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The Intermembrane Space of Mitochondria

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Abstract

Mitochondria contain two aqueous compartments: the matrix and the intermembrane space. Whereas many of the biologic functions of the matrix were well characterized in the past, it became clear very recently that the intermembrane space plays a pivotal role in the coordination of mitochondrial activities with other cellular processes. These activities include the exchange of proteins, lipids, or metal ions between the matrix and the cytosol, the regulated initiation of apoptotic cascades, signalling pathways that regulate respiration and metabolic functions, the prevention of reactive oxygen species produced by the respiratory chain, or the control of mitochondrial morphogenesis. We focus on the different biologic functions of the intermembrane space and discuss the relevance of this fascinating compartment for cellular physiology and human health. *Antioxid. Redox Signal.* 13, 1341–1358.

Introduction

Mitochondria are essential organelles present in almost all types of eukaryotic organisms and tissues. Because of overwhelming genetic evidence, their phylogenetic origin from intracellular eubacteria is generally accepted. However, the order of events that led to the evolution of mitochondria-containing eukaryotic cells is still hotly debated. Whereas the initially proposed endosymbiont hypothesis suggested that the progenitors of mitochondria were taken up by primitive eukaryotic cells that already contained nuclei and secretory membranes (113), recent evidence from the sequencing of the genomes of several "primitive" eukaryotes challenged this idea and rather sparked the hypothesis that the acquisition of the ancestors of mitochondria by an archaebacteria had represented the initial event at the very birth of the eukaryotic cell [for discussion, see (41, 62)]. Regardless of how the initial host cell looked, the eubacterial origin of mitochondria is obvious from many features of present-day mitochondria. For example, many components that replicate or express the mitochondrial genome or carry out fundamental biosynthetic processes, like respiration, the Krebs cycle, β-oxidation, heme synthesis or the biogenesis of iron sulfur clusters, are closely related to eubacterial orthologues and, in many cases, can be functionally exchanged between mitochondria and bacteria (50, 79, 112, 167). Interestingly, these "classic" mitochondrial activities are almost exclusively carried out in the mitochondrial matrix or the inner membrane. Nevertheless, mitochondria generally retained an outer membrane, and hence the periplasm-derived intermembrane space (IMS), suggesting that functional constraints forced the cell to maintain the outer membrane during evolution.

In textbooks, the intermembrane space is often misconceived as a small hydrophilic layer between the two mitochondrial membranes that, because of the relatively large openings of the porin channels in the outer membrane, is (in its physicochemical properties) equivalent or at least very similar to the cytosol. However, during the last decade, many studies identified a number of specific functions of the IMS, clearly showing that it represents a unique and important cellular compartment that exhibits a number of exciting activities, many of which we are only beginning to understand. These activities are often unparalleled by processes in prokaryotes and therefore may have been initially overlooked. Most proteins of the IMS are of eukaryotic origin and presumably were developed during the more than 2 billion years of eukaryotic evolution to integrate mitochondrial activities into cellular functions. Several of these properties and activities are discussed here.

The Intermembrane Space: One or Two Compartments?

The concept that mitochondria are organelles that contain two structurally and functionally distinct membranes was generally accepted after pioneering electron-microscopic studies by Palade and Sjöstrand in the 1950s (138, 151, 170). These initial studies described characteristic invaginations of the inner membrane, initially termed *cristae mitochondriales,* which were interpreted as extensions of the inner membrane to enlarge its surface area. This "baffle model" was challenged

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by more-detailed analyses of serial mitochondrial sections (31), which showed that small tubular structures separate cristae membranes from the so-called inner boundary membrane, the region of the inner membrane that is in proximity to the outer membrane (Fig. 1). These tubular structures were called *pediculi cristae* or cristae junctions. Although their biochemical nature is still unclear, the structure of cristae junctions was well studied when the three-dimensional cellular architecture could be visualized by electron tomography (48, 109, 110, 130, 144). Whereas the abundance and shape of cristae strongly differs between different organisms and tissues, the cristae junctions that connect the cristae to the inner boundary membrane are relatively uniform: they represent round or oval tubules of 12 to 40 nm in diameter and about 50 nm in length. High-resolution images indicated the presence of proteins around the necks of these tubules, but their nature is not entirely clear. Among the candidate proteins proposed to constitute these necks are the ring-like complexes of prohibitins (181), the IMS-exposed protein mitofilin (Fcj1 in yeast) (74, 147), and the dynamin-related protein OPA1 (Mgm1 in yeast) (21, 49).

The Physicochemical Milieu of the IMS

The position at the interface of the cytosol and the mitochondrial matrix strongly influences the small-molecule composition of the IMS. The outer and the inner membranes of mitochondria control the exchange of ions, metabolites, and other small molecules between the cytosol and the matrix (Fig. 2). The small-molecule milieu of the IMS is therefore determined by the kinetics of these efflux and influx processes.

The major transport molecule of the outer membrane is the evolutionarily well-conserved porin that allows the free passage of molecules with a mass $\leq$5 to 6 kDa (23, 94, 131). Porins belong to the group of $\beta-$barrel membrane proteins that are present in the outer membrane of bacteria, mitochondria, and plastids, but not in other cellular membranes (197). The relatively rigid structure of these large pores presumably allows the unrestricted diffusion of most small molecules across the outer membrane, so that the solute environment of the IMS might equilibrate rapidly with that of the cytosol. Hence, the ion concentrations in the IMS should be in the range of the values present in the cytosol (*i.e.*, $[K^+] \approx 150$ m*M*; $[Ca^{2+}] \approx 100$ n*M*; $[Na^+] \approx 10$–15 m*M*; and $[Cl^-] \approx 4$ m*M*).

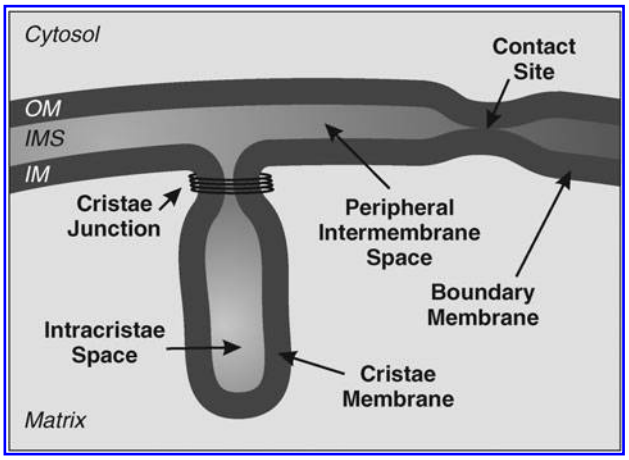

FIG. 1. Substructures of the intermembrane space of mitochondria. The IMS is defined as the small lumen between the outer (OM) and the inner (IM) mitochondrial membranes. The IMS can be structurally subdivided into two compartments, the peripheral IMS between the outer membrane and the so-called boundary membrane, and the intracristae space. The peripheral IMS and the intracristae space are in contact through small connections formed by cristae junctions. The outer and inner membranes contact each other at contact sites.

