JORGE EDUARDO DA SILVA AZEVEDO

ESTRUTURA E BIOGÉNESE DA NADH:UBIQUINONA OXIDORREDUTASE (COMPLEXO I) DA CADEIA RESPIRATÓRIA

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE DE BIOLOGIA MOLECULAR, APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR DA UNIVERSIDADE DO PORTO

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À Isabel

No cumprimento do Decreto-Lei 388/70, esclarece-se serem da nossa responsabilidade a execução das experiências apresentadas neste trabalho (excepto quando referido em contrário) assim como a sua interpretação e discussão.

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ABREVIATURAS

- cDNA; ácido desoxirribonucleico complementar
- FMN; mononucleótido de flavina
- $FMNH_2$; forma reduzida do FMN
- NADH; dinucleótido de nicotinamida e adenina
- NAD^+; forma oxidada do NADH
- e. p. r.; ressonância electrónica paramagnética
- ATP; trifosfato de adenosina
- mtDNA; DNA mitocondrial
- SDS; dodecilsulfato de sódio
- SDS-PAGE; electroforese em gel de poliacrilamida na presença de SDS
- NUO-x; subunidade do complexo I de x kDa.

Nota:

Alguns dos termos ingleses utilizados correntemente em Biologia Molecular não foram traduzidos para português pela perda de clareza inerente à tradução. Tais palavras e expressões surgem no texto entre aspas.

ÍNDICE

1- RESUMO

Vários anticorpos policlonais dirigidos contra subunidades do complexo I de *Neurospora crassa* foram preparados e utilizados no rastreio de um banco de expressão de cDNAs. Deste modo, foram isolados clones codificantes das subunidades 20.9 kDa, 17.8 kDa e 14.8 kDa. As sequências destes cDNAs e, adicionalmente, de um cDNA codificante da subunidade de 12.3 kDa, foram determinadas. A análise das estruturas primárias destas subunidades deduzidas a partir dos cDNAs respectivos, revelou, em alguns casos, a existência de homologias e similaridades com proteínas de bovinos. As implicações estruturais e funcionais destas similaridades são discutidas.

Os mecanismos de montagem das subunidades de 20.9 kDa, 17.8 kDa e 14.8 kDa foram estudados utilizando um sistema *in organello*. É demonstrado que todas estas subunidades requerem a existência de um potencial de membrana para serem eficientemente importadas por mitocôndrias isoladas. Uma vez importadas *in vitro*, as subunidades de 20.9 kDa e 17.8 kDa são encontradas na membrana interna mitocondrial apresentando características típicas das subunidades endógenas. Os dados apresentados sugerem que nenhuma destas proteínas precursoras é translocada completamente para a matriz mitocondrial. Aparentemente, estas proteínas precursoras terminam o processo de translocação membranar ao nível da membrana interna mitocondrial.

Experiências de co-imunoprecipitação e de sedimentação em gradientes de sacarose, demonstraram que uma fracção significativa da subunidade de 14.8 kDa importada *in vitro* é montada no complexo I. Esta observação sugere que toda a maquinaria enzimática necessária para a biossíntese do complexo I se encontra operacional em mitocôndrias isoladas, permitindo, assim, a utilização deste sistema *in organello* no estudo do processo de montagem da enzima.

A técnica da extracção alcalina de biomembranas, desenvolvida por Fujiki *et al.* (1982), foi utilizada com o intuito de caracterizar o tipo de interacção que várias subunidades do complexo I estabelecem com a membrana interna mitocondrial. Os resultados obtidos sugerem que as subunidades de 49 kDa e 30.4 kDa são proteínas extrínsecas de membrana; as subunidades de 20.9 kDa, 17.8 kDa e 12.3 kDa apresentam um comportamento típico de proteínas intrínsecas de membrana. Após extracção alcalina de mitocôndrias isoladas, foi

também possível isolar um subcomplexo que representa uma parte (ou a totalidade) do braço membranar do complexo I. Uma análise comparativa da composição polipeptídica deste fragmento e do subcomplexo Iα da enzima bovina (Finel *et al.*, 1992) sugere que a subunidade de 20.8 kDa [uma possível metalo-proteína (Videira *et al.*, 1990a)] e a subunidade de 9.3 kDa [um componente do sítio de ligação da ubiquinona (Heinrich & Werner, 1992; Heinrich *et al.*, 1992)], se encontram na interface dos braços membranar e periférico do complexo I.

SUMMARY

Several polyclonal antibodies were prepared against *Neurospora crassa* complex I subunits and used in the screening of a cDNA expression library. Full-length clones encoding the 20.9 kDa, 17.8 kDa e 14.8 kDa were isolated. The sequences of these cDNAs and, additionally, of a clone encoding the 12.3 kDa subunit of complex I were determined. Sequence analysis of the deduced primary structures of two of these subunits revealed interesting homologies and similarities with bovine proteins. The significance of these findings are discussed.

The biogenetic pathways of the 20.9 kDa, 17.8 kDa and 14.8 kDa subunits were studied. It is shown that all these subunits require a membrane potential to be imported into isolated mitochondria. Once imported, the 17.8 kDa and 20.9 kDa subunits are found in the mitochondrial inner membrane where they have acquired characteristics of the endogenous subunits. The data presented suggest that neither of these precursor proteins is completely translocated into the matrix, but instead, translocation of both membrane systems (*i. e.*, outer and inner membrane) is arrested at the level of the mitochondrial inner membrane.

A significant fraction of the *in vitro* imported 14.8 kDa subunit was found in completely assembled complex I demonstrating that even in isolated mitochondria all the machinery required for the assembly process is still operational. This finding will allow the use of an *in vitro* system for the study of the biogenesis of complex I.

The technique of alkaline extraction of biological membranes (Fujiki *et al.*, 1982) was employed to study the kind of interaction of several complex I subunits with the mitochondrial inner membrane. The results obtained suggest the 49 kDa and 30.4 kDa subunits are extrinsic membrane proteins; the 20.9 kDa, 17.8 kDa and 12.3 kDa behave as intrinsic membrane proteins. After alkaline extraction of mitochondria a subcomplex of the enzyme representing a fraction (or the totality) of the membrane arm of the enzyme was isolated. A comparative analysis of the polypeptide composition of this fragment with subcomplex Iα of the bovine enzyme (Finel *et al.*, 1992) suggests that the 20.8 kDa subunit [a possible iron-sulphur protein (Videira *et al.*, 1990a)] and the 9.3 kDa subunit [a polypeptide at or near the ubiquinone-binding site (Heinrich & Werner, 1992; Heinrich *et al.*, 1992)] are located in the junction of the two arms of complex I.

SOMMAIRE

Plusieurs anticorps policlonaux dirigés contre sousunités du complèxe I de *Neurospora crassa* ont été préparés et utilisés pour chercher une banque d' expréssion de cDNAs. Nous avons isolé des clones qui codifient les sousunités de 20.9 kDa, 17.8 kDa et 14.8 kDa. Les séquences de ces cDNAs et, en plus, d' un autre cDNA pour la sousunité 12.3 kDa, ont été determinés. L' analyse des structures primaires de ces sousunités a montré, dans certains cas, l'existence de homologies et similarités avec des protéines bovines.

Les méchanismes biogenétiques des sousunités 20.9 kDa, 17.8 kDa et 14.8 kDa ont été étudiés. Nous avons montré que toutes les sousunités ont besoin d' un potentiel de membrane pour être importées dans des mitochondries isolées. Aprés l' importation *in vitro*, les sousunités 20.9 kDa et 17.8 kDa apparait dans la membrane interne de la mitochondrie avec des charactéristiques que ressemblent ceux des sousunités endogénes. Les résultats indiquent que aucune de cettes sousunités est complètement transposée pour la matrice mitochondrielle.

Des expériences de co-immunoprécipitation et sédimentation ont montré que une partie significative de la sousunité 14.8 kDa importée *in vitro* est associée avec le complèxe I. Ceci indique que toute la machinerie enzymatique nécéssaire à la biosynthèse du complèxe I est encore opéracionnelle dans des mitochondries isolées, ce qui permett l' utilization de ce système *in organnelle* pour l' étude du processus de montage de l' enzyme.

La téchnique d' extraction alcaline de biomembranes devéloppée par Fujiki *et al.* (1982) a été utilisée pour charactériser l' intéraction entre les sousunités du complèxe I et la membrane interne de la mitochondrie. Les résultats obténus indiquent que les sousunités 49 kDa et 30.4 kDa sont extrinsèque à la membrane; les sousunités 20.9 kDa, 17.8 kDa et 12.3 kDa presentent un comportement de protéines intrinsèque.

Après l' extraction alcaline de mitochondries isolées, nous avons isolé un sous-complèxe que réprésente une partie (ou la totalité) du domaine membranaire du complèxe I. Une analyse comparative de la constitution en protéines de ce fragment et celle du sous-complèxe Iα de l' enzyme bovine (Finel *et al.*, 1992) indique que la sousunité 20.8 kDa [peut-être une métallo-protéine (Videira *et al.*, 1990a)] et la sousunité 9.3 kDa [un composant du local de liaison de l´ ubiquinone (Heinrich & Werner, 1992; Heinrich *et al.*, 1992)] éxistent dans la interface des domaines membranaire et périphérique du complèxe I.

2- INTRODUÇÃO

As mitocôndrias são estruturas ubíquas no reino dos eucariotas. É nestes organelos que ocorre a denominada fosforilação oxidativa, uma via metabólica complexa onde a maior parte da energia resultante da oxidação dos alimentos é convertida em ATP. A maquinaria enzimática catalizadora deste processo contém cinco complexos proteicos multiméricos fulcrais:

(1) NADH:ubiquinona oxidorredutase (complexo I);

(2) Succinato:ubiquinona oxidorredutase (complexo II)

(3) Ubiquinol:citocromo *c* oxidorredutase (complexo III);

(4) Citocromo *c* oxidase (complexo IV); e

(5) ATP sintetase (complexo V).

De um modo sequencial e com a intervenção de outros componentes (*e. g.*, ubiquinona e citocromo *c*) estes complexos catalizam a transferência de electrões do NADH e succinato para o oxigénio. Ao nível dos complexos I, III e IV, a energia libertada nas reacções redox é acoplada à extrusão de protões através da membrana interna mitocondrial (como artigos de revisão ver Hatefi, 1985; Tzagoloff & Myers, 1986). A energia associada ao gradiente electroquímico assim gerado é, então, utilizada pelo complexo V para a síntese de ATP (Mitchell, 1966).

Dada a enorme complexidade deste sistema, muito pouco é ainda conhecido a nível molecular sobre os mecanismos de catálise, biogénese, estrutura e regulação dos seus diversos componentes. A compreensão de alguns destes aspectos poderá ter importantes consequências, não só em termos de bioenergética (e as suas aplicações biotecnológicas), mas também no campo da medicina onde cada vez mais se detectam correlações entre várias doenças e anomalias da cadeia respiratória.

O trabalho aqui apresentado incide sobre a estrutura e biogénese do primeiro componente da cadeia de fosforilação oxidativa - a NADH:ubiquinona oxidorredutase. Esta enzima é, sem dúvida, o mais complexo de todos os elementos da cadeia respiratória, razão pela qual tem permanecido o menos compreendido. Na exposição seguinte vários aspectos do conhecimento actual sobre o complexo I são discutidos detalhadamente.

2.1- Distribuição filogenética do complexo I

A maior parte da informação actualmente disponível sobre a distribuição filogenética do complexo I adveio da descoberta que algumas das subunidades da enzima são codificadas e sintetizadas na mitocôndria (Chomyn *et al.*, 1985,1986). Assim, a presença no DNA mitocondrial de um dado organismo de "ORFs" ("Open Reading Frames") codificantes de proteínas homólogas às subunidades ND1-ND6 e ND4L (ver 2.2.1) tem sido considerada como evidência para a existência de complexo I nesse organismo. Explorando esta característica, foi demonstrada a existência de complexo I em diversos organismos eucarióticos desde mamíferos (Anderson *et al.*, 1981) a protozoários (Pritchard *et al.*, 1990), passando por pássaros (Desjardins & Morais, 1990), anfíbios (Roe *et al.*, 1985), peixes (Johansen *et al.*, 1990), insectos (Clary & Wolstenholme, 1985), plantas (Oda *et al.*, 1992), algas (Denovan-Wright & Lee, 1992) e fungos (Burger & Werner, 1986; Nelson & Macino, 1987). A ubiquidade filogenética do complexo I tem, no entanto, uma excepção infeliz - a levedura *Saccharomyces cerevisiae* - não permitindo, assim, a utilização de um organismo de tão fácil manipulação genética no estudo desta enzima.

Surpreendentemente, a existência de genes codificantes de proteínas homólogas a subunidades do complexo I foi também constatada no genoma de

cloroplastos de várias plantas superiores (Fearnley *et al.*, 1989; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Videira *et al.*, 1990b). Este facto, indicativo da existência de uma NADH:plastoquinona oxidorredutase em cloroplastos, veio corroborar dados bioquímicos obtidos anteriormente que sugeriam a existência nestes organelos de uma cadeia respiratória responsável pela clororespiração (Bennoun, 1982; Godde & Trebst, 1980; ver Walker, 1992).

De acordo com a teoria endossimbiótica sobre a origem da mitocôndria, não é de estranhar a presença de proteínas homólogas a subunidades do complexo I em organismos procarióticos. Assim, foi recentemente sequenciado em *Paracoccus denitrificans* um operão codificante de uma NADH:ubiquinona oxidorredutase (Xu *et al.*, 1991a,b, 1992a,b, 1993; Yagi *et al.*, 1992). Neste operão foram detectadas 20 "ORFs", 14 das quais codificam proteínas homólogas a subunidades de complexo I mitocondrial (ver tabela 1). Embora a enzima deste organismo seja visivelmente mais simples que o complexo I mitocondrial, o seu estudo irá revelar características importantes da forma mitocondrial. Por exemplo, dada a similaridade existente entre as duas enzimas em termos de espectro de e.p.r. (ver 2.2.2 e 2.4.2) e a facilidade de manipulação genética de um organismo procariótico, é de esperar a curto prazo a identificação experimental directa das subunidades que possuem centros de Fe-S.

No entanto, o aspecto mais interessante do estudo de procariotas é o facto de proteínas homólogas a subunidades do complexo I nem sempre se encontrarem associadas a NADH:ubiquinona oxidorredutases. A enzima NAD^+-hidrogenase de *Alkaligenes eutrophus* (Tran-Betcke *et al.*, 1990) ilustra bem este ponto: duas das suas subunidades apresentam homologia com 3 subunidades do complexo I, sugerindo fortemente que estes componentes representam uma unidade funcional e estrutural (ver 2.4.1).

2.2- Composição do complexo I

2.2.1- Componentes polipeptídicos

As primeiras análises electroforéticas em condições desnaturantes de preparações relativamente puras da enzima bovina sugeriram a existência de cerca de 25 subunidades polipeptídicas (ver Ragan, 1987). No entanto, à medida que a resolução dos sistemas electroforéticos melhora, este número tem vindo a aumentar. Actualmente, é unanimemente aceite que o complexo I de mamíferos é composto por cerca 40 subunidades. De facto, Walker *et al.* (1992) encontraram evidência para a existência na enzima bovina de, pelo menos, 41 subunidades polipeptídicas (ver tabela 1) e é natural que este número ainda não seja definitivo. A estes componentes (e assumindo apenas uma estequiometria unitária para cada subunidade) corresponde uma massa molecular de aproximadamente 880 kDa, superior, portanto, à massa molecular proteica de um ribossoma de *Escherichia coli* (Walker *et al.*, 1992).

No fungo *Neurospora crassa*, um dos organismos onde o complexo I se encontra, também, bem caracterizado, são detectadas cerca de 35 subunidades, 24 das quais foram já caracterizadas ao nível da estrutura primária (ver tabela 1 e Fig. 1 da secção 7.5). Cerca de 80 % das subunidades da enzima fúngica têm homólogos na enzima de mamíferos indicando que as duas enzimas são funcional e estruturalmente semelhantes possibilitando, assim, a extrapolação da informação obtida experimentalmente entre os dois sistemas.

Um aspecto interessante do complexo I reside no facto de nem todas as suas subunidades polipeptídicas serem codificadas nuclearmente. Como demonstrado por Chomin *et al.* (1985, 1986), 7 das "URFs" ("Unidentified Reading Frames") que haviam sido previamente detectadas no DNA mitocondrial humano (Anderson *et al.*, 1981) codificam na realidade

Tabela 1: Subunidades do Complexo I de vários organismos.

N. crassa	*B. taurus*	*P. denitrificans*	Referências	*N. crassa*	*B. taurus*	*P. denitrificans*	Referências
78 kDa	75 kDa (IP)	NQO3	1;2;3	n.c.	B14.5b	-	21
51 kDa	51 kDa (FP)	NQO1	1;4;3	17.8 kDa	-	-	Este trabalho
49 kDa	49 kDa (IP)	NQO4	5;6;3	14.8 kDa	B14	-	Este trabalho;12
n.c.	42 kDa	-	7	n.c.	15 kDa (IP)	-	12
40 kDa	39 kDa	-	8;7	12.3 kDa	-	-	Este trabalho
30.4 kDa	30 kDa (IP)	NQO5	9;10;3	n.c.	B12	-	12
29.9 kDa	B13	-	11;12	n.c.	13 kDa (IP)	-	12
24 kDa	24 kDa (FP)	NQO2	A;13;3	10.5 kDa	B8	-	22;12
n.c.	B22	-	12	9.3 kDa	B9	-	23;12
n.c.	TYKY	NQO9	14;3	ACP	SDAP	-	24;25
n.c.	PDSW	-	12	n.c.	MLRQ	-	12
n.c.	PSST	NQO6	15;3	n.c.	10 kDa (FP)	-	27
21.3 kDa[a]	-	-	16	n.c.	AGGG	-	12
21.3 kDa[b]	-	-	17	n.c.	MWFE	-	12
20.9 kDa	-	-	Este trabalho	n.c.	MNLL	-	12
20.8 kDa	PGIV	-	18;19	n.c.	KFYI	-	12
n.c.	ASHI	-	12	ND1	ND1	NQO8	B;3
n.c.	SGDH	-	12	ND2	ND2	NQO14	B;3
n.c.	B18	-	12	ND3	ND3	NQO7	B;3
18.3 kDa	18 kDa (IP)	-	20;12	n.c.	ND4	NQO13	B;3
n.c.	B17	-	12	ND4L	ND4L	NQO11	B;3
n.c.	B15	-	12	ND5	ND5	NQO12	B;3
n.c.	B14.5a	-	21	ND6	ND6	NQO10	B;3

Subunidades homólogas encontram-se alinhadas horizontalmente. "n.c." - subunidade não conhecida mas cuja existência não é de excluir; "-" - subunidade aparentemente inexistente nas preparações enzimáticas analisadas (ver Arizmendi *et al.*, 1992a; Yagi *et al.*, 1992). Referências: (1,5) Preis *et al.*, 1991,1990; (2,25) Runswick *et al.*, 1989,1991; (3) Xu *et al.*, 1991a,b, 1992a,b, 1993; Yagi *et al.* 1992; (4,10) Pilkington *et al.*, 1991a,b; (6,7) Fearnley *et al.*, 1989,1991; (8) Rholen *et al.*, 1991; (9,17,18) Videira *et al.*, 1990b,c,a; (11) Van der Pas *et al.*, 1991; (12) Walker *et al.*, 1992; (13) Pilkington & Walker, 1989; (14,21) Dupuis *et al.*, 1991b,a; (16) Nehls *et al.*, 1991; (20) Weidner *et al.*, 1991; (15,21) Arizmendi *et al.*, 1992b,a; (22) Duarte *et al.*, 1993; (23) Heinrich *et al.*, 1992; (24) Sackmann *et al.*, 1991; (26) Skehel *et al.*, 1991; (A) Duarte, M., Belo, J. A., Azevedo, J. E. & Videira, A. (manuscrito em preparação); (B) as sequências das subunidades codificadas mitocondrialmente das enzimas de *N. crassa* e de bovinos encontram-se compiladas em Fearnley & Walker, 1992.

subunidades do complexo I. Estas subunidades, denominadas ND1-ND6 e ND4L, têm a particularidade de serem extremamente hidrofóbicas e, como será discutido posteriormente (ver 2.3.1), encontram-se muito provavelmente embebidas na membrana interna mitocondrial.

2.2.2- Centros Fe-S e FMN

Para além de um elevado número de subunidades polipeptídicas, o complexo I contém ainda como transportadores de electrões uma molécula de FMN ligada não covalentemente e vários centros Fe-S (Hatefi *et al.*, 1962).

O número exacto de centros Fe-S existentes na enzima bovina é, ainda hoje, assunto de debate (ver Beinert & Albracht, 1982). No entanto, vários laboratórios referem consistentemente a existência de 3 centros Fe-S tetranucleares, os denominados centros N2, N3 e N4, e um centro binuclear, o centro N1b (Ingledew & Ohnishi, 1980; Beinert & Albracht, 1982). A existência de um segundo centro binuclear de potencial redox do ponto médio muito negativo (-400 mV a -500 mV), denominado centro N1a, tem também sido defendida por alguns investigadores (Ohnishi *et al.*, 1985; Ohnishi & Salerno, 1982). Evidência para um quarto centro tetranuclear (o centro N5) tem sido apresentada pelo grupo de Ohnishi (Ingledew & Ohnishi, 1980; Ohnishi, 1979; Salerno *et al.*, 1977) mas a sua existência é largamente contestada, existindo a possibilidade de se tratar de uma contaminação da preparação analisada pelos autores (ver Beinert & Albracht, 1982).

Aos centros N1a, N1b, N2-N4, e assumindo estequiometrias unitárias por FMN*, correspondem 16 átomos de ferro. Este número é ainda bastante

* O grupo de Albracht tem referido consistentemente uma estequiometria de 1 centro N1b por cada duas moléculas de FMN (ver van Belzen *et al.*, 1990). Estes resultados, não unanimemente aceites (Ohnishi, 1979), são a base de um modelo estrutural em que o complexo I, na sua forma biologicamente activa, existirá sob uma forma dimérica em que um dos monómeros não possui o centro N1b (ver van Belzen *et al.*, 1990).

inferior aos valores obtidos por análise química - 22 a 24 átomos de ferro por FMN (Ragan *et al.*, 1982b) - sendo, por isso, provável a existência de outros centros Fe-S (invisíveis por e.p.r.) na enzima bovina (ver também 2.3.3).

Análises por e.p.r. da enzima do fungo *N. crassa* revelaram a existência dos centros N2, N3, N4 e de um centro binuclear - denominado N1 - semelhante no seu potencial redox do ponto médio ao centro N1b da enzima bovina (Wang *et al.*, 1991). Nenhuma evidência para a existência de centros semelhantes aos centros N1a e N5 bovinos foi encontrada. No entanto, dadas as semelhanças estruturais existentes entre as enzimas de *N. crassa*, bovinos e *P. denitrificans* - organismo no qual também é detectado um centro N1a (Meinhardt *et al.*, 1987) - a presença de um centro do tipo N1a na enzima fúngica é altamente provável.

2.3- Estrutura do complexo I

2.3.1- Organização espacial das subunidades da enzima

Actualmente, dado que a maior parte das subunidades da enzima de bovinos e de *N. crassa* foi já caracterizada em termos de estrutura primária, a busca de informação estrutural sobre o complexo I é de uma importância fulcral para a compreensão dos mecanismos de catálise, biogénese e função da enzima.

Embora o complexo I de bovinos tenha sido isolado 23 anos antes de existir um protocolo de purificação para a enzima de *N. crassa* (Hatefi *et al.*, 1962; Ise *et al.*, 1985), a maior parte da informação disponível actualmente sobre a estrutura geral do complexo I mitocondrial foi obtida com a enzima

fúngica. Isto deve-se principalmente ao facto de a enzima de *N. crassa*, ao contrário da enzima de bovinos, ser muito estável na presença de detergentes, sendo por isso fácil de isolar num estado monodisperso, uma característica essencial para estudos de microscopia electrónica e difracção de raios X.

Muitos dos estudos estruturais realizados em complexos proteicos baseiam-se no uso de substâncias que desestabilizam de um modo relativamente suave as interacções entre proteínas (*e. g.*, agentes caotrópicos e detergentes). Aplicando esta estratégia ao estudo do complexo I, Friedrich *et al.* (1989) mostraram que, quando a enzima de *N. crassa* é incubada na presença do anião brometo, uma parte substancial das suas subunidades são dissociadas perdendo qualquer estrutura definida. No entanto, uma fracção contendo cerca de metade das subunidades da enzima resiste, de uma forma aparentemente intacta, ao tratamento com sais caotrópicos podendo ser isolada numa forma monodispersa. Este subcomplexo contém cerca de 15 subunidades codificadas nuclearmente (a maior parte das quais ainda com estrutura primária desconhecida) e, adicionalmente, todas as subunidades codificadas mitocondrialmente. O facto de subunidades altamente hidrofóbicas serem encontradas neste subcomplexo levou Tuschen *et al.* (1990) a especular que este "fragmento hidrofóbico" corresponde à parte da enzima que se encontra embebida na membrana interna mitocondrial, o que, de certo modo, foi confirmado posteriormente (ver abaixo). Por outro lado, quando o fungo *N. crassa* é crescido na presença de cloranfenicol (um inibidor da síntese proteica mitocondrial), as suas mitocôndrias acumulam um subcomplexo contendo cerca de 15 subunidades codificadas nuclearmente. Surpreendentemente, nenhuma das subunidades deste subcomplexo - denominado "pequena forma da enzima" - está presente no fragmento hidrofóbico e, em termos de composição polipeptídica, os dois fragmentos representam a totalidade da enzima fúngica (Friedrich *et al.*, 1989).

Estes resultados só foram plenamente compreendidos quando as primeiras preparações de complexo I de *N. crassa*, no seu estado monodisperso e cristalizado bidimensionalmente, foram analisadas por microscopia electrónica e difracção de raios X (Hofhaus *et al.*, 1991). Estes estudos revelaram que o complexo I tem uma estrutura em forma de L, em que um dos braços se encontra embebido na membrana interna mitocondrial e o outro, perpendicular ao primeiro, se orienta para o meio aquoso da matriz mitocondrial. Por outro lado, quando estes cristais bidimensionais são "lavados" com uma solução de brometo de sódio, praticamente todas as subunidades presentes na pequena forma da enzima são removidas, restando apenas o braço membranar cuja composição polipeptídica é semelhante à do fragmento hidrófobico. Ficou, assim, claro que a pequena forma do complexo I representa o braço mais hidrofílico (denominado braço periférico da enzima) e que o fragmento hidrofóbico é, na realidade, semelhante ao braço membranar do complexo I (Hofhaus *et al.*, 1991).

O complexo I de bovinos tem sido também alvo de estudos estruturais. Muitos dos dados obtidos com a enzima deste organismo, corroboram e, acima de tudo, refinam o modelo estrutural obtido com a enzima de *N. crassa*.

Como mostrado por Galante & Hatefi (1979), quando incubada na presença de perclorato, a enzima bovina origina 3 fragmentos: (1) a denominada FP ("Flavo-protein") constituída pelas subunidades 51 kDa (FP), 24 kDa (FP) e 10 kDa (FP), contendo todo o FMN da enzima e 6 átomos de ferro (Ragan *et al.*, 1982a); (2) a IP ("Iron-protein"), uma fracção proteica relativamente heterogénea em termos da estequiometria dos seus componentes, englobando as subunidades de 75 kDa, 49 kDa, 30 kDa, 18 kDa (IP), 15 kDa (IP), 13 kDa (IP) e B13 e ainda cerca de 10 átomos de ferro (Ragan *et al.*, 1982b; Walker, 1992); e, finalmente, (3) a HP ("Hydrophobic protein"), um aglomerado proteico insolúvel nas condições experimentais

usadas para o fraccionamento, contendo as restantes subunidades do complexo I e, pelo menos, dois centros Fe-S (Ragan *et al.*, 1982b).

Mais recentemente, Finel *et al.* (1992), explorando a labilidade estrutural do complexo I bovino na presença de detergentes, isolaram e caracterizaram dois fragmentos que, em termos de composição polipeptídica, são complementares e representam a quase totalidade da enzima bovina. O maior destes fragmentos, o denominado subcomplexo Iα, contém cerca de 25 subunidades entre as quais todas aquelas que são encontradas nos fragmentos FP e IP descritos anteriormente. Adicionalmente, este subcomplexo contém também outras subunidades que, à semelhança dos componentes das frações FP e IP, possuem uma estrutura primária típica de proteínas predominantemente hidrofílicas. No entanto, a presença neste fragmento de cinco subunidades com potencial para atravessarem um sistema membranar (uma das quais a subunidade ND-2, indiscutivelmente uma proteína de membrana) sugere que uma pequena parte deste subcomplexo se encontra embebida na membrana interna mitocondrial. Este fragmento contém, também, todos os centros Fe-S que são detectados por e.p.r. na enzima bovina.

Ao contrário do que acontece com o subcomplexo Iα, a maior parte das cerca de 15 subunidades que constituem o denominado fragmento Iβ (Finel *et al.*, 1992) possuem domínios suficientemente grandes de aminoácidos hidrofóbicos para atravessarem a membrana interna mitocondrial, sugerindo, assim, que grande parte deste fragmento se encontra embebido na membrana.

Extrapolando esta informação para o modelo estrutural obtido com a enzima de *N. crassa*, foi sugerido que o subcomplexo Iα corresponde ao braço periférico da enzima contendo, também, uma pequena porção do braço membranar; o subcomplexo Iβ representa a maior parte do braço membranar do complexo I (Finel *et al.*, 1992; Walker, 1992).

2.3.2- Localização dos sitios de ligação do NADH e FMN

Embora seja constituída por apenas três subunidades, a fracção FP do complexo I bovino (ver 2.3.1) possui actividade de NADH desidrogenase. É de salientar que os parâmetros cinéticos da ligação do NADH e NAD^+ ao fragmento FP são praticamente iguais aos obtidos com a enzima intacta, sugerindo que neste subcomplexo o sítio de ligação do NADH permanece perfeitamente funcional após o tratamento com perclorato (Dooijewaard & Slater, 1976a,b). Com o intuito de identificar qual das três subunidades da FP é responsável pela ligação do NADH, Chen & Guillory (1981) usaram um análogo radioactivo do NAD^+ em experiências de marcação por foto-afinidade. A subunidade de 51 kDa da enzima bovina era marcada significativamente sendo a incorporação do análogo inibida de uma forma competitiva por NADH. Resultados semelhantes foram também obtidos com a NADH:ubiquinona oxidorredutase de *P. denitrificans* onde um polipéptido de 50 kDa (homólogo à subunidade de 51 kDa bovina) foi identificado como constituindo o sítio de ligação do NADH (Yagi & Dinh, 1990).

