

- Kusche, M., Oscarsson, L. G., Reynertson, R., Rodén, L., & Lindahl, U. (1991) *J. Biol. Chem.* 266, 7400–7409.
- Laemmli, U. K. (1970) *Nature* 227, 608–685.
- Lam, L. H., Silbert, J. E., & Rosenberg, R. D. (1976) *Biochem. Biophys. Res. Commun.* 69, 570–577.
- Lane, D. L., Denton, J., Flynn, A. M., Thunberg, L., & Lindahl, U. (1984) *Biochem. J.* 218, 725–732.
- Linker, A., & Hovingh, P. (1972) *Biochemistry* 11, 563–568.
- McKay, E. J. (1981) *Thromb. Res.* 21, 375–382.
- Mourey, L., Samama, J. P., Delarue, M., Choay, J., Lormeau, J. C., Petitou, M., & Moras, D. (1990) *Biochimie* 72, 599–608.
- Nordenman, B., Nystrom, C., & Björk, I. (1977) *Eur. J. Biochem.* 78, 195–201.
- Nordenman, B., Danielsson, A., & Björk, I. (1978) *Eur. J. Biochem.* 90, 1–6.
- Olson, S. T., & Shore, J. (1982) *J. Biol. Chem.* 257, 14891–14895.
- Olson, S. T., & Björk, I. (1991) *J. Biol. Chem.* 266, 6353–6364.
- Olson, S. T., Halvorson, H. R., & Björk, I. (1991) *J. Biol. Chem.* 266, 6342–6352.
- Petitou, M., Duchaussoy, P., Lederman, I., & Choay, J. (1988) *Carbohydr. Res.* 179, 163–172.
- Petitou, M., Lormeau, J.-C., & Choay, J. (1991) *Semin. Thromb. Hemostasis* 17, S2 143–146.
- Pratt, C. W., & Church, F. C. (1991) *Semin. Hematol.* 28, 3–9.
- van Boeckel, C. A. A., Grootenhuys, P. D. J., & Haasnoot, C. A. G. (1991) *Trends Pharmacol. Sci.* 12, 241–243.
- Walenga, J. M., Bara, L., Petitou, M., Samama, M., Fareed, J., & Choay, J. (1988) *Thromb. Res.* 52, 553–563.

Primary Structure and Function of Novel *O*-Glycosylated Hirudins from the Leech *Hirudinaria manillensis*

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ABSTRACT: Hirudin from the leech *Hirudo medicinalis* is a most powerful anticoagulant, and many isoforms have been described. In the present work, the primary structure of two hirudins from the leech *Hirudinaria manillensis* has been elucidated. The antithrombotic activity is similar to that of *H. medicinalis* hirudins although the sequence identity is below 60%. Surprisingly, the hirudins were found to be glycosylated at one site. Sugar analysis after methanolysis yielded fucose, galactose, and *N*-acetylgalactosamine. These results combined with data from matrix-assisted laser desorption ionization mass spectrometry, plasma desorption mass spectrometry, capillary zone electrophoresis, and lectin-binding tests indicate that the sequence is Fuc-Gal β 1-3GalNAc-(*O*-threonine). This structure shows an interesting similarity to human blood group H determinants.

Hirudin, a 65 amino acid peptide produced in the salivary glands of the medicinal leech, is a most potent inhibitor of blood coagulation. It binds specifically and tightly to thrombin [$K_1 = 22$ fM] (Stone & Hofsteenge, 1986), the last regulatory enzyme of the blood clotting cascade, and thus has a high therapeutic potential for treatment of various thrombotic diseases. To date, the amino acid sequences of about 20 isohirudins are known (Dodt et al., 1984, 1986; Scharf et al., 1989; Tripiet, 1988) as well as a sequence predicted from cDNA (Harvey et al., 1986). No glycosylation has been found so far, the only posttranslational modification being sulfation at Tyr⁶³. The synthesis of a gene encoding hirudin and its expression in *Escherichia coli* or *Saccharomyces cerevisiae* has been reported by different groups (Bergmann et al., 1986; Loison et al., 1988). The three-dimensional structure of natural and recombinant hirudin has been elucidated by ¹H NMR spectroscopy (Cloue et al., 1987; Haruyama & Wüthrich, 1989; Folkers et al., 1989), and, recently, the crystal structure of the thrombin–hirudin complex has been reported

by two groups (Grütter et al., 1990; Rydel et al., 1990). These structures indicate that hirudin inhibits thrombin by a novel mechanism which does not utilize binding to the primary specificity pocket of the latter.

