RASSOW[2], Elmar WACHTER[2], Maximilian TROPSCHUG[2], Ferdinando PALMIERI[1], Walter NEUPERT[2] and Nikolaus PFANNER[2]

[1] Dipartimento Farmaco-Biologico, Laboratorio di Biochimica, Università di Bari, Italy
[2] Institut für Physiologische Chemie, Universität München, Federal Republic of Germany

(Received January 9, 1991) – EJB 91 0061

The mitochondrial phosphate carrier (P_iC) is a member of the family of inner-membrane carrier proteins which are generally synthesized without a cleavable presequence. Surprisingly, the cDNA sequences of bovine and rat P_iC suggested the existence of an amino-terminal extension sequence in the precursor of P_iC. By expressing P_iC *in vitro*, we found that P_iC is indeed synthesized as a larger precursor. This precursor was imported and proteolytically processed by mitochondria, whereby the correct amino-terminus of the mature protein was generated. Import of P_iC showed the characteristics of mitochondrial protein uptake, such as dependence on ATP and a membrane potential and involvement of contact sites between mitochondrial outer and inner membranes. The precursor imported *in vitro* was correctly assembled into the functional form, demonstrating that the authentic import and assembly pathway of P_iC was reconstituted when starting with the presequence-carrying precursor. These results are discussed in connection with the recently postulated role of P_iC as an import receptor located in the outer membrane.

The mitochondrial inner membrane contains at least nine transport systems (carriers) for metabolites [1–4]. This carrier family includes the ADP/ATP carrier, the uncoupling protein, the phosphate carrier (P_iC), and the oxoglutarate carrier. The primary structures of these proteins were determined by amino acid sequence analysis or by cDNA sequencing [5–8]. These carrier proteins are about 300 amino acid residues in length and are strongly related in amino acid sequence and structure. Each carrier consists of three homologous repeats of about 100 amino acid residues, implying that the carrier proteins are derived from a common ancestor gene that originally coded for a protein of about 100 amino acid residues.

Most mitochondrial precursor proteins that are translocated into or across the inner membrane are synthesized with amino-terminal targeting sequences (presequences) that are cleaved off by processing peptidase in the matrix [9, 10]. The precursors of the ADP/ATP carrier, the uncoupling protein, and the oxoglutarate carrier, however, were found not to carry a presequence [8, 11–15]. This demonstrated that these proteins of the carrier family contained all the targeting information in the mature protein sequence. It was therefore very surprising that the amino acid sequence of mammalian P_iC, predicted from the nucleotide sequences of bovine and rat cDNA [7, 16], suggested the existence of an amino-terminal extension sequence not present in the mature protein. This presequence exhibits general features of mitochondrial presequences, in particular a high content of positively charged amino acid residues. In this study we asked whether P_iC is indeed synthesized and imported as a larger precursor. We expressed the presequence-carrying form *in vitro* and found that it was specifically imported and processed by isolated mitochondria. P_iC imported *in vitro* was correctly assembled, demonstrating that the authentic import of P_iC was reconstituted.

Recently it was found that a putative import receptor, p32 of yeast mitochondria (which should be an outer-membrane protein), is related in primary sequence to mammalian P_iC [17, 18] and obviously identical to yeast P_iC [19, 20]. Therefore, the localization of cloned P_iC in the inner-mitochondrial membrane and their function as P_iC were questioned. We show here that the import of bovine P_iC involves specific cleavage of a presequence and requires the membrane potential ($\Delta\Psi$) across the inner membrane, strongly arguing against P_iC being an outer-membrane protein [9, 21]. P_iC imported *in vitro* acquires specific properties of the native P_iC, demonstrating that the correct P_iC was cloned. Our findings present interesting implications for the function of the p32 import receptor.

EXPERIMENTAL PROCEDURES

Subcloning and in vitro *expression of bovine P_iC*

A cDNA clone of bovine P_iC in pUC8 [7], kindly provided by Dr. J. E. Walker (MRC, Cambridge, UK), was cut with *Bam*HI and *Bgl*I. The resulting segment (2.7 kb), containing the entire coding region (plus a piece of pUC8), was treated with S1 nuclease and Klenow polymerase and cloned into a *Sma*I-cut pGEM4 transcription vector (Promega) [22], resulting in plasmid pGEM4/P_iC (Fig. 1A). Insertion was confirmed by sequencing with SP6-specific and T7-specific primers. *In vitro* transcription with SP6 RNA polymerase and subsequent translation of the capped RNA in reticulocyte

Correspondence to N. Pfanner, Institut für Physiologische Chemie, Universität München, Goethestrasse 33, W-8000 München 2, Federal Republic of Germany

Abbreviation. P_iC, phosphate carrier.

Enzyme. Proteinase K (EC 3.4.21.14).

