Sequence-Specific Deamidation: Isolation and Biochemical Characterization of Succinimide Intermediates of Recombinant Hirudin†

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ABSTRACT: Natural hirudin variant 2 with a lysine residue in position 47 (rHV2-Lys^{47}) was produced in a genetically engineered strain of *Saccharomyces cerevisiae* as a secreted protein of 65 amino acids and purified to greater than 99% homogeneity. Only reversed-phase high-performance liquid chromatography (RP-HPLC) using very shallow acetonitrile gradients indicated the presence of a component in the final product (approximately 1% of total protein) with a slightly increased retention time. Using successive RP-HPLC purification steps, this hydrophobic impurity was isolated and separated into two constituents defined as components A1 and A2 which differed from the parent molecule by mass reductions of 17.2 Da (A1) and 17.6 Da (A2), respectively, as determined by electrospray mass spectrometry (ESMS). Proteolytic digestion with endoprotease Glu-C from *Staphylococcus aureus* (V8 protease) and analysis of the peptide mixture by ESMS showed that the mass difference between rHV2-Lys^{47} and component A1 was due to a modification between amino acids 1 and 43, while the corresponding mass difference with component A2 was the result of a modification within the peptide fragment comprising residues 50–61. Further analyses using amino acid sequencing and ESMS in combination with collision-activated dissociation (CAD) detected modifications at residues Asn^{33}-Gly^{34} in component A1 and at Asn^{53}-Gly^{54} in component A2. Both of these sites were previously shown to be susceptible to spontaneous deamidation under slightly basic pH conditions. Thus, the mass reductions of approximately 17 Da and the fact that both asparagines, Asn^{33} in component A1 and Asn^{53} in component A2, proved to be resistant to Edman degradation provided strong support for them being stable succinimide intermediates of the corresponding deamidation reactions. Both intermediates were shown to have inhibition constants for human α-thrombin on the order of 1 pM, identical to that of rHV2-Lys^{47}. The isoelectric point of component A2 was determined to be within 0.01 pH unit of that of the parent molecule by isoelectric focusing in an immobilized pH gradient.

Purity evaluation and characterization of minor impurities in the final preparation of recombinant proteins destined for therapeutic use are important aspects of quality control as well as stability studies performed with the purified material. In this respect, spontaneous deamidation of Asn to Asp and to a lesser extent of Gln to Glu have been recognized as one of the major factors contributing to charge heterogeneity in purified proteins. The susceptibility of Asn to undergo deamidation has been shown to be affected by local three-dimensional structure and in particular by the amino acid C-terminal to the Asn residue. Asn followed by a Gly in synthetic peptides was shown to be most effectively deamidated with reaction rates being more than 200-fold faster than for the free amino acid (Robinson et al., 1973; Geiger & Clarke, 1987; Lura & Schirch, 1988; Stephenson & Clarke, 1989; Johnson et al., 1989b; Wright, 1991; Robinson & Robinson, 1991). An extensive survey of deamidation sites in over 1000 protein sequences has established that there is also a contribution from the amino acid N-terminal to the potential deamidation site with Ser being most favorable for deamidation (Wright, 1991). Taken together, these studies have provided substantial evidence for structural parameters playing a crucial role in determining the rate with which deamidation occurs. Neutron diffraction studies on trypsin have provided further insight into the structural requirements favoring deamidation of Asn residues indicating that hydrogen bonding aligns the side chain carbonyl carbon atom in such a way that nucleophilic attack by the nitrogen atom of the following peptide bond is facilitated (Kossiakoff, 1988).

So far, the mechanism of deamidation was mainly studied by analyzing the reaction products that were obtained after deamidation of Asn in synthetic peptides indicating that hydrolysis of the side chain amide bond involves a succinimide intermediate which subsequently hydrolyzes to give rise to peptides containing both L- and D-isoaspartyl as well as L- and D-Asp linkages (Figure 1A) (Geiger & Clarke, 1987; Lura & Schirch, 1988; Stephenson & Clarke, 1989; Aswad, 1984; Murray & Clarke, 1984). Direct supporting evidence for such a reaction mechanism was recently obtained by Teshima et al., who isolated a succinimide intermediate of recombinant

† The authors dedicate this work to the memory of the late Jean-Pierre Lecocq.

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[1] Abbreviations: rhGH, recombinant human growth hormone; rHV2-Lys^{47}, recombinant hirudin variant 2 with Lys in position 47; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; LSIMS, liquid secondary ion mass spectrometry; ESMS, electrospray mass spectrometry; V_c, sampling cone voltage; CAD, collision activated dissociation; Tris, tris(hydroxymethyl)aminomethane; PEG-6000, poly(ethylene glycol) (average molecular weight 6000); p*I*, isoelectric point.

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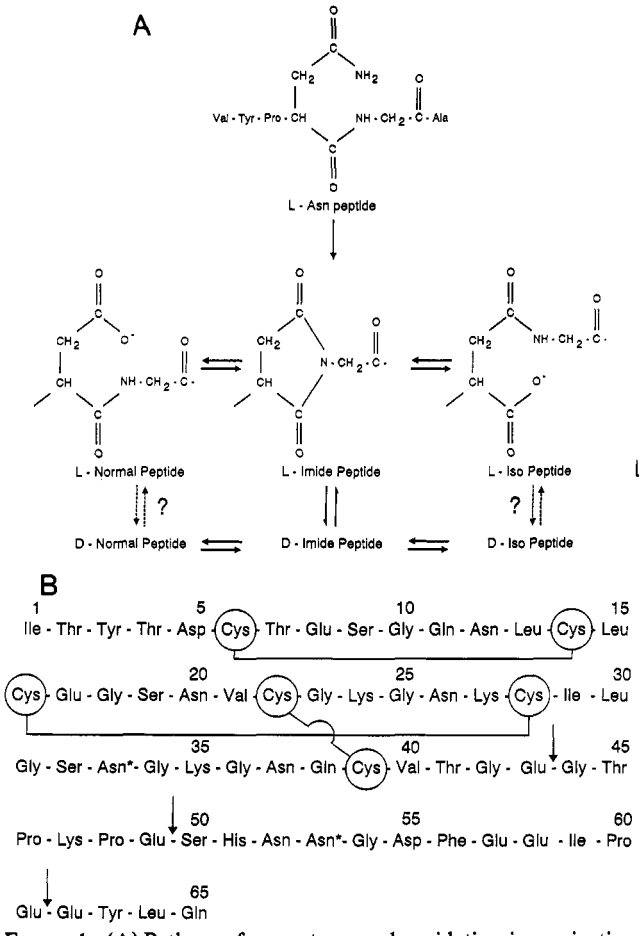


FIGURE 1: (A) Pathways for spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl hexapeptides related to adrenocorticotropin (Val-Tyr-Pro-Asn-Gly-Ala) [adapted from Geiger and Clarke (1987)]. (B) Primary structure of rHV2-Lys^{47} including disulfide bonds. The two Asn residues prone to deamidation (Asn^{33} and Asn^{53}) are marked (*), and the cleavage sites for V8 protease are indicated by arrows. Cleavages were found after Glu^{43}, Glu^{49}, and Glu^{61} but not following Glu^{8}, Glu^{17}, Glu^{57}, Glu^{58}, and Glu^{62} under the conditions described in Experimental Procedures.

human growth hormone (rhGH)[1] which had been treated at elevated temperatures in the lyophilized state (Teshima et al., 1991). In this example, the succinimide intermediate was formed by dehydration of an Asp residue that was followed by Gly showing that formation of the succinimide structure can occur either as an intermediate of the deamidation reaction of Asn or, under more drastic reaction conditions, by dehydration of Asp.

