Inhibition of Succinimide Formation in Aqueous Zn-rHirudin Suspensions[1]

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Purpose. The formation of succinimide intermediates at Asp-Gly sites and their hydrolysis products, e.g., isoAsp isomers, represents a common source of microheterogeneity in therapeutic proteins. Here we report on the stabilization effect of a zinc chloride induced precipitation of recombinant hirudin HV1 (rHir), an anticoagulant protein.

Methods. rHir was precipitated by zinc chloride at neutral pH to form a Zn-rHir suspension. An Arrhenius-type study (at 50, 40, 30, and 25°C) and a 4°C stability study were performed. Monitoring of rHir, rHir succinimides at Asp^{33}-Gly^{34} (Q5) and Asp^{53}-Gly^{54} (Q4), and further side products was by capillary electrophoresis (CE).

Results. The activation energies of rHir degradation in both aqueous rHir solution and Zn-rHir suspension were similar, i.e. 104.5 and 110.3 kJ/mol, respectively. Zn-rHir suspension demonstrated improved shelf-life stability ($t_{90\%}$, 95% confidence limit) versus rHir solution, i.e., 23 versus 3 days at 25°C and 292 versus 147 days at 4°C, respectively. In rHir solution, Q4 (Asp^{53}-Gly^{54} succinimide) levels were slightly above Q5 (Asp^{33}-Gly^{34} succinimide) levels. In Zn-rHir suspension, however, Q4 succinimide levels dropped markedly whereas Q5 levels were not affected. Correspondingly, in Zn-rHir $isoAsp^{53}$-rHir levels were reduced but not $isoAsp^{33}$-rHir levels.

Conclusions. In Zn-rHir suspensions, interactions of zinc and rHir show site-specific inhibition of succinimide formation only at Asp^{53}-Gly^{54} (Q4), located in the highly flexible C-terminal tail of rHir. In contrast, succinimide formation at Asp^{33}-Gly^{34} (Q5), located in a less flexible loop domain is not affected, reflecting steric hindrance.

KEY WORDS: recombinant hirudin; protein formulation; protein stability; capillary electrophoresis; cyclic imide.

INTRODUCTION

Hirudin is a potent and selective inhibitor of human thrombin (1). The three major variants of hirudin presently available, HV1, HV2, and HV3, have a high degree of homology (13 variable positions) but in contrast to the natural hirudin they all lack the sulfate group at Tyr^{63} (2). After injection in humans rHir has a relatively short half-life of 2 to 3 hours (3). Previously, a sustained release formulation of rHir by complex formation with zinc salts at neutral pH was proposed to form an aqueous Zn-rHir suspension (4). Its prolonged biological activity has been demonstrated in rats (4). Here we study the chemical stability of such a formulation. In particular we were interested in the mechanisms of degradation and the shelf-lives of Zn-rHir suspensions.

Previously two of the degradation products of rHir, when stored under acidic conditions, were identified as succinimides (5) forming at positions Asp^{53}-Gly^{54} and Asp^{33}-Gly^{34} (laboratory codes Q4 and Q5, respectively). Succinimides are a common source of microheterogeneity in therapeutics proteins. As observed in both peptides and proteins, succinimide intermediates hydrolyse at neutral to alkaline pH and form isoAsp and Asp containing isomers (6–9). Under such conditions $isoAsp^{53}$- and $isoAsp^{33}$-rHir are expected degradation products of rHir. Schindler *et al.* (10) identified the $isoAsp^{33}$ isomer. Deamidation of Asn^{52} leads to another potential degradation product of rHir HV1 at neutral to alkaline pH in analogy to rHir HV2. In rHir HV2, deamidation at Asn^{52} occured after the deamidation of Asn^{33} and Asn^{53} under alkaline conditions (11).

To determine the shelf-life ($t_{90\%}$) of a drug formulation at low temperature, the Arrhenius equation can be used as a tool. Many peptides and proteins follow the Arrhenius relationship, e.g., Asn-hexapeptide (12), bromelain, α-chymotrypsin, kallikrein (13), and salmon calcitonin (14).

The chemical stability of rHir in a Zn-rHir suspension (pH 7.1) was studied by capillary electrophoresis (CE). As a reference, the degradation of rHir in aqueous solution (pH 7.1) was also analyzed. The $t_{90\%}$ of the two formulations were compared and possible degradation pathways discussed. Moreover, the kinetics of succinimide formation in rHir and its inhibition in Zn-rHir suspension is studied relative to the location of the respective Asp-Gly sites in the protein, one in the highly flexible C-terminal tail and the other in a less flexible loop domain.

