Protein Splicing: Characterization of the Aminosuccinimide Residue at the Carboxyl Terminus of the Excised Intervening Sequence†

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Received April 5, 1995; Revised Manuscript Received May 25, 1995®

ABSTRACT: Protein splicing is a self-catalyzed, posttranslational process which converts a precursor polypeptide into two new proteins by the excision of an internal polypeptide segment and the ligation of the flanking polypeptides. Evidence has been presented that protein splicing involves a branched intermediate, which is resolved into the two protein products by the cyclization of an asparagine residue to aminosuccinimide [Xu, M. Q., Comb, D. G., Paulus, H., Noren, C. J., Shao, Y., & Perler, F. (1994) *EMBO J. 13,* 5517−5522]. This report describes the chemical synthesis of a peptide with a C-terminal aminosuccinimide residue, corresponding to the putative C-terminus of the excised intervening sequence (intein) derived from the thermostable DNA polymerase of *Pyrococcus* species GB-D. The synthetic aminosuccinimide peptide was compared with the C-terminal cyanogen bromide peptide of the excised intein and found to be indistinguishable in terms of its chromatographic properties, high-resolution mass spectrum, and colorimetric assay involving reaction with hydroxylamine. This establishes definitively that protein splicing is accompanied by the cyclization of asparagine to yield an aminosuccinimide residue at the C-terminus of the excised intein and that this unusual residue is therefore a natural constituent of spliced proteins. The effects of pH and temperature on the stability of the synthetic aminosuccinimide peptide are described. The stability of the C-terminal aminosuccinimide decreased with increasing pH, similar to the internal aminosuccinimide residues that occur in many proteins as intermediates in protein deamidation, but the C-terminal aminosuccinimide was 5−10 times more stable than internal aminosuccinimides, with a half-life of about 80 h at 25 °C and pH 7.4, accounting for its relative ease of isolation.

RNA splicing and protein splicing are two mechanisms by which the flow of information from a gene to its protein product can be modulated so as to yield a protein whose sequence is not strictly colinear with the gene. In both splicing processes, a portion of a primary gene product is excised in a manner that allows the rejoining of the flanking sequences. The process of protein splicing is analogous to certain types of RNA splicing in that it is self-catalyzed and involves a branched intermediate (Xu et al., 1994). In addition, it was postulated that the cyclization of an asparagine residue may play a key role in protein splicing (Cooper et al., 1993). Recently, we presented evidence that the downstream peptide bond cleavage step in protein splicing is indeed mediated by the cyclization of the asparagine residue at the carboxyl-terminal end of the intervening sequence, or intein (Perler et al., 1994), to form aminosuccinimide (Xu et al., 1994). This evidence consisted of the isolation of two CNBr peptides corresponding to the C-terminus of the spliced intein. Electron spray ionization mass spectrometry indicated that these peptides corresponded to the expected C-terminal tetrapeptide, YAHN,[1] and to a component of molecular mass 18 atomic weight units less, presumably owing to the loss of a molecule of water in the cyclization process. In this paper, we describe the synthesis of YAHN> and its comparison with the C-terminal tetrapeptide of the spliced intein in terms of chromatographic properties, reaction with hydroxylamine, and high-resolution fast atom bombardment mass spectroscopy. Our results show definitively that protein splicing is accompanied by the cyclization of asparagine to yield an aminosuccinimide residue at the C-terminus of the excised intein and that this unusual residue is therefore a natural constituent of spliced proteins. We also describe a colorimetric test for C-terminal aminosuccinimide residues as well as the effect of pH and temperature on their rate of conversion to C-terminal asparagine or isoasparagine.

EXPERIMENTAL PROCEDURES

Materials. Protected amino acids and other reagents for peptide synthesis were obtained from Applied Biosystems, except for Dnp-L-leucine, which was obtained from Sigma.

† Supported by a New England Biolabs predoctoral fellowship to Y.S.

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® Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Dnp, 2,4-dinitrophenyl; Dnp-L-leucine, *N*-(2,4-dinitrophenyl)-L-leucine; FAB/MS, fast atom bombardment mass spectroscopy; HPLC, high-performance liquid chromatography; YAHN, L-tyrosyl-L-alanyl-L-histidyl-L-asparagine; YAHN>, L-tyrosyl-L-alanyl-L-histidyl-L-aminosuccinimide; ILVA, L-isoleucyl-L-leucyl-L-valyl-L-alanine.

0006-2960/95/0434-10844$09.00/0

The construction of plasmid pMIP-36 was described earlier (Xu et al., 1994).

Peptide Synthesis. Peptides were synthesized on an Applied Biosystems Model 431 peptide synthesizer as described earlier (Xu et al., 1994). The tetrapeptide YAHN was obtained by CNBr cleavage of Dnp-AMYAHN as described below.

