Heparinase I from *Flavobacterium heparinum*. Identification of a Critical Histidine Residue Essential for Catalysis As Probed by Chemical Modification and Site-Directed Mutagenesis†

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ABSTRACT: We recently identified cysteine-135 as an important amino acid for heparinase I (EC 4.2.2.7) activity. In this study, we have identified a second residue critical for enzymatic activity. We observe concentration-dependent inactivation of heparinase I in the presence of reversible histidine-modifying diethyl pyrocarbonate (DEPC); 0.3 mM DEPC results in 95% of heparinase I inactivation in less than 3 min, and as low as 10 μM DEPC results in a 85% loss of heparinase I activity in 15 min. Heparinase I activity is restored following hydroxylamine treatment. This, along with other experiments, strongly suggests that the inactivation of heparinase I by DEPC is specific for histidine residues. Chemical modification, under nondenaturing conditions, of the histidines using nonradiolabeled and [^{14}C]DEPC indicates that between one and two histidine residues are modified. Chemical modification of the surface-accessible histidines, in the presence and absence of heparin, suggests that the histidine(s) lie(s) in or near the active site of heparinase I. The wild-type heparinase I has four histidine residues; site-directed mutagenesis of H129A, H165A, and H339A did not affect enzyme activity and the kinetic parameters, suggesting that these residues are not essential for heparinase I activity. However, H203A inactivates heparinase I while a H203D mutant has residual activity, indicating a role of this residue in catalysis. We propose that histidine-203, contained in the heparin binding site, is immediately adjacent to cysteine-135, and these residues together form the catalytic domain of heparinase I.

Heparin-like molecules are acidic polysaccharides characterized by a disaccharide repeating unit of hexosamine and uronic acid (L-iduronic or D-glucuronic acid). They are highly heterogeneous due to varying modification of sulfation of the functional groups of the repeat unit (Jackson et al., 1991; Ernst et al., 1995). Heparinases are enzymes that cleave heparin-like molecules specifically (Linhardt et al., 1986; Ernst et al., 1995). Among the different heparinases, heparinase I has been extensively studied and characterized and is used for various applications (Linker & Hovingh, 1972; Linhardt et al., 1990; Sasisekharan et al., 1993, 1994; Ernst et al., 1995). The use of heparinase I *in vitro*, for clinical monitoring of heparin concentration, has been approved by the FDA (Tejidor et al., 1993), and it is in phase I clinical trials for systemic heparin neutralization *in vivo* (*Genet. Eng. News*, 1995).

Heparinase I is a lyase that degrades heparin-like molecules through an elimination reaction (Linhardt et al., 1986; Ernst et al., 1995). There is very limited structure−function information or studies on enzyme systems in literature, involving enzymatic depolymerization through elimination (Ernst et al., 1995). As heparinase I is a model enzyme system for studying elimination reactions, we have been interested in determining the catalytic residues of heparinase I in order to understand the mechanism of heparin depolymerization as well as substrate specificity. Using chemical modification and site-directed mutagenesis experiments, we identified a surface-accessible cysteine (cysteine-135), contained in a positively charged environment, to be essential for heparinase I activity (Sasisekharan et al., 1995). Further, the observation of a decrease in the rate of heparinase I inactivation by chemical modification of cysteine-135 in the presence of heparin, along with other experiments, led to the hypothesis that a heparin binding domain is in close proximity to cysteine-135 (Sasisekharan et al., 1995). Recently, we identified and mapped the primary heparin binding site of heparinase I (Sasisekharan et al., 1996). This contains two Cardin−Weintraub (Cardin & Weintraub, 1989) heparin binding consensus sequences and a calcium coordination consensus motif (Kretsinger, 1980). Sulfhydryl-selective labeling of cysteine-135 in heparinase I protects the lysines of the heparin binding sequence from proteolytic cleavage, suggesting the close proximity of the heparin binding site to the active site (Sasisekharan et al., 1996).

In parallel, we set out to investigate other catalytically essential amino acid residues in heparinase I. It was proposed that polysaccharide lyases proceed via a base-catalyzed mechanism, where one amino acid acts as a general base by abstracting a C5 proton from the uronate of the disaccharide repeat unit of the acidic polysaccharide and a different amino acid acts as a proton donor to protonate the leaving group (Gacesa, 1987). Further, it was suggested that a histidine residue is involved in the activity of a hyaluronate lyase, possibly as an acid−base catalyst (Greiling et al.,

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1975). Histidines participate in a large number of enzymatic reactions by a variety of mechanisms, which include general acid−base catalysis and electrophilic catalysis and in the binding of substrate via hydrogen bonding and/or electrostatic interactions (Munier et al., 1992). Histidine can exert its catalytic function either by interacting directly with the substrate or by being an intermediate in a two- or three-member proton shuttle system (Munier et al., 1992). Hence, in this study, we sought to investigate the role of histidine residues in heparinase I activity. Through a combination of chemical modification and site-directed mutagenesis experiments, we demonstrate that histidine-203 is essential for heparinase I activity.

