ACHIEVEMENTS AND PERSPECTIVES OF MITOCHONDRIAL RESEARCH VOLUME II: BIOGENESIS

Proceedings of the International Symposium on Achievements and Perspectives in Mitochondrial Research held in Rosa Marina (Italy) on September 2-6, 1985.

Edited by
E. Quagliariello
E.C. Slater
F. Palmieri
C. Saccone
A.M. Kroon

1985

ELSEVIER SCIENCE PUBLISHERS
AMSTERDAM · NEW YORK · OXFORD

© 1985 Elsevier Science Publishers B.V. (Biomedical Division)

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without the prior written permission of the publisher, Elsevier Science Publishers B.V., Biomedical Division, P.O. Box 1527, 1000 BM Amsterdam, The Netherlands.
Special regulations for readers in the USA – This publication has been registered with the Copyright Clearance Center Inc. (CCC), 27 Congress Street, Salem, MA 01970, USA. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the copyright owner, Elsevier Science Publishers B.V., unless otherwise specified.

ISBN Volume II: 0-444-80746-2
ISBN Set of Volumes I and II: 0-444-80747-0

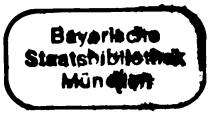
Published by:
Elsevier Science Publishers B.V. (Biomedical Division)
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Sole distributors for the USA and Canada:
Elsevier Science Publishing Company Inc.
52 Vanderbilt Avenue
New York, NY 10017
USA

Library of Congress Cataloging in Publication Data

International Symposium on Achievements and Perspectives in Mitochondrial Research (1985: Rosa Marina, Italy) Achievements and perspectives of mitochondrial research.

Bibliography: p.
Includes index.
Contents: v. 1. Bioenergetics – v. 2. Biogenesis.
1. Mitochondria – Congresses. 2. Bioenergetics – Congresses. I. Quagliariello, Ernesto. II. Title.
QH603.M5156 1985 574.87'342 85-27596
ISBN 0-444-80747-0 (set)
ISBN 0-444-80745-4 (v. 1)
ISBN 0-444-80746-2 (v. 2)



Printed in The Netherlands

CONTENTS

NUCLEOCYTOPLASMIC-MITOCHONDRIAL INTERRELATIONS; NUCLEAR GENES FOR MITOCHONDRIAL COMPONENTS

DEVELOPMENTAL ASPECTS AND MITOCHONDRIAL PATHOLOGY

© 1985 Elsevier Science Publishers B.V. (Biomedical Division)
Achievements and Perspectives of Mitochondrial Research
Volume II: Biogenesis, E. Quagliariello et al. editors

TRANSPORT OF PROTEINS INTO MITOCHONDRIA: RECEPTORS, RECOGNITION AND TRANSMEMBRANE MOVEMENT OF PRECURSORS

B. SCHMIDT, R. PFALLER, N. PFANNER, M. SCHLEYER and W. NEUPERT, Institut für Physiologische Chemie, University of Munich, Goethestr. 33, 8000 München 2, FRG

INTRODUCTION

Most of the mitochondrial proteins are coded for by nuclear genes, they are synthesized in the cytoplasm of the cell as precursor proteins and are then selectively imported by the mitochondria (1,2). The overall import appears to consist of a number of distinct sequential processes. The precursor proteins, most of which bear aminoterminal peptide extensions, bind to the mitochondrial surface and are then incorporated into the organelle. This transfer is irreversible and in most instances is dependent on an energized mitochondrial inner membrane. The next step in the assembly pathway is a processing reaction i.e. removal of the additional sequences and/or covalent modifications such as the attachment of heme in the case of cytochromes **c** and $\mathbf{c_1}$. Finally, the newly imported proteins reach their functional location and are assembled into active complexes.

Although we can distinguish individual steps of the import reaction, little is known about the components (apart from the precursor proteins) that participate in these reactions. In order to understand the molecular basis of import efforts are being made to define and characterize the components involved.

In this communication we describe experiments designed to elucidate the molecular mechanism of two aspects of protein transfer into mitochondria, viz. binding of the precursor proteins to the mitochondria, and their subsequent transmembrane movement or membrane insertion. The components responsible for binding, i.e. receptors, are proteins located on the surface of the mitochondria. Translocation directly from the binding sites is very rapid. On the other hand we demonstrate the existence of transmembrane intermediates, i.e. precursor proteins which are partly imported and span both inner and outer mitochondrial membranes. We propose an import pathway for inner membrane and matrix proteins via contact sites between inner and outer membrane.

