Characterization of the Deamidated Forms of Recombinant Hirudin

Anne Tuong,[‡] Mohamed Maftouh,[‡] Christian Ponthus,[‡] Odile Whitechurch,[§] Carolyn Roitsch,[§] and Claudine Picard*,[‡]

Sanofi Recherche, 195 route d'Espagne, 31036 Toulouse Cédex, France, and Transgène, 11 rue de Molsheim, 67084 Strasbourg, France

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ABSTRACT: Recombinant hirudin variant rHV_2-Lys 47 (MW = 6906.5) was intentionally deamidated by incubation in pH 9 phosphate buffer at 37 °C. Anion-exchange HPLC analysis showed that 11 forms could be generated. These were isolated and purified by combined anion-exchange and reversed-phase HPLC. Acid-catalyzed carboxyl methylation was used to introduce a mass shift of +15 amu per deamidated residue present in the molecule before analysis by liquid secondary ion mass spectrometry (LSIMS). Methylation enhanced, in particular, the abundance of the sequence ions in the LSIMS spectra. This permitted the determination of both the number (three) and the localization of the deamidated residues: Asn 52, Asn 53, and a residue located in the N-terminal 1–39 domain. Complementary sequencing techniques proved that the latter residue was Asn 33. Altogether four mono-, three di-, and four tri-deamidated forms were identified. The heterogeneity of the forms having identical deamidation positions but being chromatographically separable is thought to arise from the generation of α- and β-aspartyl iso forms during the nonenzymatic deamidation process.

Nonenzymatic deamidation of asparagine (Asn) and glutamine (Gln) residues in peptides and proteins may occur spontaneously at physiological temperature and pH. Robinson suggested that these reactions could serve as general molecular timers of development and of protein turnover (Robinson et al., 1970; Robinson & Rudd, 1974). Deamidation may also lead to a lowered susceptibility toward proteolytic attacks as reported for deamidated forms of human growth hormone (Lewis et al., 1986) and seminal ribonuclease (Di Donato et al., 1989).

The half-time of deamidation is generally much shorter for asparagine than for glutamine residues. In addition, it depends on the nature of the following adjacent amino acid (Capasso et al., 1989). In this context, glycine (Gly) has been shown to be a powerful activator for this type of reaction (Aswad, 1984; Murray & Clarke, 1984; Geiger & Clarke, 1987; Lura & Schirch, 1988).

Studies using short model peptides show that deamidation of Asn in the Asn-Gly sequence involves intramolecular cyclization to a succinimide intermediate, which upon hydrolysis generates a normal aspartyl (Asp) and an isoaspartyl (iso-Asp) peptide linkage in a ratio of approximately 1:3. Interestingly, the iso-Asp residue is a highly potential substrate for protein carboxymethyl transferases and has been implicated as a biological signal for the degradation or the repair of proteins (O'Connor & Clarke, 1983; Aswad, 1984; Murray & Clarke, 1984; O'Connor et al., 1984; Clarke, 1985; Johnson & Aswad, 1985).

The recombinant hirudin variant rHV_2-Lys 47 (hirudin), a specific thrombin inhibitor produced in *Saccharomyces cerevisiae* (Harvey et al., 1986; Loison et al., 1988), contains two Asn-Gly sequences: residues 33–34 and 53–54 (Figure 1). As deamidation of these residues could occur during processing and purification, there is interest in these forms from a pharmaceutical point of view.

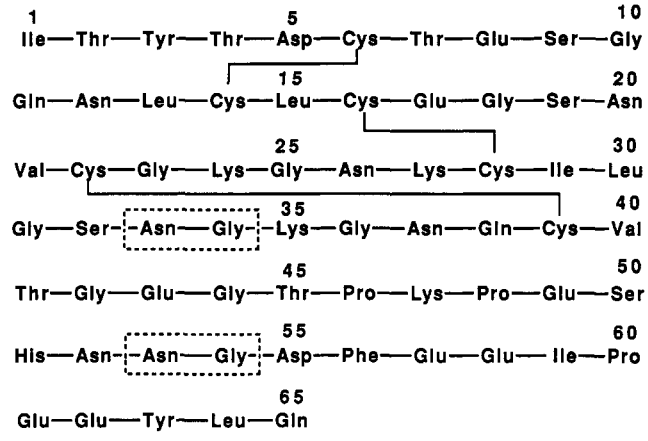

FIGURE 1: Primary structure of recombinant hirudin variant rHV_2-Lys 47, according to Harvey et al. (1986), with residue 47 as Lys instead of Asn as reported (Degryse et al., 1989). The two Asn-Gly sequences are outlined.

The first challenge was to develop an analytical system capable of separating the deamidated forms of hirudin, and the second challenge, to devise a simple way of characterizing the purified forms.

Mass spectrometry (MS) was previously shown to be a valuable tool for characterizing hirudin (MW = 6906.5) and related derivatives (Van Dorsselaer et al., 1989; Maftouh et al., 1991). However, deamidation of a single residue (Asn or Gln) results in the addition of only 1 mass unit (amu)[1] to the mass of the parent compound, and this is within the precision of the method in this high mass range regardless of the ionization mode used: liquid secondary ion mass spectrometry

* To whom correspondence should be addressed.
‡ Sanofi Recherche.
§ Transgène.

[1] Abbreviations: LSIMS, liquid secondary ion mass spectrometry; amu, atomic mass unit; EMS, electrospray mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; IE-HPLC, anion-exchange high-performance liquid chromatography; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PDMS, plasma desorption mass spectrometry; MH^+, protonated molecular ion; m/z, mass-to-charge ratio; Da, dalton.

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(LSIMS) (Richter et al., 1985; Van Dorsselaer et al., 1989) or electrospray mass spectrometry (EMS) (Van Dorsselaer et al., 1990). The objective was therefore to introduce by chemical methylation of all carboxyl groups a +15 amu shift for each deamidated residue present in the molecule, which is then easily measured.

The present study reports on the isolation of deamidated forms of hirudin by anion-exchange (IE-HPLC) and reversed-phase HPLC (RP-HPLC) and their characterization by mass spectrometry following carboxyl methylation. LSIMS analyses showed that three asparaginyl residues could undergo deamidation. In addition, esterification was very effective in enhancing the abundance of the sequence ions in the LSIMS spectra. This feature provided determination of both the number and the positions of the deamidated residues in a single analysis.