Cyanogen Bromide Cleavage of Methionine Peptides or Proteins. Polypeptides (1 mg or less) were dissolved in 1 mL of 70% formic acid and treated with 20 mg of CNBr under nitrogen in the dark for 15−20 h, followed by evaporation in a vacuum (Gross, 1967). The residue was redissolved in a small volume of water and purified by reverse-phase HPLC.

Preparation of Hydroxamic Acids. Peptide hydroxamic acids were prepared either from the methyl esters of ILVA or YAHN or from YAHN> by heating with 2 M NH_2OH, pH 9.0, for 30 min at 65 °C, essentially as described by Kwong and Harris (1994). The reaction products were then repeatedly purified by HPLC to remove traces of NH_2OH that would interfere with the colorimetric assay for hydroxamic acids described below.

Analytical Methods. The separation of peptides by HPLC employed a Rainin system with an analytical C-8 reverse-phase column (Rainin Dynamax-300A, 5-μm pores, 4.6 × 250 mm) at room temperature and a flow rate of 1 mL/min, employing linear gradients of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The following elution program was used for the separation of small peptides: sample injection; 5 min, 0% B; 10 min, 0 to 20% B; 1 min, 20 to 100% B; 2 min, 100% B; 2 min, 100 to 0% B; 5 min, 0% B.

Amino acid analysis employed a Beckman Model 7300 high-performance analyzer with a System Gold data analysis module after vapor-phase hydrolysis with HCl using a Waters PicoTag workstation.

High-resolution mass spectra were recorded on a Jeol JMS-SX102 spectrometer at the Department of Chemistry, Harvard University.

The colorimetric determination of hydroxamic acids was done by the method of Seifter et al. (1960). The sample in 156 μL of water was mixed with 665 μL of 0.73 M sodium acetate followed by 83 μL of 58 mM sulfanilic acid in 4.4 M acetic acid and 41 μL of 50 mM iodine in glacial acetic acid. After 10 min at room temperature, 16.5 μL of 0.1 M sodium thiosulfate was added to decolorize the iodine, followed by 16.5 μL of 40 mM α-naphthylamine in 5.3 M acetic acid. After 10 min at room temperature, the absorbance at 520 nm was measured in a spectrophotometer cell with a 10-mm light path. A standard curve with the hydroxamic acid of the tetrapeptide ILVA showed a linear relationship between hydroxamic acid concentration and absorbance at 520 nm from 5 to 50 nmol of ILVA-hydroxamate, with 50 nmol yielding A_{520} of 1.4 (Figure 1).

Preparation and in Vitro Splicing of MIP-36. The precursor protein MIP-36 is a fusion protein with an N-terminal *Escherichia coli* maltose binding protein domain (M) linked to the intein-1 of *Pyrococcus* GB-D DNA polymerase (I) and followed by a fragment of *Dirofilaria immitis* paramyosin (P) at the C-terminus (Xu et al., 1994). MIP-36 precursor was purified from *E. coli* ER2252/pMIP-36 by a slight modification of the procedure of Xu et al. (1993). The bacteria were grown at 30 °C to mid-exponential phase in 8 L of LB medium supplemented with 100 μg/mL ampicillin, cooled to 12 °C, supplemented with 0.3 mM isopropyl β-D-thiogalactoside, and incubated at 12−15 °C overnight. The cells were collected by centrifugation at 4 °C and resuspended in buffer S (0.5 M NaCl in 20 mM Na phosphate, pH 8.5) for sonic disruption in a Branson sonicator. Cell debris was removed by centrifugation at 15000*g* for 20 min at 4 °C, and the supernatant solution was passed through an amylose column (20 × 2.5 cm) at a flow rate not exceeding 1 mL/min. The column was washed with buffer S until the absorbance at 280 nm reached background and was then eluted with 50 mL of 10 mM maltose in buffer S. The eluate, which was monitored for protein by the method of Bradford (1976), showed a single protein peak corresponding to about 20 mg of protein, which on SDS−PAGE showed about 65% MIP and 35% MP, presumably produced by *in vivo* splicing of MIP-36. A portion of the crude MIP preparation (2.6 mg) in 2 mL was adjusted to pH 6.0 with NaH_2PO_4 and incubated at 50 °C for 3 h, conditions adequate for complete *in vitro* splicing. The mixture was then cooled to 4 °C and adjusted to 0.6 M with cold trichloroacetic acid. The precipitate was collected by centrifugation, washed twice with 1 mL of cold 0.6 M trichloroacetic acid and twice with 1 mL of ethanol, and then dried in a vacuum for storage at −20 °C.

RESULTS

Synthesis and Characterization of Peptides Corresponding to the Putative C-Terminus of the Spliced MIP-36 Intein. The C-terminal pentapeptide of the MIP-36 intervening sequence is MYAHN, and cyanogen bromide cleavage of the excised intein should thus yield YAHN or a derivative thereof. Earlier studies had tentatively identified a mixture of YAHN and YAHN> among the CNBr cleavage products of the spliced intein. Accordingly, we undertook the synthesis of YAHN and YAHN> as model peptides to compare with the peptides released from the spliced MIP-36 intein by CNBr cleavage.