A labilidade da ligação da FMN à(s) subunidade(s) do complexo I tem dificultado a identificação experimental do seu sítio de ligação (ver Ragan, 1987). Como será discutido posteriormente (ver 2.4.2), das três subunidades que constituem a fracção FP, o componente de 10 kDa não parece desempenhar um papel directo na ligação da FMN. No entanto, apesar de existir alguma (fraca) evidência baseada em análises estruturais para a atribuição do sítio de ligação da FMN à subunidade de 51 kDa (ver Walker, 1992), não é ainda possível excluir a subunidade de 24 kDa desta função.

2.3.3- Localização dos centros Fe-S

A maior parte dos estudos efectuados na tentativa de localizar os diferentes centros Fe-S do complexo I tem-se confinado à caracterização por e.p.r. dos vários fragmentos do complexo I acima descritos. Embora esta estratégia possua algumas limitações, pois após resolução da enzima muitos dos centros Fe-S perdem os seus sinais de e.p.r. característicos tornando a sua identificação difícil, foi possível localizar os centros N-1b e N-3 na fracção FP da enzima bovina (Ohnishi *et al.*, 1981, 1985; Ragan *et al.*, 1982a). Neste caso, foi mesmo possível atribuir o centro N-3 à subunidade de 51 kDa, e o centro N-1b às subunidades de 24 kDa e/ou 10 kDa (Ohnishi *et al.*, 1985; ver também 2.4.2). A presença de centros Fe-S nas subunidades de 75 kDa, 49 kDa e no heterodímero constituído pelas subunidades de 30 kDa (IP) e 13 kDa (IP) foi também sugerida (Ragan, 1987), embora nestes casos a alteração no espectro de e.p.r. resultante da técnica utilizada para a resolução da enzima não tivesse permitido a identificação desses centros metálicos.

A recente determinação da estrutura primária das subunidades do complexo I bovino forneceu mais informação sobre o possível número e a localização de centros Fe-S da enzima. Por exemplo, foi verificada a existência do motivo $CysXXCysXXCys(X)_nCysPro$ (em que X é qualquer aminoácido) nas subunidades de 75 kDa, 51 kDa e TYKY, esta última contendo mesmo duas cópias deste domínio (Runswick *et al.*, 1989; Pilkington *et al.*, 1991a; Dupuis *et al.*, 1992,b). Esta organização de resíduos de cisteínas é característica de ferrodoxinas do tipo [4Fe-4S] nas quais constituem os ligandos do centro metálico (ver Cammack, 1992). Assim, a presença de quatro destes motivos nas subunidades acima referidas, sugere fortemente a existência de quatro centros tetranucleares Fe-S no complexo I, portanto, mais um do que aqueles que são detectados por e. p. r. (Walker, 1992).

Ao contrário do que acontece com as metalo-proteínas do tipo [4Fe-4S], a identificação das subunidades do complexo I que possuem centros de Fe-S binucleares não pode ser efectuada com um grau de confiança tão elevado recorrendo apenas à análise das suas sequências. Com efeito, a distribuição dos aminoácidos que constituem os ligandos deste tipo de centros não obedece, aparentemente, a parâmetros tão rígidos. Por exemplo, a distribuição de cisteínas do polipéptido de 24 kDa (a subunidade que alberga o centro N-1b; ver 2.4.2) não é encontrada em mais nenhuma metalo-proteína do tipo [2Fe-2S] conhecida (Pilkington & Walker, 1989). Existe, no entanto, uma fracção significativa de metalo-proteínas contendo este tipo de centros Fe-S que apresentam o motivo $CysXXCys(X)_nCysXXCys$ (ver Cammack, 1992). Esta distribuição de cisteínas é encontrada na subunidade de 75 kDa, o que levou Walker (1992) a sugerir a existência de um centro binuclear neste componente do complexo I.

2.3.4- Localização do sítio de ligação da Ubiquinona

Dada a localização membranar da ubiquinona, é de esperar que o seu sítio de ligação ao complexo I resida também na membrana interna mitocondrial. A aplicação de técnicas de marcação radioactiva por análogos da ubiquinona tem sido impedida pela dificuldade em derivar quimicamente o centro redox da molécula, mantendo a sua actividade biológica. Na tentativa de circundar este problema, vários grupos tentaram identificar os sítios de acção de vários inibidores do complexo I (*e. g.*, rotenóides, petidinas e piericidinas) tendo como premissa que este tipo de compostos apresentaria uma acção inibitória competitiva com a ubiquinona (ver Friedrich *et al.*, 1990). Nestes estudos, vários investigadores demonstraram a marcação da subunidade ND-1 por análogos radioactivos e foto-activáveis da rotenona (Earley & Ragan, 1984;

Earley *et al.*, 1987). Estes resultados, juntamente com o facto de o componente ND-1 ser, de todas as subunidades codificadas mitocondrialmente, aquele que filogeneticamente se encontra melhor conservado, levou à hipótese de o sítio de ligação da ubiquinona se encontrar nesta subunidade (ver Friedrich *et al.*, 1990). No entanto, estudos cinéticos posteriores revelaram que a rotenona não actua de um modo competitivo com a ubiquinona invalidando, pelo menos parcialmente, esta conclusão (Ahmed & Krishnamoorthy, 1992; Singer & Ramsay, 1992).

Mais recentemente, Heinrich & Werner (1992) sintetizaram um análogo da ubiquinona biologicamente activo, contendo um grupo arilazido (foto-activável) na extremidade da cadeia alifática da molécula. Experiências de marcação por foto-afinidade utilizando membranas mitocondriais de *N. crassa* levaram à identificação de um polipéptido de 9.3 kDa homólogo à subunidade B9 da enzima bovina (Heinrich *et al.*, 1992). Embora não seja possível afirmar que esta subunidade é, de facto, o sítio de ligação da ubiquinona (a posição do grupo foto-reactivo na molécula encontra-se a uma distância significativa do seu centro redox; ver também 2.4.2), os resultados obtidos sugerem, no mínimo, que esta proteína se encontra extremamente próxima do sítio de redução da ubiquinona. No entanto, a localização exacta desta subunidade e, consequentemente, a localização do sítio de ligação da ubiquinona, ainda não é conhecida. Uma das experiências apresentadas neste trabalho permitiu localizar (grosseiramente) a subunidade de 9.3 kDa (ver secção 7.5).

2.4- Relações estruturais/funcionais entre o complexo I mitocondrial e enzimas procarióticas

2.4.1- A NAD^+-hidrogenase de *A. eutrophus*

A NAD^+-hidrogenase de *Alkaligenes eutrophus*, uma bactéria quimiolitotrófica, cataliza a redução do NAD^+ pelo H_2. Funcional e estruturalmente a enzima pode ser dividida em dois domínios (Tran-Betcke *et al.*, 1990): o primeiro, constituído pelas subunidades β e δ, um átomo de níquel e, possivelmente, dois centros Fe-S, retém a capacidade de redução de variadíssimas substâncias pelo H_2; o segundo, um heterodímero contendo as subunidades α e γ , uma molécula de FMN ligada não covalentemente e três ou quatro centros Fe-S, possui actividade de NADH oxidorredutase. A recente sequenciação do operão codificante desta enzima, o locus Hox S (Tran-Betcke *et al.*, 1990), permitiu constatar uma surpreendente homologia entre as subunidades α e γ da enzima procariota e as subunidades de 75 kDa, 51 kDa e 24 kDa do complexo I mitocondrial (Pilkington *et al.*, 1991a). De facto, a subunidade α representa uma fusão das subunidades de 51 kDa e 24 kDa; a subunidade γ apresenta homologia com os 200 aminoácidos N-terminais da subunidade de 75 kDa. Esta observação não só permitiu estabelecer um elo funcional entre as subunidades de 24 kDa e 51 kDa (ambas presentes na fracção FP), mas também entre estas e a subunidade de 75 kDa (um componente da fracção IP). É, assim, possível que os sítios de ligação do FMN e NADH e os centros de Fe-S N-1b e N-3 (presentes nas subunidades de 24 kDa e 51 kDa) estejam espacialmente muito próximos dos dois centros Fe-S putativamente presentes na subunidade de 75 kDa (ver 2.3.3).

2.4.2- O complexo I de *P. denitrificans*

O procariota *Paracoccus denitrificans*, quando crescido em condições aeróbicas, produz uma NADH:ubiquinona oxidorredutase fosforilativa semelhante ao complexo I mitocondrial em muitos aspectos (Yagi, 1986, 1990, 1991). De facto, a enzima deste organismo não só apresenta todos os centros Fe-S que são detectados por e. p. r. na enzima bovina (Meinhardt *et al.*, 1987), como também é inibida fortemente pelas mesmas substâncias que actuam sobre a forma enzimática mitocondrial. Assim, não deixa de ser surpreendente que o recente isolamento desta enzima tenha revelado a existência de apenas 14 subunidades estruturais, cerca de um terço dos componentes do complexo I bovino (Yagi *et al.*, 1992). Todas as subunidades da enzima de *Paracoccus* têm homólogos na enzima de bovinos, corroborando assim os dados obtidos por e. p. r. e as características enzimáticas do complexo I procariótico (Xu *et al.*, 1991a,b, 1992a,b, 1993; ver tabela 1). A simplicidade da enzima deste organismo permite, deste modo, inferir características importantes da forma enzimática mitocondrial. Por exemplo, a ausência em *Paracoccus* de uma subunidade homóloga ao componente de 10 kDa da fracção FP da enzima bovina, sugere que esta subunidade não está envolvida directamente na ligação do centro Fe-S N-1b nem na ligação do FMN. Outra observação relevante é a ausência na enzima procariótica de um homólogo das subunidades 9.3 kDa/B9 (ver 2.3.4), sugerindo que este componente não actua como mediador directo na ligação e/ou redução da ubiquinona pelo complexo I.

No entanto, talvez a característica mais importante da enzima de *Paracoccus* seja, como referido acima, a sua simplicidade. Assim, se as catorze subunidades presentes nesta enzima são suficientes para garantir uma actividade NADH:ubiquinona oxidorredutase associada a uma translocação de protões, qual é então a função das cerca de 27 subunidades da enzima bovina que não possuem homólogos no complexo de Paracoccus? É possível que

muitas destas subunidades supranumerárias tenham como função optimizar ou regular a reacção catalizada pelo complexo I mitocondrial. O envolvimento de algumas subunidades como agentes mediadores em fenómenos de "substrate-chanelling" é também plausível (ver Srivastava & Bernhard, 1986; Porpaczy *et al.* 1987). Por outro lado, é possível que o complexo I mitocondrial desempenhe funções bioquímicas adicionais, inexistentes na enzima procariótica. Como discutido seguidamente, esta última hipótese é, cada vez mais, encarada como altamente provável.

2.4.3- A "acyl-carrier protein"

Um dos factos mais salientes revelados pela determinação da estrutura primária das subunidades do complexo I foi a descoberta de uma proteína homóloga às "acyl-carrier proteins" procarióticas (ACP; Runswick *et al.*, 1991; Sackmann *et al.*, 1991). Experiências de marcação radioactiva com [^{14}C]pantotenato em *Neurospora crassa*, assim como a determinação da massa molecular da subunidade bovina, não só demonstraram a presença do grupo fosfopanteteína covalentemente ligado a esta subunidade - o centro activo das "acyl-carrier proteins" - como revelaram a existência de vários ácidos gordos ligados por uma ligação tio-éster à fosfopanteteína (Brody & Mikolajczyk, 1988; Runswick *et al.*, 1991; Sackmann *et al.*, 1991). Estes dados indicam que a ACP presente no complexo I se encontra activa.

A função de uma "acyl-carrier protein" nas mitocôndrias tem sido alvo de pesquisa. Foi verificado que o principal grupo acilo encontrado na ACP mitocondrial de *Neurospora* advém do ácido hidroximirístico (Brody & Mikolajczyk, 1988), o principal precursor da cardiolipina fúngica (Martin & Johnson, 1983). Esta observação levou Brody *et al.* (1990) a sugerirem que a ACP mitocondrial tem como função satisfazer as necessidades lipídicas

específicas da mitocôndria (a cardiolipina tem uma localização exclusivamente mitocondrial). A identificação do ácido gordo encontrado predominantemente na ACP mitocondrial de bovinos - possivelmente o ácido hidroxioctadecanoato (Runswick *et al.*, 1991) - e o facto de o grupo acilo mais abundante na cardiolipina bovina ser do tipo $C_{18:2}$ (Schlame *et al.*, 1991) corroboram esta hipótese. Existe, assim, um paralelismo entre os diferentes grupos acilo encontrados nas ACPs destes organismos e a composição das suas cardiolipinas.

Se a razão para a existência de uma ACP mitocondrial parece estar compreendida, a sua associação a um componente da cadeia respiratória permanece intrigante, levantando a hipótese de outras subunidades do complexo I estarem também associadas com a função da ACP.

2.5- O fluxo de electrões no complexo I

A cinética de redução dos diferentes transportadores electrónicos do complexo I por NADH (ou outros agentes redutores) é extremamente rápida, verificando-se a redução de todos os centros Fe-S da enzima em alguns milisegundos. Este facto, aliado à inexistência de inibidores da enzima que interrompam o fluxo de electrões entre dois grupos redox, tem dificultado a determinação experimental da sequência de redução dos diferentes centros Fe-S (como artigo de revisão ver Beinert & Albracht, 1982). Assim, o modelo actualmente aceite sobre o fluxo de electrões através do complexo I assenta, basicamente, nos potenciais redox do ponto médio dos transportadores electrónicos da enzima. Neste modelo, o FMN actua como o oxidante imediato do NADH. Do $FMNH_2$, os electrões são transferidos para o grupo isopotencial dos centros N-1b, N-3 e N-4 (Krishnamoorty & Hinkle, 1988). É interessante notar que a remoção do centro N-4 por tratamento do complexo I com N-

bromosuccinimida não altera a cinética de transferência de electrões do NADH para a ubiquinona (Krishnamoorty & Hinkle, 1988). Este facto levou estes autores a especular que o centro N-4 poderá constituir uma ramificação (opcional) do fluxo electrónico, podendo ter como função o armazenamento temporário de electrões.

Do grupo isopotencial dos centros N-1b, N-3 e N-4, os electrões são então transferidos para o centro N-2. Embora o potencial redox do ponto médio deste centro ainda não seja conhecido com exactidão (valores entre -20 mV e -120 mV têm sido descritos), é ponto assente que este centro é o mais electropositivo da enzima, razão pela qual é considerado o redutor imediato da ubiquinona (Ingledew & Onhishi, 1980; Kotlyar *et al.*, 1990; ver Beinert & Albracht, 1982).

Recentemente, foi detectada em partículas submitocondriais incubadas na presença de NADH uma ubisemiquinona cujo comportamento de relaxamento sugere uma interação com um centro Fe-S tetranuclear (provavelmente o centro N-2) (Burbaev *et al.*, 1989; Suzuki & King, 1983). Por outro lado, se o complexo I no seu estado oxidado não for pré-condicionado com NADH, verifica-se a inexistência de actividade NADH:ubiquinona oxidorredutase, assim como a incapacidade de redução do NAD^+ por transferência electrónica inversa (Kotlyar & Vinogradov, 1990). Estas observações sugerem que a transferência de electrões do centro N-2 para a "pool" de ubiquinona existente na membrana interna envolve uma ubiquinona ligada à enzima a qual oscila entre as formas $QH^{\cdot}$ (ou $Q^{\cdot -}$) e QH_2 durante o transporte electrónico (ver fig.1). A completa oxidação deste radical livre explicaria, assim, a necessidade de se pré-incubar o complexo I com NADH para activar a enzima (Burbaev *et al.*, 1989; Kotlyar & Vinogradov, 1990; Kotlyar *et al.*, 1990).

2.6- Mecanismo de transdução energética no complexo I

A elaboração de modelos sobre os mecanismos de transdução energética do complexo I tem sido um campo prolífero para bioenergeticistas. De tal modo isto é verdade, que o número de modelos actualmente existentes sobre este assunto praticamente iguala o número de autores que se debruçam sobre a matéria. Uma das razões para estas divergências reside na própria estequiometria da reacção catalizada pelo complexo I (*i. e.*, o número de protões bombeados por molécula de NADH oxidada) que cada autor considera como válida. De facto, os valores experimentais obtidos por vários grupos não são muito coerentes e razões de 3 $H^+/2\ e^-$ (De Jong & Westerhoff, 1982), 4 $H^+/2\ e^-$ (Wikstrom, 1984; Beavis, 1987; Brown & Brand, 1988) e 5 $H^+/2\ e^-$ (Lemasters, 1984; Lemasters *et al.*, 1984) têm sido descritas. Como sugerido por Murphy & Brand (1987), é muito possível que a razão $H^+/2\ e^-$ não seja um número inteiro, uma vez que a reacção catalizada pela enzima representa uma forma deslocalizada de energia. De qualquer modo, a reacção

$$NADH + ubiquinona + 5\ H^+_{(matriz)} = NAD^+ + ubiquinol + 4\ H^+_{(EIM)}$$

onde se assume que, por cada par de electrões, 4 protões são translocados da matriz mitocondrial para o espaço intermembranar (EIM) é, em geral, considerada a mais correcta (ver Weiss & Friedrich, 1991; Walker, 1992). Esta razão H^+/e^- invalida, pelo menos parcialmente, o mecanismo de "loop" proposto por Mitchel (1966), o qual prevê a translocação de dois protões por cada dois electrões transferidos do NADH para a ubiquinona.

Muitos dos modelos existentes actualmente continuam a apoiar-se na ideia base de Mitchel, procurando acoplar passos de transferência de protões às diversas reacções de oxidação/redução que ocorrem na enzima. A julgar pelos potenciais redox do ponto médio dos vários transportadores electrónicos

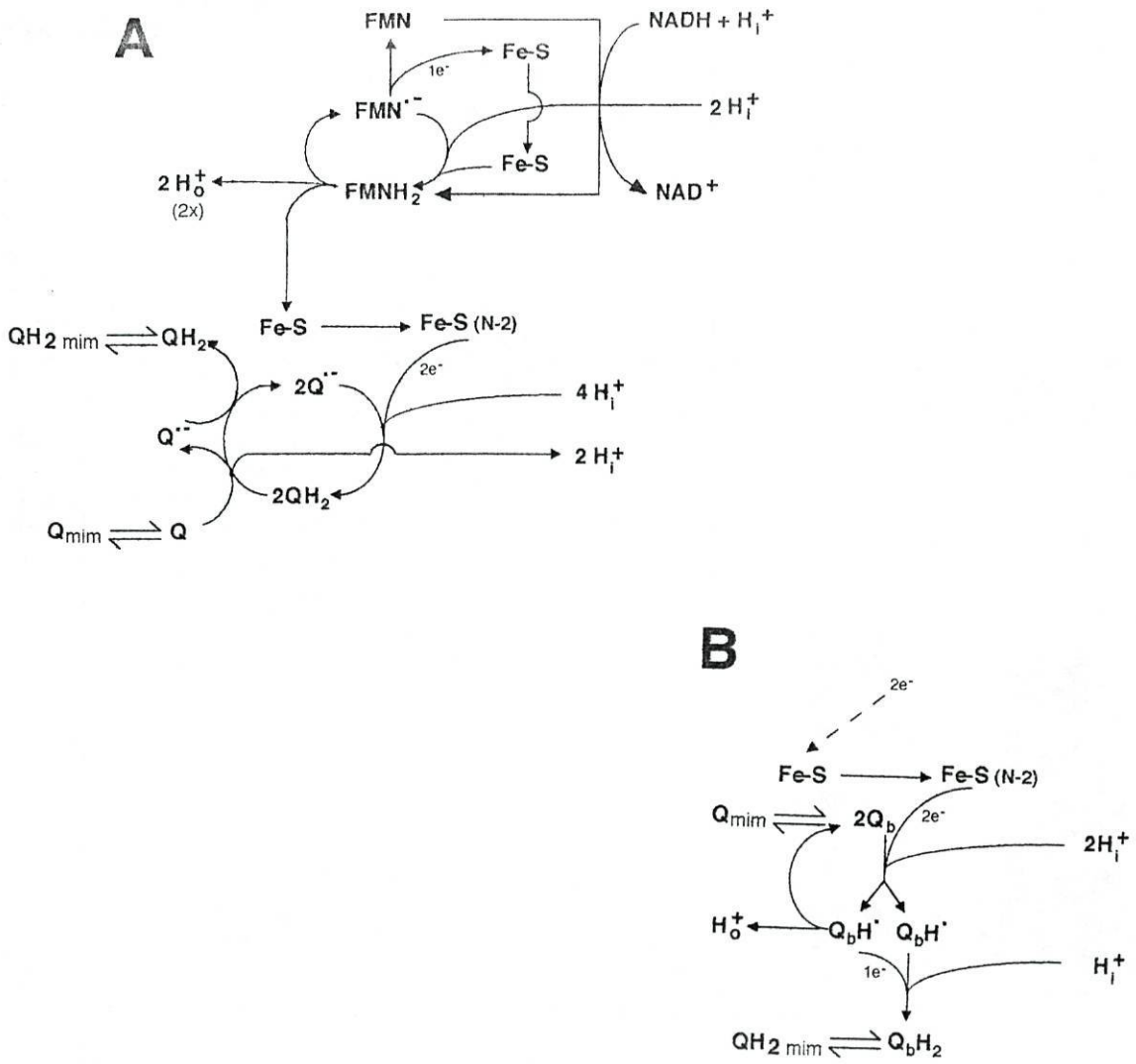

Figura 1: Possíveis mecanismos de transdução energética no complexo I.
A) Modelo proposto por Ragan (1990). Após redução do FMN pelo NADH, o $FMNH_2$ transfere um electrão para um centro Fe-S (marcado com "a") havendo, simultaneamente, translocação de dois protões para o espaço intermembranar (H_0^+). O radical livre $FMN^{\cdot -}$ assim formado é então oxidado por um outro centro Fe-S (marcado com "b") regenerando-se FMN o qual poderá ser novamente reduzido pelo NADH. Nos ciclos posteriores de oxidação/redução do FMN, a existência de um electrão armazenado nos centros Fe-S "b" e "c" possibilitará que o radical $FMN^{\cdot -}$ seja novamente reduzido captando mais dois protões da matriz (H_i^+). Este mecanismo originaria a translocação de 4 H^+ por cada 2 electrões transferidos do NADH para a ubiquinona. A substituição neste esquema do intermediário $FMN^{\cdot -}$ por $FMNH^{\cdot}$ (ver Ragan, 1990), traduzir-se-ia numa razão de 3 H^+/2 e^-. A omissão de qualquer intermediário entre o $FMNH_2$ e o FMN originaria uma razão de 2 H^+/2 e^- (Mitchel, 1966). A ubiquinona existente na membrana interna (Q_{mim}) poderá ser reduzida por uma molécula de ubiquinol ligada ao complexo I. O balanço deste passo seria a captação de dois protões da matriz mitocondrial.
B) Mecanismo alternativo para a redução da ubiquinona com transdução energética (Kotliar *et al.*, 1990). Duas moléculas de ubiquinona ligadas ao complexo I (Q_b) seriam parcialmente reduzidas pelo centro N-2. A dismutação das ubisemiquinonas assim geradas poderia resultar na extrusão de um protão para o espaço intermembranar.

do complexo I, poderia haver transdução energética na oxidação do $FMNH_2$ pelos centros isopotenciais N-1b, N-3 e N-4. Aqui, vários esquemas envolvendo ciclos de oxidação/redução da FMN têm sido propostos na tentativa de justificar razões de 3 ou 4 $H^+/2$ e- (ver Ragan, 1990; fig.1).

A transferência electrónica dos centros isopotenciais N-1b, N-3 e N-4 para o centro N-2, é também suficientemente exoenergética para suportar um processo de translocação protónica (Weiss & Friedrich, 1991). No entanto, dado o não envolvimento de protões nesta reacção redox e a ausência completa de evidência experimental, este passo não é, geralmente, englobado nos diferentes modelos de transdução energética existentes.

Finalmente, a transferência de electrões do centro N-2 para a ubiquinona poderá também estar acoplada à translocação protónica, mas apenas se se considerar um potencial redox do ponto médio para este centro de -120 mV (ver 2.5). Num dos possíveis mecanismos (Kotlyar *et al.*, 1990), o centro N-2 doaria os dois electrões a duas moléculas de ubiquinona ligadas ao complexo I (ver 2.5) as quais captariam dois protões da matriz mitocondrial. A dismutação das moléculas de ubisemiquinona assim geradas poderia traduzir-se na libertação de um protão no espaço intermembranar (ver fig. 1 B).

Como já referido, todos os mecanismos de transdução energética aqui descritos procuram acoplar, de uma forma directa, passos de transferência de protões a reacções redox. Estes modelos têm a vantagem da simplicidade e poderão mesmo, com maior ou menor facilidade, ser experimentalmente testados. No entanto, é possível que o acoplamento entre as duas actividades do complexo I (*i. e.*, actividade de NADH:ubiquinona oxidorredutase e de translocase protónica) ocorra de um modo indirecto. Por exemplo, a energia libertada na reacção redox poderia ser utilizada para provocar alterações conformacionais na enzima, alterações essas promotoras de translocação protónica. Um mecanismo deste género tem sido proposto para o complexo IV da cadeia respiratória (Citocromo *c* oxidase), uma enzima cuja conformação é

enormemente dependente do seu estado redox (como artigo de revisão ver Capaldi, 1990). Embora especulativa, a existência de um mecanismo deste tipo no complexo I não é de excluir.

2.7- Biogénese e evolução do complexo I

De acordo com a teoria endossimbiótica da origem da mitocôndria, este organelo descende de um organismo procariota que, a dado momento da evolução, estabeleceu uma relação de endossimbiose com uma célula hospedeira. Com efeito, quer estrutural quer funcionalmente, estes organelos retêm ainda hoje muitas das propriedades de um sistema procariótico (como artigos de revisão ver Tzagoloff & Myers, 1986; Attardi & Schatz, 1988). É óbvio que muitas das estruturas moleculares originalmente existentes no antecessor mitocondrial foram adaptadas à medida das necessidades da célula eucariótica, mas, pelo menos no caso do complexo I, essas alterações saldaram-se basicamente pela introdução na enzima de novos componentes. De facto, praticamente todas as subunidades do complexo I de *Paracoccus denitrificans* [organismo considerado por alguns investigadores "a free-living mitochondria" (Yagi *et al.*, 1992)], possuem homólogos na forma enzimática mitocondrial (ver tabela 1). Uma análise da estrutura primária dos componentes codificados no núcleo e pertencentes a este grupo revela uma característica interessante: todas estas subunidades possuem sequências sinal cliváveis, uma das propriedades de proteínas cujos genes foram transferidos da mitocôndria para o núcleo no decurso da evolução (ver Hartl & Neupert, 1990). Nesta perspectiva, é também relevante referir que nunca foram encontradas em procariotas proteínas homólogas às subunidades do complexo I mitocondrial sintetizadas no citoplasma e que não possuem pré-sequências. Estas observações, enquadrando-se perfeitamente na teoria endossimbiótica

da origem mitocondrial, sugerem fortemente que o domínio do complexo I com função respiratória é constituído por um "núcleo duro" de subunidades conservadas filogeneticamente (grupo das subunidades com pré-sequência e subunidades codificadas mitocondrialmente) ao qual a célula eucariótica adicionou outros componentes (subunidades sem pré-sequência).

Mesmo considerando apenas a NADH:ubiquinona oxidorredutase rotenona-sensível de *P. denitrificans* (o complexo I de mais simples composição que é conhecido), é difícil imaginar como um complexo proteico estrutural e funcionalmente tão complicado possa ter sido gerado. Uma das hipóteses mais plausíveis é que o complexo I tenha sido originado por junção de diferentes blocos ou módulos enzimáticos com actividades diferentes (Walker, 1992). Por exemplo, a dado momento da evolução, uma enzima com actividade de translocase protónica pode ter-se associado a um complexo catalizador de uma reacção redox, tendo-se criado, subsequentemente, um acoplamento entre as duas actividades. Este modelo para uma evolução modular do complexo I encontra, de facto, suporte em algumas observações efectuadas recentemente em enzimas procariotas. Por exemplo, 4 das subunidades da formato hidrogenilase de *E. coli* apresentam homologia com subunidades do complexo I (Boehm *et al.*, 1990). De um modo semelhante, e como já foi referido (ver 2.4.1), as subunidades de 75 kDa, 51 kDa e 24 kDa do complexo I são homólogas às subunidades α e γ da NAD^+-hidrogenase de *Alkaligenes eutrophus,* sugerindo que estes componentes representam um módulo enzimático responsável pela oxidação/redução do $NADH/NAD^+$ (Pilkington *et al.*, 1991a). É possível que mais enzimas com estas características venham a ser detectadas futuramente o que, indubitavelmente, aumentará o nosso conhecimento sobre a evolução, estrutura e função do complexo I.

Alguns dos trabalhos realizados sobre os mecanismos de montagem do complexo I de *Neurospora* têm também fornecido alguma evidência para uma

evolução modular do complexo I. Com efeito, um intermediário de montagem da enzima, constituído por todas as subunidades codificadas mitocondrialmente e cerca de 15 subunidades de origem nuclear, foi observado em experiências de "pulse-labelling" por Tuschen *et al.* (1990). Curiosamente, este intermediário de montagem é totalmente idêntico ao fragmento hidrofóbico da enzima (ver 2.3.1). Por outro lado, foi já referido que, quando crescidas na presença de cloranfenicol, as células de *Neurospora* produzem apenas um subcomplexo da enzima: a pequena forma do complexo I (Friedrich *et al.*, 1989; ver 2.3.1). Um resultado semelhante foi observado numa estirpe de *Neurospora* na qual o gene codificante da subunidade de 21.3 kDa do braço membranar da enzima foi inactivado (Nehls *et al.*, 1992). Estas observações levaram Weiss *et al.* (1991) não só a sugerir que os dois braços do complexo I são montados de uma forma independente, mas que este resultado poderia indicar uma origem filogenética também independente para as duas partes da enzima. No decurso da evolução, os dois braços do complexo I ter-se-iam então reunido *en bloc* para originar a enzima actual.

