ASSEMBLY OF MITOCHONDRIA

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INTRODUCTION

LUCK[1] first showed by elegant experiments with *Neurospora crassa* that mitochondria perpetuate themselves by growth and division. Recent evidences, both morphological and genetic, show that atleast in yeast, mitochondria undergo cyclic fusion and disintegration sequences during cell cycle[2, 9]. In this article, we describe the salient advances that have been made in this fascinating area of mitochondriogenesis during the intervening years. This is not intended to be an exhaustive review, but a critical assessment of the state-of-the-art. Naturally, a bias towards one's own work in the area becomes unavoidable. We have also restricted ourselves to discussing the structural entities, namely proteins and lipids.

Brief Historical Background

Ephrussi and Slonimski first showed that in yeast cells, the respiratory activity, an index of mitochondrial function, was inhibited by excess glucose in the medium and this was termed glucose repression[3]. But it was only much later that one of the prime targets of this repression was shown to be mitochondria, and in fact, the system became a powerful tool for the study of mitochondriogenesis[4, 5].

Demonstration of the presence of DNA in mitochondria by Nass and Nass was soon followed by the demonstration of the ability of isolated mitochondria to synthesise proteins, which in fact was accepted after initial reservations about bacterial contamination[6, 7]. Early enough, it was recognised that mitochondrial DNA does not have the coding capacity to account for all the proteins present in mitochondria and it has to depend on the proteins synthesised in the cytosol to achieve the full complement of proteins. Thus, the mitochondria were assigned a semi-autonomous status.

An overview of the process of mitochondriogenesis

It has been variously proposed that mitochondria perpetuate themselves (i) by *de novo* synthesis, (ii) by growth and division and (iii) from pre-existing cellular membranes[8]. The current concept based on several lines of evidence suggest that the mitochondria grow and divide.

Luck[1] gave the first experimental evidence, using ^{14}C-choline to label the mitochondrial phospholipids in a choline requiring mutant of *N. crassa* and following the labelling pattern in subsequent generations in a cold medium, that mitochondria grow and divide. It was shown in several laboratories that different components of the organelle have different turnover rates and that the lipid composition can also be altered at will[8]. Stevens[2] has reviewed morphological evidence in favour of a cyclic fusion and disintegration of mitochondria. Extensive work on mitochondrial genetics also supports the above claim[9]. Thus, growth and division is the most likely mode of the organelle perpetuation, unless a surprise is sprung. Experimentally, it would be difficult to dissect the events in many organisms that have been studied, like *N. crassa*, *Tetrahymena pyriformis*, He La cells and rat liver, since these cells have an absolute requirement for the presence of mitochondria for their survival[10-12]. Yeast, particularly *Saccharomyces cerevisiae*, offers itself for investigation by virtue of its ability to dispense with the mitochondria under anaerobic conditions or under glucose repression. Both situations being reversible, the various steps involved in mitochondriogenesis can be easily followed.

The events in yeast can be summarised very briefly as follows. Under conditions like anaerobiosis or glucose repression, there exists in the cytosol some membranous structures harbouring some mitochondrial enzymes. These have been designated as 'promitochondria'[13].

When yeast cells are released from glucose repression, mitochondriogenesis takes place in stepwise fashion involving hierarchies differing in autonomy[5]. Yeast, by virtue of being an unicellular eukaryote, offers all advantages to the geneticists in general and mitochondrial geneticists in particular, in such a way that individual events in mitochondriogenesis can be analysed and understood. No wonder, yeast has been the most popular organism among scientists interested in mitochondriogenesis.

The actual process of organellogenesis involves atleast four steps, namely, synthesis of the components, the transport of these to the site, subsequent processing and location at the proper niche. The current progress in these four aspects are reviewed in the following pages.

SYNTHESIS OF MITOCHONDRIAL COMPONENTS

As mentioned earlier, it was recognized early that the mitochondrial DNA can code only for about 5–10% of the total proteins of the mitochondria and rest of the proteins have to be synthesised in the cytosol and transported to the organelle. Linnane and his group made a breakthrough by showing that bacterial protein synthesis inhibitors like chloramphenicol, erythromycin, paramomycin etc inhibited mitochondrial protein synthesis exclusively in yeast, while cycloheximide inhibited only the cytosolic protein synthesis[14]. This made it possible to study the two protein synthesising systems independent of each other, and also for selecting mitochondrial genetic markers, a strategy which was used extensively by other workers. Using these differentially acting anti-biotics, the proteins synthesised by mitochondria themselves have been identified (table 1), and the rest of the proteins have to be imported[15].

Poyton and McKemmie[16] broke new grounds by claiming that mitochondrial proteins of cytosolic origin were made in the form of "polyprotein" precursors. Subsequent workers could not, however, substantiate this "polyprotein precursor" idea, but did find that some subunits (a partial list is given in table 2) are synthesised in precursor forms[17].

