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Static and Transient Hydrogen-Bonding Interactions in Recombinant Desulfatohirudin Studied by ^{1}H Nuclear Magnetic Resonance Measurements of Amide Proton Exchange Rates and pH-Dependent Chemical Shifts†

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ABSTRACT: With proton nuclear magnetic resonance spectroscopy at 22 °C and pD 4.5, individual exchange rates in the range from 2×10^{-5} to 1×10^{-1} min^{-1} were observed for 23 amide protons in recombinant desulfatohirudin. The remaining 38 backbone amide protons exchange more rapidly than 1×10^{-1} min^{-1}. All 23 slowly exchanging protons are located in the polypeptide segment from residue 4 to residue 42, which forms a well-defined globular domain. Three different breathing modes of this molecular region are manifested in the exchange data, which appear to be correlated with the location of the three disulfide bonds. Chemical shift changes larger than 0.15 ppm between pH 2.5 and pH 5.0 arising from through-space interactions with carboxyl groups were observed for seven backbone amide protons. Two of these shifts can be explained by hydrogen bonds in the core of the protein, Gly 25 NH–Glu 43 O$^{\epsilon}$ and Ser 32 NH–Asp 33 O$^{\delta}$, and two others by intraresidual NH–O$^{\epsilon}$ interactions in Glu 61 and Glu 62. The remaining three pH shifts for Glu 35, Cys 39, and Ile 59 imply the existence of transient interactions between the molecular core and the flexible C-terminal segment 49–65, which have so far not been characterized by nuclear Overhauser effects or other conformational constraints.

The solution conformation of recombinant desulfatohirudin determined by NMR[1] contains a well-structured globular core with residues 3–30 and 37–48 and two disordered, presumably quite flexible segments, 31–36 and 49–65 (Haruyama & Wüthrich, 1989). A similar overall structure was reported for natural hirudin (Clore et al., 1987). To further characterize the combination of well-structured and disordered flexible polypeptide segments in desulfatohirudin, the present paper describes quantitative measurements of amide proton exchange rates and the pH dependence of proton chemical shifts.

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[1] Abbreviations: NMR, nuclear magnetic resonance; 1D and 2D, one dimensional and two dimensional; COSY, two-dimensional correlated spectroscopy; 2QF-COSY, two-quantum-filtered COSY; RELAYED-COSY, two-dimensional relayed coherence transfer spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TSP, 3-(trimethylsilyl)[2,2,3,3-^{2}H$_4$]-propionate, sodium salt.

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Amide proton exchange measurements have long been used for studies of protein dynamics (Hvidt & Nielsen, 1966; Englander & Kallenbach, 1984; Woodward et al., 1982; Wagner, 1983; Wagner & Wüthrich, 1982) and for delineating hydrogen-bonding networks in polypeptide secondary structures (Wagner & Wüthrich, 1982; Wüthrich et al., 1984; Wüthrich, 1986). In the present project we make use of the fact that, in the limiting kinetic situation corresponding to exchange by an EX_2 mechanism (Hvidt & Nielsen, 1966; Roder et al., 1985a), the individual exchange rates provide a basis for distinguishing between multiple, different breathing modes in protein molecules (Wagner, 1983; Wagner et al., 1984; Wang et al., 1987). The pH dependence of amide proton chemical shifts in the range 2–5 can be related with hydrogen bonding to carboxylate groups (Bundi & Wüthrich, 1979). If the pK_a values of the acidic groups in the molecule can independently be determined, the acceptor group for a particular proton may be identified from comparison with the pK_a value for the amide proton titration. In the present context this technique is used to study long-range intramolecular interactions on a different basis than with NOEs, whereby special interest is focused on transiently formed structures in dynamic, rapid-exchange equilibria (Bundi & Wüthrich, 1979; Wüthrich & Wagner, 1979; Ebina & Wüthrich, 1984).

The experimental procedures used here have previously been applied either with well-structured globular proteins or with flexible model peptides, including a denatured form of a globular protein (Roder et al., 1985b). The present project with a polypeptide which contains extensive structured segments as well as disordered segments is a new test of the potentialities of these methods. Besides, the project is also of considerable biological interest. Since the determination of the solution conformations for hirudin (Clore et al., 1987) and recombinant desulfatohirudin (Haruyama & Wüthrich, 1989) has thus far not yielded a conclusive explanation for the reduced activity of the latter, it is conceivable that the different activities might be related to different transient, dynamic structural properties.

