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FtsH degrades kinetically stable dimers of cyclopropane fatty acid synthase via an internal degron

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Abstract

Targeted protein degradation plays important roles in stress responses in all cells. In *E. coli*, the membrane-bound AAA+ FtsH protease degrades cytoplasmic and membrane proteins. Here, we demonstrate that FtsH degrades cyclopropane fatty acid (CFA) synthase, whose synthesis is induced upon nutrient deprivation and entry into stationary phase. We find that neither the disordered N-terminal residues nor the structured C-terminal residues of the kinetically stable CFA-synthase dimer are required for FtsH recognition and degradation. Experiments with fusion proteins support a model in which an internal degron mediates FtsH recognition as a prelude to unfolding and proteolysis. These findings elucidate the terminal step in the life cycle of CFA synthase and provide new insight into FtsH function.

KEYWORDS
AAA+ protease, ATP-dependent protein degradation, substrate recognition

1 | INTRODUCTION

Proteases of the AAA+ superfamily bind specific protein targets and use the energy of ATP hydrolysis to mechanically unfold and then translocate the substrate into a sequestered proteolytic chamber for destruction (Sauer & Baker, 2011). These proteolytic machines consist of a AAA+ ring hexamer with a central axial channel and a self-compartmentalized peptidase. The AAA+ ring is responsible for substrate recognition, unfolding, and translocation. Several AAA+ proteases are upregulated in bacteria as part of the heat-shock response, helping to limit cytotoxicity by degrading misfolded or partially unfolded proteins (Meyer & Baker, 2011). In addition, AAA+ proteases degrade two transcription factors, σ^{32} and σ^{S}, the respective master regulators of heat-shock stress and nutrient-starvation stress in *Escherichia coli*, to help restore homeostasis (Herman et al., 1995; Zhou et al., 2001). After the SOS response to DNA damage, AAA+ proteases also degrade many of the induced proteins, allowing a return to homeostasis once transcription of their genes returns to pre-stress levels (Flynn et al., 2003; Neher et al., 2006; Neher, Flynn, et al., 2003; Neher, Sauer, et al., 2003).

Upon entry of *E. coli* into stationary phase, levels of cyclopropane fatty acid (CFA) synthase increase as a consequence of enhanced transcription from a σ^{S}-regulated promoter (Wang & Cronan, 1994). This enzyme converts the alkene groups within unsaturated fatty acids of the lipid membrane into cyclopropyl moieties (Law, 1971), a reaction that provides increased resistance of *E. coli* to acid shock (Chang & Cronan, 1999) and repeated freeze–thaw cycles (Grogan & Cronan, 1986). Proteolysis of CFA synthase in a σ^{32}-dependent manner subsequently helps return enzyme activity to pre-transition levels (Chang et al., 2000). Here, we report that FtsH, a membrane-anchored AAA+ protease, degrades CFA synthase in vitro and in vivo. CFA synthase is enzymatically active as a dimer (Hari et al., 2018), which we show is kinetically stable and the direct target of FtsH degradation. Like other AAA+ proteases, FtsH typically recognizes degrons consisting of disordered peptide sequences at the N- or C-termini of characterized substrates (Führer et al., 2007; Herman et al., 2003). The N-terminal residues of CFA

synthase are disordered, but we find that they play no role in FtsH recognition. The C-terminal residues of CFA synthase are inaccessible in the native dimer. Although these residues can function as an FtsH degron when appended to another protein, our mutational experiment indicate that they do not mediate degradation of CFA synthase. Thus, internal sequences in CFA synthase appear to be responsible for recognition by FtsH. These results demonstrate that FtsH plays a role in the life cycle of CFA synthase and suggest that it can degrade native proteins with substantial kinetic stability.

2 | RESULTS

2.1 | FtsH degrades CFA synthase

Using purified enzymes and substrate, we initially asked if any of the five AAA+ proteases in *E. coli* (FtsH, HslUV, Lon, ClpXP, or ClpAP) could degrade CFA synthase. In assays monitored by SDS-PAGE, FtsH degraded CFA synthase but the remaining proteases catalyzed little or no degradation (Figure 1a). FtsH did not degrade CFA synthase in the absence of ATP or if ATP-hydrolysis was inactivated by a Walker-B E252Q mutation (Figure 1b). Thus, degradation of CFA synthase is ATP dependent as well as specific with respect to the AAA+ protease involved.

A previous study showed that the intracellular half-life of CFA synthase was not affected by a *clpP* mutation (Chang et al., 2000), which prevents degradation by ClpXP or ClpAP. To analyze the stability of CFA synthase in *E. coli* strains containing single-gene knockouts of *ftsH*, *hslU*, or *lon*, we performed radiolabeled pulse-chase experiments using plasmid-borne CFA synthase (Figure 1c). Consistent with our results in vitro, chromosomal deletions of either *lon* or *hslU* had little effect on intracellular degradation of CFA synthase. However, the half-life of CFA synthase was longer in Δ*ftsH:kan* cells than in an otherwise isogenic strain, showing that FtsH plays a role in regulating intracellular levels of CFA synthase.

In *E. coli* lacking the *ftsH* gene, higher steady-state levels of CFA synthase might result in increased lipid cyclopropanation. To test this possibility, we extracted phospholipids from overnight cultures of strains AR3289 and AR3289/Δ*ftsH:kan* and analyzed lipid fatty acid methyl esters by GC/MS. Importantly, the level of the major cyclopropanated species (17:1cy) relative to its precursor with a double bond (16:1) was ~2.5-fold higher in AR3289/Δ*ftsH* than in the parental strain (Figure 1d, top panel). Adding the concentrations of the 17:1cy and 16:1 species and dividing by the concentration of the corresponding lipid species lacking a double bond (16:0) resulted in similar values in the *ftsH*$^+$ and Δ*ftsH:kan* strains (Figure 1d, bottom panel). Thus, the degree of cyclopropanation but not overall biosynthesis of these lipid variants is increased by the Δ*ftsH:kan* allele.

2.2 | FtsH degrades native dimers of CFA synthase

CFA synthase forms a dimer that is stable at μM concentrations (Hari et al., 2018). In principle, however, a small equilibrium population of native or denatured monomer could be the species recognized and degraded by FtsH. This model predicts that destabilization of the native dimer should increase the rate of FtsH degradation. To test this hypothesis, we first used Michaelis–Menten analysis to determine the steady-state kinetic parameters for FtsH degradation of ^{35}S-labeled CFA synthase (Figure 2a), yielding a K_M of 0.5 μM and a substrate turnover number (V_{max}/E_{total}) of 0.16 min^{-1} $FtsH_6^{-1}$. Next, we studied degradation of E308Q CFA synthase, which has a mutation in the dimer interface and chromatographs as a monomer in gel filtration (Hari et al., 2018). FtsH degraded the E308Q variant at about half of the maximal rate of the wild-type dimer but with a similar K_M (Figure 2a). Thus, CFA-synthase dimerization is not required for FtsH recognition. Moreover, because monomers are degraded more slowly than dimers, it is unlikely that native CFA synthase dimers must dissociate prior to recognition by FtsH.

