Heparin-binding Histidine and Lysine Residues of Rat Selenoprotein P*

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Selenoprotein P is a plasma protein that has oxidant defense properties. It binds to heparin at pH 7.0, but most of it becomes unbound as the pH is raised to 8.5. This unusual heparin binding behavior was investigated by chemical modification of the basic amino acids of the protein. Diethylpyrocarbonate (DEPC) treatment of the protein abolished its binding to heparin. DEPC and [14C]DEPC modification, coupled with amino acid sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry of peptides, identified several peptides in which histidine and lysine residues had been modified by DEPC. Two peptides from one region (residues 80–95) were identified by both methods. Moreover, the two peptides that constituted this sequence bound to heparin. Finally, when DEPC modification of the protein was carried out in the presence of heparin, these two peptides did not become modified by DEPC. Based on these results, the heparin-binding region of the protein sequence was identified as KIAHILKQVS-DHIAVY. Two other peptides (residues 178–189 and 194–234) that contain histidine-rich sequences met some but not all of the criteria of heparin-binding sites, and it is possible that they and the histidine-rich sequence between them bind to heparin under some conditions. The present results indicate that histidine is a constituent of the heparin-binding site of selenoprotein P. The presence of histidine, the pKₐ of which is 7.0, explains the release of selenoprotein P from heparin binding as pH rises above 7.0. It can be speculated that this property would lead to increased binding of selenoprotein P in tissue regions that have low pH.

Selenoprotein P is an unusual, extracellular glycoprotein that is the major form of selenium in rat plasma (1, 2). Its mRNA has 10 UGAs in the open reading frame that specify selenocysteine incorporation (3). The protein is secreted by the liver, and its mRNA is present in most other tissues, implying that it secretes it as well (4). An immunohistochemical study has shown that selenoprotein P is strongly associated with endothelial cells (5). Thus, selenoprotein P is present in extracellular fluid and bound to cells.

There is increasing evidence that selenoprotein P has a role in defense against oxidative injury. Administration of low doses of diquat to selenium-deficient rats causes lipid peroxidation, massive liver necrosis, and death within a few hours (6). These adverse events can be prevented by injection of a physiological dose of selenium 12 h before administration of the diquat. The selenium-dependent protection correlates with the appearance of selenoprotein P in plasma but not with the appearance of glutathione peroxidase in liver or in plasma (7). A subsequent study using this model showed that the initial lesion in the liver was injury of the centrilobular sinusoidal endothelial cells and loss of them within 2 h (8). Endothelial cell loss was followed within an hour by necrosis of the exposed hepatocytes. The results of these studies are compatible with protection by selenoprotein P against diquat-induced oxidative injury of endothelial cells. Two reports have appeared recently suggesting that selenoprotein P has enzymatic activities of an antioxidant nature (9, 10).

Selenoprotein P is a heparin-binding protein (11). It binds to heparin-Sepharose columns when applied at pH 7.0, but most of it is eluted when the pH is raised to 8.5. This suggests that histidine residues, which have pKₐ values of 6.5–7.0, are important in the binding. Elution of bound selenoprotein P with a pH gradient from 7.0 to 8.5 yielded several peaks, suggesting the presence of isoforms of the protein that have differing affinities for heparin (12). Two of the eluted peaks contained single proteins that shared the same N terminus. One was the full-length form of selenoprotein P, and the other was a short form that terminated at the second UGA (13). Other isoforms appear to exist, and all of them bind to heparin (12).

This report, which presents the first structural data on selenoprotein P, describes experiments carried out to locate its heparin-binding site(s). Site-directed mutagenesis is often used to assess the significance of specific amino acid residues in binding of proteins to heparin. However, expression of animal selenoproteins is generally not possible in bacteria and is even difficult to achieve in animal cells. Thus, site-directed mutagenesis could not be used to study heparin binding by selenoprotein P. The older, but classic, techniques of chemical modification, peptide mapping, and mass spectrometry were used to identify histidine and lysine residues that are responsible for the interaction of selenoprotein P with heparin.

**EXPERIMENTAL PROCEDURES**

Materials—p-Hydroxyphenylglyoxal (HPG) was from Pierce. DEPC, [14C]DEPC, TNBS, chymotrypsin, trypsin, and heparin-agarose were...
Selenoprotein P Binding to Heparin

Selenoprotein P was purified from rat plasma by a simplification of a previously described method (1). A 10-ml immunoaffinity column, made with the monoclonal antibody F8F11 (1), was equilibrated with phosphate-buffered saline containing 10 mM Tris-Cl, pH 7.0, was washed with 10 ml of buffer A. Selenoprotein P was eluted with a linear gradient from 100 mM NaCl to 1 M NaCl in buffer B.

Protection of Heparin-binding Site of Selenoprotein P from DEPC Modification—Plasma (250 µg containing selenoprotein P radiolabeled in vivo) was brought to a volume of 0.5 ml with buffer A. 4.9 M sodium phosphate buffer, pH 7.0, was used. Then dialyzed overnight at 4 °C. After dialysis, each sample was added to give 1 M NaCl to elute the remaining selenoprotein P.

