Chemical Degradation Kinetics of Recombinant Hirudin (HV1) in Aqueous Solution: Effect of pH

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Purpose. To gain information on the chemical stability pattern and the kinetics of the degradation of recombinant hirudin variant HV1 (rHir), a thrombin-specific inhibitor protein of 65 amino acids, in aqueous solution as a function of pH.

Methods. Stability of rHir was monitored at 50°C in the framework of a classical pH-stability study in aqueous buffers pH 1–9.5. Two capillary electrophoresis (CE) protocols were used; one for the kinetics of succinimide formation at Asp^{53}-Gly^{54} (C-terminal tail) and Asp^{33}-Gly^{34} (loop section), the other for the kinetics of rHir degradation. To check for potential effects of conformational changes by thermal denaturation, circular dichroism (CD) measurements were performed between 25 and 80°C.

Results. Throughout the pH range studied no effect of thermal denaturation on rHir confirmation at 50°C was observed. rHir was most stable at a neutral pH whereas, at slightly acidic pH, an intermediate stability plateau was found. Both, strongly acidic and alkaline conditions led to fast rHir degradation. Depending on the pH of degradation, rHir was found to degrade in various combinations of multiple parallel and sequential degradation patterns. Special focus was on succinimide formation at Asp^{53}-Gly^{54} (C-terminal tail) and Asp^{33}-Gly^{34} (loop) and on the potential of isoAsp formation in position 53 and 33.

Conclusions. Chemical rHir stability in the intermediate pH range depends strongly on succinimide formation. At slightly acidic conditions succinimides represent the major degradation product (up to 40%). Around neutral pH succinimides react further, presumably by isoAsp formation, and concentrations remain low. Relative preference of succinimide formation in the C-terminal tail domain versus the loop domain is explained by higher backbone flexibility in the tail.

KEY WORDS: recombinant hirudin; chemical stability; protein stability; succinimide formation; isomerization; cyclic imide formation; capillary electrophoresis; circular dichroism.

INTRODUCTION

Hirudin is a thrombin-specific inhibitor first isolated from the medicinal leech, *Hirudo medicinale* (1). Presently hirudin is produced by recombinant DNA technology. The three major variants designated HV1, HV2, and HV3 have a high degree of homology (13 variable positions only), but in contrast to the natural molecule, they lack the sulfate group at Tyr^{63} (2). For this work, recombinant hirudin HV1 was used and is designated as rHir.

The thrombin inhibitory activity of rHir is maintained even under extreme conditions as reported by Chang (3). Among the conditions which effectively and irreversibly inactivate rHir is the combination of elevated temperature and alkaline pH. Structural analysis shows that inactivation is a consequence of base-catalyzed β-elimination of the disulfide bonds. When stored under acidic conditions, two resulting congeners of rHir were characterized to be succinimide-type dehydration products involving Asp^{33}-Gly^{34} and Asp^{53}-Gly^{54}, respectively (4). As demonstrated for other peptides and proteins, hydrolysis of succinimides under near neutral to basic conditions results in either isoAsp or Asp formation (5–8). Thus, isomerization products like $isoAsp^{33}$- and $isoAsp^{53}$-rHir are expected to be involved in rHir degradation in that pH region. The $isoAsp^{33}$ analog of rHir was recently identified (9). In an analogy to rHir HV2, deamidation at Asn^{52} of rHir HV1 at neutral to alkaline pH is further expected. For rHir HV2, Tuong et al. (10) reported that deamidation at Asn^{52} occurs after deamidation at Asn^{33} and Asn^{53}.

Nordmann (11) followed the thermal denaturation of rHir by means of circular dichroism (CD). Little conformational changes were observed upon heating to 50°C; unfolding of the rHir core turned out to be a single transition with a transition midpoint temperature (T_m) of ~71–73°C at neutral pH. For rHir (11) and recombinant human interleukin-2 (12) the thermal denaturation was found to be affected by pH and/or ionic strength.

When developing therapeutic peptide or protein formulations chemical drug stability is of high interest. Often, a compromise between physiological compatibility and drug stability needs to be found. For information on the principal degradation kinetics and pathways of rHir in aqueous solution, we present its degradation profile at elevated temperature as a function of pH. A full pH stability profile of rHir is not yet available. Degradation was monitored by capillary electrophoresis (CE). The influence of several buffer systems on the thermal denaturation of rHir was studied by near-UV CD and far-UV CD to exclude partial denaturation of rHir at the temperature of the test conditions.