However, the view that porin channels facilitate unrestricted diffusion was challenged mainly on the basis of *in vitro* experiments (23, 94, 131). Because of their gating behavior in electrophysiologic setups, porins are also termed voltage-dependent anion channels (VDACs). In the open state, the porins prefer the transport of anions over that of cations. In the closed conformation, porins are still conductive but constrict and favor the transport of small cations like Ca^{2+} and K^+, while the transport of anionic metabolites such as ATP or succinate is blocked. The gating (*i.e.*, the shift between the open and the closed state) is highly voltage dependent: to close the porin channels, positive or negative voltages in the range of 20–40 mV are necessary; these most likely will not be reached *in vivo*. The physiological relevance of these *in vitro* observations is unclear, and *in vivo,* both negatively (such as ATP, ADP, and Pi) and positively charged molecules (such as Ca^{2+}, K^+, and Na^+) are easily transported across the outer membrane. It was suggested that the transport properties of porins can be adjusted to different cellular conditions (75, 92, 190), but direct experimental evidence of a physiologically relevant regulation of mitochondrial porins is still missing.

Most transporters of the inner mitochondrial membrane are members of the mitochondrial carrier family (MCF), of bacterial-type ion transporters, and of ABC transporters (139, 206). These constitute $\geq$50 membrane-embedded transport proteins that regulate the transport of metabolites, ions, cofactors, biosynthetic precursors, and other small molecules to and from the matrix. Given this large number and the overlap of substrates among many of these transporters, the physiological relevance of specific carriers and channel proteins is only poorly understood. The membrane potential, which depends on the energetic status of mitochondria, affects many transporters and thus determines the transport rates of ions and metabolites.

In support of a limited and potentially regulated transport over the inner and outer membranes, the physicochemical properties of the IMS have been reported to be distinct from those of the cytosol in several respects. This includes the pH as well as the composition of the glutathione redox buffer. The pH of the IMS has been measured with specifically targeted pH-sensitive GFP probes, both on isolated mitochondria and in intact cultured mammalian cells (24, 146). The results of these approaches were consistent and indicated the IMS to be more acidic than the cytosol by a margin of 0.2 to 0.7 pH units. The glutathione redox buffer (*i.e.*, the ratio of reduced to oxidized glutathione) has been assessed by using targeted redox-sensitive YFPs, which indicated that this buffer is at steady state more oxidizing in the IMS than that in the cytosol (71). It remains to be shown whether this is due to a regulated transport of protons and glutathione across the outer membrane or to the diffusion barrier caused by cristae junctions (see later), or both.

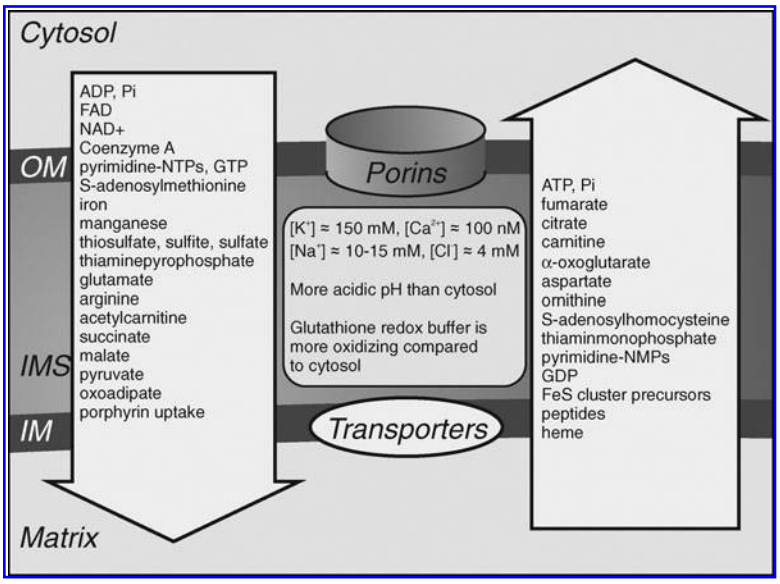

FIG. 2. Physicochemical composition of the IMS and metabolite transport through the IMS. The IMS serves as a transport hub between the cytosol and the matrix. Small molecules diffuse across the outer membrane (OM) via the relatively large openings of porins. In contrast, translocation across the inner membrane (IM) is driven by active transport processes (primary or secondary) through numerous dedicated transport systems, including members of the mitochondrial carrier family and ABC transporters. Taken together, these transport processes influence the small-molecule composition of the IMS.

Functional Separation of the Peripheral IMS and the Intracristae Lumen

The narrow openings between the cristae lumen and the external region of the IMS strongly limit the free diffusion of molecules and, hence, separate the inner membrane into two functionally distinct regions (48). The physiological consequence of the separation of cristae and inner-boundary membranes is not entirely clear. However, it appears conceivable that the constriction of the cristae junctions is used by the cell to optimize mitochondrial performance and to reduce the potential risk that is associated with respiratory activity (Fig. 3):

A. The restricted diffusion of protons through the cristae junctions restricts the leakage of protons into the cytosol and increases the performance of respiration-driven ATP synthesis.
B. The submitochondrial architecture largely restricts the production of dangerous reactive oxygen species (ROS) by the respiratory chain to the intracristae space and thereby reduces the risk of damage in extramitochondrial regions of the cell.
C. The narrow openings of cristae junctions might cause problems for an efficient exchange of ATP and ADP (111) unless they were specifically designed to facilitate this reaction; alternatively, the exchange of nucleotides might occur predominantly across the boundary membrane. Detailed analyses will be necessary in the future to localize the ATP/ADP carrier in mitochondria at high resolution;

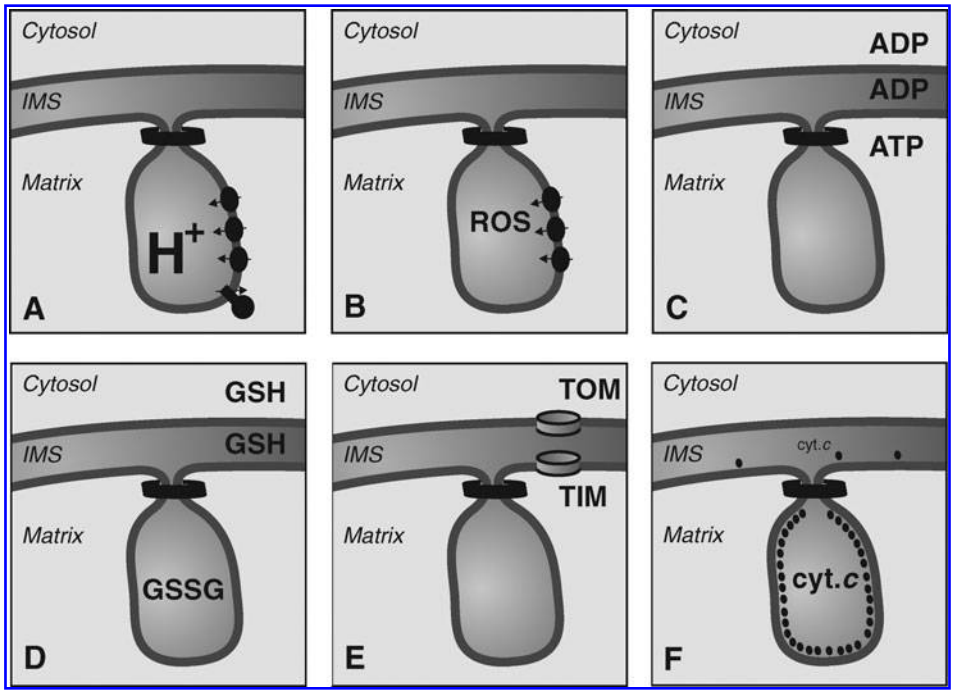

FIG. 3. The subcompartments of the intermembrane space might differ in their physiological properties. It was proposed that the cristae and the peripheral IMS differ substantially in their small-molecule environments [*e.g.*, H^+(**A**), ROS (**B**), ATP (**C**), and reduced and oxidized glutathione (**D**)], but also in the protein composition of their bordering membranes (48, 109, 110, 130, 144, 193, 198, 201). For example, respiratory chain and ATPase complexes are predominantly present in cristae membranes (**A, B**), whereas TIM complexes appear to be enriched in the boundary membrane (**E**). Moreover, cytochrome *c* was proposed to be enriched in cristae (**F**) (49).