2.8- Aspectos médicos do complexo I

Num organismo estritamente aérobico como o homem, o complexo I desempenha duas funções fulcrais: (1) contribuindo para a formação do gradiente protónico através da membrana interna mitocondrial, a enzima é responsável por quase um terço do ATP total produzido na fosforilação oxidativa**; (2) o complexo I, como NADH desidrogenase, é um importante ponto de entrada de equivalentes redutores na cadeia respiratória, sendo o principal local de regeneração do NAD^+. Não é, assim, surpreendente que uma

** Cálculo baseado na oxidação completa de uma molécula de glucose (Smith *et al.*, 1983)

alteração, mesmo que subtil, nas propriedades catalíticas da enzima possa ter um efeito catastrófico para a célula.

Nos últimos anos, várias doenças humanas etiologicamente relacionadas com deficiências do complexo I têm sido identificadas. A grande maioria destas anomalias resulta de alterações, a vários níveis, do DNA mitocondrial. A localização mitocondrial destas mutações confere características únicas a estas doenças (como artigo de revisão ver Wallace, 1989), sendo a sua transmissão genética efectuada de um modo não mendeliano, de mãe para filhos. Por outro lado, na grande maioria dos transportadores destas mutações, verifica-se a co-existência de moléculas de mtDNA normal e mutante. Durante os ciclos de divisão celular, a população mitocondrial poderá oscilar para genótipos puros havendo, assim, a possibilidade de se gerarem indivíduos completamente sãos ou homoplásmicos para a mutação.

As consequências de uma cadeia respiratória deficiente são diferentes para diferentes tecidos, segundo a sua dependência da energia gerada na mitocôndria. Por esta razão, muitas das mutações no mtDNA dão origem a encefalopatias, das quais a neuropatia óptica hereditária de Leber (NOHL) constitui um dos exemplos melhor caracterizados a nível molecular (ver Walker, 1992). Nesta doença, várias mutações nas subunidades ND1, ND2, ND4 e ND5 têm sido identificadas, contribuindo para a compreensão da função destes componentes. Por exemplo, a substituição da arginina 340 por histidina na subunidade ND4, presente em 50 % dos indivíduos portadores de NOHL, não provoca nenhuma alteração na actividade NADH:ubiquinona oxidorredutase do complexo I (Majander *et al.*, 1991; ver Wallace *et al.*, 1988). No entanto, a taxa de oxidação de vários substractos (*e. g.*, malato) por NAD^+ em mitocôndrias isoladas destes pacientes é extremamente reduzida, sugerindo que este domínio da subunidade ND4 poderá estar envolvido em fenómenos de "substrate-chanelling" (Majander *et al.*, 1991). Uma outra mutação frequentemente associada à NOHL afecta a subunidade ND1. Neste

caso, a substituição da alanina 52 por treonina reduz para 20 % a actividade de NADH:ubiquinona oxidorredutase do complexo I, talvez por alteração do sítio de ligação da ubiquinona (Howell *et al.*, 1991; Huoponen *et al.*, 1991).

Para além de afectar o sistema nervoso central, muitas mutações mitocondriais afectam também o músculo esquelético, o coração, o rim e o fígado, dando origem a quadros clínicos de encefalopatias associadas a miopatias, acidose láctica e "stroke-like episodes" (MELAS) ou de epilepsia mioclónica associada a "ragged red fibre disease" (MERF) (ver Wallace, 1989). Nestes casos, muitas das mutações identificadas por sequenciação do mtDNA são de carácter mais geral, afectando de um modo pleiotrópico vários componentes mitocondriais. Por exemplo, vários indivíduos afectados com MELAS apresentam uma mutação pontual no $tRNA^{Leu(UUR)}$ (Goto *et al.*, 1991; Ciafaloni *et al.*, 1992). Também na síndrome de Kearns-Sayre as alterações que se verificam no genótipo mitocondrial são drásticas. Esta doença é caracterizada pela existência de grandes delecções no mtDNA (delecções de 5,9 kb e 7,0 kb têm sido detectadas nestes pacientes) provocando, consequentemente, uma deficiência geral da cadeia respiratória (Zeviani *et al.*, 1988; Holt *et al.*, 1988; Moraes *et al.*, 1989).

Deficiências em complexo I associadas a um estado patológico nem sempre têm uma origem genética. Um exemplo recente é-nos dado pela doença de Parkinson. Várias observações independentes efectuadas recentemente apontam para a possibilidade de deficiências da cadeia respiratória e, especificamente, do complexo I estarem envolvidas, como causa primária, no Parkinsonismo. Por exemplo, os níveis de NADH:ubiquinona oxidorredutase em amostras de *substantia nigra* (Schapira *et al.*, 1989, 1990), músculo esquelético (Nakagawa-Hattory *et al.*, 1992) e plaquetas (Yoshino *et al.*, 1992) extraídas de indivíduos afectados com esta doença são bastante reduzidos. Alguns estudos demonstraram a presença de quantidades subestequiométricas, ou mesmo a ausência, de várias subunidades da enzima

(Bindoff *et al.*, 1989; Parker *et al.*, 1989; Schoffner *et al.*, 1991). No entanto, o argumento mais convincente para a existência de uma relação entre complexo I e Parkinsonismo adveio da compreensão do mecanismo de acção sobre o sistema nervoso central da droga MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina), uma substância-modelo para o estudo desta doença (ver Breakefield, 1992). Esta droga, uma vez no cérebro, é convertida em MPP^+, um catião lipofílico que é selectivamente captado pelo transportador de dopamina existente nas sinapses dopaminérgicas. Uma vez no citosol destes neurónios, o MPP^+ é concentrado nas mitocôndrias por um processo de difusão passiva. Apesar do MPP^+ ser um inibidor fraco do complexo I, as altas concentrações observadas na matriz mitocondrial (da ordem dos 10 mM) são suficientes para bloquear significativamente toda a cadeia respiratória, acabando por resultar na senescência da própria célula e, consequentemente, numa histopatologia em tudo semelhante à observada no Parkinsonismo (Singer *et al.*, 1987; Singer & Ramsay, 1990).

3 - **Objectivos do trabalho**

O trabalho aqui apresentado teve como objecto de estudo o complexo I de *Neurospora crassa*. As razões para a escolha de tal organismo são várias: (1º) o complexo I deste fungo é extremamente semelhante ao de bovinos (ver 2.2.1) permitindo, assim, a extrapolação de informação entre os dois sistemas; (2º) dadas as suas características morfológicas e bioquímicas (o fungo *N. crassa* é um coenócito com alto teor em mitocôndrias) é possível obter grandes quantidades de mitocôndrias intactas num curto espaço de tempo, um aspecto importante em experiências de biogénese mitocondrial *in vitro*; e (3º), relativamente a um eucariota superior, o fungo *N. crassa* é de fácil manipulação genética, um factor a considerar em experiências (futuras) de inactivação genética.

O trabalho executado poderá ser dividido em duas fases segundo o aspecto do complexo I que foi abordado. Assim, a primeira fase deste estudo consistiu na recolha de informação sobre a estrutura do complexo I de *Neurospora crassa*. Tal objectivo foi abordado experimentalmente segundo duas estratégias independentes, mas complementares, como a seguir se explicita. Primeiramente, as estruturas primárias de várias subunidades da enzima foram determinadas por sequenciação dos cDNAs respectivos. O interesse deste trabalho era múltiplo: (1º) tentar inferir o papel de um dado componente do complexo I pela eventual existência de homologias com outras proteínas de função conhecida; (2º) observar a existência de domínios nestas subunidades que poderiam, *a priori*, contribuir para o conhecimento das suas estruturas e mecanismos de montagem; e (3º) aumentar a bateria de subunidades do complexo I com estrutura primária conhecida, não só tendo em vista a realização de experiências de inactivação genética, mas também para possibilitar a localização de domínios proteicos relevantes para a função dessas subunidades, recorrendo a análises filogenéticas.

A segunda estratégia utilizada para a caracterização estrutural do complexo I consistiu na realização de várias experiências com o intuito de determinar a interacção que uma dada subunidade estabelece com a membrana interna mitocondrial assim como, se possível, a sua topologia membranar. Este conjunto de experiências culminou com o isolamento e caracterização de um subcomplexo que representa uma parte (ou mesmo a totalidade) do braço membranar do complexo I.

A segunda fase do trabalho aqui apresentado incidiu sobre a biogénese do complexo I. Neste âmbito, foram estudados os mecanismos de "sorting" de duas subunidades pertencentes ao braço membranar da enzima, uma das quais possuidora de uma pré-sequência. Finalmente, tentou-se determinar se era possível simular *in organello* todo o processo de montagem de uma subunidade. O objectivo deste trabalho era o desenvolvimento de um sistema que permitisse, de um modo rápido e eficaz, o estudo da biogénese do complexo I.

4 - **Resultados obtidos**

Tendo como premissa que o conhecimento da estrutura primária de uma proteína poderá, eventualmente, elucidar diversos aspectos da sua função e estrutura, a primeira fase deste trabalho foi dedicada à caracterização da estrutura primária de várias subunidades do complexo I. Esta estratégia pareceu-nos de particular importância uma vez que, à data de início deste projecto, apenas três das subunidades codificadas nuclearmente da enzima fúngica e 3 subunidades da enzima de bovinos haviam sido caracterizadas (Fearnley *et al.*, 1989; Pilkington & Walker, 1989; Runswick *et al.*, 1989; Videira *et al.*, 1990a,b,c).

Para este efeito, começou-se por produzir uma bateria de anticorpos mono-específicos dirigidos contra várias subunidades do complexo I de *N. crassa*. O objectivo era duplo: possibilitar a clonagem de cDNAs usando técnicas de imuno-rastreio de um banco de expressão de cDNAs construído em λgt 11 e desenvolver ferramentas para a caracterização bioquímica das várias subunidades.

Por rastreio do banco de expressão, clones codificantes das subunidades de 78 kDa (resultados não mostrados; ver Preis *et al.*, 1991), 20.9 kDa (secção 7.1), 17.8 kDa (secção 7.3), 14.8 kDa (secção 7.4) e 9.3 kDa (resultados não mostrados; ver Heinrich *et al.*, 1992) foram isolados. A sequenciação dos cDNAs codificantes das subunidades de 20.9 kDa, 17.8 kDa, 14.8 kDa e, ainda, da subunidade de 12.3 kDa (um clone isolado e mapeado geneticamente pelo grupo de A. Videira; secção 7.2) permitiu a racionalização de algumas observações efectuadas em experiências de localização e de biogénese. Estes três aspectos, *i. e.*, estrutura, localização e biogénese (quando abordada), serão descritos e discutidos conjuntamente para cada uma das subunidades caracterizadas.

4.1 - **A subunidade de 20. 9 kDa**

A estrutura primária da subunidade de 20.9 kDa (NUO-20.9) foi comparada com as sequências de proteínas compiladas em várias bases de dados. Nenhuma homologia com proteínas de função conhecida foi detectada, pelo que o seu papel no complexo I permanece por definir. É, no entanto, de referir a existência de uma similaridade de 62% entre os aminoácidos 10-72 da NUO-20.9 e os aminoácidos 283-353 da proteína M (matriz) do virus da para-influenza (Galinski *et al.*, 1987). O significado desta similaridade é, porém, desconhecido.

A análise da estrutura primária da NUO-20.9 revelou a existência de um domínio com potencial para atravessar um sistema membranar (Rao & Argos, 1986; ver secção 7.1), sugerindo que esta subunidade é uma proteína intrínseca de membrana. De facto, duas observações independentes corroboram esta possibilidade: (1º) a subunidade de 20.9 kDa não é extraível de membranas mitocondriais por tratamento alcalino, podendo, aliás, ser isolada como constituinte de um subcomplexo que representa uma parte (ou mesmo a totalidade) do braço membranar do complexo I (ver secção 7.5); (2º) a subunidade de 20.9 kDa é um componente do fragmento hidrofóbico do complexo I (ver 2.3.1; U. Nehls, comunicação pessoal).

A topologia membranar da NUO-20.9 foi também um dos parâmetros experimentalmente abordado. Os resultados obtidos com a técnica de digestão proteolítica de mitocôndrias na presença de digitononina (Hartl *et al.*, 1986; ver secção 7.1 para detalhes) sugerem que a subunidade de 20.9 kDa se encontra exposta ao meio aquoso do espaço intermembranar.

Na tentativa de elucidar os mecanismos de "sorting" desta subunidade, o cDNA codificante da NUO-20.9 foi transcrito e traduzido *in vitro* na presença de [^{35}S]metionina. O precursor radioactivo assim obtido foi então utilizado em experiências de importação mitocondrial *in vitro*. Os resultados destas

experiências demonstraram que esta subunidade não possui uma sequência-sinal clivável. O facto de o precursor sintetizado *in vitro* co-migrar em SDS-PAGE com a subunidade isolada a partir de uma preparação de complexo I (resultados não mostrados) apoia, também, esta conclusão. Assim, é óbvio que a informação que dirige este polipéptido para a mitocôndria reside na proteína matura. Tal função poderá ser desempenhada pelo domínio N-terminal da NUO-20.9 (residuos 1-17), dado esta região apresentar propriedades típicas de sequências-sinal mitocondriais (ver Hartl *et al.*, 1989).

A subunidade de 20.9 kDa não requer a presença de um potencial de membrana ($\Delta\Psi$) para interactuar com a membrana externa mitocondrial. No entanto, a translocação através deste sistema membranar só ocorre na presença de um potencial na membrana interna. Este facto sugere que a importação mitocondrial da NUO-20.9 ocorre nos sítios de contacto entre as duas membranas (ver Pfanner *et al.*, 1992).

Significativamente, cerca de 80 % da subunidade importada *in vitro* não é extraível de membranas mitocondriais por tratamento alcalino. Adicionalmente, à semelhança da subunidade endógena (*i. e.*, a subunidade presente no complexo I), este material só é sensivel à acção de proteases quando a membrana externa mitocondrial é dissolvida pela acção da digitonina. Estas duas observações sugerem fortemente que a subunidade importada *in vitro*, não só foi inserida na membrana interna mitocondrial, como adquiriu uma topologia membranar similar à da subunidade endógena. Aparentemente, esta espécie encontra-se na sua verdadeira via biossintética.

As proteínas com destino à membrana interna mitocondrial podem seguir dois mecanismos de "sorting" distintos (como artigo de revisão ver Pfanner & Neupert, 1990). Certas proteínas (*e. g.*, a translocase de ATP/ADP; Pfanner & Neupert, 1987) são inseridas na membrana interna mitocondrial imediatamente após terem translocado a membrana externa (mecanismo não conservativo). Alternativamente, algumas proteínas são, numa fase inicial, completamente

translocadas para a matriz mitocondrial e, só depois, inseridas na membrana interna (*e. g.*, a proteína Fe-S de Rieske; Hartl *et al.*, 1986; mecanismo conservativo). Qual o tipo de mecanismo seguido pela subunidade de 20.9 kDa? Duas observações sugerem que este polipéptido é inserido na membrana interna imediatamente após ter translocado a membrana externa: (1º) A subunidade de 20.9 kDa não possui nenhuma sequência-sinal clivável, uma característica unanimemente aceite como fundamental para dirigir uma dada proteína para a matriz mitocondrial (ver Hartl *et al.*, 1989); (2º) toda a NUO-20.9 importada *in vitro*, mesmo a fracção que ainda não foi inserida na membrana interna (mas que permanece associada com os sistemas membranares mitocondriais), é virtualmente encontrada exposta ao meio aquoso do espaço intermembranar mitocondrial.

Deste modo, os dados apresentados sugerem a seguinte via biossintética para a subunidade de 20.9 kDa: (i) síntese da proteína precursora nos ribossomas citosólicos; (ii) interacção com a membrana externa mitocondrial num processo $\Delta\Psi$-independente; (iii) translocação da membrana externa, provavelmente nos sítios de contacto existentes entre as duas membranas; e (iv) inserção na membrana interna, onde já apresenta características da subunidade endógena. É possível que, uma vez na membrana interna, a subunidade de 20.9 kDa se associe primeiro a outros componentes do braço membranar do complexo I e, só depois, com as restantes subunidades da enzima. De facto, a subunidade de 20.9 kDa foi detectada num intermediário biossintético do complexo I semelhante, em composição, ao braço membranar da enzima (Tuschen *et al.*, 1990; Uwe Nehls, comunicação pessoal; ver 2.7).

4.2 - **A subunidade de 12.3 kDa**

A subunidade de 12.3 kDa não é extraível de membranas mitocondriais por tratamento alcalino, sugerindo que este componente do complexo pertence à parte membranar da enzima. De facto, esta subunidade é um dos componentes do fragmento membranar caracterizado neste trabalho (ver secção 7.5). Assim, poderá parecer surpreendente que a análise da estrutura primária deste polipéptido utilizando os métodos de Kyte & Doolittle (1982), de Rao & Argos (1986) ou de Engelmann *et al.* (1986) tenha revelado uma proteína essencialmente hidrofílica, uma característica aparentemente contraditória com a evidência experimental acima descrita. Convém referir, no entanto, que todos estes algoritmos procuram detectar domínios proteicos com potencial para atravessar um sistema membranar numa conformação de α-hélice. Como é óbvio, tais métodos não são aplicáveis a proteínas que adquirem outro tipo de conformação secundária quando embebidas numa membrana biológica. A porina OMP F de *E. coli* constitui um bom exemplo para o que acabámos de referir (ver Cowan *et al.*, 1992). Por outro lado, as interações proteicas que se estabelecem entre as subunidades de qualquer complexo membranar podem possibilitar a existência de resíduos de aminoácidos não hidrofóbicos no interior da membrana. É possível que a subunidade de 12.3 kDa não seja extraível de membranas mitocondriais por tratamento alcalino devido a este tipo de interacções. Aliás, análises imunológicas efectuadas com complexo I intacto demonstraram que, pelo menos os determinantes antigénicos deste polipéptido, não são acessíveis a imunoglobulinas, sugerindo que a subunidade de 12.3 kDa se encontra escudada do meio aquoso por outras subunidades da enzima (A. Videira & S. Werner, comunicação pessoal).

A comparação da estrutura primária da subunidade de 12.3 kDa com a de proteínas compiladas em bases de dados revelou a existência de uma

similaridade com a denominada "hinge-protein" do complexo III (ubiquinol:citocromo c oxidorredutase) de mamíferos (Ohta *et al.*, 1987; Wakabayashi *et al.*, 1982). É possível que estes dois polipéptidos pertençam a uma família de proteínas com a mesma (ou similar) função (ver secção 7.2). Tal hipótese demonstraria, pela primeira vez, uma relação estrutural entre dois complexos da cadeia respiratória, sugerindo que ambas as enzimas foram sujeitas a adaptações similares durante o processo evolutivo. Só a recolha de mais informação (especificamente, a identificação de outras proteínas similares) poderá confirmar esta hipótese.

4.3 - **A subunidade de 17.8 kDa**

A sequenciação de um cDNA codificante da subunidade de 17.8 kDa revelou a presença de uma região codificante para um polipéptido de 186 aminoácidos. No entanto, os 26 aminoácidos N-terminais da estrutura primária proteica deduzida a partir do cDNA não são encontrados na subunidade matura, conforme determinado por sequenciação de Edman da proteína isolada a partir de uma preparação de complexo I. Este facto sugere que este domínio da proteína corresponde a uma sequência-sinal clivável. De facto, a abundância em argininas e aminoácidos hidroxilados, assim como a potencialidade desta região em formar uma hélice anfipática (resultados não mostrados) - características típicas de pré-sequências mitocondriais (ver Hartl *et al.*, 1989) - suportam esta hipótese (ver também abaixo).

Uma característica intrigante desta subunidade é a existência de vários resíduos de histidina agrupados na extremidade N-terminal da proteína matura. O significado deste agrupamento não é, porém, conhecido. Nenhuma similaridade foi detectada entre a subunidade de 17.8 kDa e as várias

proteínas compiladas em bases de dados, pelo que este componente é mais uma das subunidades da enzima com função desconhecida.

A análise da estrutura primária da subunidade de 17.8 kDa (NUO-17.8) revela a existência de um domínio hidrofóbico com potencial para atravessar um sistema membranar (resíduos 58-78). Considerando que esta subunidade resiste à extracção alcalina de membranas mitocondriais e que é um componente do fragmento membranar caracterizado neste trabalho (ver secção 7.5), é provável que este domínio da proteína esteja, de facto, embebido no seio lipídico da membrana interna mitocondrial.

Uma característica relevante da subunidade endógena (*i. e.*, a subunidade montada no complexo I) reside no facto de só ser acessível a proteases quando a membrana interna é destruída. Nestas condições, e na presença de proteínase K, uma fracção considerável da proteína é degradada originando um péptido de 15 kDa, o qual permanece associado à membrana interna.

O cDNA codificante da subunidade de 17.8 kDa foi transcrito e traduzido *in vitro*. O precursor da subunidade foi então utilizado em experiências de importação mitocondrial. Os resultados obtidos mostram que a proteína precursora da subunidade de 17.8 kDa é eficientemente importada por mitocôndrias isoladas sendo, posteriormente, clivada pela peptidase processadora da matriz mitocondrial (MPP; Hawlitschek *et al.*, 1988; Schneider et al., 1990).

As experiências de localização efectuadas após importação *in vitro* da pré-proteína mostraram que uma fracção da subunidade importada não só foi inserida na membrana interna, como adquiriu uma topologia membranar correcta. No entanto, este processo é relativamente ineficiente uma vez que apenas 10% do material importado alcança estas características. É interessante notar que a maioria da subunidade importada *in vitro* (presumivelmente acumulada num passo biossintético anterior) é

completamente degradada por proteínase K pelo lado citosólico da membrana interna. Aparentemente, esta população de moléculas encontra-se na membrana interna de tal modo que a pré-sequência foi já clivada na matriz pela MPP, mas a parte C-terminal da proteína ainda se encontra exposta ao meio aquoso do espaço intermembranar. O facto de 90 % da subunidade importada *in vitro* ser solúvel em meio alcalino sugere que este intermediário está ligado à membrana interna mitocondrial através de interacções proteína-proteína.

O braço membranar do complexo I de *N. crassa* contém, para além das subunidades codificadas na mitocôndria, cerca de 12 subunidades codificadas nuclearmente. Deste grupo, a estrutura primária das subunidades de 21.3 kDa[a] (Nehls *et al.*, 1991), 20.9 kDa (secção 7.1), 20.8 kDa (Videira *et al.*, 1990a), 12.3 kDa (secção 7.2) e 9.3 kDa (Heinrich *et al.*, 1992) foram já determinadas. Todas estas subunidades têm em comum o facto de não possuirem sequências-sinal cliváveis e, pelo menos no caso da subunidade de 20.9 kDa, a inserção da proteína na membrana interna mitocondrial parece ocorrer pelo lado citosólico (ver secção 4.1). O facto de a subunidade de 17.8 kDa possuir uma sequência-sinal clivável poderia sugerir que este componente da enzima segue uma via de importação diferente. Obviamente que esta subunidade requer um passo adicional antes de ser incorporada no complexo I (clivagem da pré-sequência pela MPP), mas os dados obtidos não favorecem um mecanismo de "sorting" do tipo conservativo (ver 4.1). De facto, a totalidade da subunidade importada *in vitro* (e já clivada), mas ainda não integrada na membrana interna mitocondrial, é completamente degradada por proteínase K pelo lado citosólico da membrana interna. Intermediários de importação mitocondrial com estas características foram recentemente detectados em estudos efectuados com proteínas da matriz mitocondrial (Hwang *et al.*, 1991). No entanto, em todos os exemplos documentados, estes intermediários foram obtidos em condições sub-óptimas de importação *in vitro*

(*i. e.*, concentrações baixas de ATP na matriz mitocondrial). O sistema de importação *in vitro* utilizado no estudo aqui apresentado, para além de conter ATP, contém também um sistema de regeneração de ATP (ver Pelham & Jackson, 1976). Assim, é improvável que esta seja a razão para a existência deste intermediário, sugerindo que esta espécie resulta do próprio mecanismo de importação da subunidade de 17.8 kDa. Deste modo, poder-se-á sugerir que este componente do complexo I segue um mecanismo de importação não conservativo segundo o qual o polipéptido, uma vez em contacto com a maquinaria de importação da membrana interna, escapa, por movimento lateral, para a fase lipídica da membrana.

É possível que, uma vez na membrana interna, a subunidade de 17.8 kDa siga um mecanismo de montagem similar ao proposto para a subunidade de 20.9 kDa (ver secção 4.1), mas tal possibilidade, na ausência de dados experimentais, permanece hipotética.

4.4 - **A subunidade de 14.8 kDa**

A análise da estrutura primária da subunidade de 14.8 kDa (NUO-14.8) revelou que este componente do complexo I é uma proteína essencialmente hidrofílica, sem domínios com potencial para atravessar um sistema membranar numa conformação de α-hélice. Por outro lado, esta subunidade está presente na pequena forma do complexo I, um subcomplexo de composição polipeptídica semelhante à do braço periférico da enzima (Friedrich *et al.*, 1989; Hofhaus *et al.*, 1991; ver secção 2.3.1). Adicionalmente, a subunidade de 14.8 kDa não é detectada no fragmento membranar caracterizado neste trabalho (ver secção 7.5), sendo extraível de membranas mitocondriais por tratamento alcalino (resultados não mostrados). Estas

observações sugerem que a NUO-14.8 é uma proteína de membrana extrínseca.

Das quatro subunidades caracterizadas neste estudo, a NUO-14.8 é o único componente da enzima fúngica conservado no complexo I de bovinos. Dada a distância filogenética entre estes dois organismos é de supor que este componente desempenha um papel importante na enzima. É, no entanto, de referir que esta subunidade não está presente no complexo I de *P. denitrificans* nem em enzimas procarióticas relacionadas com o complexo I (Boehm *et al.*, 1990; Tran-Betcke *et al.*, 1990; Yagi *et al.*, 1992; ver secção 2.4), sugerindo que a sua função é exclusiva à forma enzimática mitocondrial. Tal hipótese implicaria, em termos evolutivos, que a subunidade de 14.8 kDa é já uma invenção da célula detentora de mitocôndrias, o que, de certo modo, a ausência de uma sequência sinal clivável na NUO-14.8 leva a supor (ver secção 2.7).

Experiências de importação mitocondrial *in vitro* demonstraram que a subunidade de 14.8 kDa requer a existência de um potencial de membrana para ser importada por mitocôndrias isoladas. Se, previamente a uma experiência de importação, as mitocôndrias forem sujeitas a um suave tratamento proteolítico (Pfaller *et al.*, 1987), a taxa de importação da subunidade de 14.8 kDa é significativamente diminuída, sugerindo que a importação deste componente do complexo I é mediada por componentes proteináceos existentes na membrana externa mitocondrial.

Uma vez importado, uma pequena fracção do polipéptido sintetizado *in vitro* é encontrada associada com o complexo I. Várias experiências controlo demonstraram que esta associação reflecte, de facto, uma montagem autêntica da subunidade de 14.8 kDa no complexo I. Tal observação sugere que toda a maquinaria enzimática necessária para a biogénese do complexo I se encontra operacional em mitocôndrias isoladas, permitindo, assim, o uso deste sistema experimental no estudo deste fenómeno.

4.5 - Isolamento e caracterização de um fragmento membranar do complexo I

A técnica da extracção alcalina de membranas é uma metodologia que permite caracterizar o tipo de interacção que uma dada proteína estabelece com um sistema membranar (Fujiki *et al.*, 1982). Ao contrário de outras metodologias bioquímicas empregues no estudo de proteínas de membrana (*e. g.*, extracção com agentes caotrópicos, experiências de separação de fases utilizando detergentes do tipo Triton X-114, marcação com sondas hidrofóbicas) a extracção alcalina origina resultados claros e facilmente reprodutíveis. Esta foi a razão para o uso intensivo desta técnica neste trabalho. Foi, assim, possível classificar as subunidades de 78 kDa (resultados não mostrados; ver Heinrich *et al.*, 1992), 49 kDa (secção 7.3) e 30.4 kDa (secção 7.1) como proteínas de membrana extrínsecas; as subunidades de 20.9 kDa, 17.8 kDa, 12.3 kDa (ver secções 7.1 a 7.3) e 9.3 kDa (resultados não mostrados; ver Heinrich *et al.*, 1992) foram classificadas como proteínas de membrana intrínsecas.

Recentemente, verificamos também que a subunidade de 20.8 kDa (Videira *et al.*, 1990a) é resistente à extracção alcalina de membranas mitocondriais (ver abaixo). Por outro lado, anticorpos dirigidos contra esta subunidade têm a capacidade de imunoprecipitar complexo I a partir de mitocôndrias previamente solubilizadas com triton X-100. Estas observações levaram-nos a testar se, após extracção alcalina de membranas mitocondriais, era ainda possível co-imunoprecipitar subunidades do complexo I utilizando um anticorpo (denominado As-22a; Videira & Werner, 1989) que reconhece fortemente o componente de 20.8 kDa. Tal experiência poderia permitir a identificação de subunidades do complexo I que se encontram embebidas na fase lipídica da membrana interna.

Com efeito, um fragmento constituído por cerca de 18 subunidades foi obtido. A composição polipeptídica deste subcomplexo é semelhante à descrita para o denominado fragmento hidrofóbico do complexo I (Tuschen *et al.*, 1990; ver secção 2.3.1). Por exemplo, várias subunidades codificadas mitocondrialmente e a subunidade de 20.9 kDa são encontradas em ambos os fragmentos (ver secção 7.5). No entanto, os dois subcomplexos não são idênticos uma vez que, pelo menos a subunidade de 20.8 kDa, não está presente no fragmento hidrofóbico descrito por Tuschen *et al.* (1990) (ver também Weiss *et al.*, 1991).

Uma análise comparativa entre a composição polipeptídica do fragmento membranar descrito aqui e a do subcomplexo Iα da enzima bovina (Finel *et al.*, 1992; Walker, 1992; ver secção 2.3.1), revelou a existência de componentes em comum. Especificamente, as subunidades de 20.8 kDa e 9.3 kDa da enzima fúngica possuem homólogos no subcomplexo Iα: as subunidades PGIV e B9, respectivamente (Walker et al., 1992). Este resultado sugere que estes dois componentes do complexo I se encontram na interface dos braços membranar e periférico do complexo I: a sua co-purificação com um ou outro domínio da enzima depende do organismo e técnica utilizada.

Esta observação tem importantes implicações no caso da subunidade de 9.3 kDa. Este polipéptido foi identificado como fazendo parte do sítio de ligação da ubiquinona (Heinrich & Werner, 1992; Heinrich et al., 1992). É, assim, plausível assumir que o sítio de ligação da ubiquinona se encontra na junção dos dois braços do complexo I. Tal topologia para o sítio de ligação da ubiquinona poderia sugerir que os electrões não atravessam longitudinalmente o braço membranar do complexo I [um esquema que surge frequentemente em vários modelos enzimáticos para o complexo I (ver por exemplo Weiss *et al.*, 1991; Walker, 1992)] mas apenas o atravessam parcialmente (ver figura 2 na secção 7.5).