MATERIALS AND METHODS

Chemicals

Recombinant hirudin (variant rHV_2-Lys 47), produced in the yeast *S. cerevisiae*, was obtained from Sanofi Elf Bio-Recherche (Labège, Innopole, France). HPLC-grade S acetonitrile was obtained from Rathburn (Mannheim, Germany). Trifluoroacetic acid (TFA) was purchased from Janssen (Beers, Belgium) and acetyl chloride from Fluka (Buchs, Switzerland). Lichrosolv-grade methanol from Merck (Darmstadt, Germany) was dried over 4-Å molecular sieves for 48 h before use in methylation reactions. All other chemicals were of reagent grade. The aqueous solutions were made with Milli-Q purified water (Millipore, Bedford, MA).

High-Performance Liquid Chromatography (HPLC)

Anion-Exchange (IE-HPLC). The time course of deamidation was monitored by IE-HPLC using a Waters chromatograph (Millipore, MA), which consisted of two Model 510 solvent pumps, a 680 E gradient controller, a 441M fixed-wavelength detector at 214 nm, a manual U6K injector, and a 740 data integrator.

Separations and purifications were performed on a ProPac PA1 anion-exchange column (250 × 4.0 mm i.d.) and precolumn (50 × 4.0 mm i.d.) from Dionex (Sunnyvale, CA). Both contained 0.2-μm resin microbeads bonded to 10-μm polymeric particles. Solvent A was 20 mM Tris-HCl, pH 7.0, and solvent B was 0.5 M sodium chloride in A. Isocratic elution for 5 min at 28% B was followed by a linear gradient from 28% to 54% B over 60 min at a flow rate of 1.3 mL/min.

Reversed-Phase (RP-HPLC). Samples collected in the IE-HPLC mode were desalted by RP-HPLC using a Hewlett-Packard (Waldbronn, Germany) 1084B liquid chromatograph equipped with a variable-wavelength detector (set at 218 nm) and a Rheodyne 7125 injector (Cotati, CA) with a 2-mL injection loop. The column used was an Aquapore RP-300 cartridge (C8, 3 × 0.46 cm, 300 Å, 7 μm) from Brownlee Labs (Santa Clara, CA). The mobile phase was solvent A, 0.1% TFA in water, and solvent B, 0.1% TFA in water/acetonitrile (50:50, v/v). Linear gradient elution was performed from 0% B to 10% B in 1 min, then to 60% in 6 min, at a flow rate of 1.5 mL/min (conditions A).

Optimization of the methylation conditions and sample purifications was monitored on a 10-cm Aquapore column (same packing material as above) using a linear gradient from 20% B to 60% B over 30 min, at 1 mL/min (conditions B). The eluents were the same as in conditions A.

The materials collected in either RP-HPLC mode were dried in a Gyrovap vacuum centrifuge (VA Howe, London, U.K.) before further processing or analysis.

Deamidation of Hirudin

Time Course of Deamidation. Hirudin (1 mg/mL) was incubated in 0.17 M Na_2HPO_4 buffer, pH 9, at 37 °C with shaking. Ten microliter aliquots were removed at appropriate times between 1 and 168 h and analyzed by IE-HPLC.

Purification of Deamidated Forms. For micropreparative work, the deamidation process was accelerated by increasing the salt concentration of the mixture to 1 M, maintaining a 168-h reaction time. The other conditions were as above. Of this preparation, 100 μL was mixed with the same volume of hirudin incubated for 6 h in dilute buffer (0.17 M). Following dilution twice with 20 mM Tris-HCl, pH 7, the entire mixture was injected onto the anion-exchange column. Peaks were collected from successive runs and desalted by RP-HPLC (conditions A). The fractions were further purified by repeating the two HPLC steps until nearly 100 μg quantities of each peak were obtained with a purity greater than 90%.

Carboxyl Methylation

Methylation was carried out in 1.5-mL polypropylene tubes containing 2–10 nmol of dried polypeptide. One hundred microliters of 2 M acetyl chloride in dry methanol (HCl/methanol) was added. After vortexing for a few seconds, the reaction was allowed to proceed at room temperature for 15 min unless otherwise stated and was terminated by the addition of a 4-fold volume of water. Each mixture was kept on ice until analysis. To establish the optimal esterification time, aliquots of the reaction mixture were removed at designated times between 5 and 120 min and analyzed by RP-HPLC (conditions B).

Mass Spectrometry

All experiments were performed upon a double-focusing (BE geometry) ZAB-2E mass spectrometer (VG Analytical, Manchester, U.K.). The instrument was equipped with a cesium gun operating at 35-kV energy and 2-μA current. The source pressure with the cesium beam operating was approximately 5×10^{-6} Torr.

LSIMS spectra were generated by exponential down magnetic scanning over a mass interval of 1000–9000 amu with a period of 20 s. A resolving power of 800 was employed at an acceleration potential of 8 kV. Calibration and mass measurements of the processed data were carried out by using separate introduction of cesium iodide. All data were acquired, stored, and processed with Opus 2000 software on a Vax station data system.

The methylated derivatives of hirudin and its deamidated forms were dissolved in water/methanol (7:3, v/v) to a concentration of about 10 μg/μL. The matrix used was glycerol/thioglycerol/TFA (1:1:0.01, v/v/v). Appropriate peaks in the spectra were assigned according to the nomenclature of Roepstorff and Fohlman (1984).

Edman Degradation

All protein sequence data were obtained using an Applied Biosystems 470A protein sequencer with an on-line 120A PTH analyzer.

Tryptic Digestion. Deamidated forms of hirudin generated at pH 9 were digested with trypsin (Boehringer, Mannheim, Cat. No. 1047841, enzyme:substrate ratio, 20% w/w) at 37

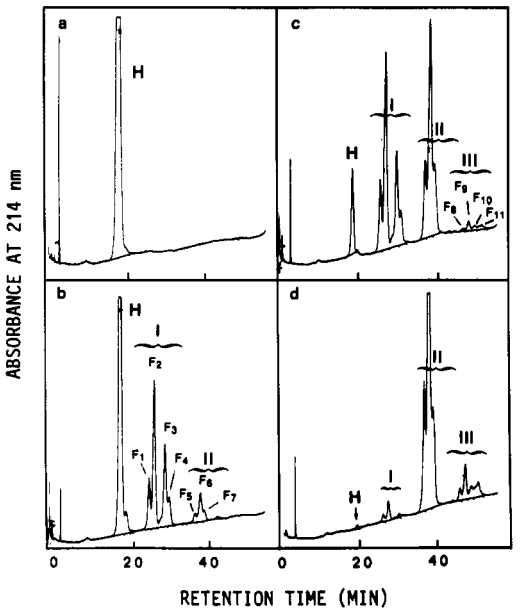

FIGURE 2: IE-HPLC monitoring of the time course of deamidation of hirudin. Representative chromatograms obtained before (a) and after 6 h (b), 24 h (c), and 168 h (d) of incubation in 0.17 M Na_2HPO_4 buffer, pH 9, at 37 °C. For the chromatographic conditions, see Materials and Methods.

