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Protein Synthesis and the Stress Response

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1. Introduction

Accuracy of protein synthesis is critical for life since a high degree of fidelity of the translation of the genetic information is required to accomplish the needs of the cellular functions as well as to preserve the variability developed by evolution. More than one hundred macromolecules are involved in this process even in the simplest organisms, including ribosomal proteins, translation factors, aminoacyl-tRNA synthetases as well as ribosomal and transfer RNAs (being rRNAs near 80% of total cellular RNAs in bacteria). Accuracy of translation of the genetic information is accomplished at different levels, being one of the most relevant the specific interaction of the aminoacyl-tRNA synthetases with their substrates, tRNA and amino acids. Either specific molecular interactions that avoid the miss-incorporation of amino acids or the hydrolysis of wrong aminoacyl-tRNAs represent strategies utilized by the aminoacyl-tRNA synthetases to reduce the formation of miss-acylated tRNAs. Additionally, the discrimination by the elongation factor Tu (EF-Tu) against miss-acylated aminoacyl-tRNAs over correctly acylated increases the accuracy of incorporation of the proper aminoacyl-tRNAs to the ribosome. The accurate decodification of the mRNA by the incorporation of the aminoacyl-tRNA with the correct anticodon ensures the fidelity of translation of the genetic information. The consequence of these discriminatory events led to an accuracy of translation of the genetic information to as low as 10^{-4} errors per amino acid incorporated. However, certain level of inaccuracy might be tolerated, even might be beneficial to the cells under certain physiological and environmental conditions.

How does the cellular translational machinery respond against stress conditions? Is protein synthesis as accurate as in normal conditions when cells are stressed? These are topics that are not well understood. Different kinds of stressing conditions might have different responses in the components of the protein synthesis system. In this chapter, we will briefly describe how bacteria respond to two stress conditions, oxidative stress and amino acid starvation, that microorganisms are commonly exposed to including in environmental conditions.

2. Oxidation of the translation machinery during oxidative stress

Oxidative stress is defined as the condition where oxidative species production is faster than the ability of the cell to eliminate them and reduce or degrade oxidized products, leading to damage [1–3]. This condition can be met when production of or exposure to oxidative species is increased, such as when bacterial cells are attacked by the immune system of a host [4–7] or when oxygen reactive species (ROS) are generated in cyanobacteria as a byproduct of photosystem II irradiation with strong light [8]. Also, “normal” ROS levels can cause oxidative stress when there is a lower level of protective enzymes as when bacteria pass from an anaerobic environment to another with oxygen [9]. As a consequence of the increase in oxidative species concentration several macromolecules can be modified, including proteins, RNA, DNA and lipids [10–12]. In this chapter we are mainly interested in the oxidation of the first two, proteins and RNA, as they are directly involved in the translation process. Several amino acids and cofactors of proteins can be target of oxidation *in vivo*. Within these, the most sensitive to oxidation are Fe/S clusters, methionines and cysteines [11,12]. Some of the oxidation states of cysteine (sulfenic acid and disulfide bond) can be reduced so this amino acid is frequently involved in regulatory processes [13–15]. The rest of the amino acids are usually considered to be more stable to oxidation, although many of them are carbonylated during oxidative stress, especially when they are located near to cations that can catalyze the oxidation reaction [11,12,16,17]. RNA oxidation is less well studied, although we know that ribonucleotide bases can be oxidized to 5-hydroxyuridine, 5-hydroxycytidine, 8-hydroxyadenosine and 8-hydroxyguanosine [18]. In addition to these oxidative modifications, nucleotides can also completely loss their bases during oxidative stress [19].

When cells enter in oxidative stress several targets are oxidized decreasing the activity of many metabolic pathways including translation in both bacteria [4,20–23] and eukaryotes [24–27]. Part of this inhibition might be due to the oxidative inactivation of several enzymes involved in the metabolism of energy and amino acid synthesis [7,11,12,28,29] both of which are essential for protein synthesis. Also, several macromolecules that participate in translation have been found to be a target of oxidation in *in vivo* or *in vitro* experiments indicating that translation is directly targeted by oxidative species. In bacteria, these target macromolecules include elongation factors Tu [30–34], Ts (EF-Ts) [32] and G (EF-G) [8,9,22,32,35], several ribosomal proteins [31,36,37], tRNA [38–42] and aminoacyl-tRNA synthetases (aaRS) [31,34,36,37,43,44] (Table 1). rRNA and mRNA has also been shown to be oxidized *in vivo* in eukaryotes [19,25,26], but in bacteria this has not been tested.

Although many macromolecules involved in translation have been found to be target of oxidation, there is little information on the effects of this oxidation on translation and the bacterial physiology (Figure 1). The effects of oxidation of any of the ribosomal proteins or of elongation factors Tu and Ts have not been studied, although we know that the deletion of *yajL* (that encodes for a chaperon/oxido-reductase that protects this and other proteins during oxidative stress) increases the error rates of translation when *Escherichia coli* is

incubated with a moderate concentration of H_2O_2 (~100 μM)[34,45]. Conversely, the effect of oxidation of EF-G has been characterized in a much greater detail. In *E. coli* cells, EF-G has been shown to be carbonylated in several stress conditions including incubation with H_2O_2 , menadione or paraquat, transference from anaerobic to aerobic conditions in high iron concentrations [9] and growth arrest conditions in strains deleted for superoxide dismutase [35]. EF-G has also been shown to be carbonylated in *Bacillus subtilis* cells growing exponentially or exposed to H_2O_2 [32]. Finally, in the cyanobacteria *Synechocystis* sp., oxidation of three isoforms of EF-G during oxidative stress caused by excessive light exposure has been shown to inhibit translation [8,20,21]

