In Vitro Import of Cytochrome *c* Peroxidase into the Intermembrane Space: Release of the Processed Form by Intact Mitochondria

Jim Kaput,* Marjorie C. Brandriss,‡ and Teresa Prussak-Wieckowska*

* Departments of Biochemistry and Cell and Structural Biology, University of Illinois College of Medicine, Urbana, Illinois 61801; and ‡ Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

Abstract. Cytochrome *c* peroxidase (CCP) is a nuclearly encoded hemoprotein located in the intermembrane space (IMS) of *Saccharomyces cerevisiae* mitochondria. Wild-type preCCP synthesized in rabbit reticulocyte lysates, however, was inefficiently translocated into isolated mitochondria and was inherently resistant to externally added proteases. To test whether premature heme addition to the apoprecursor was responsible for the protease resistance and the inability to import preCCP, site-directed mutagenesis was used to replace the axial heme ligand (His_{175}) involved in forming a pseudo-covalent link between the heme iron and CCP. Mutant proteins containing Leu, Arg, Met, or Pro at residue 175 of mature CCP were sensitive to proteolysis and were imported into isolated mitochondria as judged by proteolytic processing of the precursor. The inhibition of wild-type CCP translocation across the outer membrane may result from the inability of the heme-containing protein to unfold during the translocation process.

Although the protease responsible for cleaving preCCP to its mature form is believed to be located in the IMS, most of the processed CCP was located in the supernatant rather than the mitochondrial pellet. Since the outer membranes were shown to be intact, the anomalous localization indicated that preCCP may not have been completely translocated into the IMS before proteolytic processing or that conformationally labile proteins may not be retained by the outer membrane. Proteolytic maturation of preCCP also occurred in the presence of valinomycin, suggesting that the precursor may be completely or partially translocated across the outer mitochondrial membrane independent of a potential across the inner mitochondrial membrane.

Most mitochondrial proteins are encoded in the nucleus, synthesized as larger precursors in the cytoplasm (37), and then specifically targeted to one of four mitochondrial compartments: the outer membrane, the intermembrane space (IMS),[1] the inner membrane, or the matrix (for reviews see 14, 25, 45, 52, 59, 69). The information that targets precursors to the mitochondria is usually encoded in a cleaved presequence, although some targeting and sorting information may reside in the mature protein (for review see 58). Protein structure influences the ability of a protein to be translocated across mitochondrial membranes; i.e., precursors that have a stable tertiary structure are inefficiently imported into mitochondria (7, 15, 16, 18, 53, 70, and this report). Recent reports suggest that precursors may be maintained in a translocation-competent state by one or more cytoplasmic factors (12, 71).

Although the mechanisms involved in the translocation process are not understood, many of the events that occur during protein import into the matrix, inner membrane, and IMS have been elucidated. Precursors bind to proteinaceous receptors (23, 29, 30, 46, 49, 73) on the surface of the outer membrane and are translocated through junctions that fuse the inner and outer mitochondrial membranes (50, 59, 60). With the exception of cytochrome *c* and outer membrane proteins, the precursors examined thus far require an elec-

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Please address correspondence to J. Kaput.

1. *Abbreviations used in this paper*: CCP, cytochrome *c* peroxidase; DHFR, dihydrofolate reductase; IMS, intermembrane space. Amino acid designations following CCP indicate the amino acid at position 175 of the mature protein sequence. The wild-type sequence has His at position 175 and is referred to as CCP-His_{175} or wild-type-CCP. Precytochrome c_1-DHFR is a fusion protein consisting of the signal sequence from cytochrome c_1 fused to DHFR (66).

trochemical potential across the inner membrane (17, 48, 65, 68) and the hydrolysis of nucleotide triphosphates (6, 17, 22, 51) for in vitro import. The ATP requirement may be bypassed if the protein is incompletely folded (7, 68) or destabilized by mutations in the mature region of the polypeptide (53, 70). Most precursors are proteolytically processed by a matrix-localized protease and, for certain IMS proteins that undergo two proteolytic cleavages, by a second protease which is thought to be located on the outer face of the inner membrane (2, 10, 20, 21, 55, 56). In addition to proteolytic processing, some proteins acquire prosthetic groups or other modifications, or are assembled into complexes (for reviews see 14, 45).

We are interested in understanding the pathway and molecular mechanisms involved in translocating and sorting IMS proteins across mitochondrial membranes and into their correct intramitochondrial location. Of the four well-characterized IMS precursors—cytochrome *c* (62), cytochrome c_1 (57), cytochrome b_2 (26), and cytochrome *c* peroxidase (CCP) (32)—the latter three contain bipartite signal sequences that encode the information for targeting the proteins to the IMS. Although the import pathways of cytochrome *c* (29, 30), cytochrome c_1 (27, 28, 65–67), and cytochrome b_2 (27, 28) have been described in detail, the translocation of preCCP into isolated mitochondria has not been extensively studied.

CCP is a 33,000-D enzyme (63) of the IMS (56) that is encoded by the nuclear genome. The primary sequence of the mature protein (64) and nucleotide sequence of the cloned gene (32) revealed that CCP was synthesized with a 68-residue-long amino-terminal extension. The presequence was composed of two polar segments separated by 23 contiguous apolar amino acids. It has been proposed (27, 28, 32, 65–67) that the amino-terminal polar sequence in CCP and other IMS precursors may target the proteins to the matrix, and that the hydrophobic segment may direct sorting to the IMS. Although the structure of the signal sequence of preCCP is similar to that of the presequences of cytochromes c_1 and b_2, no CCP intermediate was detected in in vivo experiments, suggesting that preCCP was processed only by the protease on the outer face of the inner membrane and not by the matrix-localized protease (56). In addition to proteolytic processing, CCP is also modified by the addition of a heme group that may occur in the IMS during or after translocation.

We report here the results of our analysis of the import into isolated mitochondria of wild-type and mutant forms of preCCP. We show that wild-type preCCP was very inefficiently processed by isolated mitochondria and that little, if any, precursor was translocated into mitochondria. Site-specific mutations of the axial heme ligand (His_{175}) of mature CCP increased the amount of processing and import into isolated mitochondria. In addition, a large percentage of the processed forms of wild-type CCP and mutant CCP were anomalously located in the supernatant rather than in the IMS, even though the outer membrane was apparently intact during and after import. Finally, we present evidence that processing of preCCP occurs in the presence of valinomycin, suggesting that CCP may be at least partially translocated across the outer membrane in the absence of a potential across the inner mitochondrial membrane.

Materials and Methods

Reagents and Enzymes

All chemicals were reagent grade. Fraction V BSA, cytochrome *c* (Type VIII), and valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO). SP6 polymerase was obtained from Promega Biotec (Madison, WI), New England Nuclear (Boston, MA), New England Biolabs (Beverly, MA), or United States Biochemical Corp. (Cleveland, OH). T7 polymerase was purchased from United States Biochemical Corp. or Brisco, Ltd. (Winthrop, MA). Restriction enzymes were obtained from International Biotechnology, Inc. (New Haven, CT), Bethesda Research Laboratories (Gaithersburg, MD), or New England Biolabs. Zymolyase 100T was purchased from Miles Laboratories Inc. (Elkhart, IN). Proteinase K and PMSF were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Antibodies against cytochrome b_2 and cytochrome *c* oxidase subunit IV were generously provided by A. P. G. M. van Loon (University of Basel, Basel, Switzerland).

Plasmids, Phage, and Bacterial Strains

pSP64.f1 (41) and pTZ (42) phagemids were provided by Byron Kemper and David Mead (University of Illinois, Urbana, IL). These vectors are derivatives of pUC vectors and contain the intragenic region from f1 phage and either the SP6 or T7 bacteriophage promoter (for review see 40). pGEM3Z$^-$ was purchased from Promega Biotec. A plasmid containing a gene fusion between DNA encoding the amino-terminal portion of cytochrome c_1 and murine dihydrofolate reductase (DHFR) (precytochrome c_1-DHFR [66]) was a gift of A. P. G. M. van Loon. This fusion gene was subcloned into pGEM3Z$^-$ under the control of the T7 promoter (S. Kirchner, University of Illinois, Urbana, IL).