MATERIALS AND METHODS

Chemicals and Materials. Heparin, from porcine intestinal mucosa with an average molecular mass of 12 kDa and activity of 157 USP units/mg, was from Hepar (Franklin, OH). Diethyl pyrocarbonate (DEPC),[1] [^{14}C]DEPC (specific activity 2.6 mCi/mmol), and hydroxylamine hydrochloride were from Sigma Chemicals (St. Louis, MO). Molecular weight standards were obtained from GIBCO BRL/Life Technologies, MD. *Escherichia coli* BL21(DE3) host was from Novagen, WI. Molecular biology reagents and their sources are listed in the appropriate sections below.

Heparinase I Purification and Protein Analyses. Heparinase I was purified as described previously (Yang et al., 1985; Sasisekharan et al., 1993). The heparinase I used for activity measurements was extensively desalted using a Centricon P-30 (Amicon, Beverly, MA). Protein concentration was determined using Micro BCA reagent (Pierce Inc., Rockford, IL) relative to a BSA standard.

Chemical Modification with DEPC. (A) Inactivation with DEPC. Heparinase I (25 μg/mL), in 100 mM MOPS buffer (pH 7.0), was incubated with DEPC, a histidine-specific reagent, at DEPC concentrations ranging from 0.01 to 0.3 mM at room temperature. DEPC solutions were freshly diluted in ethanol just before use, from a 6 M stock solution. The amount of ethanol in the reaction mixture did not exceed 2% (v/v), and the control reaction mixtures contained the same amount of ethanol (instead of DEPC). At fixed time intervals, 25-μL aliquots were used for activity assays. The time course of inactivation was obtained by determination of the enzymatic activity retained after successive incubation intervals. Pseudo-first-order rate constants, k, for inactivation by DEPC were determined by determining the slopes (using a least-squares fit) from a plot of log percent fractional activity retained versus time.

(B) Reactivation of Modified Enzyme with Hydroxylamine. Heparinase I (25 μg/mL) was incubated with 7.5 μM DEPC for 1 min, and an aliquot was withdrawn for measuring the fractional activity retained. Hydroxylamine (in 100 mM MOPS buffer, pH 7.0) was then immediately added to the reaction mixture to a final concentration of 300 mM, and the mixture was incubated at 4 °C for about 4 h. Aliquots were taken every hour and assayed for activity. A control reaction was also performed without DEPC but with the same amount of hydroxylamine to account for the effect of hydroxylamine on the activity of heparinase I.

(C) Substrate Protection of Enzyme against Modification by DEPC. To investigate the ability of heparin to protect heparinase I against modification by DEPC, heparinase I (25 μg/mL) was incubated with 2 mg/mL heparin (final concentration 0.5 mg/mL; with and without 5 mM calcium acetate) for periods of 10 min and 1 h. The concentration of DEPC was fixed at 0.05 mM in all the experiments. Twenty-five microliter aliquots of the sample were withdrawn every 3 min, for a total time of about 15 min, for enzyme activity determination.

(D) Quantification of Histidine Residues. Quantification of DEPC-modified residues was determined by difference spectra. Two cuvettes contained heparinase I (20 μg/mL) in 100 mM MOPS buffer, pH 7.0, incubated at room temperature. At time zero, DEPC was added to the sample cuvette to a final concentration of 0.01 mM, and an equal volume of ethanol was added to the reference cuvette. The change in the absorbance at 240 nm was monitored every 5 min for 30 min. Enzyme activities of the sample and control were also measured every 5 min.

(E) Labeling with [^{14}C]DEPC and Tryptic Mapping Experiments. Heparinase I was also modified using [^{14}C]-DEPC. About 40 μg (1 nmol) of heparinase I was radiolabeled with 0.15 mM [^{14}C]DEPC for about 4 min. The reaction was stopped by adding 40 mM imidazole (pH 7.0) and immediately loaded onto a RPHPLC column to get rid of the excess radiolabel. The labeled heparinase I peak was collected and concentrated using a speed vac to get rid of acetonitrile. The sample was counted for radioactive incorporation, and the number of moles of modified residues per mole of enzyme was calculated from the specific activity. The concentrated, labeled enzyme was used for a tryptic digest as described previously (Sasisekharan et al., 1995).

Mutagenesis, Expression, and Purification of Recombinant Heparinases I. The recombinant and mutant heparinases I were expressed without the putative *F. heparinum* leader sequence, i.e., as a construct (-L *r-h*eparinase I) that reads Met-Glu$_{22}$-Glu$_{23}$-... (Sasisekharan et al., 1993). To facilitate purification, the heparinase I gene was expressed using the pET-15b system (Novagen, WI). This construct has a histidine tag and a thrombin cleavage site in a 21 amino acid N-terminal leader sequence (Sasisekharan et al., 1995).

