Heparinase I from *Flavobacterium heparinum*: The Role of the Cysteine Residue in Catalysis as Probed by Chemical Modification and Site-Directed Mutagenesis†

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ABSTRACT: Heparinase I (heparin lyase I, EC 4.2.2.7), a heparin-degrading enzyme produced by *Flavobacterium heparinum*, is used to deheparinize blood following extracorporeal procedures in surgery and in other applications. The present study of mapping and characterization of the cysteines of heparinase I represents the first structural characterization of a heparinase. [^{3}H]Iodoacetic acid labeling demonstrated that heparinase I has two free cysteines. One of the two cysteines is surface accessible and lies in a hydrophilic environment while the other is in a hydrophobic environment. Chemical modification of the cysteines, both in the presence and in the absence of heparin, suggests that the surface-accessible cysteine lies in or near the active site of heparinase I. Preferential reactivity of this cysteine with negatively charged sulfhydryl-modifying reagents and the cysteines' high reactivity to iodoacetic acid at pH 6.5 indicate that the surface-accessible cysteine is in a positively charged region. The surface-accessible cysteine (cysteine-135) was mapped as the active-site cysteine by radiolabeling with [^{3}H]iodoacetic acid and by tryptic digestion and peptide sequencing. Site-directed mutagenesis of cysteine-135 to a serine or an alanine in *r*-heparinase I demonstrates that this cysteine is essential for enzymatic activity. However, replacement of the surface-inaccessible cysteine by a serine or alanine has no effect.

Heparinase I (EC 4.2.2.7), a 42 500 Da enzyme from *Flavobacterium heparinum*, cleaves heparin in a random endolytic fashion (Linker & Hovingh, 1972; Linhardt et al., 1982). The enzyme has been utilized in the sequence determination of sugars, in the preparation of small heparin fragments for therapeutic uses, and in the removal of heparin from blood (Bernstein et al., 1988; Linhardt et al., 1990). A heparinase diagnostic, called Hepzyme, has been approved by the FDA (Teijodor et al., 1993) to monitor heparin levels in blood. Heparinase I has been cloned and expressed in *Escherichia coli* (Sasisekharan et al., 1993). Recently, heparinase I has been shown to be a potent inhibitor of neovascularization (Sasisekharan et al., 1994).

Heparinase I is believed to bind heparin through the lysine residues on the enzyme surface (Yang et al., 1985; Linhardt et al., 1982, 1990). The observation that modification by amine-reactive reagents and immobilization of heparinase I on amine-reactive supports result in extensive activity losses suggested the importance of lysines (Bernstein et al., 1988; Comfort et al., 1989; Leckband & Langer, 1991). In addition, evidence for an electrostatic nature of the interaction lies in the pH and ionic strength dependence of heparinase I activity (Yang et al., 1985). These observations, however, provide only a qualitative description of the active site or its surrounding environment.

Enzymatic response to differential chemical treatments gave some indications of important amino acid residues in heparinase I. Amine modifications resulted in loss of enzymatic activity, thus suggesting lysine involvement in enzyme activity (Comfort et al., 1989; Leckband & Langer, 1991). The effects of sulfhydryl modifications demonstrated that cysteine-reactive probes resulted in loss of enzymatic activity and the presence of a free cysteine (Leckband & Langer, 1991). In addition, earlier biochemical studies reported that heparinase I contained three to four cysteines (Yang et al., 1985) and a disulfide bond (Comfort et al., 1989). Taking all the above results together we set to address the number, the states, and the role of cysteines in heparinase I. In this study, we determine the functional role of the cysteines of heparinase I and show that the surface-accessible cysteine or cysteine-135 is an important nucleophile critical for heparinase I activity.

MATERIALS AND METHODS

Chemicals and Materials. IAA,[1] IAM, DTT, guanidine hydrochloride, ammonium bicarbonate, guanidine thiocyan-

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[1] Abbreviations: iodoacetic acid (IAA), iodoacetamide (IAM), dithiothreitol (DTT), 3-(*N*-morpholino)propanesulfonic acid (MOPS), tris(hydroxymethyl)aminomethane (Tris), *N*-ethylmaleimide (NEM), 4-vinylpyridine (4-VP), tri-*n*-butylphosphine (TBP), *p*-(chloromercuri)benzoate (PCMB), *p*-(chloromercuri)benzenesulfonic acid (PCMBS), trifluoroacetic acid (TFA), bovine serum albumin (BSA), phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), reverse-phase high-pressure liquid chromatography (RPHPLC), recombinant heparinase I), isopropyl β-D-thiogalactoside (IPTG).

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ate, glycine, QAE-Sephadex, MOPS, Tris, and NEM were from Sigma Chemical, St. Louis, MO. 4-VP, TBP, PCMB, PCMBS, and glacial acetic acid were from Aldrich, Milwaukee, WI. Urea, TFA, and acetonitrile were from Fisher Scientific, Pittsburgh, PA. Hydroxylapatite and BSA were from Bio-Rad, Hercules, CA. [^{3}H]NEM and [^{3}H]iodoacetic acid were from New England Nuclear, Boston, MA. Trypsin was from Boehringer Mannheim, Indianapolis, IN. Heparin, from porcine intestinal mucosa, 157 USP units/mg, was from Hepar, Franklin, OH. It was prepared at concentrations of 2 or 25 mg/mL in 5 mM calcium acetate, 100 mM MOPS buffer, pH 7.0. *E. coli* BL21(DE3) host was from Novagen, Madison, WI. Molecular biology reagents and their sources are listed in the appropriate section below.

Heparinase Purification and Protein Analyses. Lyophilized powdered extracts of *F. heparinum* were prepared and purified essentially according to the methods of Yang et al. (1985) and Sasisekharan et al. (1993). Protein concentrations were determined using Micro BCA reagent (Pierce Inc., Rockford, IL) relative to a BSA standard. Amino acid composition analysis was performed on an amino acid analyzer (model 420, Applied Biosystems, Foster City, CA) in Biopolymers Laboratory, MIT.

