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Stability of Hirudin, a Thrombin-specific Inhibitor

THE STRUCTURE OF ALKALINE-INACTIVATED HIRUDIN*

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Hirudin is a 65-amino acid polypeptide with three disulfide linkages. It is stable under extreme pH (1.47–12.9), high temperature (95 °C), and in the presence of denaturants (6 M guanidinium chloride or 8 M urea). The thrombin inhibitory activity of hirudin remains unaffected even after cleavage of an internal peptide bond (Lys^{36}-Asn^{37}). One condition which effectively and irreversibly inactivates hirudin is the combination of elevated temperature and alkaline pH. Structural analysis reveals that inactivation is a consequence of base-catalyzed β-elimination of the disulfide bonds. The reaction leads to the conversion of hirudin to a mixture of highly heterogeneous polymers (from monomer to heptamer) which are intra- and intermolecularly cross-linked by cystine (20%), lanthionine (50%), and lysinoalanine (30%).

Hirudin is a potent thrombin-specific inhibitor isolated from the leech *Hirudo medicinalis* (Markwardt and Walsmann, 1958; Badgy *et al.*, 1976). The thrombin inhibitory mechanism of hirudin has been extensively investigated (Chang, 1983a; Chang, 1989; Dennis *et al.*, 1990; Dodt *et al.*, 1988; Dodt *et al.*, 1990; Maraganore *et al.*, 1990; Mao *et al.*, 1988; Ni *et al.*, 1990; Niehrs *et al.*, 1990; Wallace *et al.*, 1989). The inhibitor contains two functional domains (Chang, 1983a; Gruetter *et al.*, 1990; Rydel *et al.*, 1990), a compact NH_2-terminal domain stabilized by three disulfide linkages and a disordered COOH-terminal tail. It is now known that the exceedingly high binding affinity (Stone and Hofsteenge, 1986) and specificity of the hirudin/thrombin complex is a result of cooperative binding of the two functional domains of hirudin to separate sites on thrombin (Rydel *et al.*, 1990; Gruetter *et al.*, 1990). The NH_2-terminal core domain blocks the active site of thrombin, whereas the COOH-terminal tail binds to the fibrinogen recognition site (anion binding exosite) of the enzyme. Both functional domains were also found to be active in fragment form (Chang *et al.*, 1990; Dennis *et al.*, 1990; Krstenansky and Mao, 1987; Maraganore *et al.*, 1989).

As a protein, hirudin is a remarkably stable molecule. During studies of hirudin by chemical modification (Chang, 1983a; Chang, 1989). We observed that the molecule is totally resistant to various extreme conditions. For instance, hirudin remains fully active following incubation in 50% trifluoroacetic acid at 55 °C for 15 min. The notable stability of hirudin, although shared to varied extents by numerous protease inhibitors, is nonetheless uncommon in biologically active proteins (for review, see Volkin and Klibanov, 1989). The purpose of the present study was to investigate conditions that inactivate hirudin and to elucidate the mechanism of the inactivation.

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EXPERIMENTAL PROCEDURES

Materials—Recombinant desulfato-hirudin (CGP 39393) was produced by Ciba-Geigy in collaboration with Platorgan KG (Frederal Republic of Germany) and was kindly supplied by Dr. H. Grossenbacher (Ciba-Geigy, Basel). Dimethylaminoazobenzene isothiocyanate (DABITC)[1] and dimethylaminoazobenzene sulfonyl chloride (DABS-Cl) were obtained from Fluka. Human α-thrombin was supplied by the Center for Diagnostic Products (Boston). Lysyl endopeptidase (*Achromobacter lyticus* protease I) was obtained from Wako (Japan). Asp-N and Chromozym TH were purchased from Boehringer Mannheim.

Analysis of the Stability of Hirudin—Hirudin (1 nmol) was dissolved in 40 μl (25 μM) of various solutions and incubated at various temperatures as specified in Table I. In all cases, no precipitation of hirudin took place. The treated protein was diluted to 25 nM with Tris-HCl buffer (67 mM, pH 8.0, containing 133 mM NaCl and 0.13% polyethylene glycol 6000) and was used for antithrombin (anti-amidolytic) assay. The activity of the control sample (hirudin dissolved in the Tris-HCl buffer and left at 23 °C) was taken as 100%.