Target	Type	Reference
Ribosomal proteins		
Ribosomal proteins L5, L14, L7/L12, L27, L31, S2, S4, S17 and S21	Cysteine oxidation	[31,36,37]
Ribosomal proteins S1, S2, S3, S4, S8, S10, S11, S12, S19, L2, L5, L6, L10, L11, L12, L13, L14, L27, L28	Covalent binding to chaperon/oxido-reductase protein through cysteine bond	[34]
Ribosomal proteins S2, S4, S7, S11, S13, S18, L16, L17	Disulfide bond formation	[37]
Elongator factors		
EF-Tu	Cysteine oxidation	[30,31]
EF-Tu	Covalent binding to chaperon/oxido-reductase protein through cysteine bond	[34]
EF-Tu	Carbonylation	[32,33]
EF-Ts	Carbonylation	[32]
EF-G	Cysteine oxidation	[8,20,21,46]
EF-G	Carbonylation	[9,32,35]
Aminoacyl-tRNA synthetases		
Alanyl, Phenylalanyl, Glutamyl, Glycyl, Aspartyl, Leucyl, Isoleucyl, Seryl and Threonyl-tRNA synthetase	Cysteine oxidation	[31,36,37,43,44]
Alanyl, Isoleucyl, Leucyl, Threonyl and Phenylalanyl tRNA synthetases	Covalent binding to chaperon/oxido-reductase protein through cysteine bond	[34]
tRNA		
tRNA	4-thiouridine oxidation	[41,47]
tRNA	Cross-linking of 4-thiouridine in position 8 with C8.	[42,48,49]

Table 1. Some macromolecules involved in translation that have been shown to be oxidized in bacteria.

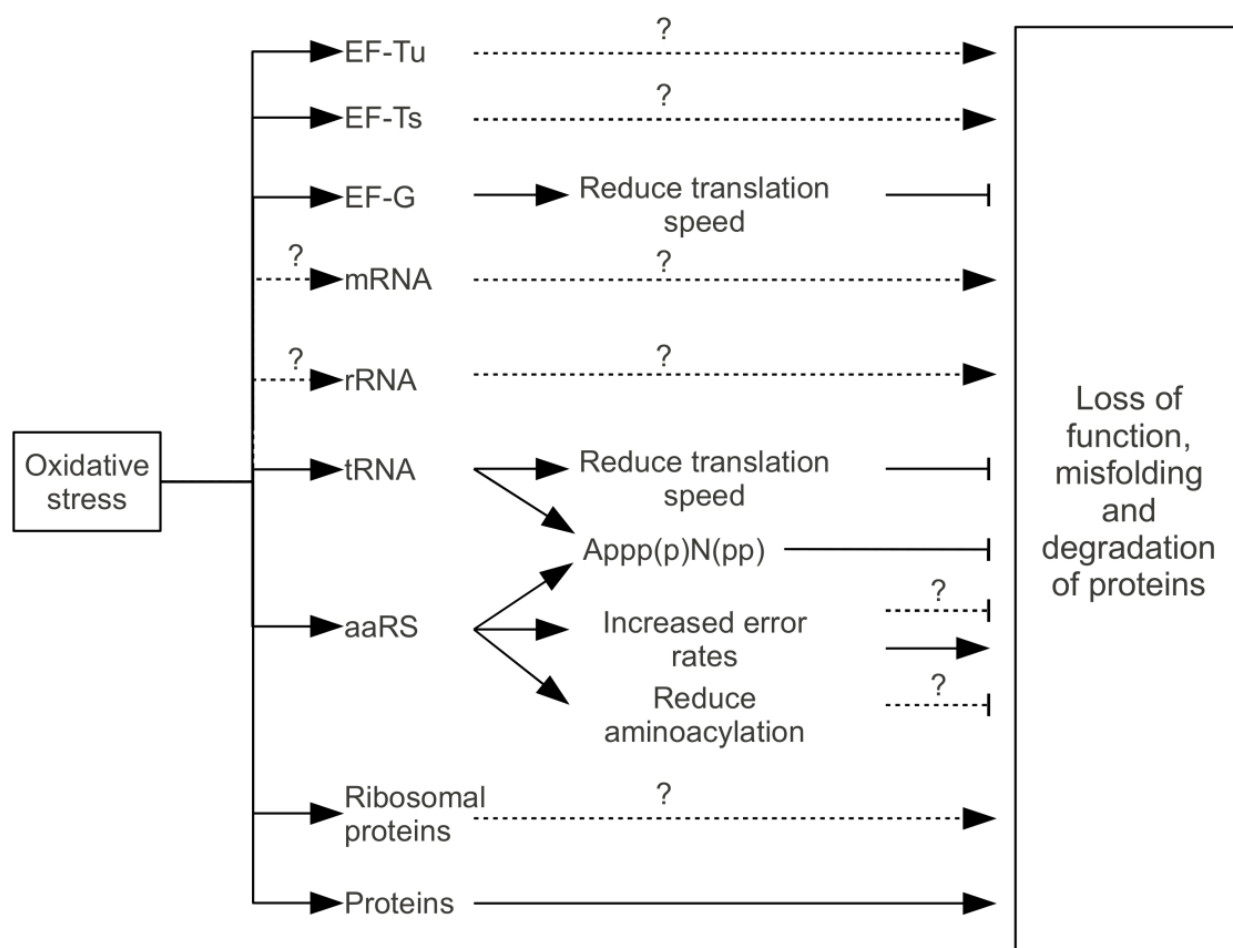


Figure 1. Effect on protein synthesis of the oxidation of translation related macromolecules. Oxidation of proteins may produce loss of function and structure which further produce protein aggregates that are toxic to the cell. Control of the translation machinery may sometimes protect the cell by decreasing the protein synthesis rate or enhancing the translation of specific proteins that protect the cell such as chaperons and proteases. Nevertheless, oxidation of the translation machinery may also increase its error rates which would produce proteins that are less stable increasing the toxicity of the oxidative stress.

