Mitochondria 1983

Nucleo-Mitochondrial Interactions

Proceedings of a Conference held in
Schliersee, Germany, July 19-23, 1983

Editors
R. J. Schweyen · K. Wolf · F. Kaudewitz

Walter de Gruyter · Berlin · New York 1983

CONTENTS

REVIEWS

REPLICATION AND TRANSCRIPTION OF mtDNA

Nuclear Control of Mitochondrial Functions

Import of Proteins into Mitochondria

TRANSPORT OF CYTOCHROME C INTO MITOCHONDRIA: INVOLVEMENT OF SPECIFIC RECEPTORS AND OF CYTOCHROME C HEME LYASE.

Bernd Hennig, Helmut Köhler and Walter Neupert
Institut für Biochemie, Universität Göttingen

The individual steps in the transport of mitochondrial proteins to their final submitochondrial destinations may differ for the various proteins (c.f. ref.1). Cytochrome c is assembled in the intermembrane space of mitochondria. It is coded by a nuclear gene, translated as an extramitochondrial precursor, apocytochrome c, on free cytoplasmic ribosomes and post-translationally transported into mitochondria. Its transport pathway is distinct from pathways used by other extramitochondrial precursors (2) and has several remarkable features: 1) The precursor becomes covalently linked to protohemin; 2) It is not proteolytically processed; 3) Its transport into mitochondria does not depend on the energized state of the inner membrane.

With aid of specific antibodies that discriminate between apo- and holocytochrome c we are investigating the details of transport and assembly of cytochrome c in cell-free, reconstituted systems prepared from Neurospora hyphae (2-7).

Previously we reported that in the cell-free system apocytochrome c is rapidly transported into mitochondria and no intermediate accumulation of apocytochrome c in mitochondria is observed (5). However, with the heme analogue deuterohemin reversible inhibition of transport of apocytochrome c has been achieved. Two steps have thus been resolved: 1) Specific binding of apocytochrome c to the mitochondrial surface. 2) Translocation of apocytochrome c across the outer membrane and attachment of heme. We have determined some characteristics of these steps. The results strongly support our suggestion that specific apocytochrome c receptors and a special converting enzyme are involved in the assembly of cytochrome c.

Mitochondria 1983

Apocytochrome c is recognized by specific receptors at the mitochondrial surface.

Apocytochrome c obtained by translation in a cell-free system from Neurospora hyphae firmly binds to mitochondria in the presence of deuterohemin. It is not removed from mitochondria by washing with isotonic buffer. By a series of experiments we have demonstrated that this binding is specific, binding sites are located at the surface of mitochondria, and binding is related to transport of apocytochrome c into mitochondria and conversion to holocytochrome c (5, 6).

First, binding is specific: Bound labelled apocytochrome c is displaced from Neurospora mitochondria by increasing amounts of unlabelled Neurospora apocytochrome c, but not by holocytochrome c. Apocytochromes c from different eucaryotic species displace bound Neurospora apocytochrome c with different efficiency (ref.6 and Tab.I). This efficiency is not correlated to the net charge of the various apocytochromes. Rather it seems to be related to an adressing signal (8) which might recognize the receptor. This assumption is supported by the finding that apocytochrome c from the procaryotic species Paracoccus denitrificans does not displace bound Neurospora apocytochrome c from Neurospora mitochondria despite the close homology of Paracoccus cytochrome c with eucaryotic cytochromes c. It is remarkable that a hydrophobic sequence which may form the addressing signal and which is invariably present in the C-terminal half of all eucaryotic cytochromes c, is lacking in Paracoccus cytochrome c .

Second, apocytochrome c is bound at the surface of mitochondria: When mitochondria with bound apocytochrome c were gently treated with trypsin, the apocytochrome c associated with mitochondria was degraded whereas endogenous holocytochrome c contained in the intermembrane space remained unaffected (5).

Third, binding is related to transfer of apocytochrome c into mitochondria: A mixture of deuterohemin-treated mitochondria containing bound apocytochrome c and of mitochondria not pretreated with deuterohemin was prepared and incubated under conditions that were appropriate for uptake and concersion of apocytochrome c. It was observed that apocytochrome c was not detached from the deuterohemin-treated mitochondria and was not transferred to the non-inhibited mitochondria where it would have been

converted to holocytochrome c. However, when protohemin had been added to the mixture, conversion of apocytochrome c was taking place. Control experiments showed that the non-inhibited mitochondria retained their activity for transport and conversion of apocytochrome c in the mixture: when freely dissolved, labelled apocytochrome c was added to the mixture, the added apocytochrome c was converted in contrast to the bound apocytochrome c (6).