MATERIALS AND METHODS

Materials

The rHir (HV1) used for the experiments was obtained from Ciba-Geigy Ltd., Basel, Switzerland. rHir consists of 65 amino acids with a molecular weight of 6,964 Da and has an isoelectric point of 3.9 (15). All chemicals were of analytical grade from Fluka-Chemie AG (Buchs, Switzerland) and Merck AG (Zürich, Switzerland). Throughout nanopure water (Milli-Q®, Millipore Corp., Bedford, MA, USA) was used. Traces of $isoAsp^{53}$-rHir and $isoAsp^{33}$-rHir were obtained from Dr. Walter Märki, Novartis Pharma AG, Basel, Switzerland.

Capillary Electrophoresis (CE)

All electrophoretic experiments were performed using a Beckman P/ACE 5010 system (Beckman Instruments, Fullerton, CA, USA). Two different protocols were used (method 1 and method 2).

The protocol of method 1 was based on Forrer *et al.* (15). Experimental and statistical details are given in (16). In brief, uncoated fused-silica capillaries with 117 cm (110 cm to UV detector) × 50 m ID × 360 μm OD were obtained from BGB Analytik AG (Rothenfluh, Switzerland). Separations were conducted at constant voltage + 28 kV (approximately 8 μA)

[1] Adapted from U. Gietz. *Therapeutic protein formulation for sustained delivery: Formulation aspects and stability.* Ph.D. Thesis ETH Zurich No. 12177, Zürich, 1997.

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ABBREVIATIONS: rHir, recombinant hirudin; Zn-rHir, zinc/recombinant hirudin suspensions; CE, capillary electrophoresis.

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and at a constant temperature of 25°C. Detection was at 200 nm, and the sample tray was cooled to 5°C to protect the samples. All concentration measurements were calculated from corrected peak areas (peak area/migration time) to compensate for variation of electroosmotic flow.

Method 2 was a modification of the protocol of Dette and Wätzig (17) avoiding its poor reproducibility and robustness. Experimental and statistical details are given in (16). In brief, neutral coated capillaries (eCap™) with 37 cm (30 cm to UV detector) × 50 μm ID were obtained from Beckman Instruments (Fullerton, CA, USA). Separations were conducted in the reverse-polarity mode (negative potential at the injection end of the capillary) at constant voltage 25 kV (approximately 26 μA) and at a constant temperature of 25°C. Detection was at 214 nm, and the sample tray was cooled to 5°C. Corrected peak areas were used throughout. For presentation of the degradation pattern of rHir, corrected peak areas of the degradation products were expressed as percentage of the sum of corrected peak area at time zero.

Kinetic Measurements

To provide sterile samples, preparation of the Zn-rHir suspension was under laminar flow. Aqueous $ZnCl_2$ (165 mM $ZnCl_2$, containing 38 mM HCl to ensure filtrability), rHir (74.1 mg/mL) and NaOH (0.6 N) solutions were sterile filtered through 0.2 μm low protein binding filters (Acrodisc®, Gelman Sciences, Ann Arbor, MI, USA). 52.8 mL of the $ZnCl_2$ solution and 29.7 mL of the rHir solution were combined and mixed in a 100 mL sterile plastic vial. 27.5 mL of the NaOH solution was then added, and by shaking the vial the Zn-rHir suspension formed. Final rHir concentration was 20 mg mL^{-1}. The pH was 7.1, and after centrifugation 99.2% of the total rHir was found in the pellet.

As a control, an aqueous rHir solution was investigated. 2.0 g of rHir were dissolved in 100.0 mL water and the pH was adjusted to 7.1 by using 0.6 N NaOH. This solution was then sterile filtered under laminar flow. Final rHir concentration was 20 mg mL^{-1}.

Samples of approximately 0.5 mL of the sterile formulations (suspension or solution) were filled into 2 mL amber borosilicate glass vials, topped with rubber stoppers and closed with aluminum seals (all from Wheaton, Millville, NJ, USA). Prior to use, the glass vials were heat sterilized and the rubber stoppers were vapor sterilized. The degradation of rHir in the two formulations was studied at 50, 40, 30, 25, and 4°C. At certain time intervals, vials were taken off and frozen at −23°C. Prior to analysis, the samples were thawed, diluted with 0.1 N HCl (1:4 for CE method 1 or 1:10 for CE method 2, to yield concentrations of 5 and 2 mg/mL, respectively), and cooled to 5°C. For each individual time point a separate vial was used.

