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Subunit *a* of proton ATPase F_0 sector is a substrate of the FtsH protease in *Escherichia coli*

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Abstract ***Escherichia coli*** **FtsH is a membrane-bound ATPase with a proteolytic activity against the SecY subunit of protein translocase. We now report that subunit *a* of the membrane-embedded F_0 part of H^+-ATPase is another substrate of FtsH. Pulse-chase experiments showed that subunit *a* is unstable when it alone (without F_0 subunits *b* and *c*) was oversynthesized and that it is stabilized in the *ftsH* mutants. Selective and ATP-dependent degradation of subunit *a* by purified FtsH protein was demonstrated in vitro. These results suggest that FtsH serves as a quality-control mechanism to avoid potentially harmful accumulation of free subunit *a* in the membrane.**

Key words: FtsH; F_1F_0-ATP synthase; Subunit *a*; Membrane protein; Protein degradation

1. Introduction

The FtsH protein of *Escherichia coli* is a cytoplasmic membrane protein having two transmembrane segments at the N-terminus and a large cytoplasmic domain which includes a region homologous to the members of the AAA ATPase family [1,2]. FtsH has ATPase and proteolytic activities [3,4]. Two apparently unrelated proteins have been shown to be substrates of the proteolytic action of FtsH. The heat shock sigma factor, σ^{32}, is unstable under ordinary growth conditions in the wild-type cells, but it is stabilized in *ftsH* mutants [3,5]. Purified FtsH degrades σ^{32} in vitro in an ATP-dependent manner [3]. Another known substrate of FtsH is the SecY protein, a multi-spanning membrane protein [4,6], which forms an integral membrane complex with SecE and SecG and functions as a central component of protein translocase [7]. SecY is normally stable in the cell but becomes very unstable under certain conditions, in which it fails to associate with SecE, the stabilizing partner [8]. Our genetic studies [6] showed that FtsH is required for this degradation of SecY and our biochemical studies [4] demonstrated that FtsH directly degrades SecY in vitro. ATPase-defective mutant forms of FtsH lacked the proteolytic activity [4]. The physiological significance of FtsH-dependent SecY degradation seems to eliminate uncomplexed SecY molecules from the membrane, since they are detrimental to the cell [6].

Proton-translocating ATPase in the cytoplasmic membrane of bacteria plays crucial roles in the interconversion between the two forms of energy, proton gradient across the membrane and ATP, and consists of F_1 and F_0 sectors [9]. While the peripheral membrane F_1 complex synthesizes or hydrolyzes ATP, the integral membrane F_0 sector serves as a proton channel. Thus, the overall architecture of proton ATPase is similar to that of the protein translocation machinery, which is composed of the SecA ATPase and the putative polypeptide-conducting channel (SecYEG complex). The F_0 complex consists of 1 *a*, 2 *b*, and 10 *c* subunits [10]. Previous studies showed that overproduction of subunit *a* is deleterious to the cell [11,12] and it does not accumulate in the membrane unless all the three subunits are co-expressed [13]. Although the failure of subunit *a* to accumulate in the membrane could be due to a failure in the integration process [13], no direct evidence supporting this notion has been presented.

In this study we addressed the questions of whether subunit *a*, like SecY, is unstable in the absence of the partner molecules and, if this is the case, whether FtsH is involved in degradation of subunit *a*. The results obtained showed that these are indeed the cases.

2. Materials and methods

2.1. Bacterial strains and plasmids

AD465 and AD466 carried Δ(*atpB-atpC*) mutation, and the former carried the *zgi-525*::IS*1*A mutation, which reduces the expression level of *ftsH* [6], as well. They were constructed from AK525 and AK519 [6] by P1 transduction using the *ilv*::Tn*10* marker. Plasmid pSTD181 carried the *atpB* gene (for subunit *a* of H^+-ATPase F_0 sector) that was placed under the *lac* promoter control. For its construction, the 1 kb *Hin*dIII-*Vsp*I fragment of pBWU13 [14] was treated with T4 DNA polymerase, and cloned into the *Sma*I site of pHSG575 [15].

2.2. Pulse-chase and immunoprecipitation experiments

Cells of AD465/pSTD181 and AD466/pSTD181 were first grown at 37°C in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml), 0.4% glucose and chloramphenicol (20 μg/ml). The cultures were then shifted to 30°C for 1 h, induced with isopropyl-β-D-thiogalactopyranoside (1 mM) and cyclic AMP (5 mM) for 10 min, and pulse-labeled for 30 s with about 2 MBq/ml of [^{35}S]methionine. Chase was initiated by adding unlabeled methionine (final 200 μg/ml), and samples were withdrawn at specified time points. Proteins were precipitated with trichloroacetic acid and subjected to immunoprecipitation with antiserum against subunit *a* as described previously [8]. After SDS-polyacrylamide gel electrophoresis [16], labeled proteins were visualized using a BAS2000 imaging analyzer.