2.1. EF-G oxidation in *Synechocystis* sp. during exposure to high intensity light

In *Synechocystis* sp., as well as in all photosynthetic organisms, light provides the energy that drives photosynthesis. Nevertheless, using photosynthesis is risky because photosynthetic transport of electrons or transfer of excitation energy to oxygen can produce ROS and subsequent oxidative stress [50]. Absorption of excessive light can also decrease the photosynthetic capacity of an organism in a process referred to as photodamage. The main target of photodamage is protein D1 which is part of photosystem II where the photochemical reaction and subsequent transport of electrons from water to plastoquinone occurs [51]. Loss of activity depends on the balance between both the speed of damage and repair of protein D1. The limiting step of repair is the *de novo* translation of the protein, thus the use of protein synthesis inhibitors like chloramphenicol has allowed to study damage separated from repair. These experiments have shown that, although oxidative stress increases the sensitivity of photosystem II to photodamage, its role is mainly to inhibit the

translation of protein D1 while damage is caused directly by light [8,20,21]. [³⁵S]-Methionine incorporation experiments confirmed that D1 protein translation is inhibited under oxidative stress and also showed that this is a rather general effect where translation of all thylakoid membrane proteins is inhibited [20,21].

Most of D1 protein translation elongation is performed in membrane bound polysomes. During oxidative stress the fraction of mRNA associated to those polysomes decrease suggesting that the inhibition of translation happens at the elongation step [8,20,21]. Further *in vitro* experiments showed that the main target of oxidation is EF-G. *Synechocystis* sp genome encodes for 3 EF-G proteins, all of which can enhance protein translation in cell extracts that have been oxidized *in vitro* with H₂O₂ [22]. Mutation analysis of one of this EF-G variant, encoded by gene slr1463 of *Synechocystis* sp. PCC6803, showed that Cys105, which is near to the GTP-binding site, is the primary target of oxidation. Mutations on this cysteine or Cys242 (with which Cys105 form a disulfide bond) bears an EF-G protein that is resistant to H₂O₂ oxidation *in vitro* as measured by its ability to stimulate translation in oxidized cell extracts [8,46]. It has not been possible to substitute the slr1463 gene by a Cys105Ser mutant. Instead the mutant protein has been expressed in a strain of *Synechocystis* sp. that maintains its copy of the wild type gene. In this system, expression of slr1463 Cys105Ser decreased the loss of photosystem II activity and increased the rate of D1 translation confirming the relevance of this cysteine during oxidative stress inhibition of translation [52].

EF-G is substrate of reduction by thioredoxin in *Synechocystis* [46,53]. Based on this observation, it has been proposed that the translation activity could be rapidly recovered after oxidative stress through the reduction of cysteines 105 and 242 by thioredoxin. This last protein in turn would be reduced by NADPH-thioredoxin reductase and ferredoxin-thioredoxin reductase with reducing power from photosystem I [8,46].

2.2. Oxidation of tRNA

Together with the oxidation of ribosomal proteins and translation factors, other macromolecules involved in translation can be oxidized during oxidative stress. In this context, oxidation of RNA has been found in eukaryotes during oxidative stress, senescence or some age related diseases [19,25,26]. This reaction would depend on the ability of RNA to bind Fe⁺² which catalyzes its oxidation. As mRNA and especially the more abundant rRNA bind Fe⁺² better than tRNAs, these molecules would be more prone to oxidize [26]. The effect of oxidation over some specific mRNAs was shown to decrease translation of those genes through either increasing ribosome stalling or decreasing translational speed [25]. Oxidation of eukaryotic ribosomes also decreases translation efficiency, although in this case it is not clear whether oxidation of rRNA or ribosomal proteins is responsible of the effect [26].

In bacteria oxidation of rRNA and mRNA has not been studied, but on the other hand tRNA is known to be subject of oxidation *in vitro* [38,47] and *in vivo* [41,42]. Many of the tRNA bases are modified after its transcription [54] and apparently those modifications are very relevant for the role that tRNA plays during oxidative stress. In *E. coli* some of these modifications have a protective effect. For example, methylation of A37 in tRNA^{Val} increases survival after an incubation of 2 hrs in 5 mM H₂O₂ [55]. The authors of this study

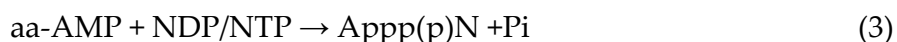
cite unpublished data that indicates that several other tRNA modifications would improve the fitness of *E. coli* in such oxidative conditions. It has also been shown that deletion of several tRNA modification enzymes affect survival of *E. coli* in a milder oxidative stress condition (0.5 mM H₂O₂) [56]. Despite these reports, it is not clear how tRNA modifications improve survival to oxidative stress. It has been suggested that tRNA modifications increase the efficiency of translation in this condition, which would be necessary to cope with the degradation of proteins due to loss of function and/or structure [55]. There are some reports in the literature that show that an increase in error rates during protein synthesis enhances protein oxidation. In theory, this would be due to an increased exposure of oxidation targets that are normally hidden in the interior of proteins [3,57]. Thus, it is also possible that the requirement of tRNA modification during oxidative stress is related with the need of decreasing error rates to hide possible oxidation targets.

Nevertheless, in the only well studied example, tRNA modification is required because it directly participates in initiating the response to oxidative stress. An important source of oxidative stress for bacteria in environments exposed to sun light is near UV irradiation (300–400 nm), which corresponds to the sun irradiation with highest energy that can cross the atmosphere [58]. Near UV irradiation of bacteria like *E. coli*, *Salmonella Thyphimurium* or *Enterobacter cloacae* produces growth arrest which depends mainly in the photochemical oxidation of the tRNA modified base 4-thiouridine (s⁴U) present in position 8 of all tRNAs [39–42]. In some tRNAs that also have a C in position 13 (50% of bulk tRNA) an internal cross-linking reaction happens that produces 5-(4'-pyrimidin 2'-one) [39,40,59]. Some cross-linked tRNAs have been shown to be poor substrates for aminoacylation [40,60,61] and in some cases also for translation [60]. The accumulation of such deacylated tRNAs can trigger the stringent response (see below). Thus, a combination of the trigger of stringent response plus the decrease in tRNA aminoacylation inhibits protein synthesis after UV exposure [40,62,63].