5 - CONCLUSÕES FINAIS

O estudo aqui apresentado foi delineado de acordo com dois objectivos principais: (i) contribuir para a compreensão da estrutura e, se possível, função de alguns dos componentes do complexo I e (ii) aprofundar os conhecimentos no domínio da biogénese desta enzima.

Especial relevo foi dado ao primeiro objectivo. A razão de tal opção é óbvia: só o conhecimento da estrutura de uma enzima possibilitará compreender plenamente a sua função, biogénese e evolução. Uma das estratégias experimentais utilizadas para este fim consistiu na determinação da estrutura primária de 4 subunidades do complexo I. As localizações destas (e de outras) subunidades da enzima foi outro dos aspectos abordados, conseguindo-se, deste modo, a racionalização de alguma da informação obtida por sequenciação. Adicionalmente, foi isolado um subcomplexo que representa uma parte (ou a totalidade) do braço membranar do complexo I, o que possibilitou não só ter uma perspectiva mais completa sobre os componentes da enzima que se encontram embebidos na membrana interna mitocondrial, como também localizar (grosseiramente) o sítio de ligação da ubiquinona.

Nenhuma das subunidades caracterizadas neste estudo apresenta homologia com proteínas de função conhecida, pelo que os seus papéis no complexo I continuam por definir. Obviamente que outro tipo de estratégia terá que ser utilizada para descortinar a função deste componentes do complexo I. Neste sentido, os cDNAs isolados neste trabalho permitirão, por exemplo, a realização de experiências de inactivação genética.

Apesar da determinação da estrutura primária de subunidades do complexo I nem sempre permitir inferir as suas funções na enzima, a informação adquirida por sequenciação, quando perspectivada filogeneticamente, pode revelar aspectos importantes da evolução e mesmo da estrutura/função do complexo I. Neste contexto, é de interesse referir que, das

4 subunidades caracterizadas neste trabalho, 3 não apresentam homologias com componentes do complexo I de bovinos, elevando, assim, para cinco o número de subunidades de *N. crassa* com estas características (ver tabela 1, secção 2.2.1). Tal observação não implica que as duas enzimas sejam significativamente diferentes. De facto, em termos de massa proteica, estas cinco subunidades representam apenas cerca de 10% da enzima de *N. crassa* [assume-se uma massa de 700 kDa para o complexo I deste organismo (ver Weiss *et al.*, 1991)]. O que é relevante notar é o facto de 4 destas subunidades pertencerem ao braço membranar do complexo I. Do mesmo modo, é intrigante que para todos os componentes codificados nuclearmente e pertencentes ao subcomplexo Iβ da enzima bovina ainda não tenham sido encontrados homólogos na enzima fúngica. Aparentemente, uma parte do braço membranar do complexo I é, filogeneticamente, bastante variável. É possível que este domínio do complexo I tenha uma contribuição menor para a função respiratória da enzima do que a desempenhada pelo braço periférico (ver também secção 7.5).

No domínio da biogénese do complexo I, o segundo objectivo deste trabalho, os mecanismos de "sorting" de duas subunidades pertencentes ao braço membranar do complexo I foram estudados. Os dados obtidos sugerem que as duas proteínas precursoras (uma das quais detentora de uma pré-sequência) seguem um mecanismo de "sorting" do tipo não conservativo. No entanto, a demonstração de que é possível utilizar um sistema *in organello* para o estudo da biogénese do complexo I foi, sem dúvida, uma das conclusões mais relevantes deste trabalho. Tal observação permitirá estudar vários aspectos da montagem da enzima que dificilmente poderiam ser abordados *in vivo*. Por exemplo, os efeitos na biogénese do complexo I de variáveis como a concentração de ATP e de iões metálicos na matriz mitocondrial e a existência de um potencial de membrana poderão, agora, ser facilmente determinados. É possível que tais estudos conduzam à identificação

de intermediários biossintéticos do complexo I aumentando, simultaneamente, o nosso conhecimento sobre a estrutura da enzima.

6 - Bibliografia

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7 - TRABALHO EXPERIMENTAL

TRABALHO 7.1

Biochem. J. (1992) 288, 29–34 (Printed in Great Britain)

Primary structure and mitochondrial import *in vitro* of the 20.9 kDa subunit of complex I from *Neurospora crassa*

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The 20.9 kDa subunit of NADH:ubiquinone oxidoreductase (complex I) from *Neurospora crassa* is a nuclear-coded component of the hydrophobic arm of the enzyme. We have determined the primary structure of this subunit by sequencing a full-length cDNA and a cleavage product of the isolated polypeptide. The deduced protein sequence is 189 amino acid residues long and contains a putative membrane-spanning domain. Striking similarity over a 60 amino-acid-residue domain with the M (matrix) protein of para-influenza virus was found. No other relationship with already known sequences could be detected, leaving the function of this subunit in complex I still undefined. The biogenetic pathway of this polypeptide was studied using a mitochondrial import system *in vitro*. The 20.9 kDa subunit synthesized *in vitro* is efficiently imported into isolated mitochondria, where it obtains distinct features of the endogenous subunit. Our results suggest that the 20.9 kDa polypeptide is made on cytosolic ribosomes lacking a cleavable targeting sequence, interacts with the mitochondrial outer membrane (in a process that does not require an energized inner membrane), and is imported into mitochondria at contact sites. The 20.9 kDa subunit is then inserted into the inner membrane acquiring a topology similar to that of the already assembled subunit.

INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.99.3), the first enzyme in the mitochondrial respiratory chain, catalyses the transfer of two electrons from NADH to ubiquinone with concomitant translocation of four or five protons across the mitochondrial inner membrane (for reviews see Ragan, 1987; Weiss *et al.*, 1991). The enzyme is a complicated assembly of about 32 subunits, seven of which are encoded and synthesized in mitochondria (Chomyn *et al.*, 1985, 1986; Videira & Werner, 1989). One FMN, a still undefined number of iron–sulphur clusters (Beinert & Albracht, 1982; Ohnishi *et al.*, 1985) and probably one internal ubiquinone (Suzuki & King, 1983; Burbaev *et al.*, 1989) comprise the redox groups of complex I.

Recently, a number of primary structures of subunits from ox and *Neurospora* complex I have been determined. In a few cases, well-known functional domains (Dupuis *et al.*, 1991; Pilkington *et al.*, 1991), or interesting sequence similarities with chloroplast (Shinozaki *et al.*, 1986; Fearnley *et al.*, 1989; Videira *et al.*, 1990*a*; Dupuis *et al.*, 1991; Masui *et al.*, 1991) and bacterial proteins (Böhm *et al.*, 1990; Pilkington *et al.*, 1991; Preis *et al.*, 1991) were found, allowing new insights into the function of these particular subunits. However, it is obvious that our knowledge about structure and function of complex I is still very limited. The recent finding of an acyl-carrier protein among complex I subunits (Runswick *et al.*, 1991; Sackmann *et al.*, 1991) illustrates this point and strongly suggests the existence of biochemical activities in this enzyme other than electron transport and proton translocation.

Because of the enormous complexity of this oligomeric protein, attempts to degrade it into smaller, easier to study, fragments have been made. *Neurospora crassa* complex I can be separated into two distinct fractions (Friedrich *et al.*, 1989; Tuschen *et al.*, 1990; Wang *et al.*, 1991): the so-called small form of complex I [an assembly of 13 nuclear-encoded subunits containing the NAD(H)-binding site and three Fe–S clusters], which is made in *Neurospora* cells poisoned with chloramphenicol; and the hydrophobic fragment, a fraction obtained by chaotropic treatment of the isolated complex, which comprises all the mitochondrially encoded subunits, about 10 nuclear-coded subunits and one Fe–S cluster. Among the latter group of constituents the ubiquinone-binding polypeptide was found (H. Heinrich, J. E. Azevedo and S. Werner, unpublished work).

Electron microscopy studies revealed that *N. crassa* complex I forms an L-shaped rod, with one arm embedded in the membrane (the hydrophobic arm), and the other (the peripheral arm) protruding into the mitochondrial matrix (Hofhaus *et al.*, 1991). Most importantly, these studies showed the equivalence between the small form of the enzyme and the peripheral arm and, on the other hand, the identity of the hydrophic fraction with the hydrophobic arm. Thus, complex I seems to be constituted of two relatively independent structural units which are thought to have independent assembly pathways also (Tuschen *et al.*, 1990).

In the present paper we describe the cloning of a cDNA encoding a 20.9 kDa subunit of the hydrophobic arm of complex I. As an attempt to elucidate the assembly of this subunit we have studied its mitochondrial import pathway *in vitro*.

MATERIALS AND METHODS

The following procedures were employed according to the references cited: growth of *N. crassa* (wild type OR74A) (Werner, 1977); preparation of complex I (Ise *et al.*, 1985; Filser & Werner, 1988); isolation of mitochondria for import *in vitro* experiments (Rassow *et al.*, 1989); SDS/PAGE (Laemmli, 1970; Videira & Werner, 1989); Western-blotting (Towbin *et al.*, 1979) and detection of horseradish peroxidase- or alkaline phosphatase-

Abbreviations used: SEM buffer (250 mM-sucrose, 1 mM-EDTA, 10 mM-Mops/KOH, pH 7.2); SEMK, SEM buffer containing 100 mM-KCl; M, matrix; cyt *c*, cytochrome *c*; α-IDH, α-isocitrate dehydrogenase.

The nucleotide sequence reported here has been submitted to the EMBL Database under the accession number X60829.

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conjugated second antibodies on Western blots (Roswell & White, 1978; Blake *et al.*, 1984); synthesis of proteins in rabbit reticulocyte lysate (Amersham International, Amersham, Bucks., U.K.) (Pelham & Jackson, 1976) in the presence of [^{35}S]-methionine (specific activity 1000 Ci/mmol; Amersham) by coupled transcription/translation (Stueber *et al.*, 1984; Hartl *et al.*, 1986); preparation of reticulocyte lysate to supplement import reactions (Hartl *et al.*, 1986); determination of protein concentration (Bradford, 1976); extraction of mitochondria with carbonate buffer (Fujiki *et al.*, 1982); automated Edman degradation of a CNBr-cleavage fragment of the isolated subunit (Wachter & Werhahn, 1979; Eckerskorn *et al.*, 1988).

The strategy used for screening a λgt11 cDNA expression library, subcloning and sequencing cDNA inserts has been described previously (Videira *et al.*, 1990*b*).

The import assays were carried out as reported (Hartl *et al.*, 1986) with the following modifications: to a microtube pre-equilibrated at 0 °C the following solutions were added: 2 μl of 0.1 M-NADH, 5 μl of 0.1 M-$MgCl_2$, 10 μl of reticulocyte lysate, 70 μl of BSA buffer [250 mM-sucrose, 3 % (w/v) lipid-free BSA, 80 mM-KCl, 10 mM-Mops/KOH, pH 7.2] and either 1 μl of 100 μM-valinomycin in ethanol or 1 μl of ethanol alone (control). Finally, 10 μl of freshly prepared mitochondria (5 mg/ml) were added. The import reaction was started by addition of 1 μl of reticulocyte lysate containing the ^{35}S-labelled 20.9 kDa subunit. The suspension was incubated for 20 min at 25 °C and divided into two parts. One half received 1 μl of proteinase K at a concentration of 1 mg/ml in SEM buffer (250 mM-sucrose, 1 mM-EDTA, 10 mM-Mops/KOH, pH 7.2) and the other half SEM buffer only. Both samples were incubated for 15 min on ice and 1 μl of 0.1 M-phenylmethanesulphonyl fluoride in ethanol was added. After a further 5 min of incubation on ice, the mitochondria were pelleted by centrifugation (10 min at 15000 *g*), resuspended gently in 200 μl of SEM buffer, centrifuged again under the same conditions and then either processed for SDS/PAGE analysis, or used for the salt and alkaline extractions.

The salt extraction was performed as follows: aliquots containing 200 μg of mitochondria from an import experiment were resuspended in 2 ml of SEM buffer containing various concentrations of NaCl (0–0.5 M). After sonicating as described (Hartl *et al.*, 1986), the samples were divided into two portions. One half was kept on ice as a control (named total sample) and the other was separated into membrane pellet and supernatant by centrifugation for 1 h at 165000 *g*. The membrane fractions were resuspended in 100 μl of 2 % (w/v) Triton X-100 in water and then 1 ml of SEM buffer containing the original NaCl concentration was added. All the other samples (i.e. the supernatants and the total samples) were supplemented with the same volume of detergent solution. Finally the protein was precipitated by adding trichloroacetic acid (100 % w/v) to reach a final 10 % (w/v) concentration. More than 90 % of the radioactive material was precipitated, as judged by liquid scintillation counting.

For the digitonin fractionation experiment (Hartl *et al.*, 1986) a 22-fold scale-up of the import reaction described above was done (i.e. 1.1 mg of energized mitochondria were used) with the following modifications: after 20 min of incubation at 25 °C, trypsin (2 mg/ml in SEM buffer) was added to a final concentration of 30 μg/ml. The suspension was incubated for 15 min on ice and a 30-fold excess (by wt.) of soy bean trypsin inhibitor was added. After a further 5 min on ice, the mitochondria were re-isolated, washed (see above) and resuspended in SEM buffer containing 100 mM-KCl (SEMK) at a protein concentration of 5 mg/ml. Aliquots (20 μl) of this suspension were added to prechilled tubes already containing 4 μl of SEMK buffer (controls), or 4 μl of various digitonin solutions in SEMK to give the final desired detergent concentrations (0.05–0.3 %). After mixing briefly, the samples were incubated for 2 min on ice and were then immediately diluted with a 20-fold volume of SEMK. Proteinase K (1 mg/ml in SEM) was added to a final concentration of 30 μg/ml and, after 15 min on ice, phenylmethanesulphonyl fluoride (0.1 M in ethanol) was added to obtain a 2 mM solution. The samples were incubated for 5 min on ice, centrifuged for 20 min at 48000 *g*, washed with 1 ml of SEMK and subjected to SDS/PAGE.

RESULTS

Isolation and sequence analysis of cDNA clones

Phages (4×10^5) of a λgt11 cDNA expression library were screened with a monospecific antibody raised an individual subunit of complex I having an apparent molecular mass of 19 kDa (see Fig. 1). Five phages giving positive results were isolated and shown to carry inserts showing sequence identity by Southern-blot analysis (results not shown). The largest insert (about 950 bp) was subcloned in the pGEM4 transcription vector (Promega), yielding the plasmid pNUO-20.9, and was sequenced (Fig. 2). The cDNA is 994 bp long and consists of a 22 bp 5′-untranslated region, an open reading frame of 567 bp and a 405 bp 3′-untranslated region. The sequence GTCACAATGT surrounding the first ATG is nearly identical to the optimum translation initiation sequence of *N. crassa* (RTCACAATGG) (Paluh *et al.*, 1988). The open reading frame encodes a 189 amino-acid-residue protein with a calculated molecular mass of

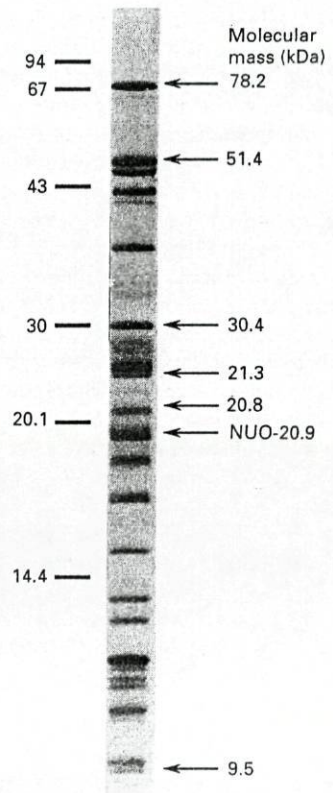

Fig. 1. SDS/PAGE of subunits of complex I from *N. crassa*

The 20.9 kDa subunit (NUO-20.9) and several subunits with already known primary structures are indicated according to the molecular masses of their mature forms [78.2 and 51.4 kDa (Preis *et al.*, 1991); 30.4 kDa (Videira *et al.*, 1990*a*); 21.3 kDa (Videira *et al.*, 1990*c*); 20.8 kDa (Videira *et al.*, 1990*b*); and the 9.5 kDa (H. Heinrich, J. E. Azevedo & S. Werner, unpublished work)]. The numbers at the left margin indicate the molecular masses of the applied standards in kDa.

(*a*)

```
-22                                                    GCAATCGTCGGCCGCCGTCACA
  1  ATGTCCAGCACTTCATCCCCGACGTACACGATCAGCAAGACACTCAATACCAATTATCCA
       M  S  S  T  S  S  P  T  Y  T  I  S  K  T  L  N  T  N  Y  P

 61  CTTATCGACAATGACCCGCACTTCCGCCGTGTAATCGGCTACGCTCGCCCGTCCGATTAT
       L  I  D  N  D  P  H  F  R  R  V  I  G  Y  A  R  P  S  D  Y

121  GTTCACGGCACCGTTGCGGGGGCTGCCGGTCCCCGCCTTCTATATCTGATGGAGAAGATG
       V  H  G  T  V  A  G  A  A  G  P  G  L  L  Y  L  M  E  K  M

181  GCACCATCAGGTGTTGGAAAGGGTGGCTTTCCCAAGGCGATGCGACTGGCGACCGCCGTG
       A  P  S  G  V  G  K  G  G  F  P  K  A  M  R  L  A  T  A  V

241  GGTTTCTTCGGCGGCTTTCTCTACTTTTACCAACGATCCATCCTCCGTTTCTATGGCATG
       G  F  F  G  G  F  L  Y  F  Y  Q  R  S  I  L  R  F  Y  G  M

301  AGCGAGAACGCCCGCGAGGTACAGATGGACATGCGTGAGATGGTTGACAAGGTCAAGGCT
       S  E  N  A  R  E  V  Q  M  D  M  R  E  M  V  D  K  V  K  A

361  GGTCAGCCACTTTACGGTGTCAGTACCCTGCCTGTCGATGTTCAAGGCATGGCTGCTCGT
       G  Q  P  L  Y  G  V  S  T  L  P  V  D  V  Q  G  M  A  A  R

421  CAGTCGAGATACTCGGCCCTTTTCTTCGCTGTCCTCCCCTGGTTCAACTTTGTGAACCAC
       Q  S  R  Y  S  A  L  F  F  A  V  L  P  W  F  N  F  V  N  H

481  AACCAGCATGGTGTCGACACAGCCAAGTACTACCAGCAGGCCGAGAGGGAGTTGGAGGCT
       N  Q  H  G  V  D  T  A  K  Y  Y  Q  Q  A  E  R  E  L  E  A

541  GAGCGCTTGGGCAAGGGAAGCTCGTCGTAAGACACGAGCATGGCGGAAGTCAAATGTGAA
       E  R  L  G  K  G  S  S  S  *

601  GGTCGAGAATGGTAGTCCGCCAGAGGGTGAGAACAGAACCTTGTTAGGCGAGAGGGCTGA
661  CCGATCGGATGATGGTGTAAACATGGAGCTTTAAACGCTGGACCAACAGAACAGAACGCA
721  AGTTAACGACCATCCCCTACCCTGATCTTTACGTGATACTTCAGGAGGTGCGTTGATTGG
781  CTGGGTTGTTAGAGGTGTTCATCACCAATAGAGAACTATGGACAAGAGGGTGGCGCTTTG
841  TGTTACGTTGATATTACGGCAGCCACTGAGATTTCGGGACCTGCCTGATGCCGCAGCCTC
901  GCTGACGTATCACTTGACAACCTCCCGATGAATTGAAGATTGGGTCTGGTTTACTCGTCA
961  AGTCTACAATCA
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(*b*)

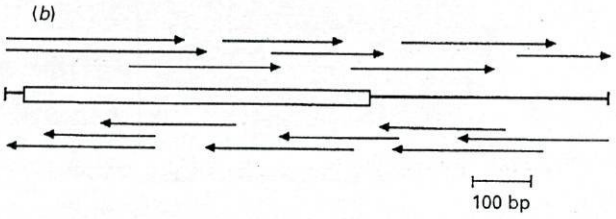

Fig. 2. cDNA sequence determination

(*a*) Nucleotide sequence and deduced protein sequence of the cDNA encoding the NUO-20.9 subunit. The amino acid sequence obtained by automated Edman degradation is underlined. A putative membrane-spanning domain is also indicated (dotted line). The stop codon is marked by an asterisk. (*b*) Sequencing strategy and structure of the cloned cDNA insert. Arrows show the direction and extent of sequence determination. The box corresponds to the coding region of the cDNA.

```
M protein (283)   SISKTLASQLVFKREICYPLMDLNPHLNLVI-WASSVEITRVDAIF
                  !***** !!         ***!* !**   ** !* ! ! ! !!
NUO-20.9 (10)     TISKTLNTN--------YPLIDNDPHFRRVIGYARPSDYVH-GTVA

