

## Receptor sites involved in posttranslational transport of apocytochrome *c* into mitochondria: Specificity, affinity, and number of sites

(cytochrome *c*/precursor protein/deuterohemim/mitochondrial biogenesis)

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**ABSTRACT** Assembly of cytochrome *c* involves a series of steps: synthesis of apocytochrome *c* on free ribosomes, specific binding of apocytochrome *c* to the mitochondrial surface, transfer across the outer membrane, covalent addition of protoheme, refolding of the polypeptide chain, and association of holocytochrome *c* with its functional sites at the inner membrane. The binding step of apocytochrome *c* to *Neurospora crassa* mitochondria was studied by inhibiting the subsequent transfer steps with the heme analogue deuterohemim. The binding sites are highly specific for mitochondrial apocytochromes *c*. Bound labeled *Neurospora* apocytochrome *c* was competitively displaced by unlabeled apocytochrome *c* from various species. These exhibited different abilities for displacement. Apocytochrome *c* from *Paracoccus denitrificans*, the amino-terminal (heme-binding) fragment of *Neurospora* apocytochrome *c*, and *Neurospora* holocytochrome *c* did not recognize the binding sites. Polylysine did not interfere with apocytochrome *c* binding. Apocytochrome *c* is reversibly bound. The binding sites are present in limited number. High-affinity binding sites were present at about 90 pmol/mg of mitochondrial protein. They displayed an association constant of  $2.2 \times 10^7 \text{ M}^{-1}$ . Apocytochrome *c* was imported into mitochondria and converted to holocytochrome *c* directly from the binding sites when inhibition by deuterohemim was relieved. We conclude that the apocytochrome *c* binding sites on mitochondria represent receptors that function in the recognition and import of this precursor by mitochondria.

Mitochondrial proteins coded for by nuclear genes are synthesized as extramitochondrial precursors and posttranslationally transported into mitochondria (see refs. 1–3). The specific interaction of extramitochondrial precursors with the mitochondrial surface is required by all mitochondrial proteins as a first step of their transfer into mitochondria. Cytochrome *c* is particularly useful for investigating this mutual recognition. Its biosynthetic precursor can be transferred into mitochondria and converted to holocytochrome *c* *in vitro* (4–6). It does not contain an additional sequence, in contrast to most other mitochondrial proteins, which are proteolytically processed during translocation into mitochondria (see refs. 1–3). This was demonstrated for cytochrome *c* from yeast (7–9), *Neurospora* (10), and rat (6, 11), both at the level of protein and at the level of DNA or RNA. The biosynthetic precursor of cytochrome *c* is apparently identical to apocytochrome *c*, which can be prepared in large amounts from the mature protein, holocytochrome *c*, by removing the covalently linked heme group. Chemically prepared apocytochrome *c* can be assembled into mitochondria *in vitro* (12, 13).

The transfer of apocytochrome *c* is inhibited by deuterohemim, which is an analogue of protohemin, the natural sub-

strate for the covalent attachment of the heme group (5). Apocytochrome *c* binds to the surface of mitochondria under this condition. We have suggested that this binding is involved in the assembly of cytochrome *c* because bound apocytochrome *c* is converted to holocytochrome *c* and translocated across the mitochondrial outer membrane when the inhibition of heme attachment is relieved (5). Our studies were now concerned with the following questions: (i) Do these mitochondrial binding sites display specificity for apocytochrome *c*? (ii) How strong is this binding and how many binding sites are there on the mitochondrial surface? (iii) Do the binding sites actually participate in the transfer of apocytochrome *c* into mitochondria?

### MATERIALS AND METHODS

**Cell Growth.** *Neurospora crassa* wild-type 74A (Fungal Genetic Stock Center no. 262) was grown at 25°C for about 14 hr in Vogel's minimal medium supplemented with 2% sucrose as described (14). Harvested cells were immediately homogenized, either in medium A for cell-free translation (5), or in 0.25 M sucrose/10 mM 4-morpholinepropanesulfonate/2 mM EDTA/0.2% bovine serum albumin/0.01% 2-mercaptoethanol, pH 7.2 (sucrose/Mops buffer) for isolation of mitochondria.