°C for 2 h in 0.05 M *N*-ethylmorpholine, pH 8.3, containing 0.1 mM calcium chloride. The reaction was stopped by addition of TFA to a final concentration of 0.1%.

RESULTS AND DISCUSSION

Deamidation. Incubation of hirudin in aqueous Na_2HPO_4 solution (0.17 M, pH 9 at 37 °C) led to the formation of a total of 11 peaks, as judged from IE-HPLC analysis (Figure 2). According to their characteristic appearance in the chromatograms they could be classified in three groups, I, II and III. The first one, consisting of four peaks, F_{1-4}, appeared rapidly within 6 h of incubation (Figure 2b). Three additional peaks, F_{5-7}, assigned to the second group were also distinguished at this time point. This group became predominant in the next 24–168 h at the expense of hirudin and the first group, both of which were barely visible at the end of the experiment (Figure 2c,d). A third group of four peaks, F_{8-11}, was detectable after 24 h of incubation but progressed only very slowly in intensity with time (Figure 2c,d).

It should be emphasized that, at any time point of incubation, the relative intensities of the peaks within each group remained essentially constant. Peaks F_{1-4} appeared, for example, in constant area-ratios of approximately 2:6:3:1, and F_{5-7} in a 1:3:1 ratio. Only the appearance/disappearance of each group was directly linked to time or the incubation conditions used. These particularities clearly indicated that deamidation was not a random process.

With the aim of accelerating the formation of the third group for isolation purposes, the effect of salt concentration and pH on its formation was investigated. Indeed, simply increasing the salt concentration from 0.17 to 1 M during the 168 h of incubation enabled production of the third group in almost the same amounts as the second one. Substitution of the 1 M pH 9 phosphate buffer with 0.1 N NaOH resulted in an even more rapid generation of this group, but as a substantial loss of material was also noticed (peptide hydrolysis?), this method was abandoned.

To associate all groups in comparable amounts for peak collections, equal volumes of two deamidation preparations of hirudin, one for 6 h in 0.17 M Na_2HPO_4 and the other for 168 h in 1 M Na_2HPO_4, were mixed just before injection into the anion-exchange column. This facilitated the preliminary isolation of all 11 peaks in a single run. However, due to the relatively poor resolution of the peaks, at least three to four sequential IE/RP-HPLC runs were required to obtain the compounds with a purity superior to 90%. Varying the elution conditions, such as the pH, had only little effect on separation. Neither did a shallower gradient provide substantial improvements. A compromise between optimal separation and speed was found with the actual elution conditions used (see Materials and Methods).

Two other anion-exchange columns were tested for their ability to separate hirudin deamidated forms, a Mono-Q column from Pharmacia and a PL-SAX column from Polymer Laboratories Ltd, but compared to the ProPac PA1 column (Dionex) used, they were both less effective (results not shown).

Carboxyl Methylation. LSIMS has been successfully applied to the characterization of peptides with molecular weights of 5–10 kDa (Van Dorsselaer et al., 1989; Maftouh et al., 1991). In order to increase sensitivity, but also to avoid misinterpretation of the monoisotopic peak within the complex molecular ion cluster, molecular weight measurements were only performed at low resolution (i.e., 800). Hence, the molecular cluster was not resolved and the average chemical mass could readily be assigned. The precision of these measurements was within 1 amu, and while excellent for mass spectrometry in the present mass range (MH^+ = 6907.5 for hirudin), it was inadequate for the purpose of establishing the exact deamidation degree of the isolated forms. Since deamidation of a single Asn or Gln residue shifts the molecular weight by only 1 amu, it is improbable that the exact number of deamidated residues could be determined unambiguously by an average mass measurement on an otherwise intact hirudin compound. To circumvent this problem, the feasibility of carboxyl esterification of hirudin was investigated. Such methodology permits the introduction of a measurable mass shift per deamidated residue present in the molecule.

Acid-catalyzed esterification of smaller peptides (MW < 2000) with benzyl or various alkyl alcohols has, for example, been reported by Naylor et al. (1986) and Falick & Matly (1989). Their purpose using derivatization was to increase the hydrophobicity of very hydrophilic peptides that are otherwise poorly detected in mass spectrometry because of impaired desorption.

Another type of derivatization, the N-acyl-N,O-permethylation, was proposed by the group of Roepstorff (Talbo & Roepstorff, 1990). The aim was, in this case, to obtain structurally informative fragment ions of peptides in plasma desorption mass spectrometry (PDMS). This method is, however, more difficult to control in that it not only includes two consecutive derivatization steps but also very often generates various artifacts such as C-methylation or quaternization of unprotected amino groups due to the use of the highly reactive dimethylsulfinyl carbanion. Nonetheless, it permitted direct and complete sequence determination of peptides containing up to 15 amino acid residues.

Kansler et al. (1988) used esterification with methanol to distinguish between the isobaric Gln and Lys residues in LSIMS, while Young & Desiderio (1976) employed methylation with the definite objective of determining the number of free carboxyl groups in dipeptides. To our knowledge, however, such a procedure has not yet been extended to polypeptides in the mass range of 7 kDa, as reported herein.

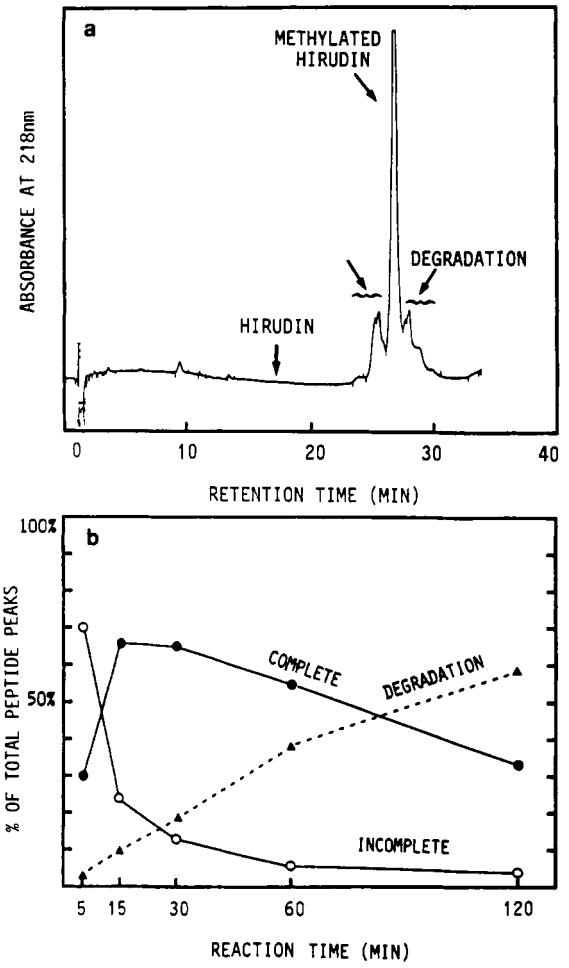

FIGURE 3: Methylation of hirudin. (a) RP-HPLC profile of hirudin reacted for 15 min with 2 M HCl/methanol. (b) A kinetic study of the reaction: (○—○) incompletely and (●—●) completely methylated hirudin; (▲- - -▲) degradation products.