YAHN was obtained from Dnp-AMYAHN by CNBr cleavage. After evaporation of the mixture, the product was isolated by HPLC and its identity was confirmed by amino acid analysis and high-resolution FAB/MS (Table 1).

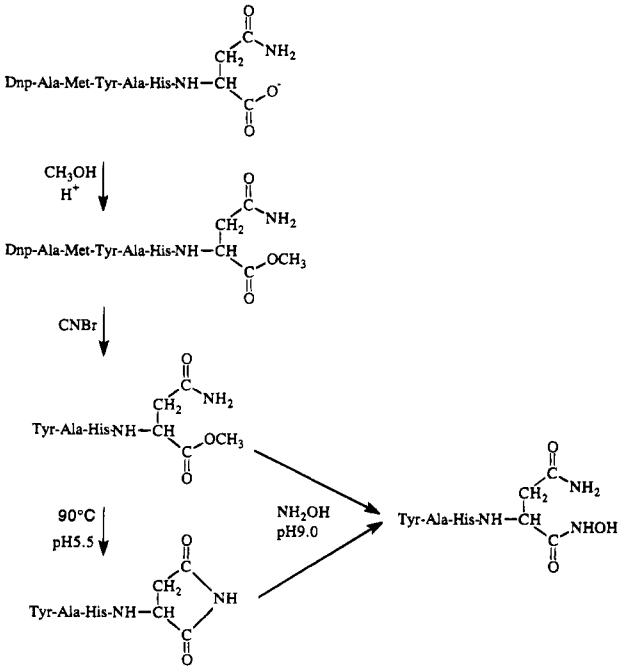
In order to synthesize YAHN>, we first prepared the methyl ester of Dnp-AMYAHN by incubating 12.25 mg of Dnp-AMYAHN and 4.8 mg of *p*-toluenesulfonic acid in 0.5 mL of absolute methanol at 60 °C for 24 h, followed by evaporation to dryness in a vacuum and purification by reverse-phase HPLC. The major Dnp-containing peak (80% yield) was collected, dried under vacuum, and subjected to CNBr cleavage. HPLC analysis showed one major tyrosine-containing peak on the basis of absorbance at 280 nm with an amino acid composition and a high-resolution mass spectrum consistent with the methyl ester of YAHN (Table 1). This material was evaporated to dryness under vacuum, redissolved in 0.1 M K phosphate, pH 5.5, and heated at 90 °C for 2 h. HPLC analysis revealed the presence of a single product, which eluted earlier than the YAHN methyl ester and had an amino acid composition and a high-resolution mass spectrum consistent with the aminosuccinimide derivative YAHN>. The reactions described are outlined in Scheme 1.

Table 1 Properties of Peptides Related to the C-Terminus of the Spliced Intein

peptide (origin)	amino acid composition (Y; A; H; D)	M + H$^+$ found (predicted)	HPLC retention time (min)	colorimetric assay ($A_{520/\mu mol}$)
YAHN (synthetic)	1.00; 1.02; 1.03; 0.97	504.2208 (504.2207)	4.8	NA
YAHN> (synthetic)	1.00; 0.92; 0.94; 0.93	486.2096 (486.2101)	5.1	NA
YAHN-OMe (synthetic)	1.00; 0.97; 0.99; 1.02	518.2352 (518.2363)	6.2	NA
YAHN-NHOH (synthetic)	1.00; 1.03; 1.05; 1.06	ND	4.9	25
peak 1 (CNBr of MIP-36)	1.00; 0.99; 1.01; 1.09	504.2184 (504.2207)	4.8	NA
peak 2 (CNBr of MIP-36)	1.00; 1.06; 0.97; 1.15	486.2099 (486.2101)	5.1	NA
YAHN-NHOH (from peak 2)	1.00; 1.05; 1.08; 1.08	ND	4.9	27

[a] NA, not applicable; ND, not done.

Scheme 1. Steps in the Synthesis of Tyr-Ala-His-aminosuccinimide[a]



[a] Note that treatment of the succinimide product with hydroxylamine is also expected to yield the hydroxamic acid of the corresponding isoasparagine peptide, which is not shown in the diagram.