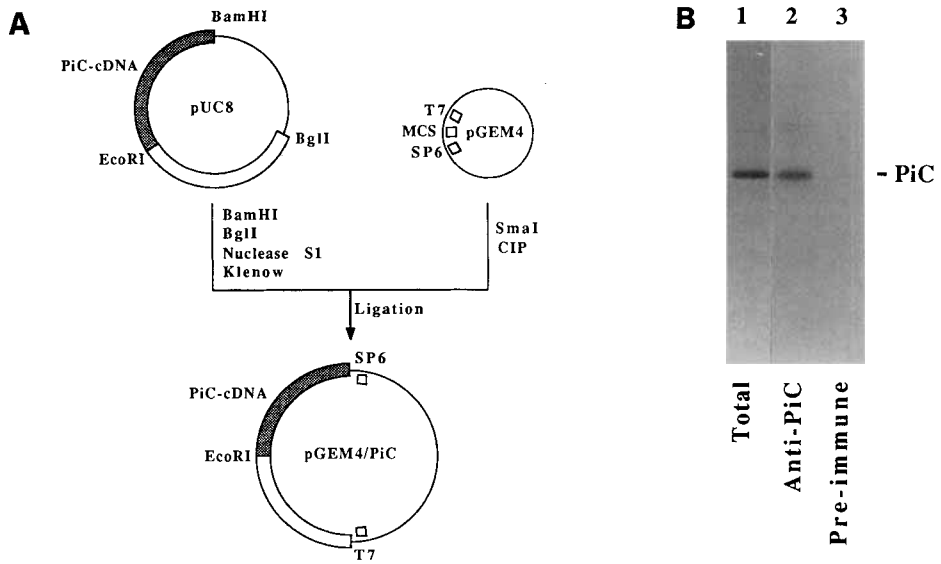

Fig. 1. *Expression of the precursor of P_iC.* (A) Construction of an *in vitro* expression vector for P_iC. (B) *In vitro* expression of P_iC precursor. Protein synthesis was carried out in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and P_iC mRNA, derived from the transcription system pGEM4, for 60 min at 30°C. Sample 1 (control), 1 µl postribosomal supernatant. Samples 2 and 3 (1 µl) were subjected to immunoprecipitation with antiserum specific for P_iC (sample 2) or preimmune serum (lane 3) using protein-A–Sepharose. The control and the immunoprecipitates were analysed by SDS/PAGE and fluorography. MCS, multiple-cloning site; CIP, calf intestinal phosphatase

lysates [23] resulted in a P_iC precursor with an apparent molecular mass of 38 kDa on SDS/PAGE.

Preparation of mitochondria

Bovine heart and rat liver mitochondria were prepared as described previously [24, 25]. Published procedures were used for the isolation of mitochondria from *Neurospora crassa* [26, 27] and *Saccharomyces cerevisiae* [28, 29].

Import of P_iC into isolated mitochondria

Isolated mitochondria (50 µg protein) were incubated in buffer A (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM Mops and 3% (mass/vol.) bovine serum albumin, adjusted to pH 7.2 with KOH [27]) and 20% (by vol.) reticulocyte lysate containing the radiolabeled precursor of P_iC in a final volume of 100 µl. NADH (Sigma; 2 mM final concentration) was added from a 50-fold-concentrated stock solution in water. Valinomycin and oligomycin (Sigma; 1 µM and 20 µM final concentrations, respectively) were added from a 100-fold-concentrated stock solution in ethanol. The samples were made chemically identical by adding the same amount of reagent-free solvent to the control samples. The duration and temperature of the incubations are given in the figure legends. For protease treatment, the samples were cooled to 0°C and proteinase K (20 units/mg; Boehringer Mannheim) was added. After incubation for 20 min at 0°C, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. Samples were further incubated for 10 min at 0°C. Mitochondria were then reisolated by centrifugation for 12 min at $18000 \times g$.

Assessment of import of P_iC by hydroxyapatite chromatography

Lysis of mitochondria and passage of samples over hydroxyapatite were performed as previously described [30–32]. P_iC was precipitated from the hydroxyapatite eluate with cold acetone at −20°C.

Sequencing

The precursor of P_iC was synthesized in reticulocyte lysate in the presence of [^{3}H] glutamic acid. Mature P_iC was prepared by import into mitochondria (4.0 mg protein) in a volume of 1.0 ml and subsequent lysis of reisolated mitochondria in 0.5 ml lysis buffer [30 mM sodium phosphate, pH 8.2, 2% (mass/vol.) SDS]. After electrophoresis and fluorography, the band of interest was excised, washed in water and extracted by electroelution for 48 h in a buffer containing 1% (mass/vol.) SDS and 30 mM sodium phosphate, pH 8.2. The electroeluted material (25000 cpm [^{3}H]glutamic-acid-labeled protein) was subjected to automated solid-phase Edman degradation [33].