2.3. In vitro proteolysis of subunit a by FtsH

Cells of AD465 carrying pSTD181 were grown at 37°C to an early log phase in M9 medium, shifted to 30°C for 1 h, induced with isopropyl-β-D-thiogalactopyranoside and cyclic AMP, and pulse-labeled with about 0.37 MBq/ml of [^{35}S]methionine for 90 s. Culture was chilled to 0°C and cells were harvested and disrupted by sonication. Membranes were prepared by ultracentrifugation and solubilized with 0.5% NP40 as described previously [4]. In vitro degradation assay was carried out essentially as described in [4]. Briefly, solubilized membrane proteins (corresponding to those from about 0.4 ml of the

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Abbreviations: SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; FtsH-His_6-Myc, hexahistidine and Myc epitope-tagged FtsH.

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culture) were mixed with ATP (final concentration, 3.3 mM), purified hexahistidine and Myc epitope-tagged FtsH (FtsH-His_6-Myc, final 35 ng/μl) [4], and incubated at 37°C. Samples were removed at intervals, and subjected to immunoprecipitation using anti-subunit *a* serum and SDS-PAGE.

3. Results and discussion

3.1. Instability of subunit a in the absence of other F_0 subunits and its stabilization in an ftsH mutant

Subunit *a* has 6 transmembrane segments with its N- and C-termini facing the cytoplasm [17]. It was reported that the expression of the chromosomally encoded subunit *a* alone in the absence of other F_0 subunits did not lead to its significant accumulation in the membrane [13]. Although Hermolin and Fillingame [13] interpreted this result to mean that integration of subunit *a* into the membrane requires the presence of the other subunits, rapid degradation of subunit *a* could also explain the result. We examined this possibility by direct pulse-chase experiments. We cloned the *atpB* gene into a low copy-number vector and placed it under the control of the *lac* promoter. Pulse-labeling experiments under the *lac*-induced conditions showed that Δ(*atpB-atpC*) cells bearing the resulting plasmid (pSTD181) synthesized subunit *a* at a rate about 2–3-fold higher than that from the chromosomal *atpB* gene in the wild-type cells (data not shown). As shown in Fig. 1, pulse-labeled subunit *a* underwent rapid degradation during the chase. Its half-life was estimated to be about 2 min (closed circle). In contrast, when pSTD181 was introduced into strain AD466, in which the expression of the *ftsH* gene had been reduced due to the *zgi-525*::IS*1*A mutation [6], subunit *a* was found to be significantly stabilized (Fig. 1, open circle). These results indicated that subunit *a* is unstable in the absence of the other F_0 subunits and that FtsH is required for the degradation.

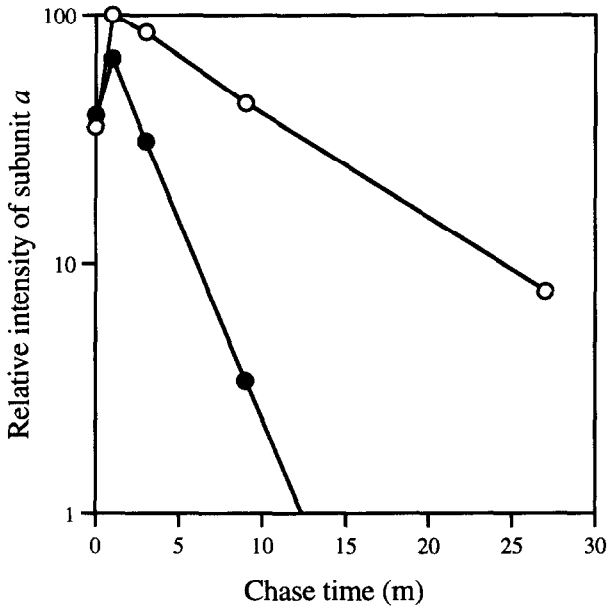

Fig. 1. Stability of subunit *a* in wild type and *ftsH* defective cells. Cells of AD465 (*zgi-525*::IS*1*A)/pSTD181 (○) or AD466 (*ftsH*$^+$)/pSTD181 (●) were grown first at 37°C and then at 30°C for 1 h, induced with isopropyl-β-D-thiogalactopyranoside and cyclic AMP for 10 min, and pulse-labeled with [^{35}S]methionine for 30 s followed by chase with unlabeled methionine for 1, 3, 9, and 27 min. Proteins of fixed total radioactivity were precipitated with trichloroacetic acid, and subunit *a* was immunoprecipitated. Radioactivities associated with subunit *a* were determined after SDS-PAGE and relative values are depicted taking that of AD465/pSTD181 at the 1 min chase point as 100.

