Biochemistry

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Intein-Promoted Cyclization of Aspartic Acid Flanking the Intein Leads to Atypical N-Terminal Cleavage

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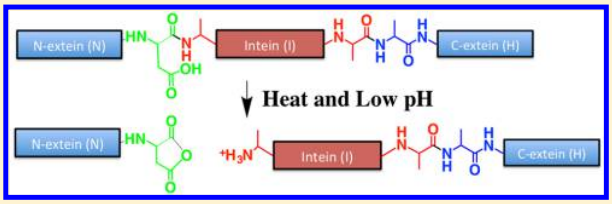
Supporting Information

ABSTRACT: Protein splicing is a post-translational reaction facilitated by an intein, or intervening protein, which involves the removal of the intein and the ligation of the flanking polypeptides, or exteins. A DNA polymerase II intein from *Pyrococcus abyssi* (*Pab* PolII intein) can promote protein splicing *in vitro* on incubation at high temperature. Mutation of active site residues Cys1, Gln185, and Cys+1 to Ala results in an inactive intein precursor, which cannot promote the steps of splicing, including cleavage of the peptide bond linking the N-extein and intein (N-terminal cleavage). Surprisingly, coupling the inactivating mutations to a change of the residue at the C-terminus of the N-extein (N-1 residue) from the native Asn to Asp reactivates N-terminal cleavage at pH 5. Similar "aspartic acid effects" have been observed in other proteins and peptides but usually only occur at lower pH values. In this case, however, the unusual N-terminal cleavage is abolished by mutations to catalytic active site residues and unfolding of the intein, indicating that this cleavage effect is mediated by the intein active site and the intein fold. We show via mass spectrometry that the reaction proceeds through cyclization of Asp resulting in anhydride formation coupled to peptide bond cleavage. Our results add to the richness of the understanding of the mechanism of protein splicing and provide insight into the stability of proteins at moderately low pH. The results also explain, and may help practitioners avoid, a side reaction that may complicate intein applications in biotechnology.



Protein splicing is a post-translational process by which an intervening protein, or intein, catalyzes its own excision from flanking polypeptides, or exteins, concomitant to the ligation of the exteins.[1,2]

The canonical mechanism of protein splicing is a four-step process[1,2] (Figure 1). First, the intein facilitates an amide-to-thioester rearrangement of the peptide bond between the C-terminal residue of the N-terminal extein (N-extein) and the first residue of the intein. (The intein in this study has N-terminal Cys, but other inteins can have Ser in this position.) Second, the first residue of the C-terminal extein (C-extein), usually Cys, Ser, or Thr, promotes a transesterification reaction by which the N-extein is transferred from the side chain of the first residue of the intein to that of the first residue of the C-extein. Third, the branched intermediate from step two is resolved by peptide bond cleavage between the intein and C-extein coupled to cyclization of the C-terminal residue, which is usually an Asn. Finally, the thioester linkage between the exteins is converted to an amide, and the C-terminal aminosuccinimide formed in step three may be resolved to asparagine or iso-asparagine.

The intein that interrupts the DNA polymerase II DP2 subunit in *Pyrococcus abyssi* (*Pab* PolII intein) can be overexpressed in frame with exogenous exteins in *Escherichia coli* and purified as an unspliced precursor protein. On *in vitro* incubation at high temperatures and in the presence of reducing agents, the intein is capable of promoting protein splicing.[3−5] On mutation of residues that promote the third step of splicing, the intein still can facilitate cleavage between the N-extein and intein, uncoupled to splicing (N-terminal cleavage). Intein-facilitated N-terminal cleavage usually occurs after steps one or two of splicing result in conversion of the amide bond linking the N-extein to the intein to a reactive ester or thioester. If the third step is prevented, this ester or thioester may be hydrolyzed to induce cleavage uncoupled from splicing.[1] Likewise, if steps one and/or two are interrupted, the intein can promote Asn cyclization uncoupled from splicing (C-terminal cleavage).[4,5]

We previously described how the *Pab* PolII intein can be used as a tool to facilitate protein purification.[6] In that work, we prevented the second and third steps of splicing by mutation of active site residues, and induced temperature-dependent N-terminal cleavage to elute maltose binding protein as an N-extein from an intein-His-tag fusion, while the His-tagged intein

Received: September 5, 2016
Revised: January 3, 2017
Published: February 6, 2017

DOI: 10.1021/acs.biochem.6b00894
Biochemistry 2017, 56, 1042−1050

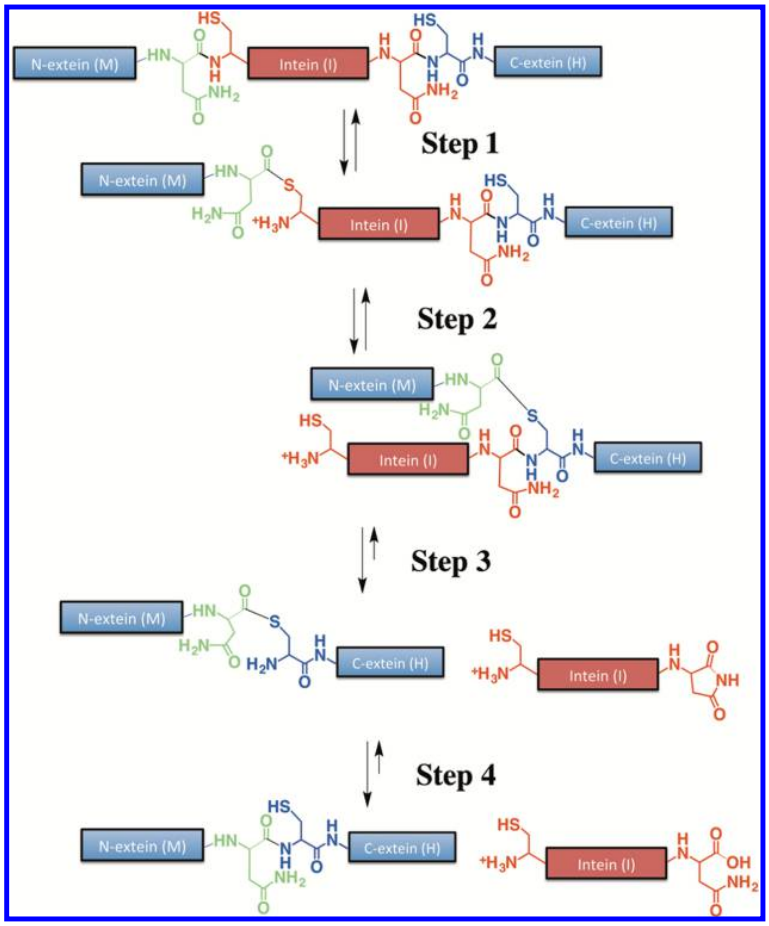


Figure 1. Mechanism of protein splicing. The chemical steps of the protein splicing mechanism that excise the intein, in red, from the N-terminal extein (N-extein) and C-terminal extein (C-extein), both in blue. The N-extein is *E. coli* MBP (M), and the C-extein is a poly-His tag (H). The C-terminal residue of the native N-extein is Asn, shown in green.