A second prediction of the monomer-degradation model is that the rate of dimer dissociation should be faster than the steady-state rate of FtsH degradation. To determine kinetic stability, we mixed equal concentrations of CFA synthase labeled separately with fluorescent donor or acceptor dyes and monitored the kinetics of heterodimer formation by FRET (Figure 2b). Under these conditions, the heterodimer concentration approaches its equilibrium value with kinetics determined by the rate of subunit dissociation (Jonsson et al., 1996). After a short lag, the half-life of subunit mixing was ~23 min at 37°C. This time is substantially longer than the ~4 min half-life of CFA synthase degradation by FtsH under V_{max} conditions and thus supports a model in which FtsH recognizes CFA-synthase dimers prior to the initiation of global unfolding and degradation. Alternatively, FtsH binding might induce more rapid dissociation of CFA-synthase dimers. However, because FtsH would have to expend energy to force dimer dissociation, this model predicts that K_M for degradation of the monomeric variant should be substantially lower than K_M for degradation of the wild-type dimer, which was not observed (Figure 2a).

We assayed the rate of ATP hydrolysis by FtsH in the absence of substrate or presence of CFA-synthase dimers or Arc-ssrA dimers at 30, 37, and 42°C (Figure 2c). At each temperature, CFA synthase reduced the rate of ATP hydrolysis by FtsH, whereas Arc-ssrA increased this rate. For other AAA+ proteases, faster ATP hydrolysis is often correlated with translocation being the rate-determining step in degradation, whereas slow ATP hydrolysis suggests that unfolding is rate limiting (Kenniston et al., 2003). FtsH degraded Arc-ssrA with a steady-state maximum velocity ~6-fold faster (see Figure 3c) than it degraded CFA synthase. The rate of ATP hydrolysis in the presence of substrate divided by V_{max} for degradation provides an estimate of the ATP cost of degradation of a single molecule of substrate. At 37°C, these values were ~460 ATPs for FtsH degradation of CFA synthase and ~140 ATPs for Arc-ssrA. Thus, FtsH uses more energy to engage, unfold, and translocate CFA synthase than Arc, which is probably a consequence of the larger size and increased stability of CFA synthase compared to Arc-ssrA. FtsH degradation of degron-tagged variants of the GlpG membrane protein, which is more stable than Arc and proteolyzed

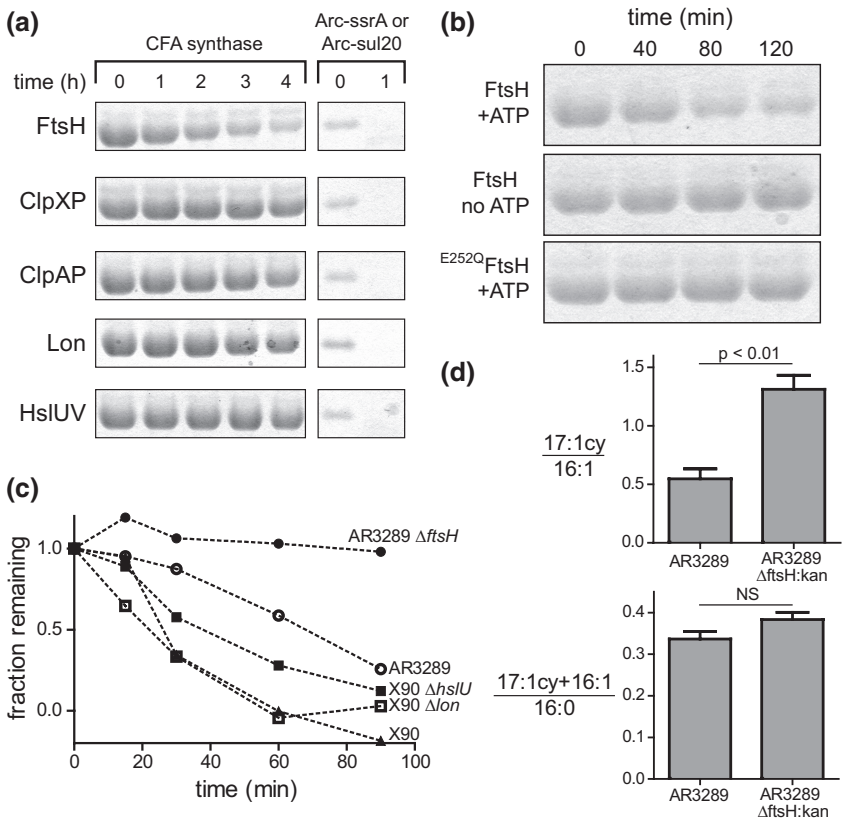

FIGURE 1 FtsH degrades CFA synthase. (a) CFA synthase (20 μM; left panel) or degron-tagged Arc repressor (20 μM; right panel) were incubated at 37°C with FtsH (0.3 μM hexamer), HslUV (0.3 μM $HslU_6$; 1 μM $HslV_{12}$), Lon (0.3 μM hexamer), ClpXP (0.3 μM $ClpX_6$; 0.9 μM $ClpP_{14}$), or ClpAP (0.3 μM $ClpA_6$; 0.9 μM $ClpP_{14}$) and degradation kinetics were monitored by SDS-PAGE. Reactions contained 4 mM ATP and a regeneration system. Arc-ssrA was used for FtsH, HslUV, ClpXP, and ClpAP. Arc-sul20 was used for Lon. The faint band above CFA synthase is creatine kinase from the regeneration system. (b) CFA synthase (20 μM) was incubated with FtsH (0.5 μM hexamer; top two panels) or ^{E252Q}FtsH (0.5 μM hexamer; bottom panel) in the presence or absence of 4 mM ATP and a regeneration system. Degradation reactions were performed and analyzed as described in panel (a). (c) After briefly expressing CFA synthase from a plasmid, ^{35}S-labeled methionine and cysteine were added to the indicated *E. coli* strains for 5 min, and a chase was initiated with unlabeled amino acids and phenyl-β-D-galactoside to represses transcription. At different times, samples were taken, separated by SDS-PAGE, and the gel was autoradiographed. Quantified bands from one replicate are shown in the plot. Based on independent experiments, the half-lives of CFA synthase were 20 and 34 min in strain X90; 34 and 29 min in strain X90 Δ*hslU*; and 12 and 18 min in strain X90 Δ*lon*. CFA synthase half-lives were 53, 52, and 60 min in strain AR3289; and >90, >90, and >90 min in strain AR3289 Δ*ftsH*. (d) Phospholipids were extracted from overnight cultures of *E. coli* strains AR3289 or AR3289 Δ*ftsH* grown at 30°C, then converted into fatty acid methyl esters and analyzed by GC/MS. NS: Not significant. Error bars represent the standard error of the mean (SEM, $n = 3$ for separately grown cultures started from the same colony).

at a rate slightly faster than CFA synthase, uses 380–550 ATPs per substrate (Yang et al., 2018).