In the following, we describe the isolation and structural characterization of two succinimide intermediates of recombinant hirudin variant 2 containing a Lys residue in position 47 (rHV2-Lys^{47}). rHV2-Lys^{47} was secreted from a recombinant strain of *Saccharomyces cerevisiae* and purified by chromatography to greater than 99%. The intermediates which could only be separated from the parent molecule by RP-HPLC using extremely shallow gradients of acetonitrile were characterized by a combination of mass spectrometric methods, amino acid sequencing, and peptide mapping showing that succinimide cycles were formed at the two Asn-Gly sites in the molecule (Asn^{33} and Asn^{53}) which had previously been shown to be susceptible to deamidation under alkaline pH conditions (Tuong et al., 1992) (Figure 1B).

EXPERIMENTAL PROCEDURES

Purification of rHV2-Lys^{47} Succinimide Intermediates. rHV2-Lys^{47} was produced in a recombinant strain of *S. cerevisiae* and secreted into the culture medium as previously described (Loison et al., 1988; Achstetter et al., 1992). Isolation was performed by a number of chromatographic steps after initial micro- and ultrafiltration of the culture supernatant. The final preparation was homogeneous as judged by analytical gel filtration, anion- and cation-exchange HPLC, and isoelectric focusing in an immobilized pH gradient between pH 3.8 and 4.8 (Bischoff et al., 1989).

For the isolation of component A (comprising components A1 and A2), 75 mg of rHV2-Lys^{47} was treated by repetitive RP-HPLC on C_{18} and C_8 stationary phases (Nucleosil, Macherey & Nagel, Düren, FRG) using acetonitrile gradients in 0.1% (v/v) aqueous TFA (gradient slope, 0.08% acetonitrile/min) giving 80 μg of component A; 120 μg of component A1 and 250 μg of component A2 were obtained from a second isolation procedure starting with 90 mg of rHV2-Lys^{47} (gradient slopes, 0.04, 0.06, and 0.08% acetonitrile/min). RP-HPLC was performed on a HP1090 liquid chromatograph equipped with a Model 1040 diode array detector and a microcomputer (Hewlett-Packard, Waldbronn, FRG). Peptides were detected at 205 nm.

Proteolytic Digestion. rHV2-Lys^{47} and the succinimide intermediates (components A1 and A2) were digested with endoprotease Glu-C from *Staphylococcus aureus* (V8 protease) (Boehringer Mannheim, Mannheim, FRG) at an enzyme to substrate ratio of 1/5 (w/w) for 3 h at 37 °C in 0.5 M sodium acetate, pH 6.5. The reaction was stopped by adding TFA to a final concentration of 0.1%. Either the digest was desalted by RP-HPLC (Vydac C_{18}: The Separation Group, Hesperia, CA) using a fast acetonitrile gradient (0–35% acetonitrile in 0.1% aqueous TFA in 7 min) or individual peptides were isolated using a gradient slope of 1% acetonitrile/min. Mass spectrometric analyses were performed by ESMS on both the peptide mixture and individual peptides.

Amino Acid Sequence Analysis. N-Terminal sequencing was performed on a Model 477A protein sequencer coupled to a Model 120A on-line phenylthiohydantoin-amino acid HPLC analyzer equipped with a microcomputer (Applied Biosystems Inc., Foster City, CA.).

Mass Spectrometry. Mass spectrometric measurements were performed using liquid secondary ion mass spectrometry (LSIMS) on a VG ZAB-SE HF instrument (VG Analytical Ltd., Manchester, U.K.) or by electrospray mass spectrometry (ESMS) on a VG Bio Tech BioQ mass spectrometer (VG Biotech Ltd., Altrincham, U.K.). LSIMS was performed in the positive ion detection mode. The ion source was equipped with a cesium gun (VG Analytical Ltd.) and was operated at 30 keV. Mass spectra were recorded with an 11-250J data system. For average or chemical mass measurements, the molecular clusters were reduced to a single peak by operating the mass spectrometer at a resolution of 1000. Cesium iodide clusters were used as calibration standards. Samples were dissolved in water containing 5% acetic acid, and thioglycerol was used as the matrix (Van Dorsselaer et al., 1989).

ESMS was performed using an electrostatic spray ion source operating at atmospheric pressure connected to a quadrupole mass analyzer with a mass range of 4000. Scanning was performed from m/z = 500 to m/z = 1500 in 10 s with the resolution adjusted so that the m/z = 998 peak from horse heart myoglobin was 1.5–2.0 Da wide on the base. High voltage (4–4.5 kV) in the positive mode was applied on the silica capillary to generate multiply-charged ions, and the voltage applied to the sampling cone (V_c) was adjusted between 10 and 150 V (for most measurements V_c was adjusted to 45 V). During the CAD process used to fragment peptides in

the ion source of the mass spectrometer V_c was 120 V. Spectra were acquired by operating the data system as a multichannel analyzer, and several scans were summed to obtain the final spectrum. For molecular species which produced a series of multiply-charged protonated molecular ions, the molecular mass was determined by simple data system routines (Mann et al., 1989). Calibration was performed using the multiply-charged ions from a separate introduction of horse heart myoglobin (16 950.5 Da). Calculated molecular masses are given as average values on the basis of the atomic masses of the elements (C = 12.011, H = 1.0079, N = 14.0067, O = 15.9994, and S = 32.06). Mass spectra were typically obtained with sample concentrations of 15–20 pmol/μL (aqueous solution with 50% methanol and 1% glacial acetic acid). Samples were introduced into the ion source by syringe pumps (Applied Biosystems Inc. Model 140A) at a flow-rate of 2 μL/min (Van Dorsselaer et al., 1990).

Sequence Prediction from Mass Spectrometric Data. Prediction of the molecular masses of all possible fragments that can be obtained upon CAD within the mass spectrometer was performed using a computer program written in Think Pascal (Symantech Corp., Cupertino, CA) adapted to MacIntosh microcomputers (Apple Computer Inc., Cupertino, CA). Measured m/z values from the mass spectra were used to interrogate the computer program for the best correlations with possible sequences.

Thrombin Inhibition. Analysis of the inhibition of human α-thrombin by hirudin was performed under steady-state conditions using chromogenic substrates to measure residual proteolytic activity. The chromogenic substrates Chromozym PL (Tosyl-glycyl-prolyl-lysine-4-nitroanilide acetate) and S-2238 (D-Phe-pipecolyl-arginine-4-nitroanilide) were purchased from Boehringer Mannheim and Kabi-Vitrum (Stockholm, Sweden), respectively. Active-site-titrated human α-thrombin was a gift of Dr. Stuart Stone (Basel, Switzerland). All assays were performed in 0.05 M Tris-HCl, pH 7.8, 0.1% PEG-6000, 0.1 M NaCl in plastic cuvettes at 37 °C. The enzyme was preincubated with various amounts of rHV2-Lys^{47} or components A1 or A2 for 5 min at 37 °C, and the reaction was initiated by addition of the chromogenic substrate. The change in absorbance resulting from the hydrolysis of the peptidyl–4-nitroanilide bond was monitored at 405 nm with a Uvikon 940 double-beam spectrophotometer (Kontron Instruments, St. Quentin-Yvelines, France).

Determination of the Molar Concentration of rHV2-Lys^{47}. The concentration of active hirudin was determined essentially as described (Wallace et al., 1989) by titrating 2 nM α-thrombin in the presence of 500 μM Chromozym PL. The steady-state velocities obtained for each amount of hirudin were fitted by weighted nonlinear regression to the following equation (Morrison, 1969; Stone & Hofsteenge, 1986):

$$V_s = V_0/2E_t[[(K'_i + \chi I_t - E_t)^2 + 4K'_iE_t]^{0.5} - (K'_i + \chi I_t - E_t)] \quad (1)$$

where V_s = steady-state velocity, V_0 = velocity in the absence of inhibitor, E_t = thrombin concentration, I_t = volume of hirudin added (microliters), K'_i = apparent inhibition constant, and χ = factor that multiplied by I_t will give the molar concentration of hirudin.