MATERIALS AND METHODS

Materials

rHir (HV1) was obtained from Ciba-Geigy Ltd., Basel, Switzerland. rHir consists of 65 amino acids (Fig. 1) with a molecular weight of 6,964 Da and an isoelectric point of 3.9 (15). (Tris-(hydroxymethyl)-aminomethan (TRIS), 4-(2-hydroxyethyl)-piperazin-1-ethansulfonic acid (HEPES), 2-morpholino-ethansulfonic acid (MES) were from Fluka-Chemie AG (Buchs, Switzerland). All other chemicals were of analytical grade from Fluka-Chemie AG (Buchs, Switzerland) and Merck AG (Zürich, Switzerland). In all cases, nanopure water (Milli-Q®, Millipore Corp., Bedford, MA, USA) was used.

Capillary Electrophoresis (CE)

All electrophoretic experiments were performed using a Beckman P/ACE 5010 system (Beckman Instruments, Fuller-

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ABBREVIATIONS: rHir, recombinant Hirudin; CE, capillary electrophoresis; CD, circular dichroism.

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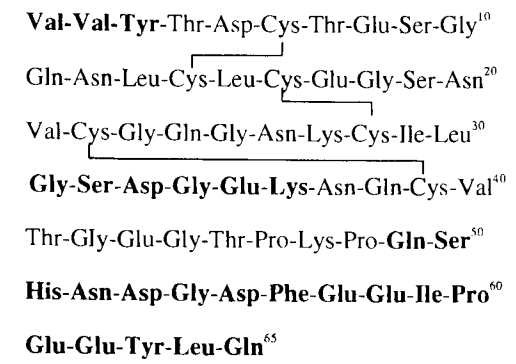

Fig. 1. Amino acid sequence of rHir (13). Bold typed segments indicate flexible domains which are not well defined by NMR data (14). Disulfide bridges are indicated by lines.

ton, California, CA). Two different protocols were used (method 1 and method 2).

Method 1

The protocol applied was according to Forrer et al. (15). Uncoated fused-silica capillaries with the dimensions 117 cm (110 cm to UV detector) × 50 μm ID × 360 μm OD were obtained from BGB Analytik AG (Rothenfluh, Switzerland). Separations were conducted at a constant voltage + 28 kV (approximately 8 μA) and a constant temperature of 25°C. Detection was at 200 nm. For sample protection the sample tray was cooled to 5°C. Injection time was 5 s at high pressure 138 kPa (= 20 psi). The buffer system consisted of 20 mM tricine, 10 mM sodium tetraborate, and ~0.27 mM 1,4-diaminobutane, resulting in a pH of 8.3. Minor migration time variations resulted from slightly variable 1,4-diaminobutane concentrations. Linearity of the analysis (main peak) was maintained between 0.05 and 6 mg/ml. All concentrations were calculated from corrected peak areas (peak area/migration time) to compensate for any variation of electroosmotic flow.

Method 2

The protocol applied was a modification of the method of Dette and Wätzig (16) to improve its low reproducibility and robustness. Neutral coated capillaries (eCap™) with dimensions 37 cm (30 cm to UV detector) × 50 μm ID were purchased from Beckman Instruments (Fullerton, CA, USA). Separations were conducted in the reverse-polarity mode (negative potential at the injection end of the capillary) at a constant voltage of 25 kV (approximately 26 μA) and at a constant temperature of 25°C. Detection was at 214 nm with the sample tray cooled to 5°C. Injection time was 5 s at high pressure 138 kPa (= 20 psi). The buffer system consisted of 60 mM sodium acetate buffer, pH 4.4, with 0.3% polyethylenglycol 20,000 and 0.1 mM zinc chloride. Prior to use, the buffer was filtered through a 0.45 μm filter. Linearity of the method (main peak) was confirmed between 0.02 and 2 mg/ml. Intraday variability (n = 10) was 0.57% relative standard deviation (RSD) for migration time, 3.35% for peak area and 3.14% for corrected peak area. Corrected peak areas were used throughout. For presentation of the degradation pattern of rHir, corrected peak areas of the degradation products were expressed as percentages of the sum of corrected peak areas at time zero.