2.3 | Terminal determinants of FtsH recognition?

Unstructured peptide degrons at either the N-terminus or C-terminus of a protein substrate are typically recognized and engaged by the axial channel of AAA+ proteases (Sauer & Baker, 2011). Internal sequences can also serve as degrons and engagement sites, albeit more rarely (Gur & Sauer, 2008; Hoskins et al., 2002; Kraut & Matouschek, 2011; Okuno et al., 2006; Piwko & Jentsch, 2006). The 12 N-terminal residues of CFA synthase are disordered in the crystal structure and thus represented a plausible degron. However, when we fused the N-terminal 15 residues of CFA synthase to the N-terminus of Arc, FtsH degraded this substrate ($N15^{CFAS}$-Arc) more slowly than parental Arc (Figure 3a). Moreover, FtsH degraded a purified CFA-synthase variant missing the 15 N-terminal residues (ΔN15-CFAS) slightly faster than wild-type CFA synthase at 30°C (Figure 3b). This temperature was used because the variant precipitated at 37°C. Thus, N-terminal residues of CFA synthase are not required for FtsH degradation.

Next, we probed the C-terminal residues of CFA synthase, as FtsH has been shown to recognize C-terminal ssrA and 108 degradation tags (Herman et al., 1998). When we appended the 11 C-terminal residues of CFA synthase to the C-terminus of Arc ($Arc\text{-}C11^{CFAS}$), FtsH degraded this substrate faster than untagged Arc and at a rate similar to degradation of Arc-ssrA (Figure 3a).

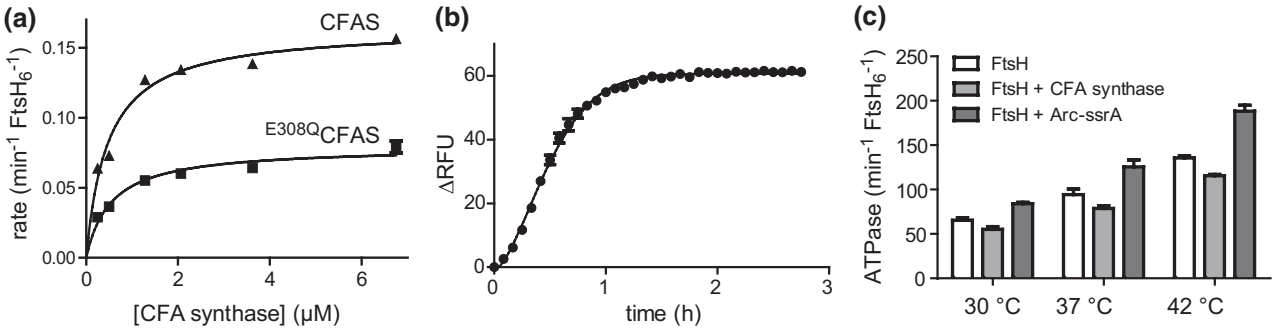

FIGURE 2 Steady-state FtsH degradation, subunit exchange, and ATP hydrolysis. (a) Rates of FtsH degradation of different concentrations of ^{35}S-labeled CFA synthase (in monomer equivalents) or ^{E308Q}QCFA synthase at 37°C were determined by scintillation counting of TCA-soluble fractions. The graph shows non-linear least squares fits of the data to the Michaelis–Menten equation (CFAS: $K_M = 0.48 \pm 0.06\,\mu M$; $V_{max} = 0.16 \pm 0.01\,min^{-1}$. ^{E308Q}CFAS: $K_M = 0.55 \pm 0.08\,\mu M$; $V_{max} = 0.079 \pm 0.003\,min^{-1}$). Error bars represent the standard error of the mean (SEM, $n = 3$ trials prepared using the same substrate and enzyme stocks). (b) Samples of CFA synthase labeled with Dylight-488 or Dylight-650 were mixed and FRET as a function of time was monitored at 37°C (excitation 480 nm; emission 675 nm). The solid line is a fit to a growth equation (Weibull, 1951) to account for the initial lag phase (see text). (c) The rate of ATPase hydrolysis by FtsH was measured alone or in the presence of either CFA synthase or Arc-ssrA (20 μM each) at different temperatures.

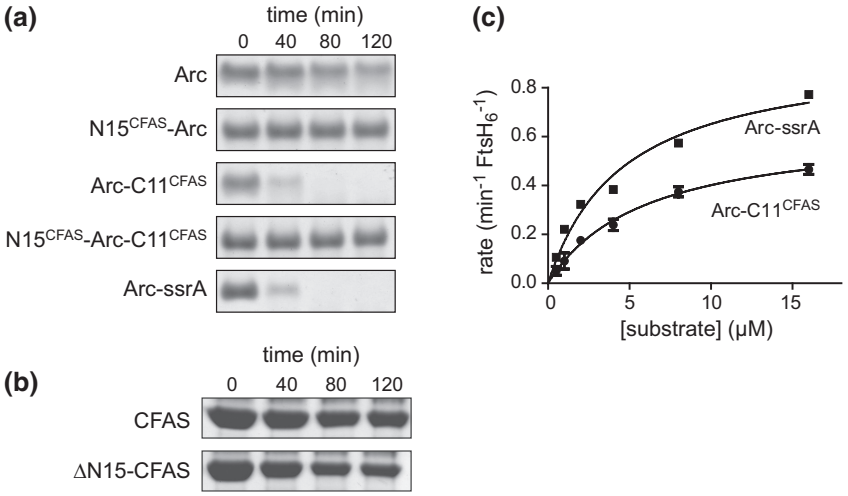

FIGURE 3 Effects of terminal CFA-synthase sequences on degradation. (a) Degradation of Arc-repressor fusions with different termini of CFA synthase by FtsH at 30°C. (b) Degradation of wild-type CFA synthase and the ΔN15 mutant by FtsH at 30°C. (c) Rates of degradation of different concentrations of Dylight-488-labeled Arc-C11^{CFAS} or Arc-ssrA by FtsH at 37°C were determined by monitoring fluorescence dequenching (Baytshtok et al., 2016). The graph shows non-linear least squares fits of the data to the Michaelis–Menten equation (Arc-C11^{CFAS}: $K_M = 5.9 \pm 1.1\,\mu M$; $V_{max} = 0.64 \pm 0.05\,min^{-1}$; Arc-ssrA: $K_M = 4.6 \pm 0.8\,\mu M$; $V_{max} = 0.95 \pm 0.07\,min^{-1}$).