The nonlinear regression program gives estimates of V_0, K'_i, and χ. The value of χ was then used to calculate the molar concentration of the hirudin stock solution.

Determination of Inhibition Constants. The apparent inhibition constant K'_i was determined using 92.4 pM α-thrombin and 100 μM S-2238. Ten data points were usually obtained and fitted to eq 1 describing tight-binding inhibition (Morrisson, 1969) with I_t being the inhibitor concentration and $\chi = 1$. The nonlinear regression program gives estimates of V_0 and K'_i. Assuming competitive inhibition, K_i and K'_i are related by the following equation: $K'_i = K_i(1 + S/K_M)$ where S is the substrate concentration and K_M is the Michaelis constant. K_i is calculated from this equation using a K_M of 3.63 μM (Stone & Hofsteenge, 1986).

RESULTS

rHV2-Lys^{47}, a potent inhibitor of human α-thrombin and thus a potential therapeutic agent to prevent thrombosis, was produced in a recombinant strain of *S. cerevisiae* and secreted into the culture medium (Achstetter et al., 1992). The protein was subsequently purified by a series of chromatographic steps resulting in a preparation that was greater than 99% pure as judged by analytical anion-, cation-exchange, and gel filtration HPLC as well as by isoelectric focusing in an immobilized pH gradient between pH 3.8 and 4.8 (Bischoff et al., 1989, 1992). Analysis by RP-HPLC, however, showed that the major peak had a shoulder toward longer retention times representing approximately 1% of total protein (hereafter defined as component A) (Figure 2A).

Isolation and Initial Characterization of Component A. In order to improve resolution between rHV2-Lys^{47} and component A, RP-HPLC with shallow gradients of acetonitrile was employed (see Experimental Procedures). Starting with 75 mg of rHV2-Lys^{47}, repetitive RP-HPLC gave 80 μg of purified component A having an increased retention time and an N-terminal amino acid sequence identical to rHV2-Lys^{47}. However, an initial series of LSIMS analyses in the m/z region between 2000 and 8000 showed that component A had a molecular mass of 6890.7 Da which was 16.8 Da lower than the measured mass of rHV2-Lys^{47} (measured mass, 6907.5 Da; expected mass, 6906.5 Da).

Previous analysis of the LSIMS spectra of rHV2-Lys^{47} had noted that C-terminal ions produced by fragmentations of the Y″-, Z′-, and X-type [nomenclature according to Roepstorff and Fohlman (1984)] could be detected (van Dorsselaer et al., 1989) (Figure 3). Comparative analyses of the Y″-fragmentation pattern of the isolated component A and rHV2-Lys^{47} was particularly instructive as it provided an observed difference in m/z of 15.9 between the Y13″-fragments of component A (m/z = 1566.9) and rHV2-Lys^{47} (m/z = 1582.8) while the Y12″-fragments of both molecules had identical m/z values (1468.7) (Figure 3A and Table I). Comparison of the Y″-fragments showed that from fragment Y13″ to Y17″ onward there was a $\Delta m/z$ of 15.9–17.4 between component A and rHV2-Lys^{47} (Table I). These data were confirmed when comparing the Z′- and X-fragments (Figure 3), although the abundance of these fragment ions was too low to detect them throughout the whole m/z range. Since the only difference between the Y13″- and the Y12″-fragments of rHV2-Lys^{47} should be the addition of Asn^{53} (see Figure 3A), it was concluded that the observed difference in m/z must be due to a modification of this residue. As Asn^{53} has been shown to be one of the prime sites of deamidation in rHV2-Lys^{47} (Tuong et al., 1992), it seemed likely that the mass difference was due to an intermediate of the deamidation reaction, and indeed the 17-Da mass reduction was in agreement with the presence of a succinimide between Asn^{53} and Gly^{54}.

A striking observation was, however, that upon close examination of the LSIMS spectrum of component A, Y″-fragment ions with molecular masses identical or very close

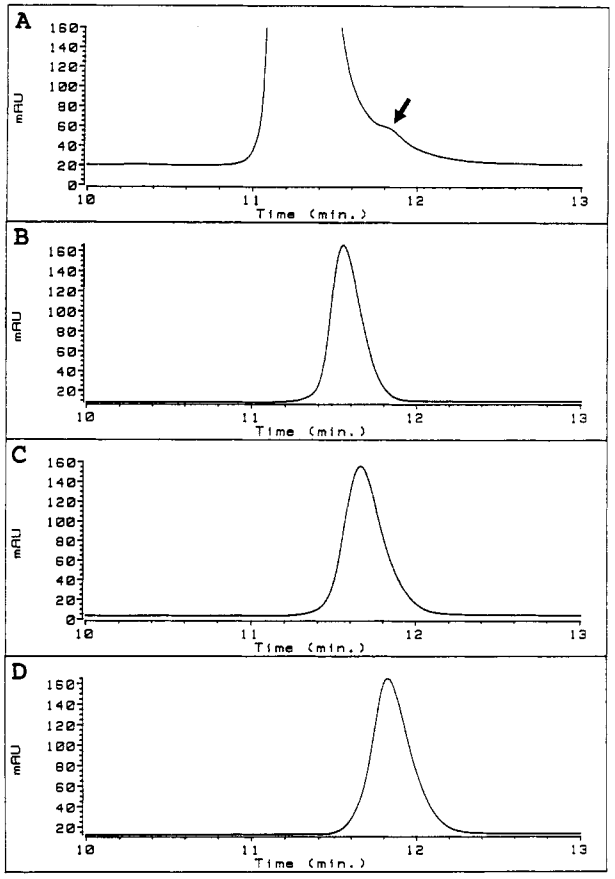


FIGURE 2: Reversed-phase HPLC of rHV2-Lys[47] and components A1 and A2. (A) Starting material from which components A1 and A2 have been prepared (component A is depicted by an arrow). (B–D) Comparative retention time analyses are shown with identical amounts of rHV2-Lys[47] after elimination of components A1 and A2 (B) (retention time, 11.55 min; measured mass, 6907.0 ± 0.2 Da; expected mass, 6906.5 Da), component A1 (C) (retention time, 11.66 min; measured mass, 6889.3 ± 0.1 Da; Δm = −17.2 Da), and component A2 (D) (retention time, 11.83 min; measured mass, 6888.9 ± 0.2 Da; Δm = −17.6 Da). Separations were performed on a Nucleosil C_8 column (4.6 × 100 mm, 3-μm particle diameter, 120-Å average pore diameter) using a gradient from 15% to 30% acetonitrile in 0.1% aqueous TFA over 15 min at a flow rate of 1 mL/min. UV absorbance was monitored at 205 nm. The molecular mass values given in parentheses were determined by ESMS. Δm depicts the mass difference between the calculated mass for rHV2-Lys[47] and the measured masses.

to those found in the spectrum of rHV2-Lys[47] were also observed (see those peaks marked with arrows next to the major Y15″- and Y16″-fragments of component A in Figure 3B at m/z = 1834.3 and 1923.0, respectively). Since the LSIMS spectrum in the higher m/z range did not indicate contamination of component A with rHV2-Lys[47], these fragment ions suggested that component A contained yet another constituent having a molecular mass reduction of about 17 Da compared to rHV2-Lys[47]. This mass reduction must, however, be located outside the region comprising Asp[55]–Glu[49] in which fragment ions were observed. Furthermore, forced deamidation studies at alkaline pH had identified a second Asn-Gly (Asn[33]-Gly[34]) sequence to be a prime site of deamidation in rHV2-Lys[47] (see Figure 1B) (Tuong et al., 1992). The subfractionation of component A was thus pursued with the aim of identifying a potential second constituent.