D. Recent studies with redox-sensitive fluorescent proteins indicated that the IMS is significantly more oxidizing than the cytosol or the mitochondrial matrix. This was astonishing, as the major cellular reductant, reduced glutathione, should be small enough to diffuse through porins. However, a limited diffusion of glutathione through cristae junctions could explain why at least certain regions of the IMS contain an increased ratio of oxidized to reduced glutathione.

E and F. The cristae junctions might also limit the diffusion of particular proteins. For example, it was proposed that in mammalian mitochondria, cytochrome *c* is sequestered in the cristae, and only after an OPA1-dependent remodeling of the inner membrane during apoptosis can cytochrome *c* escape from the intracristae space to be released into the cytosol (21, 49, 116, 202). The remodeling of the inner membrane thereby includes the opening of the cristae junctions and a mixing of the contents of both compartments of the IMS. Whether other soluble proteins of the IMS besides cytochrome *c* are unequally distributed is not known. However, for inner membrane proteins, it was shown that they can be preferentially found either in the cristae (*e.g.*, components of the respiratory chain, ATPase) or in the boundary membrane (*e.g.*, protein-import machinery) (193, 198, 201), again supporting a functional specialization of the cristae lumen and peripheral IMS.

Although these considerations are still rather hypothetical, the exciting developments in high-resolution light microscopy and fluorescent sensors should soon allow more-detailed insights into the intramitochondrial distribution of proteins, metabolites, and ions (73, 161).

Biogenesis of IMS proteins

Protein import

About 60 different soluble proteins have been identified in the IMS (Table 1). The list is steadily increasing, and, at least in mammalian mitochondria, the IMS presumably contains far more than 100 different proteins. All these proteins are encoded by nuclear genes and are initially synthesized on cytosolic ribosomes. The import of proteins into the intermembrane space was described in detail in several review articles (17, 64, 83). Matrix proteins appear consistently to use one ATP- and membrane potential–dependent import route across the translocases of the outer membrane (TOM complex) and the inner membrane (TIM23 complex) to which most of them are directed by N-terminal presequences. Conversely, proteins of the IMS embark on a number of distinct pathways that considerably differ in their energetic requirements, targeting signals and the components involved:

A. A number of IMS proteins contain typical mitochondrial presequences that direct the N termini of the proteins to the matrix. These sequences are followed by hydrophobic stretches, which can arrest protein translocation; on proteolytic cleavage of the proteins, their mature, C-terminal parts are released into the IMS or remain membrane bound (43, 54, 63, 150, 180). The classic example of proteins with such bipartite sorting signals is cytochrome b_2 (59). Furthermore, such signals are often found on proteins that are larger than 20 kDa, like the components that trigger apoptosis (AIF, Smac/Diablo, endonuclease G, or Omi/HtrA2; see Table 1) (16, 78).

B. Many if not most proteins of the IMS lack obvious presequences and are not recognized as mitochondrial proteins by prediction programs. The folding of these proteins in the IMS often appears to be of critical relevance for their import. The acquisition of a stably folded conformation was suggested to retain these proteins in the IMS and prevents them from back-translocation into the cytosol (105, 106). Folding in the IMS can be triggered by binding of a cofactor such as heme, as in the case of cytochrome *c*. In the absence of heme or of heme lyase, the enzyme that inserts heme into apo-cytochrome *c*, cytochrome *c* cannot fold and is not retained in the IMS (39, 127). Folding can also be induced by oxidation of cysteine residues in newly imported proteins. This oxidation-driven import pathway was discovered only very recently (4, 18, 118, 126, 157, 182), but presumably is of relevance for many IMS proteins. It uses two highly conserved IMS proteins, Mia40 and Erv1, and is described in detail in a review article by Toshiya Endo and co-workers in this issue (42a). Proteins that use this pathway furthermore contain a sequence motif that supports their interaction with Mia40, a so-called MISS (mitochondrial intermembrane space-sorting signal) or ITS (intermembrane space-targeting signal) (119, 168).

C. Some proteins of the IMS, like cytochrome *c* heme lyase (174), associate permanently with binding sites on the inner or outer membrane. The interaction with binding partners is critical for their stable localization in the IMS. It therefore was suggested that the binding to high-affinity binding sites in the IMS plays a central role for their targeting to mitochondria (37, 174).

It appears likely that other mechanisms might contribute to protein import into the IMS. For example, it was recently shown that the i-AAA protease of the IMS not only plays a role in processing of polynucleotide phosphorylase in human mitochondria but also actively drives its translocation across the outer membrane (150). Despite some important discoveries over the years, we still understand very little of the molecular processes underlying the translocation of proteins across the outer membrane into the IMS.

Protein folding in the IMS

Almost nothing is known about protein folding in the IMS. In contrast to almost all other cellular compartments, not a single representative of the Hsp40, Hsp60, or Hsp70 families was identified in the IMS. Nevertheless, a number of large and often multimeric IMS proteins exist for which a chaperone-assisted folding process might be expected. It was suggested that AAA proteases might be able to contribute to protein folding in addition to their role in protein degradation (5, 97). However, the evidence for this hypothesis is based mainly on observations with protease-deficient mutants of AAA proteases that might alter the mode of action of these enzymes. Also for small Tim proteins, components of the carrier-import pathway, a chaperone function in *in vitro* experiments was

Table 1. Soluble and Membrane-associated Proteins of the Intermembrane Space of Mitochondria