Michaelis–Menten analysis showed that K_M for both degradation reactions was ~5 μM and V_{max} for FtsH degradation of Arc-C11^{CFAS} was about ~30% lower that of Arc-ssrA (Figure 3c). Surprisingly, an Arc substrate containing the 15 N-terminal and 11 C-terminal residues of CFA synthase was not degraded by FtsH (Figure 3a), indicating that the N15 sequence of CFA synthase inhibits FtsH degradation of this substrate. We propose that acidic residues in N15 (NGSSSSCIEEVPDDS) might interact with basic residues in C11 (RGVENGLRVAR), as the two termini of Arc are close in space, allowing an electrostatic interaction to mask recognition of C11 by FtsH. Notably, however, the N- and C-terminal residues of CFA synthase are not close structurally, and thus this argument pertains only to the Arc-fusion protein.

Although the C-terminal 11 residues of CFA synthase can function as a modular degron for FtsH, K_M for recognition of these residues fused to Arc was ~5 μM, whereas K_M for degradation of CFA synthase was ~0.5 μM. Moreover, residues 375–382 at the C-terminus of CFA synthase pack into the dimer interface in the crystal structure (Figure 4a). For example, the side chain of Arg^{382}, the C-terminal residue in each subunit, forms multiple salt bridges and hydrogen bonds in the native structure (Figure 4b). Thus, the C-terminus of CFA synthase would only be accessible in a transiently disordered conformation. To test for Arg^{382} availability, we assayed binding of anhydrotrypsin, which binds tightly to accessible C-terminal arginines (Yokosawa & Ishii, 1977). Specifically, we immobilized different His-tagged proteins onto sensor tips

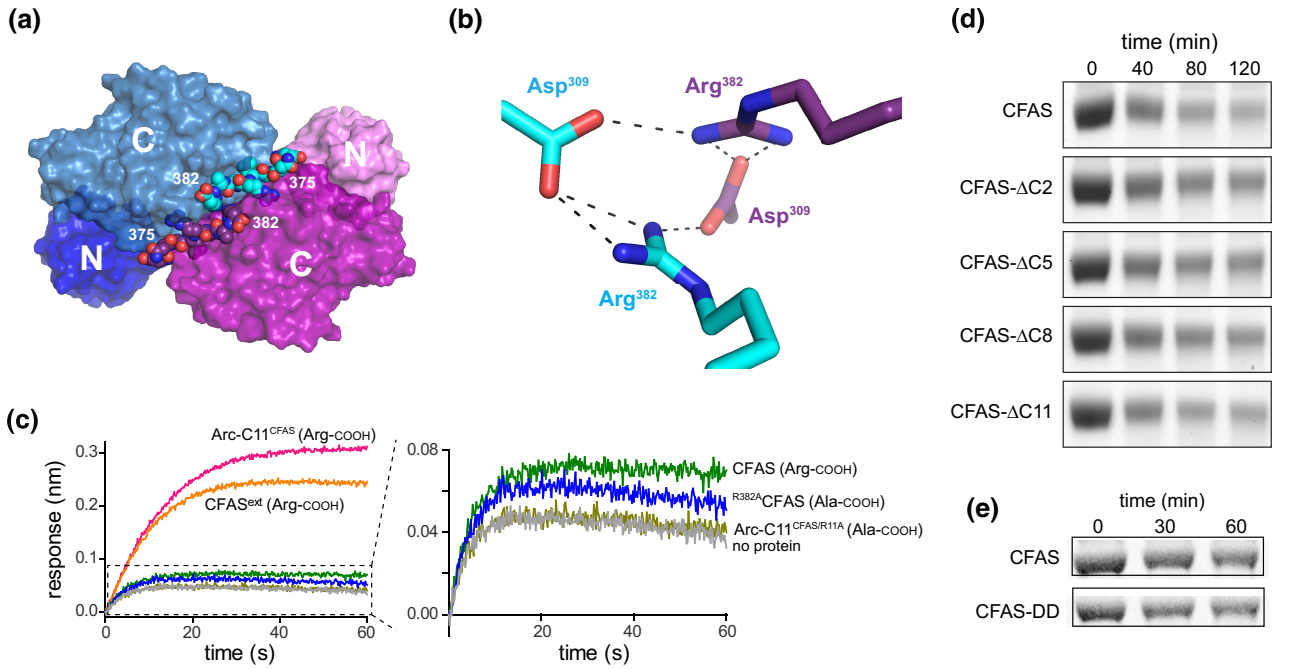

FIGURE 4 The C-terminus of CFA synthase is part of the structure and not required for FtsH degradation. (a) CFA synthase dimer (pdb code 6BQC) shown in surface representation. Subunits are colored shades of blue or purple and the N-terminal and C-terminal domains are designated. Residues 375–382 of each subunit are shown in space-filling representation and are part of the dimer interface. (b) In both subunits, the side chains of Arg^{382} point down into the dimer interface, and make multiple hydrogen-bonded salt bridges (indicated by dashed lines) with the side chains of Asp^{309}. (c) Variants of H_7-SUMO-Arc-$C11^{CFAS}$ or H_6-CFA synthase (CFAS) were loaded onto bio-layer interferometry tips coated with anti-His1K antibody and anhydrotrypsin binding was monitored. The reference control omitted His-tagged protein loading. (d) Degradation of C-terminal truncations of CFA synthase (20 μM) by FtsH (0.5 μM hexamer). (e) Degradation of CFA synthase or the Asp^{381}-Asp^{382} CFAS-DD variant (20 μM) by FtsH (0.5 μM hexamer). The experiments in panels (d) and (e) were performed at 37°C in the presence of 5 mM ATP and were monitored by SDS-PAGE.

coated with anti-His1K antibody and monitored binding using bio-layer interferometry (Abdiche et al., 2008). To validate the assay, we first compared binding responses of His_7-SUMO-Arc-$C11^{CFAS}$ (C-terminal arginine) and His_7-SUMO-Arc-$C11^{CFAS/R11A}$ (C-terminal alanine). The equilibrium response signal of anhydrotrypsin binding to His_7-SUMO-Arc-$C11^{CFAS}$ was ~6-fold greater than to the control protein, which gave a response similar to another control reaction without loaded protein (Figure 4c). We then assayed anhydrotrypsin binding to His_6-CFA synthase and to an otherwise identical R382A mutant. The response of CFA synthase was only slightly higher than the R382A mutant or His_7-SUMO-Arc-$C11^{CFAS/R11A}$, which has a C-terminal alanine. By contrast, His_6-$CFAS^{ext}$, a mutant in which the last 10 residues were duplicated to generate an exposed C-terminal arginine, produced robust anhydrotrypsin binding. Thus, conformational shielding dramatically reduces the accessibility of the C-terminus of native CFA synthase to macromolecular binding.