Escherichia coli stains RRI (for genotype see 39) and NM522 (41) were used for routine growth of plasmids. Single-stranded phagemid DNA was isolated after superinfection of NM522 cells harboring double-stranded phagemid DNA with phage strains IR1 or M13KO7 (42). CJ236 (*dut*$^-$, *ung*$^-$) was used for producing uridine-containing single-stranded DNA (35).

DNA Manipulations

Restriction enzymes were used according to the suppliers' specifications. Standard cloning techniques (39) were used for all DNA manipulations. An SP6-promoter vector containing preCCP sequences was constructed in two steps. First, a 3-kb Msp I fragment from YEp13-CCP (24) was subcloned into SP64.f1$^-$. The resulting recombinant was digested with Eco RI and partially digested with Pvu II. Intramolecular ligation of the long linear fragment resulted in the loss of a 1.3-kb fragment of 3′ noncoding DNA. This vector was designated psfCP3 and contains a 1.8-kb insert encoding preCCP (1,086 bp) with 5′ (130 nucleotides) and 3′ (~600 nucleotides) noncoding sequences. A Pst I–Eco RI fragment from psfCP3 was subcloned into the same sites in pTZ18R to yield pTCP1.

A 3-kb Pvu II–Sac I fragment from pKB8 encoding the *PUT2* gene (34) was subcloned into Sma I–Sac I–digested SP64; the resulting plasmid was designated pKB21. The *PUT2* genetic locus is believed to encode Δ^1-pyrroline-5-carboxylate dehydrogenase, a matrix-localized protein (4) involved in the conversion of proline to glutamate.

Phagemids containing single-stranded CCP sequences were isolated from IR1-infected CJ236 (*dut*$^-$, *ung*$^-$) that had been grown on luria broth agar plates containing 100 μg/ml ampicillin and 0.25 mg/ml uridine. Single-stranded DNA was isolated from phage preparations using modifications of published techniques (35, 72).

Oligonucleotide-directed Mutagenesis

Oligonucleotides used for in vitro mutagenesis procedures were made in five separate pools by Dr. Krishna Jayaraman of Eastman Kodak Co. (Rochester, NY) using an oligonucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA). The oligonucleotides were 21 residues long and the sequences in each group were identical except for positions that hybridized to the codon for His_{175}. All 20 codons were represented in the five sets of oligonucleotides. Mutagenesis was done using minor modifications of published procedures (35, 72). Since the initial sequencing screens indicated a low mutagenic rate, colonies derived from each group were prescreened with ^{32}P-labeled mutagenic oligonucleotides that had been used to construct that

pool. Single-stranded phagemid DNA from each positive colony was isolated and sequenced using standard dideoxysequencing methods (43).

SP6 Transcription and RNA Isolation

Plasmids encoding CCP and derivatives were purified from cleared lysates (9) using standard CsCl gradients (39). Control experiments indicated that SP6 transcriptions produced more RNA from highly purified templates (our unpublished observations). Therefore, CsCl-isolated DNA was further purified on a 4-step, 5–20% (wt/wt) sucrose gradient buffered with 100 mM NaCl, 10 mM Tris-Cl, pH 8, 1 mM EDTA ($\omega^2 t = \sim 5 \times 10^{11}$ rad/s). Plasmids were linearized at a unique Eco RI site that is 3′ of the CCP gene sequences. Transcriptions were done as recommended by the enzyme supplier.

DNA from transcription reactions was found to inhibit incorporation of [^{35}S]methionine in rabbit reticulocyte lysates (our unpublished observation). Therefore, RNA was purified using a modified LiCl procedure: after in vitro transcription, SDS and calf liver tRNA were added to final concentrations of 0.1% and 100 μg/ml, respectively. After chloroform/isoamyl alcohol (10:1) extractions and ethanol precipitation, nucleic acids were resuspended in 0.4 vol of H_2O. RNA was precipitated from a 3-M LiCl solution for 2–16 h at 4°C. The precipitate was pelleted by a 30-min centrifugation step, and the RNA was resuspended in H_2O, adjusted to 0.4 M sodium acetate, and precipitated with 2.2 vol cold ethanol. The ethanol pellet was resuspended in H_2O. Concentrations of RNA were determined by absorption at 260 nm ($E_{1mg/ml} = 40$).

Reticulocyte Lysate Preparation and In Vitro Translations

Standard procedures were used for preparing and using rabbit reticulocyte lysates (31) except that after nuclease treatment, lysates were desalted by centrifugation through Sephadex G25 (medium) equilibrated with 0.5 mM EGTA (Dr. V. Lingappa, University of California at San Francisco, San Francisco, CA, personal communication). Salt optima for translation of uncapped, in vitro–synthesized RNAs (final concentration 0.01 mg/ml) were 1 mM Mg^{2+} and 60–90 mM K^+. Maximal incorporation of [^{35}S]methionine into preCCP was obtained after 30 min at 28–30°C.

Mitochondrial Isolation from Yeast

Mitochondria were isolated from the wild-type strain D273-10B (α; 25657; American Type Culture Collection, Rockville, MD) using a variation of the protocol described by Gasser (20). Cells were grown to an A_{600} of 1 in lactate media (2% sodium lactate, 0.1% glucose media) (for exact formulation see 20). Harvested cells were treated with 0.125 mg/ml of Zymolyase 100T in lactate media containing 20 mM potassium phosphate plus 1.2 M sorbitol until 80–90% of the cells formed spheroplasts. Spheroplasts were collected and washed with lactate media containing 1.2 M sorbitol. Mitochondria were isolated from spheroplasts as published (20, 56).

For salt washing, mitochondria were pelleted and resuspended in mitochondrial buffer (0.6 M mannitol, 20 mM Hepes/KOH) containing 0.1, 0.5, or 1 M KCl. After a 10-min incubation at room temperature, mitochondria were reisolated and resuspended in mitochondrial buffer. Half of each supernatant was dialyzed against mitochondrial buffer.

In Vitro Import Experiments

Protein import into isolated mitochondria (20, 66) and submitochondrial fractionations (10, 21, 22, 56, 66) were done essentially as published (20, 66). Mitoplasts were prepared from mitochondria by adjusting the mannitol concentration from 0.6 M mannitol (in 20 mM Hepes/KOH, pH 7.4) to 0.1 M by addition of 10 mM Tris-Cl, pH 7.5. After a 10-min incubation on ice, mitoplasts were pelleted by a 20-min centrifugation in a centrifuge (Eppendorf; Brinkmann Instruments Co., Westbury, NY) at 4°C. Mitoplasts were resuspended in 0.1 M mannitol, 10 mM Tris-Cl, pH 7.5. Aliquots were removed and analyzed on gels or treated with proteinase K in the presence or absence of 0.1% Triton X-100. IMS and mitoplast fractions (with and without protease) were precipitated by the addition of cold 20% TCA. Mitochondrial extracts were added to IMS fractions as a coprecipitant (50 μg/ml final concentration). Protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Removal of Free Heme from Reticulocyte Lysates. To reduce the amount of free heme in reticulocyte lysates, apocytochrome b_5 which lacks the carboxy-terminal hydrophobic anchor sequence (obtained from Dr. S. Sligar, University of Illinois, Urbana, IL), hemopexin (kindly provided by Dr. M. Smith, Louisiana State University, Baton Rouge, LA), and/or apoCCP (generously given by Dr. R. Ciccarrelli, Eastman Kodak Co.) were added to translation mixes before addition of mRNA. The final concentrations were between 1 and 50 μM.

Valinomycin Experiments. Translation mixes and mitochondria were pretreated with 1–100 μg/ml valinomycin (dissolved in ethanol) in the presence of 100 mM KCl for 10 min before mixing.