MATERIALS AND METHODS

Established procedures were used to study import of proteins into mitochondria employing **in vitro** synthesized precursor proteins and isolated **Neurospora** mitochondria (3,4). The methods employed were as described elsewhere (4,5).

RESULTS

Binding of precursor proteins to mitochondria

The initial step in the import pathway subsequent to synthesis is the binding of the precursor to the mitochondria. Binding was usually observed in a reconstituted **in vitro** system when import was inhibited. This condition was achieved by destroying the membrane potential (e.g. for ADP/ATP carrier), by lowering the temperature (e.g. for porin) or by competitively inhibiting a processing step (e.g. for cytochrome **c**) (6-8). In the case of the above mentioned proteins, it was found that the binding sites (although probably distinct from each other) have common features: (i) they are saturable, (ii) they bind with high affinity, (iii) they appear to be proteins (Fig.1). The binding sites for porin (see Fig. 2) and ADP/ATP carrier appear to be located on the mitochondrial surface (6), whereas the apocytochrome **c** binding protein is located in the intermembrane space (9). The different location of these binding sites is also reflected by the differential sensitivity to

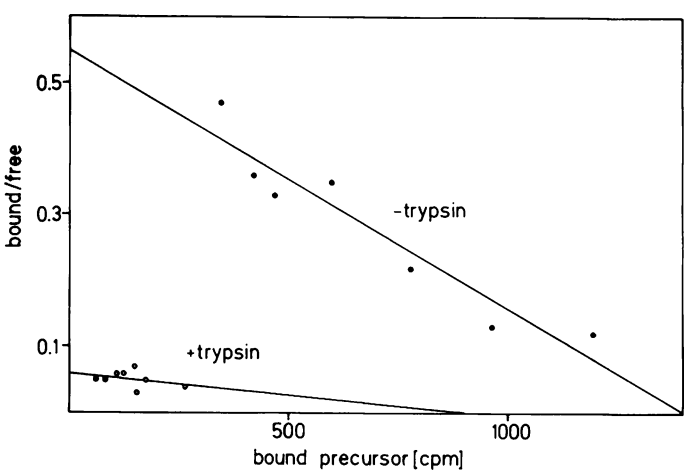


Fig. 1. High affinity binding of the precursor to the ADP/ATP carrier is mediated by proteinaceous receptors.
The precursor to ADP/ATP carrier was synthesized in a cell free system and partially purified by ammonium sulfate precipitation. Mitochondria were incubated with various concentrations of precursor under conditions where only binding takes place and subsequently reisolated. ADP/ATP carrier was immunoprecipitated from the supernatants and mitochondrial pellets. Quantification of the radioactivity associated with the carrier was carried out after excision of the carrier bands from an SDS gel. The amount of carrier bound to mitochondria is plotted versus the ratio bound/free (free = carrier recovered from the supernatants). Assuming a specific radioactivity of 3×10^4 cpm/pmol ADP/ATP carrier the number of binding sites was 1.7 pmol/mg mitochondrial protein and the K_a was $1.1 \times 10^9 M^{-1}$.
+ trypsin: mitochondria were treated with 5 µg of trypsin per/mg mitochondrial protein prior to the binding assay; - trypsin: untreated mitochondria.

