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Assembly of Cytochrome *c*. Apocytochrome *c* Is Bound to Specific Sites on Mitochondria before Its Conversion to Holocytochrome *c*

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Transport of apocytochrome *c* across the outer mitochondrial membrane and conversion to holocytochrome *c* were studied *in vitro*. Apocytochrome *c* was synthesized in a cell-free homogenate from *Neurospora crassa*. Transfer *in vitro* was accomplished in a reconstituted system consisting of the postribosomal supernatant of the cell-free homogenate and of isolated and purified mitochondria from *Neurospora*. The reconstituted system has the following characteristics:

1. Apocytochrome *c* is rapidly cleared from the supernatant and holocytochrome *c* appears in the mitochondria with the same kinetics. More than 80% of the apocytochrome *c* employed is converted to holocytochrome *c*. No transient accumulation of apocytochrome *c* is found in mitochondria.
2. The heme group becomes covalently linked to apocytochrome *c* in the reconstituted system as demonstrated by analysis of tryptic peptide maps of the apoprotein and holoprotein.
3. Deuterohemin added to the reconstituted system but not deuteroporphyrin inhibits the formation of holocytochrome *c*. This inhibition is reversed by protohemin.
4. In the presence of deuterohemin about half of the apocytochrome *c* remains in the supernatant; the other half becomes associated with the mitochondria. The latter portion is tightly bound and is specifically released upon incubation of the mitochondria with excess apocytochrome *c*. It is converted to holocytochrome *c* after addition of protohemin.

We conclude from these observations that apocytochrome *c* is transported across the outer mitochondrial membrane via receptor sites. In the presence of the heme analogue deuterohemin, binding to the receptor sites on the cytoplasmic surface of the outer mitochondrial membrane still takes place but translocation does not. The latter step is apparently coupled to the covalent linkage of the heme group. We suggest that the formation of the thioether bonds between apoprotein and heme is catalysed by an enzyme in the intermembrane space and that deuterohemin can compete with protohemin for binding to the enzyme. Finally, the data indicate that it is the heme group and not the porphyrin group which is coupled to the apoprotein.

The biosynthesis of cytochrome *c* during the assembly of the mitochondrial respiratory chain is a process which consists of several distinct steps. These occur successively in the nucleus, the cytoplasm, and the mitochondria. Cytochrome *c* is encoded by a nuclear gene [1–8]. Its expression is regulated in relation to the mitochondrial metabolism [9–13]. The protein is translated from (poly A)-containing mRNA on cytoplasmic ribosomes [10, 12, 15]. The resulting apocytochrome *c*, the polypeptide chain without heme group, first occurs as an extramitochondrial precursor [15–17] (D. Sabatini, personal communication). Recent findings have demonstrated that this precursor is synthesized on free polysomes and that it is released into the cytosolic space [15]. Apocytochrome *c* does not contain an additional 'signal' or 'leader' type sequence [7, 8, 17] in contrast to a number of other precursors of mitochondrial proteins [18–26]. Apparently, it is identical in structure to apocytochrome *c* chemically prepared from holocytochrome *c*. The final steps in the biosynthetic process are the import of apocytochrome *c* into the mitochondrial intermembrane space and the covalent addition of the heme group [27, 28]. The outer mitochondrial membrane is impermeable to holocytochrome *c* [29, 30]. Evidence available so far suggests that it is apocytochrome *c* which is recognized by the mitochondrial transport system [15, 31]. The heme group is covalently attached to the apocytochrome *c* in the mitochondria [15, 16, 32]. After acquiring its final conformation [33, 34] holocytochrome *c* becomes reversibly bound to its functional site on the inner membrane [35–37].

A number of questions remain concerning the precise mechanism of the assembly reaction. A particularly important aspect is how cytochrome *c* traverses the outer mitochondrial membrane. Is there a pore in the outer membrane through which apocytochrome *c* can diffuse in both directions or is there a specific translocating system which catalyzes a unidirectional movement? Is the translocation of the apocytochrome *c* intrinsically coupled to the covalent attachment of the heme group or are these two processes independent steps? How is the formation of the thioether bridges between heme and apoprotein achieved and in what sequence are they formed? What is the driving force for the translocation across the outer mitochondrial membrane?

We report here on the transfer *in vitro* of apocytochrome *c* into isolated mitochondria and on the inhibition of this process by interfering with the covalent linkage of the heme group to the apocytochrome *c*. This inhibition is achieved by deuteroheme, an analogue of protoheme, the natural sub-

strate required for the formation of holocytochrome *c*. The results obtained with this reversible inhibition of heme linkage suggest that there are specific binding sites ('receptor sites') on the cytoplasmic face of the outer membrane. Interaction of apocytochrome *c* with these binding sites appears to be an initial step before the translocation across the membrane starts. This translocation of the polypeptide chain across the outer membrane and the linkage of the heme group are apparently coupled processes. On the basis of these results, a mechanism for the posttranslational assembly of cytochrome *c* in the mitochondria is proposed.

MATERIALS AND METHODS

Cell Growth

Neurospora crassa, wild type 74 A, was obtained from the Fungal Genetics Stock Center (Humboldt State University, Arcata, CA). Conidia were harvested from stock cultures grown on 1.8% agar in Vogel's medium supplemented with 1% glycerol, 1% sucrose, 0.25% yeast extract, 0.1% casein hydrolysate and a collection of vitamins [38]. Hyphae were grown for 15 h at 25 °C in 2 l Vogel's medium supplemented with 2% sucrose after inoculation with 1×10^6 conidia/ml culture [39]. The cells were radioactively labelled by growth in the same medium except that sulfate was reduced to 0.08 mM and that 50 µCi [^{35}S]sulfate (10–1000 Ci/mmol, New England Nuclear, Boston, MA) was added per liter culture.

Subcellular Fractionation

Hyphae were harvested by filtration and washed with ice-cold water. Immediately after harvesting, the hyphae were homogenized at 4 °C by grinding with quartz sand in medium A. The relation of hyphae (wet weight), sand and medium A was (1:1.5:2, w/w/v). Medium A consisted of 300 mM sucrose, 30 mM KCl, 90 mM triethanolamine, 20 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.25 mM of each amino acid except the one added subsequently as radioactive precursor (leucine or cysteine, respectively). The pH was adjusted with HCl to 7.4 (20 °C). The homogenate was separated into supernatant and crude mitochondrial fraction by differential centrifugation as described [15]. The mitochondria were further purified by centrifugation into a linear gradient of 0.96 M to 1.8 M sucrose in 10 mM Tris/HCl pH 7.2 (20 °C), 2 mM EDTA [40] using a Beckman SW 60 rotor at 60000 rev./min for 60 min at 2 °C. The mitochondrial fraction was diluted with 0.45 M sucrose, 2 mM EDTA, 30 mM Tris/HCl pH 7.2 (20 °C) and sedimented in a Sorvall SS-34 rotor at 12000 rev./min (11400 × *g* at r_{av}) for 12 min at 0 °C.