(A) Mutagenesis. The mutations were introduced via 12 cycle PCR, as described previously (Sasisekharan et al., 1995), by the method of Higuchi (1990). The primers for the H203A mutation were 5′-AATATCGCCGCTGATAAAGTT-3′ and 5′-AACTTTATCAGCGGCGATATT-3′; for the H203D mutation, they were 5′-AATATCGCCGATGATAAAGTT-3′ and 5′-AACTTTATCATCGGCGATATT-3′; for the H129A mutation, they were 5′-ACCGTTTATGCTACGGCAAA-3′ and 5′-TTTGCCGTAAGCATAAACGGT-3′; for the H165A mutation, they were 5′-GCCCAATGGGCTGGTGCACCC-3′ and 5′-GGGTGCACCAGCCCATTGGGC-3′ and for the H339A mutation 5′-CAAAAAGCGGCTATCGTAAAC-3′ and 5′-GTTTACGATAGCCGCTTTTTG-3′. The mutant genes were cloned into pET-15b and sequenced as described (Sasisekharan et al., 1995).

(B) Expression and Purification. The constructs were transformed in BL21(DE3) (Novagen, WI), and the protein was purified (Sasisekharan et al., 1995). SDS−PAGE (Laemmli, 1970) was carried out using precast 12% gels and a Mini Protean II apparatus, and stained with the Silver Stain Plus kit (Bio-Rad, CA) (Sasisekharan et al., 1995).

[1] Abbreviations: DEPC, diethyl pyrocarbonate; BSA, bovine serum albumin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RPHPLC, reverse-phase high-pressure liquid chromatography.

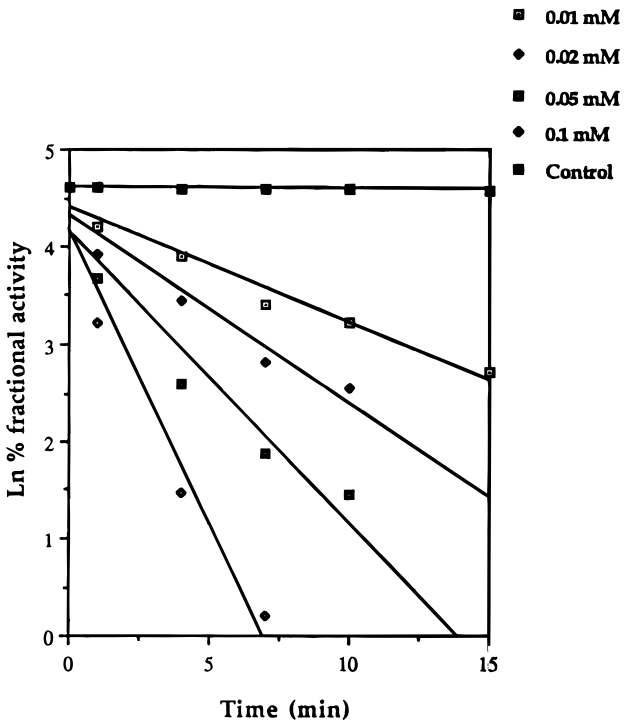

FIGURE 1: DEPC inactivation of heparinase I. Heparinase I was incubated with 0, 0.01, 0.02, 0.05, and 0.1 mM DEPC at pH 7.0 and at room temperature. The fractional activity was expressed as a percentage of the initial activity, and the natural logarithm of the fractional percent activity was plotted 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 $k = 0.119$ min^{-1} (0.01 mM DEPC), $k = 0.193$ min^{-1} (0.02 mM DEPC), $k = 0.3$ min^{-1} (0.05 mM DEPC), and $k = 0.6$ min^{-1} (0.1 mM DEPC).

Heparinase I Activity Assays. (A) UV 232 nm Assay. The UV 232 nm assay and the saccharide assay were performed as described previously (Bernstein et al., 1988; Sasisekharan et al., 1995). The enzyme activity was directly measured from the increase in absorbance at 232 nm as a function of time. All assays were performed at 30 °C, and the heparin concentration was fixed at 2 mg/mL (100 mM MOPS buffer/5 mM calcium acetate, pH 7.0). Activity is expressed as IU = μmol of product formed/min, using $\epsilon = 3800$ M^{-1}.

(B) HPLC of Heparin Oligosaccharides. Heparin (2 mg/mL) was incubated with *r*-heparinase I and mutant enzymes in 100 mM MOPS, 5 mM calcium acetate buffer, pH 7.0, for 18 h. The reaction was then subjected to anion-exchange HPLC to resolve the oligosaccharide products, as described in Sasisekharan et al. (1993).

RESULTS

Inactivation of Heparinase I with DEPC. The effect of DEPC on heparinase I activity was studied. In the presence of 0.3 mM DEPC, about 95% of heparinase I was inactivated in less than 3 min, and as low as 10 μM DEPC resulted in a 85% loss of heparinase I activity in 15 min. The inactivation rate was concentration-dependent: at 1 and 0.05 mM DEPC, inactivation was complete within 1 and 10 min, respectively. The control sample, which contained 2% v/v ethanol, showed no change in heparinase I activity. Plots of the log % residual activity versus time of incubation at different DEPC concentrations were linear, indicating that the inactivation followed pseudo-first-order kinetics (Figure 1). The pseudo-first-order rate constants, *k*, were determined from the activity after a determined time, A_t, the initial activity, A_i, and eq 1:

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

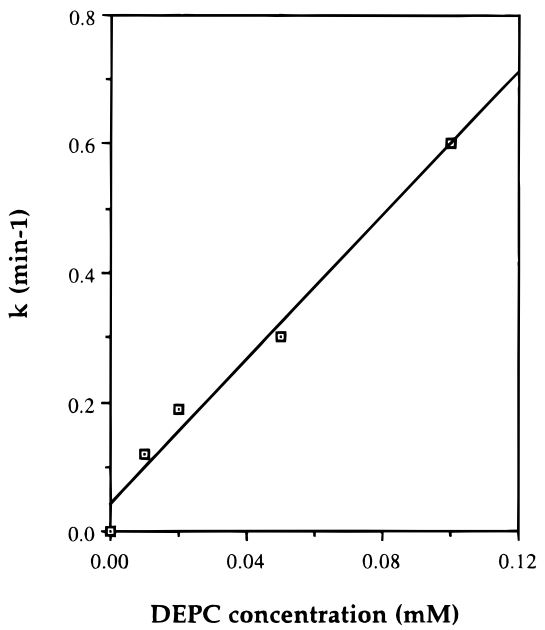

FIGURE 2: Effect of DEPC concentration on the pseudo-first-order rate constants of inactivation. The pseudo-first-order rate constants are plotted as a function of the DEPC concentration. A second-order rate constant of 5.6 min^{-1} mM^{-1} was calculated from the plot.

Table 1: Hydroxylamine Reversibility of DEPC-Based Inactivation of Heparinase I[a]

	time (min)				
	1	45	105	165	225
% initial activity	74	79	88	91	95

[a] Heparinase I inactivation was accomplished with 7.5 μM DEPC in MOPS at room temperature for 1 min. The modified enzyme was then immediately incubated with 300 mM hydroxylamine at 4 °C for about 4 h. Activity measurements were taken every hour.

A plot of the observed pseudo-first-order rate constants as a function of DEPC concentration (0.01−0.1 mM) yielded a straight line passing through the origin (Figure 2), from which a second-order rate constant of 5.6 min^{-1} mM^{-1} was obtained. A plot of log *k* versus log DEPC concentration (not shown) yielded a slope of 0.7, indicating that the rate of inactivation was first-order with respect to DEPC concentration.

Reversibility of Inactivation Using Hydroxylamine. Hydroxylamine removes the carbethoxy groups from modified histidyl and tyrosyl residues (Miles, 1977), and hence experiments were performed to determine if the loss in heparinase I activity due to modification by DEPC could be recovered by treating the enzyme with hydroxylamine. When heparinase I was incubated with 7.5 μM DEPC, the enzyme was inactivated to about 74% of its original activity in 1 min. The modified enzyme was then incubated with 300 mM hydroxylamine at 4 °C. As shown in Table 1, the enzymatic activity was restored to about 95% of its initial activity in about 4 h.

Specificity of Inactivation by DEPC. DEPC is known to react with the side chains of several amino acid residues, viz., histidine, lysine, cysteine, and tyrosine (Miles, 1977). By appropriately choosing experimental conditions, the

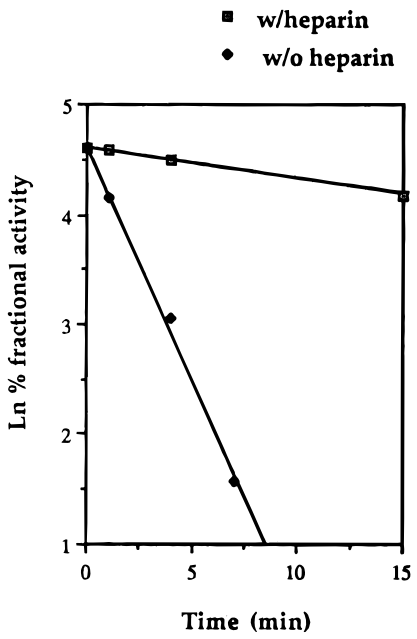

FIGURE 3: Substrate protection of DEPC inactivation of heparinase I. Heparinase I was incubated with 2 mg/mL heparin (final concentration 0.5 mg/mL) for a period of 45 min prior to the addition of 0.05 mM DEPC. A control experiment with no heparin added was also performed. Aliquots of the enzyme sample were withdrawn every 3 min, for a total time of about 15 min, for enzyme activity determination. The fractional activity was expressed as a percentage of the initial activity, and the natural logarithm of the fractional percent activity 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, to be $k = 0.43$ min^{-1} in the absence of heparin, and in the presence of 0.5 mg of heparin/mL, the rate constant was reduced by an order of magnitude to $k = 0.029$ min^{-1}.

specificity of the reagent toward a residue can be increased. For example, at pH 6.0, DEPC reacts with histidyl residues in various proteins with a high degree of specificity (Miles, 1977; Fu & Robyt, 1988). When heparinase I was incubated with 0.025 mM DEPC at pH 6.0, more than 80% of the initial enzymatic activity was lost within 15 min, suggesting specificity toward histidine residues. Further, restoration of heparinase I activity by hydroxylamine ruled out the possibility of lysine and cysteine modification and hence pointed to histidine or tyrosine modification. However, when heparinase I was incubated with DEPC, no decrease in the absorbance at 278 nm was observed (data not shown), thus precluding the possibility of tyrosine modification (Miles, 1977; Fu & Robyt, 1988). The above results taken together, and the relatively high specificity of the DEPC reaction at pH 6.0 for imidazole groups, strongly suggested that the inactivation of heparinase I by DEPC is due to the modification of the imidazole ring of histidine residues.