Protein	*Distribution*[a]	*Processing*[b]	*binding sites/Motifs*[c]
Respiratory chain			
Cox VIb/Cox12	A, F, P	−	
Cytochrome *c*	A, F, P	−	Heme
Qcr6	A, F, P	−	
Metabolic enzymes			
Acn9	A, F	−	
Adenylate kinase	A, F, P	−	
Coproporphyrinogen oxidase, CPO	A	+ (?)	Flavin
Creatine kinase	A	−	
Cytochrome b_2	F	+ (MPP and Imp1)	Heme
Cytochrome b_5-reductase, Mcr1	A, F, P	+ (MPP and Imp1)	
Nde1/Nde2, external NADH-ubiquinone dehydrogenases	F	−	
Nucleoside diphosphate kinase, NDPK	A, P	+ (?)	
Nucleoside diphosphate kinase, Ynk1	F	−	
Dihydroxy-butanone-phosphate synthase, Rib3	F, P	−	
Protein import			
Tim8/DDP	A, F, P	−	$(Cx_3C)_2$
Tim9	A, F, P	−	$(Cx_3C)_2$
Tim10	A, F, P	−	$(Cx_3C)_2$
Tim12	F	−	$(Cx_3C)_2$
Tim13	A, F, P	−	$(Cx_3C)_2$
Redox control			
Copper chaperone of Sod 1, Ccs1	A, F, P	−	Copper
Cytochrome *c* peroxidase, CCPO	F	+ (Yta10 and Pcp1)	Heme
Glutaredoxin 1	A	?	
Hot13	F	−	Zinc
Erv1/ALR	A, F, P	−	Flavin, 2x CxxC
Mia40	A, F, P	−	CPC, $(Cx_9C)_2$
Superoxide dismutase, Sod1	A, F, P	−	Copper, Zinc
Assembly of the respiratory chain			
Cmc1	A, F, P	−	$(Cx_9C)_2$
Cmc2	A, F, P	−	$(Cx_9C)_2$
Cmc3/Coa4	A, F, P	−	$(Cx_9C)_2$
Cmc4	A, F, P	−	$(Cx_9C)_2$
Cox17	A, F, P	−	Copper, $(Cx_9C)_2$
Cox19	A, F, P	−	$(Cx_9C)_2$
Cox23	A, F, P	−	$(Cx_9C)_2$
Cyt3, cytochrome *c* heme lyase	A, F, P	−	
Cyt2, cytochrome c_1 heme lyase	F	−	
Metallothionein	A, F, P	−	Copper, Zinc
Pet 191	A, F, P	−	$(Cx_9C)_2$
Proteolytic processing			
Atp23	A, F, P	−	Metal
Calpain1	A	+ (autoprocessing)	
Cym1/Mop112 protease[d]	A, F, P	?	Metal
Prd1	F, P	?	Zinc
Som1	F	−	$(Cx_9C)_2$
Apoptosis			
AIF	A, F, P	+ (?)	Flavin
Endonuclease G	A	+ (?)	
Omi/HtrA2	A	+ (?)	
Smac/DIABLO	A	+ (?)	
Morphology and distribution of mitochondria			
Gametogenetin-binding protein 1 (GGNBP1)	A	?	
Mdm35	A, F, P	−	$(Cx_9C)_2$
Mgm1/OPA1	A, F	+ (MPP, m-AAA, Pcp1)	
Ups1, Ups2, Ups3, PRELI	A, F, P	−	
Signaling			
Autophagy-related protein phosphatase, Aup1/Ptc6	A, F, P	?	
Phosphatase DSP18	A	?	
Polynucleotide phosphorylase (hPNPase)	A	+ (MPP, i-AAA)	
p66-Shc	A	−	
Sirt5, sirtuin	A	?	
Unknown			
Emi1	A, F, P	−	$(Cx_9C)_2$
Mic14	A, F, P	−	$(Cx_9C)_2$
Mic17	A, F, P	−	$(Cx_9C)_2$

[a]Proteins for which representatives in animals, fungi, and plants could be identified in the databases are depicted as A, F or P, respectively.
[b]Proteins that reach the intermembrane space after proteolytic processing of a precursor form are indicated with +. Processing peptidases if identified are depicted. MPP, mitochondrial processing peptidase.
[c]Reported cofactors of the binding sites are indicated. The presence of twin Cx_3C and twin Cx_9C motifs is indicated by $(Cx_3C)_2$ and $(Cx_9C)_2$, respectively.
[d]Cym1/Mop112 was reported to be present in the IMS in fungi, whereas its homologues in plants and animals were localized in the mitochondrial matrix.

shown (145, 192, 194). However, the role of these chaperones is to usher hydrophobic proteins from the TOM complex to their insertion sites at the outer or inner membrane (Fig. 4) (67, 84, 169, 199), and at present, no evidence suggests that small Tim proteins play a relevant role in the folding of IMS-resident proteins.

At least for certain classes of cysteine-containing proteins, the IMS protein Mia40 appears to play a crucial role in folding. Mia40 contains a surface-exposed hydrophobic cleft that directly binds unfolded substrate proteins (9, 81). Protein folding is then facilitated by an Mia40-catalyzed oxidation step, which results in the release of stably folded substrates (4, 18, 118, 124). Whether Mia40 also plays a role in the folding of proteins that lack cysteine residues is not known.

Protein degradation

The IMS of mitochondria contains a number of proteases (see Table 1). Several of these proteases recognize and degrade misfolded proteins. The i-AAA protease of yeast mitochondria might be the best-characterized example. It is formed by a homohexameric ring of the membrane protein Yme1, which exposes its proteolytically active site into the IMS (96, 195). AAA proteases exhibit an ATPase activity that presumably is used to contribute to the unfolding of substrates and to translocate them into the proteolytic cavity of the complex (95, 121). Most characterized substrates of the i-AAA protease are integral proteins of the inner membrane that expose domains into the IMS. However, the i-AAA protease was recently identified as being crucial for the import and processing of the IMS enzyme polynucleotide phosphorylase in humans (20, 150). In this case, the ATP-dependent translocation activity of the i-AAA protease might directly be used by mitochondria to import protein sequences through the TOM pore into the IMS and, potentially, to fold polynucleotide phosphorylase into its native conformation.

In addition to the i-AAA protease, several other proteases recently identified in the IMS might play a role in protein turnover or in the processing of precursor proteins (see Table 1). Moreover, it was recently proposed that IMS proteins might be degraded in the cytosol after a ubiquitin-depentent export process across the outer membrane (148). Hence, the cytosolic degradation machinery, together with IMS-resident proteases like the i-AAA protease or Omi (115), would contribute to quality control and protein turnover in the IMS.

Functions of the IMS

Mitochondria are often considered as organelles that are primarily or even exclusively used for respiration (*i.e.*, the transfer of electrons from organic metabolites to oxygen to gain the energy for ATP synthesis). Clearly, the respiratory complexes and their associated components are extremely abundant in mitochondria. Therefore, it is no surprise that cytochrome *c*, which represents the electron shuttle that connects complex III to complex IV of the respiratory chain, is the most abundant protein in the IMS (114). In addition, enzymes of the IMS are involved in the biosynthesis of heme or in the phosphorylation of creatine.

However, mitochondria exhibit many nonmetabolic functions that might be even more important than respiration. In a number of organisms, respiratory activity was lost because of their anaerobic life style; nevertheless, these organisms maintained remnants of mitochondria called mitosomes. These mitosomes still contain enzymes required for iron-sulfur–cluster biogenesis, pointing to this process as the essential function of mitochondria (57). When the mitochondrial proteome was initially systematically analyzed, it came as a surprise that only a minority of 14% of proteins plays a role in respiration (167). Particularly in the IMS, most components

