REPORT

Post-translational modification of RNase R is regulated by stress-dependent reduction in the acetylating enzyme Pka (YfiQ)

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ABSTRACT

RNase R is a processive exoribonuclease that plays an important role in degradation of structured RNAs in *Escherichia coli*. RNase R is unstable in exponential phase cells; however, under certain stress conditions, RNase R levels increase dramatically due to its stabilization. Binding of tmRNA and SmpB to the C-terminal region of RNase R is required for its instability, and this binding is regulated by acetylation of a single residue, Lys544, in exponential phase cells. RNase R is not acetylated in stationary phase. We show here that only exponential phase RNase R is acetylated because the modifying enzyme, protein lysine acetyltransferase, Pka (YfiQ), is absent from late exponential and stationary phase cells. As a consequence, newly synthesized RNase R remains unmodified. Together with the turnover of preexisting acetylated RNase R, no modified RNase R remains in stationary phase. We find that RNase R in cold-shocked cells also lacks the acetyl modification due to the absence of Pka. These data indicate that RNase R stability depends on Pka, which itself is regulated under stress conditions.

Keywords: ribonuclease; post-translational modification; protein stability; *Escherichia coli*

INTRODUCTION

Despite the critical role of ribonucleases (RNases) in RNA metabolism, little is known about the regulation of these important enzymes. One enzyme which is regulated is RNase R, a 3′-to-5′ processive exoribonuclease that participates in the degradation of structured RNAs in *Escherichia coli* (Cheng et al. 1998; Zuo and Deutscher 2001; Cheng and Deutscher 2002, 2005; Vincent and Deutscher 2006, 2009a,b). The levels of RNase R increase three- to 10-fold under certain stress conditions, such as cold shock and stationary phase (Cairrão et al. 2003; Chen and Deutscher 2005; Andrade et al. 2006), and recent work from our laboratory has helped to explain the basis for this regulation (Chen and Deutscher 2010; Liang and Deutscher 2010; Liang et al. 2011).

RNase R is an extremely unstable protein in exponential phase cells with a half-life of ~10 min (Chen and Deutscher 2010; Liang and Deutscher 2010). However, it becomes stabilized during cold shock and upon entry into stationary phase, which results in its elevation compared to the levels present in exponential phase (Chen and Deutscher 2010). RNase R instability is due to the binding of tmRNA and SmpB, two components of the *trans*-translational system, to its C-terminal region (Liang and Deutscher 2010). Elimination of either of these factors or removal of the C-terminal region stabilizes RNase R in exponential phase cells (Liang and Deutscher 2010). Analysis of RNase R purified from exponential and stationary phase cells revealed that the exponential phase protein is modified by acetylation of a single residue, Lys544, catalyzed by the product of the protein lysine acetyltransferase, *pka* (*yfiQ*) gene, and that this modification alters RNase R structure leading to tighter binding of tmRNA and SmpB and subsequent proteolytic degradation of the RNase (Liang et al. 2011). In contrast, RNase R is not modified in stationary phase cells, and as a consequence, the protein is stable (Liang et al. 2011). Taken together, these findings provided a simple explanation for the difference in stability between exponential phase and stationary phase RNase R. What was not understood, however, is why exponential phase RNase R is acetylated, whereas the stationary phase protein is not.

In this paper, we provide the explanation for this difference in acetylation. We show that acetylation of RNase R decreases as cells enter stationary phase because the

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acetylation activity is dramatically reduced in late exponential phase and it essentially disappears in stationary phase. This occurs concomitantly with the loss of Pka protein. In contrast, the deacetylase, CobB, appears to play no role in the loss of acetylation of RNase R in stationary phase. We show as well that RNase R from cold-shocked cells also lacks the acetyl modification, and this is also due to the loss of Pka. These data indicate that regulation of RNase R stability ultimately depends on Pka which itself is subject to regulation under stress conditions.

RESULTS AND DISCUSSION

Acetylation of RNase R decreases with growth

In earlier work (Liang et al. 2011), we found that RNase R is acetylated in early exponential phase cells ($A_{550} \sim 0.3$), whereas the modification is absent in RNase R isolated from overnight cultures. However, it was not known when during growth the modification begins to disappear and how this correlated with the known increase of RNase R in stationary phase compared to exponential phase cells (Chen and Deutscher 2005; Andrade et al. 2006). To answer this question, cells were isolated at multiple times from early exponential phase to late stationary phase. RNase R was immunoprecipitated from the samples, and its amount and level of acetylation was determined by immunoblotting. The Western analyses in Figure 1A and quantitation in Figure 1B show that RNase R begins to increase and acetylation begins to decrease at an A_{550} of 1.5, and that the accumulation of RNase R directly correlates with the decrease in acetylation. These data support the earlier conclusion (Liang et al. 2011) that acetylation destabilizes RNase R and that the protein is stabilized when the acetyl group is absent.