Digestion of Hirudin with Lysyl Endopeptidase—Hirudin (12 nmol) was dissolved in 240 μl of ammonium bicarbonate solution (50 mM, pH 8.0) and was treated with 10 μg of lysyl endopeptidase. Digestion was carried out at 23 °C. At each time point (30 min, 1.5, 4, 7, and 24 h), two aliquots of the digest (each containing 0.5 nmol) were withdrawn. One was freeze-dried and subjected to quantitative NH_2-terminal analysis to evaluate the extent of cleavage at the three Lys-Xaa bonds. The other was diluted to 25 nM with Tris-HCl buffer (67 mM, pH 8.0) and was used for antithrombin (anti-amidolytic) assay. A control sample without addition of the peptidase was procsessed in parallel and its anti-amidolytic activity was taken as 100%. A second control sample containing only lysyl endopeptidase was found to have no effect on thrombin's amidolytic activity.

Anti-amidolytic Assay—The anti-amidolytic activity of hirudin was measured by its ability to inhibit thrombin-catalyzed digestion of the synthetic substrate Chromozym TH. The reaction was carried out at 23 °C in 67 mM Tris-HCl buffer, pH 8.0, containing 133 mM NaCl and 0.13% polyethylene glycol 6000. The digestion was followed at 405 nm for a period of 2 min. The concentrations were: chromogenic substrate, 200 μM; thrombin, 1 nM; and hirudin, 0.9 nM. Under these conditions, the control hirudin sample inhibited 90% of the thrombin activity.

Structural Analysis of Hirudin—Amino acid composition was evaluated by the DABS-Cl method (Knecht and Chang, 1986). The DABS derivatives of lanthionine and lysinoalanine were prepared as described (Chang *et al.*, 1983). The amino-terminal sequence was obtained by the DABITC method (Chang, 1988) with double coupling (Chang, 1983b) or automatically with a gas-phase sequenator (Hunkapiller *et al.*, 1983). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). The conditions for HPLC separation of hirudin derivatives are described in the legend to Fig. 4.

[1] The abbreviations used are: DABITC, dimethylaminoazabenzene isothiocyanate; DABS-Cl, dimethylaminoazobenzene sulfonyl chloride; HPLC, high-performance liquid chromatography; Hir, hirudin.

RESULTS

Hirudin Remains Active Even after Cleavage of the Lys^{36}-Asn^{37} Bond Within Its NH_2-terminal Core Domain—Hirudin contains 3 lysyl residues, of which two are located within the NH_2-terminal core domain (Lys^{27} and Lys^{36}) and the other is situated in the hinge region of the NH_2-terminal and COOH-terminal domains (Fig. 1). All three Lys-Xaa bonds were resistant to trypsin (Chang, 1983a) and endoproteinase Lys-C (from *Lysobacter enzymogenes*) but were cleaved by lysyl endopeptidase (from *A. lyticus*). Lys^{36}-Asn was the most sensitive bond and could be selectively cleaved within 30 min of digestion (Fig. 2). Lys^{36}-Asn^{37}-cleaved hirudin retains more than 95% of its anti-amidolytic activity (Fig. 2). Thus a two-chain hirudin comprising Hir$^{1-36}$ and Hir$^{37-65}$ (held together by three disulfide linkages) was shown to maintain the active conformation. The decrease in hirudin activity was found to relate quantitatively to the cleavage of the Lys^{47}-Pro^{48} bond (Fig. 2). However, the diminished activity may possibly be attributed to the cleavage of the Lys^{27}-Cys^{28} bond which is attacked by lysyl endopeptidase as a slightly slower rate than Lys^{47}-Pro^{48}.