Proteolytic Digestion of [14C]DEPC-modified Selenoprotein P and HPLC Separation of Fragments—A 200-µg sample of selenoprotein P was dissolved in 1 ml of 0.1 M sodium phosphate buffer, pH 7.0. [14C]DEPC was added to a final concentration of 6.9 mM (specific activity 1.6 M Ci/mmol). The sample was incubated for 5 min at room temperature and then dialyzed against 4 liters of 10 mM NaHCO3 buffer for 4 h at 4 °C. This was followed by digestion with 10 µg of chymotrypsin at 5 °C for 5 h. The sample was then subjected to HPLC.

Chemical Modification of Selenoprotein P in 10 mM Tris-Cl, pH 7.0, was mixed with 2 µg of chymotrypsin, 2 µg of trypsin, or neither and incubated for 3 h at 37 °C. After incubation, the sample was loaded onto a 1-ml heparin HiTrap column equilibrated with 10 mM Tris-Cl, pH 7.0 (buffer C). The column was then washed with 10 ml of buffer C, followed by a 1-ml heparin HiTrap column equilibrated with 10 mM Tris-Cl, pH 7.0. Then 10-ml volumes of the digested mixture were analyzed using mass spectrometry.

Selenoprotein P (50 µg) was added to give a final concentration of 0.2 mM sodium phosphate buffer, pH 7.0. Modification of selenoprotein P with DEPC was done in 0.2 mM sodium phosphate buffer, pH 7.0. Modification of selenoprotein P with HPG was done in 0.2 mM sodium carbonate, pH 9.0.

Chemical Modification of Selenoprotein P Followed by Heparin-Agarose Chromatography—Four separate columns of heparin-agarose (0.8 ml) were equilibrated with 50 mM sodium carbonate, pH 7.0. Purified selenoprotein P (50 µg) was treated for 2 h with either TNBS, DEPC, or HPG, or was not treated. Modification of selenoprotein P with DEPC was done in 0.2 mM sodium phosphate buffer, pH 7.0. Modification of selenoprotein P with TNBS was done in 0.2 mM sodium carbonate, pH 8.5. Modification of selenoprotein P with HPG was done in 0.2 mM sodium bicarbonate, pH 9.0. After modification, the samples were dialyzed against 4 liters of 50 mM sodium bicarbonate, pH 7.0, for 4 h at 4 °C. After dialysis, each sample was applied to a heparin-agarose column. The columns were each washed with 11 ml of 50 mM sodium bicarbonate, pH 7.0. 75Se in the wash was determined by radioactive measurement of 1-ml aliquots of wash in an LKB Compugamma 1282 γ-counter.

Monitoring the Extent of Chemical Modification with UV-visible Spectroscopy—Carboxethylation of histidine residues by DEPC causes an increase in absorbance at 240 nm (14). Selenoprotein P radiolabeled in vivo was brought to a volume of 0.5 ml with buffer A. This sample was applied to a 1-ml heparin HiTrap column and washed with 10 ml of buffer A, pH 6.9, and successively with 10-ml volumes of the same buffer containing 0.0069, 0.069, and 6.9 M NaCl. Then 10-ml volumes of buffer A, pH 6.9, containing 1 M NaCl was passed over the column to elute the remaining selenoprotein P. This experiment is shown in Fig. 7A.

Selenoprotein P in the Plasma and Reversal of Modification by Hydroxylamine—For heparin HiTrap chromatography, 0.1-ml aliquots of plasma labeled with 3HSe diluted to 1 ml with buffer A (100 mM ammonium acetate, 50 mM Tris base, and 1 mM Na3EDTA, pH 7.0). The sample was then mixed with 6.9 mM DEPC for 5 min in the starting buffer. A second sample was modified with 6.9 mM DEPC for 5 min in the starting buffer and then treated with 1/10 volume of 2 M hydroxylamine (adjusted to pH 7.0) and incubated for 30 min at room temperature. Carboxethylation of histidine residues can be reversed by treatment with hydroxylamine (16). This reversal has previously been used to distinguish modification of histidine residues by DEPC from modification of thiol and other amino groups in proteins (17). A third sample, used as the control, was not treated with DEPC. The samples were then injected onto 1-ml heparin HiTrap columns. The flow rate was 0.25 ml/min, and the gradient was set at 2.2% buffer B.

Peptide Sequencing and MALDI-TOF Mass Spectrometry—Identification of DEPC-modified Peptides—Peptides isolated from proteolytic digests by HPLC as described above were sequenced using an Applied Biosystems 492 Procise protein sequencer.

MALDI-TOF mass spectrometry analysis was done with a Vestec Voyager Elite Time-of-Flight Mass Spectrometer using an acceleration potential of 20 kV. The instrument was calibrated with insulin as an external calibration standard.

2 µg (~40 pmol) of full-length selenoprotein P, prepared as peak 2 on a heparin HiTrap column (12), was dissolved in 20 µl of 100 mM ammonium bicarbonate. Modified trypsin (0.04 µg) was added to give an enzyme/protein ratio of 1:50, and then the solution was incubated at 37 °C for 18 h. 1 µl of the digested mixture was analyzed using mass spectrometry.

