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Artigo Original

Coupling between the proteasomal catalytic unit and the 19S regulatory unit affects mitochondrial functionality after site-specific mutations of the 20S particle

Acoplamento das unidades catalítica e regulatória do proteassomo e a funcionalidade mitocondrial em leveduras após mutações sítio-específicas na unidade catalítica do proteassomo

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ABSTRACT: A post-translational redox modification called S-glutathionylation in *S. cerevisiae* was described at Cys residues of the α5 subunit of the 20S catalytic unit of the proteasome, specifically α5-C76, posteriorly mutated to α5-C76S. The α5-C76S strain presented, as a phenotypic alteration, a higher frequency of the closed conformation of the catalytic chamber of the 20S unit and a shorter chronological life span (CLS: chronological life span). A double random mutation (DM: double mutated) in the α5 subunit (α5-S35P/C221S) induced the opening of the catalytic chamber and also increased CLS. This project aimed to assess the coupling between the catalytic and the regulatory units of the proteasome in some yeast strains (C76S, WT and DM) and to further evaluate their mitochondrial functionality. The study of the coupling of 20S-19S units was carried out in native gel electrophoresis. To determine mitochondrial functionality, the activity of citrate synthase was measured by the reaction between DTNB with CoA-SH. It was observed that in the C76S strain there was a lower degree of coupling between the 20S catalytic and 19S regulatory units, in addition to a decreased citrate synthase activity. Therefore, less coupling between the proteasomal units triggers mitochondrial dysfunction most likely due to deficient mitochondrial protein import, ultimately leading to decreased CLS, which can better our understanding in regards to the process of aging and premature cell death seen in degenerative disorders caused by protein accumulation.

Keywords: Redox biology; Mitochondria; Proteasome; Proteolysis.

RESUMO: Em leveduras da espécie *S. cerevisiae*, foi descrito uma modificação redox pós-traducional denominada S-glutationilação em resíduos Cys da subunidade α5 da unidade catalítica 20S do proteassomo, especificamente o resíduo α5-C76, posteriormente mutada para α5-C76S. A linhagem carregando essa mutação apresentou como alteração fenotípica um menor tempo de vida cronológico (CLS: chronological life span) e uma maior frequência da conformação fechada da câmara catalítica da unidade 20S. Uma dupla mutação randômica na subunidade α5 (α5-S35P/C221S) criou uma linhagem duplo-mutante (DM), a qual induziu a abertura da câmera catalítica do 20S e também aumentou o CLS da célula. O presente estudo teve como objetivo estudar o grau de acoplamento entre as unidades catalítica e regulatória do proteassomo nas linhagens de levedura (C76S, WT e DM), bem como avaliar a funcionalidade mitocondrial em

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todas elas. A análise do acoplamento foi feita com eletroforese em gel nativo. Para determinar a funcionalidade mitocondrial, foi medida a atividade da enzima citrato sintase a partir da reação entre DTNB e CoA-SH. Foi observado que na linhagem C76S com CLS reduzido havia um menor grau de acoplamento entre as unidades catalítica 20S e regulatória 19S, além de uma atividade mitocondrial diminuída. Portanto, um menor grau de acoplamento entre as unidades do proteassomo muito provavelmente provocou uma disfunção mitocondrial devido a um importe de proteínas mitocondriais defeituoso, resultando em uma CLS reduzida, fato que pode melhor elucidar a nossa compreensão acerca do processo de envelhecimento e da morte celular prematura vista em doenças degenerativas causadas por acumulação proteica.

Palavras-chave: Biologia redox; Mitocôndria; Proteassomo; Proteólise.

INTRODUCTION

Proteolysis is a process of utter importance for cellular homeostasis, given that it is through protein catalysis that the removal of aberrantly synthesized proteins and those damaged during metabolic reactions may occur flawlessly, while also recycling active cell peptides and proteins. In the mid 1980s, Aaron Ciechanover, Avram Hershko and Irwin Rose described the ubiquitin-proteasome system (UPS), responsible for the majority of cellular proteolysis. This discovery granted them the Nobel Prize in Chemistry in 2004.

The UPS is a protein degradation pathway composed of enzymes capable of tagging proteins in order to signal them for degradation. This labeling is done by ubiquitin, an 8.5 kDa protein that is linked to the proteasomal substrate by a series of enzymes. E1 (ubiquitin activating enzyme) activates ubiquitin using ATP and transfers it to E2 (conjugating enzyme). E2 then interacts with E3 (ubiquitin ligase), which allocates ubiquitin to the N-terminal lysines of the protein substrates. After the first ubiquitin is added, new ubiquitin molecules are inserted onto the existing ones, forming a poly-ubiquitin chain, which acts as a recognition signal for degradation. Once the protein is poly-ubiquitinated, it will be recognized by the proteasome.