Stability of Hirudin—The stability of hirudin under various conditions is shown in Table I. Hirudin is resistant to extreme pH at room temperature, to high temperature at pH 8.0, to a combination of low pH and high temperature, as well as to the added effects of denaturants and high temperature. In 25% trifluoroacetic acid at 70 °C, hirudin is partially inactivated. One condition which rapidly inactivates hirudin is the combined effect of elevated temperature and high pH. This phenomenon was further investigated (see below).

Hirudin Is Inactivated in Alkaline Solution—Hirudin was incubated for 15 min at various pH values (8–12.9) and temperatures. The results are presented in Fig. 3. The results clearly demonstrate that the degree of inactivation is both

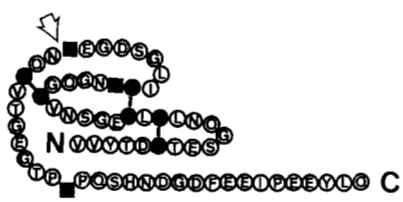

FIG. 1. **Primary structure of recombinant hirudin.** Three disulfide linkages (●—●) and three lysines (■) are denoted. The *arrow* indicates the site of cleavage by lysyl endopeptidase (*Achromobacter* protease I).

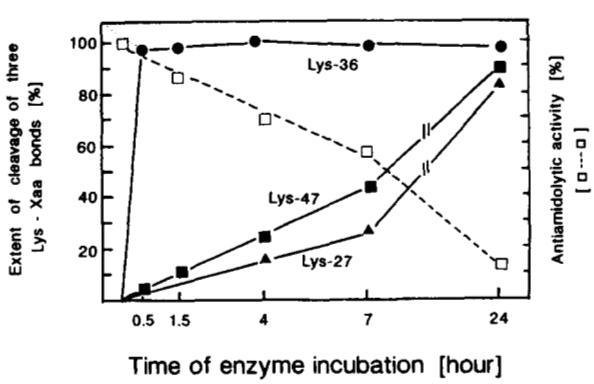

FIG. 2. **Effect of cleavages of the three lysyl bonds on the anti-amidolytic activity of hirudin.** Hirudin was digested with *Achromobacter* protease I (see "Methods"). The extent of cleavage of the three Lys-Xaa bonds was followed by quantitative NH_2-terminal analysis. Recovery of the NH_2-terminal Val^1 was used as an internal standard. The anti-amidolytic activity of time course-digested samples was measured as described in the text.

TABLE I

Stability of hirudin under extreme pH, high temperature, and in the presence of denaturants

Conditions for the treatment of hirudin	Activity[a]
	%
pH 8.0,[b] 95 °C, 10 min	100
pH 8.0, 95 °C, 30 min	95
pH 8.0, 8 M urea, 23 °C, 2 h	100
pH 8.0, 8 M urea, 70 °C, 20 min	100
pH 8.0, 6 M GdmCl, 23 °C, 2 h	100
pH 8.0, 6 M GdmCl, 70 °C, 20 min	99
pH 8.0, 1% SDS, 23 °C, 2 h	98
pH 8.0, 1% SDS, 70 °C, 20 min	97
pH 1.47,[c] 23 °C, 2 h	102
pH 1.47, 70 °C, 15 min	98
pH 12.9,[d] 23 °C, 2 h	95
pH 12.9, 70 °C, 15 min	2
25% trifluoroacetic acid, 23 °C, 2 h	98
25% trifluoroacetic acid, 70 °C, 10 min	95
25% trifluoroacetic acid, 70 °C, 30 min	72
25% dimethylformamide, 70 °C, 10 min	102
25% dimethylformamide, 70 °C, 30 min	100

[a] The specific thrombin inhibitory activity was measured as described under "Experimental Procedures." The activity of the control sample was taken as 100%.

[b] Tris-HCl buffer (67 mM, containing 133 mM NaCl and 0.13% polyethylene glycol 6000). GdmCl, guanadinium chloride; SDS, sodium dodecyl sulfate.

[c] 0.1 N HCl.

[d] 0.1 N NaOH.