In *E. cloacae*, a short period of exposure to UV light protects the cells from the growth arrest produced by a second exposition of 60 minutes. This effect coincides with a decrease in the content of s⁴U modification in tRNA [48], which could be interpreted as the presence of a protective effect mediated by the loss of this tRNA modification. Concordantly, mutation of the genes involved in s⁴U modification of tRNAs (*nuv* mutant) also protect *S. thyphimurium* cells from the lag in the growth curves produced by short exposures (15 min) to near UV light. Nevertheless, the lag in growth seems to have a protective effect in long term exposure. After 4 to 5 hours of exposure to near UV, the mutant cells died at a faster rate than wild type (over 10 fold difference of survival after 8 hrs.). Part of this increased sensitivity to UV exposure is due to the lack of stringent response, but s⁴U modification is somehow also relevant in the induction of several heat shock and oxidative stress response proteins such as alkyl hydroperoxide reductase [41]. Thus, it seems that the loss of s⁴U modification after exposure to UV could be associated with a lag in the tRNA turnover process and not with a long term protection process, although similar long term exposures have not been performed with *E. cloacae*. In accordance, tRNA sulfuretransferase activity, which is necessary for the synthesis of s⁴U tRNA modification, is inhibited in *E. coli* extracts from cells exposed to UV irradiation [49].

2.3. 5'-adenylyl dinucleotides and oxidative stress

Beside its direct effect on stringent response and the induction of several oxidative stress response enzymes, cross-linking of s⁴U also induce the synthesis of 5'-adenylyl dinucleotides of the general structure AppppN (adenosine-5', 5''-ribonucleotide tetraphosphate) and ApppN (adenosine-5', 5''-ribonucleotide triphosphate) [41]. These dinucleotides are synthesized by aminoacyl-tRNA synthetases during stressful conditions such as oxidative stress and heat shock [41,64–67]. During the synthesis of aminoacyl-tRNA, aaRS produce aminoacyl-adenylate (aa-AMP) as an intermediate of the reaction (reaction 1). In this intermediate molecule the amino acid (aa) is activated for its transference to tRNA (reaction 2) [68], but the adenylate is also activated and can be transferred to either ATP, ADP, ppGpp or other nucleotides in a side reaction that forms dinucleotides of the general structure AppppN, ApppN or ApppNpp [69–71] (reaction 3). This reaction can be catalyzed by several aaRS, but the most active are Phenylalanyl-tRNA synthetase (PheRS) and specially Lysyl-tRNA synthetase (LysRS) [64,69].



During UV irradiation cross-linking of s⁴U somehow stimulates the production of AppppA and ApppA [41], but these dinucleotides are also synthesized by bacteria under other oxidative stress conditions and during heat shock, where s⁴U state has not been evaluated [65–67,72]. Probably, an important source of the increase of these molecules concentration during heat shock is that the rate of dinucleotides synthesis by LysRS increases with temperature, while the rate of Lys-tRNA^{Lys} synthesis decreases [64]. Also, the induction at higher temperatures of a specific LysRS isoenzyme (encoded by *lysU* gene) that is more stable and active for dinucleotides synthesis may explain the increase in AppppN during heat shock [64]. A possible explanation for the increase of these dinucleotides during oxidative stress could be the inactivation of their degradation enzyme, P₁P₄-bis(5'-adenosyl) tetraphosphate pyrophosphohydrolase (ApaH), which *in vitro* is sensitive to cysteine oxidation [73]. An alternative is that oxidation of tRNA modified bases by itself activate the synthesis of the dinucleotides [65], similar to the case of UV irradiation where it has been shown that mutants that lack the s⁴U modification cannot trigger the synthesis of AppppN [41].

Originally it was thought that these dinucleotides would function as “alarmones”, that is, small molecules that are synthesized during a stress condition (in this case oxidative stress and heat shock) and trigger a fast response to it, similar to the role of (p)ppGpp during amino acid stringency (see below) [41,65–67,74]. Nevertheless, time course experiments showed that AppppA increase in concentration lags behind the synthesis of heat shock proteins during stress conditions, suggesting that it is not an alarmon in this case [72]. Also, over expression of *apaH*, the gene coding for AppppN hydrolase that eliminates these

dinucleotides *in vivo* [73], decreased significantly the levels of AppppA, but did not affect protein expression or cell survival under heat shock or H₂O₂ incubation [75]. Conversely, deletion of *apaH*, that increases the cellular level of AppppA, does affect cell survival and protein expression on several stress conditions including heat shock [76,77], UV irradiation [76], incubation with N-ethylmaleimide [76] as well during starvation [78]. It was shown that deletion of *apaH* somehow decreases expression of CAP-cAMP controlled genes which would decrease oxidative phosphorylation and limit further production of oxygen radicals. *apaH* mutant cells also showed prolonged synthesis of heat shock protein DnaK after heat shocked cells were returned to 30°C suggesting that the role of dinucleotides would be to modulate the long term response to stress conditions and not to trigger it [76]. The idea that the dinucleotides modulate the stress response is also supported by the fact that these molecules specifically bind several heat shock proteins including DnaK, GroEL [77] and ClpB [78]. Binding to DnaK inhibits its 5'-nucleotidase activity [79], but it is not known if it affects its ability to bind denatured proteins. Effects of AppppA binding to the other heat shock proteins has not been investigated, although it has been shown that increases in cellular level of Appp(p)N enhance the degradation of abnormal proteins synthesized during incubation with puromycin [78].