Derivatization of Thiol Groups: Pyridylethylation. Pyridylethylation is a cysteine-modification method that alkylates the cysteine using 4-VP (Andrews & Dixon, 1987). The alkylating group, 4-VP, is a hydrophobic residue that is stable in modified cysteines. The 4-VP-modified cysteine(s) can be characterized easily by amino acid analysis. Pyridylethylation of heparinase I (1 nmol) was achieved by reacting heparinase with TBP in the presence of 4-VP (Andrews & Dixon, 1987). Following the reaction, the protein was desalted using a Centricon P-30 microconcentrator and lyophilized. For the control reaction, 64 μL of *n*-propanol was added instead of the 5% TBP solution. Protein concentration was determined, and amino acid composition analysis was performed on ~4.2 μg (500 pmol) heparinase I samples.

PCMB and PCMBS. PCMB was essentially prepared and used as described in Glazer et al. (1975). Briefly, to 0.01 mg of enzyme/mL in 0.1 M MOPS was added 2.5−100 μM PCMB. The mixture was incubated at 4 °C for 4 h. The time course of inactivation was obtained by determination of the enzymatic activity retained after successive incubation intervals. The reversibility of the reaction was established by incubation of the PCMB-treated enzyme with 50 mM DTT. PCMB-labeling experiments determining heparinase inactivation were also carried out in the presence of different salt concentrations (50, 100 and 200 mM NaCl in 0.1 M MOPS solution). Heparinase I was also treated with PCMBS. PCMBS was added from a 10 mM stock solution to yield a final concentration of 2.5 mM (Glazer et al., 1975). In some experiments, heparinase I was pretreated with 10 μM PCMB prior to modification with [^{3}H]NEM or [^{3}H]IAA; in others, [^{3}H]IAA was added directly to the 10 μM PCMB solution to yield a concentration of 2 mM.

NEM. NEM treatment of heparinase I under nonreducing conditions was accomplished by incubation of the enzyme (0.1 mg/mL) in PBS containing 1 or 10 mM NEM at pH 7.0 and 4 °C for 8 h (Riordan & Vallee, 1972). After the labeling, heparinase I was desalted as described above. The effects of the treatment on enzymatic activity were determined as described at defined intervals. [^{3}H]NEM labeling of heparinase I under nondenaturing conditions was accomplished by incubation of the enzyme (0.1−0.2 mg/mL) with 1 mM [^{3}H]NEM (10^9−10^{10} cpm/mmol) as described for nonradioactive NEM.

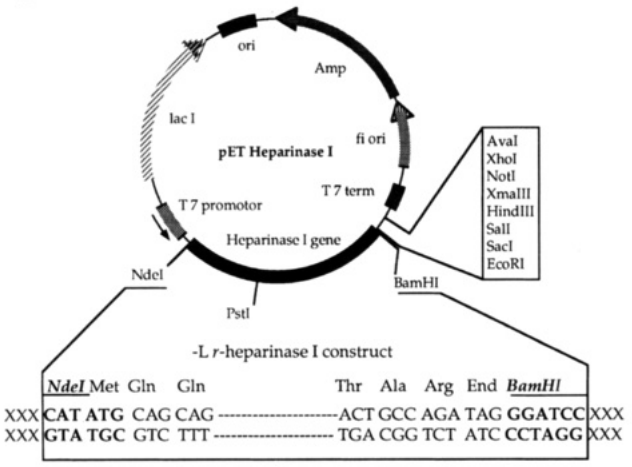
IAA. Heparinase I was alkylated with IAA by incubation of the enzyme (0.1 mg/mL) with 0.1−20 mM IAA at 4 °C for 4 h, in 50 mM MOPS at pH 7.5. Inactivation time courses were determined in the presence and absence of 2 mg of heparin/mL. The pH dependence of the alkylation reaction was determined by measurements of kinetic rates at different pH values. At each pH, the heparinase I incubation was carried out with 5 mM IAA in 50 mM MOPS at pH 6.5, 7.0, and 7.5 or in 50 mM Tris/0.1 M NaCl at pH 8.0 and 8.5. Radiolabeling of heparinase I with [^{3}H]IAA was carried out under three conditions: (1) nondenatured, nonreduced, (2) nondenatured, reduced, and (3) denatured, reduced. Heparinase I was denatured with 5 M guanidine hydrochloride at 60 °C for 15 min and reduced with 0.5 mM DTT at 4 °C under nitrogen for 4 h. The [^{3}H]IAA concentration was 0.05 M with a specific activity of 10^{11} cpm/mmol. The stock solution was prepared by adding 424 μL of 0.05 M IAA to 250 μCi of [^{3}H]IAA at 1.6 × 10^8 cpm. The reaction solution was desalted (to remove unreacted label) by passage over two successive 2 mL centrifuge columns (Falcon tubes, Becton Dickinson Laboratories, Franklin Lakes, NJ) equilibrated with 5 mM MOPS buffer (pH 7.0).

IAM. IAM treatment of the enzyme was carried out in the presence and absence of 5 mM DTT, and IAM was added from a 0.1 M aqueous stock solution. In the absence of DTT, heparinase I (0.1 mg/mL) was treated with 2, 5, 10, and 120 mM IAM in PBS at 4 °C for 4 h. The inactivation time courses were followed as with IAA-treated enzyme. IAM treatment following DTT reduction was carried out as described for PCMB modification in the presence of DTT, except that IAM was added in 2 mM excess over the DTT. The extent of cysteine labeling by IAM was also examined, where heparinase I was pretreated with IAM prior to alkylation with [^{3}H]IAA or with [^{3}H]NEM.

Tryptic Digest and Protein Sequence Analyses. Tryptic digest of the samples was performed as described previously (Sasisekharan et al., 1993). In the case of [^{3}H]IAA-labeling experiments, 1 nmol of [^{3}H]IAA-labeled heparinase I (samples labeled by the three different procedures described in the previous section with appropriate modifications) was digested by trypsin. Tryptic peptides were separated by RPHPLC and monitored at 210 and 277 nm. The peaks were collected in microfuge tubes and counted for tritium incorporation on the Beckman scintillation counter. The tritium-incorporated peptide peaks were sequenced using an Applied Biosystems Sequencer model 477 with an on-line model 120 PTH amino acid analyzer (Biopolymers Laboratory, MIT).

