Post-Translational Modifications Guard Yeast from Misaspartylation[†]

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ABSTRACT: Yeast aspartyl-tRNA synthetase (AspRS) is downregulated at the post-transcriptional level. This complex retro-inhibition mechanism causes the cell to equilibrate cellular concentrations of $tRNA^{Asp}$, AspRS, and its encoding mRNA. This strategy hinders AspRS accumulation to keep misacylation of heterologous tRNAs under control. Here, the AspRS concentration was increased artificially in vivo but did not generate $tRNA^{Asn}$ and/or $tRNA^{Glu}$ misaspartylation or the logical consecutive post-translational stress. This work allowed the detection of an additional subtle cellular lock capable of blocking AspRS toxicity. This study revealed the presence of post-translational modifications in the N-terminal extension of AspRS. We hypothesize that by neutralizing the lysine-rich motif contained in this domain, the cell mobilizes an additional strategy that considerably reduces the probability of the enzyme binding and aspartylating noncognate tRNAs and thus harming its own translation.

Accurate translation of genetic information requires precise aminoacylation of tRNAs catalyzed by aminoacyl-tRNA synthetases (aaRSs) (*1*), but tRNA aminoacylation does not always occur with absolute specificity. Misacylation of heterologous tRNAs often occurs with strong expression of a given aaRS, as explicitly shown in prokaryotic systems (*2*). Therefore, maintaining the proper equilibrium between a synthetase and its cognate tRNA could be a general strategy used by the cell to prevent the occurrence of damaging misacylation reactions and subsequent false translation. Numerous aaRS regulation pathways have been studied, especially in eubacteria, but only a few have been completely unraveled and well-understood (*3*). These mechanisms differ between aaRSs and between organisms. Expression levels of aaRS are controlled by many different transcriptional and/or post-transcriptional regulation mechanisms, with the only shared event being the involvement of tRNA or tRNA-like structured molecules (*4*).

In *Saccharomyces cerevisiae,* an original autoregulation mechanism that controls aspartyl-tRNA synthetase (AspRS) expression in the cell has been described (*5*). AspRS recognizes a unique tRNA-like structure in its own mRNA (*6*). In vivo, this interaction initiates a feedback regulation process that accounts for ultimate synchronized expression of both AspRS and $tRNA^{Asp}$ in the cell. In the proposed model, when the available $tRNA^{Asp}$ is not sufficient to complex the cytosolic AspRS fraction, the remaining free synthetase is imported into the nucleus. Once in the nucleus, the protein is (i) sequestered from the translational location and (ii) spatially restricted with both its specific binding substrates (i.e., the newly transcribed cognate $tRNA^{Asp}$ and its coding mRNA). Thus, the synthetase not only can bind the 5′ end of its own mRNA and modulate its expression but also can aminoacylate the pool of newly transcribed $tRNA^{Asp}$ and favor its export to the cytosol (*7, 8*). This model explains the coregulation we observe, in which mRNA, protein, and specific $tRNA^{Asp}$ substrate regulate each other to reach their equilibratory status. As a consequence, AspRS is never free in the cell, lowering the risk of erroneous tRNA aspartylation near the cytosolic translation site.

Interestingly, the same AspRS N-terminal domain triggers both the regulation and misaspartylation processes. This 70-amino acid helical extension is located next to the anticodon binding module of the synthetase and displays a nonspecific RNA binding motif ($_{29}$LSKKALKKLQK$_{39}$) (*9*). This motif has been shown in vitro to be essential for driving recognition between AspRS and its mRNA (*10*), but it also conveys nonspecific tRNA binding properties to the enzyme. Indeed, the motif considerably increases the affinity of AspRS not only for its cognate $tRNA^{Asp}$ but also for other tRNAs, especially $tRNA^{Asn}$ and $tRNA^{Glu}$, which are efficiently aspartylated in vitro (*11*). In vivo, such aminoacylation errors could lead to false translation, with some asparagine and glutamine residues replaced with aspartate in the synthesized proteins.

This study shows that the AspRS N-terminal extension is the site for successive post-translational modifications. The nature and functional implications for aspartylation and translational accuracy are discussed.