M protein (328)   QPSLPGEFRYYPNIIA-KGVGKIKQWN
                   !! **   * ! !* ****
NUO-20.9 (47)     GAAGPG-LLYLMEKMAPSGVGKGGFPK
```

Fig. 3. Alignment of the sequences of the NUO-20.9 and the M protein of para-influenza virus

Numbers indicate the positions within the respective polypeptides of the first amino acid residue on each line. Amino acid identities (*) and conservative substitutions (!) are indicated. Hyphens were introduced to optimize the alignment.

20981 Da. One putative membrane-spanning domain (residues 73–88) was predicted using the method of Rao & Argos (1986). A search in a protein database revealed a striking similarity, within a 60 amino-acid-residue domain, with the so-called M (matrix) protein of para-influenza virus (Galinski *et al.*, 1987; Fig. 3).

Protein-sequence analysis

Automated Edman degradation of the electrophoretically isolated 20.9 kDa subunit (NUO-20.9) failed to give any results,

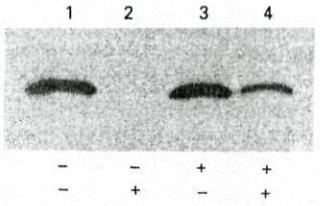

Fig. 4. Import *in vitro* of NUO-20.9 into isolated mitochondria

[^{35}S]Methionine-labelled NUO-20.9 was incubated with freshly isolated mitochondria, either in the presence (lanes 1 and 2), or absence (lanes 3 and 4) of 1 μM-valinomycin [minus and plus membrane potential ($\Delta\Psi$) respectively]. The samples in lanes 2 and 4 were treated with proteinase K (PK). After inactivation of the protease and re-isolation of the mitochondria, samples were analysed by SDS/PAGE. A fluorograph of the dried gel is shown.

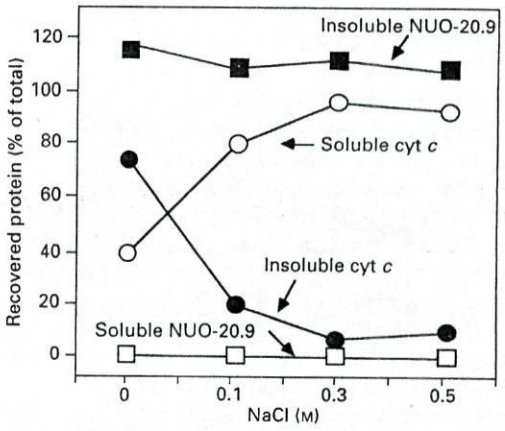

Fig. 5. NUO-20.9 imported *in vitro* is membrane bound

^{35}S-labelled NUO-20.9 was imported into mitochondria (800 μg of protein). After proteinase K treatment and re-isolation of mitochondria, aliquots containing 200 μg of mitochondrial protein were resuspended in 2 ml of SEM buffer containing the indicated NaCl concentrations. The samples were sonicated and divided into two halves. One half was kept on ice for determination of recoveries (termed 'total'). The other half was separated into a membrane fraction and a soluble fraction by centrifugation (1 h at 165000 *g*). The protein in all samples was precipitated with trichloroacetic acid (see the Materials and methods section), subjected to SDS/PAGE and blotted onto nitrocellulose. The Western-blot was first used to expose an X-ray film (to detect the subunit imported *in vitro*) and, afterwards, probed with an antiserum against cyt *c*. Membrane-associated (insoluble) NUO-20.9 and cyt *c* (■ and ● respectively), or soluble NUO-20.9 and cyt *c* (□ and ○), are expressed as a percentage of total recovered protein.

suggesting a protected *N*-terminus. Therefore, the isolated subunit was cleaved with CNBr and one fragment was partially sequenced. The sequence obtained, (M)AARQSRYSALF-FAVLPWF, matches the predicted amino acid sequence of residues 137–155, hence confirming the validity of the isolated clone.

Mitochondrial import of NUO-20.9 synthesized *in vitro*

The 20.9 kDa subunit was synthesized *in vitro* in the presence of [^{35}S]methionine and used in a mitochondrial import experiment *in vitro*. Isolated mitochondria were incubated with the NUO-20.9 subunit synthesized *in vitro*, either in the presence (Fig. 4, lanes 3 and 4) or absence (Fig. 4, lanes 1 and 2) of a membrane potential. Import into the interior of mitochondria was monitored by treatment of the import reactions with proteinase K. Proteinase-resistant subunit is found only in the presence of a

membrane potential (Fig. 4, lane 4). When the mitochondrial membrane potential is abolished no imported NUO-20.9 is observed (Fig. 4, lane 2), although binding to mitochondria is not diminished (Fig. 4, compare lanes 1 and 3). It is also clear that the imported subunit is not processed to a mature form having an appreciably different molecular mass, suggesting that this particular complex I subunit is not synthesized with a cleavable mitochondrial targeting sequence. This observation is further supported by the fact that the NUO-20.9 synthesized *in vitro* co-migrates exactly with the assembled form of the subunit upon SDS/PAGE (results not shown).

Localization of NUO-20.9

As demonstrated in the previous section NUO-20.9 can be efficiently imported into mitochondria in the presence of a membrane potential. The proteinase K resistance of NUO-20.9 observed under these conditions indicates that NUO-20.9 has at least crossed the outer membrane of mitochondria, but no more information regarding its localization can be inferred. In a first attempt at localizing the subunit imported *in vitro*, we tried to find out whether NUO-20.9 exists as a soluble or membrane-bound species inside the mitochondria. The following experiment was performed: after a standard import reaction *in vitro* in the presence of a membrane potential, mitochondria were re-suspended in SEM buffer containing various concentrations of NaCl and were sonicated; after centrifugation for 1 h at 165000 *g*, the supernatants and the membrane pellets were analysed for the presence of labelled NUO-20.9 (Fig. 5). NUO-20.9 is resistant to NaCl extraction of membranes, even at a high salt concentration (0.5 M). The *in vitro* synthesized NUO-20.9 alone subjected to the same treatment could not be sedimented under these conditions (results not shown). Thus, this result indicates that the newly imported subunit is probably tightly bound to membranes.

The nature of this interaction with membranes was investigated further by analysing the extractability of both the form imported *in vitro* and the endogenous form of NUO-20.9 with 0.1 M-Na_2CO_3 (pH 11.5). With this alkaline extraction technique it is possible to distinguish between intrinsic and peripheral membrane proteins. Mitochondria from an import *in vitro* experiment, as described above, were incubated with the carbonate solution and separated into soluble and membrane fractions by centrifugation. These fractions were analysed by Western-blotting and fluorography to detect the endogenous and the *in vitro* imported form of NUO-20.9 respectively. As shown in Fig. 6, the endogenous form of NUO-20.9 is found associated with membranes, providing strong evidence that it is an intrinsic membrane protein. This is in sharp contrast to the behaviour of the 30.4 kDa subunit of complex I, which is completely extractable under these conditions. The majority of the newly imported NUO-20.9 is also resistant to the extraction procedure, although about 20% was consistently found in the soluble fraction. When the membranes were extracted a second time with carbonate no more labelled subunit could be recovered (results not shown). Thus, we assume that the extractable protein represents an intermediate stage in the import, where the subunit interacts with the membrane (virtually all the labelled subunit is resistant to NaCl extraction), but is not yet inserted into its lipophilic environment.

To localize more specifically the subunit imported *in vitro*, as well as the endogenous NUO-20.9, we tried to assess the membrane topology of these species by subfractionation of mitochondria with digitonin. Mitochondria re-isolated from an import reaction were subjected to increasing concentrations of digitonin (in order to open successively the intermembrane space and the matrix) and a constant amount of proteinase K was added. The protease sensitivity profile of both forms of the

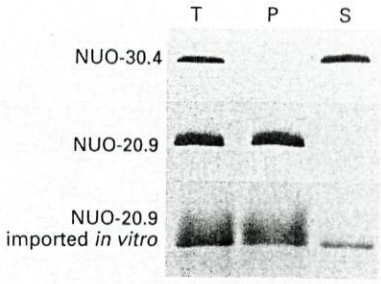

Fig. 6. Extractability of NUO-20.9 by alkaline treatment

Mitochondria (200 μg of protein) from an import *in vitro* experiment were resuspended in 2 ml of 0.1 M-Na_2CO_3 and incubated for 30 min at 0 °C. Half of the sample was kept on ice for determination of recoveries (T sample). The other half was separated into pellet (P) and supernatant (S) by centrifugation (1 h at 165000 *g*). Sample P was resuspended in 1 ml of the carbonate solution to make all solutions chemically identical. After precipitation with trichloroacetic acid [final concentration 12.5% (w/v)] the samples were subjected to SDS/PAGE. The NUO-20.9 imported *in vitro* was analysed by fluorography of a dried gel. The endogenous NUO-20.9 and the 30.4 kDa subunit were detected on Western blots with monospecific antisera.

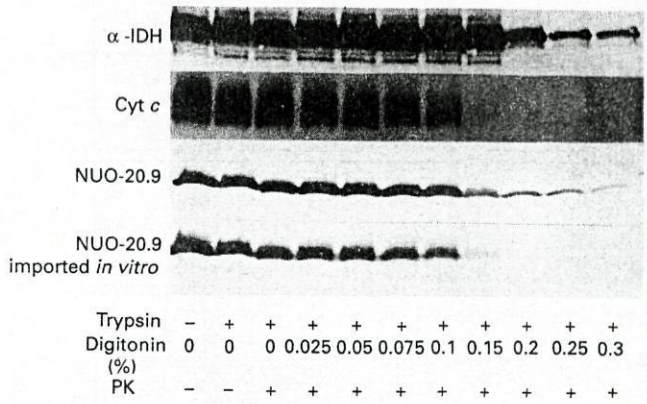

Fig. 7. Membrane topology of both the *in vitro* imported and endogenous NUO-20.9

[^{35}S]Methionine-labelled NUO-20.9 was imported into isolated mitochondria (1.1 mg of protein). An aliquot containing 100 μg of protein was kept on ice as a control (first column). The remaining mitochondria were treated with trypsin at 30 μg/ml to remove any non-imported subunit. After inactivation of the protease, mitochondria from the samples were re-isolated, washed and resuspended in SEMK buffer (see the Materials and methods section) at a protein concentration of 0.1 mg/ml. Aliquots containing 100 μg of protein were then treated for 2 min at 0 °C with the indicated amounts of digitonin and were immediately diluted 20-fold with buffer. After treatment with proteinase K (PK), as specified, mitochondria were re-isolated, washed and subjected to SDS/PAGE. The gel was blotted onto nitrocellulose and the membrane was first used to expose an X-ray film (to detect the subunit imported *in vitro*) and, afterwards, probed with antisera against cyt *c* (intermembrane space marker), α-IDH (matrix marker), and NUO-20.9.

subunit was compared with those of polypeptides of known mitochondrial localization: cytochrome *c* (cyt *c*) and α-isocitrate dehydrogenase (α-IDH) were used as markers for the intermembrane space and the matrix respectively. As shown in Fig. 7, both forms of the subunit were grossly accessible to proteinase K at a concentration of 0.15% digitonin. The same is true for cyt *c*, indicating that the intermembrane space was completely opened under these conditions. In contrast, α-IDH was not degraded at this digitonin concentration, implying that the structure of the inner membrane was still intact. However, it

should be noted that the endogenous subunit is somehow more resistant to the proteolytic action of proteinase K than the newly imported one (a fraction of the subunit remains undigested even after opening the matrix compartment). This phenomenon could reflect a more protected environment and/or a different structure of the assembled subunit. On the other hand, the existence of different subpopulations of NUO-20.9 in complex I cannot be excluded. Indeed, the stoichiometry of NUO-20.9 in relation to the 78.2 kDa and 30.4 kDa subunits is 3:1:1, as judged by laser densitometry of SDS/PAGE-resolved subunits obtained by immunoprecipitation of complex I from ^{35}S-labelled mitochondria (J. E. Azevedo & S. Werner, unpublished work).

In summary, these results strongly suggest that both the polypeptide imported *in vitro* and a considerable fraction of the endogenous form of NUO-20.9, if not all, are exposed to the mitochondrial intermembrane space.

DISCUSSION

We have cloned and sequenced a cDNA insert which encodes the 20.9 kDa subunit of complex I from *N. crassa*. In an attempt to obtain some clues about the function of this subunit we compared its deduced primary structure with the sequences compiled in the latest versions of the PIR and NBRF databases. A striking similarity over a domain of 60 amino acid residues between NUO-20.9 and the M protein of para-influenza virus (Galinski *et al.*, 1987) was found. The similarity observed is quite impressive (62%), and it is therefore possible that both regions of the two proteins may mediate the same function. However, it will be necessary to collect additional sequence data on NUO-20.9 homologues from evolutionary remote organisms to clarify this point.

To characterize the biogenetic pathway of this complex I subunit we have performed mitochondrial import experiments *in vitro*. Our results indicate that NUO-20.9 does not possess a cleavable targeting sequence. Obviously, the information to address NUO-20.9 to mitrochondria resides in the mature protein. Mitochondrial targeting sequences are generally positively charged, rich in hydroxylated residues, devoid of acidic amino acids and located at the *N*-terminus of the precursor proteins (Hartl *et al.*, 1989). The *N*-terminal region of NUO-20.9 exhibits clearly these characteristics and could therefore fulfil this function.

Furthermore we have shown that binding of the precursor protein to mitochondria is independent of a membrane potential ($\Delta\Psi$), a property of the so-called class I precursors (Hartl *et al.*, 1989). However, translocation across the outer membrane (a process that is thought to be $\Delta\Psi$-independent) is only accomplished in the presence of an energized inner membrane. This suggests that translocation across both the outer and inner membrane is a coupled event and, based on the current knowledge of import mechanisms, probably occurs at contact sites (for a review see Pfanner & Neupert, 1990).

It cannot be inferred from the import experiments *in vitro*, whether NUO-20.9 is inserted into the inner membrane at this stage (i.e. translocation at contact sites is arrested at the inner membrane), or whether the polypeptide crosses both membranes, reaches the matrix and is then redirected to the inner membrane [a mechanism that would be in agreement with the so-called conservative sorting (Pfanner & Neupert, 1990)]. The fact that we could not detect a soluble (salt-extractable) species during the import into mitochondria *in vitro* is consistent with the first pathway. However, a transient, very fast passage of NUO-20.9 through the matrix cannot be excluded. Nevertheless, it should be kept in mind that all precursor proteins sorted by the conservative pathway possess a cleavable mitochondrial targeting sequence (see Hartl *et al.*, 1989 and papers cited therein). Since this is not the case with NUO-20.9, we speculate that this subunit does not follow the conservative sorting.

Once imported, the synthesized NUO-20.9 *in vitro* mimics, in at least two aspects, the endogenous subunit: (1) it is found mainly as an intrinsic membrane protein; and (2) it acquires a membrane topology which cannot be discriminated from that of the endogenous subunit, as judged by the accessibility to proteinase K from the mitochondrial intermembrane space. Thus, these results suggest that the subunit imported *in vitro* is on its correct assembly pathway.

Pulse-labelling experiments with *N. crassa* cells (Tuschen *et al.*, 1990) demonstrated the existence of an assembly intermediate of complex I having a molecular mass of about 350 kDa. This intermediate comprises not only all the mitochondrially encoded subunits, but also a subset of nuclear-coded subunits to which the NUO-20.9 polypeptide belongs. These observations, together with our results, suggest the following biogenetic pathway for the NUO-20.9 subunit: (i) synthesis on cytosolic ribosomes of the precursor protein lacking a cleavable mitochondrial targeting sequence; (ii) interaction with the mitochondrial outer membrane in a $\Delta\Psi$-independent process; (iii) import into mitochondria, probably at contact sites; (iv) insertion into the inner membrane of the precursor protein, which already displays distinct features of the assembled form; (v) assembly into the 350 kDa intermediate; and finally, (vi) association of this intermediate with remaining nuclear-encoded subunits that, as proposed (Tuschen *et al.*, 1990) may already exist in a pre-assembled subcomplex.

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TRABALHO 7.2

The 12.3 kDa subunit of complex I (respiratory-chain NADH dehydrogenase) from *Neurospora crassa*: cDNA cloning and chromosomal mapping of the gene

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The 12.3 kDa subunit of complex I (respiratory-chain NADH dehydrogenase) is a nuclear-coded protein of the hydrophobic fragment of the enzyme. We have isolated and sequenced a full-length cDNA clone coding for this polypeptide. The deduced protein is 104 amino acid residues long with a molecular mass of 12305 Da. This particular subunit of complex I lacks a cleavable mitochondrial targeting sequence. In agreement with its localization within complex I, we have found that this subunit behaves like an intrinsic membrane protein. Nevertheless, the deduced protein is rather hydrophilic, exhibiting no hydrophobic domain long enough to traverse a membrane in an α-helical conformation. The 12.3 kDa subunit shows a significant similarity to the hinge protein of complex III, suggesting that these two polypeptides may be involved in identical functions. This complex I subunit is coded for by a single gene. Applying restriction-fragment-length-polymorphism mapping, we located the gene on the right side of the centromere in linkage group I, linked to the *lys-4* locus.

INTRODUCTION

Respiratory-chain NADH dehydrogenase (complex I, EC 1.6.99.3) is one of the components of the inner mitochondrial membrane performing proton pumping coupled to electron transfer. It is the most complicated assembly among the oligomeric enzymes that participate in oxidative phosphorylation, both in terms of prosthetic groups and polypeptide subunits [for reviews, see Ragan (1987) and Weiss et al. (1991)]. In humans and *Neurospora*, seven complex I subunits are encoded and synthesized by mitochondria (Chomyn et al., 1986; Ise et al., 1985; Videira and Werner, 1989). However, the majority of the subunits, including most that bind prosthetic groups, are encoded by the nucleus (Chomyn et al., 1988). The DNA sequences of the human mitochondrial genes have been published for quite some time (Anderson et al., 1981). In the last years, we have participated in efforts to clone the nuclear-coded proteins.

The high similarity between complex I from fungi and mammals (Ragan, 1987; Weiss et al., 1991) makes *N. crassa* a good model for studying the enzyme. Since the cloning of a 20.8 kDa polypeptide of the fungal enzyme (Videira et al., 1990a), several other nuclear-coded subunits have also been cloned in this organism. In some cases, the existence of sequence similarities to proteins of known function provided good indications about the function of those subunits (see Weiss et al., 1991).

Still unknown at present is the organization of nuclear genes for complex I subunits. Thus we started to assign genes to specific linkage groups of the *N. crassa* genome. The gene encoding the protein described here was localized on chromosome I, close to the *lys-4* locus. Our interest in these experiments is not only to gain a general idea of the chromosomal localization of genes coding for complex I subunits, but also because these assignments might be essential for gene-disruption experiments.

Here we describe the isolation and analysis of the cDNA encoding a 12.3 kDa subunit of the hydrophobic fraction of complex I. The similarity between this protein and the so-called hinge protein of the b–c_1 complex may indicate that both polypeptides play a similar role in the respiratory chain.

MATERIALS AND METHODS

A mono-specific antiserum against the 12.3 kDa protein (Videira and Werner, 1989) was used to screen a *Neurospora* cDNA library cloned in the lambda gt11 expression vector. The procedure, as well as production of fusion proteins by recombinant phage and their use for affinity purification of antibodies, and the subcloning and sequencing of both strands of cDNA inserts, have been detailed previously (Videira et al., 1990a).

The techniques for the preparation of *N. crassa* mitochondria (Werner, 1977), carbonate extraction (Fujiki et al., 1982; Azevedo et al., 1992), protein gel electrophoresis (Laemmli, 1970; Videira and Werner, 1989) and Western blotting (Towbin et al., 1979), the detection of antigens bound to filters (Azevedo et al., 1992) as well as *in vitro* transcription and translation (Maniatis et al., 1982; Videira et al., 1990a), and methods for immunoprecipitation (Werner and Sebald, 1981; Zauner et al., 1985) have been published previously.

The 12.3 kDa protein, formerly referred to as the 14 kDa subunit, was isolated from complex I (Videira and Werner, 1989) and partially sequenced from the N-terminus by automated Edman degradation (Eckerskorn et al., 1988).

The 38 *N. crassa* strains representing the Multicent-2 cross kit

Abbreviation used: RFLP, restriction-fragment-length polymorphism.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X68965.

(Metzenberg et al., 1984 were obtained from the Fungal Genetics Stock Center (FGSC # 4450-4487). Genomic DNA was isolated essentially as described by Borges et al. (1990), except that a final step of RNAase treatment, followed by organic extraction and ethanol precipitation, was included. Southern (1975) blotting on Hybond membranes, radioactive labelling of DNA using the multiprime labelling system and hybridization were performed according to the manufacturer's (Amersham) instructions.

RESULTS AND DISCUSSION

Cloning and characterization of cDNAs encoding the 12.3 kDa polypeptide (NUO-12.3)

An *N. crassa* cDNA expression library was screened with an antiserum against a nuclear-coded subunit of complex I (Videira and Werner, 1989). Several positive phages were isolated and shown, by Southern blotting, to belong to two unrelated groups (groups I and II; results not shown). In order to determine whether or not the relevant clone was isolated, *Escherichia coli* lysogens from the two groups of phage were prepared and induced for the production of the β-galactosidase fusion proteins. These were resolved by SDS/PAGE, blotted on to nitrocellulose membranes and used to affinity-purify immunoglobulins from the crude antiserum. Only the clones from group II were able to select immunoglobulins that recognize the relevant protein on Western blots of complex I (results not shown), indicating that group II phage indeed contain the correct cDNA insert.

Several cDNA inserts from phage of group II were subcloned in the pGEM4 transcription vector and sequenced at both termini. The sequences obtained were then searched for the N-terminus of the mature protein (see below). One particular cDNA insert (no. 14) was found to be full-length and was completely sequenced (Figure 1). A coding region of 312 bp, between 53 bp and 329 bp of 5′ and 3′ untranslated regions respectively, was found. The open reading frame codes for a protein of 104 amino acid residues, with a calculated molecular mass of 12305 Da. Strikingly, the predicted primary structure reveals a rather hydrophilic protein. No obvious membrane-spanning domain is found (Figure 2). The deduced primary structure of NUO-12.3 reveals the existence of only two cysteine residues. Thus this polypeptide is not a good candidate to bind iron–sulphur clusters unless other amino acids (e.g. histidine and tyrosine) serve as appropriate ligands.

In order to gain an insight into the function of this polypeptide, a sequence search in a protein data bank was performed. A significant similarity between NUO-12.3 and the so-called hinge protein of complex III from mammals was detected (see Figure 3). The *N. crassa* and bovine proteins display 47% similarity in a domain of 36 amino acids. The hinge protein mediates the interaction of cytochrome *c* with cytochrome c_1 (Wakabayashi et al., 1982), and may regulate the electron-transfer reaction between these polypeptides (Kim et al., 1987). It may be speculated that NUO-12.3 plays a similar role in complex I. In this respect it could allow the interaction between different complex I subunits or even between complex I and other proteins [for instance, with matrix dehydrogenases, a phenomenon that was reported in mammalian mitochondria (Fukushima et al., 1989)]. The last possibility is not so likely, however, if one assumes that NUO-12.3 is surrounded by other subunits of the hydrophobic arm of complex I (see below).

Protein data

The 12.3 kDa protein was isolated from complex I of *N. crassa*, applying chaotropic agents and hydrophobic-interaction chromatography, as described previously (Videira and Werner, 1989). The purified polypeptide was subjected to automated Edman degradation, and the sequence PTPESAAFLAKKPT was obtained. This sequence matches the N-terminal sequence predicted from the clone pNUO-12.3 (amino acids 2–15; underlined in Figure 1), thus confirming unambiguously the identity of

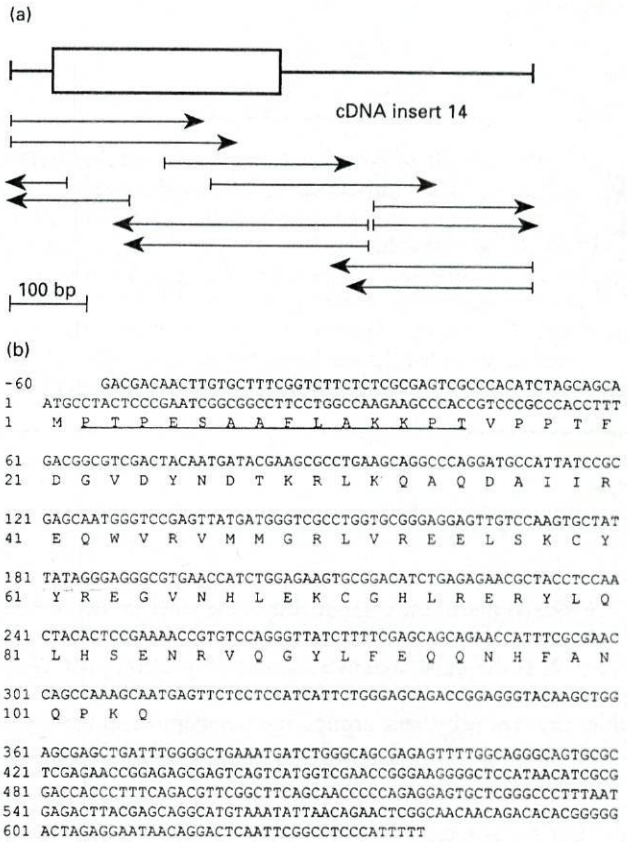

Figure 1 Nucleotide sequence of full-length cDNA and deduced primary structure of the 12.3 kDa protein

(**a**) Structure and sequencing strategy of the cDNA. The box represents the translated region. Arrows show the direction and extent of sequence determination. (**b**) cDNA and protein sequences. The amino acid positions determined also by protein sequencing (residues 2–15) are underlined.

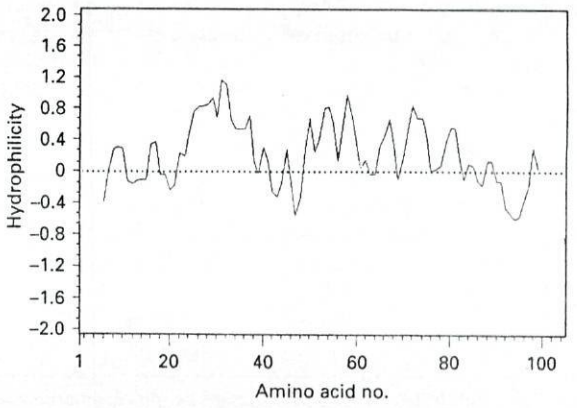

Figure 2 Hydrophilicity profile of the 12.3 kDa protein

Calculations were performed as described by Hopp and Woods (1981), using a window of 11 amino acids.

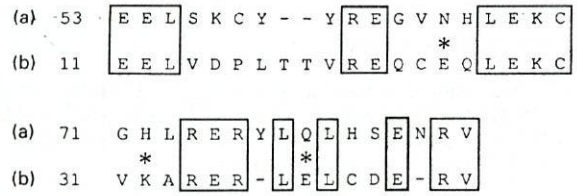

Figure 3 Alignment of the amino acid sequences of the 12.3 kDa protein (a) and the bovine hinge protein (b)

Numbers refer to positions of amino acid residues in the mature proteins. Identical residues are boxed, and conservative substitutions are indicated by asterisks. In the region shown, the human (Ohta et al., 1987) and bovine (Wakabayashi et al., 1982) hinge proteins are identical, except that the last position of the human polypeptide is occupied by aspartic acid instead of valine.

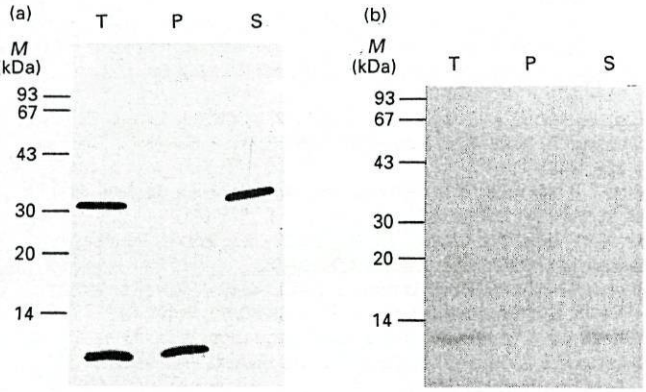

Figure 4 Localization of the 12.3 kDa protein after alkaline extraction of mitochondria

(**a**) Mitochondria were incubated with Na_2CO_3 solution, and half of the mixture was centrifuged to separate membranes (pellet) from solubilized material (supernatant). The total sample before centrifugation (T), the pellet (P) and the supernatant (S) were separated by SDS/PAGE. Then a Western blot was performed using antibodies against the 30.4 kDa and the 12.3 kDa subunits of complex I. (**b**) The 12.3 kDa polypeptide was synthesized *in vitro* in the presence of [^{35}S]methionine. The material was mixed with mitochondria and processed for Na_2CO_3 treatment as detailed in (**a**). After SDS/PAGE the gel was fluorographed. Abbreviation: *M*, molecular mass.

the isolated cDNA clone. This particular mitochondrial protein is synthesized without a cleavable targeting sequence (Videira and Werner, 1989). The initial methionine residue is, however, absent in the mature sequence.

As already mentioned, NUO-12.3 is a hydrophilic protein. Nevertheless, it is a component of the hydrophobic arm of complex I, a fragment of the enzyme that is believed to be embedded in the mitochondrial inner membrane (Tuschen et al., 1990). Thus the question is raised as to the kind of interaction through which NUO-12.3 is associated with the other subunits of the enzyme. On the basis of hydropathy data for the polypeptide one might predict that the 12.3 kDa subunit is in a hydrophilic environment. In order to test this possibility, we performed alkaline extraction of mitochondrial membranes. At the rather high pH used in this procedure (pH 11.5) protein–membrane interactions established through or with accessibility to the aqueous environment are readily disrupted. Hence, freshly isolated *N. crassa* mitochondria were incubated with 0.1 M Na_2CO_3 (pH 11.5) and separated into soluble and membrane fractions by centrifugation for 1 h at 165000 ***g***. The proteins in the pellet and supernatant were then resolved by SDS/PAGE and analysed by Western blotting with an antiserum against the 12.3 kDa subunit. As a positive control we also tested the behaviour of the 30.4 kDa subunit of complex I (Videira et al., 1990b), a protein that belongs to the peripheral arm of the enzyme (Weiss et al., 1991). This polypeptide is known to be extractable under these conditions (Azevedo et al., 1992). As shown in Figure 4(a), NUO-12.3 cannot be extracted from the mitochondrial inner membrane by applying this technique. This result strongly suggests that the interaction of NUO-12.3 with the membrane occurs in an environment not accessible to the aqueous medium. Because of this unexpected finding we still considered the possibility that the 12.3 kDa subunit *per se* would not be soluble under the conditions employed. Therefore, a parallel experiment was carried out. Immediately before treatment with the carbonate solution, mitochondria were mixed with radioactively labelled NUO-12.3 that was synthesized in a heterologous *in vitro* transcription/translation system. The samples were processed as described above, except that the gel was subjected to fluorography. As

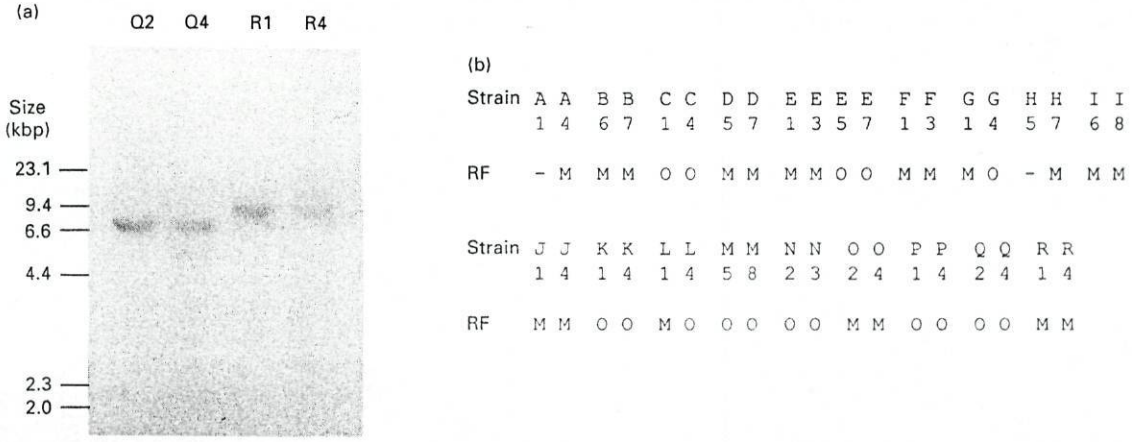

Figure 5 Chromosomal mapping of *nuo-12.3*

(**a**) Genomic DNA from the indicated strains (Q2, Q4, R1 and R4) was digested with *Eco*RI and resolved on an agarose gel. Then a Southern blot was performed using radioactively labelled cDNA insert 14 as a probe. The autoradiograph is shown. (**b**) Segregation of the RFLP among progeny of the cross between polymorphic strains. The results were obtained from an experiment as described in (**a**). Restriction fragments (RF) of the Mauriceville type (M), of the Oak Ridge type (O) and unidentified (-) are indicated.

expected, NUO-12.3 alone does not precipitate under these conditions (Figure 4b), providing evidence that only the correctly folded/assembled subunit behaves as an intrinsic membrane protein. It should be noted that the *in vitro*-synthesized subunit co-migrates with the mature/endogenous subunit upon SDS/PAGE performed under reductive or non-reductive conditions (A. Videira and S. Werner, unpublished work). Thus it is unlikely that the assembled subunit undergoes some covalent modification (e.g., lipoylation or cysteine bridging with other intrinsic membrane protein) that could explain the resistance to the alkaline extraction.

We have described the molecular cloning of the 12.3 kDa subunit of the hydrophobic arm of complex I from *N. crassa*. Notwithstanding its hydrophilic characteristics and the absence of an obvious membrane spanning domain, NUO-12.3 behaves like an intrinsic membrane protein. On the bases of double immunodiffusion (Werner and Sebald, 1981), it was already known that the protein was not recognized by the specific antiserum using intact complex I; only when isolated complex I is exposed to a detergent like SDS or a combination of deoxycholate and 2-mercaptoethanol does a precipitation reaction occur (A. Videira and S. Werner, unpublished work). Thus at least the antigenic determinants of the polypeptide seem not to be accessible to the immunoglobulins in the intact enzyme. This result, as well as the resistance of NUO-12.3 to alkaline extraction from the mitochondrial inner membrane, suggest that the polypeptide is embedded within other complex I constituents.

Chromosomal assignment of the *nuo-12.3* gene

In order to localize the gene for NUO-12.3 in the genome of *N. crassa*, the restriction-fragment-length-polymorphism (RFLP) mapping approach was applied. Genomic DNA from the individual progeny of the Multicent-2 cross kit was isolated, treated with several restriction enzymes and separated by electrophoresis in agarose gels. Southern blots were then probed with ^{32}P-labelled cDNA encoding NUO-12.3. A RFLP was found when using the enzyme *Eco*RI. The results obtained are summarized in Figure 5. Comparison with known markers (Metzenberg and Grotelueschen, 1989), revealed that the segregation pattern observed is very similar to those displayed by several genes of linkage group I. To test the linkage, the χ-square statistical test was employed. The highest value, 28.2, (considering a limit value of 3.85 for one degree of freedom), was obtained between *nuo-12.3* and *lys-4*, clearly indicating that the two genes are linked. Significant values were also obtained for other genes located in the vicinity of the *lys-4* locus.

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TRABALHO 7.3

Cloning, *in vitro* mitochondrial import and membrane assembly of the 17.8 kDa subunit of complex I from *Neurospora crassa*

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We have cloned and sequenced a cDNA encoding a 17.8 kDa subunit of the hydrophobic fragment of complex I from *Neurospora crassa*. The deduced primary structure of this subunit was partially confirmed by automated Edman degradation of the isolated polypeptide. The sequence data obtained indicate that the 17.8 kDa subunit is made as an extended precursor of 20.8 kDa. Resistance of the polypeptide to alkaline extraction from mitochondrial membranes and the existence of a putative membrane-spanning domain suggests that the 17.8 kDa subunit is an intrinsic (bitopic) membrane protein. The *in vitro* synthesized precursor of the 17.8 kDa subunit can be efficiently imported into isolated mitochondria, where it is cleaved to the mature species by the metal-dependent matrix-processing peptidase. The *in vitro* imported mature subunit is found mainly exposed to the mitochondrial intermembrane space. However, a significant fraction of the imported polypeptide acquires the same membrane topology as the endogenous subunit, indicating that correct assembly in the mitochondrial inner membrane did occur.

INTRODUCTION

The rotenone-sensitive NADH:ubiquinone oxidoreductase of the mitochondrial respiratory chain (complex I; EC 1.6.99.3) is a complicated assembly of about 34 nuclear-encoded subunits (at least 25 in *Neurospora crassa*), seven mitochondrially encoded subunits, several iron–sulphur clusters, one FMN and, possibly, an internal ubiquinone. The enzyme catalyses the transfer of electrons from NADH to ubiquinone and couples the energy of this redox reaction to the extrusion of protons across the mitochondrial inner membrane (for reviews see Ragan, 1987; Weiss et al., 1991; Walker, 1992).

Recently, electron microscopy studies have suggested that *Neurospora crassa* complex I has the form of an L-shaped rod with one arm embedded in the membrane (the hydrophobic arm) and the other (the peripheral arm) protruding into the mitochondrial matrix (Hofhaus et al., 1991). Interestingly, the two arms of the enzyme could be assigned to the two complementary subcomplexes previously characterized by Weiss and coworkers. Thus the hydrophobic arm seems to correspond to the hydrophobic fragment (Friedrich et al., 1989), a fraction of the enzyme obtained by chaotropic salt treatment of the isolated complex that comprises all mitochondrially encoded subunits, about 12 nuclear-encoded subunits, one iron–sulphur cluster (Weiss et al., 1991) and the ubiquinone binding site (Heinrich et al., 1992). The peripheral arm was shown to be similar to the so-called small form of complex I (Tuschen et al., 1990), an assembly of 13 nuclear-encoded subunits containing the NADH binding site and three Fe–S clusters (Wang et al., 1991), which is made in *Neurospora* cells poisoned with chloramphenicol.

Although knowledge about complex I is increasing rapidly, the mechanisms of catalysis, assembly and the fine structure of the enzyme are far from being understood. Moreover, the existence of other biochemical activities in this multimeric enzyme is plausible. Indeed, the finding of an active acyl carrier protein among complex I subunits (Runswick et al., 1991; Sackmann et al., 1991) may suggest additional, still unknown, functions.

As an attempt to elucidate some of these aspects, we have recently cloned several cDNAs coding for subunits of *Neurospora* complex I (Videira et al., 1990a,b,c, 1993; Preis et al., 1991; Azevedo et al., 1992; Heinrich et al., 1992). Here the cloning and sequencing of a 17.8 kDa subunit belonging to the hydrophobic fragment of the enzyme are described. Using an *in vitro* mitochondrial import system, the first steps of the biosynthetic pathway of this subunit could be followed and characterized.

MATERIALS AND METHODS

The following procedures were employed according to the references cited: growth of *Neurospora crassa* (wild-type OR74A) (Werner, 1977); preparation of complex I (Ise et al., 1985; Filser and Werner, 1988); isolation of mitochondria for *in vitro* import experiments (Rassow et al., 1989); SDS/PAGE (Laemmli, 1970; Videira and Werner, 1989); Western blotting (Towbin et al., 1979) and detection of horseradish peroxidase- or alkaline phosphatase-conjugated second antibodies on Western blots (Roswell and White, 1978; Blake et al., 1984); automated Edman degradation of proteins blotted on to glass fibre membranes (Eckerskorn et al., 1988); synthesis of proteins in rabbit reticulocyte lysate (Pelham and Jackson, 1976) in the presence of [^{35}S]methionine (specific radioactivity 1000 Ci/mmol; Amersham) by coupled transcription/translation (Stueber et al., 1984; Hartl et al., 1986); preparation of reticulocyte lysate to supplement import reactions (Rassow et al., 1989); determination of protein concentration (Bradford, 1976); alkaline extraction (Fujiki et al., 1982); and salt extraction of mitochondria (Hartl et al., 1986; Azevedo et al., 1992).

The strategy used for screening of a λgt11 cDNA expression library, subcloning and sequencing of cDNA inserts has been described previously (Videira et al., 1990b).

The import assays were carried out essentially as outlined in a previous paper (Azevedo et al., 1992). Usually, 50–100 μg of freshly prepared *Neurospora* mitochondria were added to 90 μl of import buffer, consisting of 2 mM NADH, 5 mM $MgCl_2$, 70 %

Abbreviations used: MPP, mitochondrial matrix-processing peptidase; NUO-X, NADH:ubiquinone oxidoreductase subunit of X kDa; $\Delta\Psi$, membrane potential across the mitochondrial inner membrane; CCHL, cytochrome *c* haem lyase; mtHSP70, mitochondrial 70 kDa heat shock protein.

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The nucleotide sequence data reported will appear in the EMBL, DDBJ and GenBank Nucleotide Sequence Databases under accession no. X71414.

(v/v) BSA buffer [250 mM sucrose, 3% (w/v) lipid-free BSA, 80 mM KCl, 10 mM Mops/KOH, pH 7.2] and 10% (v/v) untranslated reticulocyte lysate (Rassow et al., 1989) which contains an ATP regeneration system (10 mM phosphocreatine and 22 units/ml creatine kinase). The import reaction was started by addition of 1 μl of reticulocyte lysate containing the ^{35}S-labelled precursor of the 17.8 kDa subunit. The suspension was incubated at 25 °C for 20 min. The membrane potential ($\Delta\Psi$) was dissipated with the K^+ ionophore valinomycin (1 μM final concentration) added from a 100-fold concentrated stock solution in ethanol. The control reactions (in the presence of $\Delta\Psi$) received the same volume of ethanol. Proteinase treatment was carried out at 0 °C for 30 min with proteinase K (50 μg/ml) or trypsin (30 μg/ml). In the majority of the experiments the use of trypsin was avoided because of the fact that digestion of the precursor protein with this proteinase results in a 15.5 kDa fragment that remains associated with the mitochondrial outer membrane. The proteinases were inactivated by an incubation step at 0 °C for 5 min in the presence of 1 mM phenylmethanesulphonyl fluoride (for proteinase K), or a 30-fold excess (by wt.) of soy bean trypsin inhibitor if trypsin was used. Mitochondria were re-isolated from the import reactions by centrifugation (10 min at 15000 ***g***), resuspended in 200 μl of SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2), centrifuged again under the same conditions and then either processed for SDS/PAGE analysis or used for the salt and alkaline extractions.

The mitochondrial matrix-processing peptidase (MPP) was inhibited as reported (Hartl et al., 1986) with the following modifications: 200 μg of *Neurospora* mitochondria were preincubated for 5 min at 0 °C in import buffer containing 12 mM EDTA. *o*-Phenanthroline (20 mM in ethanol) was then added to a concentration of 0.2 mM. After 2 min at 0 °C, the import reaction was started as described above and the suspension was incubated for 10 min at 25 °C. The sample was divided into halves and the mitochondria were re-isolated. One half was resuspended in import buffer containing the above-mentioned additions to maintain the inhibition of MPP. The other half was resuspended in import buffer containing 1 mM $MnCl_2$ and 1 μM valinomycin in order to chase the already imported but still unprocessed 17.8 kDa subunit. Both samples were incubated for 10 min at 25 °C and treated with proteinase. The mitochondria were then re-isolated, washed and analysed by SDS/PAGE.

For the digitonin fractionation (Schnaitmann and Greenawalt, 1968; Glick et al., 1992), 2.2 mg of mitochondria was subjected to an import experiment in the presence of a membrane potential. An aliquot corresponding to 200 μg of mitochondrial protein was removed and mock-treated throughout the procedure. The other fraction (2.0 mg of protein) was treated with trypsin (30 μg/ml) to remove non-imported precursor. After inactivation of the proteinase, the suspension was diluted 3-fold with SEM buffer containing 100 mM KCl (SEMK buffer) and centrifuged for 10 min at 15000 ***g***. The mitochondria were then resuspended in SEMK buffer at a protein concentration of 9.2 mg/ml. An aliquot corresponding to 200 μg of mitochondria was removed and mock-treated from this stage of the procedure. Proteinase K (1 mg/ml in SEMK buffer) was added to the remaining mitochondria to a final concentration of 220 μg/ml. Immediately, aliquots (28 μl) containing 200 μg of mitochondrial protein were transferred to microtubes containing 12 μl of various digitonin dilutions in SEMK buffer to give the final desired detergent concentrations (0–0.3%). After 1 min on ice, the suspensions were diluted with 160 μl of SEMK buffer and incubated for a further 30 min on ice. The proteinase was inactivated and the protein in the samples was precipitated with trichloroacetic acid [10% (w/v) final concentration], washed with cold acetone and processed for SDS/PAGE analysis.

RESULTS

Isolation and sequence analysis of cDNA clones

About 2×10^5 phages of a λgt11 cDNA expression library were screened with a monospecific antibody prepared against the electrophoretically isolated 17.8 kDa subunit of complex I (Figure 1). Four positive phages were obtained and purified by two cycles of re-screening. The inserts were subcloned in the pGEM4 transcription vector (Promega) and sequenced. The complete sequence of the largest clone and the sequencing strategy are shown in Figure 2. The cDNA is 778 bp long and contains an open reading frame of 558 bp. Thus a 186-amino-acid protein with a calculated molecular mass of 20898 Da is obtained. The deduced protein sequence contains the 19 amino acid residues obtained by automated Edman degradation of the isolated subunit, indicating that the correct clone was indeed isolated.

The first 26 N-terminal residues of the open reading frame are not present in the mature protein. This particular domain has all the characteristics of a cleavable mitochondrial targeting sequence: an abundance of arginine and hydroxylated amino acids and the capability to form an amphipathic α-helix are well-known features of mitochondrial presequences (Hartl et al., 1989). Furthermore, the amino acid sequence around the cleavage site is in good agreement with the consensus sequence for the mitochondrial matrix peptidase (Hartl and Neupert, 1990), suggesting that the precursor protein is cleaved to a mature form of 17849 Da (see also below).

The hydropathy profile and the transfer energy plot of the mature protein (Kyte and Doolittle, 1982; Engelman et al., 1986; results not shown) revealed the existence of a single hydrophobic

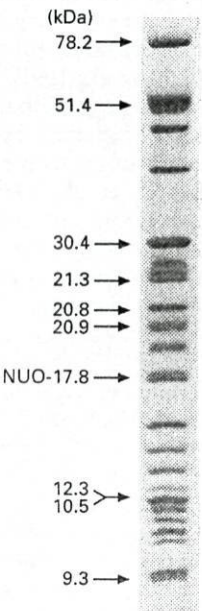

Figure 1 SDS/PAGE of subunits of complex I from *Neurospora crassa*

NUO-17.8 and several subunits of previously deduced primary structures are indicated according to the molecular masses of their mature forms: 78.2 and 51.4 kDa (Preis et al., 1991); 30.4 kDa, 21.3 kDa, 20.8 kDa and 12.3 kDa (Videira et al., 1990a,b,c, 1993); 20.9 kDa (Azevedo et al., 1992) and 9.3 kDa (Heinrich et al., 1992).

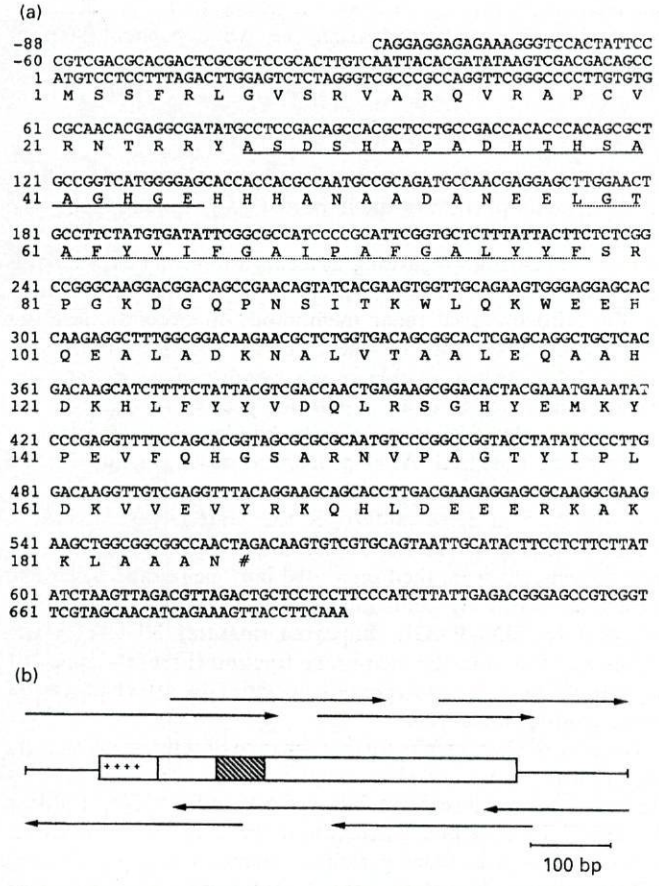

Figure 2 Nucleotide sequence and deduced protein sequence of the cDNA encoding the precursor of NUO-17.8

(**a**) The amino acid sequence obtained by automated Edman degradation of the isolated subunit is underlined. The putative membrane-spanning domain is also indicated (dotted line). The stop codon is marked by #. (**b**) Sequencing strategy and structure of the cloned cDNA. Horizontal arrows show the direction and extent of sequence determination. The open reading frame is boxed, and the presequence (+) and the putative membrane-spanning domain (▨) are indicated.

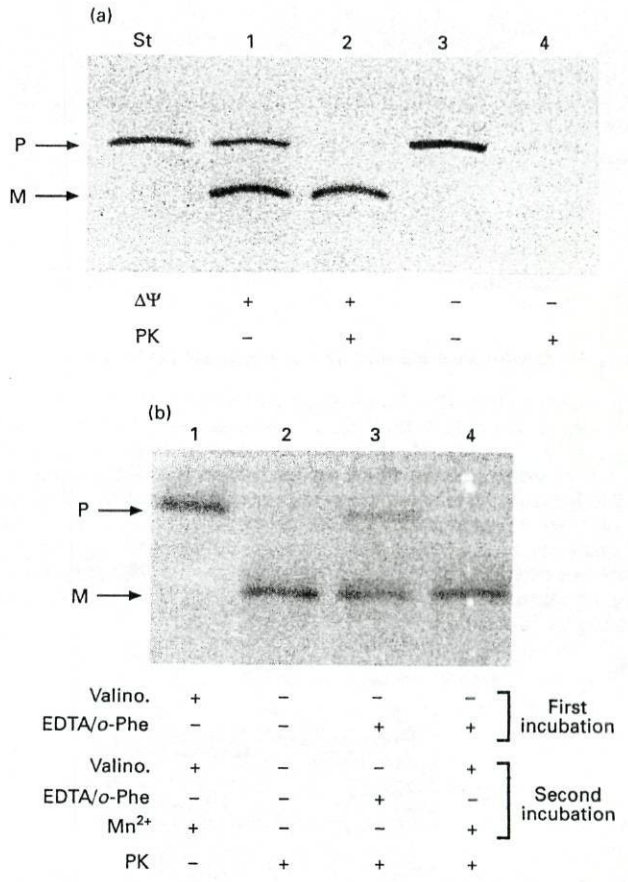

Figure 3 *In vitro* mitochondrial import and processing of preNUO-17.8