In the present study, the acid-catalyzed methylation of hirudin was performed with 2 M HCl/methanol. Although hirudin is practically insoluble in pure methanol, the reaction was, in fact, surprisingly rapid but required optimization with respect to the reaction time. Esterification with higher alcohols such as propanol and 2-propanol was also attempted. Unfortunately, however, hirudin remained intact, and assays with other alcohols were not pursued.

Monitoring of the methylation reaction of RP-HPLC (Figure 3a) coupled with LSIMS analyses of the collected peaks revealed that all 11 carboxyl groups in hirudin were esterified within 15 min (Figure 4). The yield, however, never exceeded 70%. As illustrated in Figure 3b, incomplete methylated derivatives as well as several degradation products attributable to partial peptide hydrolysis (data not shown) were always present to various degrees at any time point between 5 and 120 min. On the other hand, these were the only side reactions encountered, and no amino acid side chains other than carboxylic acids reacted. Sonication or the use of higher temperatures only accelerated degradation, while incubation at 0 °C considerably prolonged the time necessary to obtain a fully methylated peptide without gain in total yield. Finally, a 15-min reaction time at ambient temperature was chosen for all further experiments.

The derivatized samples were purified by RP-HPLC before LSIMS analysis in order to characterize well-defined compounds. This purification step appeared, however, not indispensable. Provided that the reaction yield approaches 70%,

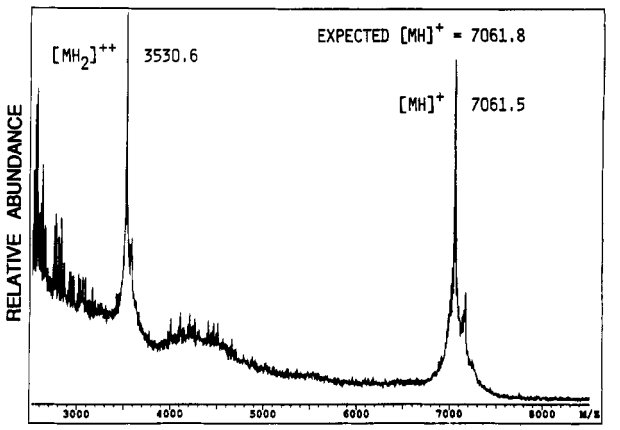

FIGURE 4: LSIMS spectrum of carboxyl-methylated hirudin. The protonated molecular ion appears at m/z 7061.5 (expected $[MH]^+$ = 7061.8) and the doubly-charged ion $[MH_2]^{2+}$ at m/z 3530.6. The signals in the mass range 4–5 kDa and below 3 kDa correspond to N-terminal and C-terminal fragment ions, respectively.

Table I: LSIMS Molecular Weight Determinations of Hirudin and Its 11 Deamidated Forms, in the Native State or after Carboxyl Methylation

	native polypeptides			carboxyl-methylated polypeptides		
compd	measd MH^+ (amu)	ΔM^a (amu)	deduced no. of deamidated residues	measd MH^+ (amu)	ΔM^a (amu)	no. of deamidated residues
hirudin	6907.5			7061.5[b]		
I						
F_1	6907.2	−0.3	0	7076.5	15.0	1
F_2	6907.5	0.0	0	7076.4	14.9	1
F_3	6907.9	0.4	0	7076.4	14.9	1
F_4	6907.8	0.3	0	7076.7	15.2	1
II						
F_5	6908.7	1.2	1–2	7090.9	29.4	2
F_6	6908.7	1.2	1–2	7091.1	29.6	2
F_7	6908.7	1.2	1–2	7091.7	30.2	2
III						
F_8	6909.9	2.4	2–3	7106.8	45.3	3
F_9	6909.8	3.3	3	7106.8	45.3	3
F_{10}	6909.8	3.3	3	7107.0	45.5	3
F_{11}	6909.5	2	2	7106.4	44.9	3

[a] Shift from the MH^+ value measured for hirudin. [b] Recombinant hirudin contains 11 carboxylic acid groups; the expected mass of the completely carboxyl methylated derivative is 7061.8.

the individual peaks of incompletely methylated or degraded derivatives are present in so small amounts relative to the fully methylated compound (see Figure 3a) that they are not likely to be detected in the LSIMS spectrum of the latter. Therefore, LSIMS analysis can be performed directly on the unpurified methylated peptide following a quick drying of the reagent under nitrogen, without addition of water to stop the reaction as indicated in Materials and Methods. This approach reduces manipulation and is less time-consuming.

Determination of the Deamidation Degree by LSIMS Analyses of the Carboxyl-Methylated Derivatives. A comparison of the molecular weight values obtained for the native and the methylated derivatives is presented in Table I. The potential of methyl esterification to ascertain the number of deamidated residues is clearly demonstrated. For example, compounds F_{1-4}, which belong to the first group of peaks detected in IE-HPLC, all exhibited MH^+ values similar to those of the parent compound when analyzed in the native state. But after methylation, the gain in 15 amu comparative to hirudin demonstrated that all four compounds possessed a deamidated residue.

