An internal region of the RpoH heat shock transcription factor is critical for rapid degradation by the FtsH protease

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Abstract The proteolysis of regulatory proteins plays an important role in the control of gene expression. The *Escherichia coli* heat shock sigma factor RpoH (σ^{32}) is highly unstable. Its instability is determined by interactions with the DnaK chaperone machine, RNA polymerase and the ATP-dependent protease FtsH. *Bradyrhizobium japonicum* expresses three RpoH proteins of which $RpoH_1$ is highly stable. To determine which regions of *E. coli* RpoH determine protein lability, we generated a number of truncated versions and hybrid proteins. Truncation of N-terminal amino acids had no, and deletion of C-terminal amino acids only a minor effect on stability of RpoH. A major determinant of RpoH lability was mapped to a region of about 85 amino acids (residues 36–122) roughly comprising the sigma factor region 2. This is the first demonstration of an internal RpoH region being responsible for FtsH-mediated degradation.

Key words: AAA family; FtsH protease; Heat shock regulation; Proteolysis; RpoH transcription factor; σ^{32}

1. Introduction

The induction of heat shock proteins after temperature upshift can successfully be attained by controlled proteolysis of the key regulatory factors. The *Escherichia coli* heat shock sigma factor RpoH (σ^{32}) is accumulated upon heat shock due to elevated translation and stabilization of the protein (reviewed in [1,2]). RpoH is highly unstable at low temperatures (half-life less than 1 min). It is rapidly degraded by the FtsH protease [3,4]. FtsH is a member of the highly conserved and widely distributed AAA protein family [5,6]. FtsH degrades a number of functionally and structurally unrelated proteins such as LpxC (EnvA), a key enzyme in lipopolysaccharide biosynthesis, subunit a of the F_1F_0-ATPase complex, as well as the phage λ cII and cIII proteins [7–10]. Genetic evidence suggests that the DnaK, DnaJ, GrpE chaperone team plays an important role in the rapid proteolysis of RpoH [11,12]. On the other hand, RpoH complexed with RNA polymerase is protected from degradation by FtsH [13–15].

Bradyrhizobium japonicum, the nitrogen-fixing root-nodule symbiont of soybean contains three RpoH homologues [16]. These have been designated $RpoH_1$, $RpoH_2$ and $RpoH_3$. All three RpoH proteins were found to recognize heat shock promoters in *E. coli* and to initiate transcription from the *groESL* promoter [16,17]. $RpoH_1$ is at least 10 times more stable than *E. coli* RpoH [15]. We made use of the difference in protein lability to localize protein regions that confer protein stability.

2. Materials and methods

2.1. E. coli strains and growth conditions

The Δ*rpoH* strain A7448 (MC4100 Δ*rpoH30*::kan *zhf-50*::Tn10 (λ*imm*21pF13-P*groE-lacZ*) [18] in the absence of complementing plasmids and the Δ*ftsH* strain A8926 (W3110 *sfhC21 zad220*::Tn10Δ*ftsH3*::Kan^R) [19] were grown at 20 or 30°C, respectively. The cloning host *E. coli* DH5α and the wild-type strain A8514 (W3110 *rrnD-rrnE*) were propagated at 37°C. All strains were grown in Luria–Bertani (LB) media supplemented with ampicillin (50 μg/ml) when required. Complementation assays with the temperature-sensitive Δ*rpoH* strain were carried out as follows: after transformation, the cells were plated on LB–ampicillin plates and incubated at 20°C or 37°C. Single colonies from plates incubated at 20°C were inoculated in liquid LB media containing ampicillin and grown overnight at 20°C. This pre-culture was used to inoculate a new culture (1:50 dilution) before growth at 37°C was followed.