(**a**) The ^{35}S-labelled precursor of NUO-17.8 subunit was incubated with freshly prepared *Neurospora* mitochondria (30 μg of mitochondrial protein), in either the presence (lanes 1 and 2) or absence (lanes 3 and 4) of a $\Delta\Psi$. The samples in lanes 2 and 4 were treated with proteinase K (PK). After inactivation of the proteinase, the samples were analysed by SDS/PAGE and fluorography. St indicates standard, i.e. 40% of the reticulocyte lysate containing the radioactive precursor used in lanes 1–4. (**b**) PreNUO-17.8 is processed by MPP. ^{35}S-labelled preNUO-17.8 was mixed with *Neurospora* mitochondria (100 μg of protein) in one of three different solutions: lane 1, import buffer (see the Materials and methods section) containing 1 μM valinomycin (Valino.); lane 2, import buffer alone; lanes 3 and 4, import buffer containing 12 mM EDTA and 0.2 mM *o*-phenanthroline (*o*-Phe). After incubation for 10 min at 25 °C, the mitochondria from all samples were re-isolated by centrifugation and incubated for a further 10 min at 25 °C (second incubation) in 500 μl of import buffer containing 1 μM valinomycin and 1 mM $MnCl_2$ (lanes 1 and 4), import buffer alone (lane 2) or import buffer containing 12 mM EDTA and 0.2 mM *o*-Phe (lane 3). The samples were treated with proteinase K as specified and analysed as described above. P and M indicate precursor and mature forms respectively of the 17.8 kDa subunit.

domain of sufficient length to cross a membrane in an α-helix conformation (Figure 2; residues 58–78).

No homologous proteins could be detected in the last release of the PIR database, leaving the function of this protein in complex I still undefined.

Mitochondrial import of *in vitro* synthesized 17.8 kDa subunit of NADH:ubiquinone oxidoreductase (NUO-17.8)

The 17.8 kDa subunit precursor (preNUO-17.8) was synthesized *in vitro* in the presence of [^{35}S]methionine and incubated with isolated *Neurospora* mitochondria. Import of the radiolabelled precursor was assessed by resistance of the polypeptide to externally added proteinases.

As shown in Figure 3(a), preNUO-17.8 was efficiently imported and processed to the mature form by isolated mitochondria. This process is clearly dependent on the existence of $\Delta\Psi$ (Figure 3a, lanes 1 and 2). In the absence of a $\Delta\Psi$, neither import nor processing occurred (lanes 3 and 4). However, binding of the precursor protein to the mitochondrial outer membrane was not affected (compare lane 1 with lane 3), a characteristic of the so-called class I mitochondrial precursor proteins (Hartl et al., 1989).

PreNUO-17.8 is processed to the mature form by MPP. This was demonstrated by performing *in vitro* import into energized *Neurospora* mitochondria in the presence of chelating agents (Schmidt et al., 1984). Under these conditions a strong (but not complete) inhibition of the MPP was achieved. As shown in Figure 3(b), a reasonable amount (40%) of the imported (proteinase-resistant) protein remained unprocessed (Figure 3b, lane 3), although a fraction of the imported subunit had escaped the MPP blocking. [It is interesting to note the existence of a third protein species migrating slightly slower than the mature NUO-17.8. This species may represent a polypeptide chain of between one and three amino-acid residues longer than the

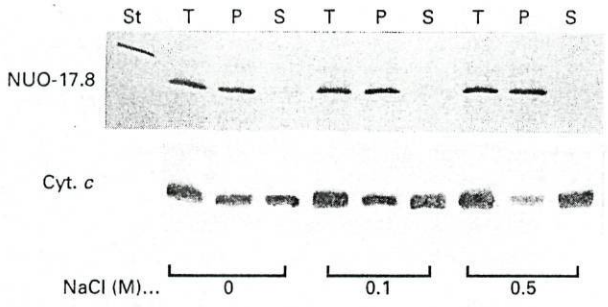

Figure 4 *In vitro* imported NUO-17.8 is membrane-associated

[^{35}S]Methionine-labelled preNUO-17.8 was incubated with energized mitochondria (600 μg of mitochondrial protein) at 25 °C for 20 min. After proteinase treatment and re-isolation of mitochondria, aliquots (200 μg of protein) were resuspended in SEM buffer (see the Materials and methods section) containing the indicated concentrations of NaCl. The samples were sonicated and divided into two halves; one half was kept on ice for determination of recoveries (sample T), and the other aliquot was separated into membrane (P) and soluble (S) fractions by centrifugation (1 h at 165000 *g*). After trichloroacetic acid precipitation, the protein in the samples was subjected to SDS/PAGE. Cytochrome *c* (Cyt. *c*) was detected on Western blots using a monospecific antiserum. St (standard), indicates 10% of the reticulocyte lysate containing the ^{35}S-labelled precursor used for each lane.

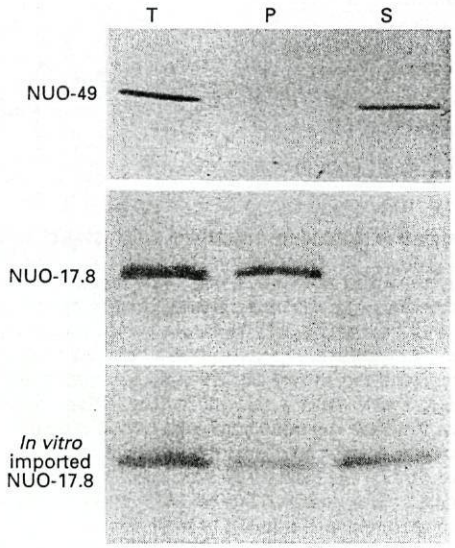

Figure 5 Alkaline extractability of *in vitro* imported NUO-17.8

Mitochondria re-isolated from an *in vitro* import experiment (200 μg of protein) were resuspended in 1 ml of 0.1 M Na_2CO_3 and incubated for 30 min on ice. Half of the sample was kept on ice (sample T), and the other half was separated into membrane (P) and soluble (S) fractions as described in the legend to Figure 4. Endogenous NUO-17.8 and the 49 kDa subunit of complex I (NUO-49) were detected on Western blots using monospecific antisera. The *in vitro* imported subunit was detected by fluorography of a dried gel.

mature subunit, and could reflect anomalous processing activity of MPP under these sub-optimal conditions. It is striking, however, that this species is apparently chased to the mature form (see below). The significance of this finding was not investigated further.] The observed uncleaved form is not a dead-end species: after the MPP activity was restored in these mitochondria (see the Materials and methods section), the precursor protein was readily chased into the mature 17.8 kDa subunit. Furthermore, this chase could take place even if the $\Delta\Psi$ across the inner membrane had been previously dissipated with valinomycin (Figure 3b, lane 4), suggesting that this uncleaved intermediate had already passed the $\Delta\Psi$-dependent step of insertion into the mitochondrial inner membrane.

Localization of the *in vitro* imported NUO-17.8

The data presented in the previous section indicate that at least the N-terminal portion of the imported polypeptide reaches the mitochondrial matrix, where it is processed by MPP. However, no more conclusions regarding its localization can be reached. Is the *in vitro* imported subunit inserted into its final localization, i.e. the mitochondrial inner membrane? In order to test this possibility, we first tried to assess whether the *in vitro* imported NUO-17.8 exists as a soluble or as a membrane-associated form inside mitochondria. Thus ^{35}S-labelled preNUO-17.8 was incubated with isolated *Neurospora* mitochondria in the presence of a membrane potential. After proteinase treatment, in order to remove non-imported protein, mitochondria were re-isolated and sonicated in SEM buffer (see the Materials and methods section) containing various concentrations of NaCl (0–0.5 M). The suspensions were then separated into membrane pellet and soluble fractions by centrifugation at 165000 *g* for 1 h and analysed by SDS/PAGE. Imported (mature) NUO-17.8 was exclusively found in the membrane fraction (Figure 4, lanes P), suggesting that the polypeptide is strongly attached to the mitochondrial membranes.

In order to characterize further the type of interaction that the imported subunit establishes with the mitochondrial membranes, we have analysed its extractability with 0.1 M Na_2CO_3 (Fujiki et al., 1982). This alkaline extraction method allows the discrimination of intrinsic from peripheral membrane proteins, since only the former class of proteins resists the extraction procedure. Thus mitochondria re-isolated from an *in vitro* import experiment, as described above, were incubated with the carbonate solution and separated into soluble and membrane fractions. The fractions were then subjected to SDS/PAGE and fluorography to detect the radiolabelled imported polypeptide. In parallel, the extractabilities of both the endogenous NUO-17.8 and the 49 kDa subunit of complex I (NUO-49; Preis et al., 1990) were also determined. As shown in Figure 5, NUO-49 was completely removed from the membranes by the alkaline treatment. This is in good agreement with the localization of this protein within complex I, since it is a component of the peripheral arm of the enzyme (Weiss et al., 1991). This result is in sharp contrast with the behaviour of endogenous NUO-17.8, which could not be extracted from the mitochondrial inner membrane by applying this technique. This finding, together with the existence of a putative membrane-spanning domain in the deduced protein sequence, provides strong evidence that NUO-17.8 is an intrinsic membrane protein.

The *in vitro* imported NUO-17.8, however, displayed more complex behaviour. The majority of the *in vitro* imported NUO-17.8 was found in the soluble fraction (Figure 5, lane S); however, about 10% of the ^{35}S-labelled polypeptide resisted the extraction procedure (Figure 5, lane P), suggesting that it had arrived at its final localization, namely the inner membrane. (It should be emphasized that this carbonate-resistant species is proteinase-protected in intact mitochondria. Furthermore, the polypeptide has already been cleaved in the matrix by MPP. Thus it is reasonable to assume that the observed behaviour indeed reflects the insertion of the ^{35}S-labelled polypeptide into the mitochondrial inner membrane and not into the outer membrane.)

In an attempt to obtain more information concerning the localization of the *in vitro* imported NUO-17.8 (which, as shown

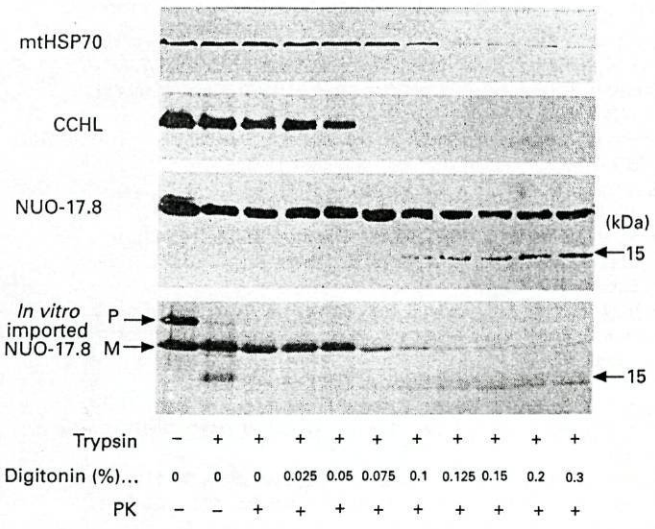

Figure 6 Membrane topology of NUO-17.8

[^{35}S]Methionine-labelled preNUO-17.8 was imported into isolated mitochondria. After trypsin treatment to remove non-imported polypeptide, mitochondria were re-isolated and resuspended in SEMK buffer (see the Materials and methods section). Aliquots containing 50 μg of protein were then treated with the indicated concentrations of digitonin in the presence of proteinase K (PK), as specified. The samples were diluted 5-fold with SEMK buffer and incubated on ice for 30 min. After inactivation of the proteinase, the protein in the samples was precipitated with trichloroacetic acid and subjected to SDS/PAGE. The gel was blotted on to nitrocellulose and the membrane was used to expose an X-ray film (to detect the *in vitro* imported subunit), and then probed with antisera directed to mtHSP70 (matrix marker), CCHL (intermembrane space marker) and NUO-17.8. P and M mark the precursor and mature forms respectively of the NUO-17.8 kDa.

above, may actually consist of at least two different subpopulations), we performed fractionation of mitochondria using digitonin (Schnaitmann and Greenawalt, 1968). By titrating intact mitochondria with this detergent in the presence of a proteinase, it is possible to cleave proteins with domains exposed in the intermembrane space before degrading proteins with domains exposed only to the mitochondrial matrix. Therefore freshly isolated *Neurospora* mitochondria were subjected to an import experiment in the presence of a membrane potential. After trypsin treatment in order to remove non-imported NUO17.8, mitochondria were re-isolated and resuspended in SEMK buffer (see the Materials and methods section). Aliquots of the suspension were then treated with several concentrations of digitonin in the presence of proteinase K. After inactivation of the proteinase, the protein in the samples was precipitated with trichloroacetic acid and analysed by SDS/PAGE. As markers for the intermembrane space and matrix, we used cytochrome *c* haem lyase (CCHL; Drygas et al., 1989; Lill et al., 1992) and the mitochondrial 70 kDa heat shock protein (mtHSP70; Kang et al., 1990) respectively. As shown in Figure 6, the endogenous/assembled NUO-17.8 is partially accessible to proteinase K only when the matrix component is open (i.e. mtHSP70 is degraded). Under these conditions, a considerable fraction of the subunit was digested to a faster-migrating species with an apparent molecular mass of 15 kDa. This fragment is still membrane-associated: when mitochondria were re-isolated by centrifugation after the digitonin treatment, the 15 kDa fragment was found in the pellet (results not shown).

As expected, the *in vitro* imported subunit displayed dual behaviour. The majority of the ^{35}S-labelled polypeptide was degraded by proteinase K as soon as the intermembrane space was opened (at a concentration of 0.075% digitonin). However, a fraction of the subunit remained undegraded at this detergent concentration. Only when the matrix compartment was open did this species become partially proteinase-sensitive. Furthermore, the observed proteolysis resulted in a 15 kDa fragment that co-migrated exactly with the one described for the endogenous subunit. These data taken together strongly indicate that the *in vitro* imported NUO-17.8 has acquired the same membrane topology as the endogenous/assembled subunit.

DISCUSSION

We have cloned and sequenced a cDNA insert encoding the 17.8 kDa subunit of complex I from *Neurospora crassa*. The deduced primary structure, together with automated Edman degradation of the isolated subunit, revealed that this polypeptide is produced as an extended precursor of 20.8 kDa which is then processed to a 17.8 kDa mature form. Interestingly, the N-terminal part of the mature protein has a rather high content of histidine residues (seven out of a total of 13 in the whole polypeptide), but the significance of this clustering is not known. No similarities with proteins of known primary structure were found, leaving the function of this subunit in complex I still undefined.

We have shown that the precursor of NUO-17.8 can be efficiently imported into isolated mitochondria, where it is cleaved to the mature form by MPP. Furthermore, localization experiments indicate that a fraction of the *in vitro* imported NUO-17.8 is inserted into the inner membrane, where it acquires the membrane topology of the endogenous/assembled subunit. However, this process is relatively inefficient: only about 10% of the *in vitro* imported subunit reaches this stage. Interestingly, the remaining portion of the subunit, presumably accumulated at some stage of the import pathway, is completely degraded by proteinase K under conditions whereby the outer membrane (but not the inner membrane) is disrupted. This species apparently becomes attached to the mitochondrial inner membrane in such a way that the presequence has already been cleaved in the matrix by MPP, but the C-terminal part is still exposed to the intermembrane space. The fact that 90% of the *in vitro* imported subunit is carbonate-extractable suggests that this intermediate is bound to the inner membrane through protein–protein interactions.

The subunit described here is a component of the so-called hydrophobic arm of complex I, a part of the enzyme that is believed to be embedded in the mitochondrial inner membrane. In agreement with this, the assembled polypeptide cannot be extracted from the membrane by alkaline treatment. The question arises of how NUO-17.8 is inserted in the mitochondrial inner membrane. The deduced primary structure of the polypeptide reveals the existence of a strongly hydrophobic domain that is long enough to cross a membrane in an α-helix conformation (no other stretch comprising more than nine hydrophobic amino acid residues is found in the mature subunit). This fact strongly suggests that NUO17.8 is a bitopic transmembrane protein. However, the transmembrane nature of NUO-17.8 could not be demonstrated by proteinase accessibility studies. It is possible that the hydrophilic domain facing the intermembrane space has intrinsic proteinase resistance and/or is protected from externally added proteinases by other components of complex I.

The hydrophobic arm of *Neurospora* complex I contains, as well as the seven mitochondrially synthesized subunits, about 12 nuclear-encoded components. Of the latter group, the primary structure is known for the following subunits: 21.3 kDa (Nehls

et al., 1990), 20.9 kDa (Azevedo et al., 1992), 20.8 kDa (Videira et al., 1990b), 12.3 kDa (Videira et al., 1993) and 9.3 kDa (Heinrich et al., 1992). All these subunits have in common the absence of a cleavable mitochondrial targeting sequence and, at least in the case of the 20.9 kDa component, insertion into the inner membrane seems to take place from the cytosolic side (Azevedo et al., 1992). The fact that NUO-17.8 has a matrix targeting sequence could suggest that this polypeptide follows a different biosynthetic pathway. Obviously, this subunit requires an additional step before being assembled in complex I (cleavage of the presequence by MPP). However, it is unclear whether NUO-17.8 really does follow a different import pathway. In particular, is the polypeptide completely translocated into the matrix before undergoing insertion into the inner membrane, a mechanism that would be in agreement with the so-called conservative sorting (for a review see Hartl et al., 1989)? The data presented here provide no evidence for such an intramitochondrial sorting pathway. For instance, no salt-extractable species could be detected after *in vitro* import experiments, suggesting that the *in vitro* imported NUO-17.8 does not exist in a soluble form in the matrix. In addition, it is quite striking that the majority of the imported (mature) subunit is found exposed to the intermembrane space. Import intermediates displaying these characteristics have been recently described for proteins *en route* to the matrix (Hwang et al., 1991). However, in all the documented examples, these intermediates were obtained by performing import under sub-optimal conditions (i.e. ATP depletion in the mitochondrial matrix). It is unlikely that this is the reason why the majority of the *in vitro* imported NUO-17.8 is found attached to the mitochondrial inner membrane ('standard' import conditions were used in these experiments; see the Materials and methods section). Rather, we think that the existence of this species reflects the specific features of the import pathway of NUO-17.8. Our results could favour a non-conservative sorting mechanism in which insertion of NUO-17.8 into the lipophilic environment of the inner membrane occurs through the inner membrane itself (probably by lateral movement of the polypeptide away from the inner membrane translocation machinery directly into the lipid bilayer). It is clear, however, that more data are needed in order to confirm the existence of such an intramitochondrial sorting pathway. In this context, it will be of major importance to determine whether or not the assembly of NUO-17.8 in the inner membrane requires the action of the matrix ATP-dependent chaperonins (Cheng et al., 1989; Kang et al., 1990). We are currently pursuing this aim.

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In organello assembly of respiratory chain complex I

Primary structure of the 14.8 kDa subunit of Neurospora crassa complex I

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Short title: *In organello* assembly of complex I

SYNOPSIS

A cDNA encoding the 14.8 kDa subunit of complex I from *Neurospora crassa* was cloned and sequenced. The deduced primary structure of this subunit reveals a predominantly hydrophilic protein containing no obvious membrane-spanning domain. In agreement with this characteristic, we have localised the 14.8 kDa subunit in the peripheral arm of the enzyme. The 14.8 kDa subunit was found to be conserved in mammalian complex I. The conservation of this subunit in such distantly related organisms suggests that the 14.8 kDa subunit is an important component of complex I. We have used an *in organello* system to study the biosynthetic pathway of this subunit. The 14.8 kDa polypeptide could be efficiently imported into isolated mitochondria. Furthermore, a fraction of the *in vitro* imported subunit was found to assemble in complex I. This is the first time that assembly in complex I of an *in vitro* synthesized subunit is demonstrated.

INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase) is the most intricated enzyme of the mitochondrial respiratory chain. It is constituted by some 30-40 subunits, 7 of which are encoded and synthesized in mitochondria. The enzyme is known to catalyse the transfer of electrons from NADH to ubiquinone coupling the energy of this redox reaction to the extrusion of protons across the mitochondrial inner membrane (for reviews see Ragan, 1987; Walker, 1992; Weiss et al., 1991). In addition, the recent finding of an acyl-carrier protein among complex I subunits (Runswick et al., 1991; Sackmann et al., 1991) suggests the existence of other, still unknown, biochemical functions in this multimeric enzyme.

Structurally, complex I from *Neurospora crassa* has the form of an L-shaped rod with one arm embedded in the mitochondrial inner membrane (the hydrophobic arm) and the other (the peripheral arm) protruding into the matrix (Friedrich et al., 1989; Hofhaus et al., 1991; Tuschen et al., 1990). In terms of polypeptide composition, the hydrophobic arm contains all the mitochondrially encoded subunits and about 15 nuclear-coded subunits; the peripheral arm comprises about 13 subunits and contains the majority of the iron-sulphur clusters of the enzyme (Wang et al., 1991).

One major goal of our work is the understanding of the biogenesis of complex I. Presently, all the information available concerning the assembly of the enzyme was obtained from pulse-labelling experiments performed in intact Neurospora cells. Using this method, an assembly intermediate of complex I corresponding to the membrane arm of the enzyme could be detected (Tuschen et al., 1990). Although elegant, this approach has an obvious drawback: the number of different factors affecting the assembly process that can be experimentally changed is quite limited. In an attempt to surround this problem we have been

trying to reproduce the biosynthetic pathway of some complex I subunits using an *in organello* system. Recently, we have shown that the 20.9 kDa subunit of Neurospora complex I could be imported into isolated mitochondria and correctly sorted to the inner membrane (Azevedo et al., 1992). Similar results were obtained with another subunit belonging to the membrane arm of the enzyme - upon mitochondrial *in vitro* import a fraction of the 17.8 kDa subunit was found correctly assembled in the inner membrane (Azevedo et al., 1993). In this report we present further studies on the structure and biogenesis of complex I. We have cloned and sequenced a cDNA encoding the 14.8 kDa subunit of *Neurospora crassa* complex I. This subunit was synthesized *in vitro* and used in mitochondrial *in vitro* import experiments. Our results show, for the first time, full assembly of an *in vitro* synthesized complex I subunit using an *in organello* system.

MATERIALS AND METHODS

The following procedures were employed according to the references cited: growth of *N. crassa* (wild type OR74A) (Werner, 1977); preparation of complex I (Wang et al., 1991); production of antisera in rabbits (Videira & Werner, 1989); isolation of mitochondria for *in vitro* import experiments (Rassow et al., 1989); determination of protein concentration (Bradford, 1976); sucrose gradient centrifugation of detergent-solubilized mitochondrial proteins and determination of NADH:$[Fe(CN)_6]^{3-}$ oxidoreductase activity (Tuschen et al., 1990; Friedrich et al., 1989); sodium dodecylsulfate gel electrophoresis (Laemmli, 1970; Videira & Werner, 1989): western-blotting (Towbin et al., 1979); synthesis of proteins in rabbit reticulocyte lysate (Amersham International, Amersham, Bucks., UK.) (Pelham & Jackson, 1976) in the presence of [^{35}S]methionine (specific activity 1000 Ci/mmol; Amersham) by coupled transcription/translation (Stueber et al., 1984; Hartl et al., 1986); automated Edman degradation (Eckerskorn et al., 1988).

The small form of complex I (Friedrich et al., 1989) was kindly provided by Dr. Hans Weiss, University of Dusseldorf, Germany.

The strategy used for screening a λgt11 cDNA expression library, subcloning and sequencing cDNA inserts has been detailed previously (Videira et al., 1990c; Heinrich et al., 1992).

The mitochondrial *in vitro* import experiments (usually 50 µg of mitochondrial protein in a 100 µl reaction) were performed in import buffer: 2 mM NADH, 5 mM $MgCl_2$, 250 mM sucrose, 2 % (w/v) lipid-free bovine serum albumin, 80 mM KCl, 10 mM Mops/KOH pH 7.2 and 10 % (v/v) of "untranslated" reticulocyte lysate (see Rassow et al., 1989) which contains an ATP-regeneration system (10 mM creatine phosphate and 22 units/ml creatine

kinase). Protease treatment of import reactions and addition of inhibitors were performed exactly as described recently (Azevedo et al., 1993).

The immunoprecipitation experiments were performed as follows: mitochondria (generally 50 μg of protein) were ressuspended in 1 ml of Triton buffer [1% (w/v) Triton X-100, 0.3 M NaCl, 5 mM EDTA, 20 mM Tris.HCl pH 7.0 and 1 mM phenylmethylsulfonyl fluoride (PMSF) freshly added from a 200 mM stock solution in ethanol] and incubated on ice for 10 minutes. The samples were subjected to a clarifying spin (15 000 X g, 10 minutes) to remove insoluble material. Five mg of protein A sepharose preincubated for one hour with 10 μl of the relevant antisera in 500 μl of Triton buffer, were then added to each sample. The suspension was gently rocked for 2-3 hours at 4° C. The protein A sepharose beads were centrifuged (1 minute at 10 000 X g), washed four times with 1 ml of Triton buffer and, finally, with 1 ml of 20 mM Tris/HCl pH 7.0. The immunoprecipitated proteins were denatured in Laemmli sample buffer (Laemmli, 1972) and subjected to SDS-PAGE.

RESULTS AND DISCUSSION

Sequence analysis and localization of the 14.8 kDa subunit of complex I

The cDNA encoding the 14.8 kDa subunit of Neurospora complex I (NUO-14.8) was isolated by immunoscreening of a λgt 11 expression library. The complete sequence of this clone is shown in figure 1. The cDNA is 515 bp long and contains an open reading frame encoding a polypeptide with a calculated molecular mass of 14 814 Da. Residues 2 to 17 of the deduced protein sequence match completely the amino acid sequence obtained by automated Edman degradation of the purified complex I subunit indicating that indeed the correct clone was isolated.

The codon for the N-terminal residue of the mature subunit is immediately preceded by ATG. This triplet most likely represents the initiator methionine since the DNA sequence surrounding this ATG is in good agreement with the optimum translation initiation sequence of *N. Crassa* (Pahlu et al., 1989) and, most importantly, the polypeptide encoded by this cDNA has all the information necessary to be efficiently imported into isolated mitochondria (see below). These data also imply that the 14.8 kDa subunit is synthesized without a cleavable mitochondrial targeting sequence.

In an attempt to get some information concerning the function of NUO-14.8 we have compared its deduced primary structure with proteins of known sequence. A high similarity (53%) was found with a recently described subunit of bovine complex I, the B14 polypeptide (Walker et al., 1992, see fig. 2). The conservation of this subunit in such distantly related organisms suggests that NUO-14.8 is an important component of complex I although its function in the enzyme cannot yet be ascertained. [It is worth mentioning that almost one third

of the nuclear encoded subunits of *N. crassa* complex I sequenced to date (5 subunits out of 18) do not have mammalian counterparts]. This observation, however, may not be valid for complex I from prokaryotic organisms - no homologue of NUO-14.8 was detected so far in operons encoding complex I or related enzymes (Böhm et al., 1990; Tran-Betcke et al., 1990; Yagi et al., 1992). It is possible that NUO-14.8 is exclusively found in mitochondrial complex I. The absence of a cleavable targeting sequence in NUO-14.8 (and its mammalian counterpart) could also favour this idea[1].

NUO-14.8 is a component of the so-called small form of complex I (see fig. 3), a fragment of the enzyme which is made in Neurospora cells grown in the presence of chloramphenicol (Friedrich et al., 1989). This functional/structural unit of complex I is identical to the peripheral arm of the enzyme, a part of complex I which is constituted by a small membrane anchor and a globular domain protruding into the mitochondrial matrix (Hofhaus et al., 1991). Taking into consideration the absence of a membrane spanning domain in NUO-14.8, it is quite likely that this subunit belongs to the globular part of the peripheral arm, and so that it exists in the close neighbourhood of components such as the 78.2 kDa, 51.4 kDa and 30.4 kDa subunits of complex I (for the polypeptide composition of the peripheral arm see Weiss et al., 1991).

Mitochondrial import *in vitro* of NUO-14.8

The 14.8 kDa subunit was synthesized *in vitro* in the presence of [^{35}S]methionine by coupled transcription-translation. The radiolabelled polypeptide was then incubated with freshly isolated Neurospora mitochondria either in the presence or absence of a membrane potential ($\Delta\Psi$). After reisolation of the organelles, aliquots from the two import reactions were treated with various amounts of proteinase K and analysed by SDS-PAGE. The integrity of

the mitochondrial outer membrane was monitored by the inaccessibility to the protease of cytochrome *c* haem lyase - an intermembrane space protein highly sensitive to proteinase K (Lill et al., 1992).

As shown in fig. 4A, when the [^{35}S]-labelled polypeptide is incubated with energized mitochondria, about 60 % of the material which was co-sedimented with mitochondria (lane 1, $+\Delta\Psi$) becomes protease-protected over a wide concentration range of proteinase K (lanes 2-6, $+\Delta\Psi$). If the mitochondria were solubilized with a mild detergent before the protease treatment, NUO-14.8 was almost completely degraded (lane 7, $+\Delta\Psi$).

In the absence of a membrane potential the amount of radiolabelled NUO-14.8 associated with mitochondria (lane 1, $-\Delta\Psi$) is approximately the same as the one obtained in the presence of a $\Delta\Psi$. However, 80 % of the bound material is readily degraded at the lowest protease concentration used (lane 2,$-\Delta\Psi$). The remaining protease-resistant subunit does not reflect imported material since it can be further degraded by increasing the protease concentration (lanes 3-6, $-\Delta\Psi$). It should be mentioned that the proteinase K concentrations used in this experiment exceed at least 10-fold the concentration of protease required to degrade more than 95 % of the labelled polypeptide under the same experimental conditions but in the absence of mitochondria (data not shown). Thus, the observed increased resistance of NUO-14.8 to proteolysis most likely arises from a $\Delta\Psi$-independent interaction of the subunit with the mitochondrial outer membrane.

In summary, these results indicate that the import of NUO-14.8 into mitochondria is dependent on the existence of a potential across the mitochondrial inner membrane.

The import of most mitochondrial precursor proteins is mediated by proteinaceous components located on the mitochondrial outer membrane (for a review see Pfanner and Neupert, 1990). By subjecting mitochondria to a mild

protease treatment part of this import machinery can be knocked-out leading to a block in the import of those precursor proteins (Pfaller et al., 1988). There is, however, a class of mitochondrial proteins that apparently do not need these protease-accessible components for an efficient import *in vitro* (Nicholson et al., 1988; Miller and Cumsky, 1991). In order to determine to which class NUO-14.8 belongs, freshly isolated Neurospora mitochondria were pre-treated with a low concentration of trypsin (30μg/ml) and subjected to an import experiment in the presence of a membrane potential. The import reaction was allowed to proceed for 6 minutes, a time point within the linear range of import of NUO-14.8 (data not shown) and the β subunit of F_1-ATPase ($F_1\beta$; Pfanner et al., 1987) - a control used in this experiment.

As shown in figure 4B, pre-trypsinization of mitochondria results in a significant decrease of the total amount of imported (protease-protected) NUO-14.8. Thus, these data suggest that the import of NUO-14.8 is dependent on proteinaceous components of the mitochondrial outer membrane.

Assembly of *in vitro* imported NUO-14.8

As described in the previous section, NUO-14.8 can be efficiently imported into mitochondria using an *in vitro* system. This process requires the existence of a membrane potential and seems to be mediated by proteinaceous components of the outer membrane. We then tried to know whether the *in vitro* imported subunit could undergo assembly in complex I. For this purpose [^{35}S]-labelled NUO-14.8 was incubated with Neurospora mitochondria either in the presence or absence of a membrane potential. After protease treatment, as specified (see fig. 5), the organelles were reisolated and solubilized with Triton buffer (see legend to fig. 5 for details). Aliquots of this material were then mixed with protein A sepharose beads containing an antibody (As-22a; Videira

et al., 1989) which is able to immunoprecipitate whole complex I but that does not cross-react with the 14.8 kDa subunit. As shown in figure 5, *in vitro* synthesized NUO-14.8 can be co-immunoprecipitated by As-22a only when energized mitochondria are used (fig. 5, lanes 5 and 6). In the absence of a $\Delta\Psi$ (lanes 7 and 8) no radiolabelled subunit is detected indicating that the antibody used indeed does not recognise NUO-14.8 *per se*. [This conclusion is further supported by the observation that no [^{35}S]-labelled material is precipitated when the mitochondrial proteins are incubated in the presence of 2 % SDS (to dissociate complex I subunits) prior to the immunoprecipitation procedure (lanes 1-4)]. The fact that no signal is detected in the absence of a $\Delta\Psi$ also indicates that the observed co-immunoprecipitation arises from an authentic assembly of NUO-14.8 during the import reaction and not from an association of the subunit with complex I (or some of its components) after the solubilization step of mitochondria.

The antibody used in this experiment recognises two subunits of complex I: the 20.8 kDa (Videira et al., 1990c) and the 29.9 kDa subunits (Van der Pas et al., 1991). Thus, it can not be inferred from the results presented above at what stage(s) of assembly is the *in vitro* imported subunit (full assembly of NUO-14.8 into complex I or association of the radiolabelled polypeptide with just one of the subunits recognised by the antibody would lead to the same experimental result). In order to clarify this point, the following experiment was performed: [^{35}S]-labelled NUO-14.8 was incubated with Neurospora mitochondria in the presence or absence (as a negative control) of a membrane potential. After reisolation of the mitochondria, the samples were solubilized with triton X-100 and subjected to sucrose gradient centrifugation. The conditions of the centrifugation were such that complex I sedimented through two thirds of the gradient, well separated from proteins with a molecular mass smaller than 50 kDa which remained at the top of the gradient (Tuschen et al., 1990; data not

shown). Aliquots of the gradients were then analysed for complex I activity (measured as NADH:$[Fe(CN)_6]^{3-}$ oxidoreductase activity) and the distribution of $[^{35}S]$-labelled material was monitored. As shown in the graphic of figure 6, complex I activity is almost completely recovered in fraction 10 of the gradients. As expected, in the absence of a membrane potential all the $[^{35}S]$-labelled material is found at the top of the gradients (fractions 1-3), representing non-assembled NUO-14.8. In the presence of a $\Delta\Psi$ the majority of the *in vitro* imported subunit is also detected in this region of the gradient. However, a small amount of radioactive material is visible in the fraction enriched in complex I activity (fraction 10, $+\Delta\Psi$) suggesting that assembly of NUO-14.8 into complex I has occurred

In order to prove unambiguously that the observed radiolabelled material co-sedimenting with complex I represents NUO-14.8 fully assembled in the enzyme (and not just some fortuitous $\Delta\Psi$-dependent aggregation of the radioactively labelled subunit), all the fractions from the two gradients were subjected to immunoprecipitation using the antibody mentioned above. The proteins in the immunoprecipitates were then analysed by SDS-PAGE, fluorography and probed with monospecific antibodies directed to the 14.8 and 20.8 kDa subunits of complex I. As shown in figure 6, both the distribution of the enzymatic activity and the profile of the radioactively labelled protein in the gradients correlate well with the pattern of "bands" obtained in the western-blot and fluorograph. This result indicates that the *in vitro* imported NUO-14.8 co-sedimenting with complex I is indeed assembled in the enzyme.

Finally, it is worth mentioning that no intermediates of assembly containing the radiolabelled subunit are detected in this experiment: the *in vitro* imported NUO-14.8 could only be found in the low molecular region of the gradients and in the fractions containing fully assembled complex I. There may be two reasons for this behaviour: either the biosynthetic pathway of this subunit is too fast to

allow the detection of assembly intermediates under the experimental conditions applied (20 minutes of import at 25°C) or, NUO-14.8 may be one of the last components to be assembled into the enzyme. This and other questions can now be easily addressed since an appropriate *in organello* system has been developed.

In this article we describe and characterise in terms of localization and biosynthetic pathway the 14.8 kDa subunit of *N. crassa* complex I. Our data suggest that NUO-14.8, the fungal counterpart of the bovine B14 subunit (Walker *et al.*, 1992), is a component of the peripheral arm of the enzyme (Hofhaus *et al.*, 1991). This complex I subunit is synthesized without a cleavable targeting sequence and requires a $\Delta\Psi$ to be imported into mitochondria. In addition, import of NUO-14.8 seems to be mediated by proteinaceous components located in the outer membrane. However, the most important aspect of the work presented here arises from the co-immunoprecipitation and sedimentation experiments. For the first time it is shown that the assembly process of a complex I subunit can be reproduced using an *in organello* system, opening new perspectives for the study of complex I biogenesis.

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Footnotes

Abbreviations used: $\Delta\Psi$, mitochondrial inner membrane potential; NUO-X, NADH:ubiquinone subunit of X kDa; CCHL, cytochrome *c* haem lyase.

[1] According to the endossymbiotic theory for the origin of mitochondria, mitochondrial nuclear-encoded proteins originally encoded in the genome of the ancestral organelle have acquired presequences in order to enable them to be targeted back to mitochondria (for a review see Hartl et al., 1989). Although there are some exceptions to this rule (e. g., cytochrome *c* haem lyase and cytochrome *c*), it is noteworthy that all nuclearly encoded complex I subunits having homologues in prokaryotes have a cleavable targeting sequence. Homologues of nuclear-coded subunits lacking a presequence were never found in prokaryotes.

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Legends to the figures

Figure 1: Nucleotide sequence and deduced protein sequence of the cDNA encoding NUO-14.8. (a) The amino acid sequence obtained by automated Edman degradation of the isolated complex I subunit is underlined. The stop codon is marked by an asterisk. (b) Sequencing strategy and structure of the cloned cDNA. Horizontal arrows show the direction and extent of sequence determination. The open reading frame is boxed.

Figure 2: Alignment of the protein sequences of NUO-14.8 and the B14 polypeptide from bovine complex I. Amino acid identities (*) and conservative substitutions (!) are indicated. Gaps were introduced to optimise the alignment. The sequence of the B14 protein is taken from Walker et al., 1992.

Figure 3: NUO-14.8 is a component of the small form of complex I.
The subunits of complex I (25 μg/lane; lanes 1 and 3) and of the small form of complex I (15 μg/lane; lanes 2 and 4) were resolved by SDS-PAGE and blotted onto nitrocelulose. Lanes 1 and 2 were stained with amido-black; the nitrocelulose strip containing lanes 3 and 4 was immunodecorated with antisera directed to the 14.8 kDa subunit. The positions of some complex I subunits of known primary structure are also marked - 78.2 kDa and 51.4 kDa (Preis et al., 1991); 30.4 kDa, 21.3 kDa, 20.8 kDa and 12.3 kDa (Videira et al., 1990a,b,c,1993); 20.9 kDa and 17.8 kDa (Azevedo et al., 1992,1993); and the 9.3 kDa subunit (Heinrich et al., 1992).

Figure 4: Mitochondrial *in vitro* import of NUO-14.8.

A) [^{35}S]Methionine-labelled NUO-14.8 was incubated with freshly isolated mitochondria (350 μg of protein) either in the presence (- $\Delta\Psi$) or absence (+ $\Delta\Psi$) of 1 μM valinomycin. At the end of the incubation (20 minutes at 25° C) the sample "+$\Delta\Psi$" received the same amount of valinomycin. The organelles from the two samples were re-isolated by centrifugation (10 minutes, 12 000 X g) washed with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH pH 7.2) and gently resuspended in the same buffer. Aliquots (50 μg protein) were then treated with the indicated amounts of proteinase K, but the organelles in lane 7 were first dissolved with 2% Triton X-100 prior to the treatment. After 25 minutes at 0° C, proteinase K was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride and the protein in all the aliquots was precipitated with trichloroacetic acid (10% final concentration), dissolved in Laemmli sample buffer and subjected to SDS-PAGE. The protein in the gel was then blotted onto nitrocellulose and the membrane was first used to expose an X-ray film (to detect the *in vitro* imported subunit) and, afterwards probed with an antiserum directed to cytochrome *c* haem lyase (CCHL).

B) Freshly isolated Neurospora mitochondria [50 μg of protein in SEM buffer (see above)] were incubated for 15 minutes at 0° C in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 30 μg/ml trypsin. After addition of soy bean trypsin inhibitor (1 mg/ml final concentration), the organelles were reisolated, washed and resuspended in import buffer (see materials and methods) containing the [^{35}S]-labelled precursors of NUO-14.8 and the β subunit of F1 ATPase (F1β). Import was allowed to proceed for 6 minutes at 25° C. The import reactions were then stopped by adding valinomycin to 1 μM. The samples applied to lanes 2 and 4 were treated with proteinase K (PK; 100 μg/ml final concentration) as described above. Finally, the samples were analysed by SDS-PAGE. A fluorograph of the dried gel is shown. P and M indicate the precursor and mature form of the β subunit of F1 ATPase,

respectively.

Figure 5: *In vitro* imported NUO-14.8 undergoes assembly.
[^{35}S]-Labelled NUO-14.8 was incubated with freshly isolated mitochondria (50 µg protein) either in the presence (+$\Delta\Psi$) or absence (-$\Delta\Psi$) of a membrane potential, as indicated. After 20 minutes at 25° C, the samples "+$\Delta\Psi$" received valinomycin to 1 µM to stop further import. Then, the samples in lanes 2, 4, 6, 8, 10 and 12 were treated with proteinase K (+PK; 100 µg/ml final concentration). After inactivation of the protease, mitochondria in all the samples were reisolated and washed with SEM buffer (for details see legend to fig. 4). The organelles were then either resuspended in 1 ml of Triton buffer (see materials and methods) containing 0.08 % SDS (lanes 5-12) or first incubated in 30 µl of a denaturing solution (2.7% SDS, 300 mM NaCl, 5 mM EDTA, 20 mM Tris/HCl pH 7.2) for 10 minutes at 50 °C to dissociate complex I subunits and, afterwards, diluted with 970 µl of Triton buffer (lanes 1-4). The samples were then subjected to immunoprecipitation (see materials and methods for details) using either an antiserum that is able to immunoprecipitate complex I (As-22a; lanes 1-8) or a pre-immune serum (PI; lanes 9-12). A fluorograph of the dried SDS-gel is shown.

Figure 6: *In vitro* imported NUO-14.8 is found in fully assembled complex I.
Two mg of Neurospora mitochondria were incubated with [^{35}S]-labelled NUO-14.8 for 20 minutes at 25°C either in the presence (+$\Delta\Psi$) or absence (-$\Delta\Psi$) of a membrane potential, as indicated. At the end of the incubation the sample "+$\Delta\Psi$" received valinomycin (1µM final concentration) and was subjected to proteinase K treatment (100 µg/ml final concentration) to remove non-imported NUO-14.8. The organelles from both samples were then reisolated, washed with SEM buffer (as detailed in the legend to fig. 4) and resuspended in 500 µl

of 2 % Triton X-100 in 0.1 M sodium phosphate, pH 7.0, 1 mM PMSF (see materials and methods). After a centrifugation step (15 000 X g, 15 minutes) to remove insoluble material, the samples were applied to the top of sucrose gradients (7.5-25 % sucrose, 0.05 % Triton X-100, 50 mM Tris/acetic acid pH 7.5) and centrifuged at 36 000 X g for 16 hours at 2° C. The gradients were fractionated into twelve fractions (approx. 900 µl each). The distribution of the radioactive material in the gradients (indicated in the graphic by black circles) was assessed by liquid scintillation counting of 10 µl aliquots from each fraction. The NADH:$[Fe(CN)_6]^{3-}$ oxidoreductase activity (indicated by open circles) was measured on 50 µl aliquots from the fractions. The remaining portions of the fractions were subjected to immunoprecipitation using the antiserum As-22a. The immunoprecipitated proteins were then resuspended in Laemmli sample buffer and analysed by SDS-PAGE. The gel was blotted onto nitrocellulose and the membrane was first used to expose an X-ray film (to detect the *in vitro* imported subunit) and, afterwards, probed with mono-specific antisera directed to the 14.8 and 20.8 kDa subunits of complex I.

