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Review

Oxidation-driven protein import into mitochondria: Insights and blind spots ☆

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ABSTRACT

The intermembrane space of mitochondria contains a dedicated machinery for the introduction of disulfide bonds into proteins. In this case, oxidative protein folding is believed to drive the vectorial translocation of polypeptides after their synthesis in the cytosol across the mitochondrial outer membrane. Substrates of this system are recognized by a hydrophobic binding cleft of the oxidoreductase Mia40 which converts them into an oxidized stably folded conformation. Mia40 is maintained in an oxidized, active conformation by the sulfhydryl oxidase Erv1, a homodimeric flavoenzyme, which can form disulfide bonds *de novo*. Erv1 passes electrons on to cytochrome *c* and further to the respiratory chain. The components of this system, their structures and the mechanisms of disulfide bond formation were analyzed only very recently. This review discusses our knowledge about this system as well as open questions which still wait to be addressed. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

1.1. Cysteine residues have unique properties

The exceptional reactivity and chemical versatility of cysteine residues sets this amino acid clearly apart from the other 19 amino

Abbreviations: Ccs1, copper chaperone for Sod1; GSH, reduced glutathione; IMS, intermembrane space; ITS, IMS-targeting signal; MISS, mitochondria IMS-sorting signal; MTS, matrix targeting signal; Sod1, superoxide dismutase 1; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane

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acids found in proteins. Numerous modifications of the thiol (-SH) moiety of cysteines have been identified. Examples are oxidations to sulfenic acid, sulfinic acid or sulfonic acid [1], glutathionylation [2,3], nitrosylation [4,5], and farnesylation [6]. Although many examples for these modifications were reported, we still have a very poor understanding of the extent by which cysteine residues are modified under physiological conditions. Recent proteomic data suggest that a large fraction of thiols is indeed modified *in vivo* [7]. For example, about 10% of all cysteine residues in proteins of human cells are glutathionylated under normal growth conditions; and upon treatment with the oxidizing reagent diamide up to half of all cysteine residues can be modified with glutathione molecules [7]. In addition to these modifications, cysteine residues in proteins can react with each other by oxidation to form intra- or intermolecular disulfide bonds (-SS-). These covalent interactions—if present at the right place—can considerably stabilize the structure of a protein. However, in all other cases, they potentially interfere with the fold of a protein and compromise its activity. These special features of cysteines have two consequences: First, cysteines are by far less abundant in proteins than most other amino acid residues: on average, only 2 out of 100 residues are cysteines [7,8]. Second, many of the cysteine residues found in proteins are evolutionary conserved, pointing to a specific function of these residues at the specific position. The recent development of techniques to analyse the amino acid modifications at specific sites of individual proteins or on proteome-wide scales will enable us to explore the physiological relevance of cysteine chemistry.

1.2. The formation of disulfide bonds in proteins is tightly controlled

To reduce the risk of detrimental cysteine oxidation, most cellular compartments prevent the formation of disulfide bonds. For example, in the cytosol, in the matrix of mitochondria or in the nucleus, thioredoxins and glutaredoxins together with a high concentration of reduced glutathione counteract the formation of disulfide bonds. In these compartments, only very few typically oxidation-sensing proteins are known to form disulfide bonds under physiological, healthy conditions [9,10].

In some compartments, however, the oxidation of cysteine residues is actively promoted due to the presence of oxidizing enzymes. These compartments are the periplasm of bacteria, the endoplasmic reticulum and the intermembrane space of mitochondria (IMS) [11–14]. The oxidation machinery of the latter was identified only recently and will be described in the following.

2. The mitochondrial disulfide relay—what we know

Mitochondria contain two membranes, the outer (OM) and the inner (IM) membrane, enclosing two hydrophilic compartments, the matrix and the IMS. The matrix harbours hundreds of proteins which are involved in many functions including respiration, metabolic conversions, the biogenesis of iron-sulfur clusters, or the propagation and expression of the mitochondrial genome. Almost all of these proteins are initially synthesized as precursor proteins on cytosolic ribosomes. These precursors carry aminoterminal matrix targeting signals (MTSs) which direct them into mitochondria and which are proteolytically removed in the matrix by the matrix processing peptidase [15–18]. As far as we know all matrix proteins embark on a common import route that employs the translocase of the outer membrane (TOM complex) and the translocase of the inner membrane (TIM23 complex). Translocation is driven by the membrane potential across the inner membrane and the hydrolysis of ATP in the matrix which is used by the import motor of the TIM23 complex to thread proteins into the matrix [19–22].