Recombinant Heparinase I. *r*-Heparinase I is produced as a soluble protein in BL21(DE3) *E. coli* host, using the pET 15b system (Novagen, WI). This construct has a histidine tag (six consecutive histidines) and a thrombin cleavage site in a 21 amino acid N-terminal leader sequence, which constitutes a high-affinity site for Ni^{2+}. The C135S, C297S, C135A, and C297A mutations were introduced by the overlap extension PCR methodology developed by Higuchi et al. (1990). The primers used for making the mutations are shown in Figure 1. After being amplified and cloned in pET-15b vector, the sequences of the mutated genes

Expression vector



Mutants and the primers

C135S

5' primer	5' AAA GGG ATT TCT GAA CAG GGG 3'
3' primer	5' CCC CTG TTC AGA AAT CCC TTT 3'

C135A

5' primer	5' AAA GGG ATT GCT GAA CAG GGG 3'
3' primer	5' CCC CTG TTC AGC AAT CCC TTT 3'

C297S

5' primer	5' C CCT AAA GAT TCC TGG ATT AC 3'
3' primer	5' GT AAT CCA GGA ATC TTT AGG G 3'

C297A

5' primer	5' C CCT AAA GAT GCC TGG ATT AC 3'
3' primer	5' GT AAT CCA GGC ATC TTT AGG G 3'

FIGURE 1: *r*-Heparinase I gene construct. Figure shows the pET 15b plasmid construct expressing *r*-heparinase I (see Materials and Methods). Also shown is the primer design for the mutant heparinase I [for DNA sequence of heparinase I, refer to Sasisekharan et al. (1993) or to Genbank, acc. no. L12534].

were verified using Sequenase (U.S. Biochemicals Inc., Cleveland, OH). The plasmid containing the gene in pET-15b was isolated, purified, and used to transform the host cells BL21(DE3).

Expression, Isolation, and Purification of Mutant r-Heparinase I in E. coli. Overnight cultures containing the *r*-heparinase I genes in pET-15b were induced with IPTG and harvested as described in Sasisekharan et al. (1993). The cell pellet was resuspended in one-twentieth of the volume of the binding buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole). The resuspended culture was placed in an ice bath, sonicated for 2 min using a Branson sonicator (model 450, power 3, 50% pulse; Branson, Danbury, CT), and centrifuged at 4 °C and 15 000*g* for 30 min. The supernatant was assayed for activity and purified by Ni^{2+} affinity chromatography using Sepharose 6B Fast Flow resin covalently linked to nitrilotriacetic acid (Novagen, WI). Briefly, the resin was charged with five column volumes of 200 mM $NiSO_4$ and equilibrated with five column volumes of binding buffer. The 6−10 mL sample was then applied followed by 12 mL of binding buffer, 9 mL of 15% elution buffer (20 mM Tris, 500 mM NaCl, 200 mM imidazole), and 10 mL of 100% elution buffer. Heparinase I was recovered in 4 mL of the 100% elution step, desalted on two PD10 columns (Bio-Rad, CA), and incubated overnight at 4 °C with 0.5 units of thrombin (Novagen, WI). After stripping (20 mM Tris, 500 mM NaCl, 100 mM EDTA) and charging the metal chelate column, the cleaved heparinase I was applied and collected in the flow-through fraction.

Table 1: Rate Constants of Heparinase I Inactivation by Iodoacetic Acid as a Function of pH[a]

pH	6.5	7.0	7.5	8.0	8.5
rate (h^{-1})[b]	0.18	0.15	0.24	0.20	0.30

[a] Heparinase I inactivation was accomplished with 500 μM iodoacetic acid in PBS at 4 °C. [b] Rate constants were determined as described in the legend to Figure 2.

Purity of the *r*-heparinase I was determined by SDS−PAGE Laemmli (1970) with a Mini Protean II electrophoresis apparatus (Bio-Rad, CA). Visualization of proteins in gels (12% gels) was accomplished with 0.1% Coomassie Blue stain or using silver stain (Bio-Rad, CA).

Heparinase I Assay. The UV 232 nm assay and the saccharide assay were performed essentially as described previously (Bernstein, et al., 1988; Sasisekharan et al., 1993). The enzyme activity was directly measured from the increase in absorbance at 232 nm as a function of time. Activity is expressed as IU = μmol of product formed/min, using ϵ = 3800 M^{-1}.

RESULTS

Thiol Modification Inactivates Heparinase I. In the presence of 2 mM IAA, 95% ± 5% of heparinase I was inactivated within 10 min. The inactivation rate was concentration dependent: at 1 mM and 0.1 mM IAA, inactivation was complete within 15 min and 15 h, respectively. Pretreatment of the enzyme for 4 h at 4 °C with DTT under nitrogen had no effect on the modification.

It is known that the IAA-reactive form of cysteine is the mercaptide anion and that the reaction rate increases with increasing pH (Torchinsky, 1981). In particular, the relative free cysteine alkylation rates at pH 5.6, 7.02, and 8.36 are 0.14, 1, and 2.1, respectively (Torchinsky, 1981). If the heparinase I cysteine was unaffected by the presence of nearby amino acids in the protein, then the IAA inactivation rates at pH 6.5 and 8.0 would be expected to vary by an order of magnitude. The pseudo-first-order heparinase I inactivation rate constants obtained at pH 6.5, 7.0, 7.5, 8.0, and 8.5 varied very little over the pH range (Table 1). The rate constants, k, were determined from the activity at a determined time, A_t, the initial activity $A_i = (A_0 - A_\infty)$ the residual activity at infinite time, A_∞, and eq 1:

$$A_t = A_\infty + A_i \exp(-kt) \quad (1)$$

The small or insignificant variation in the rate of the inactivation suggests that the cysteine was activated by the presence of nearby basic amino acids (Hammond & Gutfreund, 1959; Rabin & Watts, 1960). Further, the sensitivity of the IAA binding site to the presence of 2 mg of heparin/mL was demonstrated by the decrease in the inactivation rate in 500 μM IAA from (3 ± 1) × 10^{-3} min^{-1} to (5 ± 2) × 10^{-4} min^{-1}.