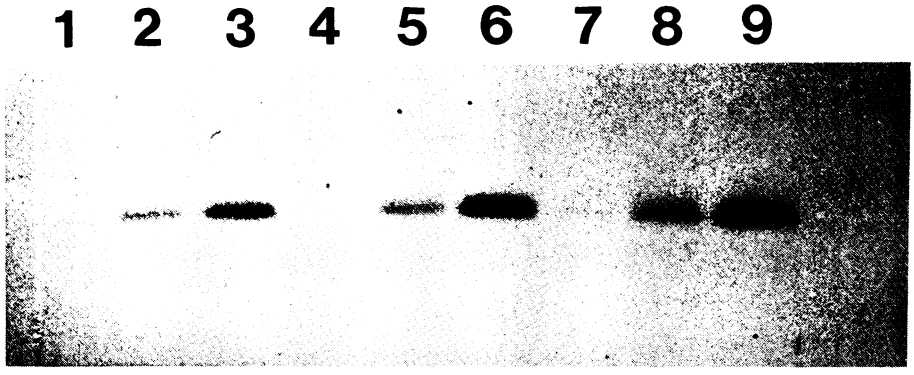


Fig. 2. Binding of water-soluble porin to isolated mitochondria.
Binding of porin to its receptor was studied in a simplified assay system, in which isolated mitochondria and a water-soluble form of mitochondrial porin were incubated in buffer. The water-soluble porin was prepared from porin isolated from mitochondria with detergents, by removal of detergent and lipids and by controlled denaturation and renaturation (7). The water-soluble form acquired a number of properties of the membrane form after insertion into artificial lipid bilayers. After radioactive labelling by reductive methylation this water-soluble porin was employed to measure specific receptor sites on mitochondria.
A mitochondrial preparation (2 mg protein) in 1 ml 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2 (SET) was divided into two halves. To one half 100 µg trypsin inhibitor was added together with 5 µg trypsin and incubation performed for 15 min at 25 °C. To the second half first 5 µg trypsin were added, the sample was incubated for 15 min at 25 °C and then 100 µg trypsin inhibitor were added. Both samples were incubated for 3 min at 25 °C and then for 10 min at 0 °C. Mitochondria were re-isolated, washed once in SET containing 100 µg/ml soybean trypsin inhibitor and resuspended in SET at a final concentration of 2 mg/ml. Binding of water-soluble porin to mitochondria was determined in samples which were prepared by mixing 125 µl reticulocyte lysate, 125 µl SET and 18 µl 0.1 M NaP_i, pH 6.8, containing 22 pmol water-soluble porin labelled with ^{14}C by reductive methylation (specific radioactivity 82 µCi/µmol). Three different amounts of mitochondria were employed: 2,3 : 50 µg; 5,6 : 100 µg; 8,9 : 250 µg. Samples 1, 4 and 7 did not contain mitochondria and served to control binding to tube walls. Samples 2, 5 and 8 contained mitochondria pretreated with trypsin. Samples 3, 6 and 9 contained mitochondria pretreated with trypsin in the presence of trypsin inhibitor. After incubation for 20 min at 25 °C samples were centrifuged, the pellets resuspended, transferred to new tubes and centrifuged again. All were then dissolved in SDS containing buffer and subjected to gel electrophoresis. The dried gel was fluorographed.

proteases. It is reasonable to conclude that different types of interaction between precursor and receptor may exist depending on the structure and the location of the receptor.

That the receptor bound state is a true intermediate state in the assembly pathway has been shown by the fact that bound precursors can be directly

imported from the binding sites. This import is efficient and rapid.In the case of the ADP/ATP carrier the import is 20-30 times faster than the observed import of the free precursor (Fig.3) The receptors identified appear to be essential components of the import pathway and represent the initial mediators of the overall process.

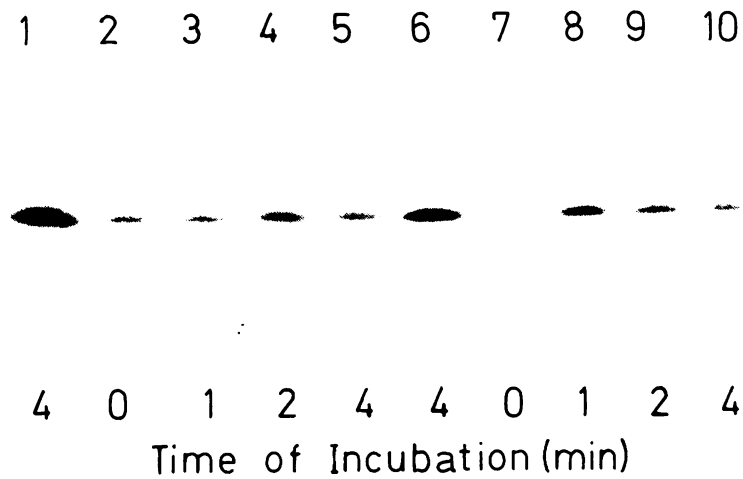


Fig. 3. Kinetics of import of ADP/ATP carrier from receptor-bound state. Mitochondria were allowed to bind the precursor to the ADP/ATP carrier in the absence of a membrane potential (lanes 2-5, 7-10). As a control, import was allowed in lanes 1 and 6. After this first incubation mitochondria were reisolated and resuspended in fresh medium containing no precursors. The membrane potential was reestablished and import allowed for the indicated times. Then the samples were halved and either the ADP/ATP carrier directly immunoprecipitated (lanes 1-5) or assayed for assembled carrier as described in ref. 5 (lanes 6-10). The immunoprecipitates were resolved on SDS-PAGE and the gel was fluorographed.