Experimental procedures for preparation of apocytochrome *c*, subcellular fractionation, *Neurospora* cell-free translation system, mitochondrial *in vitro* transfer system, reaction of apocytochrome *c* and holocytochrome *c* with specific antibodies, polyacrylamide gel electrophoresis, and determination of radioactivity have been described elsewhere (5, 14).

**Sources of Holocytochrome *c*.** The various eukaryotic cytochromes *c* were isolated from homogenates according to established procedures (14–16) or purchased (Sigma). Only the native forms, purified by chromatography on Whatman CM 32-cellulose (16), were used. Cytochrome *c* from parsnip, *Pastinaca sativa*, was kindly donated by D. Boulter (Durham, England). Cytochrome *c* from *Paracoccus denitrificans* was prepared from bacteria that had been grown anaerobically in the presence of 1% nitrate (17). The purity of the cytochromes *c* was determined by amino acid analysis with reference to the known amino acid compositions (18).

**Radioactive Labeling by Reductive Methylation.** *Neurospora* apocytochrome *c* was treated in 150 mM potassium phosphate, pH 7.2, with [<sup>14</sup>C]formaldehyde (60 Ci/mmol; New England Nuclear; 1 Ci =  $3.7 \times 10^{10}$  Bq) and sodium cyanoborohydride (Serva, Heidelberg, Federal Republic of Germany) for 1 hr at 25°C (19), dialyzed enclosed in Visking 18/32 membranes (Union Carbide, Chicago, IL) against 250 mM NH<sub>4</sub>HCO<sub>3</sub>/0.01% 2-mercaptoethanol, and subjected to a renaturation cycle (14).

Abbreviations: Mops, 4-morpholinepropanesulfonate; BNPS-skatole, bromonitrophenylsulfenylskatole.

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**Isolation of the Amino-Terminal Bromonitrophenylsulfenylskatole (BNPS-skatole) Fragment.** Holocytochrome *c* was treated in 66% (vol/vol) acetic acid with BNPS-skatole (Pierce) for 48 hr at 25°C (20). The fragments were isolated by chromatography on Sephadex G-50 (1.6 × 80 cm, 10% acetic acid) and Whatman CM-32 CM-cellulose (0.9 × 40 cm, linear gradient from 10 to 500 mM ammonium acetate, pH 6.5) and identified by amino acid analysis.

## RESULTS

**Apocytochrome *c* Binding Sites on Mitochondria Display High Ligand Specificity.** We studied the specificity of the apocytochrome *c* binding sites on *Neurospora* mitochondria by employing various different proteins related to *Neurospora* apocytochrome *c*. Binding of chemically prepared apocytochromes *c* from *Neurospora crassa*, *Saccharomyces cerevisiae* (iso-1-cytochrome *c*), horse, parsnip, and *Paracoccus denitrificans* was compared. The relative binding affinities of these apocytochromes to the apocytochrome *c* binding sites on *Neurospora* mitochondria were determined by competitive displacement of bound biosynthetic *Neurospora* apocytochrome *c*. The following experimental approach was used: radioactively labeled *Neurospora* apocytochrome *c* synthesized in a cell-free translation system was bound to isolated *Neurospora* mitochondria that had been pretreated with deuterohemin. Mitochondria were reisolated and washed. Control experiments confirmed that the labeled apocytochrome *c* remained bound and was not converted. The deuterohemin-treated mitochondria with the labeled bound apocytochrome *c* were resuspended in postribosomal supernatants from *Neurospora* to which various amounts of un-