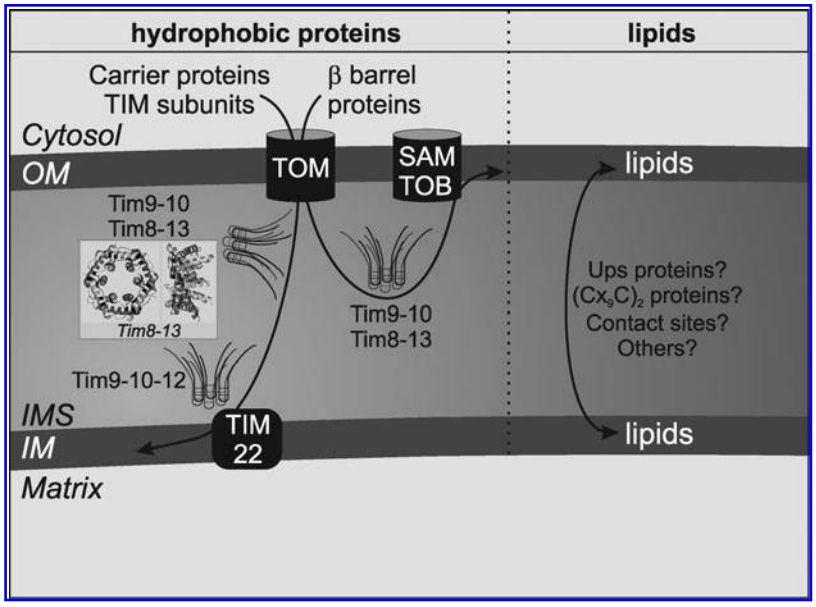

FIG. 4. Transport of hydrophobic molecules across the IMS. (*Left*) β-Barrel proteins of the outer membrane are initially imported into the IMS through the protein-conducting channel of the TOM complex before they are inserted into the outer membrane by the SAM/TOB complex. During their passage through the IMS, they bind to soluble hexamers formed by small Tim proteins (194). Two of these hexamers have been identified, consisting either of Tim9 and Tim10 or of Tim 8 and Tim13. The same complexes also usher carrier proteins and membrane-embedded subunits of the TIM translocases through the IMS. These inner membrane proteins are inserted into the membrane by the TIM22 complex, which contains a permanently bound, specialized small Tim complex consisting of Tim9, Tim10, and Tim12. (*Right*) The transport of lipids between both mitochondrial membranes is not well understood. Initially it was suggested that direct contacts of both membranes facilitate the exchange of lipid molecules. Electron-microscopic images suggest the existence of such contact sites. More recently, small proteins of the IMS were suggested to play a role in lipid distribution, in particular, proteins of the PRELI/Ups family and some members of the twin Cx_9C proteins.

might not exhibit functions in respiration and other metabolic processes but rather are relevant for the communication of mitochondria with the rest of the cell (Fig. 5).

The IMS as a Logistics Hub

Mitochondria developed from intracellular bacteria. Although initially both the bacterial ancestors and the host cells were able to live autonomously, they developed into biologic systems of absolute mutual interdependence. During evolution, the initially redundant biosynthetic capabilities were reduced so that in present cells, most components are either synthesized in mitochondria (ATP from respiration, heme, iron-sulfur clusters, ubiquinol, urea, arginine, biotin, cardiolipin, and many others) or in the rest of the cell (most proteins, several amino acids, most lipids), but not in both locations. This specialization made necessary a coordinated exchange of components into and out of the mitochondria, a process in which components of the IMS are particularly relevant. Several examples of these transport and coordination factors of the IMS are described in the following.

Protein sorting through the IMS

All nuclear-encoded proteins of the matrix and the inner membrane, as well as the β-barrel proteins of the outer membrane, have to be transported across the IMS [for review, see (17, 42, 101, 128)]. These proteins use the translocase of the outer membrane (TOM) complex as the entry gate to mitochondria. To reach the matrix or the inner membrane, two translocases of the inner membrane (TIM complexes) can be used. Proteins containing N-terminal presequences are further transported into the matrix by the TIM23 complex. During translocation, the TOM and TIM23 translocases presumably closely align and even might constitute one continuous protein-conducting channel across both mitochondrial membranes. Thereby, both membranes apparently come into close proximity, forming so-called contact sites, which also can be observed in electron-microscopic pictures (19, 35, 153, 163). Because of this direct contact of the TOM and TIM23 translocases, incoming polypeptides might not be accessible from the IMS, explaining why no soluble IMS factors were identified that contribute to the import of proteins into the mitochondrial matrix.

However, not all proteins of the inner membrane contain matrix-targeting presequences: Members of the carrier family and subunits of the TIM translocases comprise hairpin-like pairs of transmembrane domains, which are inserted into the inner membrane by the second inner membrane translocase, the TIM22 complex. These hydrophobic substrate proteins form sorting intermediates in the IMS (84, 169). A number of highly conserved IMS proteins maintain these proteins in a translocation-competent conformation and, thus, play an essential role in the import of carrier proteins. Five of these small Tim proteins are ubiquitously present in mitochondria of fungi and animals: Tim9, Tim10, and Tim12 (critical for the import of carriers), and Tim8 and Tim13, which presumably are specialized for the import of TIM subunits and some carriers that expose large hydrophilic domains into the IMS (29, 158). Small Tim proteins range from 9 to 13 kDa and form hexameric complexes. The structure of the Tim9–Tim10 complex was recently solved, showing a ringlike organization with six alternating subunits exposing 12 flexible termini like tentacles of a jellyfish (194). These termini presumably embrace the hydrophobic regions of its substrates and thereby prevent them from unproductive interactions and aggregation (12) (Fig. 4). Tim9–Tim10 and Tim8–Tim13 heterohexamers are also involved in the transfer of β-barrel proteins to the sorting and assembly machinery of the outer membrane (SAM or TOB complex). Initially, precursors of β-barrel proteins are translocated to the IMS through the TOM complex. Subsequently, small Tim proteins escort β-barrel preproteins to the SAM complex, where the two essential proteins, Sam50/Tob55 and Sam35/Tob38, cooperate in the insertion of the precursors into the outer membrane (72, 87).

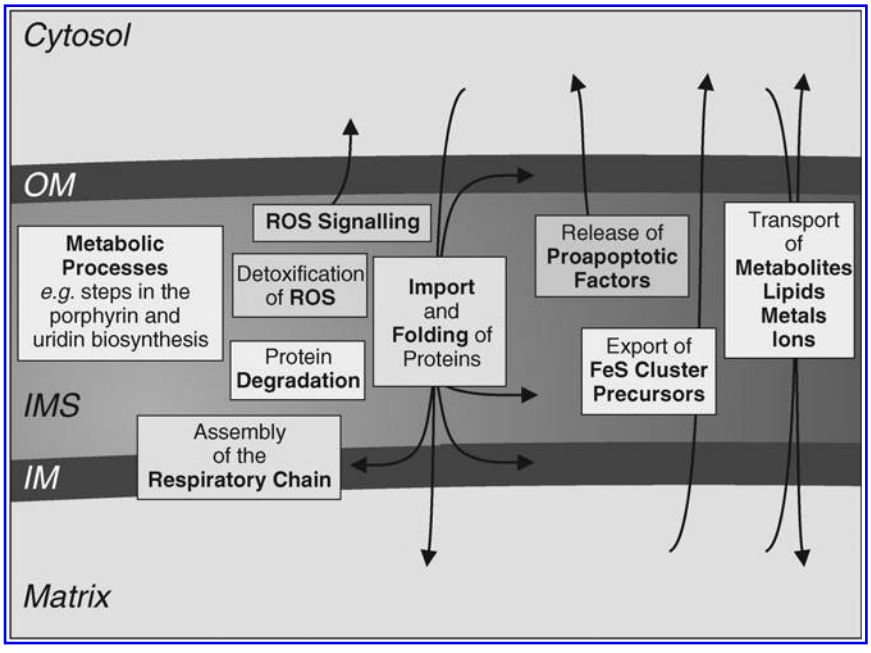

FIG. 5. Functions of the intermembrane space. The IMS is linked to numerous physiologic functions. Because of its localization at the interface of cytosol and matrix, it serves as a transport and signaling hub, and harbors numerous metabolic enzymes (see text for details).