Translocation of precursor proteins across mitochondrial membranes

We have also studied the translocation of precursors across the mitochondrial membranes, in particular the movement of proteins destined for the inner membrane and the matrix. This step requires an energized mitochondrial inner membrane. Recent studies have revealed that the import of ADP/ATP carrier into the inner membrane can be driven by a valinomycin mediated K^+ diffusion potential (Fig.4). This import cannot be abolished by addition of a protonophore. Furthermore, creation of a pH gradient does not stimulate import. These findings suggest that the membrane potential $\Delta\psi$ per se and not the total protonmotive force Δp (with the components $\Delta\psi$ and ΔpH) is the driving force for import (10). The molecular mechanism of the import, however, is still enigmatic and little is known at the moment about the putative constituents of a translocation machinery. Nevertheless, some details of the actual

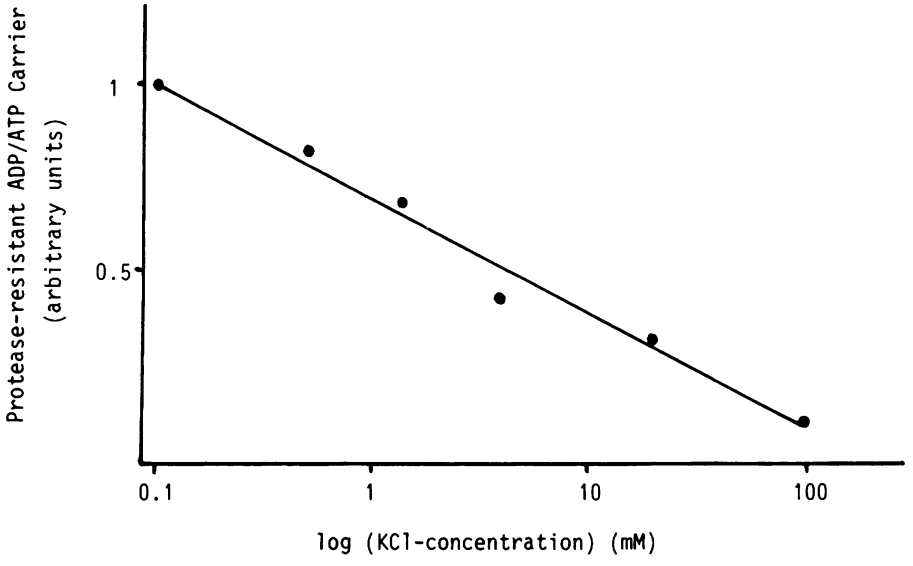


Fig. 4. Import of ADP/ATP carrier driven by a K^+ diffusion potential. The precursor to ADP/ATP carrier was bound to mitochondria in the absence of a membrane potential. Mitochondria were reisolated and resuspended in fresh medium containing valinomycin and the indicated concentrations of KCl. After incubation, samples were treated with Proteinase K to assay for imported carrier which was then immunoprecipitated. The immunoprecipitates were resolved on SDS-PAGE and the gel was fluorographed. Results were quantitatively evaluated by densitometry.

translocation process have been unravelled. First of all, the membrane traversing step does not depend on nor is it obligatorily coupled to proteolytic processing (11). Secondly, the translocation appears to occur at sites of contact between inner and outer membrane. Translocation intermediates of the ß-subunit of F_0F_1-ATPase and of cytochrome c_1 have been observed, which are accessible from both the cytoplasmic and the matrix side of the membranes (4). These intermediates were observed when transfer **in vitro** of precursor proteins into mitochondria was carried out at low temperature (7°C). They could also be observed by coupling the precursor to its antibody prior to import and allow the complex to react with mitochondria (Fig.5). The complex between antibody and precursor is only partially imported even at room temperature. The orientation of these intermediates is such that the amino terminus is located in the matrix and thus can be cleaved by processing peptidase. The major part of the polypeptide chain remains in the cytoplasm and therefore is accessible to externally added protease. Interestingly the requirement of a membrane potential extends only to the formation of such a

transmembrane orientated intermediate (4). The rest of the polypeptide chain is translocated even in the absence of a membrane potential. Thus the membrane potential may have a triggering role in transmembrane movement of precursors.