remained bound to a metal affinity resin. We tested the influence of all 20 residues as the C-terminal residue of the N-extein (i.e., the N-1 residue). Although inteins with each N-1 residue promoted N-terminal cleavage to greater or lesser extents, substitution of the native N-1 Asn to Asp produced products of unusual migration, suggesting a competing mechanism of cleavage.[6]

Peptide bond cleavage after Asp is often observed on incubation of proteins or peptides at low pH, including during sample treatment for mass spectrometry.[7−13] As it has been suggested that inteins may promote the cleavage of the N-terminal scissile bond by inducing catalytic strain at the scissile bond,[14−17] we hypothesized that Asp in the position immediately upstream of the potentially strained N-extein−intein peptide bond might promote a similar aspartic acid effect and therefore provide an alternative mechanism for intein-mediated N-terminal cleavage. This potential side reaction previously has been proposed to explain unexpected cleavage reactions in other inteins;[18] our results below provide direct evidence of this mechanism. This side reaction also may play a role in promoting undesired intein precursor cleavage in intein-based biotechnology applications.

MATERIALS AND METHODS

Plasmid Construction and Protein Expression. Plasmid pMIHPab encodes fusion protein MIHPab, with N-terminal *E. coli* maltose-binding protein fused to the seven C-terminal residues of the N-extein, the 185 residue *Pab* PolII intein, the six N-terminal residues of the C-extein, and a C-terminal poly-His tag. We previously described the preparation of the plasmid (then termed pPolWT) and relevant intein mutants.[5] Plasmid pNIHPab encodes fusion protein NIHPab, with an N-terminal sequence of MKLKRRN fused to the 185 residue *Pab* PolII intein, the six N-terminal residues of the C-extein, and a poly His tag. Its preparation (as pPabC1AWT, with a Cys1Ala mutation) was previously described.[4] Further mutations were produced by site-directed mutagenesis using appropriate oligonucleotide pairs listed in Table S1 in the Supporting Information.

We used *E. coli* BL21(DE3) to express recombinant protein by induction with 1 mM isopropyl-β-D-1-thiogalactoside at mid log phase and incubation with shaking for 16 h at 20 °C. We resuspended frozen cell pellets from 50 mL of culture in buffer A (20 mM HEPES (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid), pH 7.5, 500 mM NaCl) supplemented with BugBuster extraction reagent (Novagen-Merck Biosciences), 12 units/mL benzonase nuclease, 100 μM phenylmethylsulfonyl fluoride, and 10 μL of Protease Inhibitor Cocktail P8849

DOI: 10.1021/acs.biochem.6b00894
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(Sigma-Aldrich). We purified the soluble extract using Talon metal affinity resin (Clontech) prewashed with buffer A with a settled bed volume of about 300 μL. We washed the loaded resin with 10 mL of buffer A supplemented with 10 mM imidazole and 0.1% Tween-20 and then with 2 × 10 mL of buffer A supplemented with 10 mM imidazole. We eluted the protein from the resin with 3 × 500 μL of buffer A supplemented with 200 mM imidazole and determined the protein concentration via the Bradford method.[19] We exchanged purified proteins against the appropriate buffers noted below with a Millipore Ultracel-0.5 centrifugal filter with a 3000 molecular weight cut off.

Protein Analysis by SDS-PAGE, Electrospray Ionization-Fourier Transform-Resonance Mass Spectrometry (ESI-FT-MS), and Edman Degradation. To study intein-mediated protein cleavage, we exchanged precursor fusion protein into buffer C5, C6, or C7 (50 mM citric acid, 500 mM NaCl, with pH 5.0, 6.0, or 7.0, respectively). In some cases, we added 0.1% Tween-20 to make buffers C5T, C6T, or C7T. To induce cleavage, we made mixtures with approximately 2.8 μM or 4.6 μM protein in the appropriate buffer, supplemented with 5 mM EDTA, and, in some instances, guanidinium chloride, DTT, hydroxylamine, or aniline to the final concentrations indicated in the figure legends. To test activity under denaturing conditions, the protein sample was exchanged into buffer C5 buffer containing 6 M guanidinium chloride. Subsequent to treatment, the guanidinium chloride was removed using a SDS-PAGE Sample Prep kit (Thermo Fisher).

For analysis by SDS-PAGE, we used precast 4−20% gradient Tris-glycine gels (Lonza) by the Laemmli method.[20] We supplemented the sample buffer with 100 mM DTT and used BenchMark protein ladder (Thermo Fisher). In some cases, we stained gels first with InVision His-tag in-gel stain (Life Technologies) and visualized with an 8 s exposure with a BioRad Gel Doc EZ Imager with UV tray, and then stained the gels with Coomassie Blue (NuSep). Otherwise, gels were stained directly with Coomassie Blue.

For analysis by electrospray ionization-resonance mass spectrometry (ESI-MS), we prepared the samples as above, but Tween-20 was excluded from the wash buffer. We used a C18 column (BioBasic-18, 150 mm × 2.1 mm, 5 μm, Thermo Fisher Scientific) to separate the samples. Mobile phase A was 0.2% formic acid in water (HPLC grade, Sigma-Aldrich), and the mobile phase B was 0.2% formic acid in acetonitrile (HPLC grade, Sigma-Aldrich). After injection of 4 μL of 12 μM sample using an Agilent 1200 autosampler, we used an HPLC binary pump to deliver the gradient at a flow rate of 250 μL/min. The gradient was set as 0−5 min, 3% B; 5−8 min, 3−40% B; 8−13 min, 40−45% B; 13−20 min 45−90% B. The LC column was directly connected online to the standard ESI source of an LTQ-Orbitrap XL FT MS (Thermo Fisher Scientific). The parameters for FT-MS detection included a spray voltage of 4.5 kV, a capillary voltage of 44 V, a capillary temperature of 275 °C, a tube lens of 150 V, a sheath flow rate of 45, and an auxiliary gas flow rate of 20. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. We acquired all FT mass spectra at a resolution of 60 000 with 200−1800 m/z mass range. For components with large molecular weight, we performed the deconvolution using MagTran to obtain the average molecular weight.[21]

For N-terminal sequencing via Edman degradation, we blotted onto polyvinylidene difluoride, stained with 0.1% Ponceau S in 1% acetic acid, destained with 50% methanol/10% acetic acid, and washed with water. We excised the bands and sent them for analysis to the Tufts University Core Facility.

For analysis by NMR, the *P. abyssi* PolII intein sample was purified as previously described.[22,23] The NMR sample contains approximately 5 mM protein, 20 mM sodium phosphate, 0.5 mM ethylenediaminetetraacetic acid, and 0.05 mM sodium azide in 90% H_2O/10% D_2O. All spectra were acquired on a Bruker Advance II 800 MHz or Bruker Advance II 600 MHz (^1H) spectrometer, each equipped with a cryogenic probe. Spectra were processed with nmrPipe software[24] and analyzed using Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco, USA).

RESULTS AND DISCUSSION

N-Terminal Cleavage of Linear Thioester. In order to study N-terminal cleavage separately from protein splicing, we used fusion protein MIHPab with mutations of the C-terminal residue Gln185 and residue Cys+1 to Ala (QACA, Figure 2).