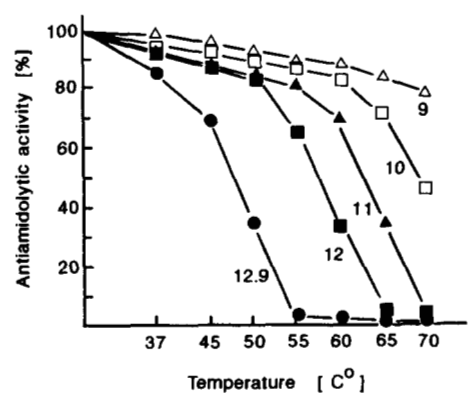

FIG. 3. **Temperature-dependent inactivation of hirudin at various pH values.** Buffers were prepared by mixture of 0.1 N $NaHCO_3$, pH 8.3, and 0.1 N NaOH, pH 12.9. Hirudin (1 nmol) was dissolved in 40 μl of alkaline buffer and heated at various temperature for 15 min. The treated samples were diluted 1000-fold with 67 mM Tris-HCl buffer, pH 8.0, and their anti-amidolytic activities were analyzed. The activity of the control sample was taken as 100%.

pH- and temperature-dependent. At a specific pH, there exists a temperature range which appears to induce the rapid inactivation of hirudin. The higher the pH, the lower the temperature range required for this accelerated meltdown. For instance, at pH 11 and 12.9, the temperature ranges of melting were 60–70 °C and 45–55 °C, respectively. The time course of inactivation of hirudin at different pH values and temperature was also studied. The inactivation obeyed first-order kinetics; the half-lives are given in Table II.

The Covalent Structure of Aklakine-inactivated Hirudin—A completely inactivated sample of hirudin and intact hirudin were characterized in parallel. Quantitative NH_2-terminal analysis of inactivated hirudin revealed Val as the only NH_2-terminal residue. The recovery of Val as compared with that obtained from intact hirudin was quantitative. These results confirmed that neither peptide bond cleavage nor blocking of

TABLE II

Half-lives of hirudin at different pH and temperature

Measurements of anti-amidolytic activity were carried out as described under "Experimental Procedures." The specific activity of the control sample was taken as 100%.

	pH 8	pH 9	pH 10	pH 11	pH 12	pH 12.9
				h		
37 °C	>24	>24	>24	>24	>24	5.0
50 °C	>24	>24	23	5.4	1.3	<0.25
60 °C	>24	7.8	1.5	0.55	<0.25	<0.25
70 °C	>24	0.82	<0.25	<0.25	<0.25	<0.25

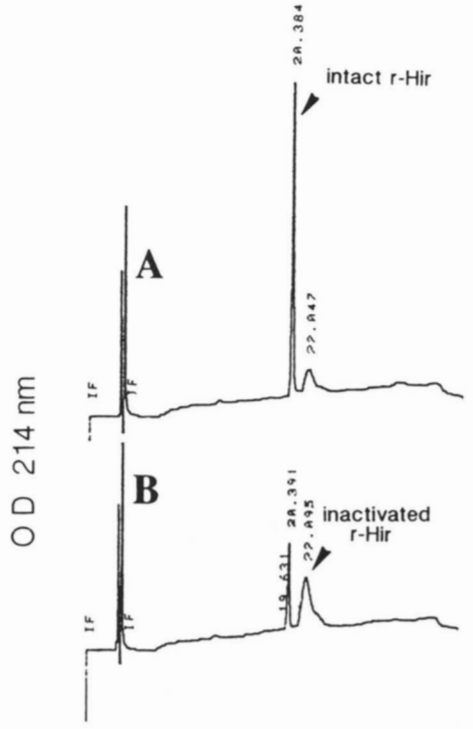

FIG. 4. **HPLC separation of time course-inactivated hirudin.** Hirudin (200 μg/50 μl) was dissolved in 0.1 N $NaHCO_3$-NaOH buffer, pH 12.0, and heated at 55 °C for 15 min (*A*) and 1 h (*B*). The treated samples were diluted 10-fold with 1% trifluoroacetic acid and subjected to HPLC analysis. The column was Vydac C-18, 5 μm; column temperature, 23 °C. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was 10 to 48% solvent B (linear) in 30 min. Reduced and carboxymethylated hirudin was eluted at the position of inactivated hirudin (22.09 min) but with a peak shape as sharp as for intact hirudin. r-Hir, recombinant hirudin.