The reversible, sulfhydryl-specific anion, PCMB, was utilized to confirm the sulfhydryl selective inactivation of heparinase I. Heparinase I treatment with PCMB at 2.5−100 μM and 4 °C resulted in a reversible loss of 95% ± 5% of enzyme activity. Upon addition of 10 mM DTT, up to 90% of the lost enzyme activity is recovered within 1 h at 4 °C, verifying the sulfhydryl specificity of the reaction. The fractional activity of heparinase I was plotted as a function

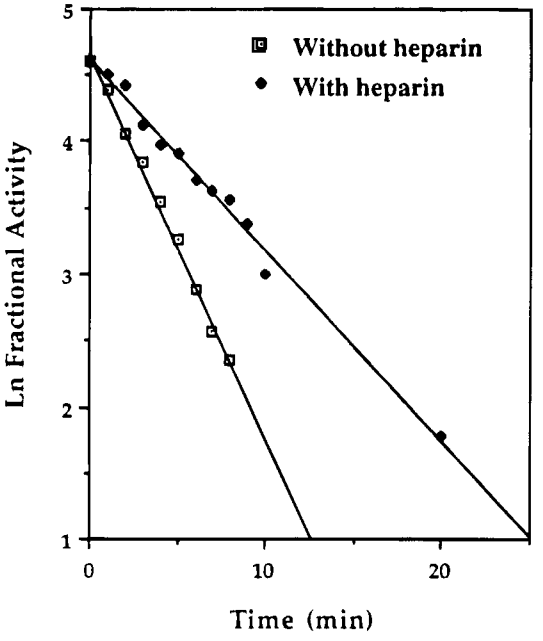


FIGURE 2: PCMB inactivation of heparinase I in the presence and absence of heparin. Heparinase I was incubated with 2.5 μM PCMB in 10 mM MOPS (pH 7.0) at 4 °C with 0.5 mg of heparin/mL and without heparin. The fractional activity was calculated by subtracting the residual activity (A_∞) from the activity at a given time t (A_t) and normalizing with the initial activity (A_i). The natural logarithm of the fractional activity (expressed as %) was plotted as a function of time (the values are averages of triplicate determinations). The pseudo-first-order rate constant was calculated by fitting the data to eq 1, obtaining $k = 0.28$ min^{-1} in the absence of heparin. In the presence of heparin, the rate constant k was reduced to 0.14 min^{-1}.

of time (Figure 2). In the presence of 0.5 mg of heparin/mL ($\sim 5K_m$), the rate of inactivation was significantly decreased. Rate constants were determined by assuming pseudo-first-order kinetics and fitting the data to eq 1. The rate constant in the absence of heparin was $k = 0.28$ min^{-1}, and in the presence of 0.5 mg of heparin/mL was $k = 0.14$ min^{-1}. The heparin concentration in the assay medium was much larger than the K_m of 0.1 mg/mL (Yang et al., 1985); consequently, any additional heparin introduced did not alter the kinetics.

Radiolabeling with [^{3}H]Iodoacetic Acid. Carboxymethylation of heparinase I cysteines with [^{3}H]IAA was used to quantify and map the cysteines. Heparinase I that had been treated with 2 mM [^{3}H]IAA in the presence of guanidine hydrochloride and DTT had $(2.2 \pm 0.05) \times 10^5$ cpm of [^{3}H]-IAA/nmol of heparinase I. The results from pyridylethylation of heparinase I are consistent with the [^{3}H]IAA labeling.[2] In order to verify that the PCMB-reactive cysteine was at the same site as the iodoacetic acid-binding cysteine, [^{3}H]-IAA was reacted with nondenatured PCMB-modified heparinase I, and the amount of label incorporated was compared to that of nondenatured, untreated heparinase I labeled with [^{3}H]IAA under identical conditions. In these experiments, about $(1.2 \pm 0.08) \times 10^5$ cpm of [^{3}H]IAA/nmol of heparinase I was labeled in the case of nondenatured, untreated (PCMB) heparinase I, while prior treatment of heparinase I with PCMB reduced [^{3}H]IAA labeling by 80%. This suggested that the IAA binding site and the PCMB binding site are identical. This was conclusively determined by trypsin digestion, peptide mapping, and amino acid sequencing of the modified heparinase I cysteine as discussed below.

Tryptic Mapping of the Cysteines of Heparinase I. Physical mapping of the cysteines of heparinase I was performed by peptide mapping with trypsin, followed by amino acid sequencing. Figure 3 shows the HPLC profile of the tryptic digest of heparinase I. In the figure, the [^{3}H]-labeled peptides were the peak eluting at 38.8 min (which corresponded to cysteine-135) and the peaks eluting at 85.5 and 89 min (both of which corresponded to cysteine-297). The sequences of the three peptides are shown in the figure legend.

In order to map the PCMB reactive cysteine, the PCMB-labeled heparinase I was isolated, denatured, and then reacted with IAM to block the other cysteine. Subsequently, the enzyme was treated with DTT to remove the bound PCMB and then labeled with [^{3}H]IAA. Modified heparinase I was digested with trypsin, and the tryptic peptides were separated. Only one cysteine, cysteine-135, was selectively labeled by [^{3}H]IAA. Cysteine-297 was not labeled by [^{3}H]IAA in this experiment. In another experiment, heparinase I was first labeled at the reactive cysteine with PCMB. The enzyme was then denatured, labeled with [^{3}H]IAA, and rechromatographed to remove the excess radiolabel. Following this, the enzyme was digested with trypsin and the tryptic peptides were separated by RPHPLC. In this experiment, cysteine-297 was selectively [^{3}H] labeled while cysteine-135 was not. The results of the above experiments, taken together, confirm that cysteine-135 was the PCMB-labeled or the active-site cysteine (Table 2). Note that [^{3}H]IAA labeling had little or no cross-reactivity and was selective in labeling the cysteines.

Thiol Reactivity. As the above results suggested that the reactive cysteine is perhaps activated by the presence of nearby basic amino acids, further investigation was carried out in an attempt to elucidate structural influences on the cysteine reactivities.