2.4. Role of aminoacyl-tRNA synthetases during oxidative stress

As discussed previously, LysRS and PheRS participate in the modulation of oxidative stress response through the synthesis of 5'-adenylyl dinucleotides. Many other aaRS that are oxidized during diverse oxidative stress conditions (Table 1) could also have relevant roles. Unfortunately, the effect of oxidation on most of these enzymes has not been characterized, although from some studied examples we know that oxidation can inactivate the enzyme [44] or increase its error rates [43]. In the case of human histidyl-tRNA synthetase (HisRS) apparently oxidation can even activate the enzyme [80], although we lack a thorough biochemical characterization of this oxidized enzyme.

In *E. coli*, cysteines from glutamyl-tRNA synthetase (GluRS) have been shown to be oxidized *in vivo* in cells lacking a periplasmic disulfuro isomerase (DsbA) [36]. The specific oxidized cysteines as well as the effect of their oxidation have not been characterized. Nevertheless, there are reports of the effect of *in vitro* oxidation on GluRS1 from *Acidithiobacillus ferrooxidans*. This enzyme has 4 cysteines one of which is near the active site, while the others form part of a Zn⁺² binding domain. Oxidation of the Zn⁺² binding cysteines inactivates the enzyme and releases part of the Zn⁺² [44]. Inactivation of the enzyme has been observed *in vivo* during excessive synthesis of tetrapyrroles like heme and it is supposed to regulate the synthesis of these molecules through modulation of the intracellular levels of its precursor, Glu-tRNA^{Glu} [81]. Nevertheless, it is not known if the *in vivo* inactivation is due to oxidation and thus, if oxidative reactions are involved in this regulatory process.

The best characterized example of oxidation of a bacterial aaRS is the case of Threonyl-tRNA synthetase (ThrRS) which increases its error rates after *in vivo* or *in vitro* oxidation. ThrRS active site normally charges a small fraction of the tRNA^{Thr} with serine (Ser). This Ser-

tRNA^{Thr} could be potentially toxic to cells due to miss-incorporation of Ser in position of threonine (Thr) during protein translation. To prevent this mis-incorporation, ThrRS has an editing site that deacylate the mis-aminoacylated tRNA^{Thr} [82]. Incubation of the enzyme with H₂O₂ inactivates its editing site, but does not affect the aminoacylation site. Thus, in these conditions the enzyme mis-acylation rate increases. Incubation of *E. coli* cells with H₂O₂ enhances the miss-incorporation of Ser in Thr positions confirming that oxidative stress do increase the error rates of the enzyme *in vivo* [43]. A similar example has been observed in eukaryotic cells where oxidative stress also increases the error rates of Methionyl-tRNA synthetase (MetRS), although instead of using the incorrect amino acid, the enzyme utilize non-cognate tRNAs [83]. Apparently, these mis-incorporation also happens in bacteria, where *E. coli* methionyl-tRNA synthetase (MetRS) is able to aminoacylate non-cognate tRNAs with Met, mainly tRNA^{Arg}_{CCU} and tRNA^{Thr}_{CGU} [84]. Unfortunately, there is no data with respect to the effect of oxidation on the mis-incorporation of Met in bacteria. It is expected that the mis-incorporation of Met could protect proteins from oxidative damage by oxidizing them before relevant targets [83]. This is in striking difference with the case of ThrRS, where it has been shown that mis-incorporation of Ser produces a lag in cell growth curves and a higher susceptibility to deletion of proteases [43].

2.5. Final remarks on the effects of oxidative stress on protein translation

While the increase of error rate by MetRS apparently protects the cell from further damage, a similar behavior by ThrRS decrease the fitness of cells to this stressful environment [43,83]. These contradictory effects of very similar phenomena are representative of what happens with all the components of the translation machinery and sometimes makes it difficult to determine whether an oxidized molecule is part of the control of protective mechanism or is itself a target of oxidation with deleterious consequences. At the same time, some of these effects can be deleterious or advantageous depending on the extent of the oxidative insult. An example of this is the role of s⁴U modification on tRNA. The absence of this modification prevents the lag on replication after a short near UV irradiation and thus increases the cell fitness on these conditions. Nevertheless, when exposed for longer times to near UV, bacteria survive better when are able to produce the modification.

Cells cannot avoid using reactive molecules that are necessary for catalysis, but at the same time are easy targets of oxidative species. In many cases oxidation inactivates proteins that use this molecules and also destabilize their structure enhancing the formation of protein aggregates that are toxic to the cell. Thus, bacteria also need to have systems that protect them from further damage. An important step in cell protection is to prevent the synthesis of new proteins that could be targets of oxidation by inactivating several macromolecules involved in translation (Figure 1). At the same time, cells need to activate the translation of proteins that participate in the synthesis and usage of antioxidant molecules, as well as proteases and chaperons that prevent the formation of aggregates that are toxic to bacteria. After the stressful condition has passed, translation has to be re-initiated and proteases have to be inactivated in order to prevent the degradation of newly synthesized proteins that are folded correctly. The data presented in this chapter shows that the translation system has a

central role in all this process. At one side is by itself a very important target of oxidation, which affects the rate of translation and its fidelity. This has profound effects on the cellular physiology some of which are protective and others deleterious. Also, as a target of oxidation, it appears that the translation system participate in the modulation of the response to stress. We still lack enough information in order to fully understand what is the specific effect of oxidation on each of the components of the system. At the same time, although we do have some hints, we do not understand how all these components interact between them during oxidative stress or how they coordinate with other oxidative stress response components.