Thermal Denaturation

Thermal denaturation of rHir upon changes in conformation was monitored by circular dichroism (CD) at 225 nm and 265 nm using a JASCO J-720 instrument (Jasco international Co., Tokyo, Japan) equipped with a Neslab RTE-111 water bath (Neslab Instruments, Portsmouth, USA). Thermostated cylindrical cells with optical path lengths of 0.1 and 1 cm were used. The instrument was programmed for a cuvette temperature increase from 25 to 80°C at a rate of 0.5°C/min. The CD signal was recorded every 0.1°C. Prior to use, the cells were cleaned with 6 N nitric acid, rinsed several times in distilled water, followed by methanol, and dried in air. The data was averaged over 0.5°C and means were connected.

Kinetics of pH Dependent Degradation

The degradation of rHir was studied at 50°C in aqueous solution. The following buffers were used: pH 1.0 and 2.0 (0.1 and 0.01N) HCl; pH 3.0 (0.05, 0.1, 0.2 and 0.3 M) sodium formate buffer; pH 4.0 and 5.0 (0.05, 0.1, 0.2 and 0.3 M) sodium acetate buffer; pH 5.5 (0.05, 0.1, 0.2, 0.3 M) MES buffer; pH 6.5 (0.05, 0.1, 0.2 and 0.3 M) HEPES buffer; pH 7.5 (0.05, 0.1, 0.2 and 0.3 M) TRIS buffer; pH 8.5 and pH 9.5 (0.05, 0.1, 0.2 and 0.3 M) sodium borate buffer. Constant ionic strength of 0.4 was maintained for each buffer by adding an appropriate amount of NaCl. The pH of the buffer solutions was determined at 50°C.

rHir was dissolved in buffer to give a final concentration of 1 mg/ml. Under the laminar flow, sterile HPLC vials (approximately 2 ml; Infochroma, Zug, Switzerland) were filled completely with sterile filtered rHir solution and tightly closed with sterile crimp sealings. The sealed vials were stored at 50°C. The tightness of the HPLC vials was tested at 50°C with water and a weight loss of maximally 1.5% (w/w) was found after 20 days.

At various times, vials were sampled and frozen at −23°C. Prior to CE analysis, the samples were thawed and diluted with water (1:4) to lower the salt concentration of the samples.

Determination of Degradation Rate Constants (k_{obs}, k_0)

The degradation of aqueous rHir solution at various pH values was monitored by CE method 2 as a function of time. Each of the kinetic profiles was recorded within one day and related to a calibration curve of the same day. The decrease of rHir concentration at all pH values was found to follow pseudo first-order kinetics. Degradation rate constants (k_{obs}) were calculated from the slopes of the semilog plot of time versus remaining rHir concentration. The extrapolated rate constant at zero buffer concentration (k_0) is given by the y axis intercept of the linear regression of k_{obs} versus buffer concentration.

RESULTS

Capillary Electrophoresis (CE)

Two CE methods were tested to determine the stability of rHir. CE method 1 was developed by Forrer et al. (15). CE method 2 was a modification of the method of Dette and Wätzig (16) which showed wall adsorption and performed poorly with respect to reproducibility and capillary robustness. Replacement