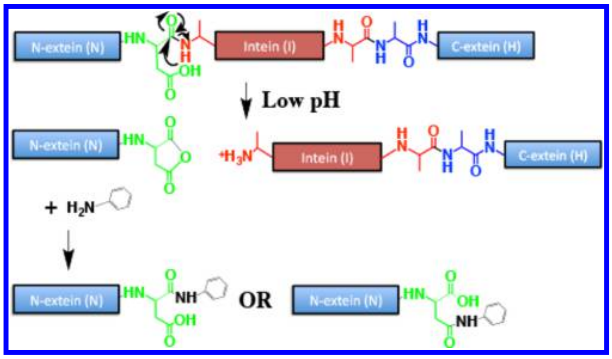

Figure 2. N-terminal cleavage in the absence of active site residues. Conversion to Ala of the N-terminal Cys of the intein, the C-terminal Gln of the intein, and the N-terminal Cys of the C-extein blocks steps one, three, and two of protein splicing, respectively. Conversion of the native C-terminal Asn of the N-extein to Asp (in green) promotes bond cleavage between the N-extein and intein by anhydride formation. Reaction of the anhydride intermediate with aniline results in a C-terminal anilide. (The intein residues are numbered 1 to 185. The C-extein residues start at +1 and proceed downstream. The N-extein numbering scheme begins are residue −1 and proceeds upstream. In this case the N-extein is labeled as "N," as in the experiment in Figures 6 and 7.)

The Cys+1Ala mutation prevents step two of the mechanism, and the Gln185Ala mutation prevents step three. Therefore, this double mutant should allow study of step one of the mechanism in isolation. We have previously shown that at saturating concentrations (greater than 100 mM) of 1,4-dithiothreitol (DTT), the rate of thiolysis of the nascent thioester can approximate the rate of the first step of splicing, and that this N-terminal cleavage is most favorable at pH 7.5, with significant decreases in the extent of cleavage at pH 6 or pH 8.[4]

Overexpression of the double mutant results in isolation of unreacted fusion protein MIH (Figure 3, lane 1 top panel). Incubation at 50 °C for 16 h in the presence of 150 mM DTT induces N-terminal cleavage, converting the precursor MIH (66.5 kDa) to M (43.7 kDa) and IH (22.9 kDa) (Figure 3, lanes QACA). Incubation without DTT also induces cleavage, likely by hydrolysis, although less efficiently. The same effect is seen with incubation at pH 5 (Figure 3, bottom panel).

DOI: 10.1021/acs.biochem.6b00894
Biochemistry 2017, 56, 1042−1050

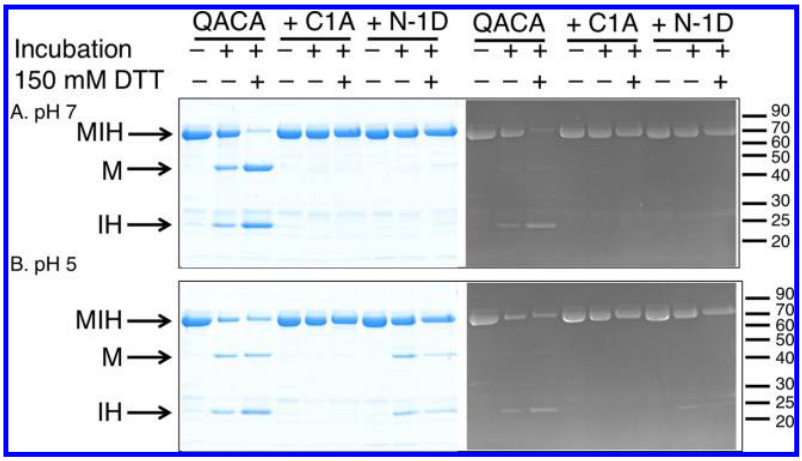

Figure 3. Influence of pH and DTT on N-terminal cleavage. Induced N-terminal cleavage at (A) pH 7.0 and (B) pH 5.0. The fusion protein MIHPab was used, with mutations (Q185A/C+1A = QACA), (C1A/Q185A/C+1A = +C1A), or (N-1D/C1A/Q185A/C+1A = +N-1D). We incubated 4.6 μM proteins for 16 h at 50 °C in buffer C7 (panel A) or buffer C5 (panel B) in lanes indicated by (+) and as described in the Materials and Methods; proteins in lanes indicated by (−) were exchanged into the appropriate buffer but not incubated. Conversion of MIH (66.5 kDa) to M (43.7 kDa) and IH (22.9 kDa) indicates N-terminal cleavage. SDS-PAGE stained by Coomassie Blue on the left and with His-tag specific InVision stain on the right, with InVision reacting with bands corresponding to MIH and IH, but not to M.

N-Terminal Cleavage of Peptide Bond Linking N-Extein and Intein at Low pH. A third mutation of Cys1 to Ala should prevent N-terminal cleavage, as it prevents conversion of the N-terminal peptide bond to a thioester. As expected, incubation of the triple mutant (C1A-QACA) at 50 °C, either with or without DTT, and pH 5 or pH 7, does not promote N-terminal cleavage (Figure 3, lanes + C1A).

However, substitution of the Asn at position N-1 for Asp, concomitant with the inactivating mutations C1A-QACA, results in an intein that can promote cleavage of MIH to M and IH. (For clarity, we will refer to this mutation as the cleavage mutant below.) This cleavage occurs preferentially at pH 5 and is not dependent on DTT (Figure 3, bottom panel, lanes + N-1D). We verified the identity of the cleavage bands in Figure 3, bottom panel, for the cleavage mutant by three separate methods. First, we stained the SDS-PAGE with InVision protein reagent, which selectively reacts with poly-His tags. The stain labeled bands MIH and IH, but not M. Second, we analyzed a sample of a 50 °C reaction of the cleavage mutant by intact protein LC-ESI mass spectrometry. The molecular weights of deconvoluted peaks are within less than 0.03% for the products of cleavage and within 0.2% for the small concentration of remaining precursor protein:[21] MIH (expected M_r 66 550, observed m/z 66 430), M (expected M_r 43 708, observed m/z 43 691), and IH (expected M_r 22 860, observed m/z 22 861) (Figure S1, A–C, respectively). Third, we analyzed the N-terminal sequence of the bands assigned to "M" and "IH" by Edman degradation and obtained spectra consistent with the predicted N-terminal sequences of M-K-I-E-E for band M and A-F-P-G-D-T for band IH.

It is well-known that cleavage after Asp can occur at very low pH in both peptides and proteins.[8−11,25−38] Given that it is apparent that our intein fusion protein can cleave in its active site downstream from a non-native Asp, we were interested in addressing two questions:

1. Although conditions of very low pH can facilitate cleavage after Asp in peptides, is this particular cleavage dependent on the catalytic action of a folded intein?
2. Although other inteins have shown unusual cleavage activity when flanked by Asp at their N-terminus, is the cleavage in this case clearly attributed to Asp cyclization and anhydride formation?