```
-48                GGAACGGCGACAACCTCCAAAGGTCCTCAAGTCACGAATTGGCCCAAA
  1 ATGCCCATCACACCTACCAAGTACGCCATCACCACCAGGCAGTCGGCAAACTGGAGCGAC
    M  P  I  T  P  T  K  Y  A  I  T  T  R  Q  S  A  N  W  S  D

 61 GCCAAGCGGAGGGTGTTCGCCCTTTATCGGCGATGGCTTCGTTCGACACCCGAGATGCAG
    A  K  R  R  V  F  A  L  Y  R  R  W  L  R  S  T  P  E  M  Q

121 TCTATGTACTCACTACCCCTCCCCATTTCGGTCATTCGCACTCGCATCAGGCAGGAATTC
    S  M  Y  S  L  P  L  P  I  S  V  I  R  T  R  I  R  Q  E  F

181 GAGCGCAACCGCTTCGTTAACAAGTTGCCCGTGGTGGATGTTCTGCTCACAAAGGGCCAT
    E  R  N  R  F  V  N  K  L  P  V  V  D  V  L  L  T  K  G  H

241 GCCGACTATCAAGAAACCATGAACTTTTGGCGCCAGACGACTCACATGATGTCGTACTTC
    A  D  Y  Q  E  T  M  N  F  W  R  Q  T  T  H  M  M  S  Y  F

301 AACGAGGAGAGCTTCCGTGGAGCCAAGAGACTTCCCTCCAGCTTTATCGATGGCTTCCTG
    N  E  E  S  F  R  G  A  K  R  L  P  S  S  F  I  D  G  F  L

361 CAGGGTCGCAACTAAGAAGAGTAATAACGAAGGGTATCAATGTCAGCCTTTCCTCTAGAG
    Q  G  R  N  #

421 CCTATGTATATAACGCAAGCCAGATGAACATCCATGGACGCAGCCGC
```

FIGURE 1A

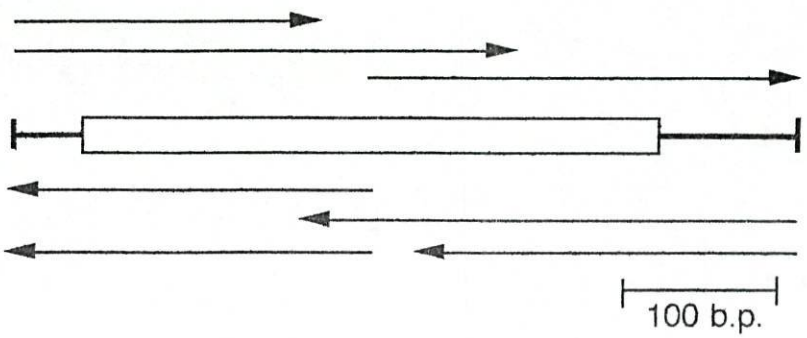

FIGURE 1B

```
NUO-14.8 (1)        MPITPTKYAITTRQSANWSDAKRRVFALYR
                     !  !!  !! !  * !  !*****  ***
B14      (1)MAASGLRQAAVAASTSVKPIFSRDMNEAKRRVRELYR

NUO-14.8    RWLRSTPEMQSMYSLPLPISV--IRTRIRQEFERNRF
             * *  *!   !!   * ***   * !!*! * !*
B14         AWYREVPNTVHLFQ--LDISVKQGRDKVREMFKKNAH

NUO-14.8    VNKLPVVDVLLTKGHADYQETMNFWRQTTHMMSYFNE
            !    ***!*! **! ! !**!  *!* **!* !* *
B14         ITDPRVVDLLVIKGKMELEETIKVWKQRTHVMRFFHE