Miscellaneous

Immunoprecipitation of P_iC, SDS/PAGE, fluorography and quantitation of the ^{35}S-labeled P_iC were performed as described [26, 34, 35].

RESULTS

Synthesis of the precursor of P_iC in vitro

A cDNA-encoding P_iC of bovine heart mitochondria was subcloned into pGEM4, resulting in plasmid pGEM4/P_iC, as described in Experimental Procedures (Fig. 1A). By *in vitro* transcription with SP6 polymerase and translation in rabbit reticulocyte lysates in the presence of [^{35}S]methionine, a protein of 38 kDa was synthesized (Fig. 1B, lane 1). This putative precursor of P_iC was specifically recognized by antibodies prepared against purified mature P_iC (Fig. 1B, lane 2).

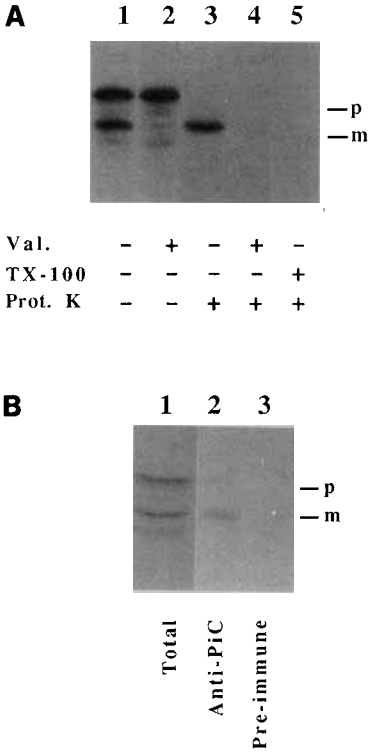

Fig. 2. *Import of P_iC* in vitro. (A) Import of P_iC into rat liver mitochondria. The import was performed as described under Experimental Procedures. Reticulocyte lysate containing ^{35}S-labeled P_iC and isolated rat liver mitochondria were incubated in the presence of 2 mM NADH (lanes 1, 3 and 5) or 1 μM valinomycin and 20 μM oligomycin (lanes 2 and 4) for 15 min at 25° C. Lanes 3, 4 and 5 were treated with proteinase K (25 μg/ml) for 20 min at 0° C after the import. Lane 5 received 1% Triton X-100 before the treatment with protease. The mitochondria (lanes 1–4) were reisolated and analysed by SDS/PAGE and fluorography. Lane 5 was subjected to precipitation with trichloroacetic acid prior to SDS/PAGE. (B) Immunoprecipitation of precursor and mature P_iC after import into rat liver mitochondria. Reticulocyte lysate containing ^{35}S-labeled P_iC and isolated rat liver mitochondria were incubated in the presence of 2 mM NADH for 15 min at 25° C. The mitochondria were then reisolated, solubilized in Triton X-100-containing buffer (see Experimental Procedures) and divided into three aliquots. Sample 1 was precipitated by acetone, samples 2 and 3 received 10 μl of anti-P_iC serum or preimmune serum, respectively. Immunoprecipitation was then performed as described under Experimental Procedures. Val., valinomycin; TX-100, Triton X-100; Prot. K, proteinase K

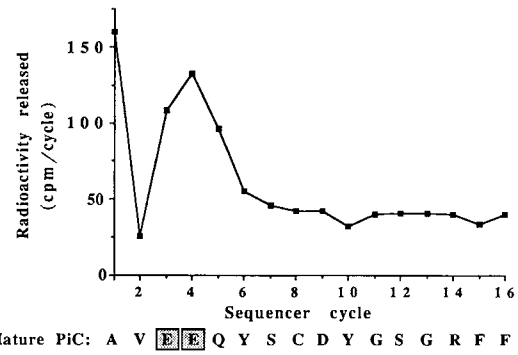

Fig. 3. *Sequencing of radioactively labeled imported mature P_iC.* The precursor of P_iC was synthesized in rabbit reticulocyte lysate in the presence of [^{3}H]glutamic acid and imported into isolated mitochondria. The processed mature protein was then subjected to sequencing as described under Experimental Procedures. The amino-terminal sequence of mature P_iC, deduced from direct amino acid analysis of the purified protein, is displayed in the one-letter code. Glutamate residues were found at positions 3 and 4. Radioactivity found in degradation step 1 is merely due to adsorbed (but not covalently bound) material