Cell-Free Protein Synthesis and Reconstituted System

The postmitochondrial supernatant of the cell homogenate was supplemented to a final concentration of 11 mM creatine phosphate, 1.35 mM GTP, 5.5 mM ATP and 9 mM $MgCl_2$. 50 µg creatine kinase (Boehringer, Mannheim) and 0.5 mCi of L-[^{3}H]leucine (55 Ci/mmol, New England Nuclear) or L-[^{35}S]cysteine (1000 Ci/mmol, New England Nuclear) in 0.5 ml 0.002 M KCl, respectively, were added per ml of postmitochondrial supernatant. The mixture was incubated for 10 min at 25 °C and then cycloheximide was added to a final concentration of 0.4 mM. Radioactivity incorporated into protein was determined in 50-µl aliquots [15]. A postribosomal supernatant was prepared by centrifugation of the cell-free homogenate in a Beckman 50 Ti rotor at 50000 rev./min (170000 × *g* at r_{av}) for 60 min. The purified mitochondria were recombined with the postribosomal supernatant by gentle resuspension in a glass-Teflon homogenizer. The mitochondria (about 1 mg mitochondrial protein) were taken up in 1 ml supernatant which corresponds roughly to the relation in the original homogenate. In the experiments to investigate the effects of the various heme and porphyrin compounds the mitochondria were resuspended in 50 µl medium A diluted with H_2O (2:1, v/v). Then 10 nmol of the respective dissolved heme or porphyrin derivative were added. The suspension was incubated first for 5 min at 25 °C and then 1 ml of the postribosomal supernatant was added. Stock solutions of protohemin (Calbiochem, Lucerne, Switzerland), deuterohemin (Porphyrin Products, Logan, UT), hematohemin and protoporphyrin IX (Sigma, St Louis, MO) were prepared by dissolving each in 10 µl 1 M NaOH, followed by dilution to 1 ml with 100 mM Tris/HCl pH 7.5 (20 °C).

In the experiments to analyse the effect of excess unlabelled apocytochrome *c* the mitochondria were incubated in the labelled postribosomal supernatant in the presence of deuterohemin for 15 min at 25 °C. The unlabelled apocytochrome *c* was added in various amounts. The quantity of the apocytochrome *c* was determined by amino acid analysis after hydrolysis in 6 M HCl, 0.05% 2-mercaptoethanol for 20 h at 108 °C. The reconstituted system was incubated at 25 °C for the various time periods indicated. Then the mitochondria and the supernatant were separated again by centrifugation at 4 °C and 12000 rev./min in a Sorvall SS-34 rotor (11400 × *g* at r_{av}) for 12 min. The mitochondria were resuspended in 0.44 M sucrose, 2 mM EDTA, 30 mM Tris/HCl pH 7.2 and sedimented as before. In the experiments to reverse the inhibition due to deuterohemin by addition of protohemin initially the mitochondria were incubated in the presence of deuterohemin as described above. Then the mitochondria were separated from the supernatant by centrifugation at 12000 rev./min for 12 min (11400 × *g* at r_{av}). They were resuspended in an unlabelled postribosomal supernatant containing protohemin and were incubated at 25 °C for the various time periods indicated. Then the mitochondria were isolated and washed as described above.

Protease Treatment of Subcellular Fractions

The mitochondria containing ^{3}H-labelled apocytochrome *c* bound in the presence of deuterohemin were prepared as described above. These mitochondria were resuspended in unlabelled postribosomal supernatant containing 1 mM $K_3[Fe(CN)_6]$. Trypsin (221 U/mg, Worthington Biochem. Corp., Freehold, NJ) was added to a final concentration of 50 µg/ml. Incubation was performed at 25 °C. After 15 min soy bean trypsin inhibitor (Merck, Darmstadt) was added to a final concentration of 150 µg/ml. Then 120 µl 20% Triton X-100 and 220 µl 0.3 M KCl, 10 mM Tris/HCl pH 7.5 (20 °C) were added and the samples were clarified by centrifugation at 15000 rev./min for 15 min. In one half of each aliquot apocytochrome *c* was immunoprecipitated, and in the other half holocytochrome *c* was immunoprecipitated.

Protein synthesis in the presence of [^{3}H]leucine was carried out in a cell-free homogenate from *Neurospora hyphae* grown on [^{35}S]sulfate. The postribosomal supernatant was prepared. $K_3[Fe(CN)_6]$ was added to a final concentration of 1 mM. After addition of 50 µg/ml of trypsin the sample was incubated for 15 min at 25 °C. Then trypsin inhibitor was added, the

sample was halved and immunoprecipitation was performed as described below.

Isolation and Purification of Neurospora *Cytochrome* c

Holocytochrome *c* was prepared from log-phase hyphae (after 15 h growth at 25 °C) as described [15]. The absorption spectrum and the ratio of $A_{410\,nm}/A_{280\,nm} = 4.7$ of the oxidized cytochrome *c* indicated its purity. Also, according to gel electrophoresis in the presence of dodecylsulfate, no contaminating protein was present at a load of cytochrome *c* sufficiently high to reveal 1% by-product. Apocytochrome *c* was prepared from holocytochrome *c* in 8 M urea, 0.1 M NaCl, adjusted with HCl to pH 2.0, by reaction with 70 mM $HgCl_2$ for 16 h at 25 °C according to Ambler et al. [41]. The purification of the protein on Sephadex G-25 and the special precautions against denaturation of the apocytochrome *c* have been described [31]. For each experimental series a new frozen aliquot of the apocytochrome *c* was taken and used only once immediately after thawing.

Immunoprecipitation

The preparation of rabbit antibodies directed against holocytochrome *c* and apocytochrome *c*, respectively, has been described [31]. The antisera were used without further purification after they had been tested for specificity by Ouchterlony double-immunodiffusion in 2% agar and for quantitative precipitation by titration. Holocytochrome *c* was immunoprecipitated in the presence of 0.5 nmol unlabelled holocytochrome *c* added as a carrier at 4 °C overnight. Apocytochrome *c* was usually isolated by incubation with antiserum and application of (protein A)-Sepharose (Pharmacia, Uppsala, Sweden) as described [31]. For preparation of the peptide maps of apocytochrome *c* immunoprecipitation was carried out employing unlabelled apocytochrome *c* (1 nmol) as a carrier.