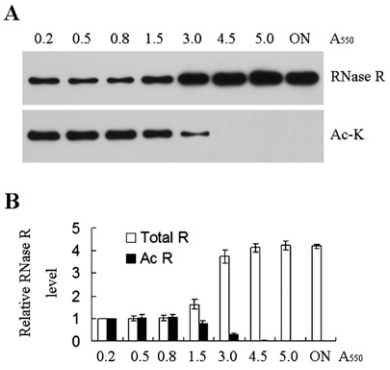

FIGURE 1. Growth phase-dependent acetylation of RNase R. Strain MG1655(Seq)*rph*$^+$ was grown in YT medium at 37°C, and samples were taken at the indicated A_{550} values. RNase R was immunoprecipitated from cell extracts using purified RNase R antibody as described in Materials and Methods, and then analyzed with RNase R antibody and anti-acetylated-lysine monoclonal antibody (Ac-K) after separation on 8% SDS-PAGE. (*A*) Representative Western blots of RNase R and acetylated RNase R from samples prepared at the indicated growth stages. (*B*) Quantitation of three independent experiments carried out as shown in panel *A*. The amount of total RNase R and acetylated RNase R were set at 1 for cells at $A_{550} \sim 0.2$. ON is a sample taken after 24 h of growth.

Acetylating activity decreases with growth

To determine why the level of RNase R acetylation decreases as cells approach late exponential phase, we directly assayed extracts prepared from cells at various stages of growth for their ability to acetylate unmodified stationary phase RNase R. Figure 2A,B shows that the activity responsible for acetylating RNase R begins to decrease at an A_{550} of 1.5 and is almost completely eliminated in cells at an A_{550} of 3.0. Stationary phase cells are devoid of this activity. Based on these data, it appears that the decrease in acetylation of RNase R is due to elimination of the activity responsible for the modification.

The data in Figure 2C,D show that the loss of acetylation activity is a direct consequence of a decrease in the amount of Pka protein, which we found previously to be responsible for RNase R acetylation (Liang et al. 2011). Interestingly, the loss of Pka protein very slightly lags the loss of acetylating activity (cf. Fig. 2C,D and Fig. 2A,B), raising the possibility that the enzymatic activity may be inactivated prior to elimination of the protein. Alternatively, this may simply be due to greater sensitivity of the FLAG antibody compared to the acetylated lysine antibody.

Deacetylation is not involved in loss of RNase R acetylation

Acetyl groups can be removed from proteins by an active deacetylation process (Blander and Guarente 2004; Escalante-Semerena 2010; Hu et al. 2010). Thus, the decrease in acetylation of RNase R may also be modulated by removal of the acetyl modification. To determine whether deacetylation might contribute to loss of the acetyl group from RNase R, we examined the effect of removing the product of the *cobB* gene. CobB plays a major role in deacetylation in *Salmonella* (Wang et al. 2010) and is the only known protein deacetylase in *E. coli* (Li et al. 2010). However, as shown in Supplemental Fig. S1A, elimination of CobB had no effect on acetylation of exponential phase RNase R, and its absence did not prevent loss of the acetyl group in stationary phase RNase R. Moreover, purified CobB was unable to remove the acetyl group from exponential phase RNase R in vitro (Supplemental Fig. S1B). Likewise, the acetyl group could not be removed by incubation of exponential phase RNase R with a crude extract from stationary phase cells (data not shown). These data strongly suggest that deacetylation does not play a role in loss of the acetyl group from RNase R and that the decrease of the acetylating enzyme is the primary determinant.