Question 1: Is the pH-Dependent Cleavage Due to the Action of the Intein? *Cleavage of the N-Terminal Splice Junction Depends on N-1 Asp, and There Is No Cleavage at the Other Asp Residues in the Fusion Protein.* The atypical N-terminal cleavage of the N-extein−intein bond is promoted by incubation at low pH. For MIHPab C1A-QACA, we observed pH dependent N-terminal cleavage with N-1 Asp (Figure 4B) but not with the native N-1 Asn (Figure 4A). There are 23 other Asp residues in the N-extein (two followed by Ala and two by Gly), 14 Asp residues in the intein (two followed by Gly), and three in the C-extein (one followed by Gly). This suggests that the cleavage of this particular Asp−Ala bond is promoted by the intein and thus that the active site remains organized at pH 5. We also have shown by NMR that the dispersion is comparable in $^{15}N-^{1}H$ HSQC spectra across a broad pH range, suggesting that the active site is likely not disrupted and that there is not a major structural rearrangement as a function of temperature (Figure S2). The alternative cleavage is specific to N-1 Asp, as it is not observed with the native Asn nor with substitution to Glu or Gln (Figure 4C).

Alternative Cleavage Requires Conserved Catalytic Residues in Intein Block B. For native inteins that facilitate off-pathway N-terminal cleavage, the cleavage is usually due to the formation of a reactive ester or thioester at the N-terminal splice junction that is not resolved by the subsequent splicing steps. We previously have shown that N-terminal cleavage of the *P. abyssi* PolII intein is temperature dependent.[4,5] Although the hydrolysis or thiolysis reaction may itself be temperature dependent, it is likely that the temperature dependence of the structural dynamics of the intein also plays a role in promoting the alternative N-terminal cleavage. Residues T90 and H93 are highly conserved residues in intein block B.[39] Their mutation to Ala influences typical intein activity of the *P. abyssi* PolII intein, and both residues are in close proximity to the N-terminal Cys.[22] It has been proposed for other inteins that these residues

DOI: 10.1021/acs.biochem.6b00894
Biochemistry 2017, 56, 1042−1050

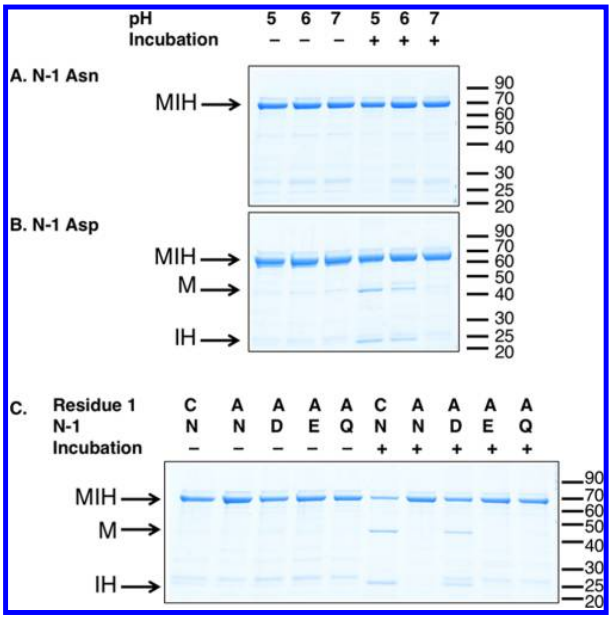

Figure 4. Influence of pH and the N-1 residue on N-terminal cleavage. Induced N-terminal cleavage of (A) MIHPab (C1A/Q185A/C+1A) and (B) MIHPab (C1A/Q185A/C+1A *and* N-1D). We incubated the 2.8 μM protein for 16 h at 50 °C in buffer C5T, C6T, or C7T in lanes indicated by (+). (C) Induced N-terminal cleavage of fusion protein MIHPab QACA; mutations as position 1 and N-1 indicated above lanes. We incubated 2.8 μM proteins for 16 h at 50 °C in buffer C5T in lanes indicated by (+). For all panels, proteins in lanes indicated by (−) were exchanged into the appropriate buffer but not incubated. Conversion of MIH (66.5 kDa) to M (43.7 kDa) and IH (22.9 kDa) indicates N-terminal cleavage. SDS-PAGE stained by Coomassie Blue.

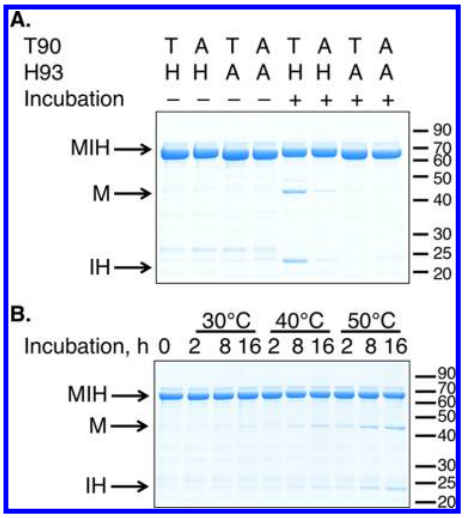

Figure 5. Influence of block B residues, temperature, and time on N-terminal cleavage. (A) Induced N-terminal cleavage of fusion protein MIHPab. Each fusion protein has the mutations N-1D and C1A-QACA; mutations of conserved residues T90 and H93 indicated above lanes. We incubated 2.8 μM proteins for 16 h at 50 °C in buffer C5T in lanes indicated by (+) and as described in the Materials and Methods; proteins in lanes indicated by (−) were exchanged into the appropriate buffer but not incubated. (B) Induced N-terminal cleavage of fusion protein MIHPab N-1D, C1A-QACA. We incubated 2.8 μM proteins in buffer C5T at the times and temperatures indicated as described in the Materials and Methods. For both panels, analysis by Coomassie Blue-stained SDS-PAGE shows conversion of MIH (66.5 kDa) to M (43.7 kDa) and IH (22.9 kDa), indicating N-terminal cleavage.

may influence the protonation state of the N-terminal nucleophile or the protonation of the leaving group in the amide-ester rearrangement.[40,41] Alternatively, it has been suggested that the block B His can promote bond strain in the N-extein−intein peptide bond, shifting the unfavorable equilibrium of the amide-ester rearrangement more toward ester formation.[16] It is likely that the relative magnitude of these effects varies from intein to intein. Mutations of the block B Thr to Ala decreases the efficiency of the alternative N-terminal cleavage from 27% to 5%, and mutation of the block B His to Ala, either alone or in combination with the Thr-Ala mutation, reduces the cleavage percentage to about 1% (Figure 5A, percentage calculated using ImageJ[42]). This indicates that the intein active site must be properly structured to promote cleavage of the peptide bond, likely by inducing catalytic strain.

The bond cleavage is also temperature- and time-dependent (Figure 5B). The traditional N-terminal cleavage mediated by thioester formation is known to be temperature and time dependent as well.[43] In that case, it is likely that the thermophilic intein, which we have shown by NMR analysis to be highly conformationally rigid at room temperature,[22] requires higher temperatures to have sufficient flexibility in the active site to promote the amide-to-thioester rearrangement.