Import and assembly of P_iC in isolated mitochondria

To prove that the authentic precursor of P_iC had been synthesized, specific import into mitochondria was demonstrated. Reticulocyte lysate containing the precursor of P_iC was incubated with energized rat liver mitochondria for 15 min at 25° C. The mitochondria were then pelleted and the proteins resolved by SDS/PAGE. A fluorograph of the dried gel shows the conversion of P_iC precursor to a protein of 33 kDa, corresponding to the apparent molecular mass of mature P_iC (Fig. 2A, lanes 1 and 3). The difference of about 5 kDa in the apparent molecular masses of precursor and mature forms agrees well with the predicted length of the presequence of 49 amino acid residues. The processed form of P_iC was imported into mitochondria and protected against externally added proteinase K (Fig. 2A, lane 3) unless the mitochondria were disrupted by detergent (Fig. 2A, lane 5). The precursor associated with mitochondria was digested by proteinase K, indicating that it had not been fully imported. Dissipation of the mitochondrial membrane potential by the potassium ionophore valinomycin completely blocked the import of P_iC, demonstrating that the import depended on an energized inner membrane, as was found with all other precursor proteins imported into the mitochondrial inner membrane or matrix (Fig. 2A, lanes 2 and 4) [9, 10, 21]. (Oligomycin was included to prevent a decrease of ATP levels and the generation of a membrane potential via the F_0F_1-ATPase.) Both the precursor and mature form of P_iC were immunoprecipitated with anti-P_iC antibodies (Fig. 2B, lane 2). No reactivity was found with preimmune serum (Fig. 2B, lane 3).

The precursor of P_iC was similarly imported into isolated bovine heart mitochondria, but not into mitochondria from *N. crassa* or the yeast *S. cerevisiae* (data not shown). This indicates a high specificity for recognition of the P_iC precursor by mammalian mitochondria. In contrast, several other precursor proteins such as the ADP/ATP carrier, the F_0-ATPase subunit 9 and ornithine transcarbamylase had previously been found to be efficiently imported into both mammalian and fungal mitochondria [21, 26, 36].

To determine whether the correct mature form had been produced after import and processing of the P_iC precursor by rat liver mitochondria, we determined the processing site by sequencing the mature radioactively labeled P_iC protein. The P_iC precursor was synthesized in reticulocyte lysate containing [^{3}H]glutamic acid. After import into rat liver mitochondria, the mature P_iC was obtained by electroelution from SDS/polyacrylamide gels and subjected to automated solid-phase Edman degradation. Fig. 3 shows the sequence of the mature protein. We observed peaks at position 3 (Glu3) and at position 4 (Glu4). This demonstrates that the processing site is after the amino acid residue at position 49 of the precursor. Thus, the amino-terminus of the mature P_iC, generated during import and processing by rat liver mitochondria, is identical to the amino-terminus of the mature P_iC purified by hydroxyapatite chromatography from solubilized bovine heart mitochondria [37, 38] (V. Zara, unpublished results).

In order to analyse whether P_iC was imported to its functional location, we made use of a unique property of the

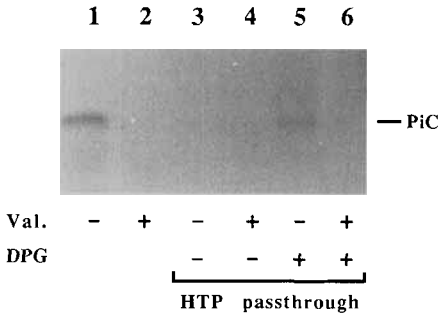

Fig. 4. *Assembly of* in vitro *imported P_iC.* Import of the P_iC precursor into mitochondria in the presence of NADH (lanes 1, 3 and 5) or valinomycin (lanes 2, 4 and 6) was performed as described under Experimental Procedures. The mitochondria were reisolated and treated as follows: samples 1 and 2 were treated with proteinase K (25 μg/ml) for 20 min at 0°C; samples 3–6 were solubilized with Triton X-114 buffer in the presence (samples 5 and 6) or in the absence (samples 3 and 4) of 5 mg/ml of cardiolipin, then chromatographed on hydroxyapatite [30–32]. Analysis of reisolated mitochondria and of acetone-precipitated eluates was performed by SDS/PAGE and fluorography. DPG, D-glycerate 2,3-bisphosphate; HTP, hydroxyapatite; Val., valinomycin