Gel Electrophoresis and Liquid Scintillation Counting

The immunoprecipitates were dissociated in 50 µl 2% dodecylsulfate, 0.35 M 2-mercaptoethanol, 10 mM Tris/HCl pH 7.5 (20 °C) at 95 °C for 10 min. For measuring the radioactivity in cytochrome *c*, 20 µl were subjected to electrophoresis in horizontal polyacrylamide slab gels (15% acrylamide, 0.4% bisacrylamide, 0.5% dodecylsulfate, 0.1 M Tris/HCl pH 8.0) for 4 h at 18 mA/cm. The gels were sliced into 1-mm fractions, extracted with 0.15 ml 1% dodecylsulfate, 0.1 M Tris/HCl pH 8.0 for at least 5 h at 60 °C and counted in 1.2 ml 0.6% Permablend III, 30% Triton X-100, 70% toluene in a Tri-Carb Scintillation Counter (Packard Instr. Chicago, IL).

Peptide Maps and Autoradiography

The immunoprecipitates of [^{35}S]cysteine-labelled holocytochrome *c* or apocytochrome *c*, respectively, were suspended with additional 20 µg unlabelled holocytochrome *c* in 50 µl 12.8 mM 2-mercaptoethanol and heated for 2 min at 95 °C. Then 100 µl ethanol were added and the precipitated protein was centrifuged for 5 min at 20000 rev./min in a Sorvall SS-34 rotor (31000 × *g* at r_{av}). The pellets were suspended in 50 µl H_2O by gentle sonication and $K_3[Fe(CN)_6]$ was added to a final concentration of 1 mM. The protein was again precipitated by addition of 100 µl ethanol followed by centrifugation. The pellets were washed twice with 70% ethanol and dried *in vacuo*. For tryptic cleavage the pellets were suspended in 20 µl 100 mM NH_4HCO_3. Parallel cleavages were performed of pure holocytochrome *c* and apocytochrome *c* and these were adjusted to 100 µg cytochrome *c* per 20 µl solution. Trypsin (221 U/mg, Worthington Biochem. Corp., Freehold, NJ) was dissolved in 20 mM $CaCl_2$, 1 mM HCl and added to a final concentration of 1 µg trypsin per 20 µl cytochrome *c* solution. Samples were incubated for 4 h at 37 °C and then dried *in vacuo*. They were applied onto thin-layer plates (10 × 10 cm) of microcrystalline cellulose (G 1440, Schleicher & Schüll, Dassel) and subjected to two-dimensional separation [42]: First, electrophoresis was performed in 10% pyridine acetate pH 4.7 at − 10 °C for 30 min. Then chromatography was performed in the second dimension using 1-butanol/H_2O/pyridine/acetic acid (75/60/50/15) for 1 h. The dried plates mapping the radioactively labelled peptides were sprayed with 0.4% 2.5-diphenyloxazole in 90% 2-methylnaphthalene, 10% toluene in order to enhance autoradiographic sensitivity [43] and then they were laid onto X-ray film (Curix RP 1, Agfa-Gevaert) for 3 days at − 80 °C. The plates mapping the peptides of pure cytochrome *c* were sprayed with 0.25% fluorescamine in acetone followed by 0.05% trimethylamine in acetone [44].

RESULTS

Kinetics of Import of Cytochrome c *into Mitochondria* in vitro

The import of cytochrome *c* into mitochondria was studied *in vitro* according to the following procedure. A cell-free postmitochondrial fraction was prepared from hyphae of *Neurospora crassa* and this was used to carry out protein synthesis *in vitro*, in the presence of [^{3}H]leucine. Apocytochrome *c* was synthesized in an amount of about 0.1 pmol/ml cell-free homogenate. After protein synthesis was terminated by addition of cycloheximide, the postribosomal supernatant was prepared and combined with isolated mitochondria. These had been purified by sucrose-density-gradient centrifugation to remove contaminating cell-wall fragments and lysophospholipase activity [45]. This reconstituted system was then incubated to allow apocytochrome *c* present in the extramitochondrial fraction to be taken up by mitochondria and to be converted to holocytochrome *c*. The reaction was stopped after various time periods by chilling the samples; mitochondria and supernatant were separated again. Apocytochrome *c* and holocytochrome *c* were immunoprecipitated from both the lysed mitochondria and the supernatant by antisera specifically for each of these two forms of cytochrome *c*. The dissociated immunoprecipitates were analysed by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. Radioactivity was determined in the cytochrome *c* peaks after slicing the gels.

Discrimination between preexistent cytochrome *c* and cytochrome *c* that was newly synthesized *in vitro* and imported into mitochondria was achieved by the following experimental set-up. The mitochondria employed in the reconstitution system were isolated from cells labelled *in vivo* with [^{35}S]sulfate and therefore contained ^{35}S-labelled holocytochrome *c*. The cell-free system used to label newly synthesized proteins with [^{3}H]leucine was prepared from unlabelled cells. This ensured that the supernatant from the cell-free system contained no preexistent ^{35}S-labelled holocytochrome *c*. Thus two different aspects could be followed simultaneously: first, the import of apocytochrome *c* and the conversion of apo-

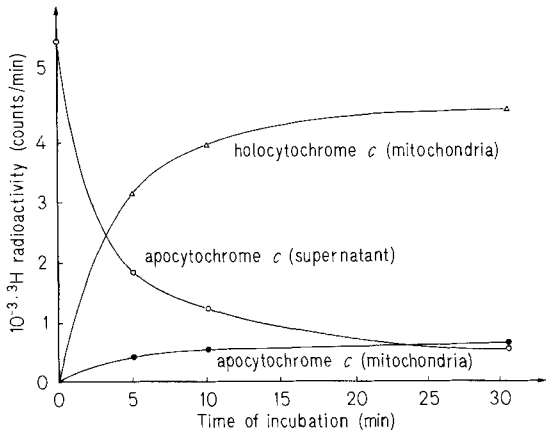

Fig. 1. *Kinetics of formation of holocytochrome* c *from apocytochrome* c *in a reconstituted system.* A post-mitochondrial supernatant was prepared from *Neurospora* cells and incubated for 15 min with [^{3}H]leucine. Then cycloheximide was added and the postribosomal supernatant was prepared. Mitochondria isolated from a different culture were resuspended and incubated in this supernatant for the time periods indicated. Then mitochondria and supernatant were separated again. Apocytochrome *c* was immunoprecipitated from the supernatant fraction and from the lysed mitochondria. Holocytochrome *c* was immunoprecipitated from the lysed mitochondria. The immunoprecipitates were analysed by gel electrophoresis in the presence of dodecylsulfate and the radioactivities in the cytochrome *c* peaks were determined. (○) Apocytochrome *c* (supernatant); (●) apocytochrome *c* (mitochondria); (△) holocytochrome *c* (mitochondria)