The proteasome is a protein complex composed of two units. The catalytic unit, known as the 20S (20SPT), is composed of four stacked heptameric rings, forming a barrel-like structure. There are two beta rings (made up of beta subunits) flanked on either side by two alpha rings (made up of alpha subunits). While the alpha rings control the access of substrates into the proteasome, the beta rings make up the catalytic chamber, with three catalytically distinct subunits in each ring: beta 1, with caspase-like activity; beta 2, with trypsin-like activity and beta 5, with chymotrypsin-like activity.

The other unit of the proteasome is the regulatory unit, known as the 19S (19SPT), capable of interacting with the alpha subunits of the 20S and modulating the activity of the proteasome. It is made up of nineteen subunits, organized into a lid and a base. In the base, there are six subunits with ATPase activity, which use energy to unfold protein substrates and to transport them into the catalytic unit, in addition to subunits that function as ubiquitin receptors. In the lid, there are proteases capable of removing ubiquitin from the substrates [1].

The proteasome can be found with the catalytic and regulatory units coupled together, forming either the 26S proteasome (when there is only one 19S unit associated with the 20S), or the 30S proteasome (when there are two 19S units associated with the 20S). However, the proteasome can also be composed by the catalytic unit (20S) in its free form. Thus, 20S deprived of 19S is responsible for the degradation of mainly non-ubiquitinated proteins, most of which have naturally unfolded sequences, or are partially denatured by oxidative stress[1].

Figure 1 illustrates the different forms in which the proteasome can be found.

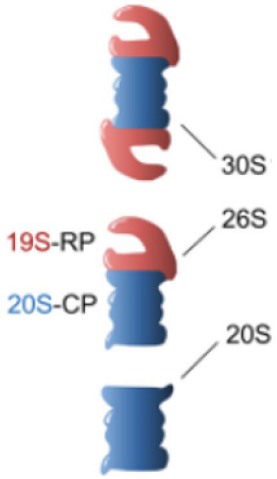

Figure 1. A schematic representation of the different forms of the proteasome[2].

It was found that the S-glutathionylation of the Cys76 residue in the α5 subunit of the 20S proteasome in yeasts of the species *Saccharomyces cerevisiae*[3,4] is critical for the opening of the catalytic chamber, since mutation at this site for Ser residues, α5-Cys76S, resulted in a higher percentage of the 20S in its closed conformation and in a strain with a shorter chronological life span (CLS: Chronological Life Span), compared to wild type. The same was observed in a strain with mutation in α5-Cys221S. Cells that had concomitant mutations in both residues, α5-Cys76S and α5-Cys221S proved to be unviable[5]. In contrast, a double random mutation of the α5 subunit (S35P/C221S) caused a higher frequency of the catalytic chamber in its open conformation and a higher

CLS. Furthermore, this double mutant (DM) strain also showed an increase in resistance to oxidative stress, with the 20S catalytic unit more active. From the aforementioned studies, it was concluded that CLS is closely related to the frequency of the open conformation of the 20S proteasome. However, the analysis of the degree of coupling between the catalytic unit (20S) and the regulatory unit (19S) of the proteasome was not carried out. Since the maintenance of CLS is based on the degradation of signaling proteins via polyubiquitination, it is speculated that deficits in the coupling between catalytic and regulatory units may result in lower rates of proteolysis by the UPS, which would lead to a proteotoxicity due to the accumulation of ubiquitinated proteins, consequently, determining a decreased CLS[6].

Currently, it is known that the association between 20S and 19S units depends, among many factors (eg, cytoplasmic chaperones - Hsp70, Hsp90 and Ecm29)[7] on mitochondrial functionality. Being responsible for the production of ATP through oxidative phosphorylation, mitochondria are of fundamental importance for UPS activation, since both the ubiquitination process and the unfolding of protein substrates before entering the catalytic unit require ATP. In order to properly function, mitochondria necessitate mitochondrial proteins, most of which are encoded by nuclear DNA, synthesized in the cytosol and imported into the organelle through translocases present in the outer mitochondrial membrane (TOM: Translocase of Outer Membrane)[8]. During the import process, some mitochondrial proteins may undergo premature folding or aggregate with other proteins, which may cause the TOM complex to become clogged. In both cases, the UPS is essential in the quality control of mitochondrial proteins, as it degrades proteins that would obstruct TOM, freeing it for protein import without structural defects[7]. In addition to this, there is a retro-translocation system of intramitochondrial proteins, damaged by the intrinsic oxidative metabolism of the organelle, to the cytosol where they can be ubiquitinated and degraded by the UPS[1]. In other words, while the mitochondria guarantees the availability of ATP for the proper functioning of the UPS, the UPS, in turn, assists in protein import, a process of paramount importance for mitochondrial functionality. Figure 2 illustrates the mechanisms described above.