2 µg (~40 pmol) of full-length selenoprotein P was dissolved as above, and DEPC was added to it at a concentration of 6.9 mM. The reaction was incubated at room temperature for 5 min and then dialyzed against water for 6 h. It was then dried using a SpeedVac and resuspended in 20 µl of 100 mM ammonium bicarbonate. Digestion and mass spectrometric analysis was performed as described for the unmodified selenoprotein P.

Tryptophan Fluorescence of Native and DEPC-modified Protein—In order to measure the structural integrity of the DEPC-modified protein, tryptophan emission spectra were taken of the modified and native proteins. Excitation was done at 295 nm, and the tryptophan emission was recorded between 300 and 420 nm. Spectra were recorded in 0.2 nm steps.
Selenoprotein P was modified with DEPC, TNBS, and HPG as described under “Experimental Procedures.” The modified protein was then passed over a heparin-agarose column, and the column was washed with pH 7.0 buffer as described under “Experimental Procedures.”

TABLE I

| Selenoprotein P treatment | % 25Se bound to column |
|--------------------------|------------------------|
| None                     | 97                     |
| DEPC                     | 3                      |
| TNBS                     | 31                     |
| HPG                      | 86                     |

Table I shows the binding of chemically modified selenoprotein P to a heparin-agarose column. Selenoprotein P was modified with DEPC, TNBS, and HPG as described under “Experimental Procedures.” The modified protein was then passed over a heparin-agarose column, and the column was washed with pH 7.0 buffer as described under “Experimental Procedures.”

RESULTS

The Effect of Chemical Modification of Basic Amino Acids on the Affinity of Selenoprotein P for Heparin—Heparin binding by proteins is mediated by basic amino acids arranged to form a heparin-binding site. In order to determine which basic amino acids are present in the heparin-binding site(s) of selenoprotein P, the 25Se-labeled protein was treated with reagents that selectively modify basic residues. Then the protein was passed over a heparin-agarose column equilibrated with buffer of pH 7.0. It was reasoned that modification of residues in the binding site would interfere with heparin binding of the protein. The amount of selenoprotein P bound to the column was determined by subtracting the amount of 25Se that passed through the column from the amount of 25Se applied to the column.

Selenoprotein P was modified with DEPC, TNBS, and HPG in separate experiments. DEPC binds to unprotonated histidine residues, but it can also modify tyrosine, lysine, and cysteine residues (18). TNBS modifies primary amino groups in proteins, especially lysine residues. HPG is highly specific for modifying arginine residues (19).

The results in Table I show that DEPC treatment of selenoprotein P abolished its heparin-binding property at pH 7.0. TNBS treatment blocked most heparin binding. However, modification of selenoprotein P by HPG had only a small effect on the affinity of the protein for heparin. These results implicate histidine and lysine residues in the binding of selenoprotein P to heparin.

Monitoring Extent of Modification of Histidine Residues with DEPC Using UV Spectroscopy—The modification of histidine and tyrosine residues by DEPC can be followed spectrophotometrically. When histidine becomes carbethoxylated, there is a corresponding increase in absorbance at 240 nm (14). Modification of tyrosine side chains produces a decrease in absorbance at 278 nm (20). In order to assess histidine and tyrosine modification, purified selenoprotein P was treated with DEPC, and UV spectra were recorded. A progressive increase in absorbance at 240 nm occurred with time, but no decrease took place at 278 nm (spectra not shown). Therefore, modification of histidine occurs, but no evidence was found for modification of tyrosine side chains.

When selenoprotein P was modified with DEPC, histidine binding reached saturation in about 2 min at pH 7.0 (Fig. 1). When selenoprotein P was modified in the presence of heparin, the increase in absorbance at 240 nm was not as great as seen with the unliganded protein (Fig. 1). This indicates that the presence of heparin prevents DEPC from modifying some of the histidine residues, implicating histidine in the heparin binding.

DEPC reacts with unprotonated histidine residues. In order to approximate the pK of the modifiable histidine residues, the modification of selenoprotein P by DEPC was carried out at varying pH values. The modification of selenoprotein P in sodium phosphate buffer with pH values ranging from 5.5 to 8.0 is shown in Fig. 2. A marked increase in reaction rate occurred when the pH reached 6.8. The data from Fig. 2 indicate that the pK values of the modified histidine residues are in the range of 6.4–6.8.

Identification of Selenoprotein P Peptides and Residues Labeled with [14C]DEPC—Peptides that become modified by DEPC treatment of the native protein are predicted to be solvent-exposed and thus might be involved in binding of selenoprotein P to heparin. To determine which peptides of the native protein can be labeled by DEPC, [14C]DEPC was employed. After labeling, the purified selenoprotein P sample was dialyzed to remove excess [14C]DEPC. Then it was digested with chymotrypsin. Peptides were separated by reverse phase HPLC (chromatogram not shown). Peaks that contained 14C were submitted for amino acid sequence analysis. The peptides identified are shown in Table II. Two of them are KNHILSKQKQVISDIHAVY, which correspond to residues 80–84 and 85–95, respectively. Although it has been reported that loss of label is usually observed during the Edman degradation procedure (21), Lemaire and colleagues (22) observed an additional peak that did not correspond to any standard PTH-derivative when sequencing a peptide containing a single histidine residue that had been labeled with [14C]DEPC. Such a peak appeared along with a PTH-derivatized histidine residue during its turn in the Edman cycle. Similarly, we were able to identify a secondary peak with a retention time slightly less than PTH-proline that was always associated with DEPC-labeled histidine. A potential interpretation of this result is that some of the carbethoxylated histidine residues survived the sequence analysis and appeared as a new peak in the sequencing chromatogram. These sequencing results (not shown) indicate that His-81, His-83, and His-81 become carbethoxylated upon treatment of the protein with DEPC (Table II).