Protease Digestions. Protease reactions were incubated for 30 min at 4°C, and the concentration of proteinase K added is listed in the figure legends. Mitochondria or mitochondrial extracts equivalent to the amount of total mitochondrial proteins in the import reactions (50 μg/ml final concentration) were added to mock import tubes immediately after the addition of proteinase K. Proteolysis was stopped by the addition of 1 mM PMSF.

Integrity of the Outer Membrane

Reduction of Exogenously Added Cytochrome c. The integrity of outer mitochondrial membrane was estimated by following the reduction of exogenously added cytochrome *c* at 550 nm using the method of Douce et al. (13). The reductase activity in duplicate or triplicate samples was determined for mitochondria (two preparations) treated in the following manner: isolated mitochondria were incubated in import buffer and components plus 10% reticulocyte lysate or in identical buffer conditions without reticulocyte lysate (control mitochondria). Aliquots of the import reaction (plus reticulocyte lysate) were treated with 50 μg/ml proteinase K for 30 min at 4°C. Mitochondria were pelleted and resuspended in 0.6 M mannitol, 20 mM Hepes/KOH. Outer membranes were lysed by adjusting the concentration of mannitol to 0.1 M (56) with 10 mM Tris-Cl, pH 8.

Release of Soluble IMS Proteins during Incubation with Isolated Mitochondria. Isolated mitochondria were incubated for 30 min in a mock import reaction (20) containing 10% reticulocyte lysate. An aliquot of the reaction was centrifuged for 10 min at 4°C; the supernatant was removed and the pellet resuspended in 0.1 M mannitol, 20 mM Hepes/KOH. Since the volumes of mitochondria, supernatant, and pellet fractions were different, the same percentage (by volume) of each fraction was subjected to electrophoresis on 12 or 15% polyacrylamide gels and subsequently electroblotted to nitrocellulose (5). Mitoplasts were prepared from control or reticulocyte lysate–treated mitochondria by adjusting the mannitol concentration from 0.6 to 0.1 M (56). After a 10-min incubation in ice, mitoplasts were centrifuged for 20 min at 4°C. In one control experiment, the mannitol concentration was adjusted to 0.6 M mannitol before pelleting the mitoplasts to ensure that the concentration of mannitol was equivalent to the normal import buffer. Mitochondria, mitoplast, and supernatant fractions were analyzed by Western blotting to determine the release of soluble proteins from mitochondria. The blots were probed with anti-CCP, anti-cytochrome b_2, or anti-COX IV antibodies. ^{125}I-Labeled protein A was used to detect the immune complexes. Electroblotting, antibody and ^{125}I-protein A incubations, and filter washing were done essentially as published (5).

Gel Electrophoresis and Autoradiography

To account for all reaction products, we analyzed the same percentage (by volume) of each reaction or of each treated fraction. For example, identical aliquots of translated products were added to reactions (with and without mitochondria), and equivalent volumes of each of these reactions were subsequently analyzed. This method of analysis was also done for fractions that had to be manipulated (e.g., by centrifugation and resuspension of pellets) to assess processing, import, or localization of the processed forms. All samples were adjusted to 6.6% SDS, 0.22 M sucrose, 0.05% bromophenol blue, 0.66 M DTT, boiled for 5 min, and subjected to electrophoresis on SDS–12% polyacrylamide gels with a 5% stacking gel (36). Gels were stained in 35% methanol, 10% acetic acid containing Coomassie blue and destained in the same solution without the dye. Gels were dried and exposed to XAR5 film (Eastman Kodak Co.) for 5–24 h.

Quantitative Data Analyses. Autoradiograms were scanned using a laser densitometer (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD). Peak areas of the precursor and mature forms of CCP were used to calculate percent processing and association with the mitochondria or IMS. Reticulocyte lysates produced a form of CCP that comigrated with authentic CCP; this background processing by lysates was subtracted from the amount

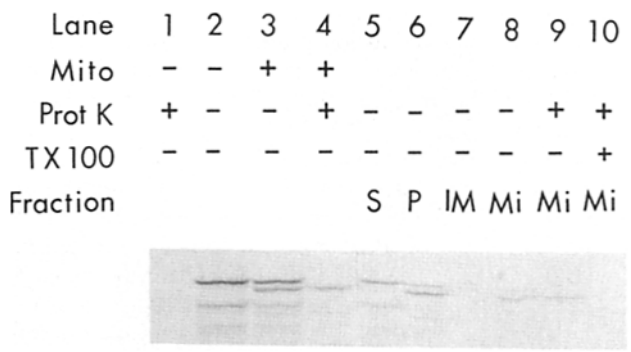

Figure 1. Import of pre*PUT2* gene product into isolated mitochondria. mRNA transcribed from pKB21 was used to program a reticulocyte lysate translation system. Translated products were incubated in the absence (lanes *1* and *2*) or presence (lanes *3* and *4*) of 50 μg/ml (final concentration) of total mitochondrial protein for 30 min at 30°C. After import, samples were analyzed for protease resistance by the addition of proteinase K (100 μg/ml final concentration) at 4°C for 30 min (lanes *1* and *4*). Approximately 50 μg/ml (final concentration) of mitochondrial proteins were added to mock import reaction (lane *1*) immediately after addition of protease (see Materials and Methods). Import was also tested by centrifugation (lane *5*, supernatant; lane *6*, pellet fraction). IMS (lane *7*, *IM*) and mitoplast (lanes *8* and *9*, *Mi*) fractions were prepared as described in Materials and Methods. Proteinase K (100 μg/ml) was added to mitoplast fraction in the absence (lane *9*) or presence (lane *10*) of Triton X-100. Equivalent percentages of each fraction were analyzed on 10% polyacrylamide gels. The precursor of pre*PUT2* is 62,000 D and the processed protein ~60,000 D. Additional bands in the *PUT2* lanes may result from prematurely terminated translation, proteolytic degradation, or initiations at internal methionines.

of processing by isolated mitochondria. Since different controls were analyzed in each experiment, the number (n) of replicate samples testing different import parameters varied from 2 to 28. For example, in 13 separate experiments, we analyzed 21 samples for processing of mutant preCCP by isolated mitochondria and 8 IMS fractions. A large standard deviation (in some cases ±20%) was observed and may be due to the number of manipulations required for analyses and variation in activity of different mitochondrial preparations.

Results

In Vitro Import of PrePUT2 into the Mitochondrial Matrix

For the subsequent analyses of in vitro import of the IMS protein, CCP, we first tested the capacity of our import protocol to import the matrix-localized enzyme (4), Δ¹-pyrroline-5-carboxylate dehydrogenase. Fig. 1 shows that total translation products synthesized from RNA transcripts derived from the plasmid pKB21 consist of a predominant protein of 62,000 D (largest protein; lane *2*) which was completely proteinase K sensitive (lane *1*). The other bands seen in lanes *2* and *3* were likely to be the results of initiation events at downstream AUGs, premature termination products, and/or proteolytic degradation products. All of these lower molecular mass species were sensitive to proteinase K (lanes *1* and *4*). The 62,000-D protein was imported into mitochondria and was processed to a 60,000-D form (lane *3*). In most experiments, ~50% of the precursor was processed. The 60,000-D species was protected from proteinase K digestion by the presence of mitochondria (lane *4*). The processed form, but not the precursor, pelleted with mitochondria (lanes *5* and *6*) and mitoplasts (lanes *7* and *8*). The mitoplast-associated form was protected from externally added proteases (lane *9*) unless the mitoplasts were disrupted by detergent (lane *10*).

These results confirm the in vivo localization of the processed form of the *PUT2* gene product (4) and demonstrate the translocation competence of our assay system. In the experiments described in this report, pre*PUT2* was used as a positive control for activity of the mitochondrial preparations.