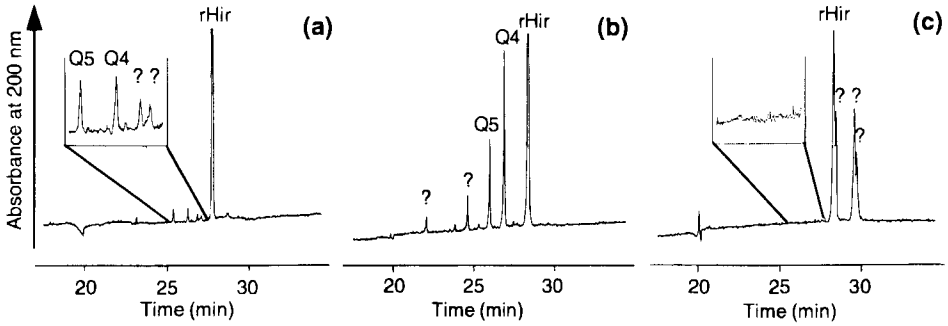

Fig. 2. CE analysis of rHir degradation. CE method 1. (a) Freshly prepared aqueous solution, (b) degraded aqueous solution (pH 4.0; I = 0.4; 1.5 days at 50°C), and (c) degraded aqueous solution (pH 7.5; I = 0.4; 8 days at 50°C). Insert shows the cyclic succinimides of rHir formed at Asp^{53}-Gly^{54} (Q4) and Asp^{33}-Gly^{34} (Q5). Initial rHir concentration was 0.25 mg/ml. For CE conditions, see Materials and Methods. Question marks indicate unidentified peaks.

of the uncoated fused silica capillary by a neutral coated capillary and changing the polarity mode to reverse polarity ensured good reproducibility and analytical robustness. The rank order of the peak migration times was inverse to that of the method of Dette and Wätzig (16), due to the reverse polarity mode used.

Figure 2 illustrates electropherograms using CE method 1. A typical electropherogram of freshly prepared rHir solution is shown in Fig. 2a. Apart from the main peak (rHir), the two known congeners of rHir, succinimides at position Asp^{53}- Gly^{54} and Asp^{33}-Gly^{34}, laboratory code Q4 and Q5 (4), were well separated (Fig. 2a, insert) as described by Forrer et al. (15). Two unknown substances were also present in small quantities (Fig. 2a, insert). At pH 4.0 good separation of the degradation products of rHir was achieved with CE method 1 (Fig. 2b). Storage at pH 7.5 resulted in degradation products which were not well separable by CE method 1 (Fig. 2c). The insert of Fig. 2c shows that Q4 and Q5 were below the detection limit at this pH and the concentration used (0.25 mg/ml).

CE method 2 based electropherograms of rHir and degraded rHir are illustrated in Fig. 3. Fig. 3a represents a typical electropherogram of a freshly prepared rHir solution. The rather broad peak at ~19 min resulted from a combination of Q4 and Q5. To prove this assumption, degraded samples containing various amounts of Q4 and Q5 were analyzed with both CE methods. A linear correlation was found between the concentrations derived from the two methods, i.e. from the peak at ~19 min (CE method 2) and the combined peaks of Q4 and Q5 (CE method 1) (n = 10; slope: 1.06, intercept: −0.003 mg/ml, r = 0.987; (17)). Based on the slope of ~1, the CE signals for the combination of Q4 and Q5 (CE method 1) and the joint peak at ~19 min (CE method 2) were equivalent.

Freshly prepared aqueous rHir solution showed no degradation products except some Q4/Q5 (Fig. 3a). The degradation products of degraded rHir at pH 4 and pH 7.5 were well separated and designated as peaks 1 to 5 (Figs. 3b and 3c). According to the characteristic migration times of the main degradation products of rHir analyzed by CE method 2, the peaks were classified in three groups, I, II, and III. The first group consisted mainly of the Q4/Q5 peak (Fig. 3b). Peaks 4 and 5 were assigned to the second group and peaks 1 to 3 to the third group (Fig. 3c). Except for the Q4/Q5 peak, all other degradation products have not yet been identified.

Slight changes in the migration times using CE method 2 were due to the variability of the commercially available neutral coated capillaries (Fig. 3). The intraday variability of the migration time (n = 10) was 0.6% RSD (same capillary) whereas the interday variability determined with different capillaries was 5.9% RSD (n = 17). To eliminate this variability, each of