We also found evidence for lysine modification by DEPC. Again, the sequence analysis provided the evidence. The PTH-derivatized lysine residues in the peptides in Table II did not elute in the position for PTH-lysine but were shifted so that the retention of the modified lysine was slightly greater than that of PTH-valine. A previous study that used DEPC to modify chick liver glutathione S-transferase demonstrated that DEPC modified lysine residues and that the N-carbethoxylated PTH-lysine eluted in a similar position to that reported here (23). Thus, Lys-80, Lys-85, Lys-86, Lys-112, and Lys-261 became...
modified with DEPC based on the sequencing chromatograms (not shown).

Identification of DEPC-labeled Peptides and Residues by MALDI-TOF Mass Spectrometry—A second approach that used MALDI-TOF mass spectrometry for detecting DEPC-labeled peptides was employed. It has been reported that peptides containing carbethoxylated histidine residues can be detected by using MALDI-TOF mass spectrometry (24–27). Fig. 3A shows the mass spectrum of a total tryptic digest of the native full-length isoform of selenoprotein P. 12 peaks with the predicted masses of tryptic peptides could be identified in the spectrum. After treatment with DEPC and subsequent digestion, shifts of 72 mass units, corresponding to the addition of a selenoethyl group, were detected in eight peptides (Fig. 3B). These results demonstrate that at least five stretches of the selenoprotein P sequence are accessible to modification by DEPC in the native protein. These stretches are residues 81–120, 178–193, 233–238, 256–262, and 292–297. These results do not indicate that no other parts of the molecule interact with heparin. The largest peptide detected (residues 190–232 in Fig. 3A, HGGHEHLSKPSNQGPGALVETSLLPSQ-LHVVVVHHHK) represents the sequence between the second and third stretches identified as potential heparin-binding sites. Because of its richness in histidines, this peptide might be expected to bind to heparin. After DEPC modification of the protein, this peptide could not be detected in the trypsin digest (no peak in Fig. 3B). Peptides on either side of it were detected, so it must have been present in the digest. This result implies that the peptide had been modified by DEPC. It seems likely that the modification impaired the ability of the peptide to be ionized and therefore detected. Thus, this stretch of the sequence would appear to be a candidate for a heparin-binding site along with the adjacent histidine-rich sequences.

Identification of Proteolytic Fragments of Selenoprotein P That Bind to Heparin—In order to determine directly the regions of selenoprotein P that can interact with heparin, the protein was digested with chymotrypsin or trypsin, and the resulting fragments were loaded onto a heparin HiTrap column, which was washed with 10 mM Tris-Cl, pH 7.0. This approach has been used to identify the heparin-binding sites of several proteins (28, 29). Fragments that bound to the column were eluted with a NaCl gradient and detected by their absorbance at 214 nm. The results of the heparin HiTrap chromatography of the chymotrypsin and trypsin digests are shown in Fig. 5, A and B, respectively. In the chymotrypsin digest, two prominent peaks eluted from the heparin column at salt concentrations of 220 (peak I) and 270 mM NaCl (peak II), respectively. These peaks were collected and subjected to electrospray ionization mass spectrometry that was coupled to a reverse phase C18 column (liquid chromatography-mass spectrometry). The peptides were identified by the mass of the chymotryptic fragments. The results are summarized in Table IV. The results of the peptide mass mapping show that the peptides KHAHL (elutes in peaks I and II) and KKQVSDHIAYV (elutes in peak II) bound to heparin.

The protein was also digested with trypsin, and the resulting chromatogram is shown in Fig. 5B. There are two very prominent peaks in this chromatogram, labeled III and IV. The peptide in peak III was determined to be TTEPSEEEHHHK. This peptide had been shown to be labeled with DEPC in the previous MALDI-TOF experiment (Fig. 3). Peak IV contained two peptides having the sequences HAHLLK and HAHILKKK. These two peptides overlap with the chymotryptic fragments in Fig. 5A. This suggests that His-81, His-83, Lys-85, and Lys-86 are capable of being involved in binding selenoprotein P to heparin. Lys-80 might also be important for the binding interaction, because trypsin removes this residue from the peptide KHAHL (Fig. 5A, peak I). The elution of the undigested protein is shown in Fig. 5C as a comparison. Several other peptides were detected as binding to heparin (Table IV), but the only one of them that had been shown to be labeled by DEPC was HKGQHR. The histidine and lysine residues in the unlabeled peptides are probably not on the surface of the protein, and those peptides are thus not likely to be significant to the heparin-binding properties of the protein.