Exchange of metals

Many enzymes of the inner membrane and the matrix contain metal cofactors, in particular iron, copper, and zinc. Our knowledge of the mechanisms by which metal ions are distributed to different cellular locations is still scarce. In the IMS of mammalian mitochondria, metallothioneins were identified that appear to play a critical role in mitochondrial metal homeostasis (203). These proteins are small factors (3 to 14 kDa) that are rich in cysteine residues, allowing a superstoichiometric binding of metal ions, in particular of zinc and copper. In addition, the IMS contains the conserved protein Cox17, which mediates the transfer of copper to the Cu_A- and Cu_B-binding sites of cytochrome oxidase (1, 53, 69). Like metallothioneins, Cox17 is a small polypeptide (8 kDa) that, at least *in vitro*, binds copper and zinc ions (6, 140). Interestingly, *in vitro,* the binding capacity of Cox17 for copper depends on the redox state of the protein. In its reduced form, Cox17 binds up to four copper ions per molecule, whereas in its completely oxidized state, it does not bind metals. However, the predominant state of Cox17 might be a partially oxidized form that binds one copper ion. It was suggested that *in vivo,* a redox-controlled reaction cycle triggers the binding and release of copper to and from Cox17 (10, 22, 68). Although the binding and release of copper to and from Cox17 had been demonstrated, *in vivo* evidence for a correlation with different redox states of the protein is still missing. Notably, Cox17 does not pass on the copper ion directly to cytochrome oxidase but through specific transfer proteins: Cox11, in the Cu_A site of Cox1, and Sco1 and Sco2 in the Cu_B site of Cox2 (8, 164, 183). Interestingly, the structures of Sco1 and Sco2 are related to that of thioredoxins, and experimental evidence suggests a redox control of the metal binding to Sco1 and Sco2 (2, 89, 91, 200). Mutations in Sco1 and Sco2 were shown to lead to a cytochrome oxidase deficiency in humans (141, 166, 186). It will be interesting in the future to unravel the molecular basis of the redox processes underlying the metal transfer in the IMS and the particular defects in patients with Sco1 or Sco2 deficiencies.

Cox17 belongs to a class of IMS proteins known as twin Cx_9C proteins. These factors are characterized by the presence of two pairs of cysteine residues that are each spaced by nine amino acid residues. In yeast mitochondria, 14 twin Cx_9C proteins were identified, of which 13 have homologues in mammalian cells (51, 104). The deletion of most of these components leads to respiration defects and a loss of cytochrome oxidase activity (51, 104). It was proposed that, like Cox17, other members of this family contribute to the metallation of cytochrome oxidase. However, experimental evidence for metal binding to these proteins is still lacking.

The transfer of zinc across the outer membrane is even less understood. The IMS protein Hot13 was recently shown to play a role in the removal of zinc from the reduced forms of Mia40 and Erv1 (28, 117, 122). Hot13, like metallothioneins, is a small acidic protein (13.6 kDa) that contains numerous cysteine residues. Whether Hot13 plays a specialized role in the demetallation of components of the mitochondrial redox relay or whether it exhibits a more general function in mitochondrial zinc homeostasis is not known.

Lipid homeostasis

The inner membrane of mitochondria has a characteristic composition of lipids that significantly differs from that of other cellular membranes [for review, see (56, 76, 162)]. Cardiolipin is a unique lipid that stabilizes the complexes of the respiratory chain and is critical for mitochondrial functionality. It is found mainly in the inner membrane but also in the outer membrane, albeit in smaller amounts (52, 70). Cardiolipin is synthesized in the inner membrane of mitochondria. Like many cellular lipids, cardiolipin is synthesized from phosphatidylserine that is initially produced in the endoplasmic reticulum (ER). A fraction of the phosphatidylserine is transported to the mitochondrial inner membrane, where it is decarboxylated to phosphatidylethanolamine. Phosphatidylethanolamine can remain in mitochondria, where it is converted to other lipids, including cardiolipin. Alternatively, it can be transported back to the ER, where it is used for the synthesis of further lipids, such as phosphatidylcholine. The mechanisms that allow this vital exchange of lipids between the ER and the outer and inner membrane of mitochondria are still poorly understood. Zones of close contact between the ER and the outer membrane can be observed in electron microscopy (110, 171); moreover, mitochondria-associated membrane (MAM) fractions can represent an active interaction compartment that allows the exchange of lipids and other components between compartments of the secretory pathway and the outer membrane (3, 60, 159). Recent studies suggest that specific protein complexes tether mitochondria to the ER and thereby allow—directly or indirectly—the transfer of lipids (34, 61, 85).

Because the outer and inner membranes of mitochondria significantly differ in their composition, dedicated transport factors might control the exchange of lipids across the lumen of the IMS (Fig. 4). Recently, a family of IMS proteins was identified for which such an exchange function was proposed: These proteins are characterized by the presence of conserved PRELI (protein of relevant evolutionary and lymphoid interest) domains. Three members of this family are present in yeast mitochondria: Ups1, Ups2, and Ups3 (136, 165, 179). These proteins are $\sim$20 kDa in size and form complexes in the IMS of $\sim$60, 100, and 60 kDa, respectively, that are associated with the inner membrane. Deletion of Ups1 leads to decreased levels of cardiolipin but increased levels of phosphatidylserine in the inner membrane. These defects are suppressed by simultaneous deletion of Ups2, indicating an antagonistic function of both proteins. Homologues of these proteins are present in mammalian mitochondria (47), which on expression in yeast, can complement Ups1-deficient yeast mutants (179). The molecular function of these proteins is not entirely clear, and it remains to be shown whether they influence the transfer of phospholipids across the IMS lumen or rather regulate the synthesis or turnover of phospholipids in mitochondria.

Export of iron-sulfur–cluster precursors

Mitochondria play a critical role in the biogenesis of iron-sulfur clusters in both mitochondrial and nonmitochondrial proteins. To synthesize iron–sulfur clusters in the cytosol, a still enigmatic precursor molecule has to be exported from mitochondria (99). Export of this precursor depends on ABC transporters in the inner membrane (82), as well as on the activity of the IMS protein Erv1 (88). The precise role of Erv1 in this process is still unclear, but because Erv1 functions as a sulfhydryl oxidase (93, 118), it appears conceivable that it has

to oxidize a potentially sulfur-containing compound to facilitate its export from mitochondria or its ability to be incorporated into cytosolic apoproteins.

Signaling from the IMS

Besides their function in energy metabolism and in the exchange of molecules, mitochondria also fulfil essential functions in cellular signaling, such as signaling via ROS and the release of proapoptotic factors. These processes essentially involve the IMS.

Signaling via reactive oxygen species

Complexes I and III of the respiratory chain are the main cellular producers of ROS (125). The major redox species generated is the superoxide radical (O_2^-), which is rapidly disproportionated to hydrogen peroxide, either spontaneously or enzymatically, by the IMS-localized enzyme superoxide dismutase 1 (Sod1, see later). Further processes ensure a rapid detoxification of different ROS species in the cell, thereby preventing deleterious side reactions that might impair essential cellular structures and functions. However, ROS are not only toxic side products, but they also serve as signaling molecules to induce transcription factor activation, gene expression, cell growth, and apoptosis (175).

In contrast to the superoxide radical, hydrogen peroxide is membrane permeable and serves as a signal to the outside that eventually triggers responses that protect cells against oxidative stress and reestablish "redox homeostasis." One example is the activation of the Yap1 transcription factor in yeast (86, 134). The oxidative activation of Yap1 operates at the posttranslational level and involves the transfer of Yap1 from the cytosol to the nucleus. This translocation is controlled by a cysteine-rich domain within Yap1 that is reduced or oxidized in response to the local redox state. The response of yeast to hydrogen peroxide revealed an increase in the expression of >100 proteins, but also the decrease in the expression of ~50 proteins (55, 107, 196). The induced proteins include many antioxidative proteins like Sod1 and Sod2, glutathione reductase, catalase, thioredoxin reductase, and cytochrome *c* peroxidase (55, 107, 196). In addition, carbohydrate metabolism is rapidly switched to the regeneration of NADPH at the expense of glycolysis (55). Moreover, in higher eukaryotes, ROS are used for regulation of the vascular tone and signal transduction from membrane receptors in various physiologic processes (30, 108).