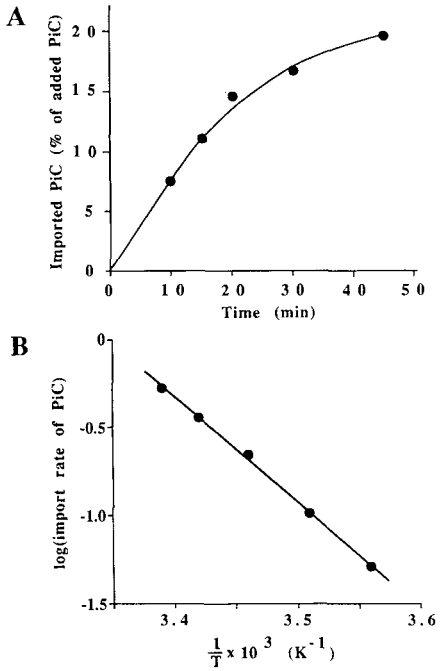

Fig. 5. *Temperature dependence of the import of P_iC. (A) Kinetics of transfer of P_iC into mitochondria.* Import of P_iC synthesized in reticulocyte lysate into mitochondria was performed at 25°C for the times indicated as described in the legend of Fig. 2. The amount of ^{35}S-labeled P_iC transferred into a proteinase-K-resistant location was determined. The reisolated mitochondria were analysed by SDS/PAGE and fluorography. Imported P_iC was quantified by laser densitometry [35]. The total amount of radiolabeled P_iC added to the import reaction was set to 100%. (B) Arrhenius plot of the temperature dependence of the import of P_iC into mitochondria. Import of P_iC into mitochondria was performed at the indicated temperatures for 15 min as described in the legend of Fig. 2. The rates of import were calculated as described for (A). Import rate of 1, 1% imported P_iC/min

mature P_iC. P_iC can be purified by a one-step chromatography by passing it over hydroxyapatite in the presence of cardiolipin [31]. In contrast, without added cardiolipin, P_iC is absent from the hydroxyapatite eluate (at a mitochondrial protein concentration of 1–3 mg/ml). Fig. 4 shows that P_iC imported *in vitro* (lane 1) bound to hydroxyapatite in the absence of cardiolipin (lane 3) and was eluted in the presence of 5 mg/ml cardiolipin (lane 5). The non-imported precursor form of P_iC was retained by hydroxyapatite even in the presence of 5 mg/ml cardiolipin (compare lanes 3 and 5 of Fig. 4 to lane 1 of Fig. 2A; data not shown). We conclude that P_iC can be expressed *in vitro* and specifically imported and assembled in mitochondria.

Characteristics of the import pathway of P_iC

Fig. 5A shows a time course for the import of P_iC at 25°C that is comparable to those of other mitochondrial precursor proteins *in vitro* [35]. The import rate was linearly dependent on the time of incubation up to at least 15 min. For the experiment shown in Fig. 5B, the import of P_iC was performed for 15 min at different temperatures and evaluated by an Arrhenius plot. There was a linear dependence between log (import rate) and $1/T$. The activation energy derived was 114 kJ/mol (27 kcal/mol).

What are the energy sources that drive the transport of P_iC into mitochondria? In Fig. 2 we showed that a potential across the inner membrane was required. Hydrolysis of ATP is necessary for the import of a number of mitochondrial precursor proteins [39–42]. To directly test if ATP was required for the import of P_iC, the reticulocyte lysate containing P_iC was depleted of endogenous ATP and ADP by incubation with apyrase (an ATPase and ADPase from potato). Oligomycin was included to prevent synthesis of ATP and subsequent dissipation of the membrane potential by F_0F_1-ATPase [39]. Lane 2 of Fig. 6 shows that removal of ATP led to a block in P_iC import. Re-addition of ATP, but not of GTP, restored the import (Fig. 6, lanes 3–6), indicating that the import of P_iC depended on the addition of ATP.

Translocation of P_iC through sites of close contact between mitochondrial outer and inner membranes (translocation contact sites) was investigated [43–47]. When the import reaction was performed at low temperature (8°C) the precursor of P_iC associated with mitochondria, yet remained accessible to externally added protease (Fig. 7) under conditions where the mitochondrial membranes remained intact [29, 48, 49]. The membrane potential was then dissipated by addition of valinomycin and a second incubation at 25°C was performed. P_iC was thereby processed and imported into mitochondria as assessed by its protection against proteolysis by proteinase K (Fig. 7). Since the presence of a membrane potential is a prerequisite for the entry of precursor proteins into the inner membrane (Fig. 2A) [43, 50], the precursor of P_iC accumulated at 8°C must have been in an intermediate location. The precursor must have been beyond the $\Delta\Psi$-dependent step, i.e. it had entered the inner membrane. The protease accessibility demonstrates that a portion of the precursor was located outside the outer membrane. We conclude that the intermediate of P_iC, observed at 8°C, bridges both mitochondrial membranes, thereby spanning contact sites [43, 50]. The chase of the precursor to the processed and fully imported form confirms that a true translocation intermediate had been generated.