the NH_2 terminus accompany the inactivation of hirudin. Hirudins were reduced and carboxymethylated, digested with V8 protease (Houmard and Drapeau, 1972) and Asp-N (Drapeau, 198) followed by quantitative NH_2-terminal analysis. "NH_2-terminal maps" (Chang, 1988) obtained from inactivated and intact hirudins exhibited no notable difference. This finding suggested that deamination of Asn and Gln does not occur to a significant extent (<5%), because if it did, additional Xaa-Asp and Glu-Xaa cleavages would appear in the NH_2-terminal map from the inactivated sample.

On reversed-phase HPLC, alkaline-inactivated hirudin eluted as an asymmetrical broad peak 1.7 min after intact hirudin (Fig. 4). Reduced-carboxymethylated hirudin had an identical elution time to the alkaline-inactivated sample, but displayed a peak shape as sharp as that from intact hirudin (chromatogram not shown). Analysis of the time-course of inactivation revealed direct transformation of intact into inactivated hirudin without intermediates (Fig. 4). The broadened peak nonetheless suggests that inactivated hirudin may be a heterogeneous product.

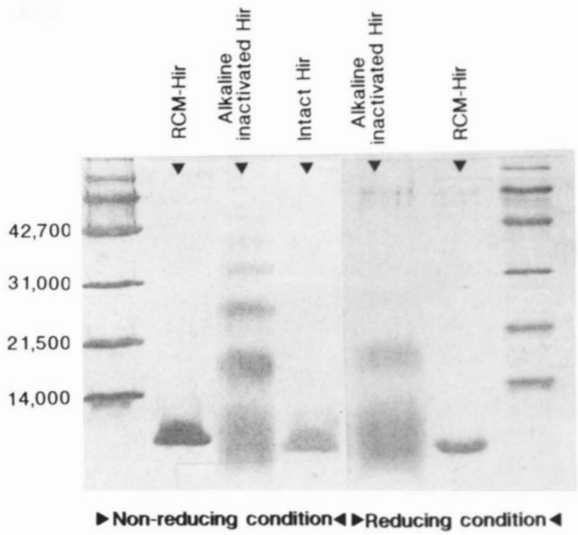

FIG. 5. **SDS-gel (15%) electrophoresis of reduced and carboxymethylated (*RCM*) hirudin, alkaline-inactivated hirudin, and intact hirudin.** Analyses were performed under both nonreducing and reducing conditions. Samples were overloaded (20 μg) to identify the polymers of inactivated hirudin. Under reducing conditions, substantial amounts of the polymers of inactivated hirudin were degraded to monomer and dimer, indicating that cystine was also involved in hirudin polymerization.

SDS-polyacrylamide gel electrophoresis (15%) revealed the heterogeneity of inactivated hirudin (Fig. 5). The inactivated sample was found to be a mixture of various hirudin polymers. Polymers as large as heptamers were visible on the gel. It is likely that the inactivated monomer itself is highly heterogeneous because of the width of the band in comparison to reduced carboxymethylated hirudin (Fig. 5).

The results of amino acid composition analyses indicated that in the inactivated sample the yield of cystine was reduced by 79% from 3 to 0.63 residue/molecule (Fig. 6). Lysine also diminished by 36% from 3 to 1.9 residues. On the other hand, two new amino acids, lanthionine (1.51 residues) and lysinoalanine (0.9 residue), were recovered. Together, the recoveries of lanthionine and lysinoalanine accounted for 83% of the loss of cystine. This number derives from the following calculation.

$$\frac{1.51\ \text{(lanthionine)} + 0.45\ \text{(half of lysinoalanine)}}{2.37\ \text{(the loss of cystine)}}$$

Since cystine, lanthionine, and lysinoalanine are the only amino acids responsible for intra- and intermolecular cross-linking of inactivated hirudin, their molar contents reflect their relative contributions to the cross-linking of inactivated hirudin. By this measure, the percentage contributions of cystine, lanthionine, and lysinoalanine were 20% [0.6/(0.6 + 1.5 + 0.9)], 50% [1.5/(0.6 + 1.5 + 0.9)], and 30% [0.9/(0.6 + 1.5 + 0.9)], respectively.