Isolation and Characterization of Components A1 and A2. RP-HPLC using gradient slopes of 0.04–0.08% acetonitrile/min showed that component A was indeed composed of at least two molecular species having slightly different retention times. They will be referred to as component A1 and component A2, respectively, according to their elution order from RP-HPLC (Figure 2). Starting with 90 mg of 99% pure rHV2-Lys[47], 120 μg of component A1 and 250 μg of component A2 were isolated indicating that component A2 was the major constituent of component A. Analyses by RP-HPLC (Figure 2C,D) and ESMS showed that cross-contamination between the two components and rHV2-Lys[47] was low and that both had a molecular mass which was approximately 17 Da lower than that of rHV2-Lys[47] supporting the LSIMS results described above.

To obtain more detailed information as to where these mass differences resided, components A1 and A2 were digested with endoprotease Glu-C from *S. aureus* (V8 protease). Isoelectric focusing in an immobilized pH gradient between pH 3.8 and 4.8 was employed to optimize the conditions for proteolytic digestion with regard to preserving potential succinimide structures, since hydrolysis of succinimide intermediates to deamidated forms of rHV2-Lys[47] would be easily detectable with this method (Bischoff et al., 1992). Analysis of component A2 in a gradient between pH 4.0 and 4.4 showed it to be homogeneous with no detectable deamidated forms. Its pI was slightly higher than for rHV2-Lys[47] (ΔpI ≃ 0.01 pH unit), stressing the similarity in surface charge of the two molecules. On the contrary, incubation of component A for 2 h at pH 7.8 and 37 °C (the first pH optimum of V8 protease) resulted in the appearance of a major new protein band with a more acidic isoelectric point (ΔpI = 0.15 pH unit), indicating that deamidation had occurred (not shown). rHV2-Lys[47] proved to be stable under identical incubation conditions. These results gave further credit to the hypothesis that component A contained base-sensitive succinimide structures. Incubation of component A with V8 protease at pH 4, the second pH optimum of the enzyme, did not give any cleavages. Therefore, digestion of components A1 and A2 was performed at pH 6.5 where only minimal deamidation was detected and the crude digest was desalted by RP-HPLC using a rapid acetonitrile gradient in 0.1% aqueous TFA (gradient slope, 5% acetonitrile/min).

The mixture of peptide fragments was subsequently analyzed as such by ESMS in comparison to a digest of rHV2-Lys[47] obtained under identical reaction conditions (Figure 4). The results provided evidence that the mass difference of 17 Da between component A1 and rHV2-Lys[47] resided in a peptide fragment comprising amino acids 1–43 (Figure 4B) while the

Table I: LSIMS m/z Measurements of rHV2–Lys[47] and Component A C-Terminal Fragments[a]

fragments	Y10″	Y11″	Y12″	Y13″	Y14″	Y15″	Y16″	Y17″
calculated m/z rHV2-Lys[47] [b]	1297.4	1412.5	1469.5	1583.6	1697.8	1834.9	1922.0	2051.1
measured m/z[c] rHV2-Lys[47]	1296.5	1411.6	1468.7	1582.8	1696.9	1834.1	1921.4	2050.6
measured m/z[c] component A	1296.6	1411.6	1468.7	1566.9	1679.9	1817.5	1904.5	2033.2
$\Delta m/z$[d]	0.1	0	0	−15.9	−17.0	−16.6	−16.9	−17.4

[a] Nomenclature according to Roepstorff and Fohlman (1984). [b] Calculated m/z values are based on the expected fragments of unmodified rHV2-Lys[47]. All calculated values represent average m/z ratios. [c] Taken from Figure 3. [d] Difference between the measured m/z values of Y″ fragments of rHV2-Lys[47] and component A.

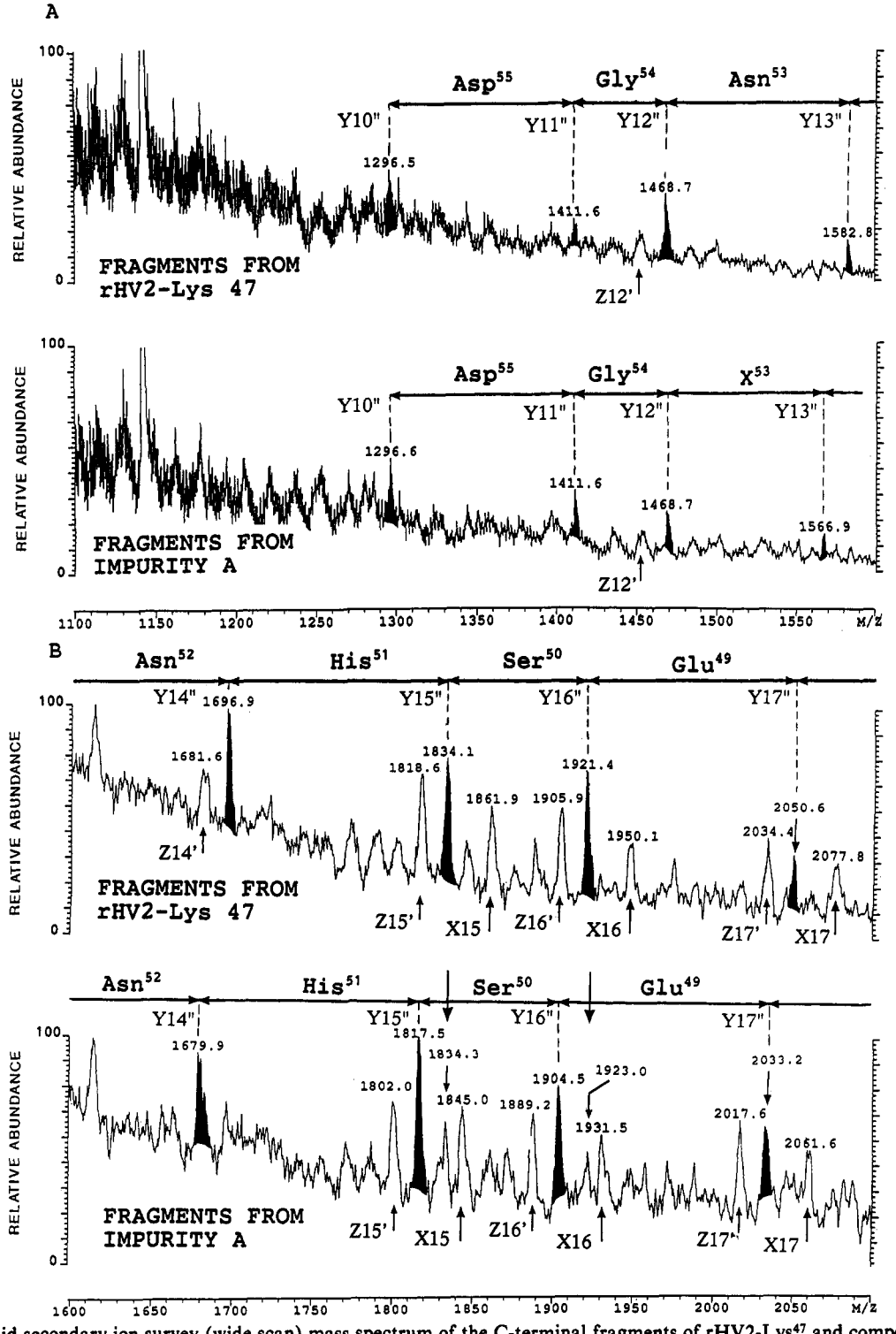
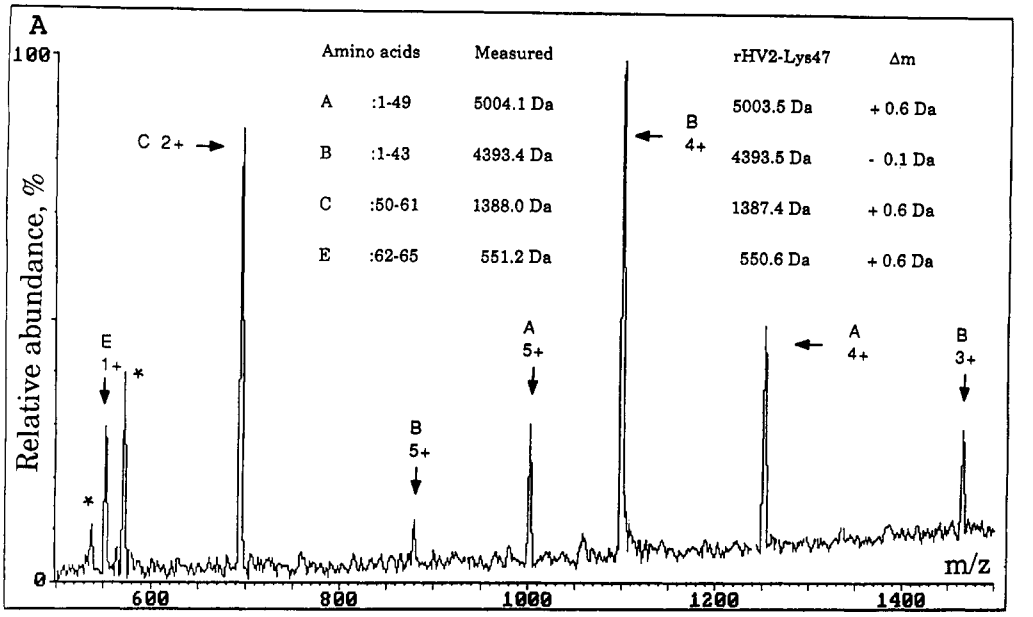
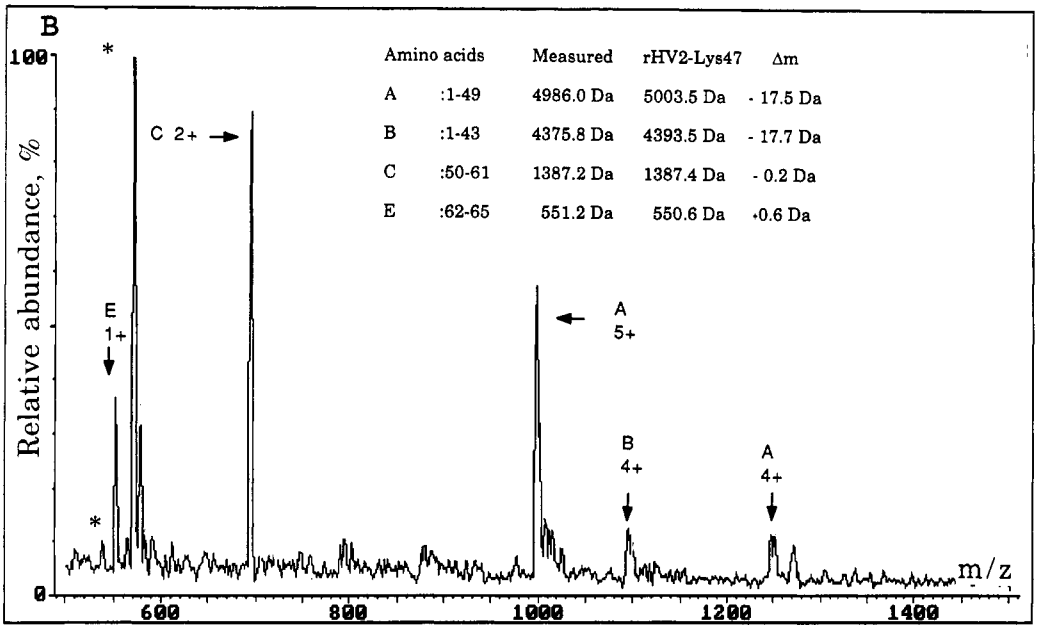