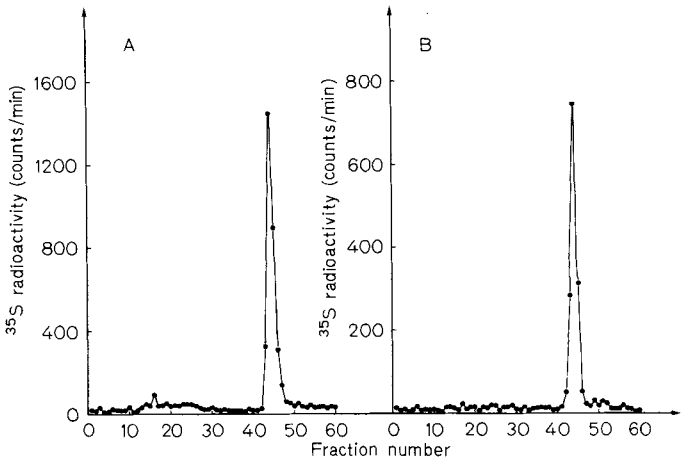

Fig. 2. *Gel electrophoretic analysis of apocytochrome* c *and holocytochrome* c *synthesized* in vitro *and isolated by immunoprecipitation.* A cell-free postmitochondrial homogenate was incubated with [^{35}S]cysteine for 15 min and then a postribosomal supernatant was prepared as described for Fig. 1. One half of the supernatant was subjected to immunoprecipitation with antibodies against apocytochrome *c*. The other half was incubated with mitochondria for 30 min. Then mitochondria were reisolated, lysed and immunoprecipitation with antibodies against holocytochrome *c* was performed. The immunoprecipitates were dissociated with dodecylsulfate and analysed by gel electrophoresis in the presence of dodecylsulfate. The gels were sliced and ^{35}S-radioactivity determined. (A) Apocytochrome *c*; (B) holocytochrome *c*

cytochrome *c* to holocytochrome *c*; second, the amount of mitochondria in the various samples and their integrity throughout the experiment.

The kinetics of the import of cytochrome *c* into mitochondria as measured by uptake of apocytochrome *c* and its conversion to holocytochrome *c* are shown in Fig. 1. Apocytochrome *c* was rapidly imported into the mitochondria and concurrently converted to holocytochrome *c*. Within 15 min import was practically complete. About 80% of the apocytochrome *c* employed was recovered as holocytochrome *c*. In the extramitochondrial fraction no [^{3}H]holocytochrome *c* could be detected. This fraction contained, however, unlabelled holocytochrome *c* at a concentration of about 0.4–0.8 nmol/ml, which was released from the mitochondria during preparation of the supernatant fraction. If the heme group had been added to apocytochrome *c* in the extramitochondrial space, the newly formed labelled holocytochrome *c* would have been diluted by a large excess of unlabelled holocytochrome *c*. The amount of holocytochrome *c* found in the supernatant fraction was not changed during incubation with mitochondria. This shows that the heme was linked to apocytochrome *c* in mitochondria, but not in the cytosol or on the mitochondrial surface. This has been noted already in an earlier report [15]. Furthermore, [^{35}S]holocytochrome *c* could not be detected in the extramitochondrial fraction of the reconstituted system, demonstrating that holocytochrome *c* was not released from the intermembrane space of the mitochondria and hence that the mitochondria had remained intact during the period of incubation. A small portion of apocytochrome *c* (ca. 10% of total) was found associated with mitochondria. The amount of this fraction did not decrease even after import activity had ceased. The meaning of this fraction is not understood. It may represent binding of apocytochrome *c* to specific sites on inactive mitochondria or to unspecific sites on the active mitochondria from which it cannot be imported, or denatured apocytochrome *c*, or binding to structures other than mitochondria. No transient accumulation of apocytochrome *c* was observed in mitochondria. Also, there was no lag between the appearance of holocytochrome *c* in the mitochondria and the decrease of apocytochrome *c* in the supernatant.

It was concluded from Fig. 1 that transport through the outer membrane and conversion to holocytochrome *c* are closely coupled with respect to time. The kinetics of the import of cytochrome *c* into mitochondria therefore suggested that one of the following two alternatives regarding the relationship between transport and conversion is true: either the transport of apocytochrome *c* into mitochondria and the conversion of apocytochrome *c* to holocytochrome *c* are independent processes and it is the transport which is the rate-limiting step of the assembly process; or the transport of apocytochrome *c* through the outer mitochondrial membrane and its conversion to holocytochrome *c* are intrinsically coupled processes, i.e. conversion is part of the translocation reaction.

The Heme Group Becomes Covalently Linked to Apocytochrome c *in the Reconstituted System*

So far the evidence that apocytochrome *c* is converted to holocytochrome *c in vitro* is based solely on the selective immunoprecipitation of apocytochrome *c* and holocytochrome *c* by specific antisera. Since the reliability of this conclusion is most important, we have tried to obtain definite proof by peptide mapping of tryptic digests of apocytochrome *c* and holocytochrome *c* synthesized *in vitro*. The chromatographic behaviour of the heme peptide of holocyto-