2.2. DNA manipulations

Recombinant DNA techniques were performed by standard protocols [20]. The RpoH derivatives constructed in this work originated from plasmids pEC5006 and pEC5007 carrying *E. coli rpoH* and from pRJ5000 and pRJ5001 harboring *B. japonicum* $rpoH_1$ [16]. Truncated *rpoH* genes were generated by PCR using appropriate primers that added a *Kpn*I restriction site to the N-terminal deletions or a *Hin*dIII restriction site to the C-terminal deletions. Upon digestion with the corresponding restriction enzymes, the PCR fragments were used to replace a 152 bp *Nco*I–*Kpn*I or a 720 bp *Hin*dIII–*Pst*I fragment in pEC5007 for N-terminal or C-terminal truncations, respectively. Plasmids carrying chimeric *rpoH* genes, as well as the Del and Add versions, were constructed by a similar strategy replacing internal fragments of pEC5006, pEC5007, pRJ5000 or pRJ5001 by PCR products which had appropriate restriction sites added to their ends. The *Sac*I and the *Hin*dIII sites of *B. japonicum* $rpoH_1$ (corresponding to amino acid residues 35 and 134, respectively, in Fig. 2) and the *Pst*I site of *E. coli rpoH* (amino acid residue 202) were used to create gene fusions. To construct the Add variant, a threonine and a serine codon in accordance with the *B. japonicum* $rpoH_1$ sequence replaced the carboxy-terminal alanine codon of *E. coli rpoH*. The *Spe*I site generated by this modification was then used to add the 3′ end of $rpoH_1$. The sequence of all final plasmids was confirmed by automated sequencing.

2.3. β-Galactosidase assay

Cells were grown in LB media at 20°C and assayed for β-galactosidase activity at 30°C according to standard procedures [21].

2.4. RpoH stability tests and immunoblot analysis

Degradation of RpoH factors in *E. coli* was examined at 30°C as

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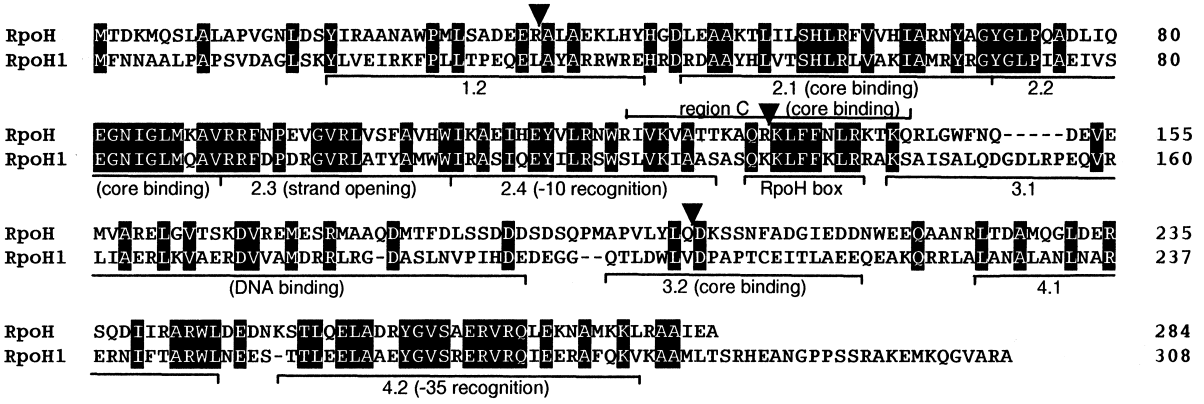

Fig. 1. Sequence comparison of *E. coli* RpoH and *B. japonicum* $RpoH_1$. Identical residues are shaded in black. Generally conserved sigma factor regions and their proposed functions are shown [26,30,31]. Region C and RpoH box define regions highly conserved in RpoH proteins but absent in other sigma factors [32]. Arrowheads indicate the positions at which chimeric proteins were fused.

described previously [15]. Whenever possible, the *rpoH* deletion strain A7448 served as host to facilitate immunodetection of the plasmid-encoded RpoH factors. The wild-type strain A8514 was used for poorly functional or non-functional RpoH variants. Cells were grown to an OD_{600} of 0.5–0.6 before IPTG (1 mM final concentration) was added for 15 min. Protein synthesis was then stopped by the addition of chloramphenicol (200 μg/ml) and the decay of RpoH protein was monitored by Western blot analysis. Crude extracts of *E. coli* cells were prepared, separated on sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose or poly(vinylidene difluoride) membranes. Rabbit anti-(*E. coli* RpoH) serum was used at a 2000-fold dilution. Purified rabbit anti-(*B. japonicum* $RpoH_1$ peptide) IgGs were used as 300-fold solutions. Bound peroxidase-coupled secondary anti-(rabbit IgG) antibodies were visualized by chemiluminescence using the Pierce SuperSignal kit.