Colorimetric Assay for Peptides with C-Terminal Aminosuccinimide. The colorimetric determination of peptides containing a C-terminal aminosuccinimide residue was based on the ability of succinimides to react with hydroxylamine to form hydroxamic acids. The latter can be estimated by the method of Seifter et al. (1960), which entails oxidation by iodine to nitrite, followed by a diazotiation reaction involving sulfanilic acid and α-naphthylamine. The assay was highly sensitive, with 10 nmol of ILVA-hydroxamate yielding an absorbance at 520 nm of 0.28. Figure 2 shows the HPLC elution profiles of YAHN-hydroxamate prepared either from YAHN methyl ester (Figure 2A) or from YAHN> (Figure 2B), both in terms of absorbance of the peptides at 220 nm and of absorbance at 520 nm obtained when corresponding fractions were subjected to the colorimetric assay described above. The color yields of the hydroxamic acids from either YAHN methyl ester or YAHN> agreed within 10% with that obtained with ILVA-hydroxamate, indicating that both peptides were converted to monohydroxamates as shown in Scheme 2. When the underivatized peptide YAHN was treated with hydroxylamine and carried through the same procedure, no absorbance at 520 nm was observed.

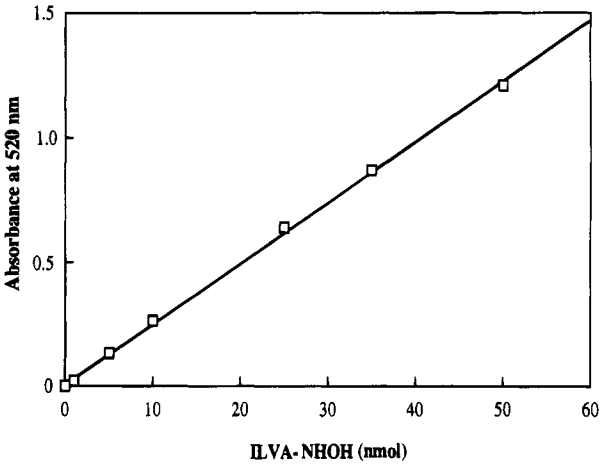


FIGURE 1: Standard curve for the colorimetric assay of hydroxamic acids.

Identification of the C-Terminal Peptides of the Spliced MIP-36 Intein. When the *in vitro* splicing products of MIP-36 were subjected to CNBr cleavage followed by HPLC, the first two peptide components eluted at 4.8 min (peak I) and 5.1 min (peak II), closely corresponding to the elution positions of synthetic YAHN and YAHN>, respectively. When synthetic YAHN was treated with CNBr under identical conditions, only material eluting at the position of peak I was observed (data not shown). Peaks I and II were present in approximately equal amounts, and acid hydrolysis of both yielded equimolar amounts of tyrosine, alanine, histidine, and aspartate (Table 1). High-resolution FAB/MS of peaks I and II yielded values of (M + H$^+$) of 504.2184 and 486.2099, respectively, in good agreement with the values of 504.2207 and 486.2101 predicted for YAHN and its aminosuccinimide derivative, YAHN>, respectively (Table 1). When peak 2 was treated with hydroxylamine, a derivative was formed, which comigrated with YAHN-hydroxamic acid on HPLC and had a similar color yield at 520 nm when subjected to the colorimetric assay procedure for hydroxamic acids (Figure 3; Table 1).

Effect of pH and Temperature on the Stability of Aminosuccinimide Peptides. The stability of chemically synthesized YAHN>, the aminosuccinimide derivative of the tetrapeptide YAHN, was examined as a function of pH and temperature by measuring the rate of conversion of YAHN> to YAHN, using reverse phase HPLC to separate the two peptides. Under all conditions studied, the hydrolysis of YAHN> was a first-order process. As shown in Figure 4A, the aminosuccinimide peptide was more stable at lower pH values, with rate constants at pH 5.5, 6.5, 7.4, and 8.5 being 3.3×10^{-5}, 2.8×10^{-4}, 7.0×10^{-4}, and 4.3×10^{-3} min^{-1},