With about 50–100 proteins, the IMS of mitochondria contains a smaller number of proteins than the matrix. Nevertheless, these proteins carry out a number of important functions in particular in the transport of metabolites, proteins or lipids between both mitochondrial membranes, in the processing or assembly of mitochondrial proteins, in the communication between mitochondria and the rest of the cell or in the regulation of apoptosis. All proteins of the IMS are nuclear encoded and synthesized in the cytosol. Most of these proteins lack MTS sequences and employ diverse routes for mitochondrial import [23,24]. In many of these proteins internal targeting signals containing cysteine residues are critical for mitochondrial import. These targeting sequences were named MISS (mitochondria IMS-sorting signal) or ITS (IMS-targeting signal) and are both sufficient and necessary for the transport of proteins into the IMS [25,26]. The cysteine residues in these signals are recognized by the IMS-localized oxidoreductase Mia40 which serves as an intramitochondrial import receptor (Fig. 1). Mia40 contains a redox-active cysteine pair which is maintained in an oxidized state by the sulfhydryl oxidase Erv1. Mia40 and Erv1 constitute the mitochondrial disulfide relay system and are evolutionary conserved among plants, fungi and animals.

2.1. Substrates of the mitochondrial disulfide relay

The substrates that rely on Mia40 and Erv1 for mitochondrial import fall into several groups which will be introduced in the following.

2.1.1. Small Tim proteins (twin CX_3C proteins)

These proteins form hexameric complexes which facilitate the translocation of hydrophobic proteins across the lumen of the IMS. In mitochondria of baker's yeast, five Tim proteins were identified: Tim8, Tim9, Tim10, Tim12 and Tim13. The numbers indicate the approximate molecular weight of these small proteins. All these proteins consistently have a helix-loop-helix fold (Fig. 1A, left). Each helix contains two cysteine residues separated by three residues which gave rise to the name of twin CX_3C proteins. The helices are of antiparallel orientation and are stabilized by two parallel disulfide bonds connecting cysteine residues 1–4 and 2–3. Six small Tim proteins form heteromultimeric complexes ($[Tim9\text{-}Tim10]_3$, $[Tim8\text{-}Tim13]_3$) of about 70 kDa in which the loop regions are in close proximity to each other and the termini remain flexible similar to a jellyfish with 12 tentacles [27,28]. It was suggested that these tentacles can embrace substrate proteins to usher them across the IMS [28]. The cysteine residues are critical for the import and folding of the small Tims as well as for their assembly [25,28–31]. A mutation of the fourth cysteine residue of the human homolog of Tim8, DDP1, leads to absence of the DDP1-containing complexes causing the progressive neurodegenerative disease Mohr Tranebjaerg syndrome in affected individuals [32,33].

2.1.2. Twin CX_9C proteins

Like small Tim proteins, these proteins share a helix-loop-helix structure. Here, the cysteine residues are spaced by nine residues (Fig. 1A). The best characterized representative of this group is Cox17, a copper-binding protein that plays a role in copper transfer to cytochrome *c* oxidase [34,35]. It was proposed that the Cox17-mediated copper transfer is associated with a change of the number of disulfide bonds present in the protein [36]. However, experimental evidence is still lacking that could support a cycling of Cox17 through different redox states *in vivo*.

Besides Cox17, 13 additional proteins with twin CX_9C motifs were identified in yeast, most of which have homologs in mammals [37,38]. Many of these proteins are required for the assembly or stability of complexes of the respiratory chain, but their exact molecular function is still unknown.

2.1.3. Other substrates

In addition to these helix-loop-helix proteins, a small number of additional proteins that rely on the disulfide relay for mitochondrial