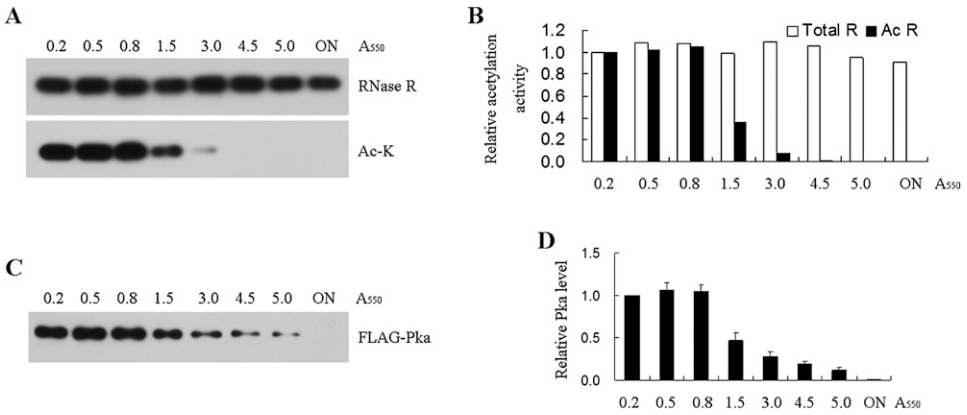

FIGURE 2. In vitro acetylation activity and amount of Pka throughout growth. (*A*) Acetylation of purified stationary phase RNase R by extracts of MG1655(Seq)*rph*$^+$ *rnr* prepared from cells throughout growth. Stationary phase RNase R was incubated for 1 h at 37°C as described in Materials and Methods. The mixtures were resolved on 8% SDS-PAGE and then analyzed with RNase R antibody or anti-acetylated-lysine monoclonal antibody (Ac-K). (*B*) Quantitation of data in panel *A*. The amounts of total RNase R and acetylated RNase R were set at 1 for cells at $A_{550} \sim 0.2$. ON is an extract prepared from cells after 24 h of growth. (*C*) Western blot analysis of FLAG-Pka throughout growth. Samples were taken at the indicated A_{550} and analyzed as described in Materials and Methods. (*D*) Quantitation of three independent experiments carried out as shown in panel *C*. The amount of Pka in cells at $A_{550} \sim 0.2$ was set at 1. ON is a sample taken after 24 h of growth.

New RNase R synthesis results in unmodified RNase R in stationary phase cells

Acetylated RNase R is an extremely unstable protein with a half-life of only 10 min (Chen and Deutscher 2010; Liang and Deutscher 2010). Moreover, as shown above, acetylated RNase R is unlikely to be subsequently deacetylated. Consequently, the unmodified RNase R that accumulates in stationary phase cells would all have to arise by new synthesis and not be acetylated. To determine whether such a scenario is feasible, the rate of RNase R synthesis was measured at various times throughout growth using a 3-min pulse of radioactive amino acids followed by immunoprecipitation (see Materials and Methods) (Fig. 3), and that was related to the amount of acetylation activity (Figs. 2A,B).

Although RNase R synthesis decreases as cells approach stationary phase (Fig. 3), the enzyme continues to be produced at a substantial rate. Meanwhile, the acetylating activity is undergoing considerable reduction. For example, at an A_{550} of 1.5, RNase R synthesis is still 80% of the maximum rate (Fig. 3), while the acetylating activity has dropped to ~40% of the maximum level (Fig. 2A,B). At an A_{550} of 3.0, the difference is even more pronounced. RNase R synthesis is still close to 60% of maximum (Fig. 3), whereas the acetylating activity is now only 10% of maximum (Fig. 2A,B). As a consequence of these relative synthesis and acetylation rates, as well as the continued turnover of acetylated RNase R, the percentage of modified RNase R is reduced to ~50% at an A_{550} of 1.5 and to <10% at an A_{550} of 3.0, a time when RNase R is approximately fourfold elevated (Fig. 1). Thus, the combination of breakdown of preexisting acetylated RNase R and synthesis of new RNase R, which cannot be modified due to the low level of acetylating activity, leads in short order to a dramatic shift in the RNase R population from fully acetylated to completely unacetylated. Since unmodified RNase R is stable (Liang et al. 2011), it continues to accumulate relative to the level of the exponential phase enzyme.

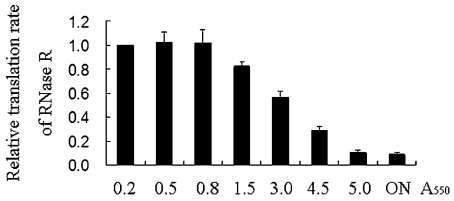

FIGURE 3. Rate of RNase R synthesis throughout growth. Strain MG1655(Seq)*rph*$^+$ was grown in YT medium at 37°C. Samples were taken at different A_{550}, as indicated, after labeling with ^{3}H amino acids, as described in Materials and Methods. RNase R was immunoprecipitated with purified RNase R antibody, and the radioactivity in the eluant was determined with a scintillation counter. The synthesis rate of RNase R at $A_{550} \sim 0.2$ was set at 1. Quantitation of three independent experiments is shown. ON is a sample from cells labeled at 24 h of growth.