3. Results

Sequence comparison between *E. coli* RpoH and *B. japonicum* $RpoH_1$ shows that these proteins display approximately 40% sequence identity (Fig. 1). Identical residues are distributed along the protein with two extended regions that are highly conserved (from region 2.1 to RpoH box, and from C-terminal end of 4.1 to 4.2). In addition, the alignment demonstrates that $RpoH_1$ possesses 23 additional residues at the C-terminus.

3.1. Importance of the N- and C-termini for RpoH stability

To test the importance of the N- and C-termini for RpoH activity and stability, we generated four truncations. The ability of these proteins to complement an *E. coli rpoH* deletion

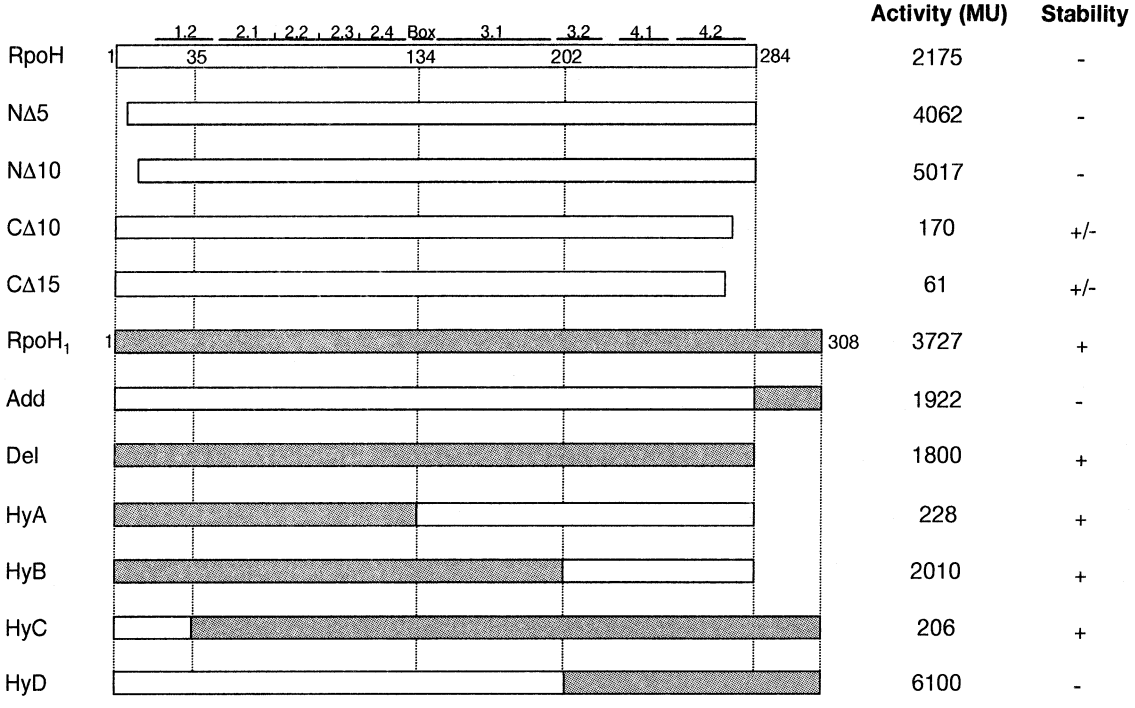

Fig. 2. Schematic representation of RpoH variants. RpoH of *E. coli* (white bar) and $RpoH_1$ of *B. japonicum* (gray bar) and their derivatives that were constructed in this work are shown. On top, the known sigma factor regions are marked. Numbers in the RpoH bar indicate the fusion sites on amino acid level. Sigma factor activity is given in Miller units obtained from a chromosomally integrated RpoH-dependent *groE-lacZ* fusion. Stable RpoH proteins are represented by (+), partially stabilized proteins by (+/−), and rapidly degraded proteins by (−).

strain which is unable to grow at temperatures higher than 20°C [18] was tested. The introduction of NΔ5 and NΔ10 conferred the ability to grow at 37°C. In contrast, CΔ10 and CΔ15 permitted only limited or no cell growth at 37°C. To assess the sigma factor activity of the truncated proteins quantitatively, we monitored the β-galactosidase activity originating from the RpoH-dependent *groE-lacZ* fusion. The N-terminal deletions showed even higher activity than the wild-type protein, whereas the C-terminal deletions had very low activity (Fig. 2). These results demonstrate that the distal end of region 4.2 is important for activity.