Determination of Observed Degradation Rate Constants (k_{obs})

At various temperatures, the degradation of rHir in the aqueous solution and in the Zn-rHir suspension was determined as a function of time. Analyses were done with CE method 2. 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 with storage time was found to follow pseudo first order at all temperatures. The slope of the semilog plot of remaining rHir concentration versus time is the observed degradation rate constant (k_{obs}).

Determination of Confidence Limits

One sided confidence limits were calculated according to standard statistics. The lower confidence limits of rHir degradation profiles at 25 and 4°C were used to estimate minimum shelf-lives ($t_{90\%,min}$). The upper confidence limit of the Arrhenius plot was taken to calculate the highest possible degradation rate constant (k_{max}) at 4°C. Calculations were based on the standard error of the regression of the experimental data. In addition, using first order kinetics, shelf-lives ($t_{90\%}$) were also calculated from both k_{max} and k_{obs}.

Determination of the Activation Energy from the Arrhenius Plot

The activation energy (E_a) of degradation was calculated from the Arrhenius equation. The physical significance of the Arrhenius approach to predict rHir stability was previously verified by circular dichroism studies (16). Only minor secondary structure changes of the protein were observed up to ~60°C.

RESULTS

Capillary Electrophoresis

Figure 1 represents typical electropherograms of fresh rHir solution, degraded rHir solution and degraded Zn-rHir suspension at pH 7.1 using CE method 1. Figure 1a is from a freshly prepared rHir solution. The main peak (rHir) and the two known congeners of rHir, the succinimides at Asp^{53}-Gly^{54} and Asp^{33}-Gly^{34}, laboratory code Q4 and Q5 (5), respectively, were well separated (Fig. 1a, insert) as described by Forrer *et al.* (15). However, the electropherogram of degraded rHir solution showed poor separation of rHir and the major degradation products (Fig. 1b). In addition to Q4 and Q5, another but unknown degradation product was observed (Fig. 1b, insert). A degraded Zn-rHir suspension is represented in Fig. 1c. Again, the major degradation products of the Zn-rHir suspension and rHir were not well separated, equivalent to the degradation of aqueous rHir solution. In contrast to degraded rHir solution, degraded Zn-rHir suspension showed much less Q4 than Q5, and the unknown peak was relatively smaller (Fig. 1b and 1c, inserts).

Electropherograms of fresh rHir, degraded rHir solution and degraded Zn-rHir suspension at pH 7.1 using CE method 2 are given in Fig. 2. Figure 2a represents a typical electropherogram of a freshly prepared rHir solution. The broad peak at ~19 min was previously demonstrated to contain both Q4 and Q5 (16). Freshly prepared rHir solution showed no degradation products except Q4/Q5 (Fig. 2a), whereas degraded rHir solution showed five main and well separated degradation products, designated as peaks I to 5 (Fig. 2b). The electropherogram of degraded Zn-rHir suspension (Fig. 2c) showed similar degradation products as compared to degraded rHir solution. In degraded rHir solution peak 4 and peak 5 were similar in height, but peak 5 dropped significantly in degraded Zn-rHir suspension.

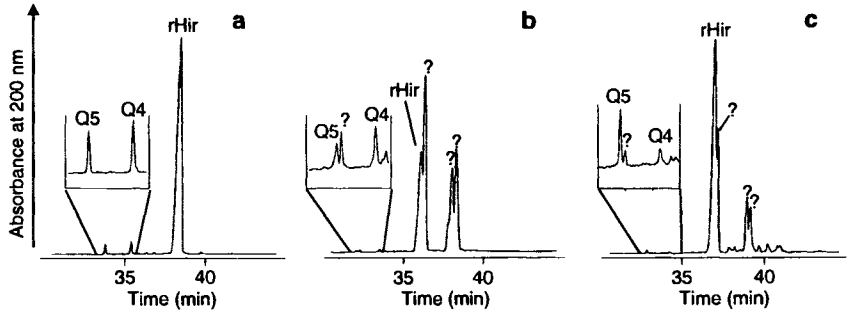

Fig. 1. Typical CE chromatograms illustrating rHir degradation. CE method 1 is used. (a) Freshly prepared rHir solution, (b) degraded rHir solution (pH 7.1; 374 days at 30°C), and (c) degraded Zn-rHir suspension (pH 7.1; 323 days at 30°C). Inserts show succinimide variants of rHir at Asp^{53}-Gly^{54} (Q4) and Asp^{33}-Gly^{34} (Q5). For the electrophoretic conditions, see materials and methods. Question marks indicate unknown peaks.