labeled apocytochromes *c* from different species had been added. Displacement of the labeled apocytochrome *c* by the unlabeled one was allowed to proceed until equilibrium between free and bound apocytochrome *c* was reached (15 min). The amount of labeled apocytochrome *c* that remained associated with the mitochondria was determined by immunoadsorption using antibodies against *Neurospora* apocytochrome *c*. Fig. 1 shows that the various apocytochromes *c* have different abilities to displace *Neurospora* apocytochrome *c* bound to *Neurospora* mitochondria, indicating that the various apocytochromes *c* have different affinities to the mitochondrial apocytochrome *c* binding sites. The following conclusions can be drawn: (i) The bacterial apocytochrome *c* (*Paracoccus*) does not compete with the eukaryotic apocytochrome *c* (*Neurospora*) for these binding sites, despite its extensive structural homology to mitochondrial cytochromes *c*. (ii) Binding sites on *Neurospora* mitochondria exhibit an affinity for heterologous apocytochromes *c* (e.g., from yeast, horse, and parsnip) 1/5th to 1/10th of that for the homologous one. Lower affinities, about 1/5th of homologous, were also determined for apocytochromes *c* from chicken, rat, and *Drosophila hydei* (data not shown).

The eukaryotic apocytochromes *c* employed are strongly positively charged proteins, with isoelectric points close to 10. In contrast, *Paracoccus* apocytochrome *c* is a slightly negatively charged protein with an isoelectric point of about 4.6 (21). Therefore it was necessary to rule out the possibility that the binding of eukaryotic apocytochromes *c* to mitochondria is simply due to unspecific electrostatic interaction with negatively charged membrane components. Preincubation of mitochondria with excess *Neurospora* holocytochrome *c* (10 nmol/ml), which has practically the same positive net charge as apocy-