Unfolding of the Intein by Guanidinium Chloride Prevents N-Terminal Cleavage at Low pH. Finally, we show that this cleavage depends on the intein by unfolding the intein with 6 M guanidinium chloride. On incubation at 60 °C and pH 5 for 16 h, the MIHPab cleavage mutant facilitates cleavage of MIH into M and IH in the absence of denaturing agent, but does not in the presence of guanidinium chloride (Figure 6A). The same is true in the presence of the nucleophiles aniline or hydroxylamine hydrochloride, discussed further below. For experiments to detect the anhydride that we describe below, we replaced the N-extein M with a short polypeptide to create NIHPab. In this context, guanidinium chloride also prevents the cleavage reaction (Figure 6B).

Question 2: Does the Intein-Facilitated Cleavage Reaction Proceed through Asp Cyclization? *Other Inteins Cleave after Asp, but It Is Unclear whether Cleavage Proceeds via Asp Cyclization.* Other inteins promote N-terminal cleavage adjacent to Asp. For instance, protein splicing of both the *Mycobacterium xenopi* GyrA intein and the *Saccharomyces cerevisiae* VMA intein is diverted to N-terminal cleavage when their native N-1 Ala is mutated to Asp. Unlike the atypical cleavage reported here, both inteins have Cys at position 1, such that this cleavage may still be mediated by the cleavage of a reactive thioester.[44,45] For the fused *Synechocystis* sp. PCC6803 DnaE intein, enhanced N-terminal cleavage instead of splicing was observed with substitution of N-1 Tyr to Asp. Cleavage required Cys1 but not Cys+1.[18] In the last study, the authors suggested the possibility of a mechanism by which cyclization of N-1 Asp drives cleavage of the linear thioester formed in step one, and note that inteins with a native N-1 Asp must have evolved a means to suppress this side reaction.

In addition, although the third step of splicing usually is facilitated by Asn, and in some cases Gln, an intein from *Carboxydothermus hydrogenoformans* can promote protein splicing with C-terminal Asp, possibly by Asp cyclization coupled to bond cleavage between the intein and C-extein.[46]

DOI: 10.1021/acs.biochem.6b00894
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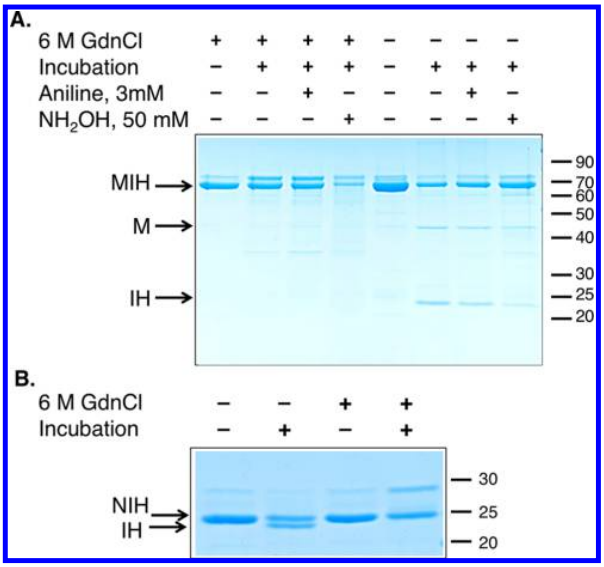

Figure 6. Influence of unfolding of the intein on N-terminal cleavage. Induced N-terminal cleavage of fusion protein cleavage mutants of MIHPab (A) and NIHPab (B). We incubated 2.8 μM proteins for 16 h at 60 °C in buffer C5T, with the concentrations of guanidinium chloride, aniline, or hydroxylamine noted, in lanes indicated by (+) and as described in the Materials and Methods; proteins in lanes indicated by (−) were exchanged into the appropriate buffer but not incubated. Samples were exchanged out of guanidinium chloride before SDS-PAGE as described in Materials and Methods.

However, a C-terminal anhydride was not detected, and the intein can promote C-terminal cleavage with substitution of Asp to Asn, Gln, or Glu, so the mechanism of C-terminal cleavage is not only due to Asp cyclization.

Direct and Indirect Evidence of an Anhydride Intermediate. We hypothesized that the alternative cleavage of the *Pab* PolII intein was through anhydride formation coupled to peptide bond cleavage. If this is the case, we should be able to detect the anhydride intermediate directly by mass spectroscopy. Alternatively, we should be able to react the anhydride with either aniline or hydroxylamine and detect the resulting anilide or hydroxamate by mass spectroscopy (Figure 2). If the intein cleavage is promoted at the same first order rate constant with or without the added nucleophile, this would suggest that the anilide or hydroxamate formation is due to attack of the weak nucleophiles on the electrophilic anhydride rather than directly on the strained peptide bond. To facilitate detection of the peptides by mass spectrometry, we used NIHPab in place of MIHPab, given the reduced size of the N-extein. (We used MIHPab in previous experiments so that both products of cleavage, M and IH, are sufficiently large to visualize by SDS-PAGE.)

As seen in Figure 7A, treatment at pH 5 with aniline or hydroxylamine did not induce cleavage of NIHPab C1A-QACA, suggesting that the amide bond linking the N-extein and intein is not sufficiently electrophilic or strained to be cleaved by these weak nucleophiles directly. However, cleavage of the NIHPab Asp-1 cleavage mutant is promoted equally in either the presence or absence of aniline or hydroxylamine. This suggests that the peptide bond cleavage is promoted by cyclization of Asp coupled to peptide bond cleavage, resulting in a C-terminal succinic anhydride, which is sufficiently electrophilic to react with the weak nitrogen nucleophiles.[7]

Further, we were able to detect expected cleavage products using ESI-MS. (See Figure S3A for separation in the total ion chromatography.) The NIHPab cleavage mutant precursor has a predicted average molecular weight of 23787.7. The C-terminal segment has a predicted average molecular weight of 22859.6. ESI-MS analysis of the treated samples reveals peaks of the appropriate mass. For samples treated without added nucleophiles, we observe peaks with molecular weight of 23 787 (Figure S3B) and 22 858 (Figure S3C); with aniline we observe peaks with molecular weight of 23 785 (Figure S3D) and 22 859 (Figure S3E); and with hydroxylamine we observe peaks with molecular weight of 23 789 (Figure S3F) and 22 859 (Figure S3G).