According to the characteristic migration pattern of the main degradation products of rHir as analyzed by CE method 2, the occurring peaks were classified in three groups, i.e. I, II, and III (Fig. 2b). The first group consisted mainly of the Q4/Q5 peak. Peaks 4 and 5 were assigned to a second group and peaks 1 to 3 to a third group (see discussion). Except for Q4/Q5, all other degradation products are not identified. Spiking of samples with suitable isoAsp-rHir traces suggested that peaks 4 and 5 in group II indicate $isoAsp^{33}$-rHir and iso Asp^{53}-rHir, respectively (data not shown).

CE method 1 resolved Q4 and Q5, whereas method 2 did not. Because CE method 2 separated rHir from the major degradation products (Fig. 1 versus Fig. 2), contrasting to the overlap of method 1, method 2 was selected to monitor the degradation kinetics of rHir, whereas the concentrations of Q4 and Q5 were analyzed by method 1.

Arrhenius Plots

Both the degradation of the rHir solution and the Zn-rHir suspension followed pseudo first-order kinetics at all temperatures investigated. Degradation of rHir was analyzed at least up to 50% rHir loss. Arrhenius plots (50, 40, 30, and 25°C) are represented in Fig. 3. For the rHir solution, statistical variability was lower (r^2 = 0.996) than for the Zn-rHir suspension (r^2 = 0.989). Degradation rates at 4°C were also included in Fig. 3 (given in parentheses). At this temperature, degradation was monitored only up to 20% rHir loss in rHir solution and 5% in Zn-rHir suspension, respectively. Because of the resulting higher statistical error, the 4°C data were not included in the Arrhenius plot calculations. By inclusion of the 4°C data, both profiles seem to suggest a slight curvature which would indicate a two step process with a shift at around 30°C.

By linear regression of the Arrhenius plot, the overall activation energy (E_a) of rHir degradation was calculated as 104.5 kJ/mol (24.9 kcal/mol) for the rHir solution and 110.3 kJ/mol (26.3 kcal/mol) for the Zn-rHir suspension. These activation energies are in good agreement with other proteins and peptides (6,12–14).

Degradation rate constants and shelf-lives of the rHir solution and the Zn-rHir suspension at 4°C were calculated by extrapolation of the Arrhenius plot. Using the upper 95% confidence limit of the Arrhenius plot, the maximum degradation rate con-

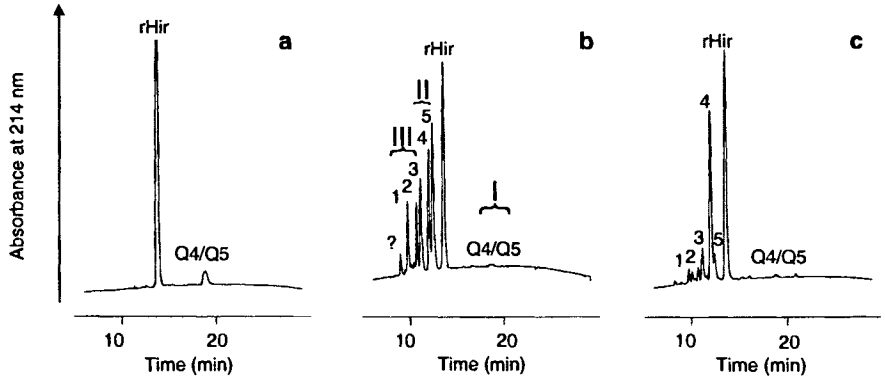

Fig. 2. Typical CE chromatograms illustrating rHir degradation. CE method 2 is used. (a) Freshly prepared rHir solution, (b) degraded rHir solution (pH 7.1; 234 days at 25°C), and (c) degraded Zn-rHir suspension (pH 7.1; 374 days at 25°C). For the electrophoretic conditions, see materials and methods. There are three groups of peaks. Group I contains a broadened peak consisting of both Q4 and Q5 (16). Group II is suggested to consist of $isoAsp^{33}$;-rHir (peak 4) and $isoAsp^{53}$-rHir (peak 5). By analogy to (17) group III, consisting of peaks 1–3, may represent deamidation products. Question marks indicate small and unknown peaks.