Table 1 *Catalogue of mitochondrially synthesised proteins in yeast*[45]

Protein	Mol. weight (daltons)
Cytochrome oxidase:	
Subunit I	40,000
Subunit II	33,000
Subunit III	23,000
Cytochrome b	30,000
F_0ATPase:	
Subunit 6	20,000
Subunit 9	7,600
Ribosomal protein	43,000
(Var-1)	47,000

The question as to whether the mitochondrially synthesised proteins are first made as precursors is not resolved yet. However, the report of Javed Ashraf and Jayaraman would suggest that some of them are atleast made as precursors[18].

PROCESSING AND TRANSPORT

Structure of Mitochondria

It is pertinent at this stage to recapitulate the work on the molecular architecture of mitochondria, as known for some crucial enzymes. Starting with the ATPase complex, it has been elegantly established that the F_1-ATPase moiety protrude on the inner side of the inner membrane into the matrix, anchored to the membrane through the proteolipid and F_0 which are embedded in the membrane. Eytan *et al*[20], using hydrophobic photolabelling techniques, showed that cytochrome oxidase spans the mitochondrial inner membrane and of the seven subunits, subunits I–III are coded by mitochondria and the rest by nucleus. All subunits, except IV and VI, are at least partially embedded in the lipid bilayer of yeast mitochondria[20, 21].

These are specific examples. In addition, there are several other enzymes (*i.e.* fatty acid oxidation enzymes, urea cycle enzymes, dehydrogenases, besides the mitochondrial ribosomal proteins) which have to be synthesised cytosolically and

Table 2 *Cytoplasmically synthesised mitochondrial proteins*[17]

Protein location	Protein	Organism	Apparent molecular size		Import observed	
			Mature	Precursor	*In vivo*	*In vitro*
Matrix	Adrenodoxin	Cattle	12	20		+
	δ-Aminolevulinate synthase	Rat	45	51	+	
	Aspartate amino-transferase	Chicken	44.5	47	+	
	F_1ATPase:					
	α-subunit	Yeast	58	64	+	+
	β-subunit	Yeast	54	56	+	+
	γ-subunit	Yeast	34	40	+	+
	Citrate synthase	Yeast	47	50		+
	L-Glutamate dehydrogenase	Rat	54	60		
	Malate dehydrogenase	Rat	37	38		
Inner membrane	Adenine nucleotide translocator	*N. crassa*	32	32	+	+
	Cytochrome *c* oxidase:					
	subunit IV	Yeast	14	17		
	subunit V	Yeast	12.5	15	+	+
	subunit VI	Yeast	12.5	17–20		
	subunit VII	Yeast	5–7.5	5–7.5		
	subunit IV	Rat	16.5	18–19.5	+	
	subunit V	Rat	12.5	15.5		
Inter membrane space	Adenylate kinase	Chicken	28	28		
	Cytochrome *c*	*N. crassa*	12	12	+	+
Outer membrane	Monoamine oxidase	Rat	59	59		
	Porin	*N. crassa*	31	31		+

imported into mitochondria. Thus a scenario emerges wherein most of the proteins of cytosolic origin are localised deep inside the organelle structure and consequently their transport across the membranes to reach their proper niche acquires importance.

Transport of cytosolic proteins

To explain the transport of proteins, Butow and his group proposed that cytoplasmic ribosomes attach themselves to the mitochondrial membrane, and as the nascent peptide is formed with its hydrophobic leader sequence, the protein is translocated across the membrane[22]. But Stevens, in an extensive electron microscopic study of yeast mitochondria, could observe such membrane attached ribosomes only very rarely[2]. Blobel has put forth the hypothesis of 'topogenic sequences' which holds that a relatively small number of conserved sequences or conformations shared by proteins with a common destiny direct their intracellular movement[23].

Further, evidence has accumulated that many proteins like cytochrome c, the outer membrane porin and several matrix located proteins are formed on free polysomes. In a recent study, Fenton *et al* have elegantly demonstrated that methyl malonyl CoA mutase, a cytosolically synthesised mitochondrial matrix enzyme, is translated in a cell free system programmed with rat liver RNA as a large precursor, which appears to be 3–4 kd larger than the subunit of purified mutase (77.5 kd). When this premutase was incubated with intact rat liver mitochondria, it was taken up rapidly and proteolytically processed to the mature subunit[24].

The strategy employed in general has been to incubate isolated mitochondria or mitochondrial subfractions with radiolabelled protein precursors synthesised *in vitro*[25, 26]. These approaches

have revealed that most mitochondrial proteins are synthesised as precursors of large molecular weight and that import involves (i) specific interactions with mitochondrial outer membranes (ii) energy dependent translocation of polypeptides across one or both mitochondrial membranes and (iii) processing by matrix located proteases[17]. The molecular mechanisms of these events are not known, but the phenomenology is fairly well investigated. The transport and processing are closely interlinked and they have to be taken together for any discussion.