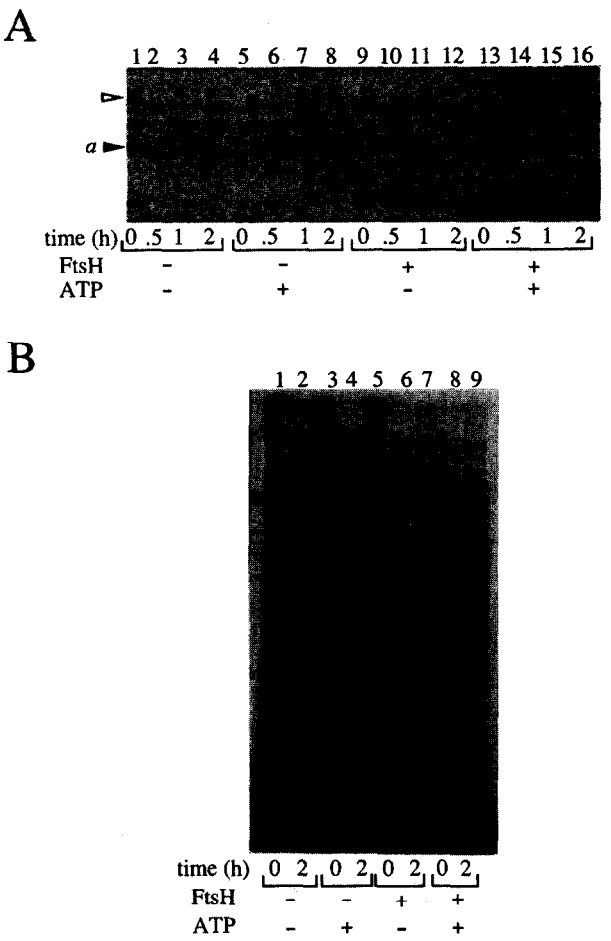

Fig. 2. Degradation of subunit *a* and membrane proteins in crude detergent extracts by purified FtsH. Cells of AD465/pSTD181 were grown first at 37°C and then at 30°C for 1 h, induced with isopropyl-β-D-thiogalactopyranoside and cyclic AMP for 10 min, and pulse-labeled with [^{35}S]methionine for 90 s. Total membranes were prepared, solubilized with 0.5% NP40, and incubated at 37°C in the presence or absence of FtsH-His_6-Myc and ATP as indicated. Samples were withdrawn at the indicated time points and subjected either directly to SDS-PAGE (B) or to anti-subunit *a* immunoprecipitation before SDS-PAGE (A). Open arrowhead indicates an unidentified background protein that was not digested by FtsH, thus serving as a control for sample loading. Lane 1 of (B) was for molecular mass markers (97.4, 69, 46, 30 and 18.4 kDa from top to bottom).

3.2. Subunit a is a substrate of the selective proteolytic action of FtsH

To examine whether FtsH directly degrades subunit *a*, membranes were prepared from induced and pulse-labeled cells of AD466/pSTD181. Membrane proteins were then solubilized with NP40 and incubated at 37°C with a purified preparation of FtsH-His_6-Myc [4], in the presence or absence of ATP (Fig. 2A). Labeled subunit *a* was immunoprecipitated. It was found that subunit *a* was degraded in the presence of ATP, but not in its absence. The electrophoretic pattern of the total membrane proteins showed that the majority of them, especially those of high molecular weight, were not degraded

under the conditions used (Fig. 2B). None of the cytoplasmic soluble proteins, as identified as SDS-PAGE bands, were degraded when incubated with FtsH-His_6-Myc and ATP (data not shown). A background protein shown in Fig. 2A (open arrowhead) was completely resistant to FtsH while subunit *a* in the same reaction was completely digested. These results indicate that FtsH-mediated proteolysis was selective. The observation of Hermolin and Fillingame [13] that subunit *a* did not accumulate in the membrane without subunits *b* and *c* may have been due to its rapid degradation.

The results presented in this paper show that the FtsH protease in *E. coli* is responsible for the proteolytic degradation of unassembled subunit *a* of F_0. Involvement of FtsH homologs in yeast, Yta10 (Afg3) and Yta12, in the degradation of F_0 subunits of the mitochondrial proton ATPase has also been reported [18,19].

Like the unassembled SecY [6], subunit *a* was shown to be toxic when it alone was overexpressed [11,12]. Whereas the proteolytic function of FtsH is important in determining cellular levels of certain intrinsically unstable soluble proteins such as σ^{32} [3,5] and λ cII protein [20,21], it may also be acting to quality-control certain integral membrane complexes. Now the latter example was expanded to include the F_0 complex. The targets of FtsH in the membrane might include incomplete assemblies of some other membrane proteins as well. Such proteins might normally be involved in crucial membrane functions (such as protein export and proton conductance), but their unregulated subreactions might act to disrupt the integrity of the membrane. Thus, a system to ensure the rapid elimination of these incomplete assemblies might have evolved. It remains to be investigated what features of uncomplexed SecY or subunit *a* determine their susceptibility to the protease action of FtsH. We proposed previously that the protease activity of FtsH is regulated by another membrane protein, HflKC [22], and FtsH may have functions that cannot be ascribed to its proteolytic activity [23]. The present results suggest that FtsH constitutes an important quality-control machinery for some membrane proteins. Undoubtedly, its proteolytic activity is crucial for this function of FtsH, but its postulated molecular chaperone-like functions [23] could also be important in the selection of substrate proteins and their presentation for degradation.

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