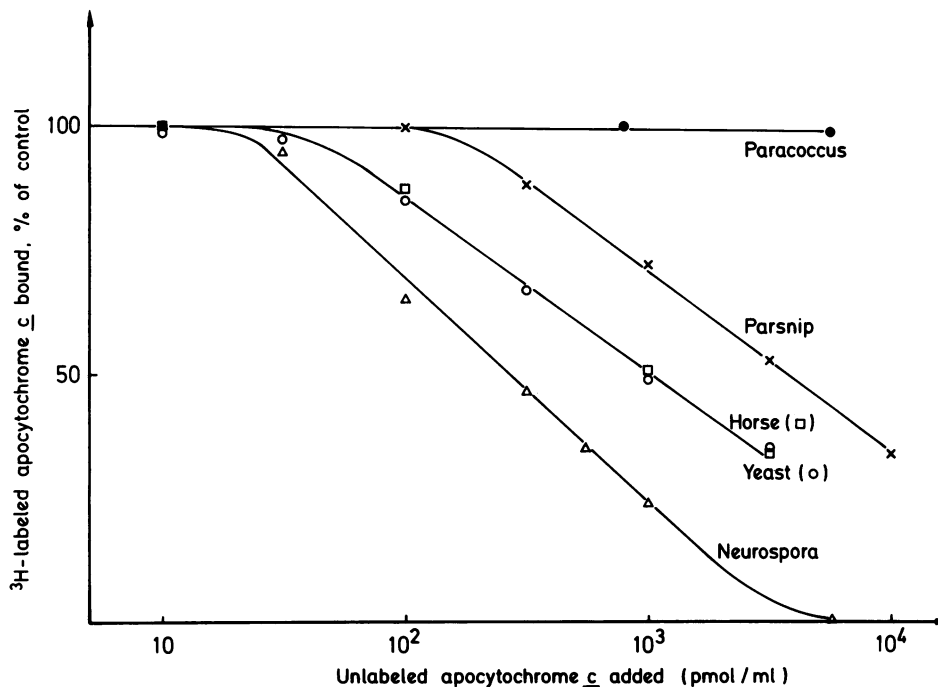


FIG. 1. Competitive displacement of labeled *Neurospora* apocytochrome *c* from *Neurospora* mitochondria by unlabeled apocytochromes *c* from various species. Mitochondria were isolated from a *Neurospora* homogenate and incubated (1 mg of mitochondrial protein per ml) for 5 min at 25°C with deuterohemin at 10 nmol/ml. Cell-free protein synthesis was carried out in a *Neurospora* postmitochondrial supernatant in the presence of 0.33 mCi of L-[<sup>3</sup>H]leucine (50 Ci/mmol) for 10 min at 25°C and terminated by cycloheximide. The postribosomal supernatant containing newly synthesized [<sup>3</sup>H]apocytochrome *c* was prepared and mixed with the preincubated mitochondria. After incubation for 15 min at 25°C mitochondria were reisolated and washed twice with sucrose/Mops buffer. The deuterohemin-treated mitochondria containing bound [<sup>3</sup>H]apocytochrome *c* were resuspended in unlabeled postribosomal supernatant and separated into 1-ml aliquots. Different amounts of unlabeled apocytochrome *c* from the various species were added from calibrated stock solutions as indicated. After equilibration of free and bound apocytochrome *c* (15 min at 25°C) mitochondria were reisolated, washed twice with sucrose/Mops buffer, and lysed with 1% Triton X-100. Apocytochrome *c* was collected from the lysates with specific antibodies against apocytochrome *c* by immunoadsorption onto staphylococcal protein A-agarose. The adsorbed immunocomplex was dissociated with 2% sodium dodecyl sulfate at 95°C and subjected to electrophoresis on 15% polyacrylamide slab gels. Radioactivity was determined in the gel slices by scintillation counting.

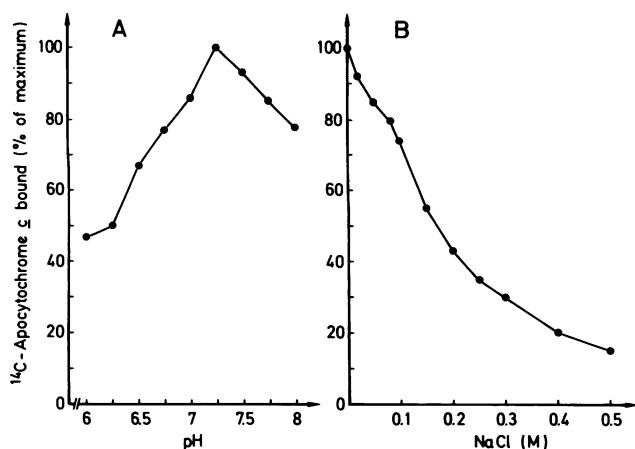


FIG. 2. Dependence of apocytochrome *c* binding to mitochondria on pH (A) and ionic strength (B). Mitochondria were prepared from *Neurospora* hyphae homogenized in sucrose/Mops buffer and purified by isopycnic centrifugation for 30 min at  $100,000 \times g$  (at the average radius) on a Percoll gradient (14). They were treated with deuterohemin (50 nmol/ml) in sucrose/Mops buffer for 5 min at 25°C and reisolated. Then 60 pmol of *Neurospora* <sup>14</sup>C-labeled apocytochrome *c* prepared by reductive methylation was added from a calibrated stock solution to samples of 250  $\mu$ l of sucrose/Mops buffer that had been adjusted to the pH values (at 25°C) or NaCl concentrations indicated in the figure. Mitochondria (0.20 mg of protein) were resuspended in this mixture. After 15-min incubation at 25°C mitochondria were reisolated by centrifugation in an Airfuge (Beckman) for 5 min at  $100,000 \times g$ . Mitochondria were washed with a medium identical to the respective incubation mixture, transferred into new tubes, and sedimented by centrifugation. The pellets were lysed with 1% Triton X-100 and radioactivity was determined by scintillation counting.

tochrome *c*, did not prevent the subsequent binding of *Neurospora* apocytochrome *c*. Also, the amino-terminal fragment of cytochrome *c* (residues 1–63) obtained by cleavage of *Neurospora* cytochrome *c* at the single tryptophan residue with BNPS-skatole, which additionally removes the heme group from the protein, did not interfere with binding of *Neurospora* apocytochrome *c* to mitochondria, although it is identical with the amino-terminal half of apocytochrome *c* and has the same net positive charge as the whole protein. In addition, polylysine (average chain length about 60 residues) did not interfere with binding of *Neurospora* apocytochrome *c* to mitochondria when it was employed up to a concentration of 1 nmol/ml. Obviously the apocytochrome *c* binding sites on the mitochondrial surface recognize the precursor of mitochondrial cytochrome *c* with high specificity.