DISCUSSION

We report here that the bovine P_iC is synthesized as precursor protein carrying an amino-terminal extension sequence

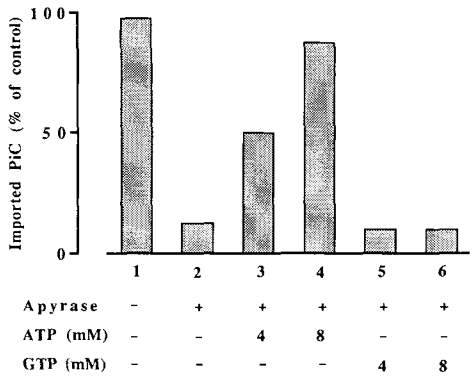

Fig. 6. *Import of P_iC requires ATP.* Reticulocyte lysate containing P_iC was treated for 15 min at 25°C with apyrase (0.5 units/ml in samples 2–6) or an apyrase preparation that had been heated to 95°C for 10 min prior to use (corresponding to 0.5 units/ml; sample 1). Isolated mitochondria, NADH and oligomycin were added. Samples 3 and 4 received ATP and samples 5 and 6 GTP. After incubation for 15 min at 25°C, the samples were treated with proteinase K (25 μg/ml) for 20 min at 0°C. Then the mitochondria were reisolated and analysed by SDS/PAGE, fluorography and densitometry. The amount of P_iC imported without treatment with apyrase was set to 100%

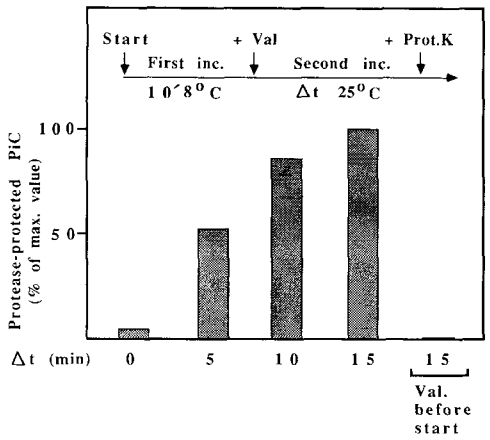

Fig. 7. *The precursor of P_iC is imported via contact sites.* Reticulocyte lysate, mitochondria and NADH were incubated for 10 min at 8°C. 1 μM valinomycin was added and a second incubation at 25°C was performed for the indicated times. One sample received valinomycin before the first incubation. After the second incubation, the samples were treated with proteinase K (25 μg/ml). The mitochondria were reisolated and analysed by SDS/PAGE, fluorography and densitometry. The amount of P_iC imported after 15 min at 25°C was set to 100%. Val., valinomycin; inc., incubation; Prot. K, proteinase K

(presequence). In contrast, the other members of the mitochondrial carrier family analysed so far such as the ADP/ATP carrier, the uncoupling protein and the oxoglutarate carrier, are synthesized without a presequence. Analysis of the transport pathway of the P_iC precursor revealed the characteristics of general mitochondrial import. The import of P_iC into rat liver and bovine heart mitochondria required the presence of ATP (the requirement for ATP was specific because the addition of GTP did not restore the import of P_iC into mitochondria). The import of P_iC also required the membrane potential, $\Delta\Psi$, across the inner membrane and occurred at membrane contact sites between outer and inner membranes, the major site for mitochondrial protein uptake. The imported protein was processed, whereby the correct amino-terminus of the mature protein was generated, demonstrating that the entire 49-amino-acid-residue presequence was removed. Finally, P_iC was correctly assembled. Assay for assembly of P_iC imported *in vitro*, binding to hydroxyapatite at low protein concentration and release in the presence of cardiolipin is highly specific; a similar assay for assembly of low amounts of proteins imported *in vitro* was previously only reported for the ADP/ATP carrier [32].

The P_iC precursor was not imported into mitochondria from yeast and *N. crassa*, suggesting that the import of bovine P_iC is restricted to mammalian mitochondria. This is probably due to a different specificity of the recognition machinery of mammalian and fungal mitochondria. Additionally, we have found that the purified matrix processing peptidase from *N. crassa* [51] was not able to process the bovine P_iC precursor, while a matrix fraction derived from rat liver mitochondria specifically removed the presequence of P_iC (V. Zara, unpublished results).

In summary, the biogenesis pathway of mammalian P_iC is unusual in comparison with that of the other mitochondrial carriers studied so far, as it involves the synthesis of a cleavable amino-terminal presequence. Obviously members of this family of homologous proteins have developed different ways of mitochondrial targeting. The exact nature of the targeting signals located in the mature parts of the ADP/ATP carrier, the uncoupling protein, and the oxoglutarate carrier is unknown, although preliminary evidence suggests the presence of at least three non-coherent internal signal sequences [52–55]. Future studies will have to identify these putative targeting sequences and compare them to the corresponding regions in P_iC. It may be speculated that the putative internal signals of P_iC were not sufficient to direct efficient import of the precursor. Among the possible functions of the presequence of P_iC are involvement in interaction with cytosolic cofactors [56] and mitochondrial surface receptors [57, 58], and the response to the membrane potential across the inner membrane (negative inside) [27].