Substrate Protection of DEPC Inactivation. One could argue that if a histidine residue plays a role in enzymatic activity (such as in substrate binding or catalysis), the rate of DEPC inactivation should decrease in the presence of substrate. To investigate the ability of heparin (substrate) to protect heparinase I inactivation by DEPC, the enzyme was preincubated with heparin or heparin fragments (see Discussion) for about 45 min. As seen in Figure 3, in the presence of 0.5 mg of heparin/mL (~5 K_m) and a DEPC concentration of 0.05 mM, the rate of inactivation significantly decreased. Rate constants were determined by assuming pseudo-first-order kinetics and fitting data to eq 1. The rate constant in the absence of heparin was $k = 0.43$ min^{-1}, and in the presence of 0.5 mg of heparin/mL, the rate constant was reduced by an order of magnitude to $k = 0.029$ min^{-1}. Since the heparin concentration in the assay medium was much larger than the K_m of 0.1 mg/mL (Yang et al., 1985), any additional heparin introduced did not alter the kinetics. When the experiment was done by incubating heparinase I in the presence of heparin fragments (primarily disaccharides and tetrasaccharides which are heparinase I derived heparin cleavage products), the inactivation rate did not change significantly ($k = 0.017$ min^{-1}) (see Discussion). In addition, a smaller incubation time of heparinase I with heparin (10 min) was sufficient to see significant protection (data not shown).

Quantification of Histidine Residues. To determine the number of modified histidine residues, the inactivation of heparinase I by DEPC was plotted against the number of modified histidine residues (calculated from the absorbance increase at 240 nm using an extinction coefficient of 3200 M^{-1} cm^{-1}) (data not shown). The plot was nonlinear, indicating that not all the histidine residues are modified at the same rate, since modification is dependent on accessibility of the residues (Fu & Robyt, 1988). Extrapolation of the first linear phase of the plot (where the enzyme is about 40% inactivated) to zero activity indicated that between one and two histidine residues were modified per mole of heparinase I. However, this does not determine the number of histidine residues essential for enzyme activity but rather only suggests that these residues are modified at a faster rate. To further corroborate the number of histidine residue(s) modified, radiolabeling of heparinase I with [^{14}C]DEPC was performed.

Radiolabeling with [^{14}C]DEPC and Tryptic Mapping of the [^{14}C]Histidines of Heparinase I. To investigate the relationship between the extent of DEPC modification and heparinase I inactivation, the amount of isotope incorporation when heparinase I was treated with [^{14}C]DEPC was measured. Heparinase I which had been treated with 0.15 mM [^{14}C]DEPC incorporated (~0.33 ± 0.05) $\times 10^3$ cpm of [^{14}C]-DEPC/μg of heparinase I or (1.3 ± 0.05) $\times 10^4$ cpm of [^{14}C]-DEPC/nmol of heparinase I. The average ^{14}C incorporation from four different experiments was about 1.4 mol of modified residues/mol of heparinase I. Thus, the results consistently pointed toward one or possibly two histidine residue(s) being modified for every mole of nondenatured heparinase I in its native conformation. This is in good agreement with the stoichiometry calculated from the absorbance changes.

Heparinase I modified by nonradiolabeled or [^{14}C]DEPC was digested with trypsin, and the tryptic peptides were separated using RPHPLC. When the tryptic digests of the modified enzyme were compared to a control (unmodified) heparinase I tryptic digest, the maps were almost identical, with minor alterations in a few defined regions (data not shown). However, under the experimental conditions tested, the tryptic mapping experiments, leading to the identification of the histidine residue(s) essential for enzymatic activity, were inconclusive (see Discussion). Hence, in order to identify the histidine residue(s) critical for enzyme activity, site-directed mutagenesis was performed. Wild-type heparinase I has four histidine residues (Sasiekharan et al., 1993), and each of these were individually modified to alanines.

Site-Directed Mutagenesis of Heparinase I. Four mutant recombinant heparinases were constructed (H129A, H165A,

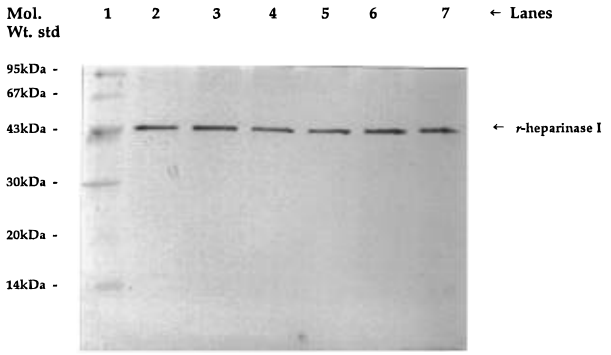

FIGURE 4: SDS−PAGE of *r*-heparinase I and mutant *r*-heparinase I. A 12% SDS−PAGE of heparinase I. Lanes are described from left to right. Lane 1: Molecular weight standards. Lane 2: Ni column purified H129A mutant *r*-heparinase I. Lane 3: Ni column purified H165A mutant *r*-heparinase I. Lane 4: Ni column purified H203A mutant *r*-heparinase I. Lane 5: Ni column purified H203D mutant *r*-heparinase I. Lane 6: Ni column purified H339A mutant *r*-heparinase I. Lane 7: Ni column purified *r*-heparinase I.