MATERIALS AND METHODS

Cloning and Purification of Aspartyl-tRNA Synthetase. The gene encoding yeast cytosolic AspRS was amplified by polymerase chain reaction (PCR) from pQE70-AspRS (encoding 6His-AspRS) (*9*) and subcloned in pGEX-2T fused

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to the 3′ end of the glutathione *S*-transferase (GST) encoding gene (Amersham BioSciences, Saclay, France). The construct was sequenced and transformed in the *Escherichia coli* Top10 strain, and the fusion protein was overexpressed following the same protocol that was used for 6His-AspRS. Cells were harvested, washed in phosphate-buffered saline (PBS), suspended in buffer A [50 mM phosphate buffer (pH 8.0) and 300 mM NaCl], and sonicated (Branson Ultrasons Annemasse, Haute-Savoie, France) on ice. After ultracentrifugation, the supernatant was loaded on 2 mL of glutathione-Sepharose resin (Amersham Biosciences) previously equilibrated in buffer A. The resin was washed with 60 mL of buffer A, and AspRS was freed from the resin-bound GST by thrombin cleavage. The reaction was performed for 2 h at 37 °C in 4 mL of buffer B [25 mM Na_2HPO_4 (pH 8.3), 100 mM NaCl, and 10% glycerol] with 250 units of thrombin from bovine plasma (Sigma-Aldrich, Lyon, France), yielding an AspRS with a short additional amino acid stretch at its N-terminus. The theoretical molecular mass of the recombinant protein was 64366.09 Da. The protein then was loaded in buffer B on a UnoS-6 column (Bio-Rad, Marnes La Coquette, France) and eluted with a shallow salt gradient (60 mL from 100 to 600 mM NaCl). When used for two-dimensional gel analysis, the fractions were pooled, concentrated on a microcon 30K apparatus (Millipore, Molsheim, France), and frozen at −80 °C. When used for functional assays (binding and aminoacylation), the protein was dialyzed overnight at 4 °C against 50 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 10 mM β-mercaptoethanol, 150 mM KCl, and 50% glycerol buffer. In all cases, AspRSs were quantified via a Coomassie blue dye binding assay (*12*) (Bio-Rad) before being stored.

In Vivo Constructs. (i) Strains and Vectors. The YBC-603 strain (*ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*) was transformed either with the pRS-416-TEF plasmid (centromeric, Ura+, gene expression under the EF1-α promoter) containing the gene encoding yeast AspRS or with pRS-416-TEF without the insert as a negative control. Positive clones were selected on supplemented minimal medium lacking uracil. Care was taken not to use strong promoters because of the observed toxicity when AspRS is strongly overexpressed in yeast.

(ii) AspRS Mutagenesis. Both AspRS variants were prepared with the Quick Change site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The catalytic site mutant R485K was obtained by simple codon replacement. The lysine-rich RNA binding motif in the N-terminal extension of AspRS was altered by introducing a +1 frameshift (insertion) at position 83 in the coding sequence of AspRS mRNA and re-establishing the reading frame (deletion) at position 147. Thus, the wild-type sequence $_{30}$SKKALKKLQKEQEKQRKKE$_{48}$ was replaced with the mutant sequence $_{30}$LVQEGLEEIAERARETEKEG$_{49}$ deprived of any RNA affinity (*5*).

(iii) Geneticin Resistance as a Reporter System for Misaspartylation. The wild-type or mutated aph-3′-IIa gene (confers aminoaglycoside resistance to prokaryotes and eukaryotes) was cloned into pRS425 (2μ, Leu+, under the control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter) and cotransformed with pRS416-TEF plasmids (encoding AspRS or without insert) in YBC-603 cells. Cells were selected and grown in minimal medium supplemented with all amino acids with the exception of leucine and lacking uracil. Then, 10^7 cells were plated on a Geneticin continuous gradient diffusion test (with an initial concentration of 100 mg/mL in the center of the plate) and analyzed after incubation for 30 h at 30 °C.

Yeast Cell Growth, Lysis, and Proteomic Analysis. (i) Growth Curves. YBC-603 cells transformed with pRS416-TEF plasmids (encoding AspRS or without insert) were inoculated in the corresponding selective minimal medium (deprived of uracil) at a density of 2×10^6 cells/mL and grown at 30 °C under vigorous shaking (170 rpm). Growth curves were determined by measuring the optical density at 600 nm (OD_{600}) every 2 h for a total of 30 h.

(ii) Proteomic Analysis. Cells were grown until the stationary phase (~7 OD units/mL), harvested by centrifugation, and washed with PBS. Cells (~100 OD units) then were suspended in 60 μL of 100 mM Tris-HCl (pH 8.0) and Fastprep lysed (5FP 120, Thermo Scientific, Courtaboeuf, France). Five successive cycles of 30 s each in the presence of glass beads (0.2 mm diameter) were performed. Then, 1 volume of 100 mM Tris-HCl (pH 8.0), 700 mM β-mercaptoethanol, and 0.6% sodium dodecyl sulfate (SDS) was added, and the protein extract was incubated at 95 °C for 30 s, followed by 1 min on ice. The subsequent steps were performed at room temperature to prevent SDS precipitation. Digestions with RNase A (2 units) and DNase I (20 units) were achieved after 1 min, and two successive centrifugations allowed for removal of the beads (5 min at 5000 rpm) and the residual cellular debris (10 min at 12000 rpm). The yeast extract then was quantified by Coomassie blue dye binding assay (*12*) (Bio-Rad) and stored at −80 °C; 300 μg of protein extract was loaded on two-dimensional polyacrylamide−SDS gels (*13*) and stained with colloidal Coomassie blue (*14*), and comparative analysis was performed with PD-Quest (Bio-Rad). For Western blot analysis, 20 μg of proteins was separated on gel, transferred to Immobilon-P transfer membranes (Millipore), and probed with a 1:20000 dilution of anti-AspRS antibody. Primary antibody was detected by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham Biosciences).