Tab. I. Specificity of apocytochrome c binding to mitochondria.

Unlabelled protein used for displacement of bound labelled apocytochrome c		Relative affinity (% displacement of labelled apocytochrome c)
Species	Net charge at pH 7	
Neurospora apocytochrome c	+4	100
Horse apocytochrome c	+9	70
Rat apocytochrome c	+9	70
Yeast iso-1-apocytochrome c	+8	70
Parsnip apocytochrome c	+7	50
Paracoccus denitrificans apocytochrome c	-4	0
N-terminal fragment (Pos.-5/+59) of Neurospora apocytochrome c	+4	0
Neurospora holocytochrome c	+7	0
Polylysine (1 nmol/ml) (average chain length 60 residues)	+60	0

Neurospora ^{3}H-apocytochrome c was synthesized by cell-free translation and bound to deuterohemin-treated Neurospora mitochondria. Samples were mixed with 10 - 10^{4} pmoles unlabelled apocytochrome c/ml and incubated 15 min at 25°C for equilibration between free and bound apocytochrome c. Mitochondria were reisolated by centrifugation, lysed with Triton X-100 and the ^{3}H-apocytochrome c was adsorbed onto Protein A-agarose using specific antibodies. The immunoprecipitate was subjected to electrophoresis by SDS-PAGE and radioactivity determined in gel slices. The displacement measured at 10 nmol unlabelled protein/ml is shown (c.f. also ref. 6).

In order to determine precisely the binding parameters it is necessary to have sufficiently large amounts of apocytochrome c with known specific radioactivity. Reductive methylation of apocytochrome c by ^{14}C-formaldehyde and cyanoborohydride results in modification of all but a few lysine residues by mono- and dimethylation. This was found to be a convenient method as methylation does not distort binding (6, 7).

Mitochondrial binding sites were titrated employing this apocytochrome c until complete saturation was obtained. Free and bound apocytochrome c was determined after equilibration. The results were transformed into a Scatchard plot in order to calculate binding parameters. A biphasic curvature is observed demonstrating the presence of two kinds of binding sites (ref.6 and Fig. 1): some 90 pmol high-affinity binding sites per mg mitochondrial protein with an association constant of $K_a = 2.2 \times 10^7$ M^{-1} and a larger number of low-affinity binding sites with $K_a = 4 \times 10^6$ M^{-1}. It is the high-affinity binding sites that represent the specific binding sites involved in transport of apocytochrome c into mitochondria. The low-affinity binding sites presumably represent unspecific binding.

The assay for the presence of the high-affinity binding sites is a suitable experimental approach for defining the binding and for isolation of the corresponding receptor. The interaction between apocytochrome c and its mitochondrial receptor appears to be essentially hydrophobic. The presence of very low amounts of non-ionic detergents (0.01 % Triton X-100), that did not solubilize the mitochondrial membranes, significantly reduced binding of apocytochrome c to mitochondria, but binding was recovered after removal of the detergent from the mitochondrial suspension. In contrast, neither the polycationic peptide polylysine (1 nmol/ml) nor low concentrations of the divalent cations Mg^{2+} and Ca^{2+} (1-5 mmol/l) did interfere with apocytochrome c binding. Gentle treatment of mitochondria with trypsin completely abolished the high-affinity binding (Fig.1). This indicates that the receptor is a protein.

Extraction of the receptor from mitochondria has been achieved using the detergent octylglucoside (0.8-1.7 %). When the extracted material was inserted into liposomes prepared from asolectin, the specific high-affinity binding sites were recovered. High-affinity binding of apocytochrome c is not an intrinsic property of the liposomes themselves. Differential extraction of mitochondria and purification of the apocytochrome c recep-

tor by column chromatography is now under study and so far has succeeded up to a 45-fold enrichement.