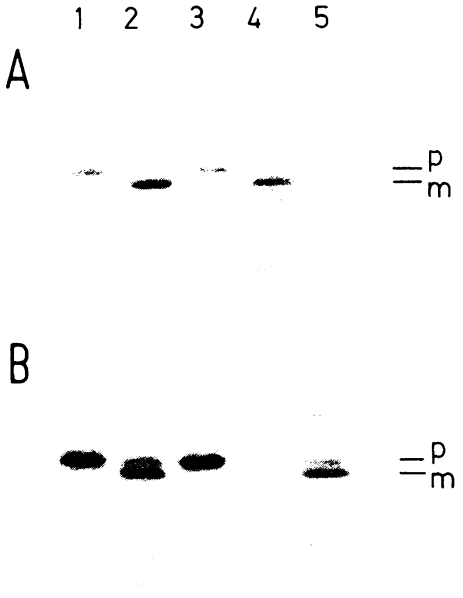


Fig. 5. Transport into mitochondria of $F_1\beta$ precursor bound to its antibody.
Import of the precursor to $F_1\beta$ into isolated mitochondria was carried out with free precursor (A) or with precursor bound to its antibody (B). After incubation of the precursors with mitochondria, mitochondria were reisolated. $F_1\beta$ was immunoprecipitated from the various reactions under the usual conditions (antibody + protein A-Sepharose) (A) or by just adding protein A-Sepharose (B). The immunoprecipitates were resolved by SDS-PAGE and fluorographed.
Lane 1: supernatant of reaction 2; lane 2: import into mitochondria; lane 3: binding to mitochondria in the absence of a membrane potential; lane 4: import into mitochondria and subsequent treatment with Proteinase K; lane 5: import into mitochondria, immunoprecipitation in the presence of an excess of antigen. p, precursor; m, mature $F_1\beta$.

DISCUSSION

Two of the components involved in the assembly of mitochondrial proteins have been characterized in some detail. These are the processing peptidase (12,13) and the receptors for precursor proteins. The import receptors recognize the precursor proteins and channel them to the mitochondria. It has been shown that the aminoterminal part of the precursor is responsible for the interaction with the mitochondrial import machinery (14,15). The nature of the interaction between precursor and receptor remains ill-defined.

Another unresolved question is: how do the precursors move from the receptor to a contact site and which proteins are involved in forming these sites. Possibly only the precursor and the receptor are required to form a contact site thus enabling the precursor to reach the inner membrane or the matrix. Since at least one of the translocation intermediates appears to be stable during fractionation of mitochondria efforts are being made to enrich and isolate these contact sites (16). The isolation and characterisation of the proteins constituting the transport machinery, would allow the reconstitution of import from isolated components and could lead to an understanding of the molecular basis of protein sorting and import by mitochondria.

ACKNOWLEDGEMENTS
We thank G. Karl, S. Meier, and C. Wagenpfeil for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Ne-101/19-1) and by the Fonds der Chemischen Industrie.

REFERENCES

1. Harmey, M.A. and Neupert, W. (1985) in: The Enzymes of Biological Membranes, A. Martonosi ed., Plenum Publ. Co. New York, **4**, 431-464.

2. Hay, R., Böhni, P., and Gasser, S. (1984) Biochim. Biophys. Acta, **779**, 65-87

3. Zwizinski, C., Schleyer, M., and Neupert, W. (1984) J. Biol. Chem. **259**, 7850-7856

4. Schleyer, M., and Neupert, W. (1985) Cell, in press

5. Schleyer, M., and Neupert, W. (1984) J. Biol. Chem. **259**, 3487-3491

6. Zwizinski, C., Schleyer, M., and Neupert, W. (1983) J. Biol. Chem. **258**, 4071-4074

7. Pfaller, R., Freitag, H., Harmey, M.A., Benz, R., and Neupert, W. (1985) J. Biol. Chem. **260**, 8188-8193

8. Hennig, B., and Neupert, W. (1981) Eur. J. Biochem **121**, 203-212

9. Köhler, H., Stuart, R., and Neupert, W. (1985) manuscript in preparation

10. Pfanner, N., and Neupert, W., (1985), EMBO J., in press

11. Zwizinski, C., and Neupert, W. (1983) J. Biol. Chem **258**, 13340-13346

12. Böhni, P., Gasser, S., Leaver, C., and Schatz, G. (1980) in: The Organization and Expression of the Mitochondrial Genome (Kroon, A.M., and Saccone, C., eds.), Elsevier/North-Holland, Amsterdam, pp. 432-433

13. Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984) Eur. J. Biochem. **144**, 581-588

14. Hurt, E.C., Pesold-Hurt, B., and Schatz, G. (1984) EMBO J. **3**, 3149-3156

15. Horwich, A.L., Kalousek, F., Mellman, I., and Rosenberg, L.E. (1985) EMBO J. **4**, 1129-1135.

16. Schwaiger, H., and Neupert, W. (1985) manuscript in preparation

AUTHOR INDEX

SUBJECT INDEX

Bayerische
Staatsbibliothek
München