The use of PCMBS resulted in inactivation kinetics similar to that of PCMB (with and without heparin; data not shown). However, treatment of heparinase I with 1 mM NEM at pH 7.0 showed little change in activity. The fractional activity of heparinase when treated with NEM was plotted as a function of time (Figure 4). Further, heparinase I treated with 1 mM NEM overnight at 4 °C resulted in an activity loss of about only 15%. In addition, no significant levels of [^{3}H]NEM bound heparinase I, even under denaturing conditions. The effect of reagent charge on cysteine reactivity was also investigated by using IAA's neutral analog, IAM. In addition to their charge difference, the reactivity of IAM with free cysteine was 5−7 times faster than IAA in aqueous media (MacQuarrie & Bernhard, 1971); consequently, a reduction in the IAM reactivity would provide strong evidence for the requirement of negative charge on the labeling reagent. Heparinase I incubated with 2, 5, 10, and 120 mM IAM in PBS at pH 7.0 and 4 °C for up to 24 h exhibited little change in activity. A 15% inactivation occurs only after a 24 h incubation in the presence of 120 mM IAM. Further, the labeling of IAM was not different under denaturing conditions. IAM, therefore, does not significantly modify heparinase I.

[2] Amino acid analyses on the 4-VP cysteine heparinase I indicated the presence of 2.14 ± 0.2 cysteines. There was no increase in pyridylethyl cysteine content following treatment with DTT, which suggested the absence of any disulfide bonds. This result was independent of the use of TBP in the labeling.

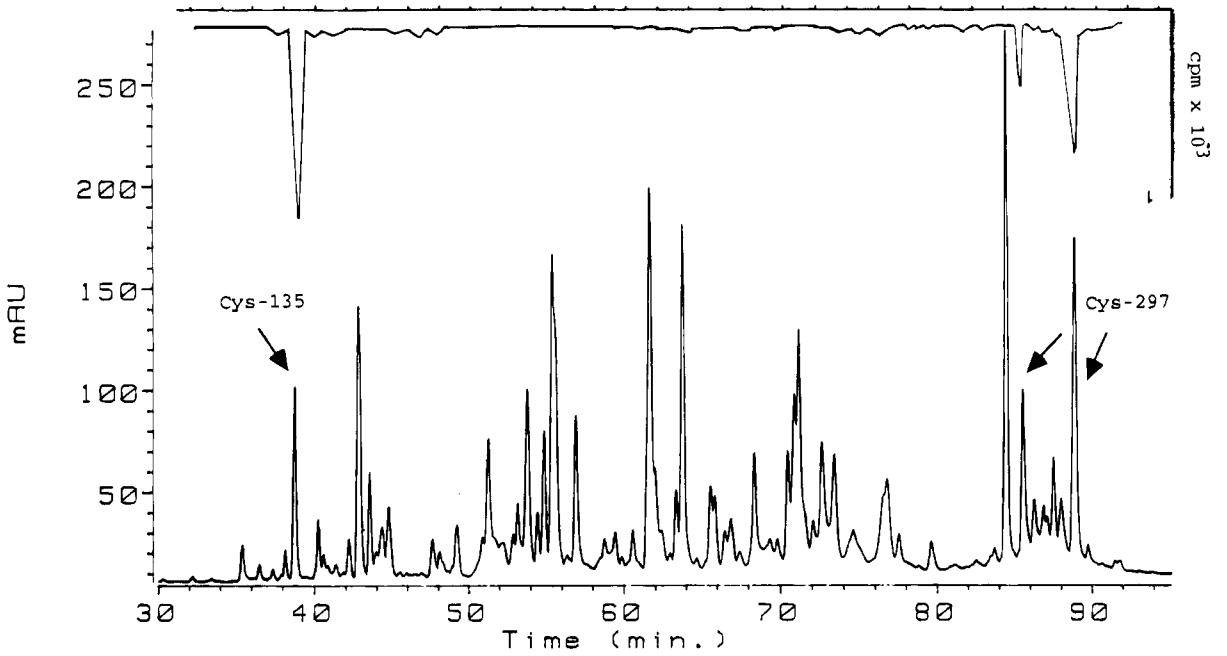


FIGURE 3: C_{18} reverse-phase HPLC profile of the tryptic peptides of [^{3}H]heparinase I. Heparinase I was digested with trypsin essentially as described in Sasisekharan et al. (1993). After trypsin digestion, the tryptic peptides were separated by RPHPLC with a 10 min isocratic run followed by 0%−80% acetonitrile (in 0.1% TFA) for 120 min. Tryptic peptides were monitored at 210 and 277 nm (in mAU), collected in microfuge tubes, and counted for tritium incorporation (shown on top, to scale in cpm × 10^{-3}). The tritium-incorporated peptide peaks were sequenced. The peak eluting at 38.8 min corresponded to the tryptic peptide K G I C* E Q G S S R containing cysteine-135. The peak at 85.5 min corresponded to tryptic peptide K D I C* W I T F D V A I D W T K, and the peak eluting at 89 min corresponded to the tryptic peptide K M P F A Q F P K D I C* W I T F D V A I D W T K. Both of these peaks contain cysteine-297. The peak eluting at 89 min appears as a partial digest of the peak eluting at 85.5 min (the peaks are marked by arrows).

Table 2: PCMB Labeling and Tryptic Mapping of Heparinase I [^{3}H]Cysteines[a]

						results	
treatment	PCMB	IAM	DTT	[^{3}H]IAA	tryptic digest	cysteine-135	cysteine-297
expt 1	+	+	+	+	+	[^{3}H] labeled	not labeled
expt 2	+	−	−	+	+	not labeled	[^{3}H] labeled

[a] Two experiments were carried out to determine the PCMB-reactive cysteine of heparinase I. The top row describes the sequence of the treatments. In experiment 1 after PCMB labeling, excess label was removed prior to denaturing heparinase I and labeling with IAM. After the IAM labeling, heparinase I was reduced to remove PCMB and then labeled with [^{3}H]IAA to tryptic map the [^{3}H]cysteine. In experiment 2, PCMB-labeled heparinase I was treated with [^{3}H]IAA (without reduction) and digested with trypsin in order to map the [^{3}H]cysteine.

In order to show that the positively charged environment around the reactive cysteine influences the labeling of the negatively charged PCMB, cysteine labeling by PCMB was performed under different salt concentrations. Figure 5 shows the time course of inactivation of heparinase I by PCMB with increasing salt concentrations. The heparinase I inactivation rate by PCMB was significantly reduced with increasing salt concentration. This result is consistent with the observation that the environment around the reactive cysteine is positively charged.