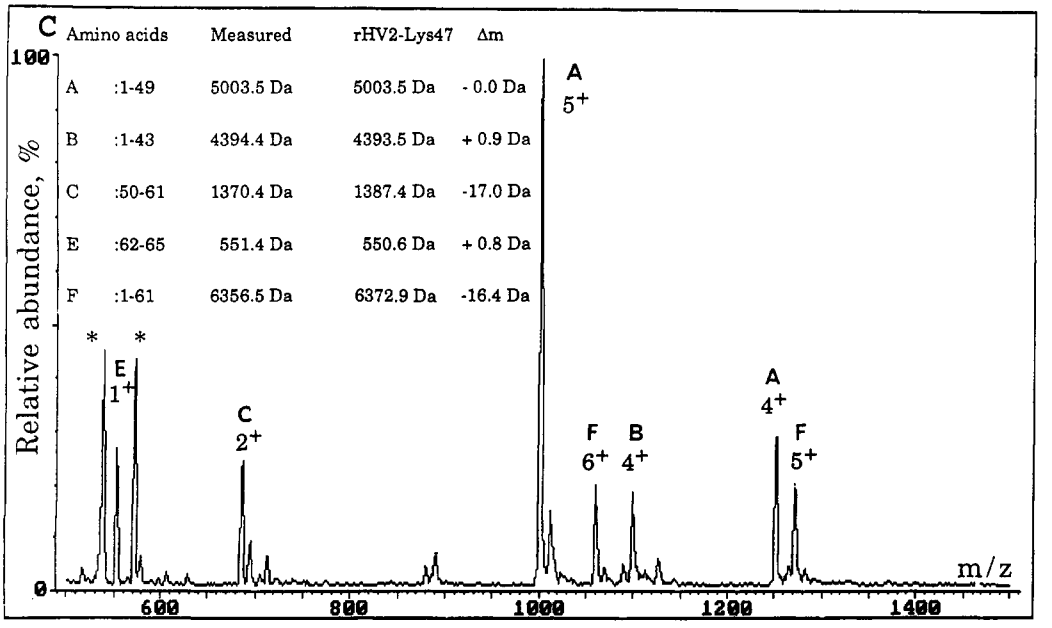
FIGURE 3: Liquid secondary ion survey (wide scan) mass spectrum of the C-terminal fragments of rHV2-Lys[47] and component A. Spectra were recorded at a resolution of 1000 by magnetic scanning of m/z ratios from 2100 to 1100. Fragments of type Y″ (filled-in peaks), Z′, and X [according to Roepstorff and Fohlman (1984)] were observed allowing the reading of the amino acid sequence between Asp[55] and Glu[49] for both samples. The difference in m/z between fragments Y13″ and Y12″ corresponds to that of an Asn residue in the case of rHV2-Lys[47] (measured $\Delta m/z$ = 114.1; expected $\Delta m/z$ = 114.1) but is 15.9 mass units lower for component A (marked at X[53]) (see also Table I). The measured molecular masses for component A and rHV2-Lys[47] (expected mass; 6906.5 Da) are 6890.7 Da and 6907.5 Da, respectively, as determined from scanning of the m/z range between 2000 and 8000 (not shown). The arrows in panel B (m/z = 1834.3 and 1923.0) point to Y″ fragment ions of component A which are also detected in unmodified rHV2-Lys[47], indicating that component A contains another constituent with a mass reduction of 17 Da.

corresponding mass difference between component A2 and rHV2-Lys[47] was localized in the fragment covering amino acids 50–61 (Figure 4C). It was thus established that component A was indeed composed of two different molecules each having a molecular mass which was 17 Da lower than for rHV2-Lys[47] and that these mass differences were located in separate parts of the molecule.