Heparin Protects Peptides KHAHL and KKQVSDHIAYV from Modification with [14C]DEPC—The results from peptide mapping with DEPC indicated that several peptides containing lysine and histidine residues were modified with DEPC (Table II and Fig. 4). Among these were the peptides KHAHL and KKQVSDHIAYV. Table IV summarizes the assessment of the heparin-binding properties of these peptides and indicates that these same two peptides, KHAHL and KKQVSDHIAYV, had the strongest affinity of the peptides for heparin.

Selenoprotein P was labeled with [14C]DEPC in the presence and in the absence of heparin. The protein was labeled as described under “Experimental Procedures” and digested with chymotrypsin. Peptides were separated using HPLC. The [14C] content of each chromatographic fraction was determined by liquid scintillation. The resulting chromatogram is shown as Fig. 6. Radioactive peptides were submitted for sequencing. The peaks that correspond to the peptides KHAHL and KKQVSDHIAYV did not appear in the presence of 5 mg/ml heparin as is shown in Fig. 6. This result demonstrates that heparin protects these two peptides from modification and further implicates them in the binding of selenoprotein P to heparin.
Protection of Heparin-binding Site of Selenoprotein P from DEPC Modification by Binding to Heparin HiTrap Column—Radiolabeled selenoprotein P was bound to a heparin HiTrap column at pH 6.9, and then DEPC at concentrations from 0.0069 to 6.9 mM was applied to the column (Fig. 7A). When the DEPC was washed off the column, no selenoprotein P eluted with it. Selenoprotein P eluted when 1M NaCl was added to the buffer. The eluted radiolabeled selenoprotein P was buffer-exchanged and applied to a second heparin HiTrap column at pH 6.9. It bound to the column and behaved like native selenoprotein P, being partially eluted when buffer of pH 8.5 was applied and completely eluted by 1M NaCl (Fig. 7B). This demonstrates that binding to heparin protects the heparin-binding site(s) of selenoprotein P from DEPC modification.

Reversal of Modification by Hydroxylamine—The results of the above experiments demonstrated that the peptides KHAHL and KKQVSDHIAVY can bind to heparin and that the lysine and histidine residues of these peptides become modified with DEPC. The reversibility of DEPC modification was tested by treating the modified protein with hydroxylamine. DEPC bound to histidine in a 1:1 ratio can be removed from it by treatment with hydroxylamine. Therefore, restoration of the activity of an enzyme by hydroxylamine after its inactivation by DEPC has been used as evidence that the enzyme activity is dependent on histidine (16, 18). For this experiment, selenoprotein P was modified with DEPC, and the modified protein was treated with hydroxylamine. The unmodified peptides were identified by their sequence numbers in Table III.

Table III

| Peak | Residues | Peptide sequence | Unmodified mass | Modified mass | No. of residues modified |
|------|----------|------------------|----------------|--------------|-------------------------|
|      |          |                  | m/z<sub>obs</sub> | m/z<sub>calc</sub> | m/z<sub>obs</sub> | m/z<sub>calc</sub> |                     |
| a    | 81–86    | HAHLLKK          | 733.62         | 733.45       | 805.67                 | 805.47             | 1                     |
| b    | 233–238  | HKQVHHR          | 732.76         | 732.41       | 834.87                 | 834.43             | 2                     |
| c    | 292–297  | HLIFEK           | 786.92         | 786.45       | 858.68                 | 858.47             | 1                     |
| d    | 87–96    | QVSHVHYVR        | 1188.01        | 1187.62      | 1260.20                | 1259.64            | 1                     |
| e    | 86–96    | KQVSHVHYVR       | 1316.02        | 1315.71      | 1387.94                | 1387.73            | 2                     |
| f    | 178–189  | TTEPSEEHHHK      | 1445.95        | 1445.64      | 1518.10                | 1517.66            | 1                     |
| g    | 178–193  | TTEPSEEHHHHKHDK  | 1963.25        | 1962.88      | 2035.22                | 2034.90            | 2                     |
| h    | 97–120   | QDEHQTDDWLLNGKDDFLYDR | 2937.68 | 2937.15 | 3009.86 | 3009.15 | 2 |
tein P was modified directly in the plasma with 6.9 mM DEPC, and the plasma sample was injected onto a heparin HiTrap column. Untreated plasma was used as the control (Fig. 8A).

As is shown in Fig. 8B, after treatment with DEPC, the selenoprotein P in the plasma elutes in the flow-through volume. This occurs after a 5-min treatment with DEPC. Thus, the protein can be modified rapidly, abolishing its ability to bind to heparin. The heparin binding capability of selenoprotein P can be restored after DEPC modification by treating the sample with 200 mM hydroxylamine at pH 7.0 for 30 min (Fig. 8C). This reversibility of DEPC modification by hydroxylamine demonstrates several points. First, at this ratio of protein/DEPC, modification of histidine residues with two DEPC molecules apparently does not occur, because a doubly modified histidine residue is irreversibly modified (18). Second, the reversibility and restoration of heparin binding implies that the protein structure is not altered upon modification. Although reversibility of DEPC modification has been used as a test to distinguish between the importance to function of modified lysine and histidine residues (17), it has been shown that

FIG. 5. A, heparin HiTrap chromatography of chymotrypsin digest. Selenoprotein P peptides resulting from digestion with chymotrypsin were loaded onto the column and washed with 10 mM Tris-Cl, pH 7.0. Bound peptides were eluted with a NaCl gradient and detected at 214 nm. Two prominent peaks, labeled as I and II, were visible in the chromatogram. The peaks were collected and submitted for mass mapping analysis (Table IV). B, heparin chromatography of trypsin fragments. Peaks III and IV are prominent. The peptides in IV, HAHLLK and HAHLLHHK, overlap with the chymotryptic digest in A. C, elution of the undigested protein (peak VI) as a comparison. This shows that the intact protein elutes at a higher salt concentration than the peptide fragments.