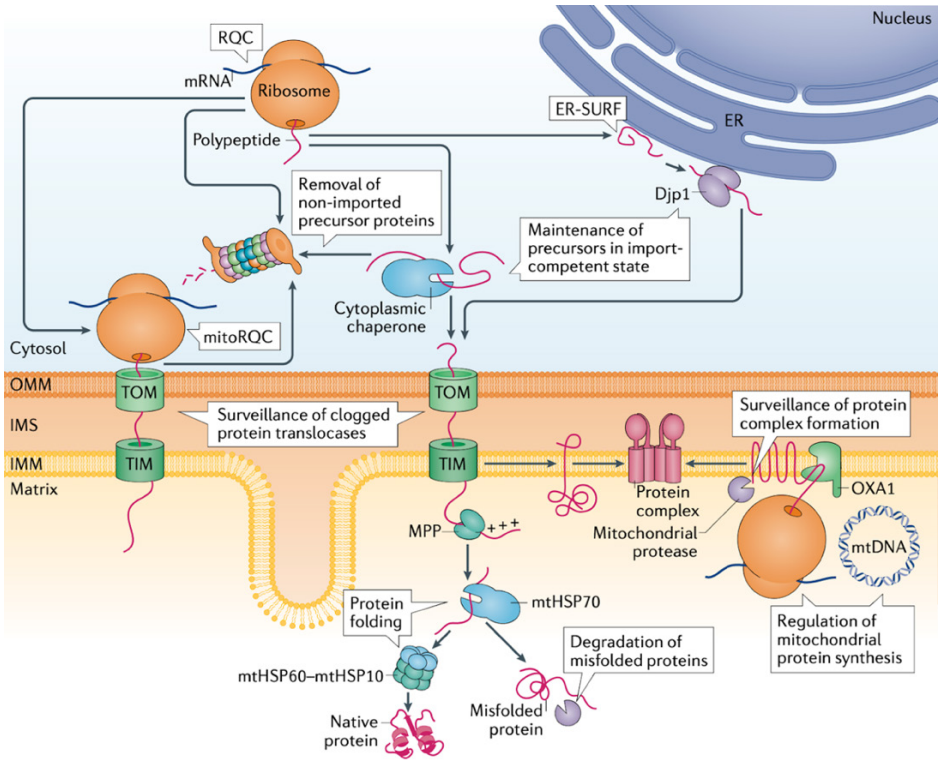

Figure 2. Representation of the mitochondrial protein quality control done by the proteasome[9]

If the quality control of mitochondrial protein import is compromised, there can be an increase in the concentration of reactive oxygen species, unfavoring the coupling between the 20S and 19S units of the proteasome, which would culminate in an increase in the 20S in its free form[10]. It is speculated that this phenomenon is a cellular defense mechanism against oxidative stress, since the catalytic unit is more resistant to oxidative damage than the 19S (and, therefore, than the 26S and the 30S) and is largely responsible for proteolysis in the absence of ATP

of oxidized and non-ubiquitinated proteins. Thus, through uncoupling, cells can redirect proteolysis to oxidized proteins rather than ubiquitinated proteins[1].
Thus, in light of the information mentioned above, our hypothesis was that CLS differences among the studied *Saccharomyces cerevisiae* strains are due to differences in coupling between catalytic and regulatory units of the proteasome, which could alter mitochondrial functionality and, therefore, affect CLS. This project aimed to evaluate the coupling of 20S-19S units in mutant strains compared to the wild-type strain and to assess mitochondrial functionality.

OBJECTIVE

The main objective of this project was to evaluate the coupling of the 20S with the regulatory unit 19S in the mutant strains mentioned above. The mitochondrial function in these strains was also assessed, since the proteasome has a fundamental role in the quality control of mitochondrial proteins.

Specific objectives

1. Assess coupling between the catalytic unit 20S and the regulatory unit 19S of the proteasome in the following strains: WT, double-mutant (DM: S35P/C221S) and C76S, using native gel electrophoresis.