In order to identify the modified sites in components A1 and A2, complementary information from ESMS with CAD as well as from amino acid sequencing was obtained. To this end, component A2 (the major constituent of component A) was digested with V8 protease, and the peptide fragment


A
100
Relative abundance, %
0
Amino acids Measured rHV2-Lys47 Δm
A :1-49 5004.1 Da 5003.5 Da + 0.6 Da
B :1-43 4393.4 Da 4393.5 Da - 0.1 Da
C :50-61 1388.0 Da 1387.4 Da + 0.6 Da
E :62-65 551.2 Da 550.6 Da + 0.6 Da
C 2+
B 4+
E 1+
A 5+
A 4+
B 3+
B 5+
600
800
1000
1200
1400
m/z


B
100
Relative abundance, %
0
Amino acids Measured rHV2-Lys47 Δm
A :1-49 4986.0 Da 5003.5 Da - 17.5 Da
B :1-43 4375.8 Da 4393.5 Da - 17.7 Da
C :50-61 1387.2 Da 1387.4 Da - 0.2 Da
E :62-65 551.2 Da 550.6 Da +0.6 Da
C 2+
E 1+
A 5+
B 4+
A 4+
600
800
1000
1200
1400
m/z


C
100
Relative abundance, %
0
Amino acids Measured rHV2-Lys47 Δm
A :1-49 5003.5 Da 5003.5 Da - 0.0 Da
B :1-43 4394.4 Da 4393.5 Da + 0.9 Da
C :50-61 1370.4 Da 1387.4 Da -17.0 Da
E :62-65 551.4 Da 550.6 Da + 0.8 Da
F :1-61 6356.5 Da 6372.9 Da -16.4 Da
A 5+
E 1+
C 2+
F 6+
B 4+
A 4+
F 5+
600
800
1000
1200
1400
m/z

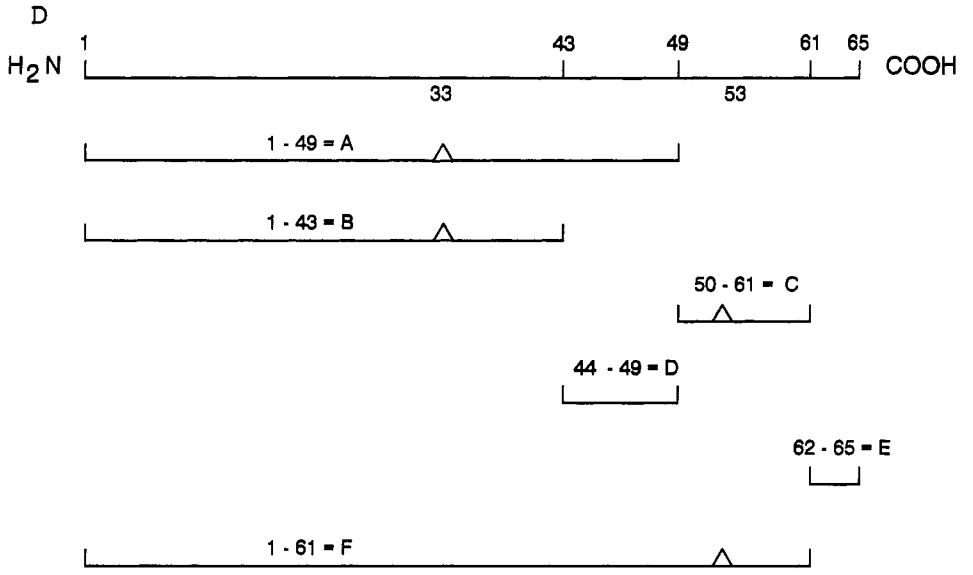


FIGURE 4: Electrospray mass spectra of the V8 proteolytic digests of rHV2-Lys[47] (A), component A1 (B), and component A2 (C). (D) Nomenclature of peptide fragments according to Van Dorsselaer et al. (1990). Twenty-five micrograms of both rHV2-Lys[47] and component A2 and 10 μg of component A1 were digested and desalted by RP-HPLC as described in Experimental Procedures. The samples were then injected into the ion source at concentrations between 10 and 20 pmol/μL at a flow rate of 2 μL/min. The measured molecular masses of fragments corresponding to the labeled peaks are listed in comparison to expected values derived from rHV2-Lys[47]. Cleavages were found after Glu[43], Glu[49], and Glu[61] but not following Glu[8], Glu[17], Glu[57], Glu[58], and Glu[62] under the conditions used (see Figure 1B and Experimental Procedures). Peaks labeled with asterisks were derived from the internal calibration standard gramicidin S which was continuously injected during the measurements to ensure correct performance of the mass spectrometer. Fragment D (m/z = 627.7) was not observed during these analyses. Triangles symbolize the locations of succinimides at Asn[33] and Asn[53], respectively (D).

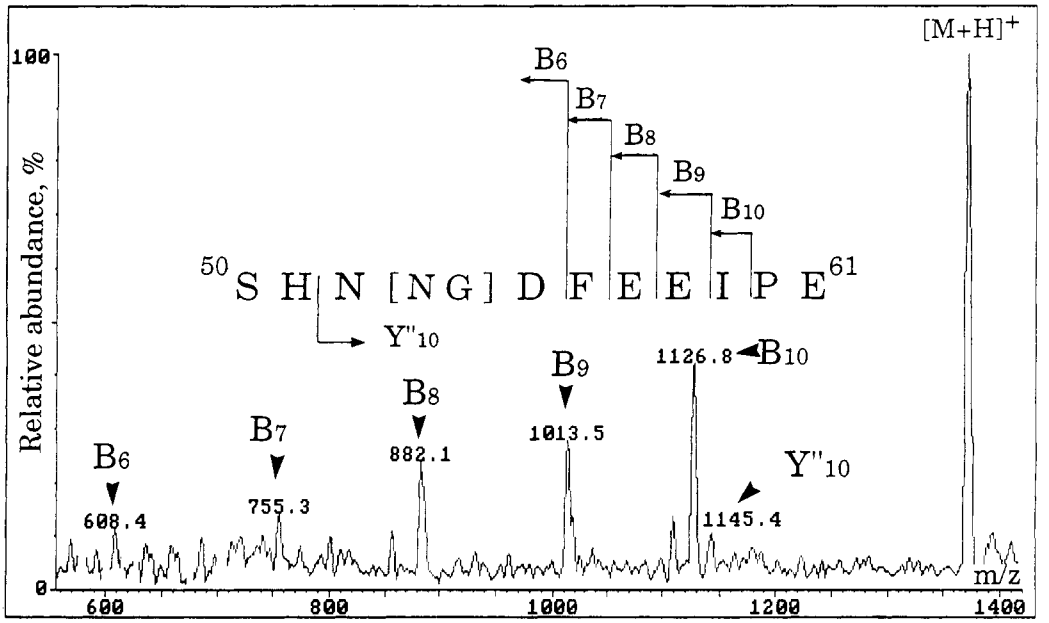


FIGURE 5: Collision activated dissociation (CAD) mass spectrum of the fragment Ser[50]–Glu[61] obtained after proteolytic digestion of component A2 with V8 protease. C- and N-terminally truncated fragments were identified on the basis of the m/z values for the respective $[M + H]^+$ ions. The measured m/z ratios corresponded well to the expected values of fragments from rHV2-Lys[47] with a reduced m/z of 17 [fragment definition as in Roepstorff and Fohlman (1984)]: B6 (measured m/z = 608.4; expected m/z = 625.4; $\Delta m/z$ = –17.0); B7 (measured m/z = 755.3; expected m/z = 772.3; $\Delta m/z$ = –17.0); B8 (measured m/z = 882.1; expected m/z = 901.3; $\Delta m/z$ = –19.2); B9 (measured m/z = 1013.5; expected m/z = 1030.4; $\Delta m/z$ = –16.9); B10 (measured m/z = 1126.8; expected m/z = 1143.5; $\Delta m/z$ = –16.7); and Y10″ (measured m/z = 1145.4; expected m/z = 1161.5; $\Delta m/z$ = –16.1). The molecular mass of the peptide fragment ($[M + H]^+$) was 1370.3 ± 0.1 Da which is 17.1 Da lower than the expected mass for the same peptide fragment from rHV2-Lys[47]. The Y″9 fragment ion (expected m/z 1030.4) was not observed. Asn[53] and Gly[54] are written in brackets, since they are assumed to be linked by a succinimide ring structure. The single-letter amino acid code is used.