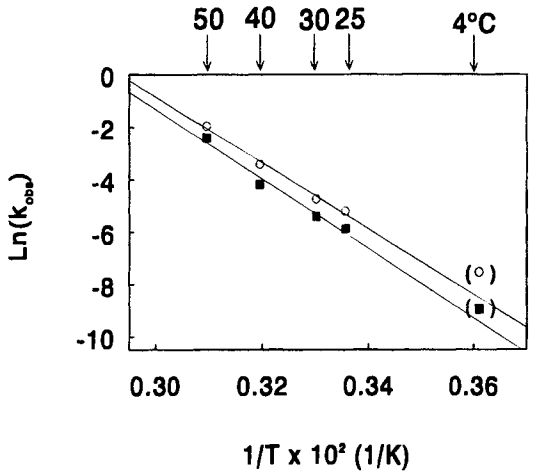

Fig. 3. Arrhenius plots of rHir degradation: ○ rHir solution; ■ Zn-rHir suspension. Symbols given in parentheses indicate k_{obs} at 4°C, calculated from profiles of ~20% and ~5% maximum rHir degradation in rHir solution and Zn-rHir suspension, respectively. 4°C values were not included in linear regression analysis. Degradation data are based on CE method 2.

Table I. Summary of Degradation Rate Constants (k_{obs}, k_{max}) and Shelf-lives ($t_{90\%}$,$t_{90\%,min}$) of rHir Solution and Zn-rHir Suspension at 25 and 4°C

°C		rHir solution pH 7.1	Zn-rHir suspension pH 7.1
25	k_{obs} (day $^{-1}$, × 10^4)	56.9	28.0
	$t^a_{90\%}$ (days)	18	38
	$t^b_{90\%,min}$ (days)	3	23
4	k_{obs} (day $^{-1}$, × 10^4)	5.4	1.3
	$t^a_{90\%}$ (days)	194	808
	$t^b_{90\%,min}$ (days)	147	292
4	k^c_{max} (day $^{-1}$, × 10^4)	5.4	4.7
	$t^d_{90\%}$ (days)	194	224

[a] Shelf-life $t_{90\%}$ calculated from k_{obs} of semilog degradation profile.
[b] Shelf-life $t_{90\%,min}$ according to lower confidence limit (95%, one-sided) of semilog degradation profile.
[c] Maximum degradation rate (k_{max}) using upper confidence limit (95%, one-sided) of Arrhenius plot (25–50°C).
[d] Shelf-life $t_{90\%}$ calculated from k_{max}.

stants (k_{max}) at 4°C were calculated and listed in Table I together with the corresponding $t_{90\%}$.

Degradation at 25 and 4°C

In addition to the Arrhenius approach, shelf-life extrapolations for rHir solution and Zn-rHir suspension were also made on the basis of the 25 and 4°C degradation profiles. Both observed degradation rates (k_{obs}) and corresponding shelf-lives ($t_{90\%}$, $t_{90\%,min}$, 95% confidence limits) were calculated (Table I).

Degradation Pattern of rHir

Figure 4 shows the degradation pattern of the aqueous rHir solution at the four temperatures selected. The principal degradation patterns at all temperatures were similar. The total corrected peak area stayed close to 100% throughout the investigated time period. The main degradation products (peaks 4 and