**Affinity and Number of Mitochondrial Binding Sites for Apocytochrome *c*.** The parameters of apocytochrome *c* binding to mitochondria were determined by employing apocytochrome *c* that had been labeled by reductive methylation. This approach had several important advantages over the assay using radioactively labeled apocytochrome *c* synthesized in cell-free systems. First, the specific radioactivity of [<sup>14</sup>C]methylated apocytochrome *c* can be precisely determined. This was on the average 0.9 Ci/mmol of protein in various preparations. Second, optimal conditions for binding could be explored, because binding could be tested in defined buffers.

Apocytochrome *c* that had been prepared from holocytochrome *c* and then labeled by reductive methylation displayed the same behavior as the biosynthetic apocytochrome *c* with respect to binding to mitochondria. This was suggested by the following observations (data not shown): Methylated apocytochrome *c* was bound to mitochondria in the presence of deuterohemin. It displaced bound biosynthetic apocytochrome *c* with the same ability as unmethylated apocytochrome *c* and reacted with antibodies against unmethylated apocytochrome *c*.

Remarkably, when holocytochrome *c* was first subjected to reductive methylation and then the heme group was cleaved off, the resulting apocytochrome *c* had a strongly reduced affinity for the mitochondrial binding sites.

First, we determined the optimal conditions for apocytochrome *c* binding, in particular the dependence on pH and on ionic strength. Optimal binding of apocytochrome *c* with respect to pH (at 25°C) was observed at pH 7.2 (Fig. 2A). Furthermore, binding of apocytochrome *c* was optimal in isotonic sucrose/Mops buffer without added sodium chloride (Fig. 2B). Binding continuously declined with increasing ionic strength, with 50% efficiency at 0.17 M NaCl. At the highest NaCl concentration tested (0.5 M) a residual binding of about 15% was observed. When mitochondria were suspended in the high-ionic strength buffer, then washed and resuspended in the buffer without added NaCl, only the low binding capacity found with the high-ionic strength buffer was left. This result indicates that irreversible destruction of the receptor sites had occurred. Divalent cations such as Mg<sup>2+</sup> or Ca<sup>2+</sup> did not enhance the binding of apocytochrome *c* when added at concentrations up to 10 mM Mg<sup>2+</sup> and 2 mM Ca<sup>2+</sup>.

Under the conditions for optimal binding (0.3 M sucrose/10 mM Mops, pH 7.20, 15 min at 25°C) saturable binding was observed (Fig. 3). In order to calculate the association constant and the number of binding sites, a Scatchard plot was constructed from the titration data. This revealed a biphasic curve indicating a high-affinity binding site and also low-affinity binding (Fig. 3). The binding parameters were calculated after correcting for the biphasic curvature (22). The high-affinity binding sites of *Neurospora* mitochondria had an association constant,  $K_a$ , of  $2.2 \times 10^7 \text{ M}^{-1}$  for *Neurospora* apocytochrome *c*. Their number was 90 pmol per mg of mitochondrial protein. The low-affinity binding displayed an association constant of  $4 \times 10^6 \text{ M}^{-1}$  and the corresponding capacity was 230 pmol per mg of mitochondrial protein.

Pretreatment of mitochondria with low concentrations of trypsin (40  $\mu$ g/ml, 20 min at 25°C, then of soybean trypsin inhibitor at 120  $\mu$ g/ml) led to complete loss of the high-affinity binding sites. This may indicate that the specific binding is mediated by a protein on the mitochondrial surface. It has been reported that the uptake of apocytochrome *c* by rat liver mitochondria shows a similar sensitivity to protease treatment (6).