Isolated Mitochondria Do Not Import Wild-Type CCP

Although in vitro (38) and in vivo (56) studies have shown that CCP is an IMS protein, preCCP synthesized in rabbit reticulocyte lysates was inefficiently processed and imported into isolated mitochondria. Typical results are shown in Fig. 2. In vitro translation of CCP mRNA resulted in the synthesis of a 39,000-D protein (Fig. 2, lane *2*), which was consistent with the predicted molecular mass of preCCP calculated from the DNA sequence (32). Additional proteins between 33,000 and 39,000 D (Fig. 3 *A*, lane *2*) were routinely observed on longer exposures, were precipitated by CCP antibodies (not shown), and were probably nonspecific proteolytic degradation products of preCCP. In addition, a specific CCP band often appeared at the position of authentic CCP (i.e., at ~33,000 D) even in the absence of mitochondria or mitochondrial extracts (Fig. 3, lane *2*). The amount of protein that migrated at this position varied from experiment to experiment but averaged ~10% of the input precursor (based on densitometric scans of 12 separate gel lanes).

Addition of proteinase K to the wild-type preCCP resulted in formation of a 33,000 ± 2,000-D form (Figs. 2 and 3 *A*, lanes *1*) that comigrated with authentic CCP (not shown). Similar results were obtained using trypsin or chymotrypsin (not shown). Control experiments demonstrated that authentic CCP (obtained from R. Ciccarrelli) was also resistant to high concentrations of proteinase K (not shown) which suggests, but does not prove, that the 33,000-D form of the protein observed in lanes *1* of Figs. 2 and 3 was the mature protein.

Addition of mitochondria to in vitro–synthesized preCCP resulted in an 8% (n = 16, from 8 experiments) increase in the amount of processed CCP over background (Fig. 2, cf. lanes *2* and *3*). We could not analyze the amount of protein imported into isolated mitochondria using protease resistance since proteinase K treatment produced a resistant frag-

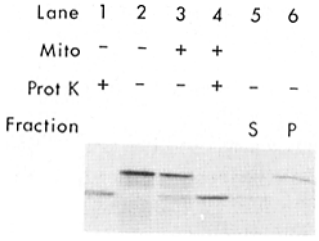

Figure 2. Incubation of wild-type PreCCP with isolated mitochondria. Wild-type pre-CCP-His_{175} translated in rabbit reticulocyte lysates was imported and analyzed as in Fig. 1. However, IMS and mitoplast fractions are not shown. The additional band between preCCP and CCP was generated in reticulocyte lysates before the addition of mitochondria and probably represents a proteolytic degradation product of preCCP.

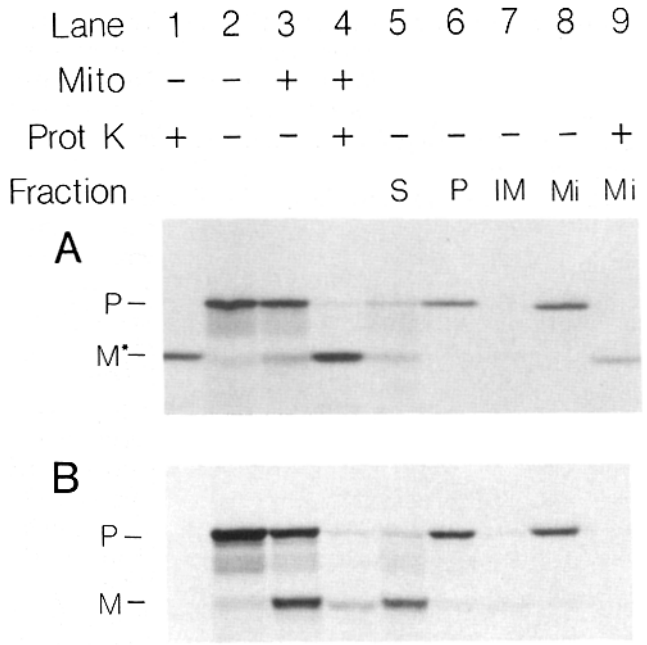

Figure 3. Import of wild-type preCCP in the presence of apoprecursors and import of preCCP-Pro$_{175}$ into isolated mitochondria. (*A*) Wild-type preCCP-His$_{175}$ translated in the presence of apoCCP or (*B*) preCCP-Pro$_{175}$ incubated in the absence (lanes *1* and *2*) or presence (lanes *3* and *4*) of 50 μg/ml (final concentration) of total mitochondrial protein for 30 min at 30°C. After import, samples were analyzed as in Fig. 1. Equivalent percentages (by volume) of each fraction were analyzed on 12% polyacrylamide gels. *P*, the 39,000-D precursor; *M*, the 33,000-D processed form; and *, a protease-resistant form of CCP that comigrates with isolated CCP. Proteins migrating between 39,000 and 33,000 D may be degradation products. Reticulocyte lysates produced a form of CCP that comigrated with the authentic protein (lane *2*). *A* and *B* are data from two separate gels—gel *B* was electrophoresed for a slightly longer period of time.

ment of CCP that comigrated with the processed form (Figs. 2 and 3 *A*, cf. lanes *1*, *3*, and *4*). We attempted to determine the amount of processed CCP imported by analyzing the amount of the protein associated with mitochondria after a 10-min centrifugation of the import reaction (Fig. 2, lanes *5* and *6*). The supernatant fraction (lane *5*) contained both precursor and processed forms while the pellet (lane *6*) contained only the precursor form. Identical results were obtained in five additional experiments. Based on the results of these experiments, therefore, we conclude that the wild-type precursor was inefficiently processed and that little, if any, preCCP was imported into isolated mitochondria.

Inhibition of Import of CCP May Be Caused by Heme Binding to the Apoprecursor

The existence of a protease-resistant fragment of CCP (Fig. 2, lanes *1* and *4*) that comigrated with the processed form of the protein hindered the analyses of preCCP import into isolated mitochondria. However, the protease resistance of CCP was reminiscent of the trypsin resistance of ferrous holocytochrome *c* compared with apocytochrome *c* (1); isolated mitochondria import apocytochrome *c* but not holocytochrome *c* (29, 33, 44). Several laboratories have recently reported that the conformation of a precursor influences the efficiency of import into mitochondria (e.g., 61). Methotrexate blocked the import of fusion proteins containing (DHFR) (15), and copper binding interfered with import of a fusion protein containing metallotheinin (8). We reasoned that the protease resistance of CCP and its inability to be imported may be explained if preCCP bound heme during synthesis in the reticulocyte lysate.

To test this possibility, we used two different experimental approaches. First, we added the apo form of the heme proteins hemopexin (a gift of Dr. M. Smith), cytochrome b_5 (obtained from Dr. S. Sligar), and/or CCP (provided by Dr. R. Ciccarrelli) to the in vitro translation system to compete for binding to free heme. We also introduced site-specific mutations into preCCP that were predicted to reduce the affinity of heme for apoCCP.

Import of Wild-type PreCCP in the Presence of Apoproteins. Addition of 10 μM (final concentration) of apoCCP to reticulocyte lysates synthesizing preCCP resulted in a slight increase (to ~12%) in precursor processing (Fig. 3 *A*, lane *3*) by isolated mitochondria compared with the amount processed in the absence of apoprotein (Fig. 2, lane *3*). Furthermore, a small, but detectable amount (~5%) of the input preCCP was associated with the mitochondrial pellet (Fig. 3 *A*, lane *6*) and was localized in the IMS (Fig. 3 *A*, lane *7*). The processed form in these fractions is visible in overexposures of Fig. 3 *A*. Similar results were obtained with apocytochrome b_5 and hemopexin (not shown). However, most of the CCP synthesized in the presence of the apoproteins was still resistant to externally added proteases (Fig. 3 *A*, lanes *1* and *4*).

Site-directed Mutagenesis of Wild-type PreCCP. Since the apoproteins did not effectively compete with apopreCCP for heme, we introduced mutations into the CCP coding sequence that were expected to reduce the affinity of heme for apoprecursor. Heme binds to CCP through multiple hydrophobic interactions and through a pseudocovalent bond between the imidazole of His$_{175}$ and the heme iron (3). Hence, mutating His$_{175}$ to other amino acids residues would be expected to decrease or eliminate heme binding. Five pools of oligonucleotides were used for in vitro mutagenesis of the gene encoding preCCP (see Materials and Methods). Seven mutant preCCP genes, which encoded Pro, Leu, Arg, Met, Val, Lys, and Asp at position 175, were obtained. RNA transcribed from four of these mutant CCP genes (Pro, Leu, Arg, and Met) was separately translated in reticulocyte lysates and analyzed for protease sensitivity. We show here the results obtained with preCCP-Pro$_{175}$; similar results were obtained with the other point mutants (not shown). Fig. 3 *B* shows that a 39,000-D protein was synthesized (lane 2) and that this protein was completely sensitive to proteinase K (lane *1*). The increased protease sensitivity indicated that mutations at His$_{175}$ reduced the stability of CCP and suggests, but does not prove, that heme binding was at least partially responsible for the inherent protease resistance of wild-type preCCP.