3. Bacterial response to amino acid starvation

During evolution, living organisms have acquired various systems to survive under adverse environmental conditions. Upon nutrient starvation, bacteria slow down all processes related to cell growth and increase the functionality of processes that overcome nutrient deficit. This generalized process is known as the stringent response and occurs in cells designated as *rel⁺*. The stringent response is triggered by the increase in the cellular levels of (p)ppGpp, also known as the “alarmone” or “magic spot” [85]. The level of these G nucleotide derivatives is regulated in *E. coli* by the activities of RelA and SpoT, two distinct but homologous enzymes. Under conditions of amino acid starvation, RelA senses uncharged tRNA stalled in the ribosome and synthesizes (p)ppGpp by pyrophosphorylation of GDP (or GTP) using ATP as the donor of pyrophosphate [86]. SpoT is a bifunctional enzyme that either synthesizes or degrades ppGpp [87] and its function is regulated in response to carbon, fatty acids or iron limitation. Catalytic activities in both enzymes are oriented to the amino termini regions that show conservation of the amino acid sequence. Conversely, carboxy termini are idiosyncratic since they are specific for each enzyme and their function is related to the signaling activities. Carboxy terminal domain (CTD) from RelA interacts with the ribosome probably sensing the uncharged tRNA [88]. SpoT contains in the CTD a region that interacts with the acyl carrier protein in the activation process [89]. RelA/SpoT related proteins have been found in all bacteria including the recently discovered “small alarmone synthetases” (SAS). These proteins seem to have complementary roles to RelA/SpoT. They are never alone but always in addition to RelA/SpoT in different combinations. RelA/SpoT homologous proteins have also been found in chloroplast probably with functions similar as in bacteria. Another ppGpp synthetase was found in chloroplasts of land plants that is sensitive to Ca^{++} [90]. Also in metazoan, another SpoT related protein, Mesh1, was recently identified [91]. The gene encoding Mesh1 compensates SpoT deficiencies in bacteria and *Drosophila* deficient in this protein show several impairments related to starvation. These findings widen the horizons of the functions of RelA/SpoT proteins to all kingdoms and also provide new relationships on signaling networks to control response to starvation.

3.1. Biosynthesis of (p)ppGpp in the ribosome

Aminoacyl-transfer RNAs (aa-tRNAs) are essential to cell physiology since they provide the amino acids to the ribosome for the translation of the genetic information encoded in the

mRNA. Aa-tRNAs are synthesized by the aminoacyl-tRNA synthetases and are delivered to the ribosome by the elongation factor EF-Tu (in bacteria) in the ternary complex aa-tRNA/EF-Tu/GTP. The ternary complex is positioned in the A site of the ribosome and as long as a correct pairing of the anticodon of tRNA with the codon in the mRNA is achieved, EF-Tu is released from the complex after hydrolysis of GTP. Upon formation of the peptide bond, the deacylated tRNA is released from the ribosome and is rapidly aminoacylated again by the corresponding aminoacyl-tRNA synthetase. Thus under normal growth conditions, the majority of tRNAs are aminoacylated and actively participating in protein synthesis. In contrast, under amino acid starvation, an important accumulation of deacylated tRNA takes place since the aminoacylation reaction is reduced. Under these conditions, an increasing number of A sites in the ribosome become empty or loaded with deacylated-tRNA and pausing of translation at these sites takes place [92-94]. Binding of deacylated-tRNA in the A site of the ribosome induces the formation of the RelA Activating Complex (RAC). RelA binds to RAC and catalyzes the transference of the β - γ pyrophosphate from ATP to either GTP or GDP for the formation of pppGpp or ppGpp respectively [93] (pppGpp is rapidly transformed to ppGpp, thus we will refer as ppGpp). Once ppGpp is formed, RelA is released from the ribosome but the deacylated tRNA might remain bound being released passively and independent from RelA. While deacylated-tRNA is still bound to the ribosome, it is unable to accommodate an incoming aa-tRNA/EF-Tu/GTP complex, thus it is stalled for protein synthesis. As long as RAC is active, new RelA molecules can bind and catalyze the formation of ppGpp [86]. As deacylated tRNA passively dissociates from the ribosome the stability of the interaction with the ribosome is a critical factor that influences the formation of ppGpp and thus the stringent response [95]. Recent data on the activation of RelA has shown that stalled ribosomes loaded with weakly bound deacylated-tRNAs require higher concentrations of enzyme than those loaded with tightly bound deacylated-tRNAs [96], suggesting that the recovery of cells from stringent response might be dependent on the type of starved amino acid.

3.2. Role of ppGpp in the transcription of stable RNAs and amino acids biosynthesis genes

The most well known effect of an increase in the concentration of ppGpp is the down regulation of the rRNA and tRNA transcription and thus of ribosomes and protein biosynthesis upon amino acid starvation. This is primarily an effect at the transcription level (reviewed in [97, 95]) and requires a direct interaction of the “alarmone” with the β and β' subunits of the RNA polymerase affecting several activities, but mainly reducing transcription of rRNA genes. Biochemical, genetic and structural data indicate that ppGpp binds near the active site of RNA polymerase suggesting that the vicinity of this interaction might be involved in some of the observed effects [99-101]. There seems to be a reduced stability in the interaction between RNA polymerase and DNA in the open complex upon binding of ppGpp to the β and β' subunits. Open complex at rRNA promoters is particularly unstable, thus this might be a requirement for the observed effect [102, 103]. However some stable open complexes are also affected by ppGpp suggesting that other

mechanisms contribute to the effect of ppGpp in the activity of RNA polymerase at this level [104]. Other steps might be affected upon binding of ppGpp to RNA polymerase such as promoter clearance, open complex formation, pausing of transcription elongation and competition between ppGpp and other nucleotide substrates. These effects are not mutually exclusive and might take place at the same time.

Although the effect on stable RNAs is the major and the most well known effect on gene expression, a number of other functions related to cell growth are also affected upon ppGpp increase in the cell. Ribosomal proteins and elongation factors gene expression are negatively affected as well as fatty acids and cell wall biosynthesis. DNA biosynthesis is particularly sensitive to ppGpp and thus to amino acid starvation since in *E. coli* its progression stops soon after induction of ppGpp accumulation [105, 106]. ppGpp binds directly to DNA primase inhibiting initiation of DNA replication at both lagging and leading strands [107].