To test more directly if the C-terminal residues play a role in FtsH degradation, we truncated two, five, eight, or eleven residues from CFA synthase and observed little effect on degradation (Figure 4d). FtsH does not degrade an Arc-ssrA variant with the C-terminal Ala-Ala dipeptide changed to Asp-Asp (Herman et al., 2003). However, changing the C-terminal dipeptide of CFA synthase from Ala-Arg to Asp-Asp (CFAS-DD) did not slow FtsH degradation (Figure 4e). Hence, neither the N- nor C-terminal residues of CFA synthase appear to be required for FtsH degradation.

Consistent with our conclusion that neither terminus of CFA synthase is recognized for FtsH degradation, we found that fusing SUMO protein to the N-terminus (SUMO-CFAS) or C-terminus (CFAS-SUMO) did not block degradation (Figure 5a). Moreover, FtsH did not degrade SUMO as a free protein (Figure 5b), ruling out the possibility that degradation initiated with the SUMO portion of the fusion proteins. We also fused an *E. coli* dihydrofolate reductase (DHFR) variant with an N-terminal affinity tag to the N-terminus (DHFR-CFAS) or C-terminus (CFAS-DHFR) of CFA synthase. In the presence of methotrexate, which prevents FtsH proteolysis of DHFR (Koodathingal et al., 2009), FtsH degradation of DHFR-CFAS produced a partially degraded species, which electrophoresed in SDS-PAGE at a molecular weight slightly larger than DHFR (Figure 5c, left side). After excising this species from the gel, sequential Edman degradation revealed an N-terminal sequence (Gly-Ser-Ser-His-His) identical to that of the DHFR construct used for this experiment. These results support a model in which FtsH degradation of DHFR-CFAS begins at a site within CFA-synthase but cannot proceed through DHFR. Somewhat surprisingly, little FtsH degradation of the CFAS-DHFR fusion protein was observed (Figure 5c, right side), likely because fusion of DHFR protein to the C-terminus, unlike SUMO, interferes with FtsH recognition of CFA synthase.

2.4 | Internal recognition

Based upon the results presented, FtsH probably recognizes an internal and unstructured segment of CFA synthase prior to engagement and degradation. In principle, this internal sequence could be part of the native protein or a partially folded enzyme species in equilibrium with the native protein. Based upon the crystal structure (Hari et al., 2018), the only unstructured region in native CFA synthase is residues 100–120, which serve as a linker to connect the N- and C-terminal domains. We hypothesized that FtsH might recognize and/or initiate degradation within this linker and then degrade CFA synthase in both C-to-N and N-to-C directions, as proposed for other substrates (Chiba et al., 2002; Okuno et al., 2006). We tested this model in two ways. First, we replaced the wild-type ARLFNLQSKKRAWIVGKEHY^{120} linker sequence with a GGGSGSGGGGSGSGGGGSGS^{120} segment composed solely of glycines and serines. Notably, FtsH degraded this mutant (GS^{linker}-CFAS) as fast as it degraded wild-type CFA synthase (Figure 6a). Thus, the sequence of the wild-type linker is neither required for recognition by FtsH nor for initiation of degradation. The distance

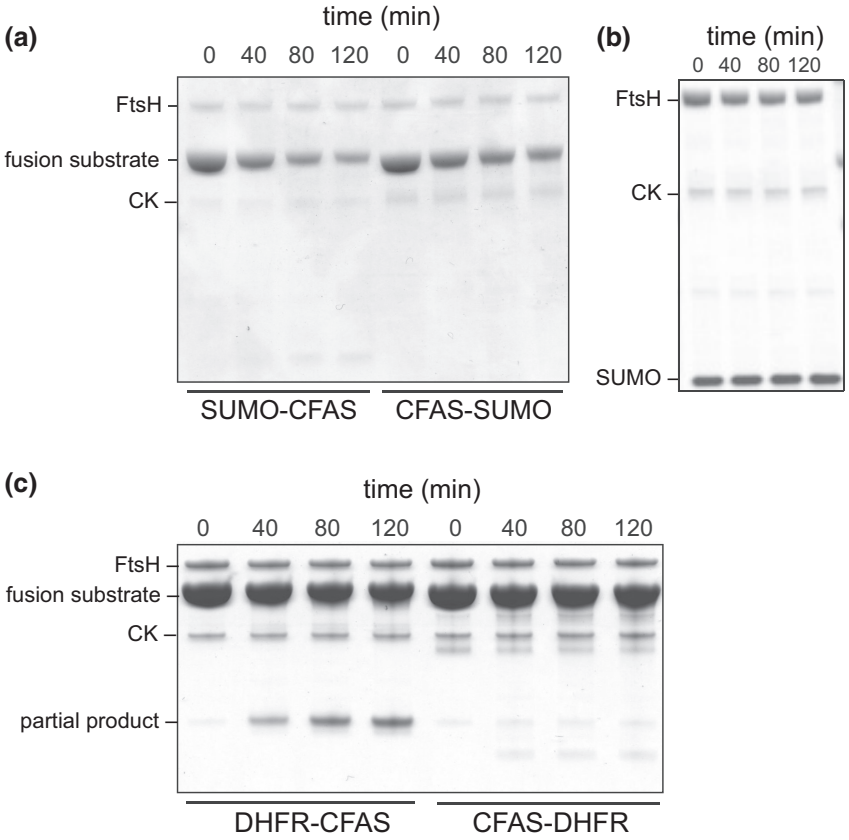

FIGURE 5 Degradation of fusion proteins. (a) Degradation of SUMO-CFAS and CFAS-SUMO (20 μM each) by FtsH (0.5 μM hexamer) at 37°C. (b) SUMO was purified by size-exclusion chromatography following overnight cleavage of SUMO-CFAS with Ulp1 (1:100 Mol. Eq.) and 20 μM was incubated with FtsH (0.5 μM hexamer) at 37°C. (c) Incubation of DHFR-CFAS and CFAS-DHFR (20 μM each plus 40 μM methotrexate) with FtsH (0.5 μM hexamer) at 37°C. In all panels, CK is creatine kinase from the ATP-regeneration system.