For identification of lysyl residues which partook in forming lysinoalanine, intact and inactivated hirudin were reduced and carboxymethylated, digested overnight at 37 °C with lysyl endopeptidase, and analyzed by quantitative peptide mapping (Chang, 1988). Comparison of the recoveries of the three amino acids Ile^{29},[2] Asn^{37}, and Pro^{48} (derived from cleavage of

[2] The extent of cleavage of Lys^{27}-Cys^{28} was evaluated by the recovery of Ile^{29} (second cycle of quantitative NH_2-terminal analysis) because of the involvement of Cys^{28} in forming lysinoalanine.

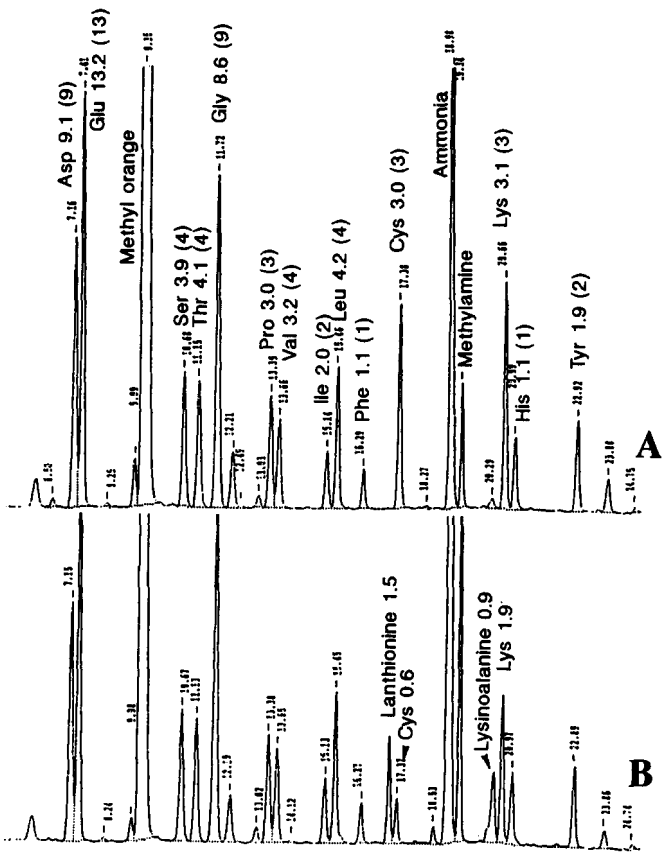

FIG. 6. **Amino acid composition analysis of intact hirudin (*A*) and alkaline-inactivated hirudin (*B*).** Analysis was performed with the DABS-Cl precolumn derivatization method (Knecht and Chang, 1986). The numbers given over the amino acid are residues per hirudin molecule derived from the average of three independent analyses (with deviations <±5%). Those in parentheses are values expected from the amino acid sequence of hirudin. The results obtained from inactivated hirudin (*B*) showed that the decrease of cystine and lysine was accompanied by the recovery of two unusual amino acids, lanthionine and lysinoalanine. The mechanism of cystine destruction is discussed in the text. The column was Lichrosphere 100 CH-18, 5 μm (12.5 cm × 4 mm inner diameter from Merck). Column temperature was 40 °C. Solvent A was 25 mM sodium acetate, pH 6.4, containing 4% dimethylformamide. Solvent B was acetonitrile. The gradient was 18 to 23% solvent B in 2 min, 23 to 30% solvent B from 2 to 8 min, 30 to 41% B from 8 to 13 min, 41 to 51% solvent B from 13 to 15 min, 51 to 54% solvent B from 15 to 17 min, 54 to 64% solvent B from 17 to 19 min, 64 to 90 solvent B from 19 to 20 min, and then 90 back to 18% solvent B from 20 to 22 min. The flow rate was 1 ml/min. The detector wavelength was 436 nm.