Yaffe and Schatz[27] recently isolated two nuclear mutants which are temperature sensitive for import of mitochondrial proteins. These mutants, designated *mas* 1 and *mas* 2, reduce the import of the precursor of subunit of F_1ATPase at 37°C by factors of 250 times and 15 times respectively. They also reduce the import of other proteins, like citrate synthase, cyt b_2, a 90 kd matrix polypeptide to different degrees. The exact lesion is being worked out, although indications are that the temperature sensitive step occurs before the arrival of precursors in the matrix[27].

It is clear now that the processing of precursors takes place in the mitochondrial matrix. For example, Fenton *et al* have used isolated mitochondria or matrix subfraction and showed that the precursor of methyl malonyl CoA mutase was processed by either of them[24].

It has been suggested that mitochondria have specific import receptors which determine the binding of cytosolically synthesised proteins. The most extensive evidence for such an import receptor comes from Neupert's work on the biosynthesis and import of cyt *c*[28].

An intriguing result has been reported by Matocha and Waterman[29]. These workers synthesised cholesterol side chain cleaving cyt P_{450} (P_{450} *scc*) and the iron-sulfur protein, adrenodoxin, in a cell free system using bovine adrenocortical poly(A^+)RNA. They found that the precursor of P_{450} *scc* was taken up and processed by adrenocortical mitochondria but not by heart mitochondria. Such specificity was not however shown for adrenodoxin precursor[29]. Omura *et al* have recently reported that the precursor to adrenodoxin is imported and cleaved by rat liver mitochondria[30].

Such studies as discussed by Matocha and Waterman, raised the concept of receptors on mitochondrial membrane and processing by proteases as a secondary event[29].

Requirements for transport

The question whether energy is required for the import of proteins is not fully resolved. There are proteins such as cytochrome *c* and porin which apparently do not show any energy requirement. Nelson and Schatz showed that import of precursors of F_1ATPase, α, β, γ subunits, as of two subunits of ubiquinol-cyt *c* reductase, is blocked by *m*CCCP and also by depleting the cells of ATP. In general, inhibitors of mitochondrial energy metabolism, including uncouplers (DNP), proton ionophores (FCCP), electron transport inhibitors (rotenone and antimycin) rhodamine 6G (a mitochondria-specific dye which inhibits adenine nucleotide transport) prevented processing of labelled precursor by the mitochondria.

But studies with *Neurospora*, rat and yeast mitochondria have shown that it is the electrochemical gradient $\Delta\bar{\mu}H^+$, which is generated during electron transport, that is essential for the transport of some proteins.

The evidence available today suggests the existence of the following types of transport mechanisms shown in table 3 (adapted from Hay *et al*)[17, 29].

PROCESSING OF PRECURSOR PROTEINS

The fact that many mitochondrial proteins are formed in the cytosol as precursors and then transported to their site of action inside the membrane, necessitates processing. A matrix-located metal chelator sensitive protease has been shown by two groups[32, 33]. Using partially purified enzymes, high specificity has been shown. It cleaves a variety of *in vitro* synthesised mitochondrial precursor proteins

Table 3 *Some requirements for protein assembly*

Component	Location	Requirements for Receptor	$\Delta\mu$ H^+	Protease	Additional
cyt c	IMS	+	−	−	Heme
cyt b_2	IMS	+	+	+	IMS protease
F_1ATPase	Matrix	+	+	+	
Porin	OM	−	−	−	
Methyl CoA mutase	Matrix	−	+	−	
P_{450} scc	IMS	Tissue specific	+		
Adrenodoxin	IMS	-do-	+		

but not *in vitro* synthesised non-mitochondrial proteins. The cleavage was restricted only to converting precursors to mature forms. There also appears to be little species specificity. Kinetic studies on the proteolytic cleaving of precursors of yeast cyt b_2, cyt *c* and probably cyt *c* peroxidase indicate that the cleavage takes place in two discrete steps. It is not clear whether the two cleavages are catalysed by the same enzyme or by different enzymes[17].

Using an entirely different approach, Javed Ashraf and Jayaraman labelled yeast cells with ^{14}C-formate in the presence of cycloheximide with the contention that, (resembling a procaryote), mitochondrial protein synthesis should be initiated with N-formyl methionine. They found this to be true and interestingly the labelling was enhanced 3-fold in presence of 1, 10, phenanthroline (a proteolytic inhibitor). Gel analysis revealed several proteins labelled in presence of 1, 10, phenanthroline[18]. These experiments have raised the possibility that some mitochondrially synthesised proteins can also be made in precursor forms.