By employing anti-idiotypic antibodies, which were raised using a synthetic mitochondrial presequence, Blobel and colleagues identified a 32 kDa protein from yeast mitochondria (p32) as a putative import receptor [17]. F_{ab} fragments directed against p32 inhibited protein import into isolated mitochondria, indicating that the receptor was exposed on the mitochondrial surface, i.e. represented an outer-membrane protein. Unexpectedly, the primary sequence of p32 was found to be identical to that of the yeast P_iC [18–20]. It was thus speculated that the cloned proteins reported to be the P_iC were not located in the mitochondrial inner membrane and in fact should not function as P_iC [18]. Our results show the $\Delta\Psi$-dependent import and proteolytic processing of mammalian P_iC, clearly excluding an outer-membrane location of P_iC, since outer-membrane proteins do not carry cleavable extension sequences and are imported without a requirement for $\Delta\Psi$ [9, 21, 58, 59]. Furthermore, we demonstrate that the cloned P_iC acquires specific properties of native P_iC (passage over hydroxyapatite in the presence of cardiolipin [31]) after import into mitochondria, confirming that indeed the authentic P_iC had been cloned. A possible explanation is that the anti-p32 antibodies recognize several members of a family of carrier proteins some of which are in the outer membrane and some in the inner membrane. Secondly, the anti-p32 antibodies might recognize some low-abundance outer-membrane proteins in addition to p32. In fact, the 38 kDa protein, MOM38, that forms part of the general insertion site in the outer mem-

brane, contains a sequence of about 70 amino acid residues with remarkable similarity to P_iC [59]. As MOM38 is about 30–100-fold less abundant than p32/P_iC [19, 59], some antibody preparations that are apparently specifically directed against p32/P_iC could also recognize MOM38 and thereby inhibit the import of precursor proteins. In any case, unravelling the exact relation between the outer-membrane import receptor described by Pain et al. [17] and the inner-membrane P_iC will be a major challenge in the analysis of mitochondrial protein uptake and mitochondrial structure.

We are very grateful to Dr. John E. Walker (MRC, Cambridge, UK) for providing a cDNA clone of bovine P_iC. This study was supported by the *Deutsche Forschungsgemeinschaft* (Sonderforschungsbereich 184, projects B1 and Z2), the *Ministero dell'Università e della Ricerca Scientifica e Tecnologica* (MURST), the target project Biotechnology and Bioinstrumentation of *Consiglio Nazionale delle Ricerche* (CNR), the Genzentrum München, and the *Fonds der Chemischen Industrie*.