**The Binding Sites Are Involved in Translocation of Apocytochrome *c* into Mitochondria.** We had previously demonstrated that bound apocytochrome *c* is converted to holocytochrome *c* and translocated into the intermembrane space of mitochondria when inhibition by deuterohemin is relieved (5). Now we investigated whether apocytochrome *c* is transferred into mitochondria directly via the occupied binding sites. The following experimental approach was used to decide whether or not apocytochrome *c* has to dissociate from these binding sites in order to become translocated into the mitochondria: Mitochondria were treated with deuterohemin and then radioactively labeled apocytochrome *c* was bound to them. They were then mixed with an equal amount of mitochondria that had not been treated with deuterohemin and thus should actively take up and convert apocytochrome *c*. The combined mitochondria were incubated in a postribosomal supernatant and it was determined whether holocytochrome *c* was formed during this incubation at expense of the bound labeled apocytochrome *c*. This was not the case, as shown in Table 1. A control experiment demonstrated that the mitochondria in the mixture that had not been treated with deuterohemin actually had retained their ability to import apocytochrome *c* and to convert it to holocytochrome *c*. Another control experiment demonstrated that the deuterohemin-treated mitochondria in the mixture recovered the ability to import and convert their bound labeled

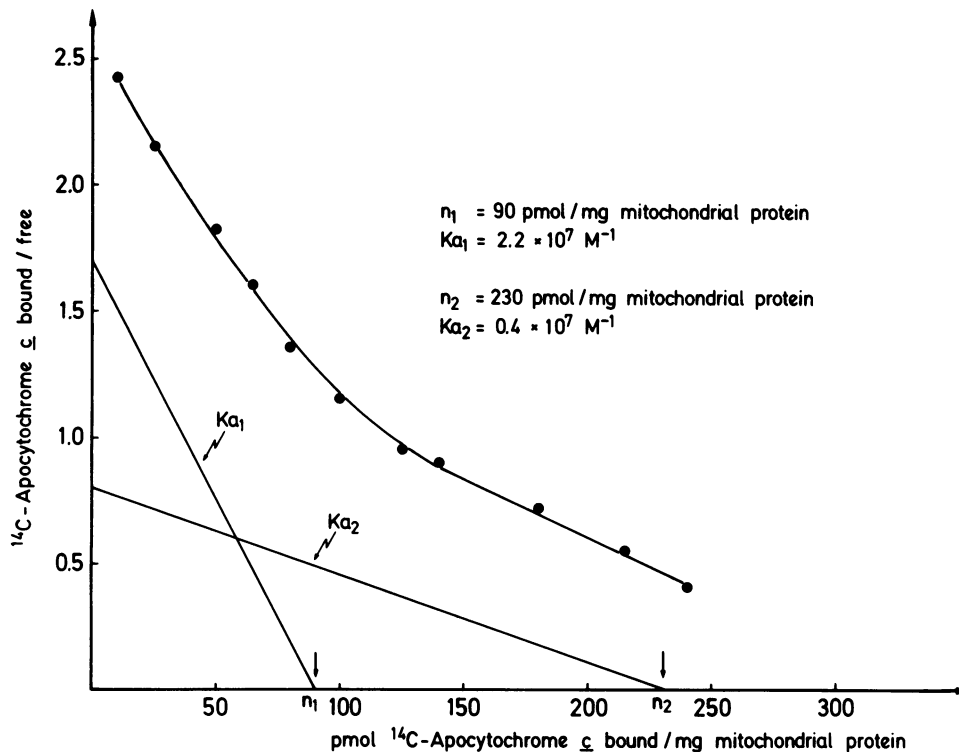


FIG. 3. Titration of apocytochrome *c* binding sites on mitochondria (Scatchard plot). Mitochondria were isolated and purified and the binding assay was performed as described for Fig. 2, employing  $^{14}\text{C}$ -labeled apocytochrome *c* that had been prepared by reductive methylation. After incubation for 15 min at  $25^\circ\text{C}$  the mixture was centrifuged and the distribution of free and bound apocytochrome *c* was determined by measuring radioactivity. Free apocytochrome *c* was determined in aliquots of the supernatants. Bound apocytochrome *c* was determined after washing the mitochondria, transferring them into new vials, and lysing them with 1% Triton X-100. The association constants ( $K_{a1}$  and  $K_{a2}$ ) and the corresponding numbers of binding sites were calculated from the biphasic curvature of the Scatchard plot by graphic parameter fitting (22).  $K_a$  values were calculated with respect to the actual mitochondrial protein concentration in the assay determined with the Folin-Ciocalteu phenol reagent with serum albumin as a standard. Specific radioactivity of the labeled apocytochrome *c* was determined by scintillation counting and amino acid analysis.

apocytochrome *c* when the inhibition was relieved by protohemin (Table 1).