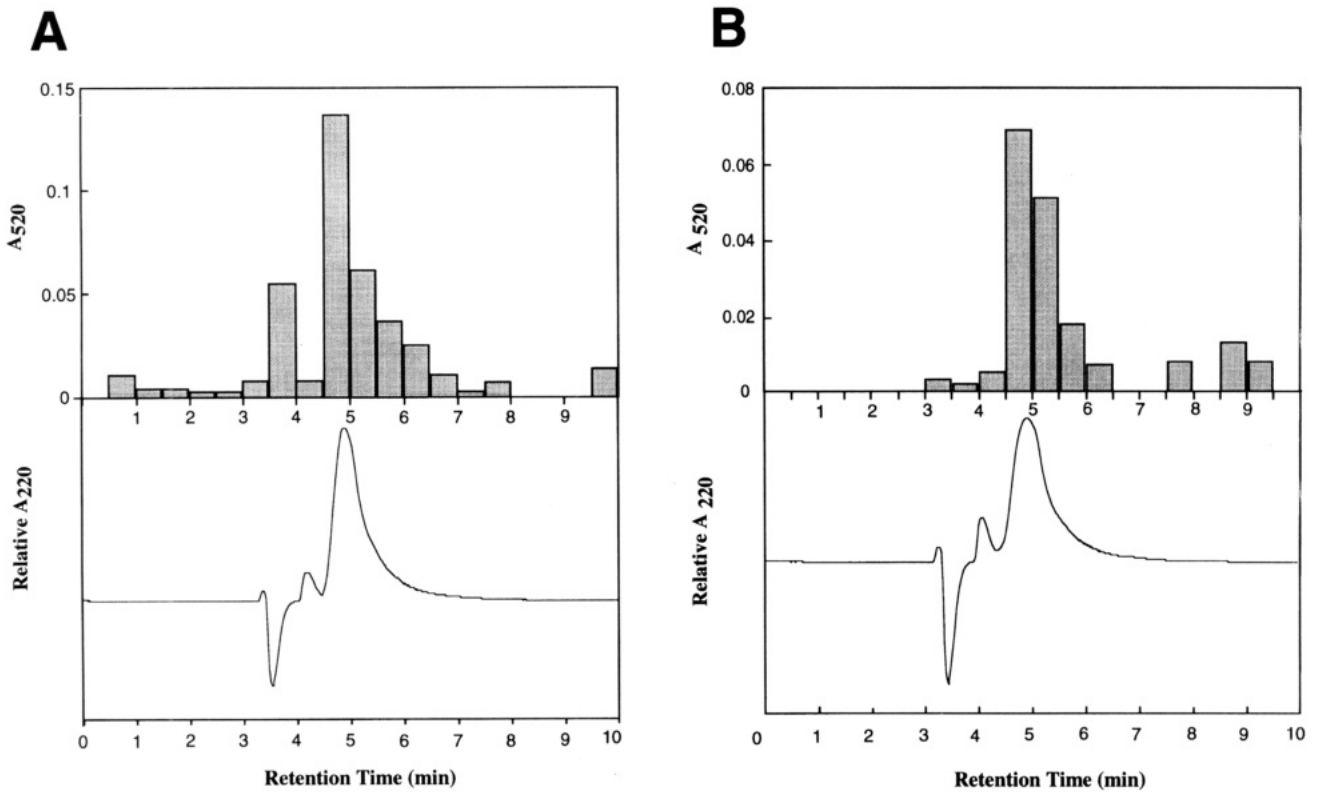
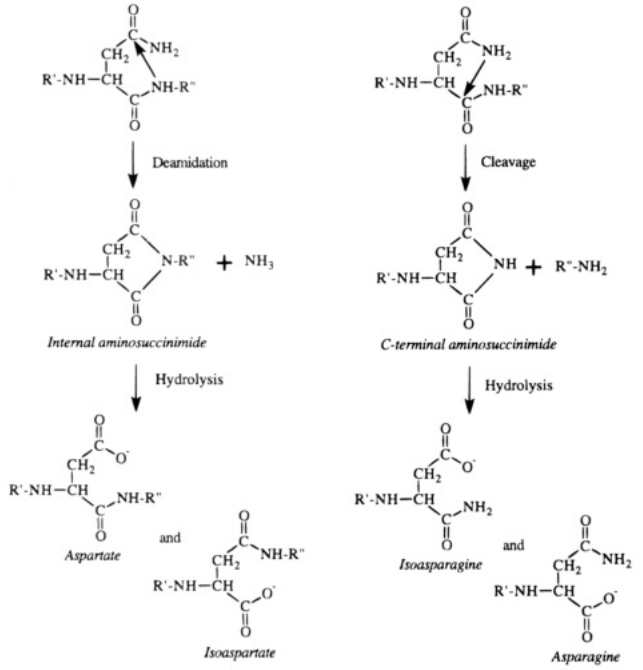


FIGURE 2: HPLC elution profiles and colorimetric assay of YAHN hydroxamic acid: (A) hydroxamic acid prepared from YAHN-OMe; (B) hydroxamic acid prepared from YAHN>. The hydroxamic acids were subjected to HPLC, and the eluate was monitored by its ultraviolet absorbance (bottom panels), with fractions being collected every 0.5 min for the colorimetric determination of hydroxamic acids as described under Experimental Procedures. The calculated color yields in experiments A and B were 30 and 25 A_{520}/μmol, respectively.

Scheme 2. Alternate Routes for the Formation and Hydrolysis of Aminosuccinimide Residues in Proteins, which can Lead to Either Protein Deamidation *(left)* or Cleavage *(right)* [after Voorter et al. (1988)][a]



[a] Results presented in this paper suggest that the pathway on the right may also be involved in protein splicing.

respectively, at 37 °C. Study of the effect of temperature on aminosuccinimide hydrolysis at pH 7.4 (Figure 4B) yielded an Arrhenius activation energy of 23.8 kcal/mol (100 kJ/mol). Under ordinary laboratory conditions (pH 7.4 at 25 °C) aminosuccinimide peptides are therefore relatively stable entities, with a half-life of about 80 h.