For glycoprotein detection, 150 μg of proteins was necessary for subsequent analysis in the presence of concanavalin-A peroxidase conjugate (ICN Pharmaceuticals, Orsay, France).

For methyllysine, acetyllysine, and biotin detection, 2.5 μg of purified AspRS overexpressed in *E. coli* was used in the presence of a 1:500 dilution of the corresponding antibody (abcam, Cambridge, U.K.).

Functional Studies. Band-shift experiments and aminoacylation reactions were performed as described in ref *9*.

RESULTS

Several Approaches to Detecting Putative Misaspartylation in Vivo. (i) AspRS Toxicity. When the AspRS gene is cloned for its overexpression in yeast, only the sequence corresponding to the open reading frame was used. Under these conditions, the gene is deprived of its 5′ untranslated region that is essential for directing mRNA recognition by the protein and initiating the retro-inhibition process (*5*). Thus, the cell no longer can control AspRS expression, and the protein can accumulate. Yeast cells were transformed with different vectors to overexpress different amounts of

cytosolic AspRS, and high levels of expression were shown to be toxic for cell growth. This observation led us to use a low-copy number plasmid with a moderate-strength promoter (pRS416-TEF) that allowed us to monitor yeast growth for several days with increased cellular AspRS concentrations.

(ii) Growth Rate of AspRS-Overexpressing Yeast. When compared with that of the wild-type strain (transformed with pRS-416-TEF), no difference in the growth rate of AspRS-overexpressing cells was observed, even when the experiment was conducted over 8 consecutive days (data not shown), indicating that moderate AspRS accumulation in the cell is not deleterious.

(iii) Geneticin Resistance as a Reporter System for Misaspartylation. The aph-3′-IIa gene encodes the aminoglycoside-3′-phosphotransferase that is responsible for aminoglycoside resistance in prokaryotes and eukaryotes (*15*). This allows efficient growth of yeast cells in the presence of Geneticin only when the enzyme catalytic site displays an essential aspartate residue at position 190 (*16*). On the basis of this observation, an in vivo reporter system was constructed, in which Asp190 was replaced with alanine (Ala190 as a negative control), asparagine (Asn190), or glutamate (Glu190). Alanine substitution was known to inhibit the resistance of *E. coli* to kanamycin (*17*), whereas asparagine and glutamate insertions at position 190 were first tested in this study. As expected, none of the mutated aminoglycoside-3′-phosphotransferases allowed yeast cells to grow efficiently in the presence of Geneticin, although insertion of glutamate rescued antibiotic resistance to some extent (data not shown). The setup of the mutations was based on previous studies showing that increased concentrations of AspRS lead to the accumulation of significant amounts of Asp-tRNA^{Asn} and Asp-tRNA^{Glu} in vitro (*11*). Thus, if this would occur in vivo, overexpression of AspRS should be responsible for the translation of an active aminoglycoside-3′-phosphotransferase via insertion of the essential aspartate residue in Asn190- and Glu190-mutated aph-3′-IIa genes. However, the mutant phenotype was not changed when the cell was cotransformed with the plasmid overexpressing AspRS (data not shown), indicating that misaspartylation of tRNA^{Asn} and tRNA^{Glu} does not exist in vivo or is not sufficient to allow the synthesis of enough aminoglycoside-3′-phosphotransferase in the cell to overcome antibiotic toxicity.

(iv) Proteome Analysis. This technique combined isoelectric focusing with SDS−polyacrylamide gel electrophoresis (PAGE) and allowed separation of nearly identical proteins. When compared with the wild-type strain, the proteome from the yeast strain overexpressing AspRS did not exhibit significant variations. The absence of a disrupted proteome indicated that misaspartylation activity was minimized and that overexpression of AspRS did not trigger any translational stress in the cell. Instead, both proteomes were comparable with the exception of a few discrete spots restricted to a well-defined area on the gel (Figure 1). Their appearance was a direct consequence of AspRS overexpression because tryptic peptide mapping and mass spectrometry allowed identification of these additional spots as cytosolic AspRSs. Thus, the only major variation in the proteome was local and showed clearly that the overproduced protein was modified such that its migration was significantly affected on a two-dimensional gel.