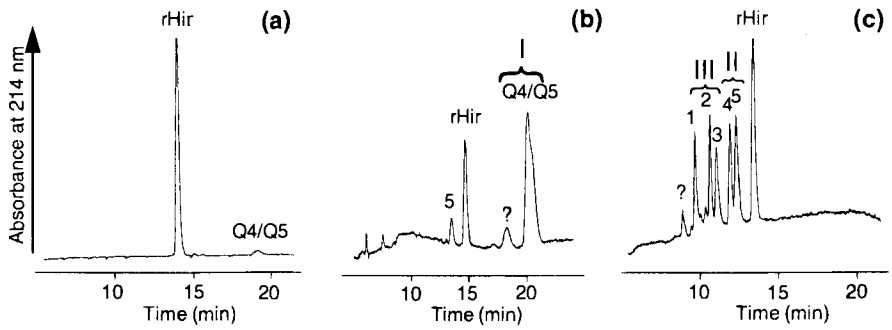

Fig. 3. CE analysis of rHir degradation. CE method 2. (a) Freshly prepared aqueous solution, (b) degraded aqueous solution (pH 4.0; I = 0.4; 4 days at 50°C), and (c) degraded aqueous solution (pH 7.5; I = 0.4; 8 days at 50°C). Initial rHir concentration was 0.25 mg/ml. For CE conditions, see Material and Methods. Unknown peaks designated as peaks 1 to 5 are further commented in the text. Question marks indicate small and unidentified peaks which were not further considered. I, II, and III refer to comments in the text.

the kinetic profiles was analyzed within one day and related to a calibration curve determined on the same day.

Based on the ability of CE method 2 to separate rHir from the major degradation products (Fig. 2 versus 3), method 2 was selected to monitor the degradation kinetics of rHir. Method 1 showed interferences of the rHir peak with other degradation products but discriminated efficiently between Q4 and Q5, which was impossible with method 2. Therefore, method 1 was used to focus on Q4 and Q5 separately.

Degradation Kinetics of rHir

Table I summarizes the observed degradation rate constants (k_{obs}) of rHir at different pH values, buffer substances, and molarities. In all samples the ionic strength was 0.4. At all pH values and buffer concentrations studied, the degradation of rHir followed pseudo first-order kinetics with correlation coefficients indicated in Table I. The rate constants at zero buffer concentration (k_0) were calculated for all pH values. No buffer concentration effect was observed with formate buffer, pH 3.0, and HEPES buffer, pH 6.5, whereas the buffer concentration of all the other buffers affected the rate constants.

Table I. Summary of the Rate Constants for the Degradation of rHir in Buffer Solutions at Different pH Values (50°C, I = 0.4)

pH	Buffer	Buffer conc. (M)	Observed rate constant, k_{obs}[a] (day^{-1})	Coefficient of correlation r^2	Rate constant at zero buffer concentration, k_0[a] (day^{-1})
1.0	HCl	0.1	2.56	0.988	
2.0	HCl	0.01	0.419	0.990	
3.0	Formate	0.05	0.373	0.998	0.378
		0.1	0.380	0.997	
		0.2	0.378	0.990	
		0.3	0.369	0.994	
4.0	Acetate	0.05	0.453	0.998	0.413
		0.1	0.450	0.998	
		0.2	0.562	0.999	
		0.3	0.586	0.999	
5.0	Acetate	0.05	0.311	0.997	0.317
		0.1	0.362	0.995	
		0.2	0.364	0.991	
		0.3	0.381	0.999	
6.5	HEPES	0.05	0.143	0.989	0.145
		0.1	0.148	0.995	
		0.2	0.147	0.995	
		0.3	0.145	0.985	
7.5	TRIS	0.05	0.159	0.989	0.143
		0.1	0.215	0.898	
		0.2	0.260	0.997	
		0.3	0.310	0.992	
8.5	Borate	0.05	0.316	0.978	0.311
		0.1	0.334	0.994	
		0.2	0.351	0.999	
		0.3	0.365	0.993	
9.5	Borate	0.05	0.993	0.987	1.02
		0.1	1.04	0.997	
		0.2	0.799	0.998	
		0.3	0.889	0.994	

[a] For calculation, see Materials and Methods.

The pH dependence of k_0 at 50°C is shown in Fig. 4. rHir was most stable at pH 6.5 and pH 7.5. Between pH 2.0 and pH 5.0, the degradation rate was approximately constant and at least 2-fold higher than at pH 6.5 and pH 7.5 (see Table I). Under strong acidic and basic conditions, the degradation rates were largely increased as compared to the intermediate pH values.