**TABLE IV**  
Mass mapping analysis of peptides bound to heparin  
The peptides shown in this table eluted from the heparin HiTrap column as shown in Fig. 5, A and B, respectively. The peaks are labeled with roman numerals. Residues in boldface type have been identified as being labeled with DEPC in a previous experiment.

| Peptide sequence       | NaCl concentration needed for elution | Peak from heparin column in Fig. 5 | m/z <sup>a</sup> | m/z <sup>b</sup> |
|------------------------|---------------------------------------|-----------------------------------|------------------|------------------|
| RIKLENQGYF             | 220                                   | I                                 | 1269.2           | 1268.5           |
| KHAHL                  | 220                                   | I and II                         | 605.4            | 605.7            |
| KKQVSDHIAYV            | 270                                   | II                                | 1289.2           | 1288.5           |
| TTEPEEHNNHK            | 130                                   | III                               | 1447.2           | 1446.5           |
| HAHLL                  | 150                                   | IV                                | 605.4            | 605.7            |
| HAHLLK                 | 150                                   | IV                                | 733.8            | 733.9            |
| HKGQHR                 | 178                                   | V                                 | 763.4            | 762.9            |
| Undigested Se-P        | 300                                   | VI                                | ND <sup>c</sup>  | ND <sup>c</sup>  |

<sup>a</sup> The observed mass to charge ratio.  
<sup>b</sup> The calculated mass to charge ratio.  
<sup>c</sup> ND, not determined.

FIG. 6. Heparin protection of peptides KHAHL and KKQVSDHIAYV from modification with [14C]DEPC. Purified protein was modified in the presence and absence of 5 mg/ml heparin in 0.1 M sodium phosphate buffer, pH 7.0. The digests (heparin present (solid line) and heparin absent (broken line)) were subjected to HPLC. Peaks in the chromatogram that were protected from modification by heparin correspond to KHAHL and KKQVSDHIAYV.
hydroxylamine treatment can also reverse modification of lysine residues (30). This should be especially true in the case of highly reactive lysine residues. This last point will be addressed under “Discussion.”

**Structural Integrity of Native and Modified Selenoprotein P as Judged by Fluorescence Spectroscopy**—Since modification of selenoprotein P with DEPC caused loss of its heparin binding, a method was needed to assess the structural integrity of selenoprotein P upon chemical modification. It seemed possible that modification of selenoprotein P with DEPC could cause the protein to unfold, thereby abolishing binding to heparin. Selenoprotein P contains two tryptophan residues in the N terminus of the protein, near many of the potentially modifiable histidine residues. A wavelength of 295 nm was chosen for excitation in order to eliminate emission from tyrosine residues. The emission spectra of native and DEPC-modified selenoprotein P were essentially identical (not shown). Both native and modified selenoprotein P had a $\lambda_{max}$ near 345 nm. If selenoprotein P were to be unfolded upon modification with DEPC, the tryptophan emission would be expected to be shifted to longer wavelengths. That was not observed. At the very least, it can be concluded that the local environment of the tryptophan residues did not change upon modification of selenoprotein P with DEPC. Further evidence that modification of selenoprotein P with DEPC did not cause structural damage to the protein is provided by reversibility of DEPC modification by hydroxylamine.

**DISCUSSION**

The major conclusion of this study is that a motif located in residues 80–95 of selenoprotein P mediates binding of the protein to heparin. Several lines of evidence have been presented to support this conclusion. First, when selenoprotein P was modified with DEPC, the protein lost its ability to bind to heparin. DEPC forms adducts with unprotonated histidine and lysine residues, and these are the basic amino acids in the putative binding sequence. Such adduct formation would be expected to block heparin binding by preventing protonation of histidine and lysine side chains. Moreover, histidine and lysine residues of the putative binding sequence were shown to become modified when selenoprotein P was treated with DEPC. This was shown by peptide sequence analysis using Edman chemistry (Table II) and by labeled peptide identification using MALDI-TOF mass spectrometry (Fig. 3).

Second, the peptides that make up the putative binding sequence bound to heparin (Fig. 5). Third, the presence of heparin protected the histidine and lysine residues in the putative binding sequence from modification by DEPC treatment of selenoprotein P (Fig. 6). In a complementary experiment, DEPC modification of selenoprotein P bound to a heparin HiTrap column did not abolish its subsequent ability to bind to another heparin HiTrap column (Fig. 7).