We expect the liberated N-extein to have an exact mass (monoisotopic) of 945.5542 with C-terminal Asp. With incubation at pH 5.0 without a nitrogen nucleophile, we observe ESI-FT-MS peaks with m/z values of 473.7847 (z = 2)

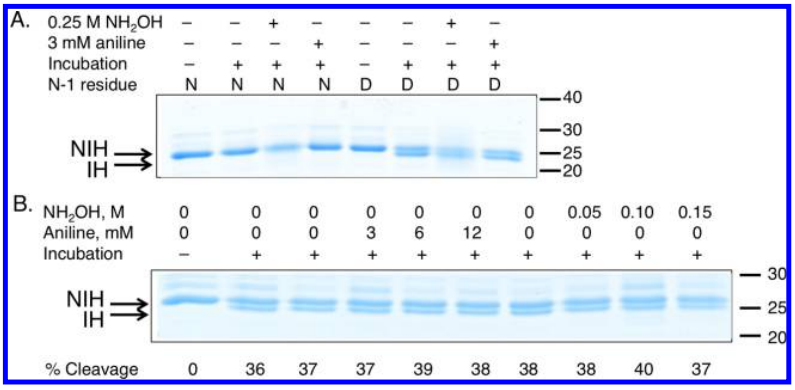

Figure 7. Induced N-terminal cleavage of fusion protein NIHPab as a function of the N-1 residue (A) or as a function of nucleophile concentration (B). Each fusion protein has the mutations C1A-QACA. In A, residue N-1 is either native Asn (N) or the reactive Asp (D); in B, residue N-1 is Asp in all lanes. We incubated 2.8 μM proteins for 16 h at 60 °C in buffer C5 in lanes indicated by (+) and as described in the Materials and Methods; proteins in lanes indicated by (−) were exchanged into the appropriate buffer but not incubated. Hydroxylamine or aniline, adjusted to pH 5, was added to the lanes at the concentrations indicated. Analysis by Coomassie Blue-stained SDS−PAGE shows conversion of NIH (23.8 kDa) to IH (22.9 kDa), indicating N-terminal cleavage. We analyzed the cleavage via SDS−PAGE and estimated the relative concentration via densitometry using ImageJ.[42]

DOI: 10.1021/acs.biochem.6b00894
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and 464.7788 (z = 2) (Figure S4A, Table S2 for calculated molecular weights). The smaller m/z value is suggestive of an anhydride. The presence of an anhydride intermediate is supported by the results obtained when running the reaction in the presence of nitrogen nucleophiles. On incubation with aniline, we expect the addition of a peak at an exact mass of 1020.6015 for the anilide derivative, but only with prior anhydride formation. We observed peaks with m/z values of 473.7845 (z = 2) (the hydrolyzed anhydride), 511.3089 (z = 2) (the anilide), and 464.7795 (z = 2) (anhydride) (Figure S4B, Table S2). On incubation with hydroxylamine, we expect the addition of a peak with an exact mass of 960.5651 for the hydroxamate. We observe peaks with m/z values of 473.7843 (z = 2) (the hydrolyzed anhydride) and 481.2896 (z = 2) (the hydroxamate) (Figure S4C, Table S2). For both the anilide and the hydroxamate, the amine nucleophile could theoretically react with either carbonyl of the anhydride yielding different products that would have the same mass; such distinction would not change our conclusion that an anhydride intermediate was involved in at least a substantial fraction of the observed cleavage.

We wished to further verify that the anilide or hydroxamate products are created by anhydride formation followed by aminolysis, rather than by attack of the weak nitrogen nucleophiles on the strained peptide bond, resulting in direct bond cleavage. To test this, we measured the time dependence of cleavage at pH 5.0 in the absence of added nucleophiles and in the presence of an excess of aniline (3 mM) or hydroxylamine (0.25 M) (Figure S5). Neither nucleophile greatly increases the first-order rate constant of cleavage, with first-order rate constants of 4.6×10^{-2} h^{-1} in the standard buffer, 5.3×10^{-2} h^{-1} with the addition of aniline, and 4.7×10^{-2} h^{-1} with the addition of hydroxylamine. In addition, we showed that the extent of cleavage is not dependent on the concentration of aniline or hydroxylamine (Figure 7B.) These experiments strongly suggest that the rate-limiting step is the slow first order anhydride formation.

Significance of the Cleavage Reaction. This cleavage mechanism is not unprecedented, as it has long been known that conditions of extremely low pH can result in cleavage of proteins specifically after Asp residues, or after acid-catalyzed deamidation of Asn residues.[8,25,33,38] Previous work provides some evidence for the catalysis of cleavage adjacent to Asp. Cleavage of glucagon after three separate Asp residues has been reported,[9,10] and a combination of pK_a determinations by NMR, kinetic solvent isotope studies, and pH-rate profiles supports a mechanism of nucleophilic attack by a deprotonated side chain on a protonated backbone carbonyl, with peptide bond cleavage coupled to succinic anhydride formation.[30] Computational and experimental studies on model peptides suggest that a locally mobile proton may be transferred from the side chain of a protonated Asp to either the backbone carbonyl oxygen, or, more likely, to the backbone amide nitrogen of the scissile bond, concomitant to anhydride formation and peptide bond cleavage.[29,37] A different model peptide study suggests a similar mechanism, with proton transfer from the side chain Asp either directly to the backbone carbonyl oxygen concomitant with cyclization to form the tetrahedral intermediate, or, more favorably, by involvement of a water molecule in proton transfer. In this model, the proton is transferred by water molecule from the oxygen to the amino leaving group during collapse of the tetrahedral intermediate.[26] Intein C-terminal cleavage via Asn cyclization, including by the *P. abyssi* PolII intein, also is usually promoted at low pH.[4] This is the case for the *Mycobacterium tuberculosis* RecA mini-intein,[47,48] and subsequent quantum mechanical calculations suggest that this pH-dependent cleavage may be driven by *a priori* protonation of the backbone amide nitrogen of the scissile bond.[49]

For the cleavage we observe here, the propensity for cleavage of the Asp−Ala bond at the intein N-terminus indicates that it is catalyzed by the intein, particularly since peptide bond cleavage after Asp, in most cases, occurs at pH values below the pK_a of Asp, or between particularly labile Asp−Pro bonds.[13,31,32,34−36] It is possible that the typical first step of splicing is promoted by shifting the equilibrium of the amide-ester rearrangement by catalytic bond strain of the amide peptide bond, by improving the electrophilicity of the carbonyl of the peptide bond by protonating the carbonyl oxygen, or by facilitating protonation of the amino leaving group either prior or concomitant to amide bond scission. Each catalytic strategy also would increase the susceptibility of the scissile N-terminal splicing junction to cleavage via an aspartic acid effect. Belfort and co-workers have suggested that inteins with native N-1 Asp must have evolved means to compensate for this potential side reaction.[18] It also has been observed, albeit for attack of the backbone amide nitrogen on the side-chain carbonyls of Asp or Asn, that most Asp or Asn side chains are not found in the proper conformation to allow nucleophilic attack, suggesting that unusual or flexible conformations might promote side chain cyclization.[50] This may account for the increase in cleavage efficiency when N-extein "M" is replaced with the smaller N-extein "N" in our experiments; the shorter extein presumably has more flexibility to adopt the appropriate conformation to facilitate cleavage.

Inteins have found wide use in biotechnology applications, including peptide bond cleavage facilitating affinity tag-traceless protein purification.[51,52] This alternative cleavage reaction, conditional upon treatment in mild acidic conditions, might prove useful in such applications as it allows for specific peptide bond cleavage without the use of a protease. However, the cleavage reaction is not as efficient as ester or thioester-based cleavage, and formation of a C-terminal succinic anhydride likely resolves in a mixture of Asp and iso-Asp (Figure 2). In addition, it has been shown that succinimide formation can result in racemization of the alpha-carbon;[50] it is likely that succinic anhydride formation would increase the acidity of the proton on the alpha-carbon to a greater extent. However, understanding this alternative cleavage mechanism can help scientists avoid it when designing intein-based biotechnology tools. Our results also likely explain unusual N-terminal cleavage found after Asp in other inteins,[18,44,45] and add to the understanding of the stability of peptide bonds at moderately acidic pH.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00894.