2. Assess mitochondrial function in the three yeast strains through the activity of the mitochondrial enzyme citrate synthase.

METHODS

Cellular growth

S. cerevisiae cells of the wild-type RJD1144 strain and mutants (S35P/C221S and C76S) are grown in YPG rich medium (Yeast extract 1%, Peptone 2%, Glycerol 2%, Ethanol 2%) at 30°C, 200 RPM for 60h. After growth, the cells were centrifuged for 10 minutes at 4,500 x g and 4°C. The obtained pellets were washed in distilled water and stored at -80°C until cell lysis.

Cell lysis and separation of mitochondria

After obtaining the pelleted cells, the cell wall was removed by incubating the cells with Zymolyase (1 mg/mL) in 3 mL of buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA and 1.2 M sorbitol) per gram of pellet. The mixture was incubated for 2 h at 37ºC, at 300 rpm. After centrifugation and washing with the buffer described above, the spheroblasts thus obtained were lysed with a buffer 50 mM Tris pH 7.4 containing: 0.5% NP-40; 1 mM EGTA, 10% glycerol, 5 mM ATP and 1 mM MgCl2. After adding the buffer (3 mL/g spheroblast), 15 aspiration and ejection movements with syringe and insulin needle were needed for cell lysis to occur. The samples were then centrifuged at 3,000 x g for 5 min. The resulting supernatant is centrifuged at 15,000 x g for 10 min. After centrifugation, the supernatant is used for native gel electrophoresis (described below) and the pellet, made up of mitochondria, is used for assessment of mitochondrial functionality. The entire procedure is performed at 4 ºC [11].

Coupling between the catalytic unit 20S and the regulatory unit 19S of the proteasome

The coupling between the 19S-20S units of the proteasome was studied using cell extracts from the yeast strains for native gel electrophoresis. This method made it possible to assess the presence of 19S-20S-19S (30S proteasome), 19S-20S (26S proteasome) and 20S in its free form (20S proteasome) in the cell extract after analyses of in-gel proteasomal activity[8].
In summary: 20 µg of protein were applied in the native gel (5% and 3% polyacrylamide for resolving and stacking gel, respectively). The electrophoresis was done in 6h, at 150V, at 4ºC. Once finished, the gel was transferred to a gel imager (L-PIX EX) and the proteasome substrate (s-LLVY-AMC) was spread onto the gel (100 µM), together with ATP (5mM) and MgCl2 (1mM). After 15 min, proteasomal activity was seen via fluorescence.

Citrate synthase activity

In order to analyze mitochondrial functionality, we observed the activity of the mitochondrial enzyme citrate synthase. The assessment of its activity is based on the formation of CoA-SH, once citrate synthase catalyzes the reaction between acetyl-CoA and oxaloacetate acid, with citrate and CoA-SH as final products. The measurement of activity of citrate synthase is fundamented on the reaction between CoA-SH and the sulfhydryl reagent DTNB (5,5'-dithiobis-2-nitrobenzoic acid). One of the products of this reaction (TNB) is determined by a colorimetric method (absorbance at 412 nm): CoA-SH + DTNB → TNB + CoA-S-S-TNB.
In summary: 5 µg of mitochondria were added to 20 mM Tris buffer pH = 7.5 (with 0.2 % Tritton), containing 10 mM of acetyl-CoA and 1 mM of DTNB. At the beginning of the experiment, 10 mM of oxaloacetate was added to the solution with a multichannel pipette. From that moment forward, the reaction was monitored for 3 min at 412 nm, during which the absorbance was measured[12].

RESULTS

Figure 3 displays the results obtained in native gel experiments performed with yeast strains grown in YPG

(Yeast extract 1%, Peptone 2%, Glycerol 2%, Ethanol 2%). It was observed that the C76S strain has a lower concentration of the 26S and 30S complexes (B) and, more importantly, it presented reduced proteasomal activity in both of the complexes and in the 20S unit in its free form (A) when compared to the WT and the double mutant (DM) strains.