Apart from hirudins isolated from the leech *Hirudo medicinalis*, no complete hirudin structure from another leech has been described to date although knowledge of conserved regions could be of interest for the design of new thrombin inhibitors. For our study, we chose the leech *Hirudinaria manillensis*, called “buffalo leech”, which is reported to be more specialized for mammalian parasitism than *H. medicinalis* (Sawyer, 1986). *H. manillensis* is prevalent in southeast Asia and like *H. medicinalis* belongs to the Hirudidae family (Mann, 1962). We report the complete amino acid sequence of two *O*-glycosylated hirudins (designated P6 and P18) from *H. manillensis*, the characterization of their carbohydrate moiety, and their inhibitory activity.

MATERIALS AND METHODS

Isolation and Purification of P6 and P18. Hirudin P6 and P18 were isolated and purified to homogeneity as described (Steiner et al., 1990).

Amino Acid Analysis. Approximately 1.5 μ g of each reduced and *S*-pyridylethylated isoform was hydrolyzed with 6

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M HCl for 24 h at 110 °C. The hydrolysate was derivatized with DABS-Cl¹ (Fluka) as described by Knecht and Chang (1986).

Preparation of Reduced and Alkylated P6 and P18. To 100 µg of hirudin, 5 mg of DTT and 250 µL of buffer, pH 8.6 (6 M guanidine-HCl, 0.5 M Tris, 0.2% EDTA), were added, flushed with nitrogen, and kept for 6 h at 75 °C. After the mixture was cooled to room temperature and flushed with nitrogen, 3 µL of 4-vinylpyridine (Fluka) was added. The reaction mixture was shaken for 2 h in the dark. Desalting was carried out with a PD 10 column (Pharmacia) with doubly distilled water as the eluent.

Chemical Cleavage. Twenty-eight micrograms (4 nmol) of reduced and S-pyridylethylated P18 and 100 µL of 70% formic acid containing 2 mg/mL CNBr were added and kept for 20 h in the dark at room temperature. After a 15-fold dilution with water, the sample was dried in a vacuum centrifuge.

Enzyme-Catalyzed Cleavages. For digestion with endoproteinase Lys-C (Boehringer), hirudin was dissolved in buffer, pH 8.1 (0.1 M 4-ethylmorpholine acetate + 0.01% β-mercaptoethanol), and the enzyme was added (1:20 w/w). The sample was kept overnight at 37 °C. For digestion with *Staphylococcus aureus* V8 protease (Sigma), hirudin was dissolved in buffer, pH 7.8 (0.1 M NH₄HCO₃ in tap water for Ca²⁺ ions), and the enzyme was added (1:20 w/w). The sample was kept overnight at 37 °C.

Isolation of Fragments. The fragments were isolated using a Waters HPLC system equipped with a Nucleosil C₁₈ column (4.6 × 150 mm; 5-µm particle size) and employing a gradient of acetonitrile into dilute aqueous TFA. The runs were monitored at 214, 260, and 280 nm.

C-Terminal Sequencing with Carboxypeptidase Y. One nanomole (7 µg) of protein was dissolved in 30 µL of 50 mM sodium acetate, pH 5.4. Digestion was started by adding 7 µL of carboxypeptidase Y solution (Cooper Biomedical, 0.04 µg/µL, room temperature). After 0, 5, 20, 60, and 120 min, 7-µL aliquots were taken, immediately frozen, and lyophilized. The released amino acids were derivatized with DABS-Cl and analyzed as described by Knecht and Chang (1986).