3.3. Role of DksA in the regulation by ppGpp

DksA is a protein that was discovered as a suppressor, when overexpressed, of the thermo sensitivity of *dnaK* mutants [108]. In addition it has many other functions, among these being the need of this protein and ppGpp to stimulate the accumulation of RpoS (the stationary phase and stress response σ factor) at the translational level [109]. A direct involvement of DksA potentiating the effect of ppGpp on the stringent response was discovered as one of its major functions [110, 111]. DksA is a structural homolog of the transcription elongator factors GreA and GreB [112]. These proteins bind directly to RNA polymerase particularly to the secondary channel of the enzyme inducing the cleavage of RNA in arrested enzymes rescuing them and restoring the polymerization activity. DksA seems to bind to RNA polymerase in a similar way, but without the induction of cleavage of RNA. Binding of DksA is believed to stabilize the interaction of RNA polymerase with ppGpp [112]. DksA can compensate the effect of a ppGpp⁰ mutation (complete absence of ppGpp) reinforcing the notion that these two factors are synergistic both in positive and negative regulation. But DksA has also some other roles that are opposite to ppGpp, for instance in cellular adhesion, indicating that although compensatory, these two factors might have their own role in the stringent response [114].

Along with the pronounced inhibition of stable RNA transcription, positive effects on gene expression have also been observed upon increase of ppGpp levels. Two major ways to activate transcription have been proposed, direct and indirect activation. Direct activation implies the interaction of RNA polymerase with an effector such as ppGpp, DksA or both to activate transcription from a promoter. Transcription of several operons for the biosynthesis of amino acids, responding to the housekeeping σ^{70} factor, is activated by a direct mechanism. Promoters for the *hisG*, *thrABC* and *argI* are activated *in vitro* by a combination of ppGpp and DksA [112]. It is proposed that a step in the isomerization during the formation of the open complex is favored in the direct activation of these promoters.

Indirect activation of a specific promoter might be the result of the inhibition of other promoter, usually a strong one, that increases the availability of RNA polymerase to activate

the target promoter [115, 116]. Evaluation of indirect activation of certain promoters comes mainly from *in vivo* studies. Activation of several σ factors other than σ^{70} also requires ppGpp. A competition mechanism that implies a reduced affinity of the core RNA polymerase for σ^{70} upon binding of ppGpp and/or DksA has been proposed, allowing to other σ subunits to bind to the core enzyme [117, 118]. It is speculated that RNA polymerase bound to strong promoters is released upon binding of ppGpp/DksA thus increasing the availability of the enzyme and also lowering the affinity to σ^{70} making the core enzyme available to the alternative σ factors.

In general speaking, ppGpp inhibits σ^{70} promoters of genes involved in cell proliferation and growth and activates promoters of genes involved in stress response and maintenance.

3.4. Targets for control of translation

The major effect of ppGpp in protein synthesis is certainly the biosynthesis of stable RNAs being inhibition of the transcription of rRNA and tRNA the targets for this effect. A marked reduction of the general translation of mRNAs as a result of the reduction of ribosomes as well as tRNAs is the major response against starvation of amino acids as well as other nutrients. In addition to this generalized response, other components of the translation machinery are also affected by the stringent response. Particularly translation factors that use guanine nucleotides are also target of ppGpp. As G proteins, these are the factors that have been the subject of analysis on the effect of (p)ppGpp at the translation level. G proteins are generally small proteins that bind GTP. The hydrolysis of this nucleotide, generally assisted by a G activating protein (GAP), to form GDP that remains bound to the protein, is required for the function to take place. The removal of GDP and its exchange for GTP is generally catalyzed by additional exchange proteins (GEP) that form part of the G proteins cycle [119].

Three proteins play important roles in the initiation step of translation, IF1, IF2 and IF3, being IF2 a G protein. IF3 binds to the ribosomal 30S subunit in the 70S ribosome releasing it from the 50S subunit to initiate a new cycle of elongation for the translation of an mRNA. IF1 assists IF3 in the releasing of the 30S subunit and also allows to the fMet-tRNA^{fMet} to be positioned in the correct P site to initiate translation. IF2 is a small G protein that in complex with GTP (IF2-GTP) binds the initiator fMet-tRNA^{fMet}. This ternary complex docks the fMet-tRNA^{fMet} in the small ribosome subunit. As the mRNA binds, IF3 helps to correctly position the complex such that the fMet-tRNA^{fMet} interacts by base pairing with the initiation codon in the mRNA. The mRNA is correctly positioned, assisted by the interaction of the Shine-Dalgarno sequence with the 16S rRNA, in the small 30S subunit. As the large 50S ribosomal subunit binds to the initiation complex, it participates as a GAP, thus GTP bound to the IF2 is hydrolyzed and released from the complex as IF2-GDP.

Elongation step of translation also requires in part the participation of the G-proteins EF-Tu and EF-G to take place. EF-Tu-GTP binds all aminoacyl-tRNAs with approximately the same affinity and delivers them to the A site of the ribosome in the elongation step of protein synthesis. Once a correct codon-anticodon interaction is detected by the ribosome, a

conformational change in the ribosome takes place that induces the release of the EF-Tu factor along with the hydrolysis of GTP, thus the ribosome in this conformation acts as the GAP for the EF-Tu-GTP complex. EF-Ts is the GEP that assists EF-Tu-GDP to exchange GDP for GTP to initiate another elongation cycle.

EF-G is a G protein factor that complexed with GTP participates in the translocation of the nascent peptidyl-tRNA in the ribosome. Peptidyl transferase activity of the 23S RNA in the 50S subunit forms the peptide bond between the newly incorporated aminoacyl-tRNA in the A site delivered by EF-Tu and the existing peptidyl-tRNA already positioned in the P site from previous elongation cycles. The new peptidyl-tRNA with one extra amino acid is translocated from the A to the P site by EF-G-GTP. This process also implies the movement of the free tRNA positioned in the P site to the E site in the ribosome. EF-G itself seems to carry its own GEP.

RF3 releasing factor is also a G protein that participates in the termination of translation. Its function will not be discussed in this article.