To assess the contribution of the RpoH termini to stability, we assayed directly for the proteolysis of the truncated proteins. The N-terminal RpoH truncations were rapidly degraded like the authentic RpoH factor from *E. coli* (Fig. 3A, compare with Fig. 3B). The C-terminal truncations were also degraded but less efficiently. This indicates that the N-terminal residues do not participate in determining the stability of RpoH whereas the integrity of the C-terminus is required for rapid, wild-type like degradation. All truncated

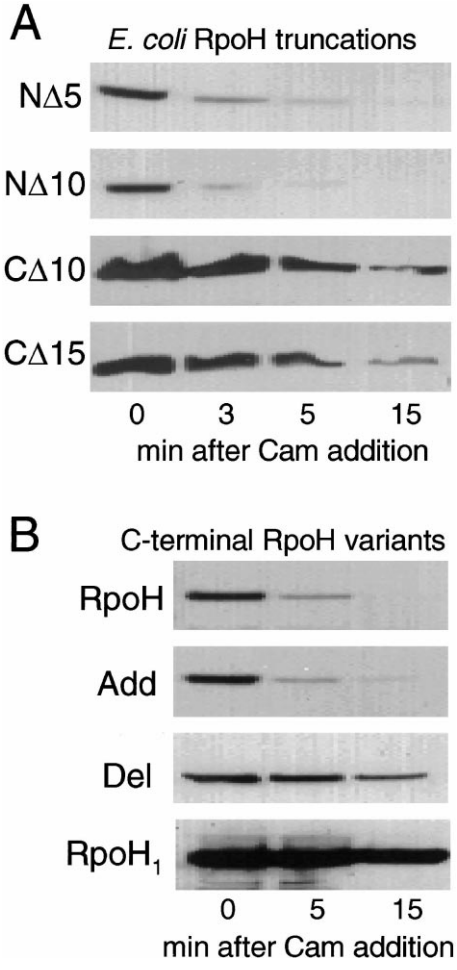

Fig. 3. Protein stability of N- and C-terminally modified RpoH proteins. The decay of RpoH proteins after chloramphenicol addition was monitored by immunoblot analysis (see Section 2). (A) Degradation of *E. coli* RpoH truncations. N-terminal deletions were analyzed in the Δ*rpoH* background, the poorly functional C-terminal truncations in strain A8514, a wild-type strain. (B) Stability of *E. coli* RpoH, *B. japonicum* $RpoH_1$ and their C-terminal variants. Degradation was monitored in *E. coli* A7448.

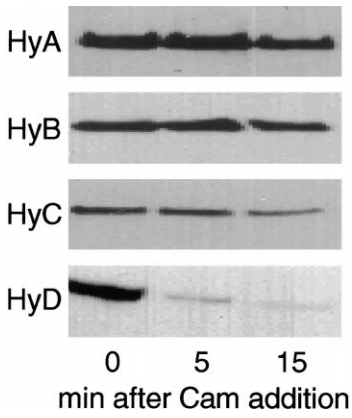

Fig. 4. Stability of RpoH hybrid proteins. Protein stability was determined as described in Fig. 3. HyB and HyD were analyzed in the *E. coli* Δ*rpoH* background. The decay of HyA and HyC was monitored in strain A8514.

RpoH proteins were stabilized in a Δ*ftsH* strain proving that their degradation was FtsH-mediated (data not shown).

To test if the extra 23 amino acid residues present at the C-terminus of $RpoH_1$ were responsible for its extreme stability, a deletion derivative (Del) and an *E. coli* RpoH version containing the extension (Add) were tested. Both proteins were highly active sigma factors (Fig. 2). Absence of the tail did not lead to a protein with reduced stability (Fig. 3B). Similarly, addition of the $RpoH_1$ tail did not increase RpoH stability. These results indicate that the C-terminus of RpoH proteins is not crucial for recognition and degradation by FtsH.