Western blot analysis of the two-dimensional gels confirmed the presence of several extra spots (at least seven), corresponding to AspRS variants that had electrophoretic characteristics that were different from those of the unmodified form (compare panels A and B of Figure 2). Modified AspRSs are more acidic (up to 0.5 pH unit), and their size appeared to be slightly increased (<1 kDa), indicating that the modifications were acidic or that they neutralized basic residues in the protein and were characterized by a low molecular mass. Moreover, additional spots were observed on the top of the gel, indicating that AspRS also was the target of higher-molecular mass modifications that will not be investigated here.

Site for Post-Translational Modification. Interestingly, the post-translational modifications were not yeast-specific because they also were detected in *E. coli*. When yeast AspRS from *E. coli* was overexpressed and purified, two-dimensional gels showed that, depending on overexpression conditions, between 20 and 80% of the recombinant enzyme displayed the same properties (pHi and size) as the modified AspRS in yeast (Figure 3A,B). The major spots corresponding to native (spot 1) and fully modified AspRSs (spots 6 and 7) in yeast are also present in AspRS overexpressed in *E. coli*; only intermediate forms do not seem to accumulate sufficiently in yeast (spots 2−5) to be visible.

Further purification of the recombinant enzyme showed that the modified fraction was eluted earlier than the unmodified fraction from a cation exchange resin. The 70-amino acid extension at the N-terminus of yeast AspRS was responsible for the retention of the protein on this type of resin, since the numerous lysine residues in this extra domain made the protein interact with negatively charged supports. Thus, eventual neutralization of these lysine residues could explain the rapid elution of the modified form of the synthetase, whereas the pure unmodified enzyme was found in the last fractions of the elution peak. The modified enzyme eluting early from the resin was the first indication of the modification site and led us to focus on the N-terminal extension of the protein.

Thus, the lysine-rich stretch in the N-terminal extension was mutated, and replacing the RNA binding motif (loss of lysine residues) in the extension led to an accumulation of a homogeneous mutant protein in vivo (Figure 2C) that was characterized by a unique spot on the two-dimensional gels. The location of this new spot was shifted to the left compared with the wild-type protein as a consequence of a more acidic amino acid sequence in the mutated protein. However, no additional spots were detected that resulted from putative post-translational modification of the mutated protein, indicating that changing the sequence in the N-terminal domain removed the modification sites.

Nature of the Modifications. (i) Mass Spectrometry Analysis. Throughout the study, we observed the same phenomena for all matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) mass spectrometry analyses of our samples. Each time a spot corresponding to the modified or unmodified AspRSs was analyzed, the N-terminal region of the protein was not covered. Tryptic digestions may explain this observation because of the large number of lysines present in this domain, but the results were the same when other proteases such as AspN or GluC that supposedly yield exploitable peptides were used. Numerous unproductive