N-Terminal Sequence Analysis. For amino acid sequencing, 0.1–0.5 nmol of the peptides was applied to an Applied Biosystems gas-phase sequencer model 477A and the PTH amino acid derivatives were analyzed on a model 120A analyzer (Applied Biosystems, Foster City, CA).

Gas Chromatographic Identification of Carbohydrates. Following the method of Chaplin (1982), 192 µg of P6 and 150 µg of P18 were subjected to methanolysis, re-N-acetylated, and silylated. The derivatives were injected into a HP 5890 gas chromatograph and separated on a fused-silica capillary (type DB-1; 30 m × 0.32 mm i.d.) coupled to a flame ionization detector and a mass spectrometer (HP 5988). The GC retention times were used for identification.

Capillary Electrophoresis. The in-house built capillary electrophoresis instrument was similar to those described in literature (Lüdi et al., 1988). The fused silica capillary had

Table I: Amino Acid Composition^a of Hirudin P6 and P18 from *H. manillensis* and Hirudin Variant 1 (HV 1; Dotd et al., 1984) from *H. medicinalis*

	HV 1	P6	P18
D + N	(9)	7.8 (8)	10.7 (10)
E + Q	(13)	13.3 (13)	9.9 (9)
S	(4)	4.1 (4)	6.5 (6)
T	(4)	3.2 (2)	4.5 (3)
G	(9)	7.8 (8)	9.6 (9)
A	(3)	2.4 (1)	1.6 (0)
R	(0)	3.2 (3)	1.3 (1)
P	(3)	2.8 (3)	2.2 (1)
V	(4)	2.2 (2)	2.5 (2)
M	(0)	1.8 (1)	1.9 (1)
I	(2)	2.2 (2)	1.3 (1)
L	(4)	1.2 (1)	2.5 (2)
F	(1)	2.3 (2)	3.7 (2)
C ^b	(6)	5.4 (6)	5.5 (6)
K	(3)	3.7 (3)	3.7 (3)
H	(1)	1.2 (1)	1.2 (1)
Y	(2)	2.6 (2)	2.6 (2)

^a Results are expressed as residues per molecule by analysis of 24-h acid hydrolysate or, in parentheses, from the sequence. Tryptophan was not determined. ^b Analyzed as S-pyridylethylcysteine.

a total length of 100 cm (80 cm to the detector) and an inside diameter of 75 µm. A small volume of P6 dissolved in water (0.4 µg/µL) was injected into the capillary by applying 5 kV for 5 s. The separations were carried out at a field strength of 280 V/cm with the detection wavelength set to 200 nm. The following electrolyte buffers were used for analysis at pH 8.3: (0 mM) 20 mM Tris, 20 mM Tricine, and 0.2 mM DAB; (10 mM) 20 mM Tricine, 10 mM sodium tetraborate, and 0.2 mM DAB; (105 mM) 90 mM boric acid, 15 mM sodium tetraborate, and 0.2 mM DAB.

Mass Spectrometry. LDI mass spectra were recorded on an in-house built time-of-flight instrument similar to that described by Beavis and Chait (1990). For sample application, 0.5 µL of 10⁻⁵ M aqueous hirudin solution was mixed with 0.5 µL of 10⁻⁴ M sinapinic acid solution (Fluka), and 0.3 µL of the mixture was given to the probe tip. The masses were calibrated with ion signals from sinapinic acid and its photo-adducts.

²⁵²Cf PD MS mass spectra were recorded on a Bio-Ion 20 mass spectrometer (Bio-Ion, Nordic, AB). For sample application, hirudin (>4 µg) was dissolved in 10 µL of water, and the solution was placed onto the sample holder (aluminum foil covered with a thin film of nitrocellulose). The masses were calibrated on the H⁺ and Na⁺ peak centroids.