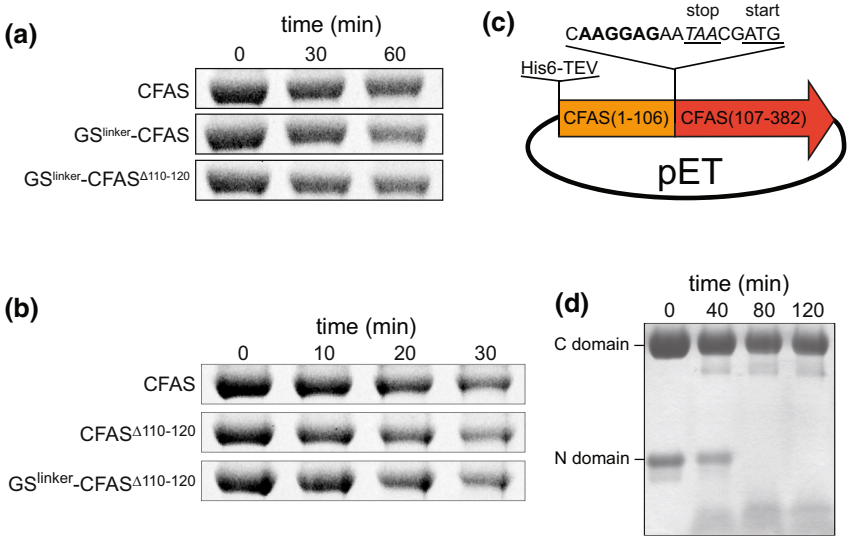

FIGURE 6 Degradation of linker variants and split CFA synthase. (a) FtsH (0.5 μM hexamer) degrades CFA synthase and GS^{linker}-CFAS (20 μM each) at similar rates. (b) FtsH degrades linker deletion variants of CFA synthase at similar rates. Conditions as in panel (a). (c) an engineered gene (Shine-Dalgarno sequence in bold) was used to express the N-terminal and C-terminal domains of CFA synthase as separate polypeptides. (d) The complex of both domains was purified by Ni^{++}-NTA affinity and incubated with FtsH under the same conditions as in Figure 1b.

between residues 99 and 121 in one subunit of the CFA synthase structure is ~25 Å, which in principle could be spanned by a linker of only nine residues. Thus, we tested if linker length was important by deleting residues 110–120 from CFA synthase or GS^{linker}-CFAS. In each case, the deletion mutant was degraded at a rate similar to wild-type CFA synthase (Figure 6b), indicating that linker length plays little role in FtsH recognition.

As described previously (Hari et al., 2018), the two domains of CFA synthase remain associated after Ni^{++}-NTA affinity purification from a strain harboring a plasmid-borne gene (Figure 6c) that separately expresses a His_6-tagged N domain and untagged C domain. When this split protein was incubated with FtsH, the N-domain was degraded rapidly and the C-domain was degraded more slowly (Figure 6d). Interestingly, C-domain degradation ceased once the N-domain was completely degraded, indicating that C-domain degradation requires the presence of the N-domain. This result could be explained if FtsH binds to a sequence within the N-domain of the split substrate and efficiently initiates degradation of this domain, while less efficiently initiating degradation of the C-domain.

3 | DISCUSSION

CFA synthase converts unsaturated fatty acids in the lipid bilayer of *E. coli* into cyclopropyl fatty acids at the onset of stationary phase (Law, 1971; Taylor & Cronan, 1979). Degradation of CFA synthase is dependent on σ^{32}, the heat-shock transcription factor, which activates biosynthesis of several AAA+ family proteases, including membrane-anchored FtsH (Chang et al., 2000; Zhao et al., 2005). Although CFA synthase was not identified as a substrate for FtsH in proteomic studies (Arends et al., 2016; Westphal et al., 2012), the experiments presented here demonstrate clearly that FtsH degrades CFA synthase both in biochemical experiments in vitro and in *E. coli*. Figure 7 shows transcriptional mechanisms at which the intracellular levels of CFA synthase are also regulated. For, example, under non-stress conditions, transcription of CFA synthase is controlled both by σ^{32} and by RydC, a small RNA that slows mRNA turnover (Fröhlich et al., 2013), whereas, σ^{S}, the stationary-phase transcription factor enhances the biosynthesis of CFA-synthase upon nutrient depletion (Wang & Cronan, 1994).

LpxC and YfgM are also degraded by FtsH during stationary phase (Bittner et al., 2017; Thomanek et al., 2019). Hence, these findings raise the possibility that FtsH plays a more global role in stress response and suggest that additional substrates might be discovered through targeted proteomic studies performed under stress conditions other than heat shock.

An important and influential early study suggested that FtsH is a "weak unfoldase" (Herman et al., 2003). For example, many of its soluble substrates (e.g., σ^{32}, degron-tagged Arc repressor, and the N-terminal domain of λ repressor) equilibrate rapidly between folded and unfolded species. Moreover, FtsH did not degrade stable native substrates, like GFP-ssrA, which can be degraded by the AAA+ ClpXP and ClpAP proteases (Herman et al., 2003). More recent investigations, however, show that FtsH can degrade integral membrane proteins, overcoming the large energetic barrier needed to dislodge these proteins from the membrane (Hari & Sauer, 2016; Langklotz et al., 2012; Yang et al., 2018, 2019). Hence, accumulating evidence suggests that FtsH may not be an inherently weak protein unfoldase. CFA synthase represents another example of FtsH degrading a protein with substantial kinetic stability. At 37°C, we find that CFA synthase can be degraded by FtsH at a rate about 5-fold faster than dimers, the predominant oligomeric species, dissociate to monomers, implying that a dimeric and not a monomeric form is the proteolytic target. As discussed below, it is possible that a dimeric CFA synthase species with a transiently unfolded region is recognized and degraded by FtsH.

AAA+ proteases need to recognize a target protein and also to engage an unstructured segment of this substrate in their axial channel to initiate the unfolding and translocation reactions that are required for degradation (Sauer & Baker, 2011). In principle, recognition and engagement could both involve the same disordered segment, as demonstrated for ClpXP and the ssrA tag (Fei et al., 2020), or recognition could involve binding regions of the AAA+ protease other than the axial channel with engagement of a peptide being relatively non-specific. FtsH and many other AAA+ proteases have been shown to recognize disordered sequences at the N-terminus or C-terminus of substrates (Chiba et al., 2002; Flynn et al., 2003; Führer et al., 2007; Gottesman et al., 1998; Herman et al., 1998, 2003; Neher, Flynn, et al., 2003; Neher, Sauer, et al., 2003; Sauer & Baker, 2011). FtsH degradation of CFA synthase, by contrast, does not depend on sequences at either protein terminus. Specifically, deletion of the N-terminal 15 residues or C-terminal 10 residues of CFA synthase does not reduce the rate of FtsH degradation, although structural context may be important as these residues substantially affect degradation when appended to Arc repressor. These results suggest that FtsH recognizes an internal degron in CFA synthase. Consistent with this proposal, blocking either terminus of CFA synthase by fusion to the SUMO protein has little effect on degradation. Fusion of DHFR to the C-terminus but not the N-terminus of CFA synthase did prevent degradation, which would normally be taken as evidence for degradation that begins at the C-terminus and proceeds to the N-terminus. We cannot rigorously eliminate this possibility, but it seems unlikely in light of the SUMO-fusion result, the structure, and our biochemical and mutational results.