These data lead us to conclude that apocytochrome *c* bound to mitochondria in the presence of deuterohemin does not detach from the binding sites before it is translocated into mitochondria and converted to holoapocytochrome *c*. Translocation appears rather to occur via the same sites to which apocytochrome *c* has been bound.

## DISCUSSION

The results demonstrate that binding sites on *Neurospora* mitochondria were recognized by apocytochromes *c* from various different eukaryotic species covering a wide range of sequence variation. On the other hand, holoapocytochrome *c* from *Neurospora* did not displace bound apocytochrome *c*. This underlines our earlier conclusion that the mature form of cytochrome *c* is not recognized by the mitochondrial transfer sites. It appears that holoapocytochrome *c* has a molecular conformation quite different from that of apocytochrome *c* (23), so that the structural part by which the precursor binds to the specific sites on the mitochondrial surface is not accessible on the surface of the mature protein. It should be mentioned in this context that the evolutionarily invariant sequence (residues 70–80 according to standardized nomenclature), which has been suggested to address apocytochrome *c* to mitochondria (6), does not expose the side chains of its amino acid residues at the surface of holoapocytochrome *c* as revealed by x-ray analysis (24).

It is remarkable that apocytochrome *c* from the bacterial species *Paracoccus denitrificans* did not compete with *Neurospora* apocytochrome *c* for binding. *Paracoccus* cytochrome *c* shares an evolutionary ancestry with eukaryotic cytochromes *c* and ex-

hibits strong structural and functional homology (25), but apparently it lacks structural features required for recognition by mitochondrial transfer sites. The invariant sequence of eukaryotic cytochromes *c* that has been suggested to address apocytochrome *c* to mitochondria is not present in *Paracoccus* cytochrome *c* (21, 26).

The different eukaryotic apocytochromes *c* bound with different affinities to the mitochondrial surface. The highest affinity to binding sites on *Neurospora* mitochondria among the diverse apocytochromes *c* was exhibited by *Neurospora* apocytochrome *c*. Similarly, rat liver mitochondria bound rat apocytochrome *c* with higher affinity than *Neurospora* apocytochrome *c* (unpublished results). This might reflect optimal adaptation of homologous apocytochromes *c* to mitochondrial binding sites.

Previously we reported results demonstrating that apocytochrome *c* uses a transfer pathway in mitochondria that is apparently different from the pathways used by the precursors of the ATP/ADP carrier and subunit 9 of mitochondrial ATPase (27). Also, cytochrome *c*<sub>1</sub> as well as porin, an abundant protein of the mitochondrial outer membrane, do not share a common transfer pathway with cytochrome *c* (ref. 28 and unpublished results). In conclusion, these and the present data demonstrate that binding of apocytochrome *c* to mitochondria is selective and highly specific.