Dot Assay for Lectin Binding. For the dot assay, 1 µL of hirudin (0.1–1 µg) was spotted onto nitrocellulose (Millipore) and fixed with 0.1% glutaraldehyde/0.4% formaldehyde. The strips were blocked for 1 h at 37 °C with phosphate-buffered saline supplemented with 1 mM MgCl₂, 1 mM CaCl₂, 0.1% bovine serum albumin, and 0.1% Tween-20 (BTB). After being washed with BTB, biotinylated lectin PNA (30 µg/mL; Boehringer) was incubated for 2 h at room temperature. Alkaline phosphatase conjugated to streptavidin was added accordingly and incubated for 1.5 h. Then 1 mL of substrate solution [nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoline phosphate (Amersham)] was added. Visual inspection of the strips was confirmed by measurement with a Gretag D142-2 reflexion densitometer. Human saliva was used as a source of blood group substances for positive controls.

Assay for Thrombin Inhibition. The effect of hirudin on the activity of human thrombin was tested as described by Stone and Hofsteenge (1986) with the following substrates: for hirudin P6, D-Phe-pipecolyl-Arg-4-nitroanilide (S-2238); and for hirudin P18, D-Val-Leu-Arg-4-nitroanilide (S-2266)

¹ Abbreviations: ²⁵²Cf PD MS, Californium-252 plasma desorption time-of-flight mass spectroscopy; CZE, capillary zone electrophoresis; DAB, 1,4-diaminobutane; DABS-Cl, (4-dimethylamino)azobenzene-sulfonyl chloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GC, gas chromatography; HPLC, high-performance liquid chromatography; LDI MS, matrix-assisted laser desorption ionization mass spectroscopy; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Tricine, N-[tris-(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)amino-methane.

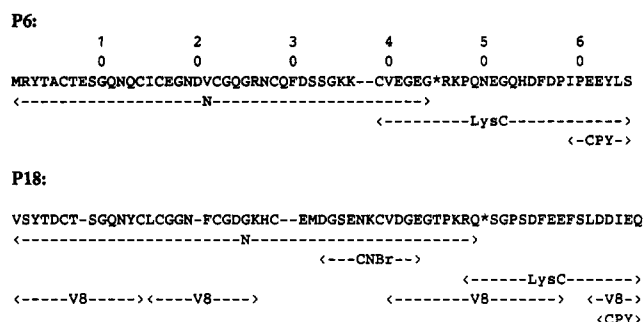


FIGURE 1: Summary of sequence determination of reduced and S-pyridylethylated P6 and P18. The lines indicate the amino acid sequences determined for peptides obtained after cleavage with endoprotease LysC (LysC), V8 protease (V8), and cyanogen bromide (CNBr); N denotes the N-terminal sequence of unfragmented P6 and P18 and CPY the C-terminal sequence determined by digestion of P6 and P18 with carboxypeptidase Y. The asterisk (*) indicates a residue unidentified by sequence analysis (glycosylation site). By amino acid analysis of the corresponding endoprotease Lys-C fragment, the residue was identified as Thr. The numbering system used corresponds to the sequence of *H. medicinalis* hirudin (see Figure 4); the minus sign (-) indicates that a gap was introduced in order to maximize homology.

(Kabi Vitrum). Hirudin P6 inhibition was assayed in the range of 0–40 pM using 20 pM thrombin and 99 μ M S-2238; for hirudin P18, 1 nM thrombin, 400 μ M S-2266, and hirudin concentrations between 0 and 2 nM were employed.

RESULTS AND DISCUSSION

Amino Acid and Sequence Analysis. In Table I, the amino acid analysis data of P6 and P18 are compared with the amino acid composition of hirudin variant 1 (Dodt et al., 1984) from the leech *H. medicinalis*. In contrast to the latter, P6 and P18 both contain arginine and methionine. The data further reveal remarkable differences in the amino acid composition of P6 and P18.

The general approach to sequence analysis is illustrated in Figure 1. Edman degradation of reduced and pyridylethylated P6 and P18 from the N-terminus identified more than 40 residues. Analysis of C-terminal fragments from endoprotease Lys-C digestion completed the sequence of the hirudins. Primary structure data of P18 fragments from V8 and CNBr cleavage agreed with those obtained above.