NUO-14.8    ESFRGAKRLPSSFIDGFLQGRN (124)
                 !*   * *  *
B14         TEAPRPKDFLSKFYVGHDP    (128)
```

FIGURE 2

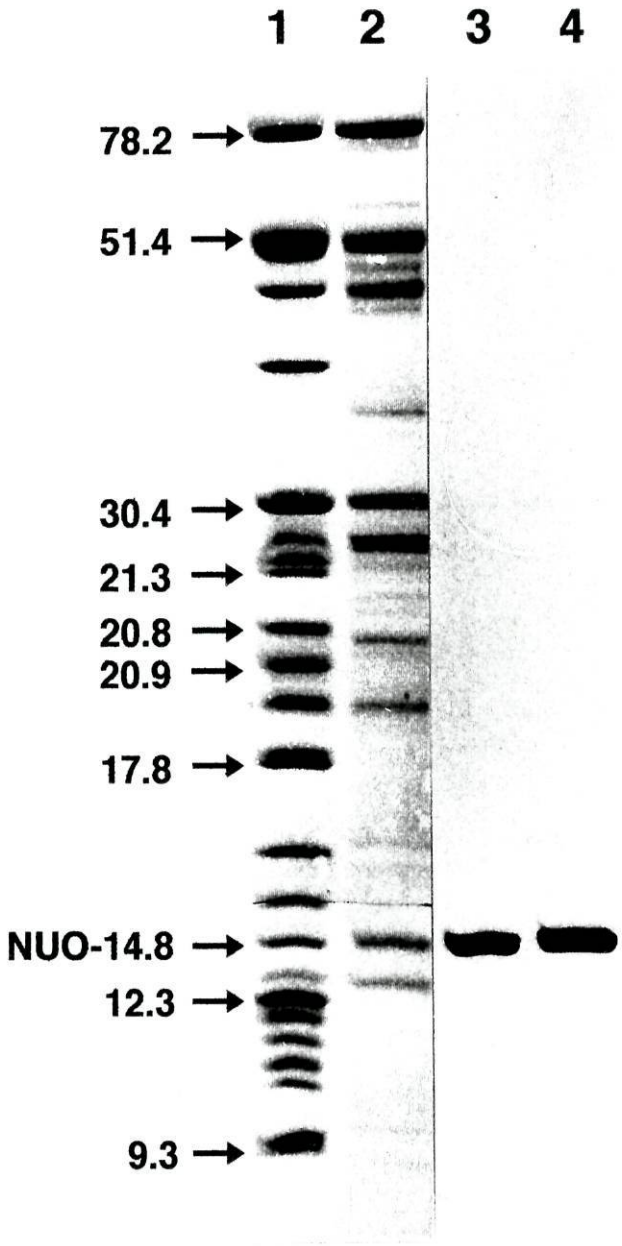

FIGURE 3

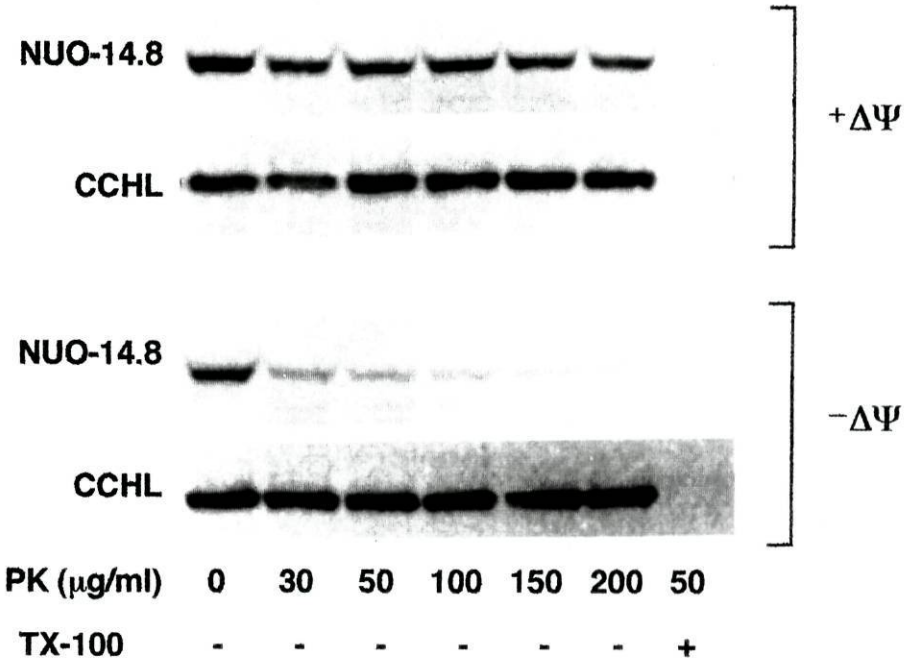

FIGURE 4A

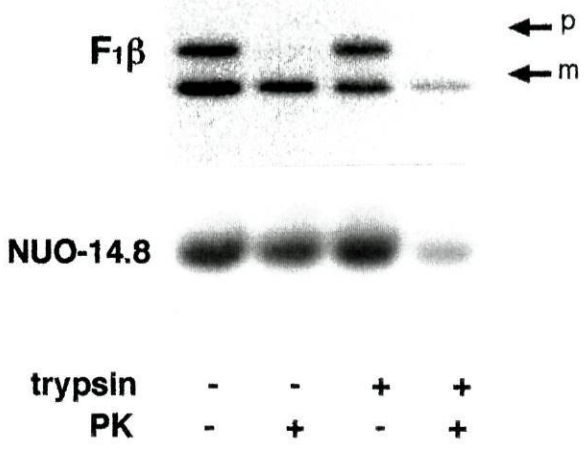

FIGURE 4B

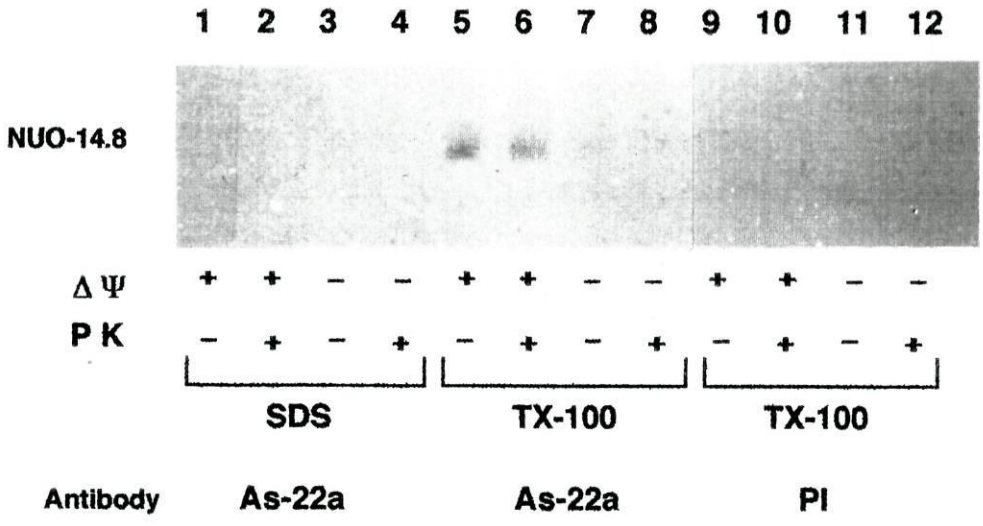
1 2 3 4 5 6 7 8 9 10 11 12
NUO-14.8
ΔΨ + + – – + + – – + + – –
PK – + – + – + – + – + – +
SDS
TX-100
TX-100
Antibody
As-22a
As-22a
PI

FIGURE 5

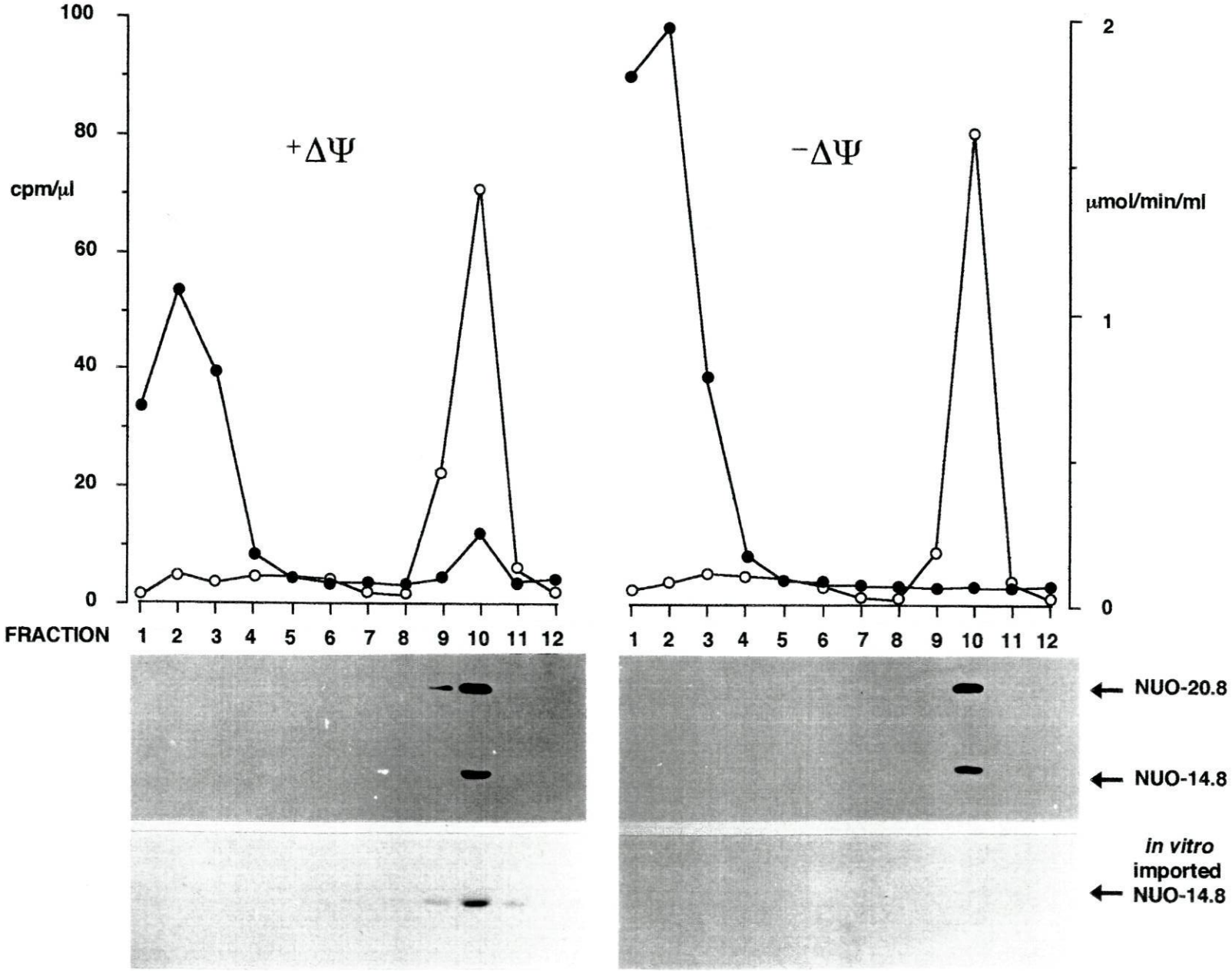
100
80
60
40
20
0
cpm/μl
+ΔΨ
−ΔΨ
2
1
0
μmol/min/ml
FRACTION
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
NUO-20.8
NUO-14.8
in vitro
imported
NUO-14.8

FIGURE 6

TRABALHO 7.5

Characterisation of a membrane fragment of respiratory chain complex I. Insights on the topology of the ubiquinone-binding site

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Running head: The hydrophobic arm of *N. crassa* complex I

Abstract

A membrane fragment of complex I from *Neurospora crassa* was isolated by immunoprecipitation from alkaline-extracted mitochondrial membranes. Analysis of the polypeptide composition of this hydrophobic domain of complex I has brought insights on the topology of two subunits of the enzyme, namely the 20.8 kDa and 9.3 kDa components. Our results indicate that the ubiquinone-binding site of complex I resides in the interface of the peripheral and membrane arms of the enzyme. The significance of these findings are discussed.

Introduction

The rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I) is a complicated assembly of about 40 subunits, 7 of which are encoded and synthesized in mitochondria. The enzyme catalyses the transfer of two electrons from NADH to ubiquinone with concomitant translocation of 4 or 5 protons across the mitochondrial inner membrane (for reviews see Walker, 1992; Weiss *et al.*, 1991).

Recently, a low resolution image of complex I from *N. crassa* was obtained by electron microscopy of two-dimensional crystals of the purified enzyme (Hofhaus *et al.*, 1991). It was concluded that complex I has an L-shaped form with one arm embedded in the membrane and the other (the peripheral arm) protruding into the mitochondrial matrix. Interestingly, when *Neurospora* cells are grown in the presence of chloramphenicol only a fragment of the enzyme is made - the so-called small form of complex I (Friedrich *et al.*, 1989). This fragment is thought to correspond to the peripheral arm of the enzyme. On the other hand, when isolated complex I is treated with chaotropic agents (Tuschen *et al.*, 1990) practically all the subunits present in the small form of the enzyme are stripped-off. However, a fraction of complex I retains some structural integrity and can be isolated. The presence in this subcomplex (the hydrophobic fragment) of many subunits with putative membrane spanning domains has led to the suggestion that this fragment represents the membrane arm of complex I (Tuschen *et al.*, 1990).

Although no data concerning the over-all shape of mammalian complex I is available, the high number of homologous subunits between the enzyme from *N. crassa* and bovine (see table 1 in the results and discussion section) suggests that mammalian complex I has a structure similar to the fungal enzyme. Attempts to resolve complex I from bovine into smaller fragments have been quite successful. Recently, Walker and co-workers could separate this enzyme into two major subcomplexes: fragments Iα and Iβ (Finel *et al.*, 1992). Subcomplex Iα contains most of the enzyme polypeptides, including several homologues of subunits of the small form of *N. crassa* complex I. However, at least one homologue of a subunit belonging

to the hydrophobic fragment of *N. crassa* complex I is found in subcomplex Iα (the ND-2 component), suggesting that this bovine fragment contains a part of the membrane arm of the enzyme (Walker, 1992). Subcomplex Iβ contains several mitochondrial encoded subunits and at least 11 nuclear encoded subunits. This fragment was proposed to represent a membrane fragment of the bovine enzyme (Finel *et al.*, 1992; Walker, 1992).

In this article, we describe the isolation and characterisation of a membrane fragment of complex I from *N. crassa*. Analysis of the polypeptide composition of this subcomplex has brought important insights on the topology of two subunits - the 20.8 kDa and 9.3 kDa components. Our results indicate that the ubiquinone-binding site of complex I is in the region where the two arms of the enzyme join together.

Materials and methods (this section may be printed in small type)

The following procedures were employed according to the references cited: preparation of complex I (Wang *et al.*, 1991); determination of protein concentration (Lowry *et al.*, 1951) and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970; Videira & Werner, 1989). The alkaline extraction of mitochondrial membranes (Fujiki *et al.*, 1982) was performed at a protein concentration of 1 mg/ml.

The immunoprecipitation experiments were performed in Triton buffer (1% Triton X-100, 0.3 M NaCl, 5 mM EDTA, 20 mM Tris.HCl pH 7.0 and 1 mM phenylmethanesulfonyl fluoride) using antibodies (Videira & Werner, 1989) cross-linked to protein A sepharose (Harlow & Lane, 1998). About 5 mg of these protein A sepharose beads were used per 1 mg of membranes. The suspension was gently rocked for 2-3 hours at 4 °C. The protein A sepharose beads were centrifuged (1 minute at 10000 *g*), washed four times with 1 ml of Triton buffer and, finally, with 1 ml of 20 mM Tris.HCl pH 7.0. The immunoprecipitated proteins were incubated in Laemmli sample buffer (Laemmli, 1972) for 30 minutes at room temperature and subjected to SDS-PAGE analysis. The gels were first stained with coomassie blue and, afterwards, with silver (Tuschen *et al.*, 1990).

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Results and discussion

The alkaline extraction of membranes (Fujiki *et al.*, 1982) has been widely used in the study of membrane proteins. Under the high pH used in this procedure, proteins acquire a very negative net charge leading to strong inter-molecular electrostatic repulsions. Thus, extrinsic membrane proteins are easily removed from membranes applying this technique. In contrast, intrinsic membrane proteins (whose interaction with the membrane occurs in an environment not accessible to the aqueous medium and is thus pH insensitive) resist the extraction procedure. On the other hand, this technique seems to be relatively mild in terms of protein tertiary structure - after alkaline extraction of peroxisomal membranes several membrane enzymes are still active (Fujiki *et al.*, 1982).

Due to the high performance of this technique, both in reproducibility and obtention of clear-cut results, we have been using it to study the mitochondrial respiratory chain complex I. It was shown that the 78 kDa, 49 kDa and 30.4 kDa subunits of complex I are extractable from membranes under alkaline pH; the 20.9 kDa, 17.8 kDa, 12.3 kDa and 9.3 kDa subunits are alkaline-resistant (Azevedo *et al.*, 1992, 1993; Heinrich *et al.*, 1992; Videira *et al.*, 1993). Here, we identify the majority of complex I subunits (if not all) that cannot be extracted from the mitochondrial inner membrane under alkaline pH. Our experiments were based on the striking observation that the 20.8 kDa subunit cannot be extracted from membranes by alkaline treatment. Like the 12.3 kDa subunit (Videira *et al.*, 1993), the 20.8 kDa subunit (Videira *et al.*, 1990a) does not contain any obvious membrane spanning domain; yet, both subunits behave as intrinsic membrane proteins. On the other hand, immunopurified antibodies directed to the 20.8 kDa subunit are able to immunoprecipitate complex I from Triton X-100 solubilized mitochondrial membranes (data not shown). These two observations led us to investigate whether, after an alkaline extraction, the 20.8 kDa component is still associated with other subunits of the enzyme. In this case, such experiment would define subunits of the enzyme which are embedded in the lipid phase of the inner membrane. For this purpose, freshly isolated *Neurospora* mitochondria were

resuspended in 0.1 M Na_2CO_3 (pH 11.5) and incubated on ice for 30 minutes. The mitochondrial membranes were isolated by centrifugation and either dissolved in 2.5 % SDS (to dissociate complex I subunits) or in 1 % Triton X-100. The protein solutions were then subjected to immunoprecipitation using an antiserum which is able to precipitate complex I but that precipitates mainly 3 subunits from the SDS-dissociated enzyme. As shown in fig. 1, when SDS-solubilized membranes are used in the immunoprecipitation experiment only the 20.8 kDa and 12.3 kDa complex I subunits are observed (the third subunit recognised by the antibody - the 29.9 kDa component - was removed by the alkaline treatment). However, if the membranes are solubilized with the mild detergent Triton X-100 and then subjected to immunoprecipitation, a large number of subunits is co-immunoprecipitated with the 20.8 kDa and 12.3 kDa subunits indicating that this fraction of the enzyme resists the extraction procedure. These subunits constitute a part (or the totality) of the membrane arm of complex I.

The polypeptide composition of this fraction of complex I (see table 1 for details) appears to be similar to the one described for the so-called hydrophobic fragment of complex I (Tuschen *et al.*, 1990). For example, several mitochondrial encoded subunits and the 20.9 kDa subunit are present in both subcomplexes (Azevedo *et al.*, 1992; Weiss *et al.*, 1991). However, the two fragments may not be identical as the 20.8 kDa subunit was assigned to the peripheral arm of the enzyme (Weiss *et al.*, 1991). If this is indeed the case, then the 20.8 kDa subunit is part of the small membrane anchor of the small form of complex I (Hofhaus *et al.*, 1991).

A comparative analysis between the polypeptide composition of the complex I fragment described here and subcomplex Iα from the bovine enzyme (Finel *et al.*, 1992; Walker, 1992), revealed some common components. In particular, the 20.8 kDa (a possible iron-sulphur protein) and 9.3 kDa subunits present in the membrane fragment of the fungal enzyme, have homologues in bovine subcomplex Iα (subunits PGIV and B9, respectively). This result indicates that these two components are localized in the interface of the membrane and peripheral arms of complex I; their co-purification with one or another arm depends on the organism and resolving technique used.

These topological considerations acquire a special importance in the case of the 9.3 kDa subunit. This polypeptide was identified using a photo-affinity ubiquinone analogue as being part of the ubiquinone-binding site (Heinrich & Werner, 1992). Thus, we assume that the ubiquinone-binding site of complex I is localized in the junction of the two arms of the enzyme. This conclusion is supported by the fact that bovine subcomplex Iα has all the e.p.r.-detectable Fe-S clusters of the enzyme (including cluster N-2, assumed to be the immediate reductor of ubiquinone) (Finel *et al.*, 1992).

The results presented here are depicted in figure 2 in the context of all the structural data presently available for complex I both from *N. crassa* and bovine [for detailed information regarding the function of some complex I components see (Walker, 1992)]. The most striking feature of this working model is that all the redox groups and substrate binding-sites of the enzyme are localized in the peripheral arm and in the part of the membrane arm in contact with it. This observation could suggest that the electrons do not flow along the membrane arm of complex I but just cross it partially. It is clear, however, that more data are needed to test this hypothesis.

Finally, it is worth mentioning that 11 out of the 12 *Neurospora* complex I subunits with known primary structure that are not resistant to carbonate extraction have counterparts in the bovine enzyme. In sharp contrast, 4 out the 6 nuclear-encoded subunits with known primary structure that belong to the membrane fragment described here have no bovine homologues. It is possible that these two domains of complex I have been under different evolutionary constraints.

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Summary

Neurospora crassa mitochondrial membranes were subjected to alkaline extraction in order to remove peripheral membrane proteins. When these membranes were solubilized with a mild non-denaturing detergent and, afterwards, subjected to immunoprecipitation using an antibody that recognises mainly three subunits of complex I, a fragment comprising about 15 subunits of the enzyme was obtained. This fragment contains a fraction (or the totality) of the membrane-embedded complex I subunits. A comparative analysis of the polypeptide composition of this hydrophobic fragment with subcomplex Iα from the bovine enzyme (Finel et al., 1992) revealed the existence of two common components: the ubiquinone-binding 9.3-kDa polypeptide (Heinrich et al., 1992) and the 20.8 kDa subunit (Videira et al., 1990a). This finding provides evidence that these two components are localized in the interface of the peripheral and membrane arms of the enzyme.

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Table 1: Characteristics of *N. crassa* complex I subunits with known primary structures

N. crassa complex I subunit	Carbonate fragment	Small form[c]	Bovine homologue[d]	References
78 kDa	no	yes	75 kDa (IP)	Preis et al., 1991
51 kDa	no	yes	51 kDa (FP)	Preis et al., 1991
49 kDa	no	yes	49 kDa (IP)	Preis et al., 1990
40 kDa	no	yes	39 kDa	Rohlen et al., 1991
30.4 kDa	no	yes	30 kDa (IP)	Videira et al., 1990b
29.9 kDa	no	yes	B13	Van der Pas et al., 1991
24 kDa*	no	n.k.	24 kDa (FP)	-
21.3 kDa[a]	n.k.	no	n.k.	Nehls et al., 1990
21.3 kDa[b]	no	yes	n.k.	Videira et al., 1990c
20.9 kDa	yes	no	n.k.	Azevedo et al., 1992
20.8 kDa	yes	yes?	PGIV	Videira et al., 1990a
18.3 kDa	no	yes	18 kDa (IP)	Weidner et al., 1991
17.8 kDa	yes	n.k.	n.k.	Azevedo et al., 1993
14.8 kDa*	no	yes	B14	-
12.3 kDa	yes	no	n.k.	Videira et al., 1993
10.5 kDa	no	yes	B8	Duarte et al., 1993
9.3 kDa	yes	n.k.	B9	Heinrich et al., 1992
ACP	no	yes	SDAP	Sackmann et al., 1991

* The sequences of these *N. crassa* subunits are unpublished; [a,b] There are two subunits with the same molecular mass; [c] The polypeptide composition of the small form of complex I is given in Weiss et al. (1991); [d] All the information regarding bovine complex I subunits is compiled in Walker (1992). n.k. - not known.

Legends to the figures

Fig.1: SDS-PAGE analysis of a membrane-embedded fragment of complex I.

Freshly isolated *Neurospora* mitochondria (5 mg of protein) were subjected to carbonate extraction as described in the materials and methods section. The membranes were resuspended in 100 µl of 0.1 M sodium phosphate buffer pH 7.2 and halved. One aliquot was diluted with 1.7 ml of Triton buffer containing 0.08 % SDS; the other half was first treated with 2.5 % SDS for 30 minutes to dissociate complex I subunits and, afterwards, diluted with 1.7 ml of Triton-buffer. The samples were centrifuged 10 minutes at 10000 *g* to remove insoluble material, subjected to immunoprecipitation (see materials and methods for details) and analysed by SDS-PAGE. Lane 1 - isolated complex I; Lane 2 - immunoprecipitate obtained from membranes solubilized with Triton X-100; Lane 3 - immunoprecipitate obtained from membranes solubilized with SDS. The positions of nuclear encoded complex I subunits with known primary structure are indicated (see table 1 for references). Several mitochondrial encoded subunits (diffuse bands indicated by the arrows at the right-side) are visible in both isolated complex I (lane 1) and membrane fragment (lane 2). The ACP and 78 kDa subunits stain poorly with silver.

Fig. 2: Working model of complex I structure.

Complex I subunits from *N. crassa* are arranged within the L-shaped form of the enzyme according to their presence in the peripheral or membrane arm. In the latter arm, 3 of the indicated subunits are part of a subdomain (shadowed box) which in bovine is co-purified with subcomplex Iα. With the exception of the 24 kDa, 51 kDa and 78 kDa subunits, which are known to constitute a functional unit (Walker, 1992), all the other components are randomly arranged within the three boxes.

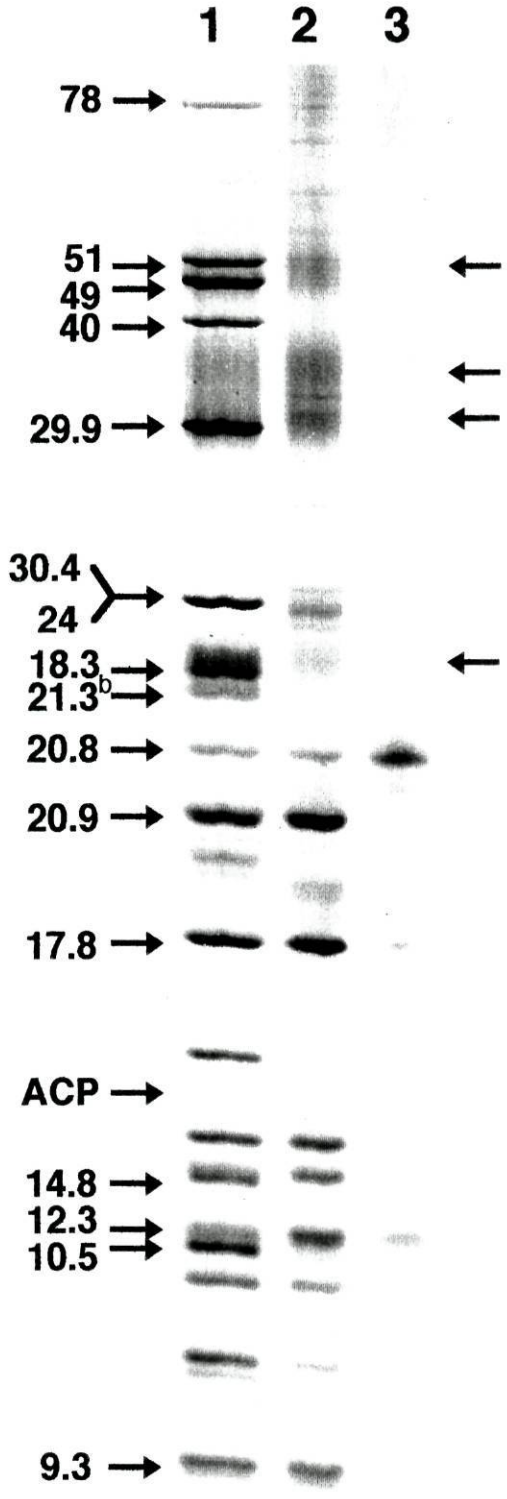

figure 1

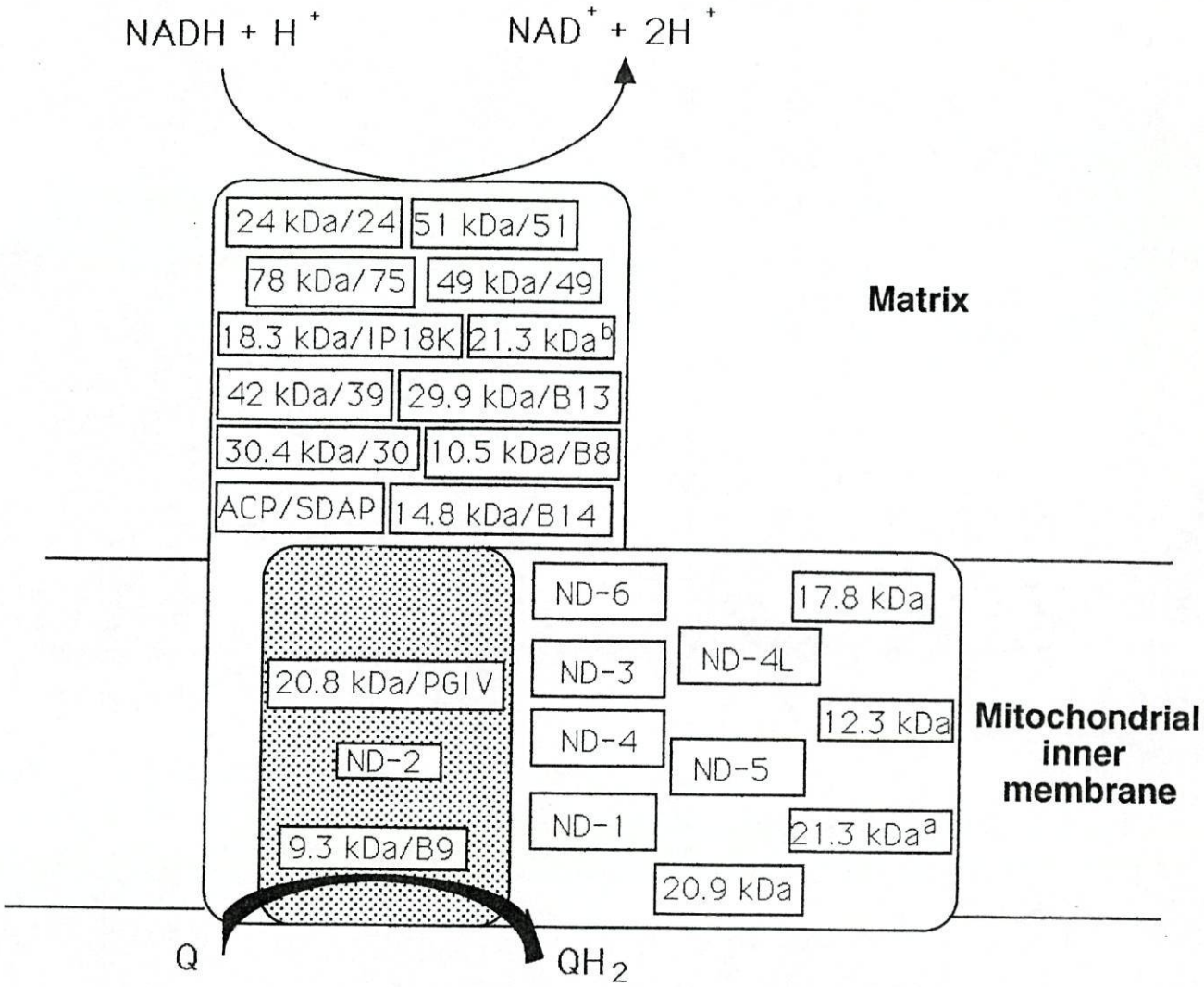
NADH + H+
NAD+ + 2H+
24 kDa/24
51 kDa/51
78 kDa/75
49 kDa/49
18.3 kDa/IP18K
21.3 kDa[b]
42 kDa/39
29.9 kDa/B13
30.4 kDa/30
10.5 kDa/B8
ACP/SDAP
14.8 kDa/B14
Matrix
20.8 kDa/PGIV
ND-2
9.3 kDa/B9
ND-6
17.8 kDa
ND-3
ND-4L
ND-4
12.3 kDa
ND-5
ND-1
21.3 kDa[a]
20.9 kDa
Mitochondrial inner membrane
Q
QH2

figure 2