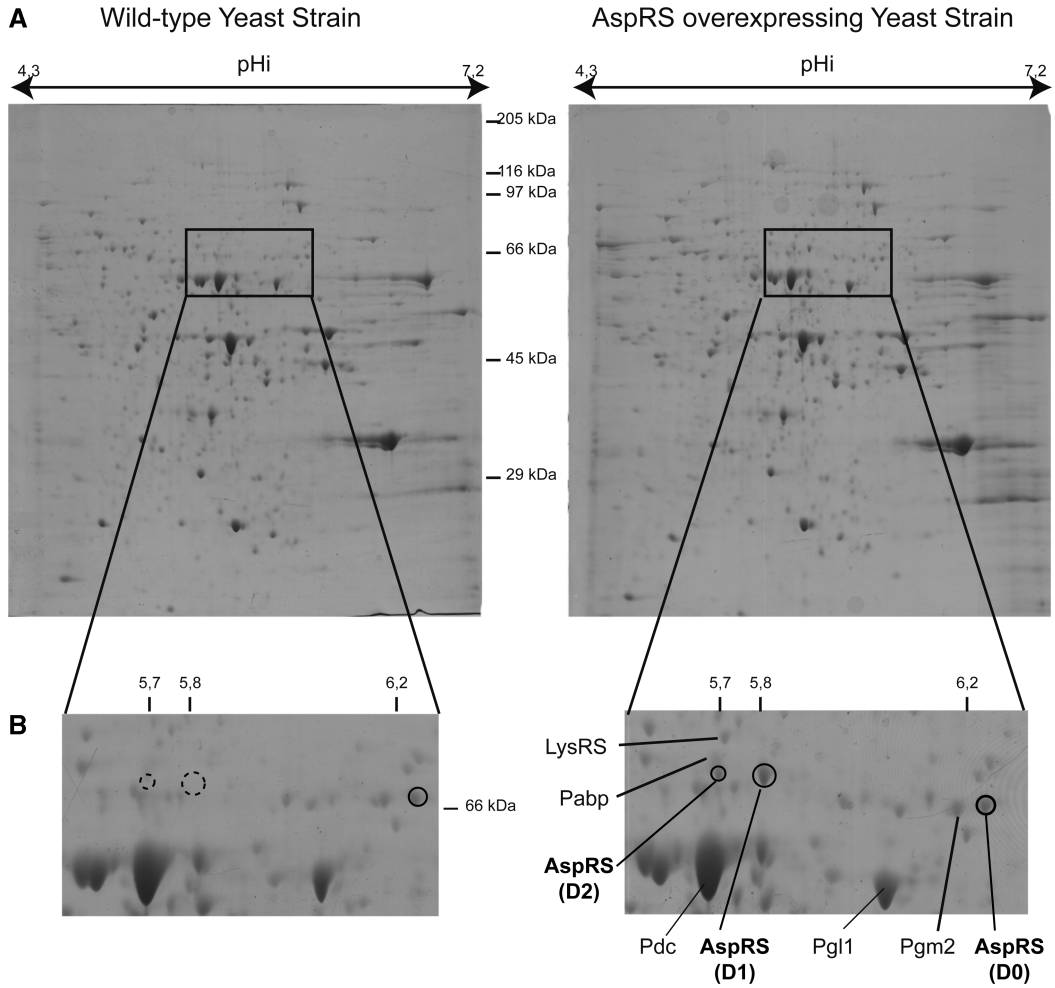

FIGURE 1: Comparative proteomics. (A) Comparison of two-dimensional gels corresponding to the control (wild-type strain, left) and the strain overexpressing AspRS (right). This approach allows the separation of proteins (300 μg total) on the basis of their pHi values (from 4 to 8) horizontally and their size vertically on a 10% SDS−polyacrylamide gel. (B) Enlargement of the region displaying significant variations between both gels. The endogenous AspRS (D0) and the two modified forms (D1 and D2) are indicated. Some invariant proteins (LysRS, Pabp, and Pgm2) were identified by mass spectrometry, whereas Pgl1 and Pdc) were assigned directly by comparisons with a reference proteone. The molecular weight was obtained by comigration of a molecular weight standard (Bio-Rad).

attempts to obtain spectra in the modified N-terminal domain of the synthetase compelled us to perform series of Western Blot analyses of AspRS and AspRS mutants using different specific antibodies raised against potential modifications.

(ii) Autoaspartylation. Previous studies demonstrated that AspRS catalyzes autoaspartylation on its own lysine residues in vitro (*18*), leading to the hypothesis that such a reaction might occur in vivo. The substitution of Arg485 with lysine in the catalytic site (R485K) was previously shown to completely inhibit aspartylation by impairing adenosine triphosphate (ATP) binding (*19*). However, this mutant still retains the capacity to be modified and shows the same pattern as wild-type AspRS on the two-dimensional gel (Figure 2D), clearly demonstrating that the modifications are not the result of autoaspartylation.

(iii) Glycosylations. Our observations, in particular the small size of the modifications, did not agree with the putative glycosylation of AspRS, and this possibility was definitively excluded experimentally by negative results obtained from a concanavalin-A peroxidase conjugate detection assay.

(iv) N-Methylation or N-Acylation of Lysines by Acetyl and Biotinyl Groups. AspRS analysis (overexpressed in *E. coli*) with anti-methyllysine and anti-acetyllysine antibodies indicates clearly that methylations and acetylations are present on the modified proteins. Methylations appear rapidely on spots 3−7 (Figure 3E), whereas acetylations are added later only on spots 6 and 7 (Figure 3C). This pattern shows that lysine residues are indeed modified in a successive manner with methyl and acetyl groups but not with biotins (although, this is a relatively rare modification) (Figure 3D).

Functional Consequences, Binding, and Aminoacylation. In *S. cerevisiae*, AspRS not only binds and aminoacylates $tRNA^{Asp}$ but also binds its own mRNA (*10*) and initiates retro-inhibition of its expression. These properties were tested in the presence or absence of post-translational modifications in the N-terminal extension.