Degradation Pattern of rHir

The pattern of rHir degradation showed a marked dependence on pH. Degradation patterns at pH 2, 3, 8.5 and 9.5 are not reported here, instead all information is given by Gietz (17). Fig. 5 represents the degradation pattern of rHir at pH 4.0, 5.0, 6.5 and 7.5 as determined by CE method 2. Degradation of rHir at pH 4.0 was dominated by the formation of succinimides, Q4/Q5. Almost no other degradation products were observed at this pH. The total corrected peak area decreased to approximately 60% of the initial peak area. The degradation pattern of rHir at pH 5.0 resulted in the formation of Q4/Q5 and peak 5; at pH 6.5, peaks 2 to 5 were observed and Q4/Q5 formation became minor. The loss of total peak area at pH 5 and 6.5 was small (approximately 10%). Degradation at pH 7.5 resulted in an approximately 20% loss of total peak area after 10 days storage at 50°C. A variety of degradation products were formed (peaks 1 to 5). All of these degradation products migrated prior to rHir.

The increase of Q4 and Q5 during the degradation of rHir at pH 4.0 was investigated by CE method 1. Figure 6 represents the separately determined concentrations of Q4 and Q5. After storage at pH 4.0 for 3 days at 50°C, the concentration of Q4 was 2-fold higher as compared to Q5. This indicates a preference of succinimide formation at Asp^{53}-Gly^{54} (Q4) as compared to Asp^{33}-Gly^{34} (Q5).

Thermal Denaturation of rHir

For information about the thermal unfolding of rHir at the storage temperature of 50°C, thermal denaturation of rHir was followed by CD. Denaturation was monitored from 25 up to 85°C in three different buffer systems. The major drop in ellipticity was shown to be above 50°C using far-UV CD (225 nm; Fig. 7a) and near-UV CD (265 nm; Fig. 7b). Thus, the major change in secondary and tertiary structure took place above 50°C. The three buffers chosen combine the full pH range of the stability study.

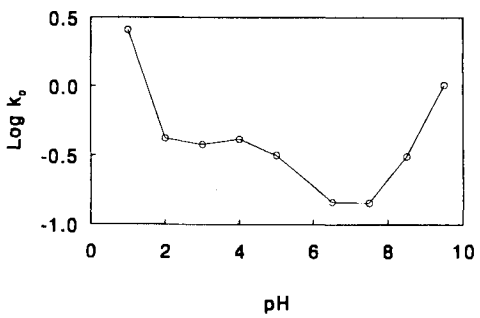

Fig. 4. pH-rate profile of rHir degradation in aqueous solution at 50°C determined by CE method 2. Rate constants corrected for zero buffer concentration were used. For calculation, see Materials and Methods.

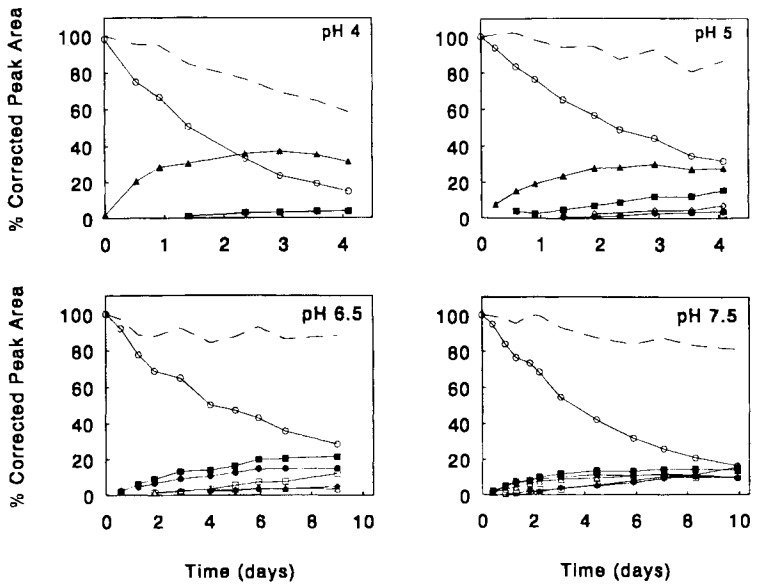

Fig. 5. Degradation pattern of rHir determined by CE method 2 at pH 4.0, 5.0, 6.5, and 7.5. Buffers were all 0.05 M and the ionic strength was 0.4. ○ rHir, ▲ Q4/Q5, ◆ peak 1, △ peak 2, □ peak 3, ● peak 4, ■ peak 5. For peak codes see Fig. 3. Broken lines indicate the sum of the corrected peak area of all peaks expressed as percentage of the total corrected peak area at time zero.