Figure S1. LC-ESI-MS of precursor and cleavage fragments from N-terminal cleavage of MIHPab. Figure S2. pH and temperature dependence of *Pab* PolII intein probed by NMR. Figure S3. LC-ESI-MS of N-terminal fragment from N-terminal cleavage of NIHPab. Figure

DOI: 10.1021/acs.biochem.6b00894
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S4. LC-ESI-MS of C-terminal fragment from N-terminal cleavage of NIHPab. Figure S5. Time dependence of N-terminal cleavage of NIHPab. Table S1. Oligonucleotides used in mutagenesis reactions. Table S2. Observed m/z values for the C-terminal cleavage products of theNIHPab cleavage mutant (PDF)

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Funding

This work was supported by the National Science Foundation (Grants MCB-1244089 and MCB-1517138 to K.V.M.), a Henry Dreyfus Teacher-Scholar Award (K.V.M.), and the Arnold and Mabel Beckman Foundation (K.M.C.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Brian Callahan for useful conversations and critical reading of the manuscript.

ABBREVIATIONS

DTT, 1,4-dithiothreitol; *Pab*, *Pyrococcus abyssi*; M, the N-extein containing an N-terminal *E. coli* maltose binding protein; N, the N-extein with the sequence MKLKRRN; I, the *Pab* PolII intein; H, the C-extein containing a C-terminal His-tag; MIHPab, the fusion protein of M, I and H; NIHPab, the fusion protein of N, I and H; QACA, mutation of Gln185 and Cys+1 to Ala; C1A-QACA, mutation of Cys1, Gln185 and Cys+1 to Ala

REFERENCES

(1) Mills, K. V., Johnson, M. A., and Perler, F. B. (2014) Protein splicing: how inteins escape from precursor proteins. *J. Biol. Chem. 289*, 14498−14505.

(2) Paulus, H. (2000) Protein splicing and related forms of protein autoprocessing. *Annu. Rev. Biochem. 69*, 447−496.

(3) Chen, W., Li, L., Du, Z., Liu, J., Reitter, J. N., Mills, K. V., Linhardt, R. J., and Wang, C. (2012) Intramolecular disulfide bond between catalytic cysteines in an intein precursor. *J. Am. Chem. Soc. 134*, 2500−2503.

(4) Mills, K. V., Dorval, D. M., and Lewandowski, K. T. (2005) Kinetic analysis of the individual steps of protein splicing for the Pyrococcus abyssi PolII intein. *J. Biol. Chem. 280*, 2714−2720.

(5) Mills, K. V., Manning, J. S., Garcia, A. M., and Wuerdeman, L. A. (2004) Protein splicing of a Pyrococcus abyssi intein with a C-terminal glutamine. *J. Biol. Chem. 279*, 20685−20691.

(6) Mills, K. V., Connor, K. R., Dorval, D. M., and Lewandowski, K. T. (2006) Protein purification via temperature-dependent, intein-mediated cleavage from an immobilized metal affinity resin. *Anal. Biochem. 356*, 86−93.

(7) Darrington, R. T., and Anderson, B. D. (1994) The role of intramolecular nucleophilic catalysis and the effects of self-association on the deamidation of human insulin at low pH. *Pharm. Res. 11*, 784−793.

(8) Inglis, A. S. (1983) Cleavage at aspartic acid. *Methods Enzymol. 91*, 324−332.

(9) Joshi, A. B., and Kirsch, L. E. (2004) The estimation of glutaminyl deamidation and aspartyl cleavage rates in glucagon. *Int. J. Pharm. 273*, 213−219.

(10) Joshi, A. B., Rus, E., and Kirsch, L. E. (2000) The degradation pathways of glucagon in acidic solutions. *Int. J. Pharm. 203*, 115−125.

(11) Lamed, R., Kenig, R., Morag, E., Yaron, S., Shoham, Y., and Bayer, E. A. (2001) Nonproteolytic cleavage of aspartyl proline bonds in the cellulosomal scaffoldin subunit from Clostridium thermocellum. *Appl. Biochem. Biotechnol. 90*, 67−73.

(12) Lyons, B., Kwan, A. H., and Truscott, R. (2014) Spontaneous cyclization of polypeptides with a penultimate Asp, Asn or isoAsp at the N-terminus and implications for cleavage by aminopeptidase. *FEBS J. 281*, 2945−2955.

(13) Segalas, I., Thai, R., and Menez Claudio Vita, R. (1995) A particularly labile Asp-Pro bond in the green mamba muscarinic toxin MTX2. Effect of protein conformation on the rate of cleavage. *FEBS Lett. 371*, 171−175.

(14) Callahan, B. P., Topilina, N. I., Stanger, M. J., Van Roey, P., and Belfort, M. (2011) Structure of catalytically competent intein caught in a redox trap with functional and evolutionary implications. *Nat. Struct. Mol. Biol. 18*, 630−633.

(15) Dearden, A. K., Callahan, B., Roey, P. V., Li, Z., Kumar, U., Belfort, M., and Nayak, S. K. (2013) A conserved threonine spring-loads precursor for intein splicing. *Protein Sci. 22*, 557−563.

(16) Romanelli, A., Shekhtman, A., Cowburn, D., and Muir, T. W. (2004) Semisynthesis of a segmental isotopically labeled protein splicing precursor: NMR evidence for an unusual peptide bond at the N-extein-intein junction. *Proc. Natl. Acad. Sci. U. S. A. 101*, 6397−6402.

(17) Schwarzer, D., Ludwig, C., Thiel, I. V., and Mootz, H. D. (2012) Probing intein-catalyzed thioester formation by unnatural amino acid substitutions in the active site. *Biochemistry 51*, 233−242.

(18) Amitai, G., Callahan, B. P., Stanger, M. J., Belfort, G., and Belfort, M. (2009) Modulation of intein activity by its neighboring extein substrates. *Proc. Natl. Acad. Sci. U. S. A. 106*, 11005−11010.

(19) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248−254.

(20) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.

(21) Zhang, Z., and Marshall, A. G. (1998) A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J. Am. Soc. Mass Spectrom. 9*, 225−233.

(22) Du, Z., Liu, J., Albracht, C. D., Hsu, A., Chen, W., Marieni, M. D., Colelli, K. M., Williams, J. E., Reitter, J. N., Mills, K. V., and Wang, C. (2011) Structural and mutational studies of a hyperthermophilic intein from DNA polymerase II of Pyrococcus abyssi. *J. Biol. Chem. 286*, 38638−38648.

(23) Liu, J., Du, Z., Albracht, C. D., Naidu, R. O., Mills, K. V., and Wang, C. (2011) 1H, 13C, and 15N NMR assignments of the Pyrococcus abyssi DNA polymerase II intein. *Biomol. NMR Assignments 5*, 233−235.