(i) Binding Experiments. Band-shift experiments performed with both AspRSs showed that the apparent affinity (K_d) between the two enzymes and $tRNA^{Asp}$ was not changed (∼25 nM), but upon closer inspection, the shifting pattern appeared to be atypical when AspRS was modified (Figure 4A). Only one band corresponding to the complex between AspRS and $tRNA^{Asp}$ could be detected. The same band was detected in the unmodified complex, but it corresponded to the earliest form that appeared only when low AspRS concentrations were used. This form would correspond to the interaction of only one tRNA with dimeric AspRS,

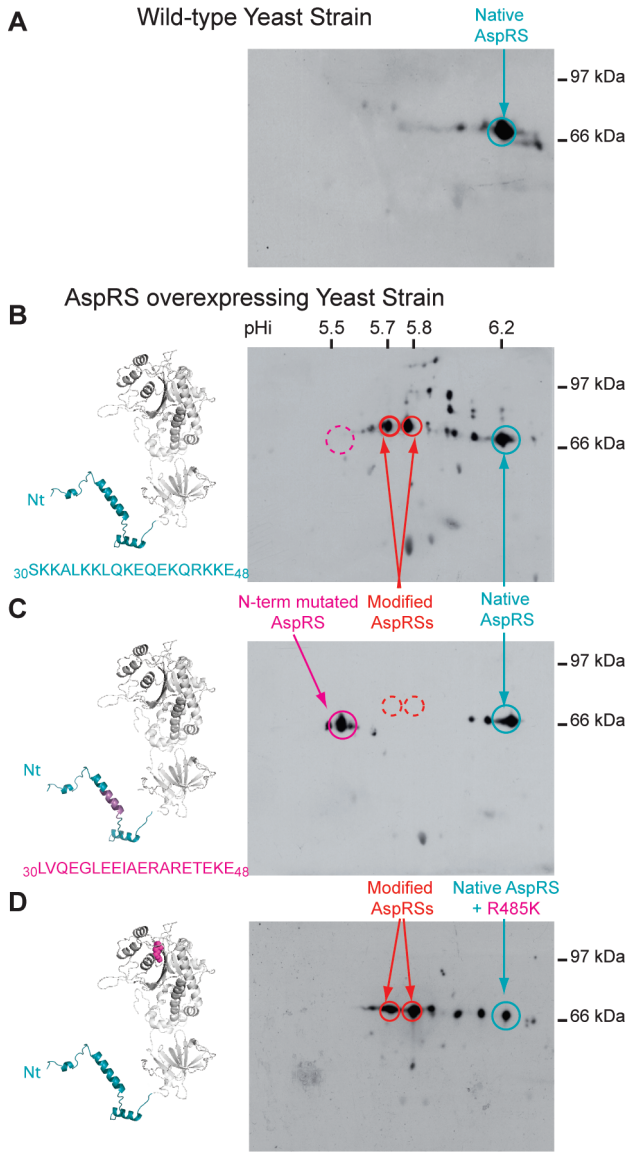

FIGURE 2: Western blot analysis of AspRS variants on two-dimensional gels. (A) Control experiment corresponding to the wild-type yeast strain. (B) Strain overproducing wild-type AspRS. (C) AspRS mutated in its N-terminal extension. (D) AspRS mutated in its catalytic domain. All gels were assessed under the same conditions. The theoretical molecular mass and pHi values were 63515 Da and 6.16 for wild-type AspRS, 63389.14 Da and 5.45 for the N-terminally mutated enzyme, and 63487 Da and 6.16 for the enzyme mutated in its catalytic site, respectively.