Incubation of mutant preCCP-Pro$_{175}$ with isolated mitochondria resulted in the formation of a protein ~6,000 D smaller than the precursor (Fig. 3 *B*, cf. lanes *2* and *3*), a result consistent with cleavage of the presequence from the precursor. Qualitative comparisons of wild-type CCP with CCP-Pro$_{175}$ import done in parallel consistently show greater processing of the mutant CCP-Pro$_{175}$ compared with the wild-type precursor (cf. Figs. 2 and 3). Quantitative analyses

of 21 samples (equivalent to Fig. 3 *B*, lanes *2* and *3*) from 13 separate import experiments indicated that 33 ± 12% of mutant preCCP (usually preCCP-Pro_{175}) was processed to a 33,000-D form. As previously stated, a much lower percentage (~8%) of the wild-type protein was processed in these import assays (see above).

Although the mutant precursors were more efficiently processed by isolated mitochondria, only a small percentage (i.e., 9 ± 6%; $n = 19$) of the processed protein was resistant to externally added proteases (Fig. 3 *B*, lanes *1* and *4*). In addition, only 11 ± 8% ($n = 16$) of the input preCCP pelleted with the mitochondria (Fig. 3 *B*, lane *5* and *6*). Subfractionation (56) of mitochondria into IMS and mitoplast fractions indicated that the processed form was located in the IMS (Fig. 3 *B*, lane *7*; this form is visible in overexposures of this gel). PreCCP-Lys_{175}, preCCP-Met_{175}, and preCCP-Leu_{175} were also tested and gave similar results (data not shown). The presumed difference between preCCP-His_{175} (i.e., the wild-type precursor; Figs. 2 and 3 *A*) and the mutant precursors (Fig. 3 *B*) is the reduced ability of the mutant to bind heme. These results are consistent with the hypothesis that addition of heme to the in vitro–synthesized wild-type precursor reduced the efficiency of proteolytic processing by isolated mitochondria and apparently prevented import of wild-type preCCP.

The data demonstrate two other characteristics of preCCP import. First, a large amount of the precursor sedimented with mitochondria (Fig. 3 *B*, lane *6*) and with mitoplasts (lane *8*). Since a portion of wild-type preCCP (Fig. 3 *A*) and all of preCCP-Pro_{175} (Fig. 3 *B*) was sensitive to proteinase K, these precursors were not inside the mitochondria. We have not determined whether the precursor was specifically bound to the outer membrane. Second, the results presented in Figs. 2 and 3 also suggest that preCCP was not processed to an intermediate during its translocation into the IMS. PreCCP (wild type and Pro_{175}) processing and import were also analyzed at 2, 5, 7.5, and 15 min at both 28 and 18°C (65), and no intermediate was detected (data not shown). These results were consistent with the observation that no intermediate of preCCP was detected in vivo (56). We have not ruled out the possibility that an intermediate similar in size to the full-length precursor may be formed but may not be resolved by the gel system used in our analyses. We are currently investigating this possibility.

Anomalous Localization of Processed CCP

Although a significantly large percentage of mutant preCCP was processed by isolated mitochondria, the majority of the mature form did not cosediment with mitochondria after import. One explanation for this result may be that the outer mitochondrial membrane was damaged during isolation procedures resulting in the formation of mitoplasts (i.e., mitochondria with broken outer membranes). To test whether mitoplasts were capable of processing preCCP, mitoplasts were prepared (56) and incubated with either preCCP-His_{175} or preCCP-Pro_{175} in normal import buffers. In three separate experiments, no detectable wild-type CCP was formed (Fig. 4). The failure to process wild-type preCCP indicates that (*a*) this precursor may not be translocated through the normal pathway; (*b*) the IMS protease was inaccessible to the supernatant fraction even in damaged mitochondria; or (*c*) the IMS protease could not cleave this precursor because of steric constraints induced by heme attachment. About 25 ± 7% ($n = 11$ from 9 separate mitoplast preparations) of the mutant precursor was processed to the 33,000-D form by mitoplasts. The fact that mitoplasts processed the mutant precursor does not directly address whether the outer membranes were damaged either during the isolation procedure or during incubation with isolated mitochondria.

Direct Tests of the Integrity of the Outer Mitochondrial Membrane

Reduction of Cytochrome c by an Inner Membrane–associated Enzyme. The structural integrity of the outer membrane was determined by assaying the reduction of exogenously added holocytochrome *c* by succinate:cytochrome *c* oxidoreductase, an enzyme associated with the outer face of the inner membrane (13, 59). This assay is based on the fact that the outer membrane is impermeable to holocytochrome *c* (13, 29, 30).

Mitochondria were incubated in a mock import reaction (i.e., all components required for translation and import except for reticulocyte lysate) or in the normal import reaction (i.e., in the presence of 10% reticulocyte lysate). Exogenously added cytochrome *c* was very slowly reduced by mitochondria when assayed at 0.6 M mannitol (Fig. 5), which may represent nonenzymatic reduction of cytochrome *c*. Identical aliquots assayed in 0.1 M mannitol had an 8–12-fold higher reductase activity, indicating that the outer membrane was broken and demonstrating that succinate:cytochrome *c* oxidoreductase was active in these mitochondrial preparations. The rate of reduction of cytochrome *c* by mitochondria treated with 10% reticulocyte lysate was equivalent to control mitochondria; hence, incubation with lysate and import buffers did not damage the outer membrane. The results suggest that the outer membranes of the majority (90–95%) of mitochondria were intact after incubation in the import reaction.

However, mitochondria incubated with lysate and subsequently treated with proteinase K showed an increase in reductase activity when assayed at 0.6 M mannitol and a lower ratio of activity (~3.5-fold increase) between 0.1 and 0.6 M mannitol relative to mitochondria that had not been proteolyzed. Incubation with lysate, treatment with proteinase K, and subsequent reisolation damaged the outer membrane of a substantial number of mitochondria. In the experiments described in this report, mitochondria in import

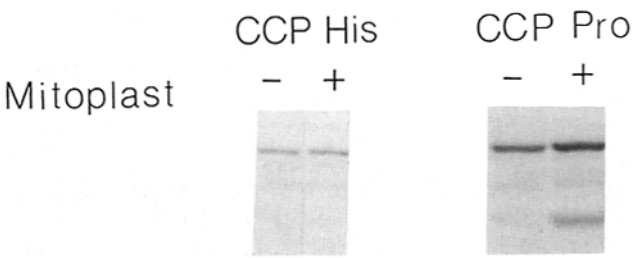

Figure 4. Cleavage of preCCP-Pro_{175} by mitoplasts. Mitoplasts were prepared as described in Materials and Methods. Wild-type preCCP or preCCP-Pro_{175} was incubated with isolated mitoplasts under normal import conditions and subsequently analyzed on separate 12% acrylamide gels as described in Materials and Methods.