Materials and Methods

Desulfatohirudin produced with recombinant DNA techniques was used as it was given to us by Ciba-Geigy AG., Basel, Switzerland (Meyhack et al., 1987; Grossenbacher et al., 1987). For the ^{1}H NMR experiments we used either a Bruker WM 500 or a Bruker AM 600 spectrometer.

Amide Proton Exchange Studies. The protein samples used for these experiments were first dissolved at a concentration of 6.5 mM in H_2O, and the pH at 22 °C was adjusted to 4.5 by addition of HCl and NaOH. After lyophilization the protein was redissolved in the same amount of D_2O at 4 °C and immediately inserted into the NMR instrument where the probe temperature had been equilibrated at the desired exchange temperature. Accumulation of the first NMR spectrum was started ca. 15 min after the protein was dissolved in D_2O. The pD values of the samples were measured after the completion of the exchange experiments, using a glass electrode at 22 °C without correction for isotope effects; in all cases they were in the range 4.5–4.7.

One of the techniques used for the exchange measurements was to record several NOESY spectra at different times after the protein was dissolved in D_2O, process the different spectra identically, and compare the relative intensities of corresponding peaks in the different spectra. Advantages of using NOESY (Boelens et al., 1985) rather than COSY (Wagner & Wüthrich, 1982) for exchange measurements were discussed elsewhere (Wang et al., 1987). Phase-sensitive NOESY spectra with a mixing time of 150 ms were recorded at 500 MHz, with time-proportional phase incrementation of the initial pulse (Marion & Wüthrich, 1983). A total of 200 t_1 values with 2048 data points in t_2 were used, resulting in a measuring time of 7 h per spectrum. The time domain data matrix was expanded to 512 points in t_1 and 4096 points in t_2 by zero filling, and the final digital resolution along ω_2 was 2.7 Hz/point. Prior to Fourier transformation, the data were multiplied in both directions with a phase-shifted sine bell. Cross sections through the NOESY spectra were plotted, and peak heights were measured relative to a well-separated low-field $C^\delta H$–$C^\epsilon H$ cross peak of Tyr 3. Exchange rate constants were then obtained from a nonlinear least-squares fit of the experimental data to a single exponential function.

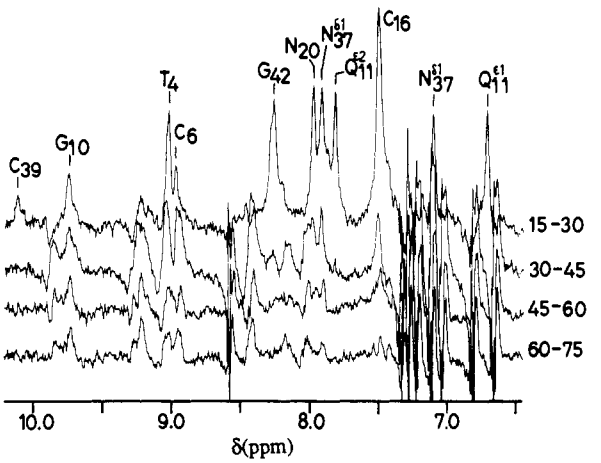

FIGURE 1: 1D difference ^{1}H NMR spectra at 600 MHz of a freshly prepared 6.5 mM solution of recombinant desulfatohirudin in D_2O, pD 4.5, T = 22 °C. The two numbers on the right of each trace indicate the start of the two experiments used to obtain the difference (in minutes after sample preparation). Each individual spectrum was recorded during 5 min. Resonance lines corresponding to polypeptide backbone amide protons are identified by the one-letter amino acid symbol and the sequence number; side-chain amide protons are further defined by a superscript.

With NOESY, exchange rates slower than ca. 1×10^{-3} min^{-1} could be measured. To expand the range to somewhat faster rates, we used 1D ^{1}H NMR difference spectra at 600 MHz with 16K data points. Starting 15 min after the protein was dissolved in D_2O, a series of recordings of 5-min duration were obtained during 4 h. For the data analysis it was assumed that the exchange of protons which were observed by NOESY is negligible during the recording of one 1D spectrum, so that the difference spectra contain only peaks corresponding to amide protons with exchange rates of the order of 10^{-2}–10^{-1} min. As an illustration, Figure 1 shows four difference spectra obtained with these experiments. Exchange rate constants were again obtained from a nonlinear least-squares fit of a single exponential.