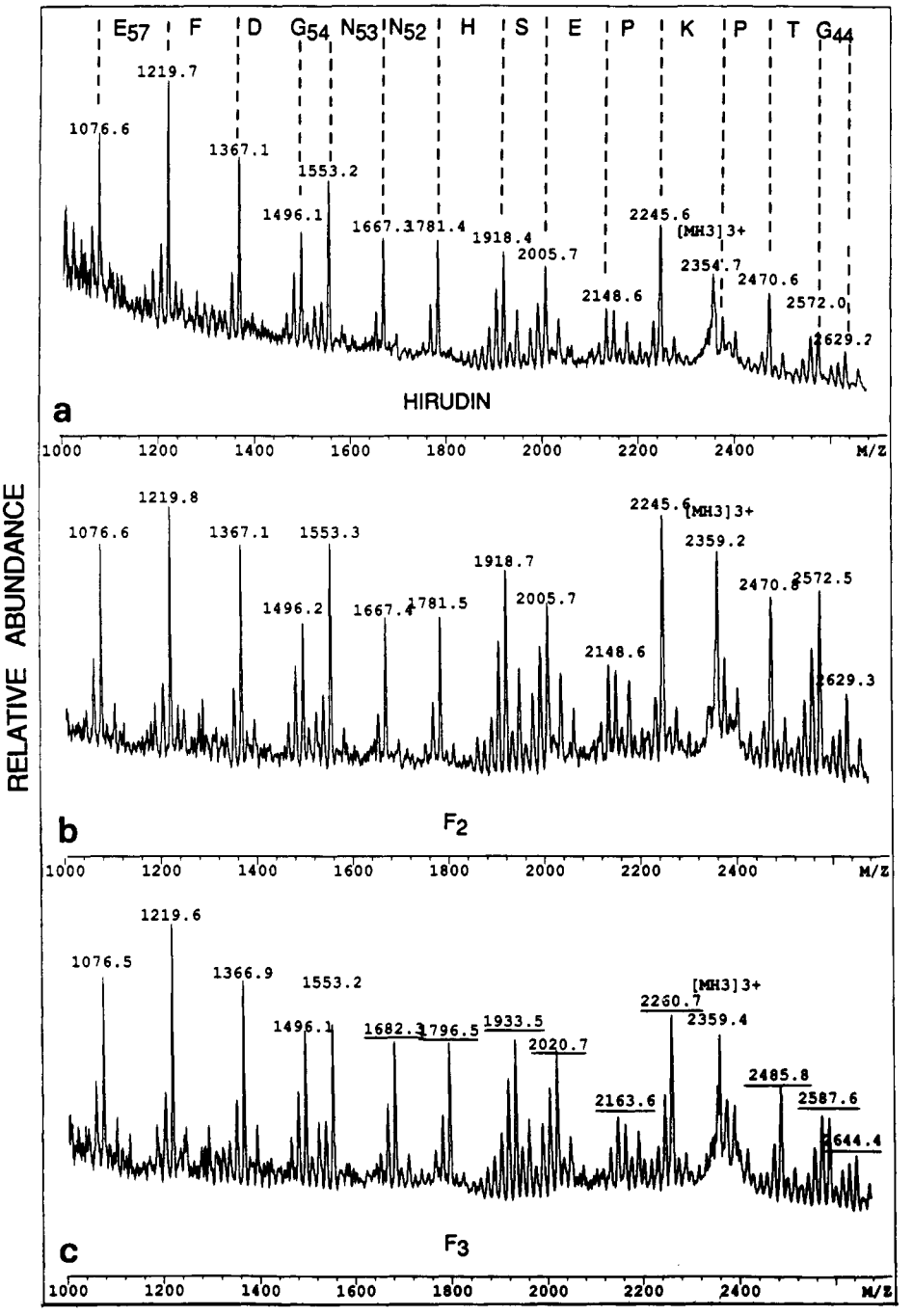

FIGURE 5: Comparison of the LSIMS spectra obtained in the 1–3-kDa region (C-terminal 44–65 moiety) for the methylated derivatives of hirudin (a) and forms F_2 (b) and F_3 (c). The ions shifted by +15 amu are underlined as in spectrum c. The assigned ions are Y″ fragment ions according to the nomenclature of Roepstorff and Fohlman (1984). The ion at 2354.7 (spectrum a) corresponds to the triply-charged methylated hirudin $[MH_3]^{3+}$.

As evidenced from these analyses, the highest number of deamidated residues generated is three (Table I). The first group of compounds (F_{1-4}) corresponds to mono-deamidated forms, whereas the second (F_{5-7}) and third (F_{8-11}) groups are di- and tri-deamidated forms, respectively.

LSIMS Fragmentation Patterns. Intense LSIMS fragment ions were observed in the spectra of native hirudin and its deamidated forms in two particular mass regions, 1–3 and 4–5 kDa, which correspond to those previously described for hirudin by Van Dorsselaer et al. (1989). The outstanding effect of methylation was to increase the abundance of these ions with a prominent gain to signal-to-noise ratio. As a result, sequence recognition in the above-mentioned mass regions was very simple.

The ions could readily be assigned according to the nomenclature proposed by Roepstorff and Fohlman (Roepstorff & Fohlman, 1984). It is interesting to note that the most intense ions observed in the 1–3-kDa region virtually all corresponded to C-terminal Y″ fragments of the 44–65 moiety (m/z 2629, Figure 5a), whereas the main ions present in the 4–5-kDa region could be assigned to A fragments of the complementary N-terminal 1–44 moiety (m/z 4462, Figure 6a). This specific rupture of the peptide bond on both sides of the Gly 44 residue indicated a particular fragility at this point of the molecule under the LSIMS conditions used. Even in the high mass domain, the low-resolution power used did not impair the precision and the reproducibility of the measurements. In all cases, precise and unambiguous inter-