Table 2: Kinetic Constants of *r*-Heparinase I and the Mutant *r*-Heparinases I[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)
wild-type *r*-heparinase I	92	10.2
H129A *r*-heparinase I	89	9.1
H165A *r*-heparinase I	88	9.2
H203A *r*-heparinase I	ND[a]	ND
H203D *r*-heparinase I	3.5	5.7
H339A *r*-heparinase I	91	9.1

[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 (Lohse & Linhardt, 1992). [b] ND = not determined since the enzyme was inactive.

H203A, H339A) and expressed in the BL21(DE3) host. *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). The purity of *r*-heparinases as determined by SDS−PAGE is estimated to be 80−90% after the first step and greater than 98% with silver stain after the second step (Figure 4). While it was found that the mutants H129A, H165A, and H339A were expressed in *E. coli* with essentially identical activity as the control -L *r*-heparinase I, the H203A mutation resulted in an enzyme with no detectable activity. Table 2 lists the kinetic parameters for the recombinant and mutant heparinases.

In order to differentiate between a catalytic and a substrate binding role for histidine-203 (see Discussion), an additional H203D mutant was expressed and purified. Aspartic acid was chosen because of its ability to act as a nucleophile but not participate in heparin binding due to its negative charge. As shown in Figure 5, this enzyme was active but with a significant (30-fold) reduction in k_{cat} (Table 2). The above results from chemical modification studies and site-directed mutagenesis experiments taken together strongly suggest the involvement of histidine-203 in heparinase I activity.

DISCUSSION

Characterization of the Functional Role of Histidine in Heparinase I. Heparinase I modification by DEPC at pH 7.0 inactivated the enzyme. The rate of inactivation followed a pseudo-first-order mechanism, which suggests the presence of a highly reactive residue critical for enzymatic activity. Furthermore, the value of the second-order rate constant for inactivation of heparinase I by DEPC (5.6 mM^{-1} min^{-1}) was high when compared with that measured for other enzymes under similar conditions (Abdulwajid & Wu, 1986; Pelton & Ganzhorn, 1992; Battaglia et al., 1994).

DEPC can react with the side chains of several amino acid residues such as cysteines, lysines, histidines, and tyrosines (Miles, 1977). The importance of lysines in heparinase I activity was demonstrated (Leckband & Langer, 1991), and we have recently shown that a cysteine residue is involved in the catalytic site (Sasisekharan et al., 1995). When heparinase I was partially inactivated by DEPC, its activity could be restored by addition of 300 mM hydroxylamine. Hence, this ruled out the involvement of lysines and cysteines and suggested the possible modification of histidine or tyrosine residues (Miles, 1977). In addition, lysine residues can only be modified by DEPC at pH values higher than 7.0 (Fu & Robyt, 1988). When heparinase I was incubated with 25 μM DEPC at pH 6.0, more than 80% of its initial activity was lost within 15 min, thus ruling out the involvement of lysines. Furthermore, the absence of a decrease in the 278 nm absorption spectra when heparinase I was incubated with DEPC indicated that tyrosines were not modified, hence pointing to the specificity of the reaction toward histidine residues.

To quantify the number of modified histidine residues, inactivation of heparinase I by DEPC was correlated with the moles of histidines modified (calculated from absorbance changes at 240 nm and from [^{14}C]DEPC incorporation) per mole of enzyme. The data from both experiments pointed toward one or two residues being modified per mole of enzyme. DEPC has been used over a wide range of concentrations, from 0.01 to 40 mM (the maximum solubility in water) depending on the reactivity or accessibility of histidyl residues in various proteins (Miles, 1977). It is interesting to note that heparinase I displayed great sensitivity to this reagent, being rapidly and completely inactivated under relatively mild conditions (i.e., as low as 0.01 mM DEPC), suggesting that the histidine residues are not only critical but also readily surface-accessible. In addition, the rate of inactivation was significantly (10-fold) reduced in the presence of heparin, suggesting the proximity of the modified residues to the active site or the substrate binding site. Similar heparin protection against inactivation was also observed previously for chemical modification of the surface-accessible active site cysteine-135 (Sasisekharan et al., 1995). It is possible that DEPC labeling could sterically hinder heparin access to the active site; another explanation is that heparin bound to heparinase I could alter the charge characteristics and hence lower the labeling kinetics. However, a mechanistic role was established through site-directed mutagenesis experiments (see below). Thus, the above results taken together, viz., pseudo-first-order inactivation by DEPC, the reversal of DEPC inactivation by hydroxylamine, and the protection against DEPC inactivation by heparin, strongly suggested a role for histidine residues in catalysis.