In the IMS, ROS not only result in protein carbonylation and lipid peroxidation, but also have shown that, for example, cysteines in complex I become oxidized to disulfides by hydrogen peroxide (204), raising the interesting possibility that ROS might serve to control the activity of proteins in the IMS by reversible oxidation (156) (see later).

Release of proteins from the IMS during apoptosis

Many events can result in regulated cell death. These include the excessive production of ROS but also physiologic events during embryonic development (32, 135). Mitochondria take a critical position within the so-called intrinsic pathway of apoptosis, where they serve as integration point for metabolic and proapoptotic signals (32, 120, 132). This often results in an amplification of death signals by the release of apoptogenic factors from the IMS, an altered electron transport through the respiratory chain, and the loss of the membrane potential over the inner membrane (32, 187). The loss of the integrity of the outer membrane is usually regarded as a point of no return in apoptosis.

The susceptibility of mitochondria to apoptotic signals is strongly influenced by the ratio of pro- and antiapoptotic members of the BCL-2 family. Various models proposed that the proapoptotic members of this family play prominent roles in the permeabilization of the outer membrane, by either (a) oligomerizing and subsequent pore formation; (b) interaction with mitochondrial proteins, concomitant triggering of permeability transition, and swelling-induced rupturing of the outer membrane; or (c) altering membrane curvature that results in lipid pores (32, 129, 187).

Among the factors released from the IMS are cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G, Smac/Diablo, and Omi/HtrA2 (38, 98, 142, 177, 178, 188, 189, 191). Besides these proteins, many more proteins have been identified to be released on apoptosis in different experimental approaches (143, 160, 172). Released proteins participate in a variety of processes during apoptosis, including the assembly of the apoptosome (cytochrome *c*, Smac/Diablo), chromatin condensation (AIF), and DNA degradation (endonuclease G, AIF) (38, 98, 142, 177, 178, 188, 189, 191). Cytochrome *c* constitutes the most important signal released from the IMS, because its release allows the formation of the apoptosome and the subsequent activation of effector caspases (155, 205). The storage of these apoptosis-inducing components in the IMS is an elegant way to increase the levels of these components rapidly in the cytosol without the need of a time-consuming protein expression. Besides their role in the induction of apoptosis, these components might exhibit additional functions in mitochondrial biogenesis. This is clearly the case for cytochrome *c*, but also for AIF, a physiologic role in redox processes in the IMS was proposed (77, 184).

Redox Control in the IMS

The IMS is a compartment in which considerable amounts of ROS are produced, presumably leading to the nonenzymatic generation of disulfide bonds. Conversely, the IMS contains the disulfide relay system that introduces disulfide bonds in various proteins. Peroxidases and glutathione-dependent reductases presumably counterbalance protein oxidation (Fig. 6). To keep these different processes in check, the redox level in the IMS must be tightly regulated. Dysfunctions in this control have been associated with numerous diseases, including neurodegenerative diseases and cancer.

The most important redox buffer is the glutathione redox buffer, defined by the ratio of reduced and oxidized glutathione. The glutathione redox buffer of the IMS is at steady state more oxidizing than the respective buffers of its neighboring compartments (cytosol and matrix, see also earlier discussion) (71). Numerous factors could contribute to this more-oxidizing IMS environment [*e.g.*, the generation of ROS by the respiratory chain; see also the review on Sod1 by Giovanni Manfredi in this issue (80a)] or the recently identified Erv1-Mia40 disulfide relay machinery [see also review by Toshi Endo in this issue (42a)], but also a limited diffusion of reduced glutathione over the outer membrane, and oxidative

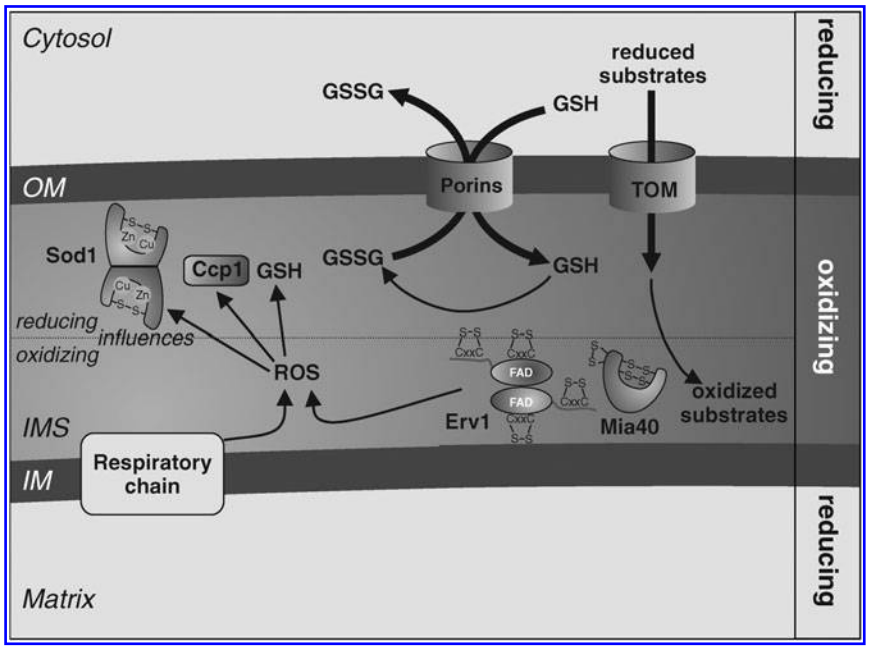

FIG. 6. Factors contributing to the redox environment of the IMS. The glutathione redox buffer of the IMS is, at steady state, more oxidizing than those of the cytosol or the mitochondrial matrix. Various processes could contribute to the oxidizing environment in the IMS, including the production of reactive oxygen species (ROS) by the respiratory chain and the disulfide relay system (Mia40, Erv1). However, these oxidizing systems are likely in a delicate dynamic balance with reducing processes such as the import of reduced glutathione from the cytosol to support stable and constant redox conditions in the IMS.

pathways currently not yet assessed in detail (e.g., Sco2) [see also review by Scott Leary in this issue (88a)].

In most cells, ROS are predominantly generated by the respiratory chain, in particular as by-products of complex I and III (125). The main ROS species produced are superoxide anions (O_2^-), which, in the case of complex III, are at least partially released into the IMS (103, 123, 125, 173). Because of its high reactivity, O_2^- can modify proteins and membrane lipids, resulting in protein carbonylation and lipid peroxidation, respectively (125). To prevent such ROS-induced damage, the IMS harbors various antioxidative components that rapidly scavenge ROS. These antioxidative systems in the IMS include the glutathione redox buffer, cytochrome *c* peroxidase (Ccp1), and superoxide dismutase (Sod1) (33, 80, 176). Glutathione mainly converts different ROS to superoxide anions. Subsequently, other antioxidative systems complete the detoxification. This coupling allows the scavenging of a broad range of ROS species. Moreover, glutathione participates in the reversal of cysteine oxidation by reducing disulfide bonds, and it protects free cysteine residues by their reversible glutathionylation to counteract irreversible protein oxidation. In other cellular compartments, deglutathionylation is catalyzed by glutaredoxins (Grx), and their presence has also been reported for the IMS, at least in mammalian cells (66, 137). Importantly, glutathione-dependent processes result in glutathione oxidation, which is reduced again by glutathione reductases present both in the cytosol and in the mitochondrial matrix.