DISCUSSION

Our earlier studies had suggested that the amino acid residue at the C-terminus of the spliced intein, derived either *in vivo* or *in vitro* from a construct (MIP-36) in which the intein of *Pyrococcus* species GB-D DNA polymerase was inserted between the *E. coli* maltose binding protein and a fragment of *D. immitis* paramyosin (Xu et al., 1993), consisted of a mixture of asparagine and its cyclic derivative, aminosuccinimide (Xu et al., 1994). To obtain further support for this conclusion, we undertook the synthesis of tetrapeptides corresponding to the C-terminus of the spliced intein, containing either a terminal asparagine or aminosuccinimide residue. In both cases, we first prepared the corresponding hexapeptides containing a methionine residue in the same position as the intein, so that the terminal tetrapeptide could be released from the synthetic peptides by cyanogen bromide cleavage in the same manner in which the terminal tetrapeptides were obtained from the natural product of the protein splicing reaction. When the normal asparagine-containing hexapeptide was subjected to cyanogen bromide cleavage, no aminosuccinimide-containing product could be detected (data not shown), indicating that no cyclization of asparagine occurs under these conditions. The synthesis of a peptide with a C-terminal aminosuccinimide residue involved the cyclization of the methyl ester of N-substituted asparagine in a manner analogous to the synthesis of peptides with an internal aminosuccinimide residue by the cyclization of peptides containing the methyl ester of aspartate or isoaspartate (McFadden & Clarke, 1986).

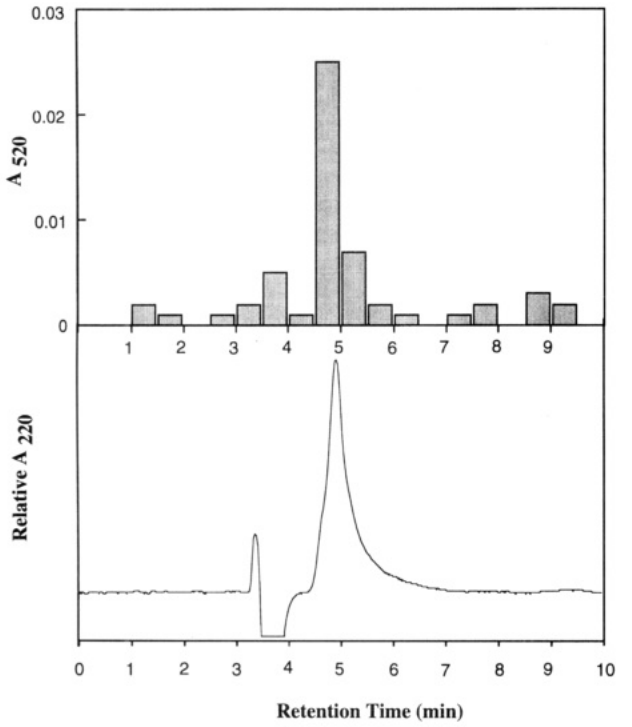


FIGURE 3: HPLC elution profile and colorimetric assay of hydroxamic acid prepared from peak 2 of the CNBr cleavage products of the excised MIP36 intein.

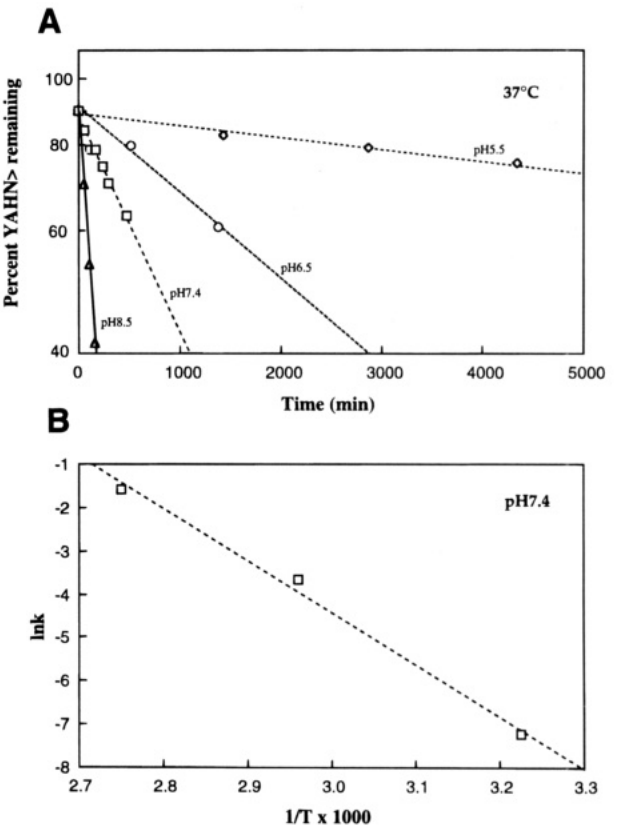


FIGURE 4: Effect of pH and temperature on the rate of hydrolysis of the aminosuccinimide peptide, YAHN>: (A) hydrolysis of YAHN> as a function of pH at 37 °C, presented as a first order plot of the logarithm of reactant remaining against time; (B) hydrolysis of YAHN> as a function of temperature at pH 7.4, presented as an Arrhenius plot of the logarithm of the first-order rate constant against the reciprocal of temperature. The course of hydrolysis of YAHN> was followed by HPLC.