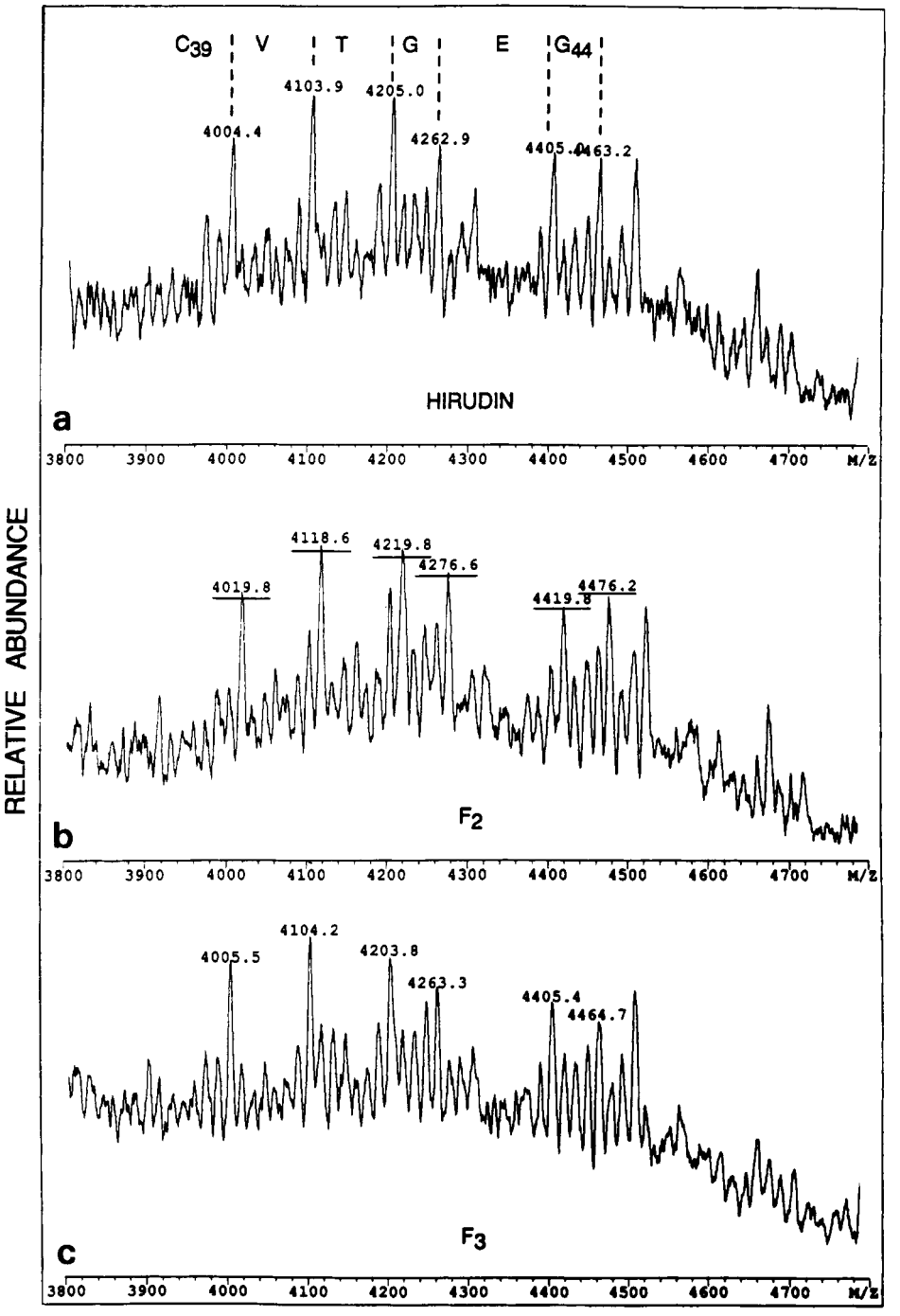

FIGURE 6: Comparison of the LSIMS spectra obtained in the 4–5-kDa region (N-terminal 1–44 moiety) for the methylated derivatives of hirudin (a) and forms F_2 (b) and F_3 (c). The ions shifted by +15 amu are underlined as in spectrum b. The assigned ions are A fragment ions according to the nomenclature of Roepstorff and Fohlman (1984).

pretation of most ions was possible. For simplicity, however, only the main Y″ or A ions were assigned. Examples of the fragmentation pattern demonstrating the correlation between the observed mass fragments and the position of cleavage along the peptidic chain of methylated hirudin are shown in Figures 5a and 6a. Most important is the finding that such a fragmentation pattern provided an excellent tool for locating the position(s) of the deamidated residue(s).

Localization of the Deamidation Sites in Hirudin by LSIMS. The homologous spectra of the methylated derivatives of hirudin and two of its mono-deamidated forms, F_2 and F_3, recorded between 1 and 3 kDa (the C-terminal domain), are compared in Figure 5a–c. Superposable spectra were obtained for hirudin and F_2 in this region (Figure 5a,b). In contrast, clear differences were noticed between those of hirudin and F_3 (Figure 5a,c). In the two spectra, all the Y″ ions were identical up to the Gly residue at position 54, but were thereafter, in the F_3 spectrum, systematically shifted by +15 amu in the N-terminal direction. This indicates that Asn 53 has been converted to a methylated Asp residue, and it was concluded that F_3 is an Asp 53 deamidated form of hirudin.

The same type of comparison was repeated with the spectra recorded between 4 and 5 kDa (the N-terminal domain) which are presented in Figure 6. This time the spectra of hirudin and F_3 were very much alike (Figure 6a,c), whereas all the A ions present in the homologous spectrum of F_2 were annotated 15 amu higher (Figure 6a,b). These figures demonstrate that F_2, but not F_3, possesses a deamidated residue within the N-terminal 1–39 domain.

Table II: Position or Localization of Deamidated Residue(s) in the 11 Deamidated Forms of Hirudin

compounds	no. of deamidated residues	position or localization
F_1, F_2[a]	1	domain 1–39
F_3, F_4	1	Asn 53
F_5, F_6, F_7	2	Asn 53, domain 1–39
F_8, F_9, F_{10}, F_{11}	3	Asn 52, Asn 53, domain 1–39

[a] The numbers refer to the elution order of the compounds in IE-HPLC (see Figure 2).

Unfortunately, the existence of the disulfide bridge Cys 22–Cys 39 totally abolished the A fragmentation beyond the Val 40 residue toward the N-terminal, which limited the structural information strictly to the determination of the number of deamidated residues within the N-terminal 1–39 domain. The three disulfide bonds in methylated hirudin were therefore submitted to reduction and alkylation before LSIMS analysis, but neither alkylation with 4-vinylpyridine nor alkylation with iodoacetamide provided sufficient interpretable fragment ions in the mass region of interest (4–5 kDa).

The 11 methylated deamidated forms were analyzed by LSIMS, the methodology outlined above being applied, and the results are summarized in Table II. It appears that only three residues among the 10 possible (seven Asn and three Gln) were found to deamidate: Asn 52, Asn 53, and a residue in the N-terminal 1–39 domain.

These results were not completely surprising as it has been previously demonstrated that Asn in the sequence Asn-Gly is particularly prone to deamidation (Geiger & Clarke, 1987). Since hirudin contains two such sequences, Asn 33-Gly 34 and Asn 53-Gly 54, it was presumed that these positions would be touched first. LSIMS confirmed one of the positions, the residue Asn 53. It is therefore conceivable that the residue located in the 1–39 domain could be Asn 33, even though the methylation methodology failed to prove it. Complementary Edman degradation studies were thus required and clearly showed both Asn 33 and Asn 53 to be preferential deamidation sites of hirudin. In fact, amino acid sequencing of tryptic peptides derived from deamidated forms of hirudin demonstrated that deamidation could be detected either at Asn 33 or Asn 53 or at both positions simultaneously (data not shown). Peaks containing more than two deamidation sites were, however, not sequenced. The third residue identified as a deamidation site by the methylation methodology, Asn 52, precedes the Asn 53-Gly 54 sequence and had the particularity of not deamidating before the reaction was completed at either of the two other sites.