In fungal mitochondria, the heme-containing enzyme Ccp1 catalyzes the conversion of hydrogen peroxide to water. During this reaction, the bound iron becomes oxidized and is reduced again by cytochrome *c* (65). Hydrogen peroxide in the IMS is mainly derived from the spontaneous or Sod1-catalyzed disproportionation of O_2^-.

Sod1 is a homodimeric copper- and zinc-containing protein with a dual localization to the cytosol and the IMS of mitochondria (80, 133, 176). Although only few percentages of the cellular Sod1 are present in mitochondria, the Sod1 concentration in the IMS is presumably much higher than that in the cytosol because of the very small volume of this compartment. Biogenesis of Sod1 requires its specific activating chaperone Ccs1 (for "copper chaperone of Sod1") that introduces the copper ion into Sod1 (27, 149). The intracellular distribution of Sod1 is presumably influenced by the distribution and activity of Ccs1 (46, 152). It is unclear whether the mitochondrial fraction of cellular Sod1 can be adapted to the amount of ROS

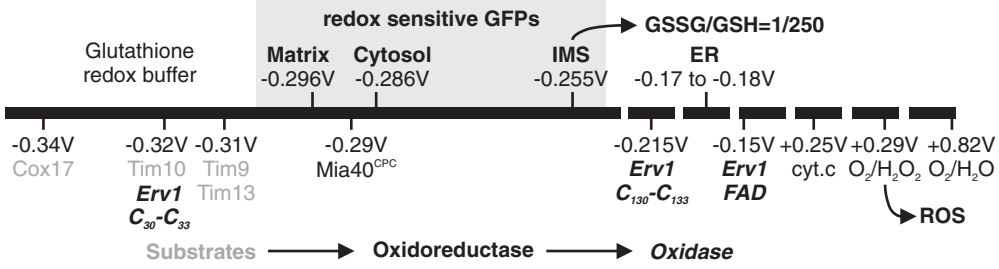

FIG. 7. Redox properties of the disulfide relay system. Redox proteins and factors linked to the mitochondrial disulfide relay are ordered according to their electrochemical potential. The composition of the glutathione redox buffer is indicated as well.

produced in certain cell types or physiologic conditions. In humans, mutations in Sod1 lead to amyotrophic lateral sclerosis (ALS), a neurodegenerative disease that ultimately results in the death of the patients (25, 100, 185). At least in certain cases, mutated Sod1 is associated with mitochondria, suggesting that a misdistribution of Sod1 to IMS can contribute to the pathogenesis of ALS (26, 36, 45, 102). In addition to Sod1, other enzymes to detoxify ROS may exist in the IMS, like peroxiredoxins, glutathione peroxidises, or catalase.

The disulfide relay influences the redox balance in the IMS

A further oxidizing influence on the IMS redox milieu is exerted by the Erv1-Mia40 disulfide relay system that mediates oxidative folding. This system introduces oxidizing equivalents into the IMS by three means: (a) by oxidation of reduced substrates (118, 156); (b) by generating ROS, presumably as a minor-site product of Erv1 activity (15, 44); and (c) by using GSH for proofreading its activity (13). In the latter case, GSH provides the electrons required for the correction of wrongly formed disulfide bonds and thereby becomes oxidized. The disulfide relay system is composed of three major components, Mia40, Erv1, and cytochrome *c*. Whereas Mia40 interacts directly with substrate proteins, Erv1 reoxidizes Mia40 by shuttling electrons from Mia40 to cytochrome *c* from where they are fed into the respiratory chain. The transfer of electrons by Erv1 involves the switch between two-electron transfers (thiol-disulfide exchange) and the transfer of one electron (cytochrome *c*). This switch is mediated by the redox cofactor of Erv1, FAD. During its redox cycle, electrons can be leaked onto molecular oxygen, resulting in ROS generation (Fig. 7).

Further factors that influence the redox states of the thiols in the IMS

Sco1 and Sco2 are conserved assembly factors for cytochrome oxidase (89, 90, 154). Structurally they belong to the thioredoxin family, which catalyzes thiol-disulfide redox reactions (7, 200). Like classic thioredoxins, they contain two conserved cysteine residues in their reaction centers, although these are spaced by three rather than by two amino acid residues, as in other thioredoxins. Nevertheless, Sco2 has been shown to influence the redox state of cysteine residues in Sco1, thereby regulating the copper-binding of Sco1 during maturation of subunit 2 of cytochrome oxidase (91). However, it is still unknown how Sco2 is subsequently reoxidized.

The conserved flavoprotein Cyc2 was recently proposed to function as an enzyme that maintains cysteine residues in newly imported *c*-type cytochromes (cytochrome c_1 of complex III and cytochrome *c*) in a reduced state to allow the covalent linkage of heme cofactors into the respective apoproteins (11, 40, 58) [see also Bonnard *et al*. in this issue (15a)]. Because the cysteine residues in *c*-type cytochromes are arranged in CxxC motifs that resemble the active site of redox-active proteins, it is conceivable that Cyc2 may also interact with other redox-active proteins in the IMS.

Another oxidoreductase of the IMS is the proapoptotic protein AIF (77). During apoptosis, AIF becomes released from the IMS and mediates processes in caspase-independent apoptosis, such as chromatin condensation and DNA degradation (187). The physiologic role of AIF is not entirely clear, but AIF uses FAD and NADH as cofactors, making a reducing activity likely. AIF participates in ROS detoxification, and it is known that its depletion mainly affects complex I in higher eukaryotes. It has been proposed to be involved in the assembly and maintenance of respiratory complexes I and III.

Consequences of Changes in the IMS Redox Environment for Regulation

Several proteins of the IMS and the inner membrane contain conserved cysteines that face the IMS. Examples of these proteins include subunits of complex I, the Rieske protein of complex III, subunits of the TIM translocases, and members of the mitochondrial carrier family (156). However, it is unclear whether the formation of disulfide bonds exerts any influence on the activity of these proteins and whether these disulfides are reversibly formed (*i.e.*, whether dedicated reductive systems exist in the IMS). It was proposed that the redox conditions in mitochondria strongly depend on the oxygen levels, respiration rates, coupling states of the inner membrane, ROS production by respiratory chain, and the glutathione redox buffer in the IMS (14). The composition of the glutathione redox buffer in any compartment is of crucial importance, because it not only protects against hyperoxidizing conditions and scavenges potentially deleterious ROS, but also because its state can influence enzymatic activities. Proteins that are susceptible to a reversible redox regulation by the glutathione redox buffer harbor nonstructural cysteines, and it is striking that numerous IMS proteins contain conserved cysteine residues in motifs deviating from the ones typically found in disulfide-bonded IMS proteins. It will be exciting to explore their specific physiological relevance in the future.

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Abbreviations Used

AIF = apoptosis-inducing factor
ER = endoplasmic reticulum
GFP = green fluorescent protein
GRx = glutaredoxin
GSH = glutathione
IMS = intermembrane space
ROS = reactive oxygen species
TIM = translocase of the inner membrane of mitochondria
TOM = translocase of the outer membrane of mitochondria

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