(24) Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR 6*, 277−293.

(25) Blackburn, S., and Lee, G. R. (1954) The liberation of aspartic acid during the acid hydrolysis of proteins. *Biochem. J. 58*, 227−231.

(26) Catak, S., Monard, G., Aviyente, V., and Ruiz-Lopez, M. F. (2008) Computational study on nonenzymatic peptide bond cleavage at asparagine and aspartic acid. *J. Phys. Chem. A 112*, 8752−8761.

(27) Fairchild, T. A., Fulton, D., Fontana, J. T., Gratton, J. P., McCabe, T. J., and Sessa, W. C. (2001) Acidic hydrolysis as a mechanism for the cleavage of the Glu(298)−>Asp variant of human endothelial nitric-oxide synthase. *J. Biol. Chem. 276*, 26674−26679.

(28) George-Nascimento, C., Lowenson, J., Borissenko, M., Calderon, M., Medina-Selby, A., Kuo, J., Clarke, S., and Randolph, A. (1990) Replacement of a labile aspartyl residue increases the stability of human epidermal growth factor. *Biochemistry 29*, 9584−9591.

(29) Herrmann, K. A., Wysocki, V. H., and Vorpagel, E. R. (2005) Computational investigation and hydrogen/deuterium exchange of the fixed charge derivative tris(2,4,6-trimethoxyphenyl) phosphonium:

DOI: 10.1021/acs.biochem.6b00894
Biochemistry 2017, 56, 1042−1050

implications for the aspartic acid cleavage mechanism. *J. Am. Soc. Mass Spectrom. 16*, 1067−1080.
(30) Joshi, A. B., Sawai, M., Kearney, W. R., and Kirsch, L. E. (2005) Studies on the mechanism of aspartic acid cleavage and glutamine deamidation in the acidic degradation of glucagon. *J. Pharm. Sci. 94*, 1912−1927.
(31) Lidell, M. E., and Hansson, G. C. (2006) Cleavage in the GDPH sequence of the C-terminal cysteine-rich part of the human MUC5AC mucin. *Biochem. J. 399*, 121−129.
(32) Lidell, M. E., Johansson, M. E., and Hansson, G. C. (2003) An autocatalytic cleavage in the C terminus of the human MUC2 mucin occurs at the low pH of the late secretory pathway. *J. Biol. Chem. 278*, 13944−13951.
(33) Oliyai, C., and Borchardt, R. T. (1993) Chemical pathways of peptide degradation. IV. Pathways, kinetics, and mechanism of degradation of an aspartyl residue in a model hexapeptide. *Pharm. Res. 10*, 95−102.
(34) Osicka, R., Prochazkova, K., Sulc, M., Linhartova, I., Havlicek, V., and Sebo, P. (2004) A novel ″clip-and-link″ activity of repeat in toxin (RTX) proteins from gram-negative pathogens. Covalent protein cross-linking by an Asp-Lys isopeptide bond upon calcium-dependent processing at an Asp-Pro bond. *J. Biol. Chem. 279*, 24944−24956.
(35) Osipiuk, J., Mulligan, R., Bargassa, M., Hamilton, J. E., Cunningham, M. A., and Joachimiak, A. (2012) Characterization of member of DUF1888 protein family, self-cleaving and self-assembling endopeptidase. *J. Biol. Chem. 287*, 19452−19461.
(36) Piszkiewicz, D., Landon, M., and Smith, E. L. (1970) Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. *Biochem. Biophys. Res. Commun. 40*, 1173−1178.
(37) Rozman, M. (2007) Aspartic acid side chain effect-experimental and theoretical insight. *J. Am. Soc. Mass Spectrom. 18*, 121−127.
(38) Schultz, J., Allison, H., and Grice, M. (1962) Specificity of the cleavage of proteins by dilute acid. I. Release of aspartic acid from insulin, ribonuclease, and glucagon. *Biochemistry 1*, 694−698.
(39) Perler, F. B. (2002) InBase: the Intein Database. *Nucleic acids research 30*, 383−384.
(40) Du, Z., Shemella, P. T., Liu, Y., McCallum, S. A., Pereira, B., Nayak, S. K., Belfort, G., Belfort, M., and Wang, C. (2009) Highly conserved histidine plays a dual catalytic role in protein splicing: a pKa shift mechanism. *J. Am. Chem. Soc. 131*, 11581−11589.
(41) Du, Z., Zheng, Y., Patterson, M., Liu, Y., and Wang, C. (2011) pK(a) coupling at the intein active site: implications for the coordination mechanism of protein splicing with a conserved aspartate. *J. Am. Chem. Soc. 133*, 10275−10282.
(42) Abramoff, M. D., Magalhaes, P. J., and Ram, S. J. (2004) Image Processing with ImageJ. *Biophotonics Int. 11*, 36−42.
(43) Dorval, D. M., Connor, K. R., and Mills, K. V. (2006) Catalysis of individual steps of protein splicing of the Pyrococcus abyssi PolII intein. *FASEB J. 20*, A40−A40.
(44) Chong, S., Williams, K. S., Wotkowicz, C., and Xu, M. Q. (1998) Modulation of protein splicing of the Saccharomyces cerevisiae vacuolar membrane ATPase intein. *J. Biol. Chem. 273*, 10567−10577.
(45) Southworth, M. W., Amaya, K., Evans, T. C., Xu, M. Q., and Perler, F. B. (1999) Purification of proteins fused to either the amino or carboxy terminus of the Mycobacterium xenopi gyrase A intein. *BioTechniques 27*, 110−120.
(46) Amitai, G., Dassa, B., and Pietrokovski, S. (2004) Protein splicing of inteins with atypical glutamine and aspartate C-terminal residues. *J. Biol. Chem. 279*, 3121−3131.
(47) Wood, D. W., Derbyshire, V., Wu, W., Chartrain, M., Belfort, M., and Belfort, G. (2000) Optimized single-step affinity purification with a self-cleaving intein applied to human acidic fibroblast growth factor. *Biotechnology progress 16*, 1055−1063.
(48) Wood, D. W., Wu, W., Belfort, G., Derbyshire, V., and Belfort, M. (1999) A genetic system yields self-cleaving inteins for bioseparations. *Nat. Biotechnol. 17*, 889−892.
(49) Shemella, P., Pereira, B., Zhang, Y., Van Roey, P., Belfort, G., Garde, S., and Nayak, S. K. (2007) Mechanism for intein C-terminal cleavage: a proposal from quantum mechanical calculations. *Biophys. J. 92*, 847−853.
(50) Clarke, S. (1987) Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int. J. Pept. Protein Res. 30*, 808−821.
(51) Topilina, N. I., and Mills, K. V. (2014) Recent advances in in vivo applications of intein-mediated protein splicing. *Mobile DNA 5*, 5.
(52) Wood, D. W., and Camarero, J. A. (2014) Intein applications: from protein purification and labeling to metabolic control methods. *J. Biol. Chem. 289*, 14512−14519.

DOI: 10.1021/acs.biochem.6b00894
Biochemistry 2017, 56, 1042−1050