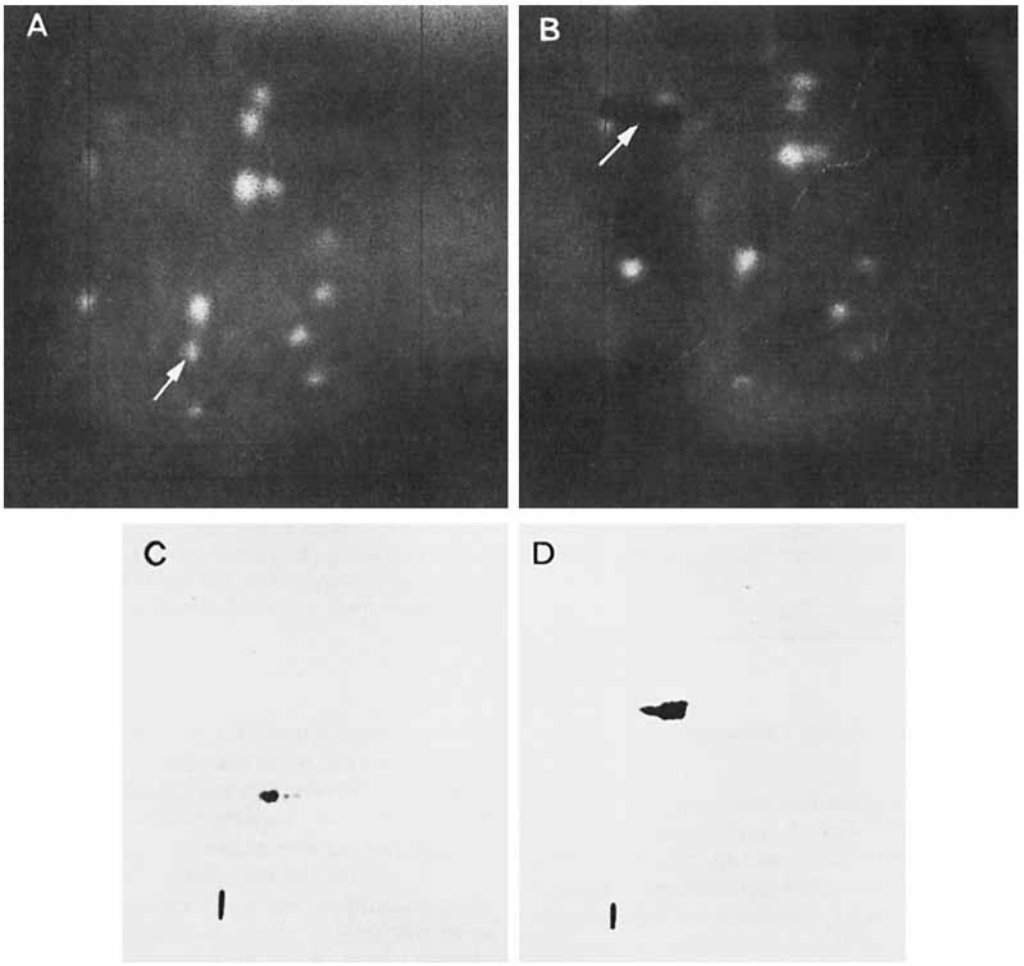

Fig. 3. *The heme group becomes covalently linked to cytochrome* c *in a cell-free reconstituted system.* Isolated apocytochrome *c* and holocytochrome *c* were subjected to digestion with trypsin. The peptides were analysed by peptide-mapping on cellulose thin-layer plates. The plates were stained with fluorescamine. Apocytochrome *c* and holocytochrome *c* synthesized *in vitro* and labelled with [^{35}S]cysteine as described in the legend to Fig. 2 were isolated as immunoprecipitates. These immunoprecipitates were denatured, precipitated with ethanol, suspended in NH_4HCO_3 and then digested with trypsin. The digests were analysed by peptide mapping and the thin-layer plates were subjected to autoradiography. (A, B) Peptide maps of isolated apocytochrome *c* and holocytochrome *c*, respectively; staining with fluorescamine. (C, D) Peptide maps of apocytochrome *c* and holocytochrome *c*, respectively, synthesized *in vitro*; autoradiographs. Arrows point to the heme-binding peptide of apocytochrome *c* and the heme-containing peptide of holocytochrome *c*, respectively

chrome *c* differs from that of the corresponding heme-binding peptide of apocytochrome *c* because of a large difference in polarity.

For these experiments, [^{35}S]cysteine was used in the cell-free translation system to synthesize labelled apocytochrome *c* and to obtain labelled holocytochrome *c* in the reconstituted system. Since *Neurospora* cytochrome *c* contains cysteine only in the heme-binding sequence (-Arg-Cys-Ser-Gln-Cys-His-) [46, 47] incorporation of [^{35}S]cysteine selectively labels the heme-binding peptide in apocytochrome *c* and the hemepeptide in holocytochrome *c*. Fig. 2 A and B show the gel electrophoretic analysis of apocytochrome *c* and holocytochrome *c*, respectively, labelled by this procedure. The sliced gels displayed a single peak in either case, demonstrating the purity of the two proteins. Comparable gel patterns were obtained when labelling was performed with [^{3}H]leucine.

Fig. 3 A and B show the tryptic peptide maps of isolated apocytochrome *c* and holocytochrome *c*, respectively. The patterns were obtained by staining with fluorescamine. The heme-containing peptide was easily visible by its intense orange colour (see arrow in Fig. 3 B). The peptide map of apocytochrome *c* did not display a spot at the position of the heme peptide (cf. Fig. 3 B) but instead showed a spot not present in the peptide map of holocytochrome *c* (see arrow in Fig. 3 A). This represented the heme-binding peptide, as confirmed by amino acid analysis of the eluted spot.

Peptide maps of the digested immunoprecipitates were subjected to autoradiography. In the case of holocytochrome *c* a radioactive spot was seen which coincided with the heme-containing peptide (Fig. 3 D). In contrast, the [^{35}S]cysteine-containing peptide observed after digestion of immunoprecipitated apocytochrome *c* coincided with the heme-binding peptide in the stained tryptic peptide map of isolated apocytochrome *c* (Fig. 3 C).

The experiments demonstrate that heme becomes covalently linked to apocytochrome *c* in the cell-free system. Hereby, our earlier conclusion [15, 16] is confirmed that antibodies raised against isolated apocytochrome *c* and holocytochrome *c*, respectively, selectively recognize these two forms of cytochrome *c* synthesized *in vitro*.

Table 1. *Effect of various heme and porphyrin compounds on the formation of holocytochrome* c *in a reconstituted system*
A postribosomal supernatant containing ^{3}H-labelled apocytochrome *c* synthesized *in vitro* was incubated with isolated mitochondria in the presence of various heme and porphyrin compounds (10 nmol/mg mitochondrial protein and ml). After 30 min incubation, mitochondria were isolated by centrifugation and holocytochrome *c* was immunoprecipitated. The immunoprecipitates were analyzed by gel electrophoresis in the presence of dodecylsulfate and the radioactivities in the cytochrome *c* peaks were determined

Addition	Holocytochrome *c* formed	
	counts × min^{-1}	% of control
None	3258	100
+ Protohemin	3198	98
+ Deuterohemin	622	19
+ Deuterohemin and protohemin	2549	78
+ Deuterohemin, 15 min, then protohemin, 15 min	2806	86
+ Protoporphyrin IX	3616	111
+ Deuteroporphyrin	2317	71
+ Deuterohemin, 15 min, then protoporphyrin IX, 15 min	586	18

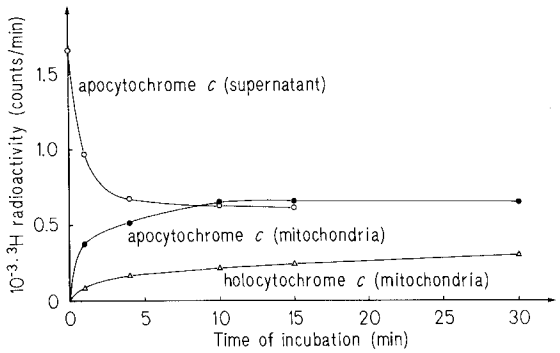

Fig. 4. *Deuterohemin inhibits the formation of holocytochrome* c in vitro. Formation of holocytochrome *c* from apocytochrome *c* synthesized *in vitro* was studied in a reconstituted system as described for Fig. 1. Isolated mitochondria were preincubated with deuterohemin (10 nmol/mg protein) for 5 min at 25 °C and then incubated in the postribosomal supernatant containing ^{3}H-labelled apocytochrome *c*. All other steps were carried out as described for Fig. 1. (○) Apocytochrome *c* (supernatant); (●) apocytochrome *c* (mitochondria); (△) holocytochrome *c* (mitochondria)