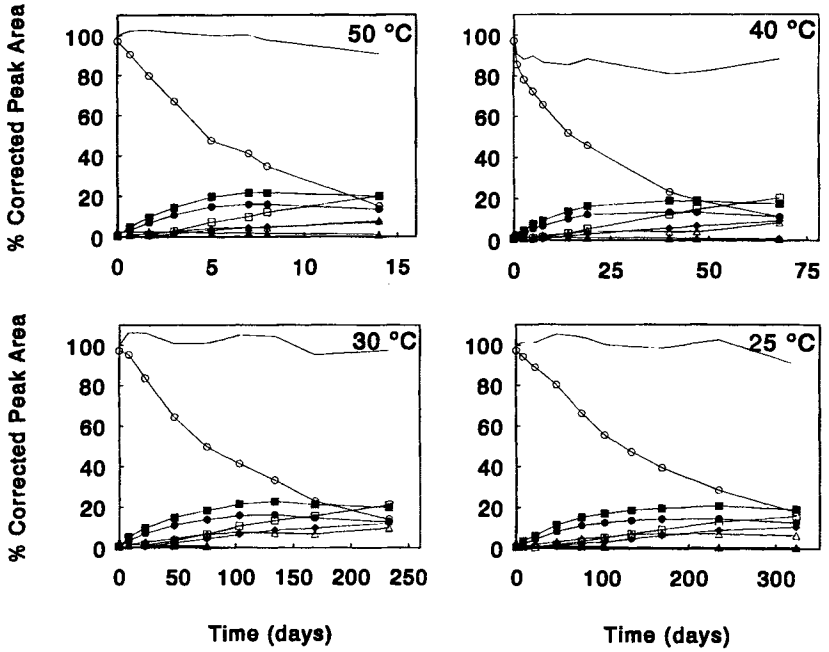

Fig. 4. Degradation pattern of rHir solution at 50, 40, 30 and 25°C monitored by CE method 2. ○ rHir, ▲ Q4/Q5, ◆ peak 1, Δ peak 2, □ peak 3, ● peak 4, ■ peak 5. For peak codes see Fig. 2. Upper lines without symbols indicate the sum of the corrected peak area of all peaks expressed as percentage of the total corrected peak area at time zero.

5) showed an initially fast formation and reached a plateau or a flat maximum (~15 and ~20%, respectively). In contrast, peak 3 steadily increased throughout the investigated time period reaching an upper level of 15–20%. Peaks 1 and 2 each stayed below 10%. The Q4/Q5 peak, consisting of both the succinimide at Asp^{53}-Gly^{54} and Asp^{33}-Gly^{34} (16), was below 2% and approximately constant during the observed time period.

Figure 5 represents the degradation of Zn-rHir suspension at the various temperatures. Again the degradation patterns at all temperatures were similar. A slight decrease (~20%) of the total corrected peak area was only observed at 50°C. For all the other temperatures, the total corrected peak area stayed close to 100% during the investigated time period. At all temperatures, the main degradation product was peak 4. An inital increase followed by a flat maximum or a plateau was observed. In contrast to the aqueous rHir solution, peak 5 was reduced to a minor fraction. Similar to the degradation pattern of the rHir solution, a steady increase of peak 3 was observed. All the other degradation products (peaks 1, 2, and 5) were formed only in small quantities (below ~10%). The combined Q4/Q5 peak was in the range of ~2% and stayed approximately constant.

Using CE method 1, both succinimides, Q4 and Q5, were determined separately. Analyses were performed at an inital rHir concentration of 5 mg/mL to detect the small amounts of Q4 and Q5. At all four temperatures studied, the degradation of the rHir solution showed slightly lower percentages of Q5 than Q4 (Fig. 6), whereas in Zn-rHir suspension much lower percentages of Q4 than Q5 were observed (Fig. 7). The overall trend of the succinimide concentrations was also different. In rHir solution the trend was negative, whereas an increase or constant level was observed with Zn-rHir suspension.

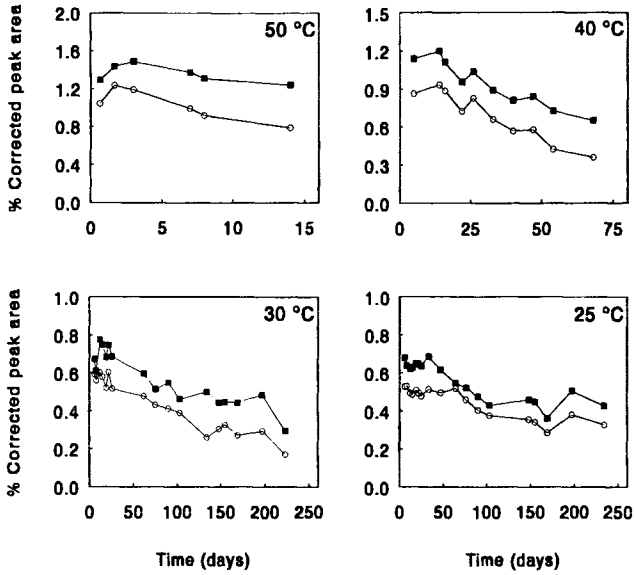

Fig. 6. Succinimide concentrations of rHir solution during the stability study at various temperatures. ■ Q4 (succinimide at Asp^{53}-Gly^{54}), ○ Q5 (succinimide at Asp^{33}-Gly^{34}). CE method 1 was used.