Possible Generation of Deamidated Iso Forms. Because only three residues were affected by deamidation, the occurrence of the 11 chromatographically separable forms, several of which shared identical deamidation positions (Table II), required explanation.

The mechanism of deamidation of Asn preceding Gly in a sequence is today well documented (Aswad, 1984; Geiger & Clarke, 1987; Lura & Schirch, 1988; Capasso et al., 1989; Teshima et al., 1991). It has been shown that this reaction proceeds via the formation of a succinimide intermediate (Figure 7) which upon hydrolysis generates both a normal aspartyl and an isoaspartyl linkage in a 1:3 ratio, respectively. It is notable that this particular area ratio was perceived in IE-HPLC especially among each pair of mono-deamidated forms, F_1/F_2 deamidated at position 33 and F_3/F_4 deamidated at position 53. Hence, it is tempting to postulate that the multiplicity of forms having identical deamidation positions (Table II) arises from Asp/iso-Asp isomerization processes, and that both F2 and F3 are isoforms according to their observed high relative intensities.

On the basis of this hypothesis, a rational scheme can be drawn (Figure 8) which accounts for the affiliation and the chromatographic intensities of the multiple mono- and di-deamidated forms detected.

From the above isomerization rule, mono-deamidation of Asn 33 and Asn 53 will in total produce four deamidated compounds, as also experimentally observed (Table II). In IE-HPLC, these forms appeared systematically in stoichiometric ratios of 2:6:3:1 with respect to the last eluting peak F_4 (group I, Figure 2). The occurrence of a second deamidation step literally splits each form into two new isomers, for which the Asp/iso-Asp ratio must again be 1:3. Given the above HPLC ratios, the relative intensity of any new di-deamidated combination can be predicted as outlined in Figure

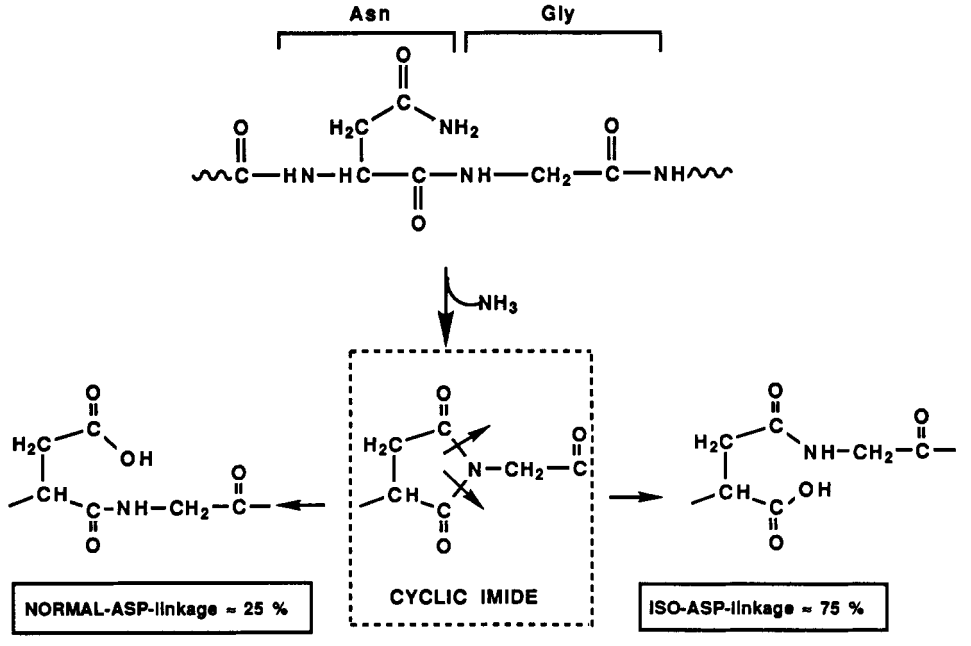

FIGURE 7: Pathways for the deamidation of Asn-Gly-containing peptides and proteins.