Deuterohemin Reversibly Inhibits the Formation of Holocytochrome c in vitro

It appears from the data presented that assembly of cytochrome *c* can only be resolved into the individual steps if it is possible to inhibit the assembly process at specific points. The conversion of apocytochrome *c* to holocytochrome *c* involves the covalent attachment of heme. Addition of hemin to the reconstituted system, however, had no influence on the conversion (Table 1). Apparently, hemin is present in mitochondria in sufficient amounts. In order to block the addition of the heme group, the analogue deuterohemin was employed. This compound has two hydrogen atoms at the positions C-3 and C-8 of the porphyrin ring (according to the IUPAC nomenclature [47a]), instead of two vinyl groups. It therefore cannot be covalently linked to the polypeptide chain. As shown in Table 1, deuterohemin strongly inhibited formation of holocytochrome *c*. This inhibition could be reversed, when deuterohemin and protohemin were added together, or when mitochondria were first incubated with deuterohemin and then protohemin was added (Table 1). The most probable explanation for these observations is that deuterohemin binds reversibly to an enzyme which catalyzes the formation of the thioether bonds and thereby competitively inhibits the reaction.

In contrast to deuterohemin, deuteroporphyrin only slightly inhibited the formation of holocytochrome *c*. Protoporphyrin was not able to reverse the inhibition exerted by deuterohemin (Table 1). This suggests that protohemin and not protoporphyrin becomes linked to the apoprotein. The same conclusion has been reached by Colleran and Jones [48] using a different approach. The slight inhibition by deuteroporphyrin may be due to a partial conversion to deuteroheme by the mitochondrial ferrochelatase [49].

What happens to the apocytochrome *c* which is not converted to holocytochrome *c* in the presence of deuterohemin? This was investigated by determining the amounts of apocytochrome *c* in the supernatant fraction and of apocytochrome *c* associated with mitochondria, as well as holocytochrome *c* in mitochondria. No holocytochrome *c* was detected in the postmitochondrial supernatant during the period of incubation. Fig. 4 shows that the formation of holocytochrome *c* was very low compared to the control (cf. Fig. 1). Apocytochrome *c* rapidly disappeared from the supernatant fraction and became associated with the mitochondria. However, in contrast to the control, only about half of the total apocytochrome *c* was taken up from the supernatant. Apparently, not only conversion of apocytochrome *c* to holocytochrome *c* is inhibited by deuterohemin, but also the transfer of apocytochrome *c* into the mitochondria.

Apocytochrome c *Reversibly Binds to Specific Sites on the Mitochondria in the Presence of Deuterohemin*

In order to analyse the submitochondrial location of the apocytochrome *c* which is associated with mitochondria in the presence of deuterohemin, the following experiments were carried out. First it was checked whether apocytochrome *c* is loosely attached or firmly bound. For this purpose, mitochondria were incubated with the supernatant of a cell-free translation system in the presence of deuterohemin for 15 min. Then mitochondria were reisolated and washed with 0.44 M sucrose, 10 mM Tris/HCl, 2 mM EDTA pH 7.5. Less than 5% of the apocytochrome *c* bound to the mitochondria was released into the wash medium. To determine whether this tight binding is a specific effect, it was tested whether the bound ^{3}H-labelled apocytochrome *c* can be specifically displaced by excess apocytochrome *c*. Mitochondria were incubated with the postribosomal supernatant containing [^{3}H]-apocytochrome *c* in the presence of deuterohemin for 15 min and then unlabelled apocytochrome *c* was added at final concentrations of 1 – 10000 pmol/ml. After incubation for 15 min, the apocytochrome *c* which remained associated with mitochondria as well as the apocytochrome *c* in the postmitochondrial supernatant were determined. As a control, preexistent ^{35}S-labelled holocytochrome *c* in the mitochondria was also determined. In a further control, the mitochondria were incubated with holocytochrome *c* instead of apocytochrome *c* employing the same concentrations. The results are

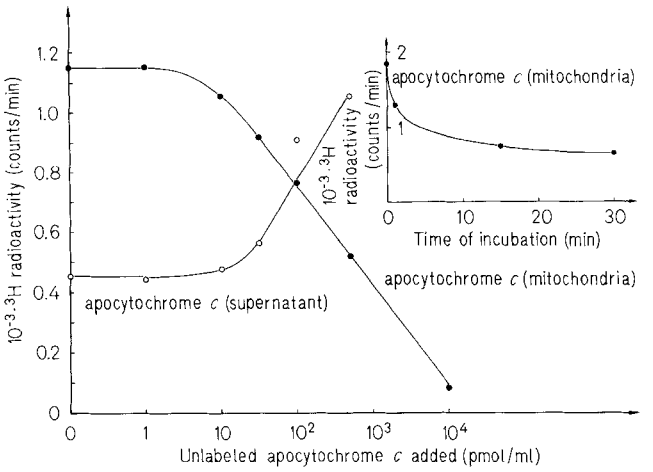

Fig. 5. *Apocytochrome* c *associated with mitochondria in the presence of deuterohemin is displaced by excess added apocytochrome* c. A postribosomal supernatant containing ^{3}H-labelled apocytochrome *c* synthesized *in vitro* was incubated with isolated mitochondria in the presence of deuterohemin. The mitochondria had been isolated from cells grown in the presence of [^{35}S]sulfate. After 15 min incubation the suspension was separated into eight aliquots of 2 ml. To these aliquots different amounts of unlabelled apocytochrome *c* were added. After incubation for 15 min the aliquots were halved. With all samples supernatant and mitochondria were separated again by centrifugation. Apocytochrome *c* was immunoprecipitated from the mitochondria. Apocytochrome *c* was also immunoprecipitated from the supernatants after adding apocytochrome *c* in such amounts that the total apocytochrome *c* concentrations was 0.5 nmol/ml in all samples. Samples with higher concentrations of apocytochrome *c* were not subjected to immunoprecipitation of apocytochrome *c* from the supernatant since under these conditions precipitation was not quantitative. Holocytochrome *c* was also immunoprecipitated from the mitochondria. Gel electrophoresis in the presence of dodecylsulfate was performed with all immunoprecipitates and the radioactivities in the cytochrome *c* peaks were measured. (●) [^{3}H]Apocytochrome *c* (mitochondria); (○) [^{3}H]apocytochrome *c* (supernatant). Insert: kinetics of displacement of apocytochrome *c* from mitochondria. Concentration of unlabelled apocytochrome *c* was 1×10^{3} pmol/ml