Lys^{27}-Cys^{28}, Lys^{36}-Asn^{37}, and Lys^{47}-Pro^{48}) relative to the yield of NH_2-terminal Val^1 provided the basis for calculating the extent of involvement of Lys^{27}, Lys^{36}, and Lys^{47} in cross-linking. The rationale was that participation of a particular lysine in forming lysinoalanine would block hydrolysis by lysyl endopeptidase. The results indicated that 53% of Lys^{27}, 43% of Lys^{36}, and 16% of Lys^{47} were involved in forming lysinoalanine. Thus, the total amount of lysine (0.53 + 0.43 + 0.16 = 1.12 residues) which engaged in cross-linking was roughly accounted for by the lysinoalanine recovered (0.9 residue).

Finally, the structure of inactivated hirudin was investigated by peptide mapping. Hirudin and inactivated hirudin were reduced and carboxymethylated, digested with chymotrypsin overnight, and the peptides separated by HPLC. The control sample gave 9–10 peptides, whereas the inactivated sample showed only two sharp peaks and a broad zone (Fig. 7). Sequence analysis of the two sharp functions revealed that they represented the NH_2-terminal (Hir$^{1-3}$) and COOH-terminal (Hir$^{50-63}$) peptide (Fig. 7). It was clear that the interior sequence of inactivated hirudin was engaged in extensive crosslinking and eluted as a broad peak two min after Hir$^{50-63}$.

DISCUSSION

Hirudin is resistant to a variety of unfavorable conditions that normally inactivate proteins (see Table I). Our data, however, do not rule out the possibility that hirudin may be reversibly denatured under such conditions. The stability of hirudin is primarily attributed to the compact structure of its NH_2-terminal core domain which is tightly folded by disulfide linkages and antiparallel β-interactions (Rydel *et al.*, 1990; Folker *et al.*, 1989). The NH_2-terminal core domain of hirudin is also highly resistant to digestive enzymes (Chang, 1990). Among proteolytic enzymes commercially available (including pepsin, chymotrypsin, thermolysin, elastase, trypsin, and Lys-C from *L. enzymogenes*), only *A. lyticus* protease I (EC 3.4.21.50) (Masaki *et al.*, 1981; Tsunasawa *et al.*, 1989) is found to cleave the internal peptide bond of the hirudin core domain. *Achromobacter* protease I is specific for lysine and hydrolyses lysyl bonds including Lys-Pro (Masaki *et al.*, 1978; Tsunasawa *et al.*, 1987). Its proteolytic activity is also an order of magnitude higher than that of bovine trypsin (Masaki *et al.*, 1981). This protease preferentially hydrolyses Lys^{36}-Asn^{37} of hirudin, generating a two-chain inhibitor which is stabilized by disulfide linkages and still possesses full thrombin inhibi-

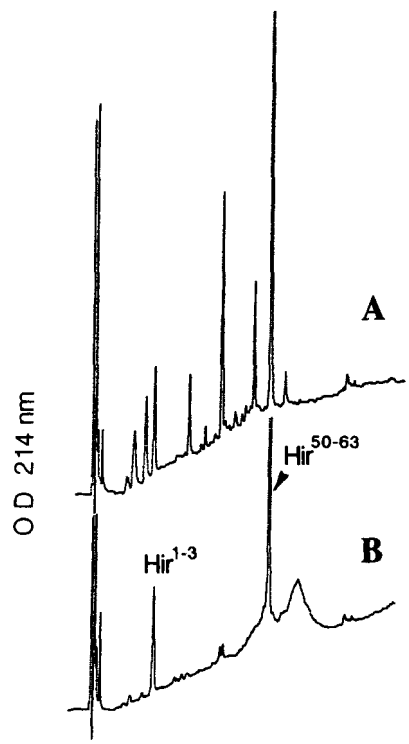

FIG. 7. **HPLC analysis of chymotryptic peptides derived from intact hirudin (*A*) and alkaline-inactivated hirudin (*B*).** Hirudins were reduced-carboxymethylated and digested with chymotrypsin (enzyme/substrate, 1:20, by weight) in 50 mM ammonium bicarbonate, pH 8.0, at 23 °C overnight. The digested samples were acidified with 1% trifluoroacetic acid and injected for HPLC analysis using similar conditions to those described in the legend of Fig. 4. The two sharp fractions recovered from the inactivated hirudin were sequenced and were found to represent the NH_2-terminal (Hir$^{1-3}$) and COOH-terminal (Hir$^{50-63}$) peptides of hirudin.