corresponding to amino acids 50–61 was isolated by RP-HPLC and subjected to CAD in the ion source of the mass spectrometer (Figure 5). Mass spectrometric analysis of the fragments obtained by CAD showed that the 17-Da mass difference between the corresponding peptide from rHV2-Lys[47] and component A2 was localized outside the region comprising Ser[50]-His[51] and Phe[56]–Glu[61] leaving the sequence Asn[52]–Asp[55] as the potential site of modification. Since earlier results obtained by LSIMS of component A had shown that a modification resided at residue Asn[53] (Table I), N-terminal amino acid sequencing of fragment 50–61 from component A2 by Edman degradation was undertaken to probe this site further. As shown in part A of Table II, a clear sequence was read from residues Ser[50] to Asn[52] which stopped at Asn[53],

Table II

(A) Amino Acid Sequencing of Fragment Ser^{50}–Glu^{61} from V8 Protease Digests of rHV2-Lys^{47} and Component A2

amino acid	Ser^{50}	His^{51}	Asn^{52}	Asn^{53}	Gly^{54}	Asp^{55}	Phe^{56}	Glu^{57}	Glu^{58}	Ile^{59}	Pro^{60}	Glu^{61}
yield: rHV2-Lys^{47} [a]	34	5	27	11	28	13	16	12	8	10	11	0.1
yield: component A2[a]	27	7	27	0	2	0	0	0	0	0	0	0

(B) N-Terminal Amino Acid Sequencing of rHV2-Lys^{47} and Component A1 (Amino Acids 1–2 and 26–37 Are Shown)

amino acid	Ile^{1}	Thr^{2}	Asn^{26} [b]	Lys^{27}	Cys^{28}	Ile^{29}	Leu^{30}	Gly^{31}	Ser^{32}	Asn^{33} [b]	Gly^{34}	Lys^{35}	Gly^{36}	Asn^{37}
yield: rHV2-Lys^{47} [c]	3419	1957	646	669	nd[d]	781	831	658	191	404	459	327	458	356
yield: component A1[c]	3449	2120	437	259	nd[d]	267	323	266	103	92	82	16	112	34

[a] The yield is given in picomoles after background subtraction. [b] Repetitive sequencing yields: between Asn^{26} and Asn^{33} (rHV2-Lys^{47}, 93.5%; component A1, 80.0%) and between Asn^{20} and Asn^{26} (rHV2-Lys^{47}, 97.2%; component A1, 97.1%). [c] The yield is given in picomoles after background subtraction and lag correction. [d] Not detectable (the samples were neither reduced nor alkylated).

indicating that Asn^{53} was involved in a structure that prevented Edman degradation from continuing. A similar amount of fragment 50–61 obtained from a V8 protease digest of rHV2-Lys^{47} gave the expected amino acid sequence without any noticeable reduction in yield at Asn^{53} (Table II, part A). The low sequencing yield of Glu^{61} in the last cycle was most likely due to loss of the amino acid from the sequencing support. Thus, the results obtained through mass measurements by LSIMS and ESMS in conjunction with CAD as well as by N-terminal sequencing provided strong evidence that component A2 was a stable succinimide intermediate of the deamidation reaction of Asn^{53} which opened during amino acid sequencing to form an isoaspartyl linkage unable to propagate the Edman degradation reactions further (Aswad, 1984).[2]

Component A1, the second constituent of component A, was also analyzed by N-terminal amino acid sequencing, as previous results by mass spectrometry had shown that a modification resided within fragment 1–43. The fact that rHV2-Lys^{47} contained five Asn residues between amino acids 1 and 43 (Asn^{12}, Asn^{20}, Asn^{26}, Asn^{33}, and Asn^{37}; Figure 1B) allowed the calculation of repetitive sequencing yields between pairs of Asn residues and the use of these as a criterion for the efficiency of the Edman degradation reactions over the first 37 sequencing cycles. This analysis showed that repetitive sequencing yields between Asn^{12}, Asn^{20}, and Asn^{26} were in the range of 93–97% for both rHV2-Lys^{47} and component A1. However, a significant drop in repetitive yield was observed between Asn^{26} and Asn^{33} of component A1 (80.0%) while no such reduction in yield was detected in the case of rHV2-Lys^{47} (93.5%) (Table II, part B). These data together with the mass spectrometric analyses indicated the presence of a succinimide ring structure between Asn^{33} and Gly^{34} as an intermediate of the deamidation reaction which had previously been shown to occur at this position (Tuong et al., 1992).

α-Thrombin inhibition by component A1 and component A2 was measured under steady-state conditions in comparison with nonmodified rHV2-Lys^{47} to assess whether the succinimide ring structure would interfere with the thrombin–hirudin interaction. The measured inhibition constants were in the range of 1 pM for all of the molecules indicating that formation of the thrombin–hirudin complex was not affected by succinimides at either Asn^{33}-Gly^{34} or Asn^{53}-Gly^{54} (Table III). As the kinetic measurements necessitated a preincubation at pH 7.8 and 37 °C for 5 min to reach equilibrium, it could not be entirely excluded that some of the succinimide structures hydrolyzed to the deamidated forms. The time course of the inhibition kinetics indicated, however, that there were no forms of rHV2-Lys^{47} in the reaction mixture which had significantly reduced inhibition constants for α-thrombin.

Table III: Inhibition of Human α-Thrombin by rHV2-Lys^{47}, Component A1, and Component A2

	K_i'[a] (pM)	K_i[b] (pM)
rHV2-Lys^{47}	32.73 ± 1.43[c]	1.15
component A1	32.85 ± 1.61	1.15
component A2	27.59 ± 1.81	0.96

[a] K_i': apparent inhibition constant (see eq 1 in Experimental Procedures). [b] K_i: inhibition constant derived from K_i' using: $K_i' = K_i(1 + S/K_M)$ with S being the substrate concentration and K_M = 3.63 μM according to Stone and Hofsteenge (1986). [c] Standard errors of the nonlinear regression analysis.

DISCUSSION

Deamidation of Asn residues in proteins may proceed without enzymatic catalysis under physiological conditions. This reaction has therefore been thought to be related to protein half-life in vivo (Robinson et al., 1970). A recent survey of deamidation sites in a large number of published protein sequences provided evidence that certain amino acid combinations which facilitate deamidation, such as Asn-Gly or Ser-Asn, were counterselected for in nature, since their natural abundance was significantly lower than the statistical average (Wright, 1991; Robinson & Robinson, 1991). These studies support the hypothesis that deamidation of Asn residues has a physiological function in controlling the in vivo half-life of proteins.

Deamidation of Asn residues has also been shown to lead to inactivation of certain proteins and peptides such as lysozyme (Ahern & Klibanov, 1985), triosephosphate isomerase (Yuan et al., 1981; Casal et al., 1987; Ahern et al., 1987), adrenocorticotropin (Graf et al., 1973), and calmodulin (Johnson et al., 1989a). In the case of the recombinant hirudin variant rHV2-Lys^{47}, no loss in inhibitory activity against human α-thrombin upon deamidation has been observed.[3] Additionally, recombinant hirudin variant 1 (HV1) which shows strong sequence homology to rHV2-Lys^{47} but contains aspartic acid residues in positions 33 and 53 proved to be a very efficient thrombin inhibitor with a K_i below 1 pM (Braun et al., 1988). The work presented here supports these results, since it shows that the two succinimide intermediates of rHV2-Lys^{47} have identical inhibitory constants compared to the parent molecule indicating that the two sites which are modified by succinimide ring structures (Asn^{33}-Gly^{34} and Asn^{53}-Gly^{54}) are not in direct contact with the protease.