The *H. medicinalis* hirudins contain a sulfated tyrosine in the third position from the C-terminus. Sequencing of P6 yielded tyrosine in the same position, whereas, in P18, tyrosine was absent at the C-terminus. However, when P6 was digested with carboxypeptidase Y, no tyrosine was found. This indicated that tyrosine was modified, too. This was verified later by mass spectrometry (see below).

No PTH-amino acid could be detected at position 45 of P6 and at position 50 of P18, and the calculated molecular masses of P6 (6876 Da) and P18 (6592 Da) were not in agreement with the values obtained by ^{252}Cf PD MS (P6, 7416 Da; P18, 7199 Da). From these findings combined with the results from amino acid analysis and ^{252}Cf PD MS of hirudin fragments, we anticipated that these positions represented heterogeneously glycosylated threonines.

Carbohydrate Analysis. Gas chromatographic carbohydrate analysis yielded for both P6 and P18, GalNAc, Gal, and Fuc. The heterogeneity of the sugar moiety was first investigated by capillary zone electrophoresis. It has been reported by Honda et al. (1989) that monosaccharides form anionic borate complexes in borate buffer. Thus, we employed different concentrations of borate buffer in CZE of P6 and P18 to achieve separation of the peptides differing only in their

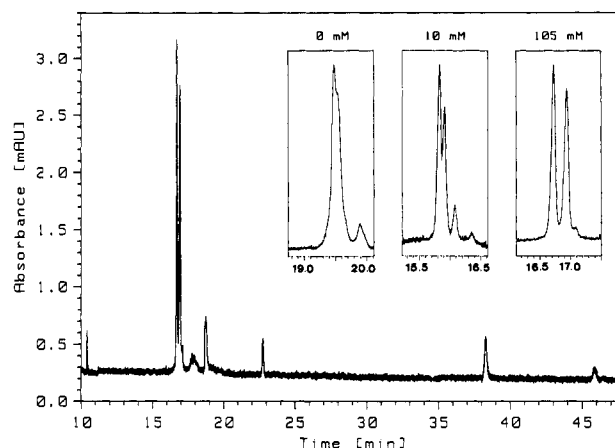


FIGURE 2: Electropherogram of hirudin P6 in 105 mM buffer resolving the inhomogeneity originating from various degrees of glycosylation at Thr⁴⁵. (Inset) Main peak at various borate buffer concentrations (see Materials and Methods for buffer composition).

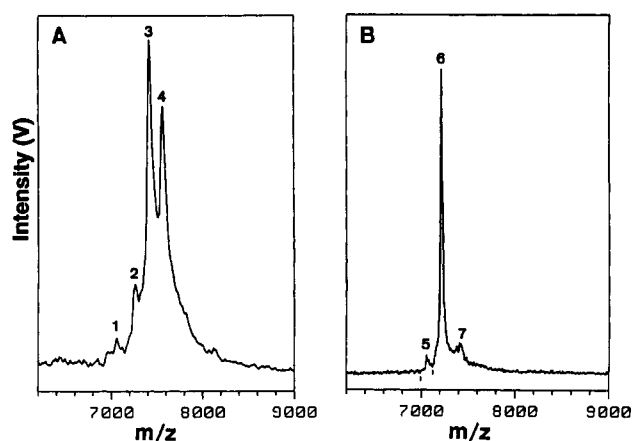


FIGURE 3: Negative LDI mass spectra of 0.1 to 0.2 μ g analyte using sinapinic acid as matrix. (A) P6; (B) P18. Peaks: 1, P6, m/z 7060.9, M_{calc} , 7056.7; 2, GalNAc-(O-Thr⁴⁵)-P6, m/z 7263.7, M_{calc} , 7259.9; 3, Gal-GalNAc-(O-Thr⁴⁵)-P6, m/z 7420.9, M_{calc} , 7422.0; 4, Fuc-Gal-GalNAc-(O-Thr⁴⁵)-P6, m/z 7564.1, M_{calc} , 7568.2; 5, Gal-GalNAc-(O-Thr⁵⁰)-P18, m/z 7056.4, M_{calc} , 7058.6; 6, Fuc-Gal-GalNAc-(O-Thr⁵⁰)-P18, m/z 7204.0, M_{calc} , 7204.7; 7, sinapinic acid photoadduct.

carbohydrate moiety, as shown in Figure 2.