Intrinsic exchange rate constants for recombinant desulfatohirudin were calculated from the parameters given by Molday et al. (1972).

pH Titration of ^{1}H Chemical Shifts. A 6.5 mM protein solution was prepared in a mixed solvent of 90% H_2O/10% D_2O and its pH value adjusted by adding small amounts of HCl or NaOH. The pH titration shifts of the amide protons, βCH_2 of Asp, and γCH_2 of Glu were followed by 2QF-COSY (Rance et al., 1984; Neuhaus et al., 1985) at 500 MHz. The experiments were carried out at 22 °C. The water signal was suppressed by homogated decoupling during the preparation period. A data matrix of 2048 points in t_2 and 512 points in t_1 was used. The digital resolution after zero filling was 2.7 Hz/point in ω_2 and 5.4 Hz/point in ω_1. A phase-shifted sine

Table I: Chemical Shifts, δ, and Exchange Rate Constants, k_m, for the Slowly Exchanging Backbone Amide Protons in Recombinant Desulfatohirudin

residue	δ (ppm) (pD 4.5, 22 °C)	k_m (10^{-4} min^{-1}) (pD 4.5, 22 °C)[a]	k_m (10^{-4} min^{-1}) (pD 4.5, 14 °C)[b]
Thr 4	9.02	520*	
Cys 6	8.96	12*	2.1
Glu 8	7.36	10*	2.2
Gly 10	9.73	96*	14
Gln 11	7.42	26*	2.7
Asn 12	8.16	29*	3.0
Leu 13	9.21	34*	4.2
Cys 14	7.29	0.69	0.11
Leu 15	9.05	0.63	0.18
Cys 16	7.49	280*	(40)
Asn 20	7.97	270*	(50)
Val 21	9.08	570*	
Cys 22	8.45	2.6	0.82
Asn 26	8.04	5.0	1.2
Lys 27	9.30	1.2	0.30
Cys 28	9.08	0.87	0.28
Ile 29	9.89	0.24	0.03
Leu 30	8.46	2.5	1.7
Gly 31	8.97	15*	3.2
Glu 35	7.82	960*	
Gln 38	8.47	2.3	0.40
Val 40	9.26	0.92	0.20
Gly 42	8.25	350*	(70)

[a] Exchange rates obtained from 1D NMR experiments are indicated by asterisks. [b] Parentheses indicate that the exchange rate was estimated from less than four data points.

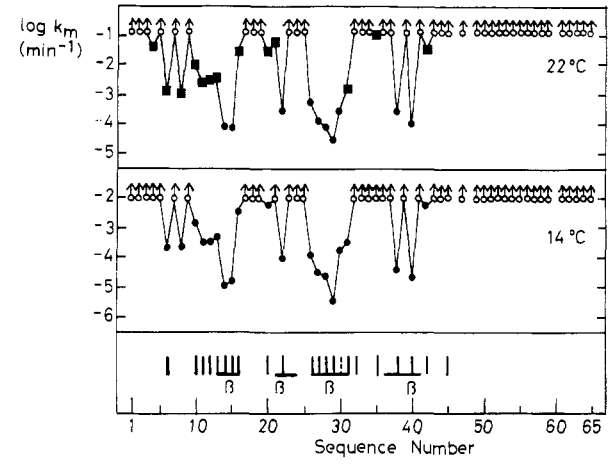

FIGURE 2: Plot of the logarithm of the amide proton exchange rates versus the amino acid sequence of recombinant desulfatohirudin. Filled squares represent data obtained from 1D ^{1}H NMR, and filled circles represent the results obtained from analysis of NOESY spectra. Open circles in the plot at 14 °C indicate exchange rates that were estimated from less than four data points. Open circles with arrows indicate a lower limit for k_m; these amide protons were observed in H_2O solutions of the protein but exchanged too rapidly to be detected in D_2O solutions. The k_m values of sequentially neighboring amide protons are connected by straight lines. At the bottom the locations of antiparallel β-strands are indicated by horizontal lines, those of hydrogen bonds between backbone amide and carbonyl groups (Haruyama & Wüthrich, 1989) as indicated by solid vertical lines, and a hydrogen bond between a backbone NH and a side-chain acceptor group is indicated by a broken line.

bell was applied in both directions prior to Fourier transformation. The chemical shifts were measured relative to internal TSP. Titration parameters were obtained by nonlinear least-squares fits of the experimental data to a one-proton titration curve (eq 1), where δ(pH) is the experimental

$$\delta(\mathrm{pH}) = (\delta_{HA} + \delta_{A^-} \times 10^{\mathrm{pH}-\mathrm{p}K_a})/(1 + 10^{\mathrm{pH}-\mathrm{p}K_a}) \quad (1)$$

chemical shift in ppm at a specified pH value, δ_{HA} is the chemical shift in the fully protonated state, and $\delta_{A^-} = \delta_{HA} + \Delta\delta$ is the chemical shift in the fully deprotonated state.