Decreased acetylation regulates RNase R under other stress conditions

In addition to stationary phase, RNase R levels also increase under other stress conditions such as cold shock (Cairrão et al. 2003; Chen and Deutscher 2005) and growth in minimal medium (Chen and Deutscher 2005). In these cases as well, the elevation of RNase R compared to exponential growth in rich medium at 37°C was shown to be due to stabilization of the otherwise unstable protein (Chen and Deutscher 2010). To determine whether the same stabilization mechanism operates under these conditions as was described above for stationary phase, we examined both the acetylation status of RNase R and the amount of Pka. The data in Figure 4A,B show that RNase R is elevated approximately

fivefold at 10°C compared to 37°C, in agreement with earlier observations (Cairrão et al. 2003; Chen and Deutscher 2005). As with stationary phase, this increase in the amount of RNase R is accompanied by the absence of lysine acetylation due to the dramatic decrease (~90%) in the acetylating enzyme, Pka. Based on these data, we conclude that the stabilization of RNase R during cold shock operates by the same mechanism as that described for stationary phase.

Although RNase R also increases during growth in minimal medium, the elevation is not as pronounced as in other stress conditions (Fig. 4C,D; Chen and Deutscher 2005). Likewise, the loss of acetylation of RNase R and the decrease in Pka are not as complete (Fig. 4C,D). Nevertheless, these data indicate that the same mechanism for RNase R regulation is in force.

The data presented here extend our previous observations on the role of lysine acetylation in regulating the stability of RNase R (Liang et al. 2011), and they provide a simple explanation for why RNase R is acetylated in exponential phase cells but not in cells under a variety of stress conditions. We show that, in each case, the common mechanism is the loss of acetylating activity due to a dramatic decrease in the amount of Pka. This, of course, raises the interesting question of how Pka itself is regulated. In *Salmonella*, the Pka paralog, termed Pat, is known to be regulated at the transcriptional level (Wang et al. 2010). Further work will be necessary to determine whether similar regulation of Pka exists in *E. coli*. Inasmuch as Pka undoubtedly plays a role in the acetylation of other proteins besides RNase R, these findings suggest that loss of acetylation due to disappearance of Pka will have global effects in the stress responses of *E. coli*.

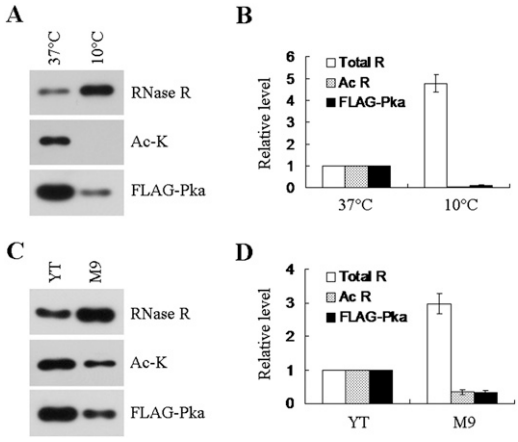

FIGURE 4. Acetylation of RNase R during cold shock and in minimal medium. (*A*) RNase R was immunoprecipitated from MG1655(Seq)*rph*$^+$ cell extracts using purified RNase R antibody and then analyzed with RNase R antibody and anti-acetylated-lysine monoclonal antibody (Ac-K). The amount of FLAG-tagged Pka in the cell extracts was determined with anti-FLAG M2 mAb. For cold shock, cells growing in YT medium at 37°C to an A_{550} ~ 0.3 were transferred to 10°C and grown for 2 h. (*B*) Quantitation of three independent experiments carried out as shown in panel *A*. The amounts of total RNase R, acetylated RNase R, and Pka were set at 1 for cells growing at 37°C. (*C*) MG1655(Seq)*rph*$^+$ cells were grown in YT medium (YT) or M9 minimal medium (M9) to an A_{550} ~ 0.3. RNase R was immunoprecipitated from cell extracts using purified RNase R antibody and then analyzed with RNase R antibody and anti-acetylated-lysine monoclonal antibody (Ac-K). The amount of Pka in cell extracts was analyzed with anti-FLAG M2 mAb. (*D*) Quantitation of three independent experiments carried out as shown in panel *C*. The amounts of total RNase R, acetylated RNase R, and Pka were set at 1 for cells growing in YT medium.