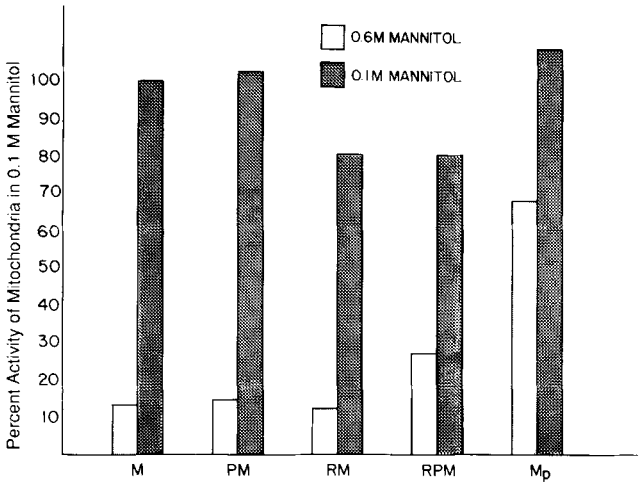

Figure 5. Reduction of exogenous cytochrome *c* by succinate: cytochrome *c* reductase. The reduction of cytochrome *c* by succinate:cytochrome *c* reductase was determined as described (13, 59). Mitochondria were incubated in import conditions in the presence of reticulocyte lysate. Control mitochondria were incubated in mock import conditions (i.e., identical buffer conditions but no reticulocyte lysate). Aliquots of mitochondria (*M*) or reticulocyte lysate-treated mitochondria (*RM*) were treated with 50 μg/ml proteinase K for 30 min at 4°C (*PM* and *RPM*, respectively). Mitochondria treated with 0.1 M mannitol to break the outer membrane were also tested (*Mp*). The activity of the reductase in the various mitochondrial preparations was assayed at 0.6 and 0.1 M mannitol. The total activity used to determine the 100% value was 80 and 567 nmol of cytochrome *c* reduced per min per mg protein at 0.1 M mannitol in experiments 1 and 2, respectively. Each value represents the average of duplicate samples from two different mitochondrial preparations.

reactions (i.e., with lysates) were either centrifuged (Figs. 1–3, lanes *5* and *6*) or treated with proteinase K (Figs. 1–3 and 5, lanes *4*), but no aliquots were treated successively with proteinase K and centrifugation.

Release of Endogenous IMS Proteins during Incubation with Reticulocyte Lysates. The above tests show that components in the IMS were inaccessible to externally added proteins. However, this assay determined the integrity of the outer membrane after incubation with reticulocyte lysates. Soluble IMS proteins or the protease responsible for cleaving preCCP to its mature form might have been released from mitochondria if the outer membrane broke during the import reaction but resealed before subsequent analysis.

To test whether a protease leaked from the mitochondria during the import reaction, a mock-translated reticulocyte lysate was incubated with mitochondria under normal import conditions. Mitochondria were pelleted and the supernatant was tested for protease activity by determining the amount of radioactive preCCP-Pro_{175} processed to the 33,000-D form. As shown in Fig. 6, the precursor was not processed by supernatant fractions, indicating that proteases were not released from the mitochondria during the import reaction.

We also measured release of the endogenous intermembrane proteins cytochrome b_2 and CCP from mitochondria incubated in import buffer containing 10% reticulocyte lysate but no radioactive precursors. The results demonstrate that soluble IMS proteins did not leak from the mitochondria during the import reaction (Fig. 7, lanes *M*, *S*, and *P*) since essentially all of the CCP and cytochrome b_2 cosedimented with mitochondria after incubation in the import buffer. Control experiments showed that most of the cytochrome b_2 and CCP, but not the inner membrane-associated Cox IV protein, were released into the supernatant by reducing the mannitol concentration from 0.6 to 0.1 M (Fig. 7, lanes *M*, *IMS*, and *Mp*) as had been previously demonstrated by Reid et al. (56). Based on these results, we conclude that the outer mitochondrial membrane was not broken during the in vitro import reaction.

Tests of the Normal Import Pathway

The exclusion of holocytochrome *c* from the enzymes on the inner membrane and the demonstration that proteases, endogenous cytochrome b_2, and CCP were not released during the import assay indicated that mitochondria used to analyze preCCP import had intact outer membranes. However, the majority of processed CCP was nonetheless located in the supernatant. These results suggested that preCCP was not being imported by the normal import pathway (65). Since it has been proposed that translocation into the IMS requires a potential across the inner mitochondrial membrane (65), the ionophore valinomycin was added to import reactions of mutant preCCP, pre*PUT2*, and the fusion protein precytochrome c_1–DHFR (obtained from A. P. G. M. van Loon) to test whether processing by mitochondria or mitoplasts occurred during the normal import pathway. The latter recombinant protein consists of 64 amino acids of precytochrome c_1 fused by a 4-amino acid linker to murine DHFR (66). Precytochrome c_1 and precytochrome c_1–DHFR are processed by a matrix-localized protease to a form that migrates between the precursor and the mature protein (i.e., an intermediate). The formation of this intermediate is thought to be an essential step in the import pathway into the IMS (28, 56, 66).

Valinomycin blocked the processing and import of pre-*PUT2* since no mature *PUT2* was formed (Fig. 8, lane *14*), and translation products incubated with valinomycin-treated mitochondria were sensitive to proteinase K (lane *15*). These results indicate that pre*PUT2* was imported by mechanisms similar to other matrix-localized proteins (for reviews see 45, 52) and that the inner membrane potential was dissipated by the ionophore. Valinomycin also greatly reduced the amount of cytochrome c_1–DHFR intermediate (Fig. 8, cf. lanes *9* and *7*) formed by mitochondria, confirming that proteolytic

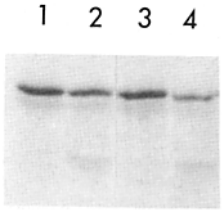

Figure 6. Analyses of supernatant fractions for proteolytic activity after mock import. Mitochondria were incubated in import buffers plus 10% reticulocyte lysate for 30 min at 28°C. Mitochondria were pelleted by a 10-min centrifugation in a centrifuge (Eppendorf; Brinkmann Instruments Co.). Supernatant was removed and the pellet resuspended in 0.6 M mannitol, 20 mM Hepes/KOH. Equivalent aliquots of the supernatant (lane *3*) and pellet (lane *4*) were added to preCCP-Pro_{175} and incubated at 28°C for 30 min. Mitochondria that had not been treated were used as control (lane *2*). Lane *1* is preCCP-Pro_{175} precursor.

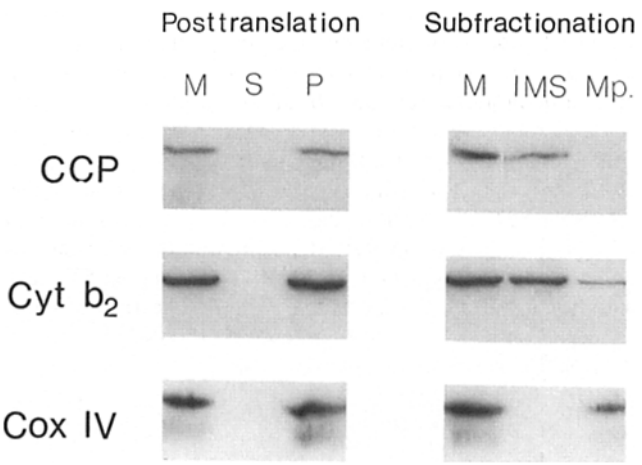

Figure 7. Western blot analysis of supernatant isolated from mock import reactions. For posttranslation analysis, mitochondria were incubated in mock import reaction as described in Materials and Methods. After incubation, mitochondria were pelleted, and equivalent fractions of the input mitochondria, supernatant, and mitochondria pellet were analyzed using Western blots. For subfractionation analysis, mitoplasts and IMS fractions were prepared from mitochondria that were isolated from the import reaction or control mitochondria using osmotic lysis of the outer membrane (see Materials and Methods). Designations are as follows: *M*, mitochondria; *S*, supernatant of a 10-min centrifugation; *P*, pellet of a 10-min centrifugation; *IMS*, supernatant of a 20-min centrifugation of mitochondria incubated in 0.1 M mannitol; and *Mp*, pellet of a 20-min centrifugation of mitochondria incubated in 0.1 M mannitol (i.e., mitoplasts).

cleavage to an intermediate depends on an energized inner membrane as shown for the precytochrome c_1–Cox IV fusion protein (65).