Residues 100–120 form a disordered linker between the N-terminal and C-terminal domains in the crystal structure of *E. coli* CFA synthase (Hari et al., 2018) and thus were a good candidate for an internal FtsH degron and/or a site at which degradation initiates. However, our mutational studies show that the sequence of residues 100–120 can be changed dramatically without affecting FtsH degradation. Moreover, no other internal CFA synthase sequences are disordered in the crystal structure. Thus, our results seem to rule out the obvious N-terminal, C-terminal, or disordered internal sequence candidates of CFA synthase as acting as degrons for FtsH degradation. How then does FtsH recognize and degrade CFA synthase? A model in which a segment of dimeric CFA synthase, probably within

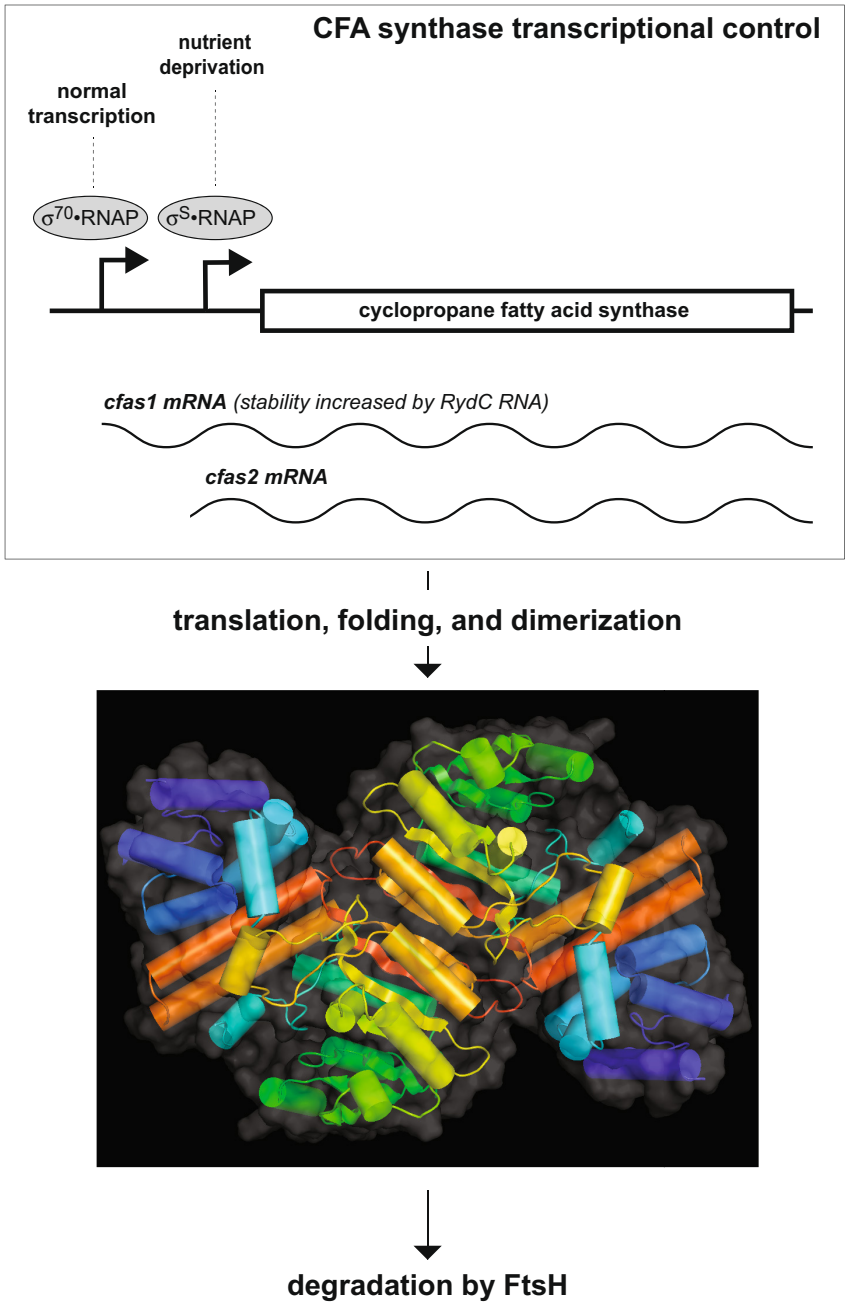

FIGURE 7 Control of intracellular CFA synthase levels. Transcription of CFA synthase can occur from a σ^{70}-dependent promotor or a σ^{S}-dependent promotor (Wang and Cronan, 1994). Degradation of the longer σ^{70}-dependent mRNA is also regulated by the small RydC RNA (Fröhlich et al., 2013). Following translation, folding, and dimerization, CFA can be degraded by the AAA+ FtsH protease, whose synthesis is controlled by the σ^{32} transcription factor.

the N domain, unfolds transiently to provide a disordered polypeptide that is recognized and engaged by the axial channel of FtsH is consistent with our experimental results. Such transient unfolding could be spontaneous or induced *in trans* by another protein. However, the identity of this segment remains to be determined. Another possibility, which is less likely in our opinion, is that CFA synthase contains redundant degrons (e.g., the 100–120 region or the C-terminal region or the N-terminal region) and thus that mutation of any one of these regions has little effect of FtsH degradation.

Our findings establish CFA synthase as a substrate of FtsH. In light of this discovery and prior work implicating AAA+ proteases in degradation of stress-induced proteins (Flynn et al., 2003; Lim et al., 2013; Neher et al., 2006; Neher, Flynn, et al., 2003), we believe that additional proteins that are upregulated during cellular responses to environmental stress are likely to be FtsH substrates.

4 | MATERIAL AND METHODS

4.1 | Bacterial strains

E. coli strain X90 (Δ*lac pro XIII*, *ara*, *nalA*, *argE*(*am*), *thi*$^{-}$, *rif*^{r}, [*F′lacI*^{q1}, *lacZY*$^{+}$, *proAB*$^{+}$]) was obtained from laboratory stocks. Deletion strains lacking *lon* or *hslU* were generated by P1 transduction and verified by Sanger sequencing. Strain AR3289 (W3110 *sfhC21 zad220::Tn10*) and AR3289 Δ*ftsH::kan* were kindly provided by Teru Ogura (Kumamoto University).