Recombinant Heparinase. Site-directed mutations were performed to confirm the role of cysteine-135 in heparinase I activity. Four mutant recombinant heparinases were designed (C135S, C297S, C135A, and C297A) and expressed in the BL21(DE3) host. The *r*-heparinase I construct devoid of the putative signal sequence (−L-*r*-heparinase I) was expressed as a control (Sasisekharan et al., 1993). The level of protein expression for all the recombinant heparinases was identical in the BL21(DE3) host (data not shown). While −L-*r*-heparinase I control was expressed as a soluble protein in *E. coli* with an activity of ~5.2 units/mg of *E. coli* crude extract (Sasisekharan et al., 1993), the C135A *r*-heparinase I was expressed in BL21(DE3) with no enzymatic activity. The C135S *r*-heparinase I, however, was expressed in BL21-(DE3) with an activity of ~0.06 units/mg of *E. coli* crude extract. Importantly, the mutations at cysteine-297 (C297S and C297A) were both expressed in the same host with no change in their enzymatic activity compared to the −L-*r*-heparinase I control. Table 3 lists the kinetic parameters for the recombinant and mutant heparinases I. The purity of *r*-heparinases (as determined by SDS−PAGE with silver stain) is estimated to be 80%−90% after the first step and greater than 98% after the second step (Figure 6). The above results taken together show that cysteine-135 is important for heparinase I activity and that altering cysteine-297 did not alter heparinase I activity.

DISCUSSION

Number of Cysteines of Heparinase I. The present work demonstrates that heparinase I contains two free cysteines. However, the open reading frame (ORF) of the cloned *F. heparinum* heparinase I gene contains three cysteines (cysteine-17, -135, and -297), with cysteine-17 in the putative 21 amino acid signal sequence (Sasisekharan et al., 1993). Since the N-terminal amino acid of the mature heparinase I from *F. heparinum* is blocked, it has not been possible to

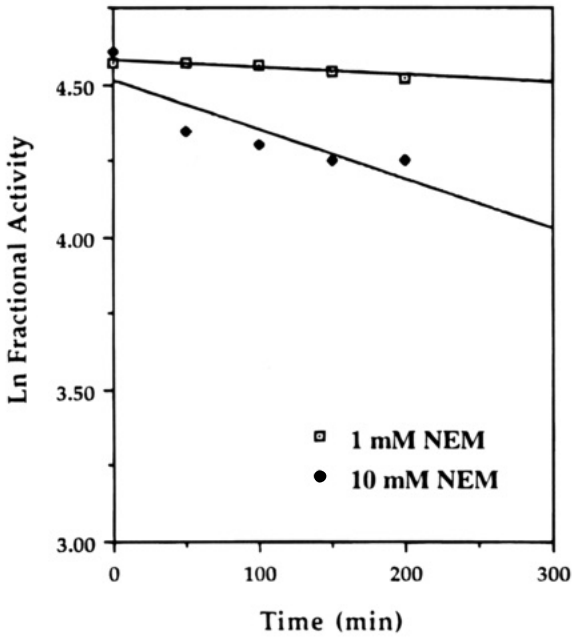


FIGURE 4: NEM inactivation of heparinase I. Heparinase I was incubated with 1 or 10 mM NEM in PBS at pH 7.0 and 4 °C. The activity was expressed as a fraction of the initial activity (expressed as %), and the natural logarithm of the fractional activity was plotted as a function of time. The values are averages of triplicate determinations. The data were fitted to eq 1; the rate constants were calculated to be $k = 0.00024$ min^{-1} (1 mM NEM) and $k = 0.0016$ min^{-1} (10 mM NEM).

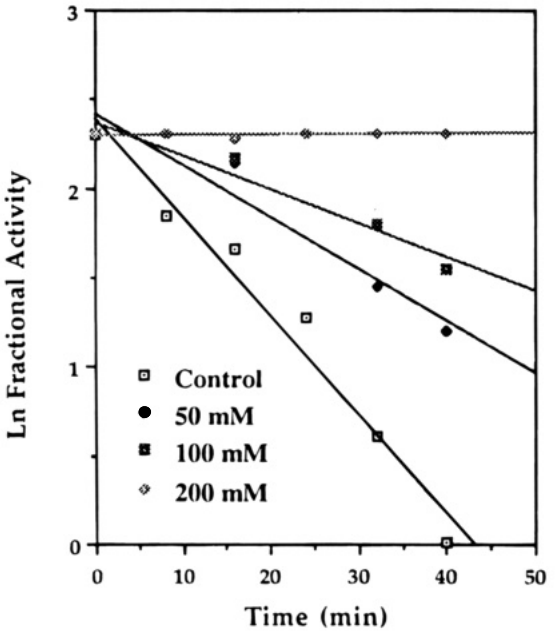


FIGURE 5: PCMB inactivation of heparinase I in varying salt concentrations. Heparinase I was incubated with 2.5 μM PCMB in 10 mM MOPS (pH 7.0) at 4 °C: (⊡) control containing no salt; (♦) containing 50 mM NaCl; (■) containing 100 mM NaCl; (lightly-shaded diamonds) 200 mM NaCl. The activity was expressed as a fraction of the initial activity, and the natural logarithm of the fractional activity (expressed as %) was plotted as a function of time. The values are averages of triplicate determinations. The salt concentration range used in this study is based on the previously studied conditions for both the enzyme stability as well as activity (Yang et al., 1985; Lohse & Linhardt, 1992).

determine if the proteolytic processing of the signal sequence occurs in *F. heparinum* at Ala_{19}-Tyr_{20}-Ala_{21} site, the classical processing site (Sasisekharan et al., 1993; Von Heijne, 1988). Further, the *F. heparinum* heparinase I signal sequence is

Table 3: Kinetic Constants of *r*-Heparinase I and the Mutant *r*-Heparinase I[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)
wild-type *r*-heparinase I	92	10.2
C135S *r*-heparinase I	2	4.2
C135A *r*-heparinase I	ND[b]	ND

[a] The kinetic constants reported in the present work for the wild-type *r*-heparinase I are consistent with previous reports for the native *F. heparinum* heparinase I (Yang et al., 1985; Loshe & Linhardt, 1992). [b] ND = not determined, because the enzyme was inactive.