3.2. Protein stability of chimeric RpoH proteins

To further probe for protein sequences that may play a role in determining RpoH stability, a set of hybrid proteins was constructed and assayed (Fig. 2). HyA and HyC were unable to allow colony formation at 37°C directly after transformation of the corresponding plasmids into the reporter strain. However, after pre-cultivation at 20°C, all hybrids promoted bacterial growth in liquid medium at 37°C. The β-galactosidase values are in line with these growth phenotypes (Fig. 2).

To define RpoH regions responsible for the differential degradation of RpoH proteins, we examined the stability of the hybrid proteins. All hybrids in which the region from amino acids 35 to 134 originated from $RpoH_1$ (HyA to HyC) were as stable as the original *B. japonicum* protein, whereas HyD carrying this region derived from RpoH was highly unstable comparable to *E. coli* RpoH (Fig. 4). All hybrids were stabilized in the Δ*ftsH* strain, indicating that in fact FtsH was responsible for their degradation. In summary, the hybrids point to a previously unrecognized internal region of RpoH proteins determining their susceptibility towards the FtsH protease.

4. Discussion

Substrate discrimination is of crucial importance for selective protein degradation by FtsH. However, little is known as to how this enzyme selects its substrate. It was shown that both N- and C-termini can be utilized by the protease [22,23]. Our present information on specific RpoH regions required for its rapid proteolysis is very limited, and the issue is complicated by the sequestration of RpoH by the DnaK chaper-

one machine and by core RNA polymerase [19]. However, the extreme difference in protein stability of *E. coli* RpoH and of *B. japonicum* $RpoH_1$ allowed us to map a previously unknown major lability determinant in *E. coli* RpoH.

First, our results demonstrate that recognition and degradation of RpoH by FtsH do not involve the residues present at the N-terminal end. Second, we show that the removal of as few as 10 or 15 residues from the C-terminal end of RpoH leads to functional inactivation. Whether C-terminal truncations of RpoH are stabilized or not is disputed [13,24]. It has been shown that proteins tagged with the short non-polar SsrA tag at their C-terminus are degraded by FtsH [25]. This finding indicates that at least abnormal proteins are removed by FtsH in a tail-specific manner. Our results show that C-terminally truncated RpoH derivatives are only slightly stabilized in vivo. It is possible that these functionally inactive proteins are somehow sequestered from degradation by FtsH, probably because they aggregate. We also found that the extended tail of 23 amino acid residues is not required for $RpoH_1$ function and does not contribute to protein stability. In summary, FtsH-mediated degradation of RpoH does not seem to occur via its termini.

By using a set of hybrid proteins between *E. coli* RpoH and *B. japonicum* $RpoH_1$, we were able to identify an internal region that plays a dominant role in determining RpoH stability. The result obtained with HyB defines the lability determinant present in RpoH to the 204 N-terminal residues. Stability of HyA reduces this region to the first 134 amino acids. Residue 134 is located in the middle of the highly conserved C region (residues R122–Q144). A role of this region in the turnover of RpoH has been ruled out previously [15,26]. Thus, the lability region of RpoH may extend only to residue 122. The stability of HyC indicates that this lability region is probably less than 100 amino acids from residue 36 to residue 122, roughly comprising region 2 (Fig. 1). The crystal structure of a σ^{70} fragment suggests that all subregions of region 2 are exposed on the surface of RpoH [27]. The functional properties support this inference as this region is intimately involved in contacting the RNA polymerase (regions 2.1 and 2.2), in DNA binding (region 2.4), or in DNA strand opening (region 2.3). In addition, region 2 contains three DnaK binding sites, as was demonstrated by peptide library scanning [28]. More precisely, DnaK contacted patches from H44 to P74, V90 to A111, and H114 to Q132. The exposed and probably flexible region 2 could also interact directly with FtsH.

Binding of RpoH to RNA polymerase or DnaK has opposing effects on RpoH stability. It is easily conceivable that the sequence of region 2 determines the equilibrium between DnaK and RNA polymerase binding of RpoH influencing its stability. Both, reduced affinity for DnaK and improved affinity for RNA polymerase should increase the stability of the transcription factor. Recent experiments with RpoH mutants deficient in binding to RNA polymerase indicate, however, that core binding is not the major effector and that DnaK is an active modulator of RpoH stability [29]. Detailed mutagenesis studies of region 2 should contribute to a deeper understanding of substrate recognition principles of FtsH.

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