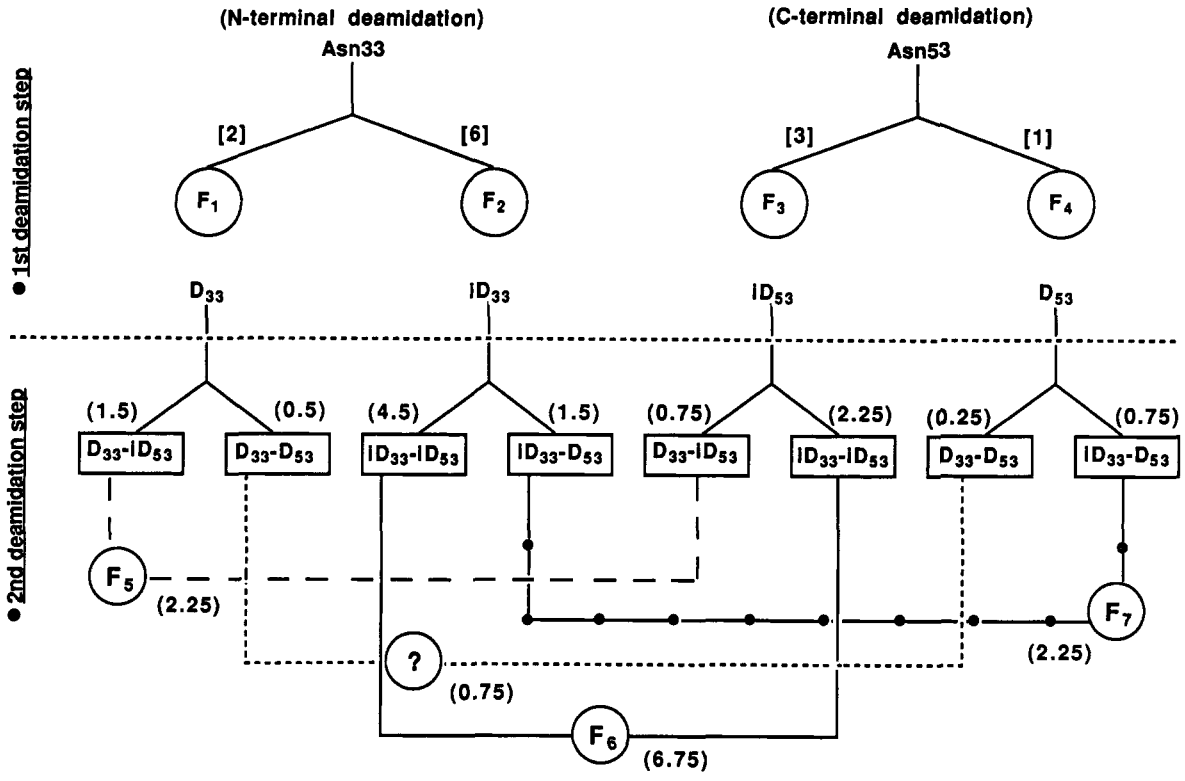

FIGURE 8: This hypothetical scheme concerns two sequential deamidation steps initiated from Asn 33 and Asn 53 in parallel. The compounds generated (circled) are ranked from left to right according to their elution order in IE-HPLC (see Figure 2), and their measured relative proportions are figured in square brackets. Since deamidation of Asn in the Asn-Gly sequence generates both Asp and iso-Asp residues in a constant 1:3 ratio, respectively, F_1 and F_4 can be assigned to normal Asp, F_2 and F_3 to iso-Asp forms according to their relative proportions within each pair. Hence the elution order becomes $D_{33} < iD_{33} < iD_{53} < D_{53}$ (Asp form = D, iso-Asp form = iD). In the second deamidation step, each form produces two new isomers which will appear in proportions that can be calculated directly by application of the 1:3 rule (results within brackets). Summing up, four di-deamidated combinations are expected. Due to the deduced elution orders from the first step (D_{33}-iD_{53} < $iD_{33}iD_{53} \approx D_{33}D_{53} < iD_{33}D_{53}$) and to the calculated relative proportions (2.25:6.75:0.75:2.25, which approaches the observed 1:3:1 ratio for the peaks F_5, F_6, and F_7 in IE-HPLC), it appears rational to assign this second group of peaks as follows: $F_5 = [D_{33}$-$iD_{53}]$, $F_6 = [iD_{33}$-$iD_{53}]$, $F_7 = [iD_{33}$-$D_{53}]$. The theoretically less abundant combination $[D_{33}$-$D_{53}]$ containing two normal residues was not identified, but possibly coelutes with one of the other peaks under the chromatographic conditions used.

8. Altogether four di-deamidated forms may possibly exist. Among these, the iso-Asp 33/iso-Asp 53 combination should proportionately be the highest and the Asp 33/Asp 53 combination the smallest of the isomers, if the above rule holds. Concomitantly, the two other combinations, Asp 33/iso-Asp 53 and iso-Asp 33/Asp 53, should appear quantitatively equivalent.

It seems also that a correlation can be drawn between this scheme and the chromatographic patterns of the di-deamidated forms (group II, Figure 2). Taking into account the deduced elution orders as well as the calculated intensities from Figure 8, we can reasonably assign F_6, the most prominent of the di-deamidated peaks, to the iso-Asp 33/iso-Asp 53 form, while F_5 and F_7 should correspond to Asp 33/iso-Asp 53 and iso-Asp 33/Asp 53 forms, respectively. It can also be predicted that the Asp 33/Asp 53 form will not be separated from the other di-deamidated compounds under the chromatographic conditions used and is probably present in too minute amounts to be detected specifically in a mixture by LSIMS.

The scheme has not been extended to cover the third deamidation step, because the mechanism by which residue Asn 52 deamidates is at present not clear. As already mentioned, it responded so slowly that both Asn 33 and Asn 53 had converted to Asp/iso-Asp residues before deamidation could be detected in this position. This residue, which is preceded by His (see Figure 1), can therefore be considered as if it were in the sequence His 51-Asn 52-Asp or iso-Asp 53.

Robinson et al. (1973) demonstrated that the deamidation half-time of Asn and Gln in synthetic pentapeptides, none of which contained an Asn-Gly sequence, was significantly shortened when the adjacent amino acids possessed a charged side chain. In particular, Arg, Asp, and His residues were shown to promote deamidation of Asn. In this context, deamidation of Asn 53 could be a prerequisite for the reaction to proceed at position 52, but His 51 might also act as the catalyzing factor.

Recently Wright (1991) reviewed the various mechanisms of the nonenzymatic deamidation and listed numerous examples of sequences in proteins for which sequence-dependent deamidation is documented or is likely to occur. It is noteworthy that among these sequences the specific His-Asn-Asp sequence (see above) is reported. According to Wright, the Asp/iso-Asp shift mechanism via cyclization, usually with glycine, appears the most important but is not the unique pathway of deamidation. Acid and base hydrolytic reactions, also catalyzed by proximate charged amino acid side chains, must be taken into account. Such reactions, however, lead exclusively to naturally occurring forms.

It was established in the present study that a high ionic strength phosphate buffer, 1 M (pH 9), was required for the

deamidation of Asn 52 to be measurable. Yet the mechanism may not be ascribed to a direct hydrolytic reaction like the one discussed by Wright, because it is incompatible with the number of generated compounds. In fact, during preparative workup of the four tri-deamidated forms, evidence was found for the presence of at least two additional compounds, visible as shoulder peaks, and although they were only imperfectly purified, no difference from the other tri-deamidated forms in terms of molecular weight or deamidation site could be detected (results not shown). The number of "identical" tri-deamidated compounds presently found points, therefore, to an Asp/iso-Asp isomerization of Asn 52 during its deamidation. Complementary work is needed to support this hypothesis.

CONCLUSION

Eleven deamidated forms of hirudin were prepared and characterized in the present work. Our methodology based on LSIMS analyses of the carboxyl-methylated derivatives of these forms provided determination of both the number (at the highest, three) and the positions of at least two of the deamidated residues in a single analysis. Altogether four mono-, three di-, and four tri-deamidated forms were identified.

Recognizing the sequence location of deamidated residues by conventional approaches involves proteolytic digestion followed by extensive HPLC purifications of the generated fragments before sequence analyses by Edman degradation. Our results illustrate, however, that most deamidated residues can be evidenced in a matter of hours when using LSIMS-based strategy directly on the intact but methylated compounds.

Of hirudin's seven Asn residues, only Asn 53 in the C-terminal and Asn 33 in the N-terminal domain were readily deamidated under basic conditions. It is noteworthy that both residues are followed by Gly in the sequence. These results are in accordance with previously published data describing this sequence as a particularly favorable site for deamidation. The only other residue that was shown to deamidate was Asn 52. This reaction occurred, however, only under special incubation conditions and never before deamidation at the two other sites was accomplished.

It was found that deamidation of a single residue in either domain of hirudin always gave rise to two chromatographically separable compounds but with identical sequences. This observation could, however, be explained when considering the documented Asp/iso-Asp isomerization process which arises from the differential cleavage of an unstable succinimide intermediate formed between Asn and Gly during the nonenzymatic deamidation reaction. According to this principle, we proposed a rational scheme which allows the ready assignment of seven of the 11 peaks observed in IE-HPLC.

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