DISCUSSION

The structure of rHir contains three flexible back bone domains (14), a short one at the N-terminal end, the Gly^{31}-Lys^{36} loop, and the large Gln^{49}-Gln^{65} C-terminal segment (Fig. 1). The two identified rHir degradation products, Q4 and Q5, are succinimides at Asp^{53}-Gly^{54} and Asp^{33}-Gly^{34}, respectively (4). They are found in the two major flexible segments, in the loop (Q5) and in the C-terminal tail (Q4). As shown in the literature, protein conformation can strongly affect succinimide formation (18,19) which requires conformational flexibility in both the main chain and the side chain of a protein. Results obtained by CE method 1 showed that during degradation at pH 4.0, the concentration of Q4 was up to 2-fold higher than of Q5 (Fig. 6). This indicates that the formation of succinimide in the free C-terminal segment (Q4) is facilitated as compared to the loop segment (Q5). This may be due to the higher flexibility of the C-terminal tail as compared to the loop consisting of 6 amino acids only, and attached to two rigid ends.

With the exception of Q4 and Q5, all other degradation products formed within the pH range studied have not yet been identified and were thus designated as peaks 1 to 5 (Fig. 3). CE migration times contain information about charge to mass ratios of the observed substances. In the reverse polarity mode (negative potential at the injection end of the capillary), compounds with a higher negative charge to mass ratios are migrating faster than compounds with a lower negative charge to mass ratios. Thus, using CE method 2, peak 1 is expected to have the greatest, and peak Q4/Q5 the smallest negative charge to mass ratios (Fig. 3b and c). The remaining products have intermediate charge to mass ratios.

The degradation products of rHir as monitored by CE method 2 were classified in three groups, I, II, and III, as indicated in Figs. 3b and 3c. The first group mainly consisted of Q4/Q5. The formation of a succinimide in the backbone results in the loss of one carboxyl group, i.e. the loss of a negative charge. Therefore, as Q4 and Q5 both lack a carboxyl group, their negative charge to mass ratio is lowered and they are expected to migrate slower than rHir, as is reflected in Fig. 3b. Group II and III indicate more negatively charged compounds migrating prior to rHir (Fig. 3c). So peaks 4 and 5, assigned to the second group, may result from the formation of one or two isoAsp residues. Isoaspartyl was previously reported to have a slightly more acidic pK value than Asp, i.e. pK_{isoAsp} 3.4 and pK_{Asp} 4.1 (5). Therefore, isoAsp formation results in a more negatively charged compound compared to rHir, and migration of isoAsp-rHir is expected to be slightly faster than rHir. The third group consisted of even more negatively charged products (peaks 1 to 3). These are likely to contain deamidated products or a mixture of deamidated and isomerized rHir. A possible deamidation site is Asn^{52}, an analogy to rHir HV2 (10).

Each of the three groups were formed at characteristic pH values. Large quantities of Q4/Q5 (group I) were observed at a slightly acidic pH (pH 3.0 to 5.0) and only small or negligible

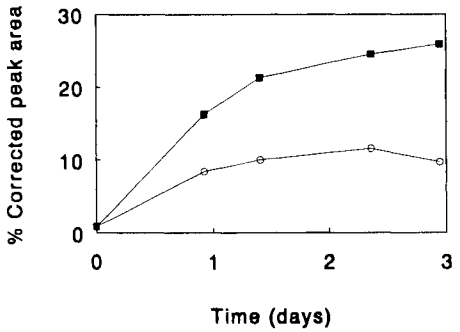

Fig. 6. Concentration of succinimides in rHir at pH 4.0 (I = 0.4). ■ Q4 (succinimide at Asp^{53}-Gly^{54}), ○ Q5 (succinimide at Asp^{33}-Gly^{34}). CE method 1 was used. Initial rHir concentration was 0.25 mg/mL.