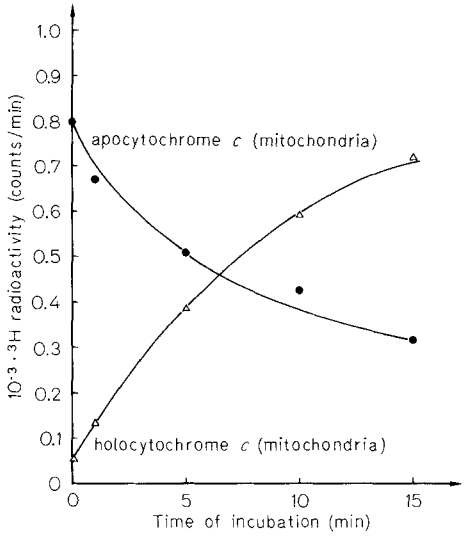

Fig. 6. *Inhibition of holocytochrome* c *formation by deuterohemin is reversed by protohemin.* Isolated mitochondria (about 1 mg protein) were first incubated with deuterohemin (10 nmol) for 5 min at 25 °C and then combined with the postribosomal supernatant, which was obtained from a cell homogenate after carrying out protein synthesis *in vitro* in the presence of [^{3}H]leucine. The mitochondria were obtained from cells grown in the presence of [^{35}S]sulfate. After incubation at 25 °C for 15 min mitochondria were reisolated by centrifugation. They were then resuspended in a postribosomal supernatant from unlabelled cells. Protohemin (10 nmol/ml) was added and the mixture incubated at 25 °C. After the times indicated aliquots were removed, the mitochondria separated by centrifugation and lysed with Triton X-100. The lysates were halved and subjected to immunoprecipitation with antibodies against holocytochrome *c* and apocytochrome *c*, respectively. Immunoprecipitates were subjected to gel electrophoresis in the presence of dodecylsulfate and the radioactivities in the cytochrome *c* peaks were determined. ^{35}S-radioactivity represents preexisting holocytochrome *c* in the isolated mitochondria. (●) [^{3}H]Apocytochrome *c* (mitochondria); (△) [^{3}H]holocytochrome *c* (mitochondria)

shown in Fig. 5. Increasing concentrations of added unlabelled apocytochrome *c* led to the displacement of [^{3}H]apocytochrome *c* from the mitochondria. The insert in Fig. 5 shows the kinetics of the reaction. About 15 min were required to equilibrate bound and free apocytochrome *c*. When holocytochrome *c* was added (1 nmol/ml), 98% of the apocytochrome *c* remained bound to the mitochondria. Also, preexistent holocytochrome *c* in the mitochondria was not released from the mitochondria (95% recovered).

These observations strongly suggest that the apocytochrome *c* associated with the mitochondria is present in high-affinity binding sites and that the binding is reversible and specific. Further support for the specificity of binding of the apocytochrome *c* was obtained by employing apocytochrome *c* from other species in the displacement analysis. For example, apocytochrome *c* from horse was found to be less effective by a factor of about 10 compared to the *Neurospora* apocytochrome *c*. Apocytochrome *c* from the bacterium *Paracoccus denitrificans* which is closely related to mitochondrial cytochrome *c*, was not effective at all (unpublished results). The concentration of the binding sites with high affinity on the mitochondria were calculated to be roughly 50 pmol × mg mitochondrial protein^{-1}.

In order to demonstrate that these binding sites are actually involved in the transfer of apocytochrome *c*, binding in the presence of deuterohemin was performed. Mitochondria were reisolated and were transferred into a supernatant of a cell-free system which did not contain labelled apocytochrome *c*. Then protohemin was added. After incubation for different time periods mitochondria were separated from the supernatant and both apocytochrome *c* and holocytochrome *c* were immunoprecipitated from the mitochondria. Fig. 6 shows that holocytochrome *c* was formed at the expense of apocytochrome *c*. During the experiments the integrity of the mitochondria was retained as demonstrated by the unchanged content of preexisting ^{35}S-labelled holocytochrome *c*.

These findings strongly suggest that in the presence of deuterohemin, when formation of holocytochrome *c* is blocked, apocytochrome *c* is bound to specific receptor sites. From these sites apocytochrome *c* can be translocated and converted to holocytochrome *c* when deuterohemin inhibition is relieved.

The question remains whether apocytochrome *c* bound to mitochondria in the presence of deuterohemin is actually present at the outer face of the outer membrane. One possibility to answer this question is to show that this apocytochrome *c* is susceptible to digestion by added protease. Mitochondria containing bound apocytochrome *c* were treated with trypsin. Immunoprecipitable apocytochrome *c* was decreased by about 80% (Table 2). Endogenous holocyto-

Table 2. *Apocytochrome* c *bound to mitochondria is degraded by added trypsin in preference to endogenous holocytochrome* c
(A) Mitochondria isolated from cells grown on [^{35}S]sulfate were incubated in the presence of deuterohemin with a postribosomal supernatant containing ^{3}H-labelled apocytochrome *c*. The mitochondria were reisolated and resuspended in unlabelled postribosomal supernatant. Trypsin was added (50 μg/ml) and incubation at 25 °C was performed for 15 min. After addition of soy-bean trypsin inhibitor, mitochondria were lysed, the lysate was halved, and immunoprecipitation with antisera against apocytochrome *c* and holocytochrome *c*, respectively, was carried out. (B) A cell-free system was prepared from cells grown on [^{35}S]sulfate and protein synthesis was carried out after adding [^{3}H]leucine. The postribosomal supernatant was prepared which contained ^{35}S-labelled holocytochrome *c* leached out from mitochondria and ^{3}H-labelled apocytochrome *c* synthesized *in vitro*. This supernatant was treated with trypsin as described above and apocytochrome *c* and holocytochrome *c* were immunoprecipitated after addition of Triton X-100

Fraction	[^{3}H]Apocytochrome *c*		[^{35}S]Holocytochrome *c*	
	counts × min^{-1}	% of control	counts × min^{-1}	% of control
A. *Mitochondria*				
Control	1348	100	2466	100
+ Trypsin	334	25	2314	94
B. *Cytosolic fraction*				
Control	6966	100	4992	100
+ Trypsin	2226	32	2834	57

chrome *c* was largely resistant to this treatment. In contrast holocytochrome *c* which leached out from the mitochondria and was present in the extramitochondrial space was largely sensitive to tryptic digestion. Also, apocytochrome *c* in the extramitochondrial space was largely degraded by added trypsin (Table 2). This suggests, that apocytochrome *c* bound to mitochondria is present in a position accessible to added protease, i.e. on the mitochondrial surface. However, caution is required locating a polypeptide by this kind of protease experiments. Binding of proteins to membranes or interaction with other components of the complex reconstituted system may alter their susceptibility to added proteases. A careful separation of outer and inner membranes and an attempt to discriminate between the two faces of the outer membrane will be necessary. Experiments in this direction have been initiated and preliminary results support the above conclusions on the location of apocytochrome *c* bound to mitochondria.