The conclusion that can be drawn from these results is that the peptide KHAHLKKQVSDHIAY is responsible for at least part of the selenoprotein P-heparin interaction at pH 7.0. Thus, lysines 80, 85, and 86 and histidines 81, 83, and 91 appear to mediate interaction between heparin and selenoprotein P.

Several consensus sequences have been determined for proteins that bind to heparin (31). One such sequence is XBXBX, where X is a hydrophobic residue and B is a basic residue. The peptide LKHAHL, which corresponds to residues 79–84 of selenoprotein P, matches this consensus sequence and has been experimentally determined to bind to heparin (see Fig. 5 and Table IV). Although chymotrypsin cleaves the peptide bond between Leu-79 and Lys-80, the resulting peptide, KHAHL, still binds to heparin.

The heparin-binding experiment shown in Fig. 5 raises the possibility of a second heparin-binding site on selenoprotein P. Another group has reported a study of the interaction of human selenoprotein P with heparin using surface plasmon resonance (32). They detected high affinity binding and low affinity binding. Interestingly, they stated that their results indicated that heparin had two binding sites for selenoprotein P. However, we feel their results are also consistent with the presence of two heparin-binding sites on the protein.

When the protein was digested with trypsin, one of the peptides that bound to heparin had the sequence TTEPSEE-HNHHK. This same peptide was modified by DEPC in the MALDI-TOF experiment (Fig. 3). Therefore, this peptide could be modified by DEPC, and it was able to bind to heparin. It is possible that this peptide is involved in the selenoprotein P-heparin interaction, although its affinity for heparin is much weaker (elutes at 130 mM NaCl) than those of peptides KHAHL and KKQVSDHIAY (Fig. 5).

Selenoprotein P has two histidine-rich regions (Fig. 4), and the peptide TTEPSEEHNHHK is in the first histidine-rich region. The second histidine-rich region contains a peptide with seven histidines in a row. When digested with trypsin, this peptide has the sequence HGHEHLGSSKPEQGPD-VEKVLPSGLHHHHHHH. This peptide appears as a peak with a mass of 4295.58 in the upper panel of Fig. 3. We were unable to detect this peptide as binding to heparin or being modified with DEPC. However, its disappearance from the mass spectrum after DEPC treatment of the protein suggests...
that it was modified. Further experiments are needed to clarify the role(s) of this region of selenoprotein P.

Two isoforms of selenoprotein P have been characterized, and two more have been postulated (12, 13). All isoforms share the same N-terminal sequence through amino acid residue 244, which is the residue just upstream from the second selenocysteine residue. The heparin-binding site should therefore be present in all isoforms. Indeed, all isoforms bind to heparin (12).

Chemical modification has been used to study the interactions between heparin and many proteins (33–36). It was used in this case because selenoprotein P cannot be expressed in bacterial systems, and therefore site-directed mutagenesis could not be employed. The majority of proteins that bind to heparin do so through the basic amino acid residues lysine and arginine (31). Chemical modification of arginine and/or lysine residues severely inhibits or completely abolishes the heparin binding property of those proteins. As far as we are aware, there is only one other example of a heparin-binding protein that is suggested to use histidine as a basic residue in the heparin-protein interaction (37). Interestingly, both selenoprotein P and that protein, histidine-rich glycoprotein, are found in the plasma and elute from heparin in a pH-dependent fashion.

A study that employed a peptide library of random 7-mers demonstrated that peptides that bound to heparin were rich in lysine and arginine residues. However, their histidine content was not distinguishable from their content of the majority of the nonbasic amino acids (38). This reinforces the perception that histidine is rarely involved in heparin binding.

Rat selenoprotein P cDNA codes for 28 histidine residues (out of 366 residues), and 16 of them are concentrated in two stretches of sequence. The first stretch is 14 residues in length (amino acid residues 185–198) with eight histidines and two lysines; the second stretch is 10 residues in length (amino acid residues 225–234) with eight histidines and two lysines. The other 12 histidine residues are not clustered in such a manner.

The lysine and histidine residues in the major heparin-binding peptides (residues 80–96) could be unambiguously identified as being labeled with DEPC. The sequencing results indicated that in the case of histidine there was an additional peak in the sequencing chromatogram that was present when histidine was being sequenced in the Edman cycle. This agrees with a previous report that identified N-carbethoxylated histidine residues by Edman degradation (22). We could also identify N-carbethoxylated lysine residues because of the shift of the N-carboxethoxylated PTH-lysine derivative in the sequencing chromatogram. This also agrees with a previous report that identifies N-carbethoxylated lysine residues (23). Therefore, several lysine residues of selenoprotein P are also important for the heparin-protein interaction.

It is interesting to note the different reactivities of the lysine residues of selenoprotein P toward DEPC and TNBS. As observed by kinetics of modification (not shown), modification of lysine residues by TNBS is slow. In contrast, when the protein is modified with DEPC for 5 min, its heparin-binding ability is abolished. The results in Tables II and III indicate that lysine residues at positions 80, 85, and 86 become modified with DEPC. In order for lysine to be modified with either DEPC or TNBS, lysine must be in its neutral, unprotonated form. The fact that DEPC is able to modify lysine residues at pH 7.0 indicates that these lysine residues are highly reactive and most likely have a lower $pK\alpha$ than is usual for lysine residues (normally near 10). The slow reaction rate of the lysine residues of selenoprotein P with TNBS is probably due to the presence of the negatively charged sulfate group of TNBS, which could mimic the sulfate groups of heparin. As the $\epsilon$-amino group of lysine would approach the sulfate group of TNBS for nucleophilic attack, the $pK\alpha$ of the $\epsilon$-amino group would increase due to the presence of the negatively charged sulfate group, making this reaction slow. The $pK\alpha$ values of these lysine residues are probably higher in the selenoprotein P-heparin complex than in the protein alone. This probably contributes to the binding of the protein with heparin.