Results

Amide Proton Exchange. The results are summarized in Table I and Figure 2. The data were collected with the following four series of measurements: At 22 °C, NOESY experiments (Jeener et al., 1979; Anil-Kumar et al., 1980) were started 15, 360, 720, 5740, 10800 and 19275 min after sample preparation. In a second series using the protein sample obtained after completion of the 1D NMR experiments (see below), additional NOESY spectra were recorded starting 280, 7000, 10000, and 17500 min after sample preparation. For 10 amide protons, cross-peak intensities could be measured in more than four of these NOESY spectra, and their exchange rates were obtained by a nonlinear least-squares fit. Their rate constants, k_m, are in the range from 2×10^{-5} to 5×10^{-4} min^{-1}. A total of 13 additional amide proton exchange rates between 1×10^{-3} and 9.6×10^{-2} min^{-1} could be measured by 1D difference ^{1}H NMR experiments. As a check on the 1D NMR data, where spectral overlap made some of the resonance assignments rather difficult (Figure 1), a series of NOESY spectra at 14 °C were started 20, 490, 1020, 1500, 1960, 8380, 18580, 30100, and 89000 min after sample preparation. Quantitative exchange rates were obtained for 17 amide protons. For three additional protons, where the peak height could only be measured in less than four spectra, estimates for k_m were obtained. All these 20 protons were also studied by 1D or 2D NMR at 22 °C. Table I shows that the relative order of the individual exchange rates is nearly identical at the two temperatures, which supports the validity of the data collected with 1D NMR.

pH Titration Shifts. Since complete assignments for the labile protons in recombinant desulfatohirudin are available at pH 4.5 and pH 3.0 (Haruyama & Wüthrich, 1989), the pH dependence of the backbone amide proton resonances could readily be followed over the pH range 2.5–5.0 in 2QF-COSY spectra. Not observed were the amino groups of Val 1 and Asp 5 for which the amide proton resonance was present only in NOESY spectra (Haruyama & Wüthrich, 1989). Figure 3 shows the fingerprint region of two 2QF-COSY spectra recorded at pH 3.0 and pH 4.5, respectively. Several NH–αH cross peaks with large chemical shift changes are identified in the figure. When going from low pH to high pH, the amide proton resonances of Asp 33 and Gln 65 shifts to higher field, while the amide proton resonances of Glu 35, Cys 39, Gly 25, and Ser 32 shift to lower filed. Additional amide proton shifts are listed in Table II, and the analysis was also extended to βCH_2 to Asp and γCH_2 of Glu. The curve fitting used to obtain the titration parameters is illustrated with the amide proton resonance of Cys 39 (Figure 4). Table II lists the titration parameters for the amide and side-chain protons of all Asp and Glu residues. For the other amino acid residues, titration parameters for the amide proton were computed only when $|\Delta\delta| = |\delta_{HA} - \delta_{A^-}|$ was greater than 0.10 ppm. Overall, titration parameters were thus obtained for the amide protons of two of the four Asp, six of the eight Glu, and seven additional residues. pK_a's for the side-chain carboxylates were obtained for 5 of the 12 Asp and Glu residues.

Discussion

As in the structure determination by NOEs and distance geometry calculations (Haruyama & Wüthrich, 1989), different polypeptide segments in the recombinant desulfatohirudin molecule show distinctly different properties also with regard to proton exchange. In the entire C-terminal segment