DISCUSSION

Regarding their typical CE migration pattern (method 2), rHir and its main degradation products may be classified in three groups (Fig. 2b). Since migration times provide information about the charge to mass ratio, group I should contain less negatively charged degradation products (Q4/Q5) as compared

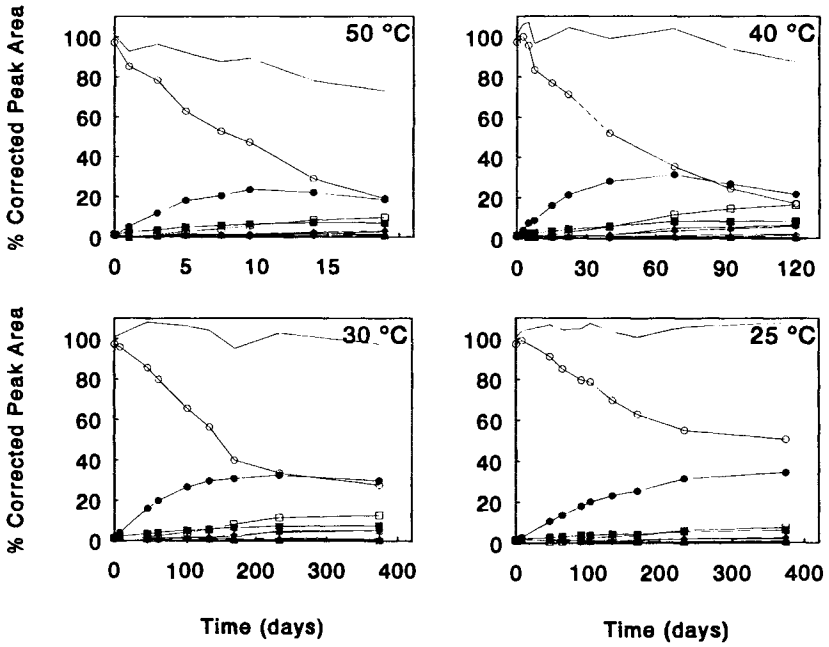

Fig. 5. Degradation pattern of Zn-rHir suspension at 50, 40, 30 and 25°C monitored by CE method 2. ○ rHir, ▲ Q4/Q5, ♦ peak 1, Δ peak 2, □ peak 3, • peak 4, ■ peak 5. For peak codes see Fig. 2. Upper lines without symbols indicate the sum of the corrected peak area of all peaks expressed as percentage of the total corrected peak area at time zero.

to rHir. Group II (peaks 4 and 5) is supposed to cover degradation products of slightly higher negative charge than rHir. They are proposed to indicate isomerized rHir (isoAsp^{33}-rHir and isoAsp^{53}-rHir, respectively), having a slightly lower pK than Asp-rHir (8,16). This assumption was confirmed using spiked samples. Finally, group III (peaks 1 to 3) is expected to consist of even more negatively charged degradation products, possibly deamidated rHir and mixtures of deamidated and isomerized rHir (17).

Stability studies of an Asp containing hexapeptide at neutral pH showed the isomerization of Asp to occur via formation of a succinimide (6). The authors concluded the formation of this intermediate to be rate limiting as compared to the ring opening reaction to Asp or isoAsp, resulting in very low succinimide concentration (0.2%) at this pH. Succinimide formation was also observed to represent the rate limiting step for the deamidation of RNase A at pH 7.2 to 9.0 (7). These observations are consistent with the results of our study showing only minor concentrations of Q4 and Q5 in both rHir solution and Zn-rHir suspension (pH 7.1; total concentration $\leq$ 2.5%). In contrast to Q4 and Q5 much higher concentrations of peaks 4 and 5 (proposed to represent isomerized rHir, see below) were found in degraded rHir solution. Thus, the formation of the succinimide intermediates is considered to be the rate limiting step for rHir isomerization at pH 7.1.

A typical feature of the degradation pattern of the rHir solution are the plateaus or flat maxima of peaks 4 and 5, whereas peak 3 followed a steady increase during the full time period. Corresponding to the suggestions of Dette and Wätzig for HV2-rHir (17), we assume peaks 4 and 5 to represent isomerized rHir, namely isoAsp^{33}-rHir and isoAsp^{53}-rHir, respectively. More evidence for this assumption was obtained by analysis of spiked samples. Peak 3 is speculated to indicate deamidated rHir. Many other studies have shown the reversibility of the isomerization process of an Asp residue and the irreversibility of the deamidation process of an Asn residue in peptides (6,12,18). These features are both reflected in the respective profiles of peak 4 and 5 (plateaus or flat maxima = reversible reaction) versus peak 3 (steady increase = irreversible reaction).