Similarly, the data in Fig. 2 demonstrate that the $pK\alpha$ values of the modifiable histidine residues are in the range of 6.4–6.8. Therefore, at pH 7.0, some of the histidine residues would be in the unprotonated form, which would not be optimal for their binding to the negatively charged sulfate groups of heparin. These histidine residues must have altered $pK\alpha$ values in the selenoprotein P-heparin complex to be responsible for binding. Alternatively, histidine residues with higher $pK\alpha$ values are responsible for heparin binding.

The reactivity of lysine residues in selenoprotein P is further demonstrated by the restoration of heparin binding by hydroxylamine treatment. Lys-80, Lys-85, and Lys-86 are clearly important for the selenoprotein P-heparin interaction, as demonstrated by the heparin-binding experiment in Fig. 5. After modification with DEPC and treatment with 200 mM hydroxylamine, the selenoprotein P-heparin interaction is restored (Fig. 8). This indicates that hydroxylamine reverses the histidine modification by DEPC and strongly suggests that it reverses the lysine modification as well. DEPC modification of highly reactive lysine residues in proteins can be reversed by hydroxylamine as reported by Pasta et al. (30), although an earlier report had concluded that reversibility was specific for histidine (16). Thus, reversal of DEPC modification of both histidine and lysine residues in the heparin-binding site of selenoprotein P seems likely because both histidine and lysine residues clearly contribute to the protein-heparin interaction. When it becomes possible to produce sufficient quantities of mutant selenoprotein P by site-directed mutagenesis, more details about the contribution of each of these residues can be learned.

It is clear that the native structure of selenoprotein P is required for the most efficient heparin binding. The peptides KHAHL and KKVQSDHIAVY bind to heparin less tightly than does the intact protein, as judged by the salt concentrations (220 and 270 mM NaCl, respectively) needed to elute them from heparin (Fig. 5). Denaturation of the protein by the addition of the reducing agent 1,4-dithiothreitol also resulted in impaired heparin binding (not shown). This indicates that the native tertiary structure of the protein is needed to align these histidine and lysine residues of selenoprotein P to maximize their interaction with heparin.

Heparin binding by proteins and peptides is defined as “tight” when a salt concentration of 1 M or greater is needed to break it (39). Displacement by 0.3–0.4 M salt is defined as “weak” binding. Selenoprotein P eluted from the heparin column at 0.3 M salt (Fig. 5C). Thus, it would be classed as having weak binding by established criteria. However, those criteria were developed for heparin-binding sites that were made up of lysine and arginine, and it is not certain whether they should be used when histidine-mediated heparin binding is being studied. It is postulated here that this “weak” heparin binding of selenoprotein P has physiological significance.

Extracellular superoxide dismutase is an appropriate protein to compare with selenoprotein P. It is an extracellular oxidant defense enzyme that also binds to heparin (40–42). Its heparin binding is postulated to be important in its function of regulating superoxide concentration near cell membranes. Extracellular superoxide dismutase is heterogeneous with respect to heparin binding, a property that it shares with selenoprotein P. The enzyme elutes from heparin in three fractions, designated as A, B, and C (41). Fraction A does not bind to heparin;
fraction B elutes at a salt concentration of 0.2 M; and fraction C elutes at a salt concentration of 0.5 M. Selenoprotein P binds more tightly than does fraction B but not as tightly as does fraction C. An additional comparison can be made between selenoprotein P and histidine-rich glycoprotein (37). Heparin binding by these two proteins is pH-sensitive. Histidine-rich glycoprotein elutes from heparin between 0.3 and 0.5 M NaCl. It has been proposed that histidine-rich glycoprotein acts as a pH sensor, interacting with negatively charged glycosaminoglycans only when it acquires a net positive charge. Based on this proposal, the histidine residues of a protein should be able to provide a mechanism for regulating binding of the protein to cell surface proteoglycans. Local pH can drop by as much as 1 pH unit in acidosis or anoxia (43), leading to an increase in protonation of histidine residues and facilitating binding.

The heparin binding behavior of extracellular superoxide dismutase is modulated by deletion of heparin-binding sites on one (fraction B) or both (fraction A) of its subunits (44). Heparin binding by selenoprotein P is modulated also. However, physical modification of selenoprotein P does not appear to be involved. Instead, heparin binding by selenoprotein P is modulated by pH.

It seems possible that the function of selenoprotein P depends on its binding to cell surface and matrix heparan sulfate proteoglycans. If it does serve as an oxidant defense as we have postulated (7), localization to areas of low pH, such as sites of inflammation, would be logical so that it could protect the host cells in those areas.

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