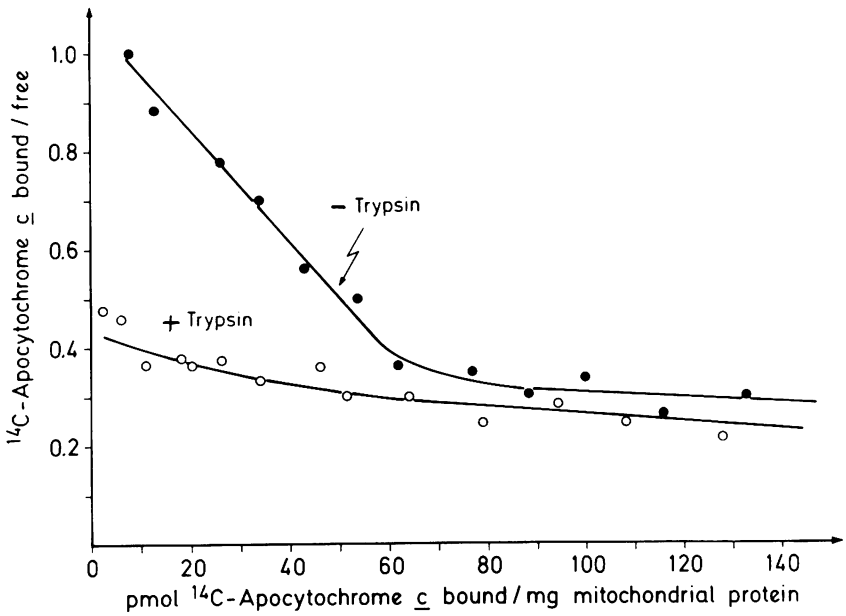


Fig. 1. Apocytochrome c binding to mitochondria (Scatchard plot) and protease sensitivity of the receptor.

Neurospora mitochondria were isolated and resuspended in isotonic buffer (sucrose/MOPS pH 7.5) containing 30 nmol deuterohemin/ml. Samples were titrated with increasing amounts of Neurospora ^{14}C-methylated apocytochrome c (0.9 Ci/mmol protein). After 15 min at 25°C mitochondria were spun down. Free and bound apocytochrome c was determined by scintillation counting of radioactivity in the supernatant and the mitochondria, respectively. Trypsin sensitivity of binding sites was investigated by treatment of mitochondria with 40 ug trypsin/ml x mg mitochondrial protein for 15 min at 25°C. After addition of soybean trypsin inhibitor mitochondria were titrated with apocytochrome c as above.

Heme attachment to apocytochrome c is mediated by an enzyme.

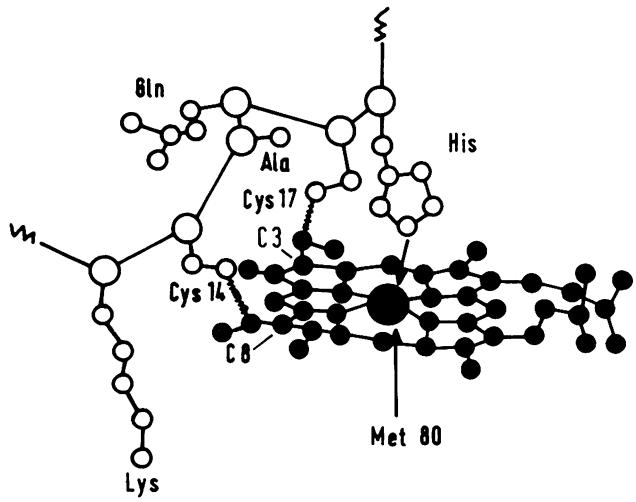
The conversion of apo- to holocytochrome c, i.e. the covalent attachment of protohemin to apocytochrome c, does not proceed spontaneously. This reaction rather requires certain conditions which strongly suggest that it is catalyzed by an enzyme. This enzyme must be either contained as a soluble protein in the intermembrane space or located at the inner side of the outer membrane (5, 9).

First, the insertion of protohemin in cytochrome c is theoretically possible in two alternative ways, but actually takes place stereospecifically (Fig. 2): the vinyl group at carbon atom C 3 of protohemin is only attached to the cysteine at position 17 of cytochrome c and the vinyl group at carbon atom C 8 to the cysteine at position 14. Only this particular stereospecific insertion leads to the native, functional conformation of holocytochrome c (10).

Second, certain heme analogues, which completely lack vinyl groups and therefore cannot be linked to apocytochrome c, compete with the physiological substrate, protohemin, and inhibit conversion. In addition, there is a remarkable structural specificity determining whether a heme derivative competes with protohemin or does not interfere with conversion (Fig. 2): Deuterohemin and mesohemin efficiently compete with protohemin. In contrast, hematohemin, which has two hydroxyethyl sidechains, does not compete at all with protohemin and does not interfere with conversion. A similar but distinct competition of porphyrin substrates is known with the ferrochelatase (11). It seems as if the hematohemin would not fit into the substrate binding site of the putative converting enzyme.

Fig. 2. Stereospecificity and substrate specificity for covalent attachment of the heme group to apocytochrome c.