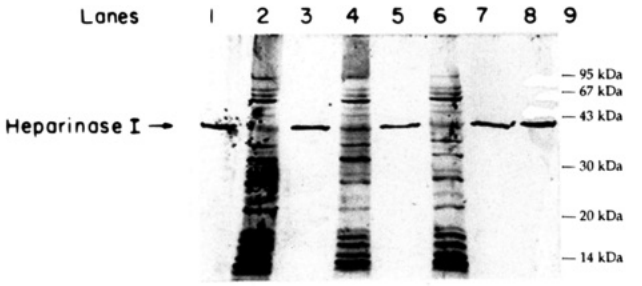


FIGURE 6: SDS−PAGE of *r*-heparinase I and mutant *r*-heparinase I. A 12% SDS−PAGE of heparinase I. Lanes are described from left to right. Lane 1: purified *F. heparinum* heparinase I. This enzyme preparation was used in the labeling and mapping studies (see Material and Methods). Lane 2: crude extract of BL21(DE3) with C135A mutant *r*-heparinase I. Lane 3: Ni column purified C135A mutant *r*-heparinase I. Lane 4: crude extract of BL21(DE3) with C135S mutant *r*-heparinase I. Lane 5: Ni column purified C135S mutant *r*-heparinase I. Lane 6: crude extract of BL2(DE3) with *r*-heparinase I. Lane 7: Ni column purified *r*-heparinase I. Lane 8: purified *F. heparinum* heparinase I. Lane 9: Molecular weight standards.

not processed in *E. coli* (Sasisekharan et al., 1993). *r*-Heparinase I starting at Gln_{22}, assuming that the leader sequence is processed at the classical Ala_{19}-Tyr_{20}-Ala_{21} site, was active, and this indicated that cysteine-17 was not essential for enzymatic activity. However, this *r*-heparinase I protein has a molecular mass >1 kDa less than the *F. heparinum* heparinase I (Sasisekharan et al., 1993). An alternate cleavage site of the signal sequence starting with Gln_{12} (hence the inclusion of cysteine-17 in the mature protein) was invoked to account for the difference in the molecular mass between the wild-type and *r*-heparinase I (Sasisekharan, 1991). This hypothesis was consistent with earlier biochemical studies of heparinase I from *F. heparinum*, which indicated the presence of three to four cysteines and a disulfide bond (Yang et al., 1985; Comfort et al., 1989).

The cysteine labeling of *F. heparinum* heparinase I in the present work, however, gave only 0.4 ± 0.1 mol % for the total cysteine content. This was independent of heparinase pretreatment with reducing agents. On the basis of an average molecular mass of 110 Da per amino acid, a cysteine content of 0.4 ± 0.1 mol % corresponded to a stoichiometry of ~2 mol of cysteine/mol of heparinase I. This was lower than the previously reported value of 0.8 mol % (Yang et al., 1985). In the previous analysis, the cysteine content was determined from the cysteic acid generation by performic acid treatment. As cysteic acid analysis can suffer from glutamic acid interference, it is possible that there was an overestimation of cysteine(s). The radiolabeling and tryptic mapping studies reported in this work demonstrate that cysteine-135 and cysteine-297 are the only cysteines of mature *F. heparinum* heparinase I.

The present work shows that cysteine-17 is not contained in the mature *F. heparinum* heparinase I. However, due to unknown protein modification(s) (Sasisekharan, 1991), the actual start site for the mature *F. heparinum* heparinase I and the basis of the difference in the molecular weights between recombinant and the wild-type heparinase I remain to be determined (Sasisekharan et al., 1993).

Characterization of the Functional Role of the Cysteines of Heparinase I. Heparinase I derivatization by IAA and sulfhydryl-specific reagent PCMB inactivated the enzyme. Chemical modification experiments described here verified that the IAA- and PCMB-reactive cysteines are identical, and this cysteine is surface accessible. In addition, protection of heparinase I with heparin during both IAA and PCMB derivatization suggested that the reactive cysteine is possibly near the active site. The surface-accessible cysteine was mapped as cysteine-135 by radiolabeling, tryptic digestion, and peptide sequencing. One possible interpretation of the sulfhydryl-labeling experiments is that the chemical modification of cysteine-135 may affect the enzymatic activity by steric impedance of heparin access to the active site. Another possible explanation is that the heparin bound to heparinase I could alter the charge characteristics near cysteine-135 and thereby reduce the labeling kinetics. In order to ascertain a mechanistic role of the surface-accessible cysteine in the enzymatic pathway, site-directed mutagenesis was performed. The replacement of cysteine-135 with alanine abolished the enzymatic activity and demonstrated the importance of this amino acid in heparinase I activity. However, replacement of cysteine-135 with serine drastically reduced heparinase I activity and suggested a nucleophilic role of this residue in the endolytic cleavage of heparin (see below; Linhardt et al., 1986).

The chemical modification experiments in this study have served both to identify cysteine-135 as an essential amino acid (which was verified by site-directed mutagenesis) and also, importantly, to aid in the elucidation of surface properties around this residue. The relative reactivities of the different sulfhydryl-reactive probes demonstrated that negatively charged reagents preferentially modify the active-site cysteine and rapidly inactivate heparinase I. Neutral sulfhydryl-reactive reagents (NEM, IAM) fail to either modify or inactivate heparinase I. However, loss in enzymatic activity at very high NEM concentrations may be due to a concentration dependent shift in the label partitioning into the active site. Additionally, the activity loss may be partially due to concentration dependent amine cross-reactivity (Jocelyn, 1987; Glazer et al., 1975; Riordan & Vallee, 1972). Heparinase I contains 41 lysine residues (Sasisekharan et al. 1993), and the modification of heparinase I amines results in extensive activity losses (Comfort et al., 1989; Leckband & Langer, 1991). Similar results also were observed with the neutral analog of IAA, IAM. In contrast, up to 50% of the total cysteine is easily accessible to negatively charged [^{3}H]IAA and to negatively charged PCMB under identical conditions. This preferential IAA reactivity persists, despite the generally greater IAM reactivity toward sulfhydryls relative to IAA. Finally, it was observed that increased salt concentrations (thus altering the electrostatic properties during PCMB labeling) reduced the rate of thiol inactivation significantly. These results strongly support the hypothesis of the presence of positive charge around the cysteine residue. Furthermore, the inability to selectively label cysteine-135 using PCMB in the presence of heparin and the results of site-directed mutagenesis experiments taken together suggest the close proximity of a heparin binding site to cysteine-135.