Heating of the tetrapeptide methyl ester at pH 5.5 led to its quantitative conversion to the aminosuccinimide peptide. The identity of the product was confirmed by high-resolution FAB/MS, with a value of $(M + H^+)$ of 486.2096, very close to the predicted value of 486.2101, and by its conversion to a hydroxamic acid, which could be purified by HPLC, measured by a colorimetric method (Figure 2), and identified by FAB/MS (Table 1).

Treatment of the *in vitro* excised MIP-36 intein with cyanogen bromide produced two low molecular weight products whose retention times on HPLC corresponded to those of the synthetic peptides YAHN and YAHN>. The identity of these products was confirmed by high-resolution FAB/MS, with the putative YAHN> yielding a value of $M + H^+$ of 486.2099 (predicted value, 486.2101). The suspected YAHN> product was further characterized by treatment with hydroxylamine, followed by isolation of the resulting hydroxamic acid and assay by the color reaction of Seifter et al. (1960), in which it yielded the same molar absorbance at 520 nm as the hydroxamic acid prepared from the synthetic peptide. These observations constitute definitive evidence that excision of the intein in the course of protein splicing is accompanied by the cyclization of asparagine to aminosuccinimide, thereby producing a protein with a C-terminal aminosuccinimide residue.

Our results suggest that polypeptides with an aminosuccinimide residue at their C-terminus are produced naturally in the course of protein splicing, and it was therefore of interest to study the stability of C-terminal succinimide residues in peptides. This was made possible by the availability of synthetic YAHN> and the ability to separate this peptide from its hydrolysis product YAHN[2] by reverse-phase HPLC. The hydrolysis of YAHN> was a pseudo-first-order process, whose rate increased strikingly with pH. At pH 5.5, YAHN> hydrolyzed with a half-life of 350 h at 37 °C, which decreased to 17 h at pH 7.4. If we compare the Gibbs free energy of activation for the hydrolysis of YAHN> at 37 °C and pH 7.4 (25.3 kcal mol^{-1}), calculated from the first-order rate constant, with the enthalpy of activation (23.2 kcal mol^{-1}) obtained from the Arrhenius plot (Figure 4B), we find that $-T\Delta S^*$ for the hydrolysis of the cyclic imide (2.1 kcal mol^{-1}) is unusually small compared to the corresponding parameter for the OH^--catalyzed hydrolysis of an amide such as glycylglycine (Lawrence & Moore, 1951). The high stability of the cyclic imide at pH 5.5 explains why heating YAHN methyl ester at pH 5.5 led to the almost quantitative conversion to the aminosuccinimide derivative. In contrast, when tetragastrin methyl ester was incubated at pH 7.4, the cyclic imide could be observed only as a transient intermediate with a half-life of about 2 h, the aspartate and isoaspartate peptides being the major products of hydrolysis (McFadden & Clarke, 1986). Even though N-substituted cyclic imide residues at internal positions of a polypeptide chain are also more stable at low pH (Murray & Clarke, 1986), they seem to be considerably less stable than the peptides with a C-terminal aminosuccinimide residue described in this paper. For example, at pH 7.4 and 37 °C,

[2] Hydrolysis of YAHN> would be expected to yield a mixture of the corresponding asparagine and isoasparagine peptides (Johnson & Aswad, 1985; McFadden & Clarke, 1986; Murray & Clarke, 1984, 1986). However, we have made no attempt to distinguish between these products, which presumably comigrate under the chromatographic conditions employed in our studies.

$t_{1/2}$ is 1.9 h for the cyclic imide of tetragastrin (McFadden & Clarke, 1986), 3 h for the cyclic imide derivative of the hexapeptide VYPDGA (Murray & Clarke, 1986), and 4.2 h for the cyclic imide derivative of adrenocorticotropin (Johnson & Aswad, 1985), compared to 17 h for YAHN>. Similarly, at pH 5.5 and 40 °C, the 101-succinimide derivative of lysozyme has a $t_{1/2}$ of 15 h (Tomizawa et al., 1994), compared to 350 h for YAHN> under similar conditions. The observation that C-terminal aminosuccinimides are considerably more stable than the N-substituted cyclic imides at internal positions of polypeptide chains explains why it was relatively easy to demonstrate their occurrence in inteins excised in the course of protein splicing, in contrast to the somewhat elusive nature of internal cyclic imides [e.g., Carter and McFadden (1994)].