^{3}H-apocytochrome c was synthesized in a cell-free system prepared from Neurospora hyphae. Mitochondria were preincubated with the various heme derivatives (10 nmol/ml) for 5 min at 25°C and then mixed with the postribosomal supernatant containing the ^{3}H-apocytochrome c. ^{3}H-holocytochrome c formed after 30 min at 25°C was immunoprecipitated and radioactivity determined as described in Tab. I.



Heme derivative added	Substitution at C 3	Substitution at C 8	Holocytochrome c formed (%)
None (= control)			100
Protohemin	$-CH=CH_2$	$-CH=CH_2$	100
Deuterohemin	$-H$	$-H$	20
Mesohemin	$-CH_2-CH_3$	$-CH_2-CH_3$	19
Hematohemin	$-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_3$	$-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_3$	95
Pemptohemin	$-H$	$-CH=CH_2$	20
Isopemptohemin	$-CH=CH_2$	$-H$	20
Deuterohemin + Protohemin			86
Deuterohemin + Protoporphyrin IX			18

Third, the simultaneous presence of two vinyl groups at the heme substrate is required for linkage to apocytochrome c . The two heme derivatives, pemptohemin and isopemptohemin, have only a single vinyl group at the carbon atoms C 8 and C 3, respectively. Theoretically, covalent attachment of pemptohemin to apocytochrome c via a thioether linkage between the single vinyl group at C 8 and the cysteine 14 would appear possible, as well as of isopemptohemin via the vinyl group at C 3 and the cysteine 17. In fact, neither pemptohemin nor isopemptohemin are used as substrates for the attachment to apocytochrome c but rather inhibit conversion like deuterohemin (Fig. 2).

Furthermore, the presence of two vinyl groups in the porphyrin system is a necessary requirement, but in addition also the iron must be present in the porphyrin molecule: protoporphyrin IX is not used as a substrate (Fig. 2) although it is otherwise identical to protohemin and artificially can be introduced in cytochrome c (12).

Tab. II. Requirement of a cytosolic factor for conversion.

Neurospora mitochondria were incubated with deuterohemin (10 nmol/ml) and mixed with a postribosomal supernatant containing ^{3}H-apocytochrome c. After incubation for 15 min at 25°C mitochondria were isolated by centrifugation and resuspended in the different media as indicated. Formation of ^{3}H-holocytochrome c was determined as described in Fig. 3. The amount is given as counts/ min x ml assay x mg mitochondrial protein:

	Treatment of mitochondria	Holocytochrome c formed
1.	Resuspension in unlabelled postribosomal cytosol	
	+ protohemin (10 nmol/ml)	3,230
2.	Resuspension in isotonic buffer (sucrose/MOPS, pH 7.5)	
	+ protohemin (10 nmol/ml)	0
3.	Resuspension in isotonic buffer (sucrose/MOPS, pH 7.5)	
	+ protohemin (10 nmol/ml)	
	+ cytosolic extract	2,045

Finally, a cytosolic cofactor is required to promote conversion. When deuterohemin-treated mitochondria associated with bound apocytochrome c were resuspended in unlabelled cytosol with addition of protohemin, conversion was resumed. However, when they were resuspended in defined isotonic buffer and excess protohemin was added, no conversion took place. Conversion was then resumed only after addition of a deproteinated extract prepared from the extramitochondrial cytosol (Tab. II). A similar finding was previously reported by others (9). We assume that the cytosolic extract contains a cofactor which is required by the putative enzyme to activate either the vinyl groups of the protohemin or the cysteine SH-groups of apocytochrome c in order to initiate the subsequent covalent coupling.

The putative enzym has to be named cytochrome c heme lyase according to the recommendations given by the IUPAC/IUB Committee on Enzyme Nomenclature. Experiments to locate the enzyme in submitochondrial fractions or even to isolate it, have failed as yet. The converting activity has been lost whenever the intact structure of mitochondria was disrupted by sonication, osmotic swelling - shrinking or lysis with non-ionic detergents.

Concluding remarks: Transfer of apocytochrome c across the outer membrane of mitochondria.

Protohemin is covalently attached to apocytochrome c within mitochondria. The reaction does obviously not take place at the mitochondrial surface. Otherwise holocytochrome c would be formed at the cytoplasmic side of the outer membrane and holocytochrome c but not apocytochrome c would be translocated across the outer membrane. This is clearly not the case. The findings unequivocally demonstrate that apocytochrome c and not holocytochrome c competes for binding and transport (2, 5, 6).