In addition to proteolytic cleavages, there are several other modifications that take place in proteins. Heme is covalently bound to cytochrome *c* and c_1, probably within the intermembranal space. Mature cyt *c* has been shown to have its amino terminal end acetylated. There is one report of ADP-ribosylation in rat liver mitochondria[34]. Glycosylation reactions preceding proteolysis have also been reported[35]. The recent study initiated in the author's laboratory on the flavoprotein dehydrogenase complexes should prove fascinating in this regard.

The Question of Assembly

The mechanisms by which the various transported components locate and fit into their proper niche in the membrane and express themselves, and whether the membrane lipids have any role in this are perhaps least understood. Considerable work needs to be done on the protein–protein and protein–lipid interactions. While the beeline orientation of single polypeptides like cytochrome *c* might itself be a complicated factor, homo-oligomeric polymers like ornithine transcarbamylase and hetero-oligomeric polymers like cytochrome oxidase, where both mitochondrially and cytosolically synthesised subunits have to be aligned with each other, requires high precision cellular manoeuvres[36]. The recent report of Douglas *et al* on the directed import of β-ATPase subunit by fusing its gene to *lac z* gene is an interesting line and may open up new avenues[37].

In the light of the recent reports on the synthesis, transport and processing of the imported proteins, some earlier observations need to be reevaluated or reinterpreted. These are as follows: (a) Tzagoloff's report that soluble F_1ATPase activity is present in chloramphenicol treated derepressing yeast cells[38]. (b) Beattie has shown that yeast cells pre-treated with chloramphenicol, and subsequently washed free of the antibiotic and transferred to cyclo-

heximide containing medium, showed depression, suggesting an accumulation of partially synthesised products[39]. (c) Jayaraman *et al* and Chandrasekaran *et al* showed that mitochondrial particles isolated from yeast cells treated with chloramphenicol and cycloheximide separately can be mixed and that the enzyme activities of cytochrome oxidase and membrane-bound ATPase were reconstituted[45, 41]. (d) Chandrasekaran *et al* have also demonstrated that a second addition of glucose to yeast cells at the onset of derepression inhibited mitochondrial translation, but some cytosolic components like succinic dehydrogenase, heme *b* and ubiquinone accumulated in the cytosol at levels more than the normal levels[41]. These are under conditions when probably even mitochondrial RNA processing is inhibited (Nithyakalyani Raghavan and J. Jayaraman, unpublished results). (e) In experiments using synchronously growing yeast cells, Somasundaram and Jayaraman[42] elegantly demonstrated a phase difference in the synthesis of cytosolic and mitochondrial components, leading to the expression of cytochrome oxidase and cold insensitive, oligomycin-sensitive ATPase in mitochondria. While in the case of the former enzyme, immunoprecipitation technique was used to show the accumulation of the cytosolically synthesised subunits in the cytosol during the S phase and their subsequent migration to the mitochondria during the G_2 phase, in the case of ATPase, a similar phenomenon was shown using immunochemical methods as well as *enzyme activities*[42].

Jayaraman *et al*[5] concluded that during glucose repression of yeast cells, there occurs not only a blockade of mitochondriogenesis, but also a breakdown of pre-existing mitochondria, Dharmalingam and Jayaraman[40] showed an enhancement in the phospholipase D activity in the repressed cells and implicated it in the breakdown of mitochondria. Working with the same system, Javed Ashraf and Jayaraman[18] found that during repression, succinic dehydrogenase was released into the cytosol and it was reintegrated in presence of cardiolipin during derepression following the exhaustion of glucose. Preliminary experiments suggest a similar pattern of behaviour for isocitrate dehydrogenase also (V. Thenmozhi and J. Jayaraman, unpublished data). Somasundaram *et al*[42, 43] confirmed the release of radioactively labelled proteins from mitochondria during glucose repression and their reintegration into the membrane. Cyclic AMP prevents this release. It is pertinent to point out that a similar release from and reintegration to periplasmic membranes of *E. coli*, of some key electron transport enzymes under the influence of glucose repression has been found in this laboratory[44].

Mitochondrial genetics have testified to the interaction of the organelles among themselves. But hardly have any studies been carried out to understand any of these phenomena.

CONCLUDING REMARKS

In this brief review, an attempt has been made to bring forth some salient features of the recent developments in the fascinating area of mitochondriogenesis. The fast progressing field of mitochondrial genetics has not been touched upon. More than a decade of research has shown the sequence of events, like synthesis of proteins at a site different from their location, their vectorial targetted translocation, processing where necessary and their specific assembly. It is to be hoped that the mechanics will soon be worked out.

30 January 1985

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Abbreviations:
DNP – 2,4, Dinitrophenol
FCCP – Carbonyl cyanide p-trifluoro methoxyphenyl hydrazone
m-CCCP – Carbonyl cyanide m-chlorophenyl hydrazone

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