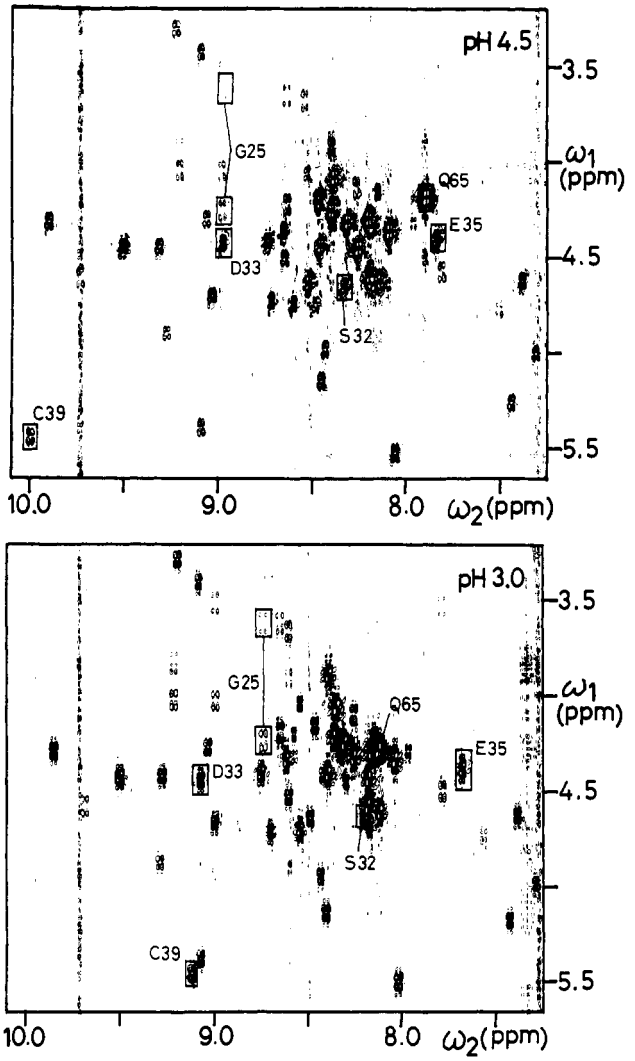

FIGURE 3: Fingerprint region of two ^{1}H 2QF-COSY spectra of recombinant desulfatohirudin in H_2O recorded respectively at pH 3.0 and 4.5 (Larmor frequency 500 MHz, temperature 22 °C). Some cross peaks with outstandingly large amide proton shift variations with pH (see Table II) are indicated by rectangular frames, and the resonance assignments are given.

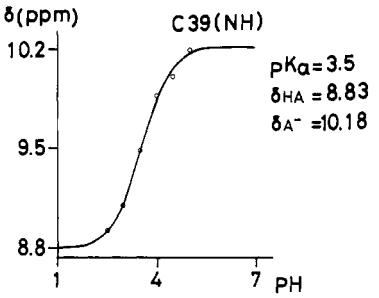

FIGURE 4: Plot of the chemical shift of the amide proton of Cys 39 versus pH. The circles represent the experimental data. The curve was determined by a nonlinear least-squares fit of the experimental data to eq 1. The titration parameters obtained from this fit are also indicated.

43–65, for which no well-defined conformation was found, the amide proton exchange is faster than 1×10^{-1} min^{-1}. This value is near to what one expects for unstructured, flexible model peptides (Molday et al., 1972; Roder et al., 1985b). It thus indicates that this "disordered" part of the polypeptide chain is devoid of hydrogen bonds and that the structural disorder is dynamic in character. Although the molecular geometry of the loop formed by residues 31–36 appears to favor intramolecular hydrogen bonding for the amide protons

Table II: Titration Parameters at 22 °C for Protons with pH-Dependent Chemical Shifts in Recombinant Desulfatohirudin

residue and proton type[a]	δ_{HA} (ppm)[b]	$\Delta\delta$ (ppm)[c]	pK_a[d]
Asp 5	8.99	−0.23	[e]
Glu 8	7.37	0.01	[e]
C$^\gamma$H	2.46	−0.12	4.6 ± 0.1
Glu 17	8.58	0.05	3.1 ± 0.2
Asn 20 (NH$^{\delta 1}$)	7.46	0.11	4.4 ± 0.1
Gly 25	8.71	0.37	4.1 ± 0.1
Ser 32	8.18	0.20	4.2 ± 0.2
Asp 33	9.07	−0.15	3.9 ± 0.2
C$^\beta$H	3.13	−0.23	4.0 ± 0.2
C$^\beta$H	2.84	−0.16	4.2 ± 0.1
Glu 35	7.64	0.23	3.7 ± 0.1
Lys 36	8.31	0.10	4.6 ± 0.5
Cys 39	8.95	1.22	3.5 ± 0.1
Glu 43	8.13	0.00	[e]
C$^\gamma$H	2.59	−0.16	4.3 ± 0.1
Asp 53	8.48	0.03	[e]
C$^\beta$H	2.95	−0.19	3.4 ± 0.2
Asp 55	8.16	−0.06	4.2 ± 0.2
C$^\beta$H	2.74	−0.17	3.9 ± 0.2
Glu 57	8.17	0.04	4.6 ± 0.4
Glu 58	8.25	0.09	4.6 ± 0.1
Ile 59	8.16	0.15	4.8 ± 0.2
Glu 61	8.34	0.15	4.0 ± 0.1
Glu 62	8.29	0.16	4.3 ± 0.2
Gln 65	8.14	−0.31	3.5 ± 0.1