On the other hand, covalent attachment of protohemin to apocytochrome c and translocation of the protein across the outer membrane must be intimately coupled events. When conversion of apo- to holocytochrome c is prevented by deuterohemin, apocytochrome c does not accumulate in the intermembrane space but remains firmly bound to the receptor sites (5). Transport into mitochondria only proceeds when conversion is resuming.

Considering these two aspects we have arrived at the following hypothesis for the assembly of cytochrome c: Binding of apocytochrome c to its receptor may arrange the precursor at the outer membrane in such away that it becomes competent for transfer across the membrane. That part of apocytochrome c, which carries the heme binding region, might then protrude through the outer membrane, perhaps via a pore close to the receptor or via a channel formed by the receptor itself. Thus the cysteine SH-groups of the heme-binding region might become exposed at the inner side of the outer membrane where they would be accessible to the converting enzyme. Attachment of protohemin by cytochrome c heme lyase would force the polypeptide to refold around the heme group. This refolding is a spontaneous process (13, 14). It may pull the polypeptide chain of cytochrome c completely across the outer membrane. It is known that refolding of a polypeptide around the heme group proceeds with a half-time of considerable length (14, 15). Refolding of the polypeptide chain after heme attachment and concomitant completion of its transfer across the outer membrane may therefore be the rate limiting step in the assembly process.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (grant He 875/4). We are grateful to Leopoldine Sander for skillful technical assistance.

REFERENCES

1. Hennig, B. and Neupert, W. (1983): Assembly of mitochondrial proteins. In: Horizons in Biochemistry and Biophysics, vol.7 , (eds. E. Quagliariello, F. Palmieri and T.P.Singer), J. Wiley / Chichester, New York (in press)

2. Zimmermann, R., Hennig, B., and Neupert, W. (1981): Different transport pathways of individual precursor proteins in mitochondria. Eur. J. Biochem. 116, 455-460

3. Korb, H. and Neupert, W. (1978): Biogenesis of cytochrome c in Neurospora crassa. Synthesis of apocytochrome c, transfer to mitochondria,

and conversion to holocytochrome c. Eur. J. Biochem. 91, 609-620

4. Zimmermann, R., Paluch, U., and Neupert, W. (1979): Cell-free synthesis of cytochrome c. FEBS Letters 108, 141-146

5. Hennig, B. and Neupert, W. (1981): Assembly of cytochrome c. Apocytochrome c is bound to specific sites on mitochondria before its conversion to holocytochrome c. Eur. J. Biochem. 121, 203-212

6. Hennig, B., Köhler, H., and Neupert, W. (1983) : Receptor sites involved in posttranslational transport of apocytochrome c into mitochondria: Specificity, affinity and number of sites. Proc. Natl. Acad. Sci. USA 80, 4963-4967

7. Hennig, B. and Neupert, W. (1983) : Biogenesis of cytochrome c in Neurospora. Methods in Enzymology 97 (in press)

8. Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D.D., and Morimoto, T. (1981): In vitro synthesis and posttranslational uptake of cytochrome c into isolated mitochondria: role of a specific addressing signal in the apocytochrome c. Proc. Natl. Acad. Sci. USA 78, 4368-4372

9. Basile, G., DiBello, C. and Taniuchi, H. (1980): Formation of an iso-1-cytochrome c-like species containing a covalently bonded heme group from the apoprotein by a yeast cell-free system in the presence of hemin. J. Biol. Chem. 255, 7181-7191

10. Takano, T. and Dickerson, R.E. (1981): Conformation change of cytochrome c. II. Ferricytochrome c refinement at 1.8 A and comparison with the ferrocytochrome c structure. J. Mol. Biol. 153, 95-115

11. Porra, R.J. and Jones, O.T. (1963): Studies on ferrochelatase. 2. An investigation of the role of ferrochelatase in the biosynthesis of various haem prosthetic groups. Biochem. J. 87, 186-192

12. Brems, D.N., Liu, Y.C. and Stellwagen, E. (1982): The conformational transition of horse heart porphyrin c. J. Biol. Chem. 257, 3864-3868

13. Stellwagen, E. (1968): The reversible unfolding of horse heart ferricytochrome c. Biochem. 7, 2893-2898

14. Leutzinger, Y. and Beychok, S. (1981): Kinetics and mechanism of heme induced refolding of human a-globin. Proc. Natl. Acad. Sci. USA 78, 780-784

15. Ridge, J.A., Baldwin, R.L., and Labhardt, A.M. (1981): Kinetics of the fast and slow refolding reactions of iron(III) cytochrome c. Biochem. 20, 1622-1630