The use of matrix-assisted laser desorption/ionization mass spectrometry (Karas et al., 1987) with a new powerful instrument constructed in our laboratories (Börnsen et al., 1990) allowed the further characterization of the sugar heterogeneity. Indeed, as shown in Figure 3A, the mass spectrum presents resolved molecular ions corresponding to P6 without sugar, GalNAc-(O-Thr⁴⁵)-P6, Gal-GalNAc-(O-Thr⁴⁵)-P6, and Fuc-Gal-GalNAc-(O-Thr⁴⁵)-P6. Note the similarity to the 10 mM borate electropherogram in Figure 2. In contrast to PD MS, the sulfate group at Tyr⁶³ was not destroyed, and no fragmentation was observed. The absence of a signal for Fuc-GalNAc-(O-Thr⁴⁵)-P6 suggests that the sequence is linear. Figure 3B reveals that about 95% of the P18 molecules contain Fuc-Gal-GalNAc-(O-Thr⁵⁰).

A dot assay for verification of binding to lectin PNA [Gal β (1-3)GalNAc specific] was positive for P6 and negative for P18. This corroborates the conclusion that the oligosaccharide sequence is linear and suggests a β (1-3) linkage between Gal and GalNAc.

The presence of carbohydrates on hirudins is reported for the first time. It is interesting to note that Thr⁴⁵, the glycosylation site of P6, is conserved in all hirudin variants described

	1	2	3	4	5	6
	0	0	0	0	0	0
1	VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
2	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
3	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
4	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
5	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
6	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
7	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
8	TYTDCIESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
9	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
10	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
11	ITTYTDCTESGQDLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
12	ITTYTDCTESGQDLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
13	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
14	VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
15	VVYTDCTESGQDLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
16	VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
17	VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
18	VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
19	ITTYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
P6	MRYTACTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEETPKPKQSHNDGDFEEIPEEYLQ					
P18	VSYYTDCI-SGQNYCLCGG-NFCGDKHCEMD--GSENKCVDEGTPKQRTSGPSDFEEFSLDDIEQ					

FIGURE 4: Comparison of the amino acid sequences of hirudins isolated from *H. manillensis* (P6 and P18) with those of the 19 variants isolated from *H. medicinalis* [1 (Dodt et al., 1984); 2–8 (Tripiet, 1988); 9 (Dodt et al., 1986); 10–18 (Scharf et al., 1989); and 19 (Harvey et al., 1986); the latter was predicted from cDNA]. The minus sign (–) indicates that a gap was introduced in order to maximize the homology. The \$ sign represents a glycosylation site. Identical residues are shaded.

to date. P18 is not glycosylated at Thr⁴⁵, but at Thr⁵⁰ instead, and all *H. medicinalis* variants contain a Ser in this position as a possible site for *O*-glycosylation. The proposed carbohydrate sequence Fuc-Gal β (1–3)GalNAc is reminiscent of the human blood group H determinants (Watkins et al., 1988), and further investigations on linkage and blood group H activity are under way.

Thrombin Inhibition. The inhibitory effect of hirudin on human α -thrombin was tested as described by Stone and Hofsteenge (1986). The kinetic constants obtained for the inhibition of human thrombin were $K_i = 58 \pm 2$ fM and $K_i = 7.8 \pm 1.1$ pM for P6 and P18, respectively. Thus, the inhibitory activity of hirudin P6 from the leech *H. manillensis* is comparable to that of hirudin from the leech *H. medicinalis* ($K_i = 22$ fM).