As it is expected, these G proteins have been the subject of attention as potential targets for the action of ppGpp in the control of translation under the stringent response. GTP is at very high concentrations in the cell reaching more than 1 mM under normal growth conditions whereas GDP reaches very low concentrations. Upon amino acid starvation ppGpp can accumulate at the expenses of GTP that lowers its concentrations to nearly 50% [120-122]. Both nucleotides reach similar concentrations, thus depending on their affinities for the binding sites in proteins, they might compete. It is expected that G proteins can be severely affected under starvation since the levels of GTP are lowered, but also because ppGpp might interfere with its function. These proteins have been target of analysis since the early periods after discovery of the alarmone as the factor that influenced the stringent response. Initial studies indicated that pppGpp was able to substitute GTP in the reactions of EF2 and EF-Tu, but not in the function of EF-G [123]. Later studies revealed that EF-Tu as well as EF-G are inhibited by ppGpp, but this inhibition is dependent on the conditions of the reaction. EF-Tu is inhibited only if EF-Ts is not present. The inhibition can be fully reversed by the presence of aminoacyl-tRNA and EF-Ts [124].

As was mentioned before, IF2 is a G protein involved in the initiation of translation. This factor interacts in the initiation process with different ligands, ribosomal subunits, fMet-tRNA^{fMet}, GTP, GDP as well as ppGpp [125]. This protein participates in the entire initiation process and it has been shown by several methodologies that different conformational changes are necessary to each step. Because of the similar affinities of IF2 with GTP and GDP (dissociation constants between 10-100 μ M), it is expected that under normal growth conditions (GTP 1 mM), IF2 binds the 30S subunit mostly with GTP bound. Hydrolysis of GTP, upon binding of the 50S subunit triggers the release of IF2-GDP from the initiation complex. Because this hydrolysis has not been proven as essential for this process, it led Milon et al. (2006) [126] to question the real role of this activity and asked about the reason for the evolutionary conservation of this process. The binding site of GTP and GDP in IF2, as well as in other G proteins involved in translation, is also the binding site for ppGpp. NMR

data illustrates that ppGpp binds basically in the same site as GDP, although some differences might account for the different structure and function. To test the role of ppGpp on the IF2 function the authors measured the effect on different steps of the initiation process, i.e. binding of the fMet-tRNA^{fMet}, dipeptide formation, and the translation from the initiation codons on mRNAs containing AUG or AUU as initiator codon (the later being more dependent on IF2). All these steps in initiation of translation are severely affected upon ppGpp binding. From these studies the authors concluded that binding of ppGpp to IF2 might represent the signal to inhibit translation under conditions of metabolic shortage [126]. Thermodynamic analysis revealed that ppGpp binds to IF2 with higher affinity than to EF-G. Binding of fMet-tRNA^{fMet} to IF2 occurs with little variation in the presence of ppGpp compared to GTP while it is very sensitive to the nucleotides when complexed with the 30S subunit [127]. These results support the notion that initiation of translation is preferentially regulated by ppGpp under conditions of nutrient starvation.

3.5. Translation accuracy in the stringent response

Translational accuracy has been a topic of debate since the discovery of the stringent response. It is known that under amino acid starvation, *rel*⁺ cells translation is at least 10 fold more accurate than in *rel*⁻ although the rate of protein synthesis is the same in either type of cell. Different interpretations for this accuracy have been proposed, i.e. increased ribosome proof reading by ppGpp upon binding of either initiation or elongation factors, alterations of A site in the ribosome by binding of uncharged tRNA and different ribosome states controlled by the binding of ppGpp. It has also been proposed that there is no need for a special mechanism to maintain accuracy of translation since under amino acid starvation concentration of charged tRNA is not reduced as much as uncharged tRNA is increased [128]. At the same time, uncharged tRNA might bind to the A site in the ribosome competing for non-cognate tRNA thus reducing the chance to enter in the A site with the incorrect codon. Reduction in the activity of EF-Tu at the A site upon binding of ppGpp might reduce the chance to an error in translation. Measurements of aminoacylation levels for several tRNA revealed that *rel*⁻ strains have at least five fold less aminoacyl-tRNAs than *rel*⁺ strains suggesting that increased inaccuracy in these strains might be explained only by the charging level of tRNAs rather than other particular mechanisms [129]. These results imply that accuracy of translation is not affected under stringent response because there are either particular mechanisms that account for it or because there is a combination of effects (based on the real concentration of aminoacyl-tRNA, deacylated-tRNA bound to the A site of ribosome and the reduction in the translation rate by inhibition of IF2 and EF-Tu functions) that minimize the possibility that non-cognate aminoacyl-tRNAs enter the A site of the ribosome.

3.6. Overview of the effects of stringent response on translation

Upon amino acid starvation a generalized response, the stringent response, is achieved in bacterial cells. The major effector of this response is the marked increase of the cellular concentration of the nucleotide ppGpp, also known as the “magic spot” or the “alarmone”.

This nucleotide is synthesized in the ribosome by the RelA protein upon activation by the presence in the A site of the ribosome of deacylated-tRNA. Two major effects on translation of the genetic information are observed. First, the dramatic reduction on the transcription of stable RNAs, i.e. rRNAs and tRNAs. The binding of ppGpp to the β and β' of RNA polymerase triggers this effect by the destabilization of the open complex between RNA polymerase and strong promoters of stable RNAs. As consequence a marked reduction in the concentration of ribosomes and tRNAs slows down the translation of mRNAs. The second effect of the increase in the concentration of ppGpp on translation is an inhibition of translation itself by the effect on initiation and elongation steps. IF2, EF-Tu as well as EF-G are affected by the binding of ppGpp, but it seems likely that the initiation of translation through the inhibition of the IF2 function is the preferred target for the action of ppGpp to modulate the translation process. Accordingly, it has been proposed that IF2 might be a sensor to modulate translation depending on the nutritional status of the cell.

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