Addition of 1 μg/ml of valinomycin, however, did not block the formation of the 33,000-D form of CCP-Pro_{175} by mitochondria (Fig. 8, cf. lanes *3* and *5*) or by mitoplasts (not shown). Densitometric analyses of 28 gel lanes from 13 separate mitochondrial preparations indicated that 24 ± 14% of preCCP-Pro_{175} was processed in the presence of 1 (Fig. 7), 5, or 10 μg/ml valinomycin compared with 33 ± 12% in the absence of valinomycin. Mitoplasts processed 18 ± 6% (n = 8) in the presence and 25 ± 7% (n = 11) in the absence of the ionophore. Similar qualitative results were obtained for precytochrome c_1–DHFR fusion protein: valinomycin prevented processing to the intermediate form, but not the 22,000-D form (Fig. 8, cf. lanes *7* and *9*) when import was done at 30°C (as opposed to 18°C [65]). Protease protection analyses showed that variable amounts of the processed proteins were sensitive to proteolysis (Fig. 8, cf. lanes *5* and *8*). Hence, we can not definitively state that the proteins were completely translocated into the mitochondria in the presence of the ionophore. The data demonstrated, however, that precytochrome c_1–DHFR and preCCP-Pro_{175} were at least partially translocated across the outer membrane since the mature size forms were produced in the presence of valinomycin.

Detectable amounts of both preCCP and precytochrome c_1–DHFR were resistant to proteolytic degradation after import into valinomycin-treated mitochondria (Fig. 8, lanes *5* and *10*), which might indicate that the outer membrane artificially protected the precursors or that the precursors were partially translocated into a protease-resistant site within the mitochondria. Since protease sensitivity is an empirical assay (i.e., the amount protected depends on the concentration of the protease, the concentration of mitochondrial proteins, and the length of incubation time) and since valinomycin might decrease the stability of the outer membrane, we can not draw unequivocal conclusions regarding the amount of either the precursor or the processed forms that were protected from proteinase K digestion.

The data in Fig. 8 also support the conclusion that the outer mitochondrial membrane was intact after import since the intermediate of cytochrome c_1–DHFR was consistently resistant to externally added proteases in the absence of valinomycin (lane *8*); this protein may be sequestered by the outer membrane or by components of the proteinacous pore (57, 58). Control experiments demonstrated that the intermediate was sensitive to proteinase K treatment if the mitochondria were incubated in 0.1 M mannitol (data not shown); hence, the intermediate was not inherently resistant to externally added proteases.

Although the dissipation of the inner membrane potential blocked processing of pre*PUT2* and the formation of the intermediate of cytochrome c_1–DHFR, processing to the 22,000-D form of DHFR and the 33,000-D form of CCP-Pro_{175} was not prevented by the presence of valinomycin. Three inde-

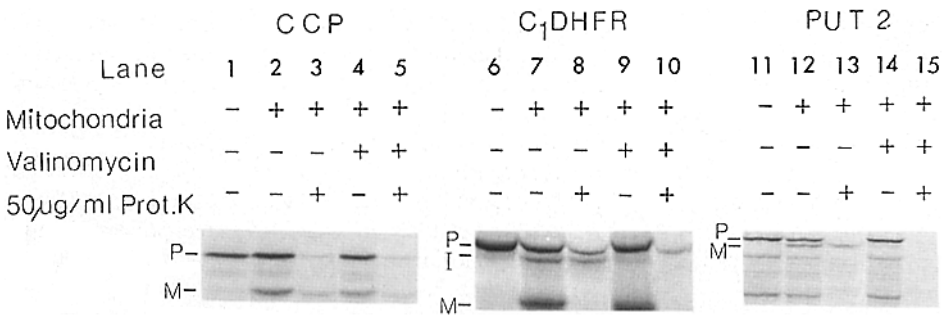

Figure 8. Import of pre*PUT2*, preCCP-Pro_{175}, and precytochrome c_1–DHFR in the presence of valinomycin. PreCCP-Pro_{175}, precytochrome c_1–DHFR, and pre-*PUT2* mRNAs were separately translated in reticulocyte lysates and the products subsequently incubated with mitochondria that had been pretreated with valinomycin in the presence of K^+. The import reactions contained 1 μg/ml valinomycin and 100 mM K^+. Incubations and protease treatments were identical to those in Fig. 1. *PUT2* import was analyzed on a 10% polyacrylamide gel (36), CCP on a 12% gel, and cytochrome c_1–DHFR on a 15% gel. The additional band between the preCCP-Pro_{175} and the processed protein is present in the minus mitochondrial lane and represents a nonspecific proteolytic cleavage of preCCP. *P*, precursor; *I*, intermediate; and *M*, processed forms. The molecular masses used to identify each of the species were determined by gel electrophoresis and the results were consistent with published values for CCP (32, 64), DHFR (66), and mature *PUT2* (4).

pendent tests (reduction of holocytochrome *c*, retention of soluble IMS proteins, and the protection of the cytochrome c_1–DHFR intermediate) indicated that the outer membrane was intact (see above). Release from mitochondria might be due to the presence of a nonmitochondrial protease that adventitiously associated with the mitochondria during isolation procedures. To test this possibility, isolated mitochondria were treated with different concentrations of KCl in mitochondria buffer for 10 min and subsequently reisolated. Half of each salt-washed supernatant was directly assayed for its ability to cleave preCCP-Pro_{175} and half was dialyzed against mitochondria buffer and then assayed for protease activity. The results (not shown) were essentially identical to those shown in Fig. 6: supernatants did not process preCCP-Pro_{175}, but the pellet fractions produced the 33,000-D form. We conclude that there was no salt-dissociable protease attached to the surface of the outer mitochondrial membrane.

The data presented are consistent with the hypothesis that preCCP and precytochrome c_1-DHFR were partially or completely translocated across the outer membrane, processed by the IMS protease, and subsequently released from intact mitochondria. In addition, translocation of these two IMS precursors across the outer membrane may be a discrete step which does not require an energized inner membrane.

Discussion

We have characterized the processing and import of the IMS protein, CCP, by isolated mitochondria. The data demonstrate that (*a*) wild-type preCCP was only poorly processed by isolated mitochondria; (*b*) a protease-resistant fragment of preCCP was generated by proteinase K treatment; (*c*) import into the IMS was highly inefficient; (*d*) most, if not all, of the processed form was released into the supernatant; and (*e*) a substantial amount of the precursor was associated with the mitochondria. The protease resistance of CCP and the inability to import the precursor may be explained by binding of heme to the apoprecursor.

To test this possibility, apoproteins were added to the translation system to bind free heme. A small increase in processing was observed and a detectable amount of processed CCP sedimented with mitochondria. Since the apoproteins did not dramatically increase processing or import, site-directed mutagenesis was used to replace the axial histidine (His_{175}) with proline, leucine, arginine, or methionine. We reasoned that replacement of this residue would drastically reduce heme binding to preCCP since the imidazole forms a pH-dependent, pseudo-covalent bond with the heme iron (3). These mutant preCCPs were sensitive to protease digestion in the absence of mitochondria, suggesting that the resistance of wild-type preCCP may be due to conformational changes induced by heme binding. Although these data are specific for CCP, the results indicate that any given protein may become inherently resistant to externally added proteases during or after import; protease resistance, therefore, should not be used as the sole test of protein translocation across membranes.

In addition to altering the protease sensitivity of the preCCP, the His_{175} replacement mutants resulted in increased processing and association of the mature mutant protein with isolated mitochondria. These data suggest that the amount of processing and import of wild-type CCP was reduced by the attachment of heme to the precursor. The results obtained with CCP were consistent with the observation that apocytochrome *c*, but not holocytochrome *c*, was imported into isolated mitochondria (29, 33). In addition, enzymatic inhibitors, which bind precursors, prevented import of these proteins into mitochondria (15) and chloroplasts (11) in vitro (11, 15) and in vivo (11). More recently, Vestweber and Schatz (70) showed that mutations that altered the conformation of DHFR (i.e., preCox IV-DHFR) increased the efficiency of in vitro import from ~10 to ~50%. Others (8, 53) have demonstrated that mutations in the mature portion of mitochondrial precursors decreased the ATP concentration needed to import proteins into the matrix. Translocation into the matrix, therefore, is increased by destabilizing the protein structure. The data presented in this report extend these conclusions to the IMS protein, CCP.