Amino Acid Sequence Homologies. In Figure 4, the sequences of P6 and P18 are aligned with the complete hirudin sequences from *H. medicinalis* published to date. Only 62% of the P6 residues and 52% of the P18 residues are identical compared to hirudin variant 1. The six cysteines and the glycines belonging to turns are conserved as well as many residues of the rigid N-terminal core domain, emphasizing the uniqueness of the 3-D structure of hirudin. With the exception of Asp⁵⁵Phe⁵⁶, the C-terminal segment was not conserved although the number and distribution of acidic residues is roughly the same. The absence of ⁶³Tyr-SO₃H in P18 should be pointed out.

P6 contains an arginine residue in the second position. Therefore, the potency of P6 is somewhat surprising in view of the site-directed mutagenesis studies with hirudin variant 1 that indicated that small hydrophobic amino acids were necessary in the first two positions of hirudin for potent inhibitory activity (Wallace et al., 1989). However, molecular modeling studies on a Met(1)–Arg(2) mutated hirudin, based on the X-ray structure of hirudin variant I (Grütter et al., 1990), indicate that the side chain of Arg(2) would fit without steric constraints into the primary specificity pocket of thrombin, similar as the Arg of the inhibitor D-Phe-Pro-

Arg-chloromethylketone (FPRCK) (Bode et al., 1989). According to our model, the Tyr(3) side chain is bound in a hydrophobic cage of thrombin consisting of the amino acids Ile(174), Trp(215), Leu(99), Tyr(60A), and Trp(60D). D-Phe of FPRCK is oriented in a similar way in the same cage (H. Rink, personal communication).

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REFERENCES

- Beavis, R. C., & Chait, B. T. (1990) *Anal. Chem.* 62, 1836.
- Bergmann, C., Dodt, J., Köhler, S., Fink, E., & Gassen, H. G. (1986) *Biol. Chem. Hoppe-Seyler* 367, 731.
- Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J.* 108, 3467.
- Börnsen, K. O., Schär, M., & Widmer, H. M. (1990) *Chimia* 44, 412.
- Chaplin, M. F. (1982) *Anal. Biochem.* 123, 336.
- Clare, G. M., Sukumaran, D. K., Nilges, M., Zarbock, J., & Gronenborn, A. (1987) *EMBO J.* 6, 529.
- Dodt, J., Müller, H.-P., Seemüller, U., & Chang, J.-Y. (1984) *FEBS Lett.* 165, 180.
- Dodt, J., Machleidt, W., Seemüller, U., Maschler, R., & Fritz, H. (1986) *Biol. Chem. Hoppe-Seyler* 367, 803.
- Folkers, P. J. M., Clare, G. M., Driscoll, P. C., Dodt, J., Köhler, S., & Gronenborn, A. M. (1989) *Biochemistry* 28, 2601.
- Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J.* 9, 2361.
- Haruyama, H., & Wüthrich, K. (1989) *Biochemistry* 28, 4301.
- Harvey, R. P., Degryse, E., Stefani, L., Schanber, F., Caze-nave, J. P., Courtney, M., Tolstoshev, P., & Lecocq, J. P.