DISCUSSION

The analysis of the molecular events in the biogenesis of cytochrome *c* largely depends on the ability to discriminate between apocytochrome *c* and holocytochrome *c*. In our studies, we have employed antibodies which differentially react with these two forms of cytochrome *c*. Under appropriate conditions, no cross reaction was observed. In order to demonstrate conclusively the specificity of the antibodies in picking up apocytochrome *c* and holocytochrome *c* synthesized *in vitro*, tryptic peptides of the immunoprecipitates were analysed. The data showed unequivocally that apocytochrome *c* imported into mitochondria actually was converted to holocytochrome *c* by covalent linkage of the heme group. This confirmed our earlier conclusions [15, 16] and also those of Basile et al. [32]. The antibodies therefore are reliable tools to study individual reactions of intracellular transport and assembly of cytochrome *c*.

Import of cytochrome *c* entails at least two processes: transport through the outer membrane and linkage of the heme group. As described in this report, import in a reconstituted system occurs in such a way that it is not possible to discriminate between these two processes. In particular, it is not possible to decide whether transfer across the outer membrane and addition of the heme group are separate steps occurring independently from each other, or whether transfer is essentially coupled to the addition of the heme group. We describe here the inhibition of the covalent addition of heme by the analogue deuteroheme. This allows a more detailed analysis of the individual steps of assembly. Deuteroheme most probably inhibits in a competitive manner the apocytochrome *c* heme lyase which supposedly catalyses the formation of the two thioether bridges between the vinyl groups of protoheme and cysteines 14 and 17 (according to standardized nomenclature) of the apoprotein. Deuteroheme lacks the vinyl groups, so covalent linkage cannot occur. Inhibition can be reversed by the addition of excess protoheme. Obviously, the inhibition is not caused by a destruction of the mitochondrial structure or of the import system.

In the presence of deuterohemin a large fraction of the apocytochrome *c* becomes associated with the mitochondria. This bound apocytochrome *c* is not released from the mitochondria in an isotonic sucrose buffer. It can, however, be released by addition of excess apocytochrome *c* yet not by excess holocytochrome *c*. From these binding sites apocytochrome *c* can be translocated and converted to holocytochrome *c* when inhibition is relieved by excess protohemin.

The binding of apocytochrome *c* to mitochondria therefore meets all criteria for a specific interaction of a protein with a receptor [51]: (a) binding of apocytochrome *c* is rapid and reversible; (b) the number of binding sites is limited and saturable; (c) the binding sites are specific for apocytochrome *c*; and (d) binding of apocytochrome *c* is directly related to the mitochondria being the target organelles and to addition of the heme group as a next step in assembly.

Where are these binding sites located? A number of studies have demonstrated that the outer mitochondrial membrane is not freely permeable to macromolecules, such as proteins and polysaccharides [52 – 54]. Especially the inability of holocytochrome *c* to diffuse through the outer membrane is documented [29, 30]. This suggests that the binding sites for apocytochrome *c* are located at the mitochondrial surface. The experiments presented here on the lability of bound apocytochrome *c* after addition of protease, in contrast to the stability of endogenous holocytochrome *c*, are in full agreement with the binding sites being on the cytoplasmic face of the outer mitochondrial membrane. A similar proteolytic sensitivity is known of the rotenone-resistant NADH-dehydrogenase on the outer membrane compared to the proteolytic resistance

of the adenylate kinase in the intermembrane space [55]. Specific but different binding sites have been demonstrated for other precursors of imported mitochondrial proteins, such as the ADP/ATP carrier and the dicyclohexylcarbodiimide-binding subunit of ATP synthase [31,56]. In these cases, protease treatment also leads to degradation of the bound precursors, but not of the assembled mature proteins.

What is the role of these binding sites in the assembly process? We speculate that they provide the stereochemical complementarity sites which direct a precursor to the target organelle, i.e. which recognize a precursor and position it for the next steps in translocation across the membrane. Apparently apocytochrome *c* is not transferred across the outer membrane when it cannot be converted to holocytochrome *c*. Rather, it remains bound to the specific sites on the outer membrane. We therefore suggest that transfer and conversion are processes which are intrinsically coupled.

How does the covalent linkage of the heme group take place? We have suggested earlier that there is an enzyme in the intermembrane space which catalyses this reaction. Destruction of mitochondrial integrity by sonication or simply by a hypotonic treatment leads to the loss of their ability to attach the heme group to the apoprotein [16]. Other aspects have led Basile et al. [32] and Colleran and Jones [48] to claim that an enzyme is involved. The above-mentioned observations would indicate that apocytochrome *c* heme lyase is a soluble enzyme in the intermembrane space. On the other hand it is not excluded that apocytochrome *c* heme lyase is involved in binding apocytochrome *c* and is associated with the outer membrane.

How is the energy provided for the transport and accumulation of cytochrome *c* into the mitochondria? Free diffusion of apocytochrome *c* across the outer membrane apparently does not occur. Cleavage of an additional peptide is not involved and energization of the mitochondria is not necessary for transfer of cytochrome *c* [31]. Also the ionophore valinomycin was found to completely inhibit transfer of e.g. the ADP/ATP Carrier, but had no effect on the transfer of apocytochrome *c* and on its conversion to holocytochrome *c* (Hennig and Neupert, unpublished). Apocytochrome *c* and holocytochrome *c* have quite different conformations [57–59]. The heme group exerts a distinctive effect on the folding of the polypeptide chain [33,60]. It is therefore reasonable to assume that the linkage of the heme group and the entailing refolding of the molecule provide the driving force to pull cytochrome *c* through the outer membrane.

Which portions of the apocytochrome *c* molecule are involved in the binding to the receptor? Apparently it is not the aminoterminal sequence. This conclusion stems from the observation on mutations of cytochrome *c* in yeast by Sherman and coworkers [61–63]. Alterations in the first 10 positions of the sequence including the complete lack of this region do not impair formation of holocytochrome *c* and import into mitochondria.

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