Titration of these binding sites revealed that there are a limited number of high-affinity binding sites. Their intrinsic association constant is comparable to the constants determined for various plasma membrane receptors for polypeptide hormones (29, 30). In addition, low-affinity binding was detected. Biphasic Scatchard plots are commonly observed in titrating

Table 1. Translocation of apocytochrome *c* into mitochondria and conversion to holoapocytochrome *c* via binding sites on mitochondria

	Incubation mixture	Holoapocytochrome <i>c</i> formed, cpm
1.	Mitochondria pretreated with deuterohemin and containing bound apocytochrome <i>c</i> + unlabeled supernatant	0
2.	Mitochondria pretreated with deuterohemin and containing bound apocytochrome <i>c</i> + mitochondria not pretreated with deuterohemin + unlabeled supernatant	0
3.	Same as 1 + protohemin	601
4.	Same as 2 + protohemin	383
5.	Mitochondria not pretreated with deuterohemin + supernatant containing apocytochrome <i>c</i>	1,629
6.	Mitochondria pretreated with deuterohemin and containing bound apocytochrome <i>c</i> + mitochondria not pretreated with deuterohemin + supernatant containing apocytochrome <i>c</i>	1,595

Mitochondria were isolated and pretreated with deuterohemin (10 nmol/ml) and [<sup>3</sup>H]apocytochrome *c* synthesized in a *Neurospora* cell-free system was bound to them as described for Fig. 1. The deuterohemin-treated mitochondria containing [<sup>3</sup>H]apocytochrome *c* were reisolated by centrifugation and aliquots (1 mg of mitochondrial protein) were resuspended in 1-ml samples of unlabeled postribosomal supernatant prepared with sucrose/Mops buffer. Mitochondria not pretreated with deuterohemin and protohemin (10 μM) were added as indicated. After incubation for 15 min at 25°C, mitochondria were reisolated by centrifugation (12,000 × *g* for 12 min at 4°C) and lysed with 1% Triton X-100, and apocytochrome *c* and holoapocytochrome *c* were determined by employing specific antibodies against these two forms of cytochrome *c*. The dissociated immunocomplexes were separated by polyacrylamide gel electrophoresis (15% acrylamide/0.1% sodium dodecyl sulfate) and radioactivity was determined by subsequent scintillation counting of gel slices. Deuterohemin at 10 μM inhibits formation of holoapocytochrome *c* with a delay but allows subsequent reconversion by protohemin more efficiently than higher concentrations. The amount of holoapocytochrome *c* initially formed in the control reaction (line 1) was therefore subtracted as a background in all reactions (628 cpm). To compare the values in reactions 1–4 with those in lines 5 and 6 it is important to note that practically all apocytochrome *c* is converted to holoapocytochrome *c* in the absence of deuterohemin and that only about 40% of the total apocytochrome *c* present in the postribosomal supernatant binds to mitochondria treated with deuterohemin.

cellular receptors with their appropriate ligands (29, 30). In general, the nature of the low-affinity binding sites has remained obscure. With respect to mitochondria, it can be expected that the high-affinity sites are the ones that are relevant to the transfer because the cytosolic pool of apocytochrome *c* appears to be far below the concentration required to occupy the low-affinity binding sites (31). On the basis of the average size of *Neurospora* mitochondria and their content in the cells (32, 33) one can calculate that some 600 high-affinity binding sites are present per μm<sup>2</sup> of mitochondrial surface.

The mitochondrial binding sites may not only selectively pick up apocytochrome *c* from the cytosolic pool of precursors but also arrange apocytochrome *c* properly at the mitochondrial outer membrane to facilitate the next step of transfer. Our results demonstrate that apocytochrome *c* remains firmly bound to mitochondria as long as inhibition of heme attachment is maintained. When this inhibition is relieved, apocytochrome *c* is converted to holoapocytochrome *c* and translocated into mitochondria by a process that apparently does not depend on a transient dissociation of apocytochrome *c* off the outer membrane. We suggest that apocytochrome *c* is translocated across the outer membrane directly from its binding sites.

Are these binding sites receptors? Distinction of a receptor

from nonreceptor binding requires the presence of a "biologically responsive system in which context such (binding) data can be placed" (34). Apocytochrome *c* binding to mitochondria meets this prerequisite. We conclude therefore that the functional characteristics of the apocytochrome *c* binding sites support our previous suggestion (5) that these sites represent mitochondrial receptors involved in the posttranslational transfer of apocytochrome *c* into mitochondria.

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