- (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 1084.
 Honda, S., Iwase, S., Makino, A., & Fujiwara, S. (1989) *Anal. Biochem.* 176, 72.
 Karas, M., Bachmann, D., Bahr, U., & Hillenkamp, F. (1987) *Int. J. Mass Spectrom. Ion Processes* 78, 53.
 Knecht, R., & Chang, J.-Y. (1986) *Anal. Chem.* 58, 2375.
 Loison, G., Findeli, A., Bernard, S., Nguyen-Juilleret, M., Marquet, M., Riehl-Bellon, N., Carvallo, D., Guerra-Santos, L., Brown, S. W., Courtney, M., Roitsch, C., & Lemoine, Y. (1988) *Biotechnology* 6, 72.
 Lüdi, H., Gassmann, E., Märki, W., & Grossenbacher, H. (1988) *Anal. Chim. Acta* 213, 215.
 Mann, K. H. (1962) in *Leeches (Hirudinea) Their Structure, Physiology, Ecology and Embryology*, p 140, Pergamon Press, Oxford.
 Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, II, J. W. (1990) *Science* 249, 277.
 Sawyer, R. T. (1986) in *Leech Biology and Behaviour*, p 420, Oxford Science Publications, Oxford.
 Scharf, M., Engels, J., & Tripier, D. (1989) *FEBS Lett.* 225, 105.
 Steiner, V., Knecht, R., Grütter, M., Raschdorf, F., Gassmann, E., & Maschler, R. (1990) *J. Chromatogr.* 530, 273.
 Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry* 25, 4622.
 Tripier, D. (1988) *Folia Haematol. (Leipzig)* 115, 30.
 Wallace, A., Dennis, S., Hofsteenge, J., & Stone, S. R. (1989) *Biochemistry* 28, 10079.
 Watkins, W. M., Greenwell, P., Yates, A. D., & Johnson, P. H. (1988) *Biochimie* 70, 1597.

pH Dependence of the Absorbance and ^{31}P NMR Spectra of *O*-Acetylserine Sulfhydrylase in the Absence and Presence of *O*-Acetyl-L-serine[†]

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ABSTRACT: *O*-Acetylserine sulfhydrylase (OASS) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme which catalyzes the final step in the biosynthesis of L-cysteine in *Salmonella*, viz., the conversion of *O*-acetyl-L-serine (OAS) and sulfide to L-cysteine and acetate. UV-visible spectra of OASS exhibit absorbance maxima at 280 and 412 nm with pH-independent extinction coefficients over the range 5.5–10.8. Addition of OAS to enzyme results in a shift in the absorbance maximum from 412 to 470 nm, indicating the formation of an α -aminoacrylate Schiff base intermediate [Cook, P. F., & Wedding, R. T. (1976) *J. Biol. Chem.* 251, 2023]. The spectrum of the intermediate is also pH independent from 5.5 to 9.2. The observed changes in absorbance at 470 nm at different concentrations of OAS were used to calculate a K_d of 3 μM for OAS at pH 6.9. As the pH decreases, the K_d increases an order of magnitude per pH unit. The ^{31}P NMR signal of the bound PLP has a pH-independent chemical shift of 5.2 ppm in the presence and absence of OAS. These results indicate that the phosphate group is present as the dianion possibly salt-bridged to positively charged groups of the protein. In agreement with this, the resonance at 5.2 ppm has a line width of 20.5 Hz, suggesting that the cofactor is tightly bound to the protein. The sulfhydrylase was also shown to catalyze an OAS deacetylase activity in which OAS is degraded to pyruvate, ammonia, and acetate. The activity was detected by a time-dependent disappearance of the 470-nm absorbance reflecting the α -aminoacrylate intermediate. The rate of disappearance of the intermediate was measured at pH values from 7 to 9.5 using equal concentrations of OAS and OASS. The rate constant for disappearance of the intermediate decreases below a $\text{p}K$ of 8.1 ± 0.1 , reflecting the deprotonation of the active-site lysine that originally formed the Schiff base with PLP in free enzyme. A possible mechanism for the deacetylase activity is presented where the lysine displaces α -aminoacrylate which decomposes to pyruvate and ammonia.

Cysteine biosynthesis in the enteric bacterium *Salmonella typhimurium* is catalyzed by two enzymes. The first, serine transacetylase, catalyzes the formation of *O*-acetyl-L-serine

(OAS)¹ from acetyl-CoA and L-serine, while the second, *O*-acetylserine sulfhydrylase, catalyzes the formation of L-cysteine from sulfide and OAS. The latter enzyme is dimeric with a subunit molecular weight of 34 450 (Levy & Danchin, 1988) and has 1 mol of PLP tightly bound per subunit (Becker et al., 1969).

Cook and Wedding (1976) have shown that the enzyme has a ping-pong mechanism. This mechanism requires that acetate

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¹ Abbreviations: OAS, *O*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5'-phosphate; F, α -aminoacrylate intermediate; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.