[2] B. Violand, poster presentation at the 5th Symposium of the Protein Society in Baltimore, MD, June 1991.

[3] O. Whitechurch and C. Roitsch, unpublished experiments.

Structural studies on hirudin by NMR spectroscopy (Folkers et al., 1989; Haruyama & Wüthrich, 1989) and on the hirudin–α-thrombin complex by X-ray crystallography (Rydel et al., 1990, 1991; Grütter et al., 1990) did not allow the definition of the regions around Asn^{33} or Asn^{53} (Rydel et al., 1991). However, the X-ray crystallographic analyses demonstrated that the loop containing Asn^{33} was completely free of interactions with α-thrombin, corroborating our results on the inhibition kinetics. The fact that no electron density could be found for either positions Asn^{33}-Gly^{34} or Asn^{53}-Gly^{54} might result from the chemical instability of these two positions leading to a slow conversion of Asn to Asp under the conditions of crystal growth (Figure 1A). The resulting molecular heterogeneity could be the reason for the ill-defined electron densities that were obtained. However, the absence of clearly defined electron densities at these two sites might also be the result of conformational flexibility in these regions suggesting that flexibility around Asn residues is an important parameter for the formation of the more rigid succinimide cycle and thus for deamidation to occur.

Another area of research where understanding of the mechanisms underlying facilitated deamidation of Asn residues plays an important role is related to protein stability. As more and more recombinant proteins are being used as human therapeutics, evaluation of their stability during fermentation, purification, and final storage is becoming an issue of increasing research efforts (Patel & Borchardt, 1990). The charge heterogeneity which is frequently observed when purified proteins are analyzed by isoelectric focusing especially in high-resolution immobilized pH gradients points to the fact that microheterogeneity is a more common phenomenon than was previously anticipated. While glycosylated proteins are well-known to be heterogeneous upon isoelectric focusing due to different amounts of sialic acid per molecule, it has also been observed that this heterogeneity was not completely eliminated after enzymatic deglycosylation (Anicetti et al., 1989). Furthermore, non-glycosylated recombinant proteins produced in *Escherichia coli* or *S. cerevisiae* have been shown to present some heterogeneity on analysis by isoelectric focusing in immobilized pH gradients (Bischoff et al., 1992, 1991). These observations emphasize the necessity of gaining a better understanding of the structural parameters favoring deamidation including the underlying reaction mechanisms. This should lead to a more rational approach toward improving the stability of purified proteins.

An important step toward elucidating the reaction mechanism of deamidation was the finding that a succinimide ring was formed by nucleophilic attack on the carbonyl carbon of the side chain amide by the peptide bond nitrogen following an Asn residue (Bornstein & Balian, 1977). Such an intermediate was consistent with the finding that the deamidation products of Asn-Gly-containing peptides contained not only L-Asp residues but also L-isoaspartyl as well as D-Asp and D-isoaspartyl linkages. These modified amino acids could have only arisen from hydrolysis and/or enolization of a putative succinimide intermediate (Figure 1A). Until recently, no such intermediate of a protein has been isolated and characterized in its purified form most likely due to the chemical lability of the succinimide ring structure and the difficulties in separating it from the parent molecule. Most recently, Teshima et al. were successful in isolating an intermediate of rhGH and in showing that it contained a succinimide cycle between Asp^{130} and Gly^{131} (Teshima et al., 1991). Succinimide formation was induced at an Asp-Gly site by heat treatment of the lyophilized protein which led to the loss of water. This result supported calculations of Geiger and Clarke showing that Asp residues should be able to form succinimide structures at pH 7.4, albeit at slower reaction rates than for an Asn residue (Geiger & Clarke, 1987). Other authors found that succinimide formation at an Asp-Gly site in recombinant porcine growth hormone was enhanced at acidic pH values (pH 3–6.5) in agreement with the fact that protonation of the side chain carboxylate of Asp should facilitate nucleophilic attack of the peptide bond nitrogen resulting in the elimination of water.[2]

The work on rHV2-Lys^{47} shows that low amounts of succinimide intermediates are formed at the two Asn-Gly sites in the molecules (Asn^{33}-Gly^{34} and Asn^{53}-Gly^{54}) and that these intermediates are sufficiently stable to be isolated and biochemically characterized. The presence of these intermediates at the end of a production process which did not use extremes of either pH or temperature indicates that their formation is facilitated by the local structure around the two Asn residues. Our results thus confirm and extend earlier findings that Asn-Gly sites are particularly susceptible to deamidation and that this reaction involves succinimide intermediates. While rHV2-Lys^{47} contains seven Asn residues, only those two followed by Gly were found to deamidate most rapidly (Tuong et al., 1992) and to form succinimides. That the succinimide cycles found in rHV2-Lys47 are generated from the Asn-Gly sequence rather than the deamidated Asp-Gly sequence is supported by the fact that no deamidated forms are found either in the starting material or in the highly purified fraction (Figure 2A). The deamidated forms are easily identified by isoelectric focusing or anion-exchange HPLC, and once formed are relatively stable. According to previous work on synthetic peptides containing an Asn-Gly site (Geiger & Clarke, 1987), it is unlikely that the succinimide cycle can form without a detectable amount of the deamidated form, since the equilibrium tends to be in favor of the deamidated form. The succinimide intermediates constituted less than 1% of total protein in the starting material, and they resembled unmodified rHV2-Lys^{47} to such an extent that all high-resolution separation techniques based on differences in surface charge (anion-exchange HPLC, cation-exchange HPLC, and isoelectric focusing) proved to be unsuccessful for their isolation. Only the slightly increased hydrophobicity of the succinimides as compared to the amide forms allowed their isolation by RP-HPLC under essentially isocratic elution conditions. The chemical stability of the succinimide structure was most surprising, as it withstood incubation in 0.1% aqueous TFA at 65 °C for at least 1 h without any sign of degradation. However, its lability at alkaline pH gave rise to the deamidated products as expected.

Our results and those of other investigators have shown that succinimide formation might be a more common phenomenon in proteins containing Asn-Gly sites than was previously anticipated and that the stability of at least some of these deamidation intermediates allows their isolation. It is mainly due to the progress of advanced analytical techniques such as mass spectrometry of high molecular mass compounds that structural characterization of such intermediates has become feasible even if they represent only 0.3% of total protein in the starting material as was the case for component A1.

Natural hirudins can only be isolated in microgram amounts (Scharf et al., 1989), making detection of potential succinimide intermediates at the level of 1% of total protein very difficult. While no such intermediates have been observed in a recent preparation of HV2-Lys^{47} from leech heads (Jaquinod et al., unpublished experiments), it is likely that they exist, since

numerous proteins have been observed to deamidate in vivo during their natural life span. Therefore, these intermediates must also exist in the living organism, albeit for a limited time period. Early investigations corroborate this fact, since dipeptides with isoaspartyl linkages have been found in the urine of healthy individuals with the dipeptide iso-Asp-Gly being by far the most abundant species (Pisano et al., 1966; Dorer et al., 1966). These results indicate that succinimide intermediates are naturally present in humans as a result of the deamidation of proteins containing Asn-Gly sites. As iso-Asp-Gly proves resistant to further proteolytic degradation, it is excreted into the urine as the major dipeptide component. Succinimide formation should thus be considered as part of the structural variety of natural proteins adding another facet to the complex picture of protein structures. It is conceivable that more of these "unusual" protein modifications will be discovered as the power of modern analytical methods increases.

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