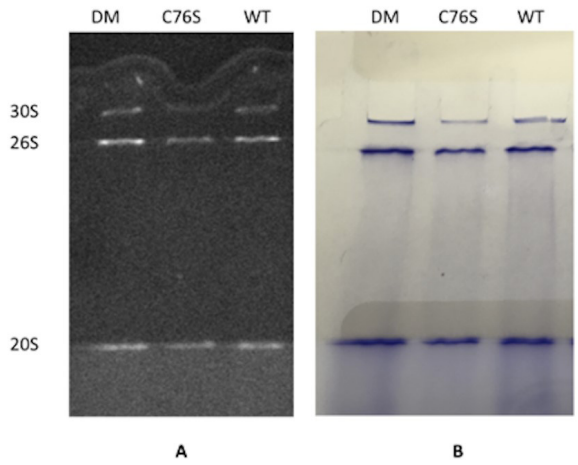

Figure 3. (A) Native gel electrophoresis using cell extract from the studied yeast strains. After the experiment (6h, 150V, 4ºC), proteasomal activity was assessed using a gel imager (L-PIX EX) that registered fluorescence once the proteasomal substrate Suc-LLVY-AMC was applied to the gel. (B) The same gel was coloured with Coomassie Blue.

Furthermore, when comparing the mitochondrial pellets of the three strains, it was noticed that the C76S, in addition to being smaller, did not have the same brownish color of the others; on the contrary, it presented a pale off-white color, which suggests an alteration in cytochrome C. The mitochondrial pellets are shown in Figure 4.

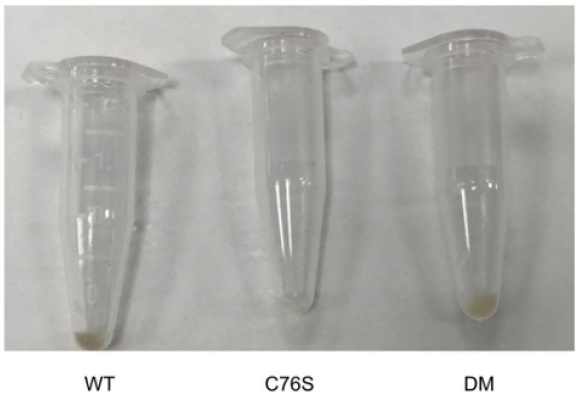

Figure 4. Mitochondrial pellets from the 3 yeast strains, which were obtained as described in Methods

Consequently, the C76S strain displayed a lower citrate synthase activity in comparison with the WT strain, while the DM strain showed a higher enzyme activity than WT, as shown in Figure 5.

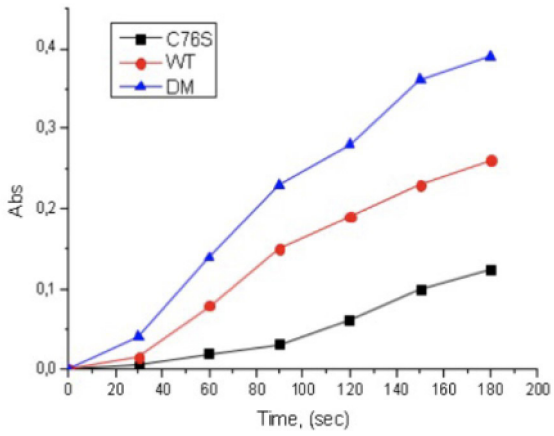

Figure 5. Graph representing the activity of citrate synthase in the three yeast strains.

CONCLUSION AND DISCUSSION

The lower degree of coupling between the 20S and 19S units of the proteasome, in conjunction with a reduced proteasomal activity observed in the C76S strain, most likely generated mitochondrial dysfunction, evidenced by the low activity of citrate synthase and the pale mitochondrial pellets, which ultimately led to a decreased CLS.

Our hypothesis is that, with reduced coupling of the proteasomal units, together with an attenuated proteasomal activity, the proteasome is unable to adequately perform its role in the quality control of mitochondrial protein import, which most likely results in a proteotoxicity caused by an accumulation of mitochondrial proteins in the cytosol. Consequently, the mitochondria is incapable to properly function, spawning a depletion of ATP and therefore an ineptitude to maintain vital cellular functions, ultimately leading to a diminished CLS. We may speculate that the knowledge behind this biochemical mechanism is paramount in unveiling some degenerative cell processes leading to anticipated cell death in disease-based protein aggregates (e.g.; some forms of dementia and transthyretin amyloid cardiomyopathy), as well as the aging process itself, which is crucial to the development of new therapeutic interventions.

In the future, we expect to deepen our understanding in regards to the relationship between the proteasome and mitochondria in the yeast strains. The next step is to assess mitochondrial protein import through immunostaining in order to determine if there is in fact protein aggregation in

the cytoplasm because of deficient import, and to study in greater detail mitochondrial dysfunctionality, by analyzing the activity of the mitochondrial enzymes cytochrome C oxidase and reductase, given that the white mitochondrial pellet from the C76S strain highly suggests alterations in cytochrome C.

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