It is of interest to note that of the four histidines in heparinase I (Sasisekharan et al., 1993), histidine-129 is in close proximity to the active site cysteine-135 in the primary sequence of heparinase I, and histidine-203 is contained in the primary heparin binding site (which was proposed to be

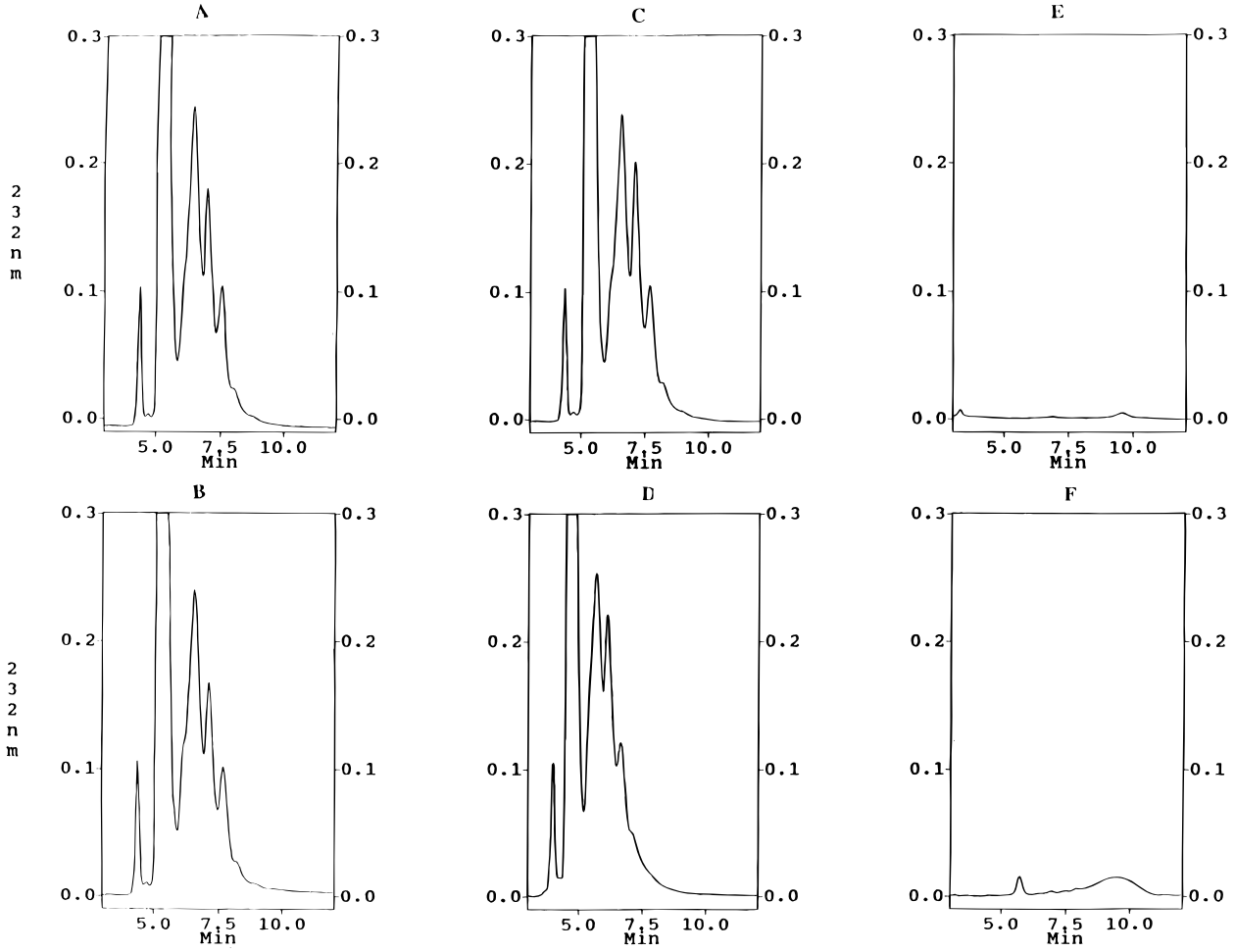

FIGURE 5: Effect of histidine mutations on activity of heparinase I. Heparin (2 mg/mL) was incubated with *r*-heparinase I and mutant *r*-heparinases I in 5 mM calcium acetate, 100 mM MOPS buffer, pH 7.0, for 18 h as described under Materials and Methods. The reaction was then subjected to anion-exchange HPLC using a POROS Q/M (4.6 mm × 100 mm) column (PerSeptive Biosystems, MA) with a salt gradient of 0−1 M NaCl in 5 min and monitored at 232 nm. Panel A shows the product profile of heparin degradation by *r*-heparinase I; panel B shows the product profile of heparin degradation by the mutant H129A; panel C shows the product profile of heparin degradation by the mutant H165A; panel D shows the product profile of heparin degradation by the mutant H339A; panel E shows the product profile of heparin degradation by the mutant H203A; and panel F shows the product profile of heparin degradation by the mutant H203D.