tory activity. These findings are further evidence for the unique stability of hirudin.

Hirudin is readily inactivated at elevated temperatures in alkaline solution. For instance, even at mildly alkaline pH (9.0), a significant fraction (22%) of hirudin is inactivated after overnight digestion at 50 °C. Inactivation is found to be due to selective destruction of cystines. Disulfide bonds are known to stabilize proteins (for review, see Wetzel, 1988). In fact, introducing disulfide linkages has been a favored approach in enhancing the stability of recombinant proteins (Villafranca *et al.*, 1983; Sauer *et al.*, 1986; Wells and Powers, 1986). There is no doubt that the three disulfide bonds of hirudin play a major role in maintaining the remarkable stability of its core domain (Chang, 1990; Rydel *et al.*, 1990), since any attempt to disrupt them leads to the concomitant loss of the inhibitory activity of hirudin. However, these disulfide bonds turn out to also be the most sensitive covalent structures in hirudin, as demonstrated in this work. The paradoxical properties of disulfide bonds (Volkin and Klibanov, 1987) suggest that a search for cystine substitutes that are resistant to heat and high pH may be required in the future design of more stable recombinant proteins.

The inactivation of hirudin is consistent with the proposed mechanism of base-catalyzed β-elimination (Nashef *et al.*, 1977; Whitaker and Feeney, 1983; Florence, 1980). In this model 1 mol of cystine is transformed into 1 mol of dehydroalanine and 1 mol of thiocysteine which then converts to cysteine. Subsequent reaction of dehydroalanine with nucleophiles (lysine and cysteine) leads to the formation of lysinoalanine and lanthionine and at the same time causes random cross-linking (polymerization) and irreversible inactivation of the protein. Structural characterization has revealed that all 3 lysines of hirudin participate to varied extents (see "Results") in forming lysinoalanine. It is surprising that the NH_2-terminal amino group of hirudin does not react with dehydroalanine at all, despite the fact that it has a lower pK_a value than the α-amino group of lysine and reacts more efficiently toward chemical modification (Chang, 1989). This may be due to the steric hindrance of the NH_2-terminal amino group. Analysis of numerous model proteins has demonstrated that lanthionine and lysinoalanine are indeed characteristic by-products of the alkaline-induced destruction of cystine.[3] These two unusual amino acids have been analyzed traditionally with an amino acid analyser (Bohak, 1964; Ziegler *et al.*, 1967) or by thin-layer chromatography (Sternberg *et al.*, 1975). We describe in this report that they may be analyzed quantitatively in a more sensitive and efficient way using the HPLC/DABS-Cl method (Knecht and Chang, 1986). Another technique which greatly facilitated this work is quantitative NH_2-terminal analysis by the DABITC method (Chang, 1988). This technique is not only useful for checking the purity of proteins, but is also useful for identifying selectively modified lysines and acidic amino acids. Although the same task can be performed with an automatic sequenator, the manual DABITC method is clearly a more efficient tool for this particular purpose because of the possibility of processing multiple samples and the quantitative recovery of Ser and Thr.

Since inactivated hirudin is a highly heterogeneous mixture of polymers, small amounts of the inactivated contaminant (less than 1–2%) may not be easily detected by HPLC, gel electrophoresis, amino acid analysis, or even capillary electrophoresis. Therefore, at any stage in the production of hirudin and other cystine-containing recombinant proteins, keeping the pH below 8–9 and the temperature no higher than 37 °C is recommended.

[3] J.-Y. Chang, unpublished results.

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