[a] Unless explicitely specified otherwise, the backbone amide proton was observed. All Asp and Glu residues are listed and all other amino acids for which the amide proton pH titration shift was bigger than |0.1| ppm. [b] δ_{HA} is the chemical shift in the protonated state. [c] $\Delta\delta = (\delta_{A^-} - \delta_{HA})$, where δ_{A^-} is the chemical shift of the deprotonated species, is the pH titration shift. Negatives numbers indicate upfield shifts upon deprotonation. [d] the pK_a is the acid dissociatison constant obtained by a least-squares fit of the pH-dependent chemical shifts in the pH range 2.5–5.0 to eq 1. [e] The pK_a value was not determined either because $\Delta\delta$ was too small or because of other technical reasons (see text).

of residues 31, 32, and 35 (Haruyama & Wüthrich, 1989), the proton exchange is fast, indicating a high degree of flexibility. This coincides with the results of the structure calculations from NOE data, which indicate the existence of multiple spatial orientations for this loop. The well-structured protein core consisting of residues 3–30 and 37–43 displays amide proton exchange patterns which are reminiscent of those found in other globular proteins [e.g., Wagner and Wüthrich (1982), Wüthrich et al. (1984), and Wang et al. (1987)] and are discussed in more detail in the following.

We consider the reaction scheme (Hvidt & Nielsen, 1966)

$$N(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow{k_{intr}} O(D) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N(D) \qquad (2)$$

where native closed states of the protein, N(H), are in equilibrium with open states, O(H). Exchange is possible only from the open states, with the intrinsic exchange rate k_{intr} (Molday et al., 1972; Roder et al., 1985b). On the basis of earlier, systematic studies of amide proton exchange mechanisms in a globular protein (Roder et al., 1985a), it can safely be concluded that, with the presently chosen experimental conditions, slow exchange in desulfatohirudin is governed by an EX_2 mechanism (Hvidt & Nielsen, 1966). The observed rate constant, k_m, can then be expressed by

$$k_m = \frac{k_1}{k_2} k_{intr} \qquad (3)$$

Since k_{intr} can be estimated with the rules by Molday et al. (1972), the equilibrium between the states N and O in eq 2 can be evaluated from the apparent exchange rates k_m (Table I). In plots of log k_m vs log k_{intr}, amide protons with identical

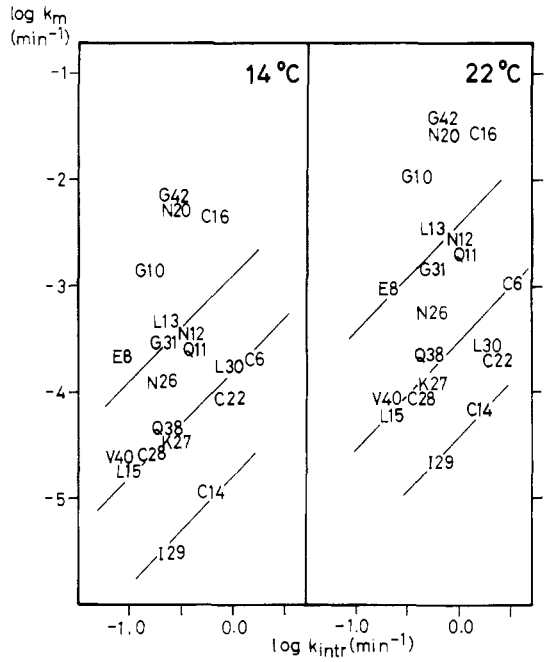

FIGURE 5: Logarithmic plots of the observed exchange rates, k_m, versus the intrinsic exchange rates, k_{intr}. The data points for the individual amide protons are in the center of the area covered by the two or three digits indicating the one-letter amino acid code and the sequence number. Three lines of slope 1 represent the average values of k_1/k_2 for three groups of amide protons (see text).