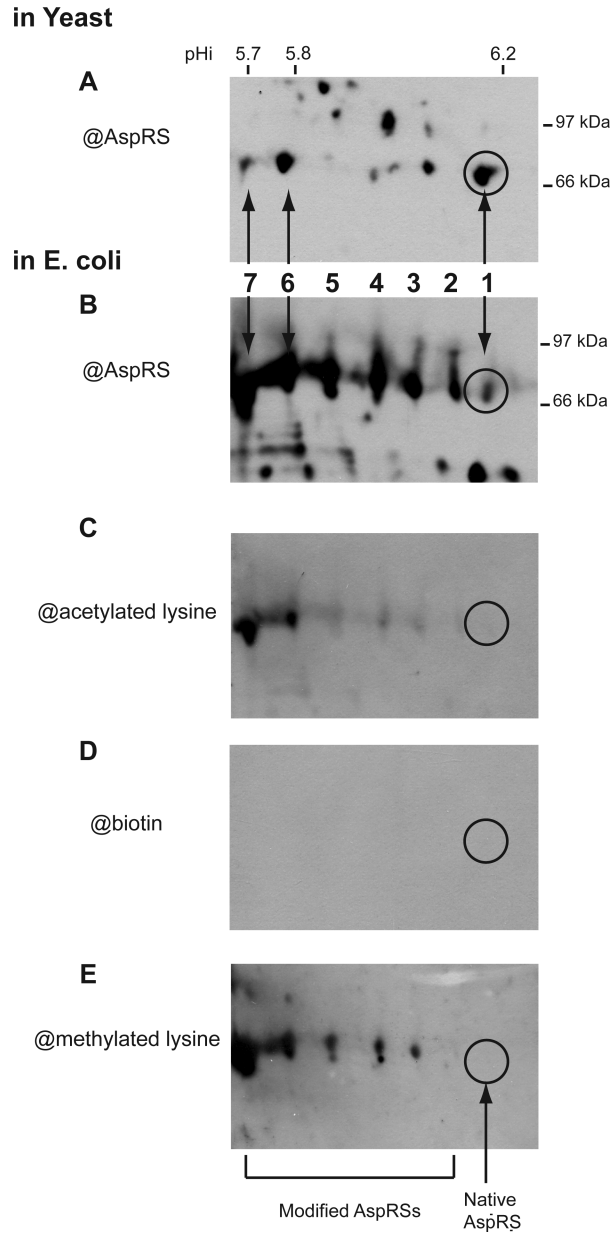

FIGURE 3: Western blot analysis of modified AspRS. Control experiments correspond to the yeast strain overproducing wild-type AspRS (A) and recombinant AspRS purified from *E. coli* (B) incubated with anti-AspRS antibodies. Spots corresponding to the native AspRS (1) and to modified versions of AspRS (2−7) are indicated in both the yeast and bacterial contexts. Recombinant AspRS was tested sequentially with antibodies raised specifically against acetyllysines (C), biotins (D), and methyllysines (E), Western blots were performed in this exact order.

whereas the fastest migrating form, observed only with higher concentrations of unmodified AspRS, would correspond to two tRNA molecules bound to ApsRS. With the modified enzyme, only the slowest migrating complex (one tRNA per dimer) was detectable, indicating that the strengthened interaction that was shown previously for the second tRNA binding (D. Kern, personal communication) no longer existed.

However, the binding affinity measured between both AspRSs and mRNA was not affected (50 nM), clearly showing that the modified enzyme still bound efficiently to the 5′ end of its own messenger and thus could initiate the regulation its own expression (Figure 4B).

(ii) Determining and Comparing Catalytic Parameters. Compared with that of the unmodified AspRS, the global aminoacylation rate of the modified AspRS was not affected, but its affinity constant for $tRNA^{Asp}$ was changed significantly. The K_M value was increased 30-fold with the modified enzyme which consequently reduced significantly $tRNA^{Asp}$ aspartylation efficiency in vitro (Table 1).

DISCUSSION

Despite the strong potency of yeast AspRS in aspartylating $tRNA^{Asn}$ and $tRNA^{Glu}$ in vitro, this study suggests an absence of misaspartylation of these tRNAs in vivo. Proteome analysis clearly showed that AspRS overexpression did not induce any translational stress that would result from aminoacylation errors. In contrast, only a few spots corresponding to modified versions of AspRS were detected. In