Cyclization of asparagine to aminosuccinimide residues, which was first described in peptides by Geiger and Clarke (1987) and in proteins by Voorter et al. (1988), is a well-known post translational modification that can lead either to spontaneous protein deamidation or to protein cleavage. However, owing to their instability, aminosuccinimide residues have been directly identified in only a few proteins, for example, adrenocorticotropin (Johnson & Aswad, 1985), methionyl human growth hormone (Teshima et al., 1991), porcine somatotropin (Violand et al., 1992), recombinant hirudin (Bischoff et al., 1993), and the excised MIP-36 intein described here. The earlier examples all involve N-substituted succinimides at an internal position in a protein, and only the example described in this paper involves an unsubstituted C-terminal succinimide. It is therefore of interest to compare the origins and fates of internal and terminal succinimide residues in proteins. As suggested earlier by Geiger and Clarke (1987) and Voorter et al. (1988), cyclization of an asparagine residue in proteins can occur either by the attack of the peptide bond amide N on the side-chain carbonyl of asparagine, yielding NH_3 and an internal N-substituted cyclic imide, or by the attack of the asparagine amide N on the peptide bond carbonyl group, producing peptide chain cleavage and a C-terminal unsubstituted cyclic imide (Scheme 2).[3] Spontaneous protein deamidation, which results from the attack of the peptide bond N on the side-chain carbonyl, seems to be governed primarily by steric factors (Clarke, 1987; Wright, 1991), although neighboring amino acid side chains may also participate (Wright, 1991). It is therefore likely that the relative frequencies of spontaneous attack on the peptide bond carbonyl and on the side-chain carbonyl, leading to deamidation and peptide chain cleavage, respectively, are governed by similar factors. On the other hand, in a specifically catalyzed process such as protein splicing, the course of the reaction is probably determined by the catalytic intervention of specific amino acid side chains, for example, the histidine residue adjacent to the asparagine residue of interest, which is conserved in all known self-splicing proteins and whose replacement by other amino acids prevents the final cleavage step in protein splicing but not the earlier steps (M.-Q. Xu, unpublished results). The protein succinimides can hydrolyze to yield either internal aspartate or isoaspartate residues or C-terminal asparagine or isoasparagine, depending on whether the initial cyclization involved the peptide or side-chain carbonyl of asparagine (Scheme 2). Assuming that hydrolysis of the cyclic imide is a spontaneous and not a catalyzed process, one would expect internal N-substituted succinimides to be more labile that C-terminal unsubstituted succinimides owing to electron withdrawal by the N-alkyl group in the former. This is consistent with the observed greater stability of the C-terminal aminosuccinimide residue described in this paper.

The C-terminal residues of the excised MIP-36 intein consisted of approximately equal amounts of aminosuccinimide and asparagine (Xu et al., 1994). It is possible that the latter was produced by hydrolysis of the cyclic imide subsequent to splicing. If that were the case, the C-terminal asparagine residue found in about one-half of the excised intein molecules should actually be a mixture of asparagine and isoasparagine. We have not been able to distinguish between these isomers by reverse-phase HPLC; however, a method was recently developed for this purpose (Brennan & Clarke, 1993), and its application to the excised inteins would answer whether the asparagine-terminated peptide is indeed the product of nonenzymatic hydrolysis of the aminosuccinimide derivative. An interesting question is whether substantial amounts of the excised inteins, which function as homing endonucleases, occur as the cyclic imide in the cell. In view of the 17-h half-life of C-terminal aminosuccinimide at pH 7.4 and 37 °C, a period much longer than the doubling time of yeast and bacteria, one would expect a relatively high level of C-terminal aminosuccinimide in the endonucleases which are excised in the course of splicing of the yeast vacuolar ATPase VMA subunits (Gu et al., 1993; Kane et al., 1990) and the bacterial RecA proteins (Davis et al., 1992, 1994). On the other hand, the aminosuccinimide termini of the endonucleases encoded by the inteins associated with the DNA polymerases of hyperthermophilic archaea (Perler et al., 1992; Xu et al., 1993) are probably predominantly hydrolyzed at the normal growth temperature of 90 °C or higher, the $t_{1/2}$ for the hydrolysis of C-terminal aminosuccinimide residues being only 3 min at pH 7.4 and 90 °C.

[3] Another possible mechanism of polypeptide chain cleavage at asparagine residues involves the formation of an isoimide derivative through attack by the amide carbonyl oxygen on the peptide carbonyl (Clarke, 1987). The isoimide and succinimide derivatives of YAHN being structural isomers, they would not be distinguished by high-resolution FAB/MS. However, hydrolysis of the isoimide would yield the asparagine peptide as the sole product and no isoasparagine, and analysis of the hydrolysis products by the method of Brennan and Clarke (1993) should thus be able to distinguish between isoimide and succinimide intermediates.

ACKNOWLEDGMENT

We thank Drs. Donald Comb and Francine Perler for valuable discussions and comments on the manuscript.

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BI950771T