REFERENCES

1. LaNoue, K. F. & Schoolwerth, A. C. (1979) *Annu. Rev. Biochem. 48*, 871–922.
2. Meijer, A. J. & Van Dam, K. (1981) in *Membrane transport* (Bonting, S. & De Pont, J., eds) pp. 235–256, Elsevier Press, Amsterdam.
3. Palmieri, F., Prezioso, G., Bisaccia, F., Indiveri, C., Zara, V., De Pinto, V. & Genchi, G. (1987) in *Advances in myochemistry* (Benzi, G., ed.) pp. 87–104, John Libbey, Eurotext Ltd.
4. Kramer, R. & Palmieri, F. (1989) *Biochim. Biophys. Acta 974*, 1–23.
5. Aquila, H., Misra, D., Eulitz, M. & Klingenberg, M. (1982) *Hoppe Seyler's Z. Physiol. Chem. 363*, 345–349.
6. Aquila, H., Link, T. A. & Klingenberg, M. (1985) *EMBO J. 4*, 2369–2376.
7. Runswick, M. J., Powell, S. J., Nyren, P. & Walker, J. E. (1987) *EMBO J. 6*, 1367–1373.
8. Runswick, M. J., Walker, J. E., Bisaccia, F., Iacobazzi, V. & Palmieri, F. (1990) *Biochemistry 29*, 11033–11040.
9. Attardi, G. & Schatz, G. (1988) *Annu. Rev. Cell Biol. 4*, 289–333.
10. Pfanner, N. & Neupert, W. (1990) *Annu. Rev. Biochem. 59*, 331–353.
11. Zimmermann, R., Paluch, U., Sprinzl, M. & Neupert, W. (1979) *Eur. J. Biochem. 99*, 247–252.
12. Freeman, K. B., Chien, S.-M., Litchfield, D. & Patel, H. V. (1983) *FEBS Lett. 158*, 325–330.
13. Ricquier, D., Thibault, J., Bouillaud, F. & Kuster, Y. (1983) *J. Biol. Chem. 258*, 6675–6677.
14. Arends, H. & Sebald, W. (1984) *EMBO J. 3*, 377–382.
15. Ridley, R. G., Patel, H. V., Gerber, G. E., Morton, R. C. & Freeman, K. B. (1986) *Nucleic Acids Res. 14*, 4025–4035.
16. Ferreira, G. C., Pratt, R. D. & Pedersen, P. L. (1989) *J. Biol. Chem. 264*, 15628–15633.
17. Pain, D., Murakami, H. & Blobel, G. (1990) *Nature 347*, 444–449.
18. Murakami, H., Blobel, G. & Pain, D. (1990) *Nature 347*, 488–491.
19. Phelps, A., Schobert, C. & Wohlrab, H. (1991) *Biochemistry 30*, 248–252.
20. Meyer, D. I. (1990) *Nature 347*, 424–425.
21. Hartl, F.-U., Pfanner, N., Nicholson, D. W. & Neupert, W. (1989) *Biochim. Biophys. Acta 988*, 1–45.
22. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.
23. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.
24. Low, H. & Vallin, I. (1963) *Biochim. Biophys. Acta 69*, 361–370.
25. Goglia, F., Liverini, G., Lanni, A., Iossa, S. & Barletta, A. (1988) *Biochem. Biophys. Res. Comm. 151*, 1241–1249.
26. Schleyer, M., Schmidt, B. & Neupert, W. (1982) *Eur. J. Biochem. 125*, 109–116.
27. Pfanner, N. & Neupert, W. (1985) *EMBO J. 4*, 2819–2825.
28. Daum, G., Gasser, S. M. & Schatz, G. (1982) *J. Biol. Chem. 257*, 13075–13080.
29. Hartl, F.-U., Ostermann, J., Guiard, B. & Neupert, W. (1987) *Cell 51*, 1027–1037.
30. Klingenberg, M., Aquila, H. & Riccio, P. (1979) *Methods Enzymol. 56*, 407–414.
31. Bisaccia, F. & Palmieri, F. (1984) *Biochim. Biophys. Acta 766*, 386–394.
32. Schleyer, M. & Neupert, W. (1984) *J. Biol. Chem. 259*, 3487–3491.
33. Wachter, E., Machleidt, W., Hofner, H. & Otto, J. (1973) *FEBS Lett. 53*, 97–102.
34. Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132–135.
35. Pfanner, N., Müller, H. K., Harmey, M. A. & Neupert, W. (1987) *EMBO J. 6*, 3449–3454.
36. Cheng, M. K., Pollock, R. A., Hendrick, J. R. & Horwich, A. L. (1987) *Proc. Natl Acad. Sci. USA 84*, 4063–4067.
37. Kolbe, H. V. J. & Wohlrab, H. (1985) *J. Biol. Chem. 260*, 15899–15906.
38. Aquila, H., Link, T. A. & Klingenberg, M. (1987) *FEBS Lett. 212*, 1–9.
39. Pfanner, N. & Neupert, W. (1986) *FEBS Lett. 209*, 152–156.
40. Pfanner, N., Tropschug, M. & Neupert, W. (1987) *Cell 49*, 815–823.
41. Chen, W.-J. & Douglas, M. G. (1987) *Cell 49*, 651–658.
42. Eilers, M., Oppliger, W. & Schatz, G. (1987) *EMBO J. 6*, 1073–1077.
43. Schleyer, M. & Neupert, W. (1985) *Cell 43*, 339–350.
44. Schwaiger, M., Herzog, V. & Neupert, W. (1987) *J. Cell Biol. 105*, 235–246.
45. Pfanner, N., Hartl, F.-U., Guiard, B. & Neupert, W. (1987) *Eur. J. Biochem. 169*, 289–293.
46. Vestweber, D. & Schatz, G. (1988) *J. Cell Biol. 107*, 2037–2043.
47. Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U. & Neupert, W. (1989) *J. Cell Biol. 109*, 1421–1428.
48. Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. & Neupert, W. (1986) *Cell 47*, 939–951.
49. Nicholson, D. W., Hergersberg, C. & Neupert, W. (1988) *J. Biol. Chem. 263*, 19034–19042.
50. Pfanner, N. & Neupert, W. (1987) *J. Biol. Chem. 262*, 7528–7536.
51. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. & Neupert, W. (1988) *Cell 53*, 795–806.
52. Adrian, G. S., McGammon, M. T., Montgomery, D. L. & Douglas, M. G. (1986) *Mol. Cell. Biol. 6*, 626–634.
53. Pfanner, N., Hoeben, P., Tropschug, M. & Neupert, W. (1987) *J. Biol. Chem. 262*, 14851–14854.
54. Liu, X., Bell, A. W., Freeman, K. B. & Shore, G. C. (1988) *J.Cell Biol. 107*, 503–509.
55. Smagula, C. S. & Douglas, M. G. (1988) *J. Cell. Biochem. 36*, 323–327.
56. Murakami, K. & Mori, M. (1990) *EMBO J. 9*, 3201–3208.
57. Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N. & Neupert, W. (1989) *Cell 59*, 1061–1070.
58. Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. & Neupert, W. (1990) *Cell 62*, 107–115.
59. Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N. & Neupert, W. (1990) *Nature 348*, 610–617.