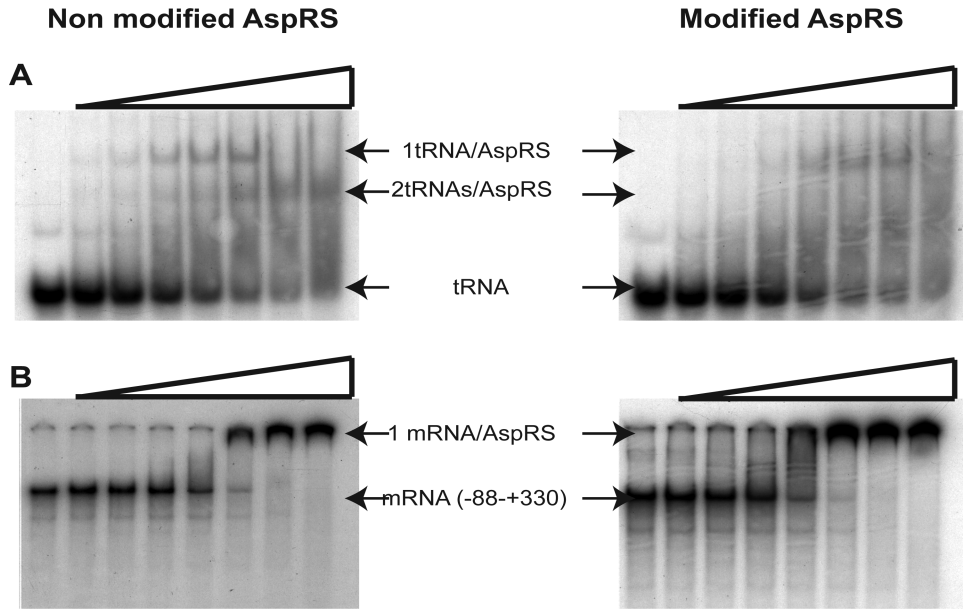

FIGURE 4: Band-shift experiments with unmodified (left) and modified (right) AspRSs toward $tRNA^{Asp}$ (A) and AspRS mRNA (B). The binding of two tRNA molecules per AspRS dimer causes the complex to enter the gel more rapidly than the one tRNA and one AspRS dimer complex, resulting from the large number of negative charges conferred by the tRNA that compensate for the moderately increased size of the complex. AspRS concentrations varied between 6 and 400 nM, and the apparent K_d values determined in these experiments were 25 nM for $tRNA^{Asp}$ and 50 nM for mRNA. The mRNA fragment used in this experiment corresponded to nucleotide −88 in the 5′ untranslated region of the mRNA to nucleotide 330 in the coding sequence (*10*).

Table 1: Comparison of Kinetic Parameters for Aspartylation of $tRNA^{Asp}$ by Unmodified and Modified *S. cerevisiae* AspRSs

AspRS	K_M (nM)	k_{cat} (s^{-1})	k_{cat}/K_M[a] ($\times 10^{-3}$)
unmodified	20 ± 5	0.18 ± 0.03	9
modified	650 ± 40	0.17 ± 0.03	0.26

[a] Aminoacylation efficiencies (k_{cat}/K_M) were calculated for each aspartylation system.

fact, two spots were visible on colloidal blue-stained gels that corresponded to the more acidic forms of the protein. However, the more sensitive Western blot analysis revealed intermediate spots that were not otherwise visible. A total of seven spots were displayed, and their presence fit well with the successive accumulation of modifications on each individual lysine residue present in the N-terminal domain of AspRS.

Proteins can be modified post-translationally by covalent attachment of one or more classes of molecules (*20, 21*). Mass spectrometry did not allow for the identification of the nature of the modification because of the systematic absence of N-terminally derived peptides in the enzymatic peptide mapping experiments. However, methylated and acetylated lysines have been experimentally detected in AspRS and may be good candidates for successive modifications on the protein N-terminal extension.

By covalently linking modifying groups, post-translational modifications change the molecular composition of the protein and provide proteins with properties different from those established for the unmodified enzyme. Here, the modified AspRS was characterized by less efficient aminoacylation properties. The protein was still effective in activating and transferring aspartate but had a significantly reduced affinity for its $tRNA^{Asp}$. This binding difference may be explained by the disappearance of the cooperativity between the two monomers in the dimeric AspRS. Indeed, dimerization is known to increase considerably the binding efficiency of the second $tRNA^{Asp}$ when the first is already in place. Moreover, the 30-fold decrease in affinity that was observed when AspRS was modified can be compared with the 100-fold decrease measured when AspRS was deprived of its complete RNA binding motif in the N-terminal domain (*9*).

Interestingly, AspRS modifications emerge only when the enzyme is overproduced compared to the available $tRNA^{Asp}$ in the cell. The unbound AspRS N-terminal extension is thus accessible for modification enzymes to add methyl and acetyl groups on the RNA binding motif, which leads to the only modification of the molecules challenging aspartylation accuracy. Indeed, the functional analysis confirmed that, when modified, the properties of lysine residues in the RNA binding motif were lost, and the N-terminal extension was no longer functional for tRNA binding. Finally, even if the N-terminal extension is not mandatory for aspartylation, the probability of the modified protein still aminoacylating heterologous tRNAs is reduced considerably.

However, the modified protein still can interact efficiently with the 5′ end of its own mRNA, indicating that even if AspRS is less efficient in aminoacylation, it still can initiate its own retro-inhibition. Indeed, minimizing the possibility of misaspartylation by neutralizing the RNA binding motif should not hinder autoregulation, which would be the most efficient method of reducing cellular concentrations of AspRS. Moreover, the presence of these modifications on a motif that was once predicted to be a nuclear localization signal (*22*) could be a "switch" to initiate AspRS confinement in the nucleus where the regulation process occurs.

Finally, the higher-molecular mass modifications that were detected on two-dimensional gels could indicate that methylation of lysine residues is only a first step in a more global process. Methylation may be a signal for further modifications, such as ubiquitinylation, that would target AspRS for degradation and therefore remove the risks for misaspartylation definitively. Modification of the AspRS

RNA binding motif could be a transient lock necessary for rapidly inhibiting any misaspartylation and consequent translational stress in the cell until the equilibrium between $tRNA^{Asp}$ and the synthetase is recovered via retro control and/or degradation.

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