Active Site of Heparinase I. It has been proposed that nucleophilic amino acids play an important role in the catalytic activity of polysaccharide lyases in general (Linhardt et al., 1986). These residues abstract the C5 proton on the uronate of the disaccharide repeat unit of the acidic polysaccharides, and initiate the elimination-based depolymerization reaction. The results presented in this work lead us to hypothesize that cysteine-135, surrounded by a positively charged environment, is an important nucleophile in the elimination reaction of heparinase I.

The thiol group of cysteines has a pK_a of 8.35 in free solution (Fersht, 1985), indicating that the residue will be fully protonated at pH 7.0, the pH optimum for heparinase I (Yang et al., 1985). It is possible, however, that a positively charged environment will tend to keep the thiol group negatively charged (i.e., lowering its pK_a) so that it can act as a base for proton abstraction. Similar behavior was observed in other systems in which the cysteine reactivity was due to a highly charged or a nonpolar environment (Riordan & Vallee, 1972). More specifically, cysteine reactivity was enhanced by the proximity of basic amino acids (Torchinsky, 1981; Rabin & Watts, 1960), which accounted for the high reactivity of cysteine-135 at pH 6.5. Further, the replacement of cysteine-135 with a weaker nucleophile (serine) using site-directed mutagenesis significantly reduced heparinase I activity, consistent with a plausible proton abstraction role (Table 3).

This model of a positively charged active-site environment is expected since the substrate heparin is a highly sulfated, negatively charged polymer (Jackson et al., 1991). The electrostatic nature of the enzyme−substrate interaction is evidenced by the high sensitivity of activity to pH and ionic strength (Yang et al., 1985). Further, heparinase I has a pK_a of 9.1, contains a heparin-binding consensus motif made up of a cluster of basic residues, and exhibits a high degree of specificity for heparin (Yang et al., 1985; Sasisekharan et al., 1993; Linhardt et al., 1982). Chemical modification studies of heparin-binding proteins such as ATIII and bFGF implicate lysines in heparin binding (Chang, 1989; Liu & Chang, 1987; Peterson et al., 1987; Sun & Chang, 1989; Li et al., 1994). Thus, it is likely that heparin binds to heparinase I via charge complementarity (with basic residues), and the active-site environment perhaps plays a key role in biasing the active-site reactivity.

In conclusion, the chemical modification experiments reported here have provided information on the physicochemical environment of the active site, which is not immediately available from site-directed mutagenesis experiments alone. Further, the identification of an essential cysteine residue through sulfhydryl-specific derivatization and mutagenesis represents the first such examination of the catalytic mechanism of a heparin-degrading enzyme.

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REFERENCES

Andrews, P. C., & Dixon, J. E. (1987) *Anal. Biochem. 161*, 524−528.

Bernstein, H., Yang, V. C., Cooney, C. L., & Langer, R. (1988) *Methods Enzymol. 137*, 515−529.

Chang, J. (1989) *J. Biol. Chem. 264*, 3111−3115.

Comfort, A. R., Albert, E., & Langer, R. (1989) *Biotechnol. Bioeng. 34*, 1383−1390.

Fersht, A. (1985) *Enzyme Structure and Mechanism* (2nd ed.) Freeman and Company, NY.

Glazer, A. N., DeLange, R. J., & Sigman, D. S. (1975) Chemical Modifications of Proteins, in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., & Work, E., Eds.) Vol. 4, pp 69−199, American Elsevier, NY.

Hammond, B. R., & Gutfreund, H. (1959) *Biochem. J. 72*, 349−353.

Higuchi, R. (1990) Recombinant PCR, in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, T. J., Eds.) Academic Press, Inc., San Diego, CA.

Jackson, R. L., Busch, S. J., & Cardin, A. D. (1991) *Physiol. Rev. 71*, 481−539.

Jocelyn, P. C. (1987) *Methods Enzymol. 143*, 44−67.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Leckband, D., & Langer, R. (1991) *Biotechnol. Bioeng. 37*, 227−237.

Li, L.-Y., Safran, M., Aviezer, D., Bohlen, P., Seddon, A., & Yayon, A. (1994) *Biochemistry 33*, 10999−11007.

Linhardt, R. J., Fitzgerald, G. L., Cooney, C. L., & Langer, R. (1982) *Biochim. Biophys. Acta 702*, 197−203.

Linhardt, R. J., Galliher, P. M., & Cooney, C. L. (1986) *Appl. Biochem. Biotechnol. 12*, 135.

Linhardt, R. J., Turnbull, J. E., Wang, H. M., Longanathan, D., & Gallagher, J. T. (1990) *Biochemistry 29*, 2611−2617.

Linker, A., & Hovingh, P. (1972) *Methods Enzymol. 28*, 902−911.

Lohse, D. L., & Linhardt, R. J. (1992) *J. Biol. Chem. 267*, 24347−24355.

Lui, C., & Chang, J. (1987) *J. Biol. Chem. 262*, 17356−17361.

MacQuarrie, R. A., & Berhard, S. A. (1971) *Biochemistry 10*, 2456−2460.

Peterson, C., Noyes, C. M., Pecon, J. M., Church, F. C., & Blackburn, M. N. (1987) *J. Biol. Chem. 262*, 8061−8065.

Rabin, B. R., & Watts, D. C. (1960) *Nature 188*, 1163−1165.

Riordan, J. F., & Vallee, B. (1972) *Methods Enzymol. 25B*, 449−456.

Sasisekharan, R. (1991) Ph.D. Thesis, Harvard University, Cambridge, MA 02138.

Sasisekharan, R., Bulmer, M., Moremen, K., Cooney, C. L., & Langer, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3660−3664.

Sasisekharan, R., Moses, M. A., Nugent, M. A., Cooney, C. L., & Langer, R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1524−1528.

Sun, X., & Chang, J. (1989) *J. Biol. Chem. 264*, 11288−11293.

Teijidor, L., Oman, D., Zimmermann, J., Russo, A. Rose, M., Borzhemskya, L., & Pelzer, H. (1993) *Thromb. Hemostasis 69*, 866.

Torchinsky, Yu. M. (1981) *Sulfur in Proteins*, Pergamon Press Inc., NY.

Von Heijne, G. (1988) *Biochim. Biophys. Acta 947*, 307−333.

Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L., & Langer, R. (1985) *J. Biol. Chem. 260*, 1849−1857.

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