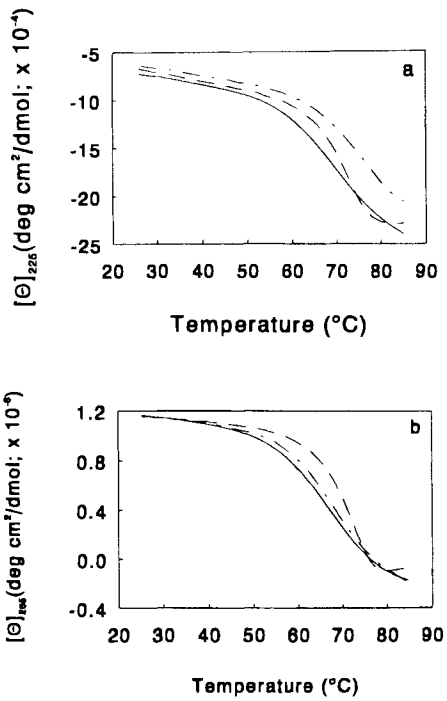

Fig. 7. Thermal denaturation of rHir monitored in the far-UV CD at 225 nm (a) and near-UV CD at 265 nm (b). —— pH 2.0 ($T_{m,225}$ = 70°C; $T_{m,265}$ = 67.1°C), — - — pH 6.5 ($T_{m,225}$ = 78.6°C; $T_{m,265}$ = 67°C), and — — pH 9.5 ($T_{m,225}$ = 72.7°C; $T_{m,265}$ = 72.2°C). Protein concentration was 0.1% and 0.2%, respectively. Buffer concentration was 0.05 M (except for pH 2.0, where 0.01N HCl was used) and ionic strength was 0.4. Optical path length was 0.1 cm and 1 cm, respectively.

amounts developed at higher pH values (pH 6.5 and 7.5). This observation corresponds with previous studies on peptides. High succinimide concentrations were found at slightly acidic pH values, whereas low succinimide concentrations were detected at near neutral to basic conditions (5,20). The main formation of peaks 4 and 5 (group II) was observed at pH 6.5 and 7.5 whereas only small amounts of succinimides (group I) were found. Under these conditions succinimides only form as intermediates which are then further hydrolyzed. This result is in agreement with previously published data on peptides. In the course of the degradation of Asp-containing peptides (6; 20,21), low succinimide and increasing isoAsp concentrations were determined at neutral to basic conditions. These observations suggest the hypothesis that peaks 4 and 5 represent isomerized rHir, i.e. isoAsp^{33} and isoAsp^{53}, as supported by corresponding conclusions with rHir HV2 (16). Without additional independent confirmation, preferably by mass spectrometry, sequence data or purified standards for CE, these suggestions remain speculative.

Deamidation is known to take place at neutral to alkaline pH as shown for lysozyme (22), human growth hormone (23), and human epidermal growth factor 1–48 (24). Fig. 5 illustrates that peaks 1 to 3 (group III) were formed in reasonable quantities only at pH 6.5 and higher, supporting the suggestion that these peaks represent deamidated rHir.

At acidic and alkaline pH values, a loss of total peak area was observed, whereas total peak area was preserved between pH 5.0 and 7.5. At acidic pH (≤ pH 4.0), degradation by peptide bond hydrolysis or multiple succinimide formation may take place. The formation of di-succinimide-containing rHir may result in a reduced CE migration rate rendering it undetectable within the monitored time frame. The loss of total peak area at alkaline pH (≥ pH 8.5), may result from cleavage of S-S bonds, as reported for lysozyme (22), and rHir (3). Chang (3) reported that alkaline inactivated rHir is a mixture of various rHir polymers as large as heptamers. Polymerization can result in very slow or even in no migration of the degradation product in the CE capillary rendering it undetectable.

The thermal unfolding of rHir (HV2) was previously studied by CD spectroscopy, and the midpoint of the transition (T_m) was observed to be around 65°C (25). For rHir (HV1), a two stage melting was observed (11). Minor effects were between 0 and 40°C and related to changes in the tail segment only. For the rHir core a T_m of ~71–73°C was determined at neutral pH. Changes of a protein T_m can occur by changing the pH or the ionic strength of the solvent (11–12,26). Because of the large pH range (pH 1.0 to 9.5) and the rather high ionic strength (0.4) of the buffers used in this stability study, the thermal denaturation of rHir was looked at in various buffer systems (Fig. 7). The T_m of the rHir core in all the buffers studied was 17–28°C above 50°C, the storage temperature used for the chemical degradation study. Therefore, major influence of partial denaturation of rHir at the temperature selected for this study should be excluded.

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