NMR studies by Nordmann[5] showed that the interactions of Zn^{2+} with rHir were site specific. Among the sites identified, she observed Asp^{53} (degradation site of Q4, located in the large highly flexible C-terminal tail rHir$^{49-65}$) to interact with Zn^{2+}, whereas Asp^{33} (degradation site of Q5, located in the less flexible loop rHir$^{31-36}$) did not. These results are consistent with our observations. In the Zn-rHir suspension, the Q5 concentration is much higher than the Q4 concentration (Fig. 7). The low Q4 concentration indicates a site specific interaction of Zn^{2+} and rHir that inhibits only the Asp^{53}-Gly^{54} succinimide (Q4) formation whereas the succinimide formation at the sterically more restricted Asp^{33}-Gly^{34} site (Q5) is not affected. In rHir solution, i.e. in the absence of Zn^{2+}, Q4 concentrations were slightly higher than Q5 concentrations (Fig. 6), reflecting a slightly higher backbone flexibility at Asp^{53} than at Asp^{33}.

[5] A. P. Nordmann. *Spectroscopic and related studies of recombinant hirudin, human neuropeptide Y and analogues*. Ph.D. Thesis, Birkbeck College, London, 1996.

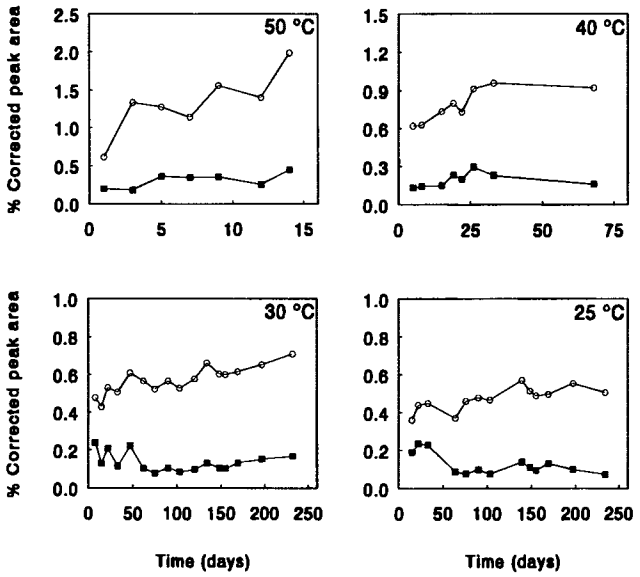

Fig. 7. Succinimide concentrations of Zn-rHir suspension during the stability study at various temperatures. ■ Q4 (succinimide at Asp^{53}-Gly^{54}), ○ Q5 (succinimide at Asp^{33}-Gly^{34}). CE method 1 was used.

Hydrolysis of a succinimide in peptides and proteins at neutral pH leading to either Asp or isoAsp formation has been the subject of several reports (e.g., 6–8). From the above discussed observation of less Q4 (Asp^{53}-Gly^{54} succinimide) than Q5 (Asp^{33}-Gly^{34} succinimide) in degraded Zn-rHir suspension (Fig. 7), the formation of less isoAsp^{53} than isoAsp^{33} would be expected. As assumed by the migration times of the CE (Fig. 2b) and as suggested by the spiked samples, peaks 4 and 5 were proposed to represent the isoAsp analogs of rHir, isoAsp^{33}-rHir and isoAsp^{53}-rHir (16). In fact, as shown in Fig. 5, smaller quantities of peak 5 (<9%) than peak 4 (up to 30%) were determined in degraded Zn-rHir suspension. Peak 5 is assumed to represent isoAsp^{53}-rHir (hydrolysate of Q4) whereas peak 4 indicates isoAsp^{33}-rHir (hydrolysate of Q5). In degraded rHir solution, slightly lower Q5 than Q4 concentrations were determined (Fig. 6), and consistent with the above assumption, slightly lower levels of peak 4 as compared to peak 5 were found.

In conclusion, an overall consistent mechanism for the observed rHir degradation pathway is suggested. Inhibition of both succinimide formation and isomerization is site-specific. The control of succinimide intermediates, Q4 and Q5, plays a pivotal role in controlling the isomerization of Asp-Gly sequences in rHir. Only in case of the Asp^{53}-Gly^{54} site can Zn^{2+} successfully inhibit succinimide formation and isomerization. No effect is observed at the Asp^{33}-Gly^{34} site indicating steric hindrance. Overall, the interaction with zinc is beneficial for the shelf-life of rHir. The deamidation pathway of rHir needs to be further evaluated.

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