4.2 | Plasmids

The *cfa* gene from *E. coli* was amplified from genomic DNA and cloned downstream of an encoded His_6 tag and TEV site into a pET21-based plasmid. It was also cloned without any tags into pTrc99a. Further modifications were made as needed by site-directed mutagenesis. All sequences were verified by Sanger sequencing. The base Arc gene used for all studies was Arc-(Cys^{54})-st11. All other plasmids were obtained from laboratory stocks.

4.3 | Proteins

Purified Lon, HslU, HslV, ClpA, and Arc-sul20 were kindly provided by V. Baytshtok (MIT). ClpX, ClpP, FtsH, and Arc-ssrA were expressed and purified as described (Hari & Sauer, 2016). CFA synthase as well as mutants and fusion proteins were purified as described (Hari et al., 2018).

^{35}S-labeled CFA synthase was prepared as follows: *E. coli* T7 Express cells harboring a pET21-based plasmid with a gene encoding His_6-TEV-CFA synthase were grown in 100 ml of minimal media (without methionine or cysteine) to log-phase and induced with 0.5 mM IPTG and EXPRE$^{35}S^{35}S$ protein labeling mix (40 μCi/ml, PerkinElmer) for 3 h at 30°C. The harvested pellet was subjected to three cycles of freeze-thawing, then resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 10% glycerol) before incubating with lysozyme and PopCulture reagent (Novagen) at 4°C for 30 min. After centrifugation, the supernatant was added to 100 μl of Ni-NTA slurry, washed extensively with lysis buffer, and the protein was eluted with lysis buffer containing 300 mM imidazole. After incubation with TEV protease and dialyzing into lysis buffer overnight at 4°C, the solution was applied to 100 μl of Ni-NTA slurry. The flow-through was concentrated and snap-frozen for storage.

4.4 | Biochemical assays

Substrates and enzymes were incubated at the indicated concentrations in PD buffer (50 mM Tris–HCl, pH 8.0, 10 mM KCl, 5 mM $MgSO_4$, 10 μM $ZnCl_2$, 10% glycerol, 1 mM DTT, 0.1% Igepal CA-630) with ATP (4 mM) and a regeneration system (16 mM creatine phosphate, 75 μg/ml creatine kinase). Samples were quenched at indicated times with SDS loading buffer, separated by SDS-PAGE, and visualized by Coomassie staining. ATP-hydrolysis rates were measured using a continuous spectrophotometric assay (Norby, 1988).

4.5 | Enzyme kinetics

Unlabeled and ^{35}S-labeled CFA synthase were mixed to the indicated concentrations and incubated with FtsH (0.1 or 0.2 μM hexamer) in PD buffer with ATP (4 mM) in a regeneration system at 37°C. Samples were quenched with TCA (12.5%) and allowed to precipitate overnight at 4°C. After centrifugation, the soluble fraction was counted by scintillation.

For quantitative kinetic measurements of Arc degradation, Cys^{54} was labeled with Dylight-488 maleimide (Thermo Fisher), and degradation was monitored by de-quenching of fluorescence (excitation 495 nm; emission 515 nm).

4.6 | Pulse-chase analysis

E. coli strains harboring untagged CFA synthase in pTrc99a were grown to early log phase in Luria-Bertani media, washed with M9 salts base, then resuspended in minimal media (without methionine or cysteine) to O.D. = 0.3. The cells were grown for an additional 30 min at 37°C before they were induced with 50 μM IPTG for 5 min, pulsed with EXPRE$^{35}S^{35}S$ protein labeling mix (20 μCi/ml) for 5 min, then chased with 5 mM phenyl-β-D-galactopyranoside and 10 mM unlabeled amino acids. Samples were taken at different times and precipitated using TCA. The pellets were washed with acetone, resolubilized in SDS loading buffer, and separated by SDS-PAGE. A sample of purified ^{35}S-labeled CFA synthase was also loaded onto the gel as a molecular-weight standard. The gels were then dried, exposed, and imaged as described above. Uninduced samples were treated in the same way but were incubated without IPTG before pulsing. Half-lives were calculated by densitometry.

4.7 | Phospholipid analysis

Liquid cultures of *E. coli* strains AR3289 and AR3289 Δ*ftsH:kan* were grown overnight, and ~10^{11} cells were harvested and washed with Tris-buffered saline (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) before freezing.

Lipids were extracted as follows: each pellet was thawed and resuspended in residual buffer before transferring to a glass vial and extracting with 3 ml chloroform: methanol (2:1). If necessary, a small amount of water was added to separate emulsions. The organic layer was washed twice with 150 mM NaCl and concentrated in vacuo to yield a clear film. Phospholipids were selectively precipitated with acetone for at least 2 h at 4°C. Precipitates were dissolved in chloroform. Typical yield was ~5 mg.

Fatty acid methyl esters (FAMEs) were prepared as described (Ichihara & Fukubayashi, 2010). Briefly, 2.5 mg of dried phospholipid extract was dissolved in toluene (0.1 ml), followed by addition of methanol (0.75 ml) and hydrochloric acid (8% v/v in methanol). The solution was vortexed and incubated at 100°C for 1 h and then cooled to room temperature. FAMEs were extracted from the solution using hexane (0.5 ml) and water (0.5 ml). GC/MS was performed by the Harvard Small Molecule Mass Spectrometry Facility.

4.8 | Subunit exchange

An aliquot of purified CFA synthase was desalted into GF buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM βME, 10% glycerol) and labeled with six equivalents of Dylight-488 or Dylight-650 maleimide

(Thermo Fisher) for 30 min at room temperature. The reactions were quenched with 2 mM DTT, and excess dye was removed by desalting. Labeled proteins (2 μM each) were mixed at equal volumes and monitored by fluorescence (excitation 480 nm; emission 675 nm). The curve was fit to a growth equation (Weibull, 1951).

4.9 | Biolayer interferometry

Assays were performed using an Octet RED96 instrument (ForteBio/Molecular Devices) at 30°C using GF buffer with 0.05 mg/ml bovine serum albumin. Biosensor tips coated with Anti-Penta-HIS (His1K) antibody were loaded with proteins (400 nM) to a response of ~0.7 nm. The tips were then moved to solutions containing anhydrotrypsin (Molecular Innovations, 150 nM), and responses were monitored at a sampling rate of 5 Hz.

AUTHOR CONTRIBUTIONS

Sanjay B. Hari: Conceptualization; investigation; methodology; resources; validation; writing – original draft; writing – review and editing. **Juhee P. Morehouse:** Investigation; methodology; writing – original draft; writing – review and editing. **Tania A. Baker:** Supervision. **Robert T. Sauer:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be provided upon request.

ETHICS STATEMENT

Data and strains will be made available upon request.

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