MATERIALS AND METHODS

Materials

Antibody against RNase R was prepared and purified as described previously (Cheng and Deutscher 2002; Liang et al. 2009). Anti-FLAG M2 mAb was from Sigma. Acetylated-lysine mouse mAb was purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse IgG HRP conjugate were obtained from Santa Cruz Biotechnology. Plasmid pET28a and Ni-NTA His-bind resin were obtained from Novagen. The protease inhibitor cocktail was purchased from Calbiochem. The coimmunoprecipitation kit was obtained from Pierce. ^{3}H-labeled arginine, leucine, lysine, tyrosine, and valine were purchased from GE Healthcare, and unlabeled amino acids were obtained from Sigma. Purified exponential phase and stationary phase RNase R and Pka (YfiQ) were prepared as previously described (Liang et al. 2011).

Bacterial strains and growth conditions

E. coli K12 strain MG1655(Seq)*rph*$^+$ and its derivatives lacking RNase R, Pka or CobB were obtained from Dr. Kenneth Rudd (Baba et al. 2006). DNA encoding the 2xFLAG sequence was fused to the N terminus of chromosomal Pka following a previously published recombineering protocol (Datsenko and Wanner 2000) using oligos Y1 and Y2 (Supplemental Table S1). Recombinants were selected on LB-kanamycin plates and were confirmed by PCR using primers Y3 and Y4 (Supplemental Table S1). The kanamycin resistance cassette was removed by plasmid pCP20 (Cherepanov and Wachernagel 1995), and the resulting gene fusion construct was confirmed by DNA sequencing.

Cells were grown at 37°C in liquid culture in YT medium or M9-glucose medium. Antibiotics, when present, were at the following concentrations: kanamycin, 50 μg/mL; ampicillin, 100 μg/mL; chloramphenicol, 34 μg/mL. Exponential phase cells were collected at an A_{550} of ~0.3, and cells grown overnight were used as stationary phase samples.

Immunoprecipitation of RNase R

Purified RNase R antibody was bound to the antibody coupling gel in the coimmunoprecipitation kit according to the manufacturer's protocol. Cells were disrupted in binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40) containing protease inhibitor cocktail as described (Liang and Deutscher 2010). After centrifugation at 12,000 × *g* for 15 min, the supernatant fraction was collected and 2 mg of soluble protein was incubated with antibody-coupled gel at 4°C for 2 h. The gel was collected using a spin column and then washed five times with 500 μL of binding

buffer containing 0.5 M NaCl to remove nonspecific contaminants. RNase R was eluted with 0.1 M glycine-HCl, pH 3.5 and immediately neutralized with 0.1 M Tris-HCl, pH 8.0.

Pulse-labeling of cells

For pulse-labeling experiments, cultures were grown at 37°C. Cells at different growth stages were labeled with a mixture of ^{3}H-labeled arginine, leucine, lysine, tyrosine, and valine (1 μCi each) for 3 min, followed by a chase with 0.2 mM each of unlabeled amino acids for 30 sec. The samples were collected and subjected to immunoprecipitation with RNase R antibody, as above. Radioactivity was determined in a scintillation counter.

Overexpression and purification of CobB

The *cobB* gene was amplified by PCR with primers C1 and C2 (Supplemental Table S1). The PCR product was purified and digested with NheI and XhoI and then cloned into the corresponding sites on pET28a. His-CobB was overexpressed and purified according to the manufacturer's protocol.

Western blot analysis

Proteins were resolved on 8% gels and subjected to immunoblotting. RNase R, acetylated RNase R, and recombinant FLAG-Pka were detected by purified RNase R antibody (1:10,000 dilution), acetylated-lysine mouse mAb (1:1000 dilution), and anti-FLAG M2 mAbs (1:1000 dilution), respectively. Underexposed films were used for quantitation by Quantity One (Bio-Rad).

In vitro acetylation of RNase R

The in vitro acetylation reaction was performed as described previously (Wang et al. 2010) using 50 ng of purified stationary phase RNase R protein as the substrate and 10 μg of cell extract. Reaction mixtures were incubated at 37°C for l h and then analyzed by immunoblotting using acetylated-lysine antibody or RNase R antibody.

CobB-mediated in vitro deacetylation

The in vitro deacetylation reaction was carried out as described previously (Wang et al. 2010) with 50 ng of purified exponential phase RNase R protein and 50 ng of purified His-CobB. Reaction mixtures were incubated at 37°C for different times and analyzed by immunoblotting, using acetylated-lysine antibody or RNase R antibody.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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