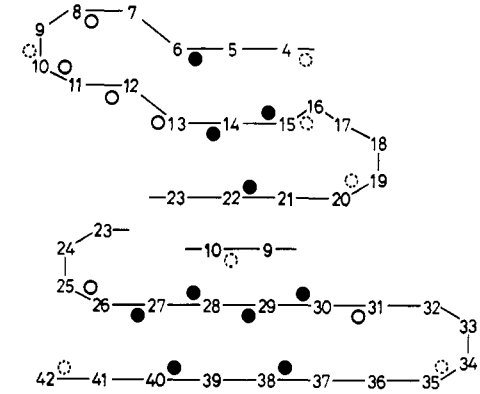

FIGURE 6: Schematic presentation of the geometric distribution of slowly exchanging amide protons in the well-structured core of recombinant desulfatohirudin in solution. The polypeptide backbone is represented by the sequence positions connected by straight lines. The filled circles identify the amide protons which are near the bottom line and the middle line in Figure 5. The solid, open circles identify the protons which are near the top line in Figure 5. The broken circles indicate the amide protons which would be located above the top line in Figure 5 but for which the exchange was nonetheless sufficiently slow so that they could be observed in D_2O.

equilibrium constants k_1/k_2 are expected to fall on the same straight line with slope 1 (Wagner, 1983; Wagner et al., 1984).

In Figure 4 the log k_m values are plotted against log k_{intr} for all those amide protons in desulfatohirudin which were observed in the experiments at both 14 and 22 °C (Figure 2). Three lines with slope 1 have been drawn in the figure to indicate that the amide protons can be grouped into classes with different values of the equilibrium constant k_1/k_2. Considering the scatter among the experimental points, this classification is somewhat arbitrary, but nonetheless four groups of protons are quite clearly distinguishable, especially at 14 °C. For the protons included in Figure 5, the locations in the secondary structure of desulfatohirudin are indicated in Figure 6. The two groups of protons with the smallest values for k_1/k_2 (Figure 5) occupy the central strands and the inside of the peripheral strands in the central region of both β-sheets. The location of the amide protons with small values of k_1/k_2 coincides also closely with the locations of the three disulfide bridges Cys 6–Cys 14, Cys 16–Cys 28, and Cys 22–Cys 39. Cys 14 NH and Ile 29 NH, which have the smallest equilibrium constants, are part of or immediately adjacent to residues involved in the disulfide bonds. The tight network of disulfide bonds thus evidently limits the breathing motions of the protein structure. The amide protons in the third class from the bottom in Figure 5 are located immediately adjacent to this most rigid core of the protein. Finally, a slight retardation of amide proton exchange relative to the values in model peptides (positions at the top of Figure 5) is observed for a few protons in the exposed loops of the polypeptide chain. Overall, a picture emerges of the desulfatohirudin molecule, where the extent of structure fluctuations increases when going from the hydrophobic core with the disulfide bonds toward the periphery of the globular part of the structure, with apparently largely unrestricted flexibility for the chain termini of residues 1–3 and 49–65. Furthermore, the fact that the equilibrium constants at 22 °C are higher than the corresponding constants at 14 °C seems to indicate that the three-dimensional structure of desulfatohirudin is stabilized primarily by hydrophilic interactions (Wagner et al., 1984).

For an interpretation of the pH titration of amide proton chemical shifts (Table II), the direction of the observed shift enables a first distinction between the different, individual protons. Upfield shifts upon deprotonation of carboxylate groups arise from interactions mediated via covalent bonds ("intrinsic titration shifts"; Bundi & Wüthrich, 1979). Such titration shifts are expected for the amide proton of the C-terminal amino acid residue and the amide protons of Asp. Absence of the intrinsic pH shifts for these protons would indicate that they are compensated by through-space interactions with other carboxylates. On this basis the upfield shifts at high pH observed for Asp 5, Asp 33, Asp 55, and Gln 65 can readily be rationalized. For Asp 55 the intrinsic pH shift appears to be compensated by other interactions, which cannot be further specified, whereas the amide protons of Asp 5, Asp 33, Asp 55, and Gln 65 seem not to be involved in hydrogen bonds or other through-space interactions with carboxylates.