in proximity to the active site cysteine-135 in the folded, tertiary structure) (Sasisekharan et al., 1996) (see below). As stated above, both nonradiolabeled as well as [^{14}C]DEPC labeling experiments pointed to one or two residues being surface-accessible, and hence this raised the possibility of either or both of the above-mentioned histidine residues being surface-accessible. However, tryptic mapping experiments with nonradiolabeled DEPC and [^{14}C]DEPC-modified enzyme showed that the reversed-phase elution of tryptic peptides td9 and td50 (Sasisekharan et al., 1993), containing histidine-129 and histidine-339, respectively, was unaltered if not identical to that of the control tryptic map, suggesting that these residues were unlikely to be modified. The effects of chemical modification on the migration of tryptic peptides containing histidine-203 and histidine-165 were inconclusive. It is possible that the very small and hydrophilic tryptic peptide containing histidine-203 might have eluted in the void volume (Sasisekharan et al., 1996). Hence, we sought to map the critical histidine residue through systematic site-directed mutagenesis experiments as described below.

Site-Directed Mutagenesis of Histidines of Heparinase I. In order to determine which histidine residue is involved in enzyme activity and to ascertain a mechanistic role, site-directed mutagenesis was performed. The H129A, H165A, and H339A mutations did not affect enzyme activity and the kinetic parameters (Table 2), suggesting that these residues are not essential for enzyme activity.

However, the most significant effect on enzyme activity was observed when histidine-203 was replaced with an alanine. The H203A mutation completely inactivated heparinase I, indicating the importance of this residue in enzyme activity. As pointed out earlier, histidine-203 belongs to the heparin binding consensus sequence (Cardin & Weintraub, 1989) of the heparin binding site in heparinase I (Sasisekharan et al., 1996). To differentiate between a nucleophilic role and a role in heparin binding, this residue was replaced with an aspartic acid, which can act as a nucleophile but is not known to participate in heparin binding due to its negative charge. This resulted in an enzyme with residual activity (30-fold reduction in k_{cat}), suggesting a nucleophilic role for this residue. It should be pointed out that when heparin protection of DEPC inactivation of heparinase I was conducted in the presence of heparin or heparin fragments such as di- and tetrasaccharides, the inactivation rates were not significantly different. The K_m of heparinase I binding to heparin (0.3 μM) differs significantly from the K_m of heparinase I binding to individual tetrasaccharides (15 μM−80 μM) (Rice & Linhardt, 1989). Hence if histidine-203 were to play a role in heparin binding, one might expect the inactivation kinetics in the presence

of heparin to be different from those in the presence of primarily di- and tetrasaccharides. The observation of more or less identical inactivation rates is consistent with the hypothesis that histidine-203 plays a role in catalysis.

The Catalytic Domain of Heparinase I. As pointed out earlier, a surface-accessible cysteine (cysteine-135), contained in a basic environment, was essential for heparinase I activity, and it was hypothesized that a heparin binding domain provided the basic environment to active site cysteine-135 (Sasisekharan et al., 1995). We identified and mapped the primary heparin binding site of heparinase I (which contains two basic clusters) and showed that sulfhydryl-selective labeling of cysteine-135 in heparinase I protected the lysines of the heparin binding sequence from proteolytic cleavage, corroborating the hypothesis of the close proximity of heparin binding site to the active site cysteine-135 (Sasisekharan et al., 1996). However, the most interesting observation from the work presented in this report, in conjunction with the above-mentioned parallel study (Sasisekharan et al., 1996), is the finding not only that histidine-203 is contained in the heparin binding site but also that it is critical for catalysis. The determination of a catalytic role for histidine-203 provides the most compelling evidence for the heparin binding domain being in close proximity to the active site cysteine-135. Thus, we propose that histidine-203 and cysteine-135 are adjacent to the scissile HI linkage during catalysis and form the active site or the catalytic domain of heparinase I.

Cysteine reactivity has been shown to be enhanced by the proximity of basic amino acids (Rabin & Watts, 1960; Torchinsky, 1981). In the case of heparinase I, it was suggested that a positively charged heparin binding site would tend to keep the thiol group of the active site cysteine-135 negatively charged (by lowering its pK_a) so that it can act as a base for proton abstraction (Sasisekharan et al., 1995). Furthermore, histidine residues in particular have been thought to be involved in promoting the reactivity of cysteine residues (Amuro et al., 1985; Miran et al., 1991) and subsequently act as an acid/base catalyst for proton abstraction and donation. In cysteine proteases such as papain, the active site contains a imidazolium−thiolate ion pair, where the histidine activates the cysteine residue for catalysis (Lewis et al., 1981). In this study, we have identified yet another residue, histidine-203, which is a part of the heparin binding site and is critical for catalysis in heparinase I. It remains to be seen if histidine-203 acts as a primary nucleophile (i.e., as a base) by abstracting a proton from the substrate or activates the cysteine-135 residue for catalysis and hence acts as part of a charge relay system, as seen in several other enzymes.

In conclusion, employing chemical modification and site-directed mutagenesis approaches, we have demonstrated the importance of histidine-203 in the enzyme's catalytic action. Thus, this study has led to the identification of the second critical residue of the catalytic domain of heparinase I.

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