Anomalous Localization of Processed IMS Proteins

About 10% of processed CCP-Pro_{175} (and less wild-type CCP) was associated with mitochondria after incubation and centrifugation, which was consistent with the percentage of import observed for other precursors (e.g., 70). The most likely explanation for the appearance of the majority of processed CCP and DHFR in the supernatant fraction was that the outer membrane was damaged before, during, or after the import reaction.

The integrity of the outer membrane was tested by determining whether exogenously added holocytochrome *c* was reduced by the inner membrane–associated enzyme, succinate:cytochrome *c* oxidoreductase (13). This assay was used to show that *Neurospora crassa* mitochondria stabilized in 0.25 M sucrose had intact outer membranes after incubation in reticulocyte lysates (59). Based on this assay, we conclude that the outer membrane was intact in the majority of isolated mitochondria. This conclusion was supported by the fact that the intermediate of cytochrome c_1–DHFR was resistant to proteinase K digestion in mitochondria but not in mitoplasts. We also analyzed whether the outer membrane was damaged and subsequently resealed during incubation in reticulocyte lysate–containing buffers. Supernatant fractions from nonradioactive, mock import reactions or from salt-washed mitochondria failed to process wild-type preCCP or preCCP-Pro_{175}. In addition, Western blot analyses demonstrated that endogenous CCP, cytochrome b_2, and Cox IV were not released during the import reaction. Control experiments demonstrated that both CCP and cytochrome b_2, but not Cox IV, were released by osmotic shock, indicating that these proteins were not retained by interactions with other IMS components or by the presence of prosthetic groups.

Based on the measurements of succinate:cytochrome *c* reductase activity, the protease protection of the intermediate of precytochrome c_1–DHFR in mitochondria but not mitoplasts, and the fact that soluble IMS proteins were retained by the mitochondria during the import reaction, the majority of mitochondria in our import reactions had intact outer membranes. The anomalous location of in vitro–synthesized, processed IMS proteins, therefore, was not due to broken outer membranes. Since there was no salt-dissociable protease on the outside of the mitochondria, we conclude that preCCP was processed during or after in vitro translocation

across the outer membrane and subsequently released from intact mitochondria. Release of processed IMS proteins from isolated mitochondria has not been previously described (27, 28, 65–67). The apparent inconsistency between our results and the results of others may be due, at least in part, to differences in methodology. We routinely compared the total amount of processing by mitochondria (i.e., Figs. 1–3, lanes *3*) to the amount of processed protein in the supernatant fraction (i.e., Figs. 1–3, lanes *5*) and pellet fractions (lanes *6*). In contrast, most reports analyzed the protease-resistant fraction (Figs. 1–3, lanes *4*), the mitochondrial pellet fraction (Figs. 1–3, lanes *6*), or a protease-treated, mitochondrial pellet fraction.

Possible Reasons for Release of Processed Proteins

The release of the processed forms by isolated mitochondria is not consistent with the known localization of wild-type cytochrome c_1 and wild-type CCP in intact yeast cells. The results may be caused by the nature of the precursor proteins or by alterations in the in vitro import mechanisms. Pfanner et al. (54) have suggested that abnormal proteins may be imported via an uncharacterized bypass mechanism. Precytochrome c_1–DHFR is an abnormal protein since it consists of the amino-terminal 64 residues of cytochrome c_1 fused via a 4-amino acid linker to murine DHFR (66); the foreign sequences adjacent to the signal sequence might adversely affect the cleavage reaction. Likewise, preCCP-Pro$_{175}$ is abnormal by virtue of the mutation at residue 175 of the mature protein. Although these proteins are different from their wild-type counterparts, they are also dramatically different from each other. Any putative bypass mechanism, therefore, must be able to recognize changes at the cleavage site (e.g., precytochrome c_1–DHFR) and also point mutations that occur at a considerable distance (in linear sequence) from a wild-type cleavage site (e.g., preCCP-Pro$_{175}$). Recognition of these two different types of "abnormal" proteins must produce the same result—i.e, release of the processed forms from intact mitochondria. Although use of an alternative pathway remains a formal possibility, we believe it is unlikely.

The abnormal protein structure may, however, alter the ability of the protein to remain in the IMS. That is, the precursors may be imported by the normal import mechanisms but the processed forms may not be retained in the IMS. Retention may require interaction with components of the IMS or a stable protein conformation. For example, CCP-Pro$_{175}$ retention may require heme binding to stabilize the structure. This may not be unique to CCP: apocytochrome *c* was not imported (29, 33, 44) or perhaps it was not retained in mitochondria that were deficient in their ability to add heme to the apoenzyme. We are currently designing reagents that may determine whether the protein is completely translocated into the IMS before proteolytic maturation.

Although the conformation of the protein might affect its ability to be retained in the IMS, a more likely explanation of the anomalous localization of the processed forms may be that translocation across the outer membrane is a kinetically slow event relative to the processing step in isolated mitochondria; i.e., preCCP and precytochrome c_1–DHFR may have been processed before complete translocation into the IMS. Garcia et al. have shown that the hepatitis B virus precore protein may be prematurely cleaved during translocation into ER vesicles, resulting in release of the processed protein into the supernatant (19). Although premature proteolytic maturation or release from mitochondria probably does not occur in vivo, these results demonstrate that complete translocation in vitro is inefficient (as observed by others [e.g., 70]) and that certain steps in the import pathway of CCP were affected by in vitro conditions.

Import Pathway of preCCP-Pro$_{175}$

There are currently two models that describe the pathway by which proteins are imported into the IMS. It has been proposed (32, 65–67) that the extreme amino termini of IMS precursors were translocated across the inner membrane, but that complete translocation into the matrix was prevented by a hydrophobic segment located in the signal sequence (i.e., the stop-transfer model). Neupert et al. (27, 28) have proposed that IMS precursors are completely translocated into the matrix, processed to an intermediate, and subsequently translocated across the inner membrane to the IMS (i.e., the "conservative sorting" model). Both models are consistent with the observations that an inner membrane potential was presumably required for translocation of the precursors to the IMS (28, 65) and that cytochromes b_2 and c_1 were processed to an intermediate by a matrix-localized protease (21, 66).

To determine whether preCCP was imported by one of these two pathways, the ionophore valinomycin was used to disrupt the inner membrane potential. Although pre*PUT2* was not imported into the matrix and precytochrome c_1–DHFR was not processed to an intermediate (as previously shown for preCox IV-DHFR fusion protein [65]), a substantially large percentage of preCCP-Pro$_{175}$ and precytochrome c_1–DHFR were processed to their mature forms in the presence of the ionophore in parallel import reactions. It is possible that these IMS precursors were processed aberrantly during import via a putative bypass pathway (47, 54). However, as discussed above, it is unlikely that the putative bypass mechanism processes different types of abnormal proteins by the same mechanisms in the presence or absence of an inner membrane potential.

Since we have not been able to detect a protease on the outside of the mitochondria that processes preCCP-Pro$_{175}$, we favor an alternative interpretation of these data. We suggest that an inner membrane potential is not required for partial and perhaps complete translocation of preCCP-Pro$_{175}$ or precytochrome c_1–DHFR precursors across the outer mitochondrial membrane. This proposal also implies that partial translocation of the extreme amino terminus (21, 32, 65–67) or complete translocation of the precursor (27, 28) across the inner mitochondrial membrane may not be obligate steps in the import pathway of these particular IMS precursors. This conclusion is supported by the facts that we and others have not been able to detect a CCP intermediate in vivo (56 and Kaput J., unpublished observations) or in vitro (this report and our unpublished data). If the interpretation of these data and observations is correct, preCCP may be translocated across the outer membrane by mechanisms similar to cytochrome *c* since this protein is also translocated across the outer membrane independent of a membrane potential (for review see 45). However, further experiments are required to test this hypothesis.

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