Large conformation-dependent pH shifts are observed for the backbone amide protons of Gly 25, Ser 32, Glu 35, Lys 36, Cys 39, Ile 59, Glu 61, and Glu 62. Structural interpretations of these shifts must rely on the identification of the carboxylate groups which act as hydrogen-bond acceptors and cause the pH shifts. Comparison of the pK_a values measured for the individual amide protons with those of the carboxylate groups (Table II) may support such acceptor group identifications. Direct pK_a measurements by titration of covalently related protons were accessible for six carboxylates (Table II). In addition, we conclude tentatively that the pK_a values of the glutamic acid residues 17, 35, 57, 58, 61, and 62 coincide with those of their amide protons and thus are 3.1, 3.7, 4.6, 4.6, 4.0, and 4.3, respectively. This assumption is based on the fact that none of the amide protons of Glu [except for Glu 35, which is in a flexible loop and for which the amide proton exchange is only slightly slower than that in model peptides (Table I)] are implicated in hydrogen bonds with other backbone groups, and on previous experience with such freely accessible amide protons of Glu in other peptides and proteins (Bundi & Wüthrich, 1979; Wüthrich & Wagner, 1979; Ebina & Wüthrich, 1984). From reference to the three-dimensional structure of desulfatohirudin (Haruyama & Wüthrich, 1989), it was then checked which of the hydrogen-bonding interac-

tions indicated by the pK_a values were sterically possible.

From this analysis, the hydrogen bonds Gly 25 NH–Glu 43 O$^\epsilon$ and Ser 32 NH–Asp 33 O$^\delta$ are implicated. The absence of a low-field shift of the amide proton of Glu 43 upon deprotonation of the side-chain carboxyl group may then also be explained by the fact that its carboxyl group is not free to form an intraresidue hydrogen bond because of the interaction with the amide proton of Gly 25. As mentioned above, the pH shifts observed for the amide protons of Glu 17, Glu 35, Glu 57, Glu 58, Glu 61, and Glu 62 could be explained by intraresidual interactions. We are then left with large, unexplained pH shifts for Lys 36, Cys 39, and Ile 59. In the case of Ile 59 the pK_a values indicate that an interaction with Glu 8, Glu 57, or Glu 58 causes the observed shift. This parameter thus indicates an intramolecular interaction either between the protein core and the flexible C-terminal part or between two residues in the flexible part, which was not observed by NOEs. For Lys 36 and Cys 39 it can be excluded from the combined information on the spatial structure and the pK_a values that the pH shift is due to interactions with carboxylate groups in the core of the protein. Here we thus have clear-cut evidence for interactions of the more rigidly structured protein core with the flexible C-terminal polypeptide segment. Such interactions are sterically possible, since the amide protons of Lys 36 and Cys 39 are both exposed on the surface of the structured, globular part of the polypeptide chain. In particular, the outstandingly large pH shift for the amide proton of Cys 39, which indicates a high population for the hydrogen-bonding interactions with carboxylate groups, must result from interactions with the C-terminus or with Asp 53. It can also not be excluded that these two carboxylates both interact with Cys 39 in a dynamic equilibrium situation (Bundi & Wüthrich, 1979). For Lys 36 NH the reaction partners in the hydrogen-bond formation must be Glu 57, or Glu 58, or both of these residues in a dynamic equilibrium.

In conclusion, with the use of quantitative amide proton exchange measurements the internal motility of the globular portion of the polypeptide chain in desulfatohirudin could be characterized in terms of regions with different breathing modes. The exchange data also confirmed the predominant flexibility and solvent exposure of the unstructured polypeptide segments, in particular residues 48–65. Amide proton pH titration shifts, which also identified two hydrogen bonds with carboxylate groups in the globular core region, then enabled the identification of transient interactions of the structured globular core of residues 3–30 and 37–48 with the flexible C-terminal segment 49–65. The implicated hydrogen bonds involving the amide protons of Lys 36 and Cys 39 and the carboxylates of Asp 53, Glu 57, Glu 58, and Gln 65 appear to be quite highly populated, as indicated by the large pH shift for Cys 39 NH (Bundi & Wüthrich, 1979). Nonetheless, their presence could so far not be observed by NOEs and is also not manifested by slow amide proton exchange rates. This implies that these intramolecular interactions are very short lived. Considering that the global conformations of natural hirudin (Clore et al., 1987) and recombinant desulfatohirudin (Haruyama & Wüthrich, 1989) appear to be very similar, it is tempting to speculate that the reduced activity of the recombinant protein might be related with these transient structural features. A direct check of this hypothesis is presently not possible, since the amide proton titration data are not available for natural hirudin. However, in view of the results obtained here for desulfatohirudin, the previously reported (Haruyama & Wüthrich, 1989) chemical shift differences for residues Asp 53, Gly 54, and Asp 55 in the two proteins are an indication that the transient interactions between protein core and the flexible C-terminal polypeptide segment might be significantly different in native hirudin and in desulfatohirudin.

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