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PURIFICATION OF HEMOPEXIN AND ITS DOMAIN FRAGMENTS BY AFFINITY CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the preparation of apohemopexin from Cohn Fraction IV-4 of human serum by one-step affinity chromatography on a heme-agarose column and separation of its tryptic domain fragments by high-performance liquid chromatography (HPLC). Limited tryptic digestion cleaved human apohemopexin after Arg-216 into half molecules and the N-terminal half was degraded very rapidly, whereas heme-saturated hemopexin was cleaved after Lys-101. These results suggest that hemopexin is composed of two domains that are connected by an exposed histidine-rich hinge-like region in apohemopoxin which becomes inaccessible to trypsin in heme-saturated hemopexin. Also described is the preparation of apohemopexin from whole rabbit serum in two steps, heme-affinity chromatography and ion-exchange HPLC, and separation of its tryptic domain fragments by HPLC. Limited tryptic digestion also cleaves rabbit apohemopexin into half-molecules but the N-terminal half is more stable than the C-terminal half in this case. This lends support to the idea of functional differences between domains.

INTRODUCTION

Hemopexin is a serum β-glycoprotein that binds heme in an equimolar ratio with much greater affinity than albumin, the other major heme-binding protein in serum[1]. Human hemopexin (63 kilodaltons, kDa) consists of a single polypeptide chain containing 439 amino acid residues with six intrachain disulfide bridges[2,3]. The N-terminal threonine residue is blocked by an O-linked galactosamine oligosaccharide, and the protein has five glucosamine oligosaccharides. The 18 tryptophans are arranged in four clusters and 12 of the tryptophan residues are conserved in homologous positions. Computer-assisted analysis of the internal homology in amino acid sequence indicates that hemopexin consists of two similar halves, thus suggesting a two-domain structure. The two-domain structure is also supported by predictions of the secondary structure and the hydrophilic–hydrophobic character. Because hemopexin consists of two domains but binds only one heme, the question arises as to whether the functions of hemopexin binding heme and of the hepatocyte receptor binding the heme–hemopexin complex reside in separate domains or are governed by

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both domains. In this report, we describe the purification of apohemopexin and the isolation by affinity chromatography and high-performance liquid chromatography (HPLC) of its domain fragments produced by limited tryptic digestion.

EXPERIMENTAL

Materials

Cohn Fraction IV-4 of human plasma was provided by Harold Gallick (Michigan Department of Public Health, Lansing, MI, U.S.A.). Pooled rabbit serum was purchased from Pel-Freez (Rogers, AR, U.S.A.). Purified human hemopexin, saturated with hemin, was obtained from Behringwerke (Marburg/Lahn, F.R.G.). L-(Tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK)-treated trypsin, soybean trypsin inhibitor and kallikrein were purchased from Worthington (Freehold, NJ, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. Antisera to whole human serum and human hemopexin were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and Atlantic Antibodies (Scarborough, ME, U.S.A.), respectively.

The sources of reagents and columns were: aminoethyl-agarose and hemin chloride (type I) (Sigma), 1,1'-carbonyldiimidazole and N,N-dimethylformamide (spectrophotometric grade) (Aldrich, Milwaukee, WI, U.S.A.), 1-propanol (Burdick and Jackson, Muskegon, MI, U.S.A.), Spherogel-TSK 2000SW (60 × 0.75 cm I.D.), 3000SW (60 × 0.75 cm I.D.) and IEX-540 DEAE (30 × 0.46 cm I.D.) columns (Altex, Berkeley, CA, U.S.A.), Synchropak RP-P (25 × 0.41 cm I.D.) column (SynChrom, Linden, IN, U.S.A.).

Analytical methods

The apparatus used for HPLC was a Model 334 gradient HPLC system (Beckman, Berkeley, CA, U.S.A.) including a 421 CRT controller, two 110A pumps, a 210A injector, and mixing chamber. The eluent was monitored at 280 nm in a 1-cm light path with a Gilson Holochrome UV monitor, connected to a linear recorder, Model 261/M. Sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS–PAGE) was performed by the method of Lambin *et al.*[4]. The method of reduction and aminoethylation of the purified protein was described by Takahashi *et al.*[2,3] Immunochemical analysis was kindly performed by Dr. S. Migita of Kanazawa University (Cancer Research Institute, Kanazawa University, Kanazawa, Japan). Absorption spectra of apo-, heme-hemopexin and of a limited digest of heme-hemopexin were obtained with a DU-7 spectrophotometer (Beckman). The methods for sequence analysis by automated Edman degradation with a Beckman 890C sequencer have been described by Takahashi *et al.*[2,3].

Purification of apohemopexin

Cohn Fraction IV-4 paste (3 g) of human serum was suspended in 300 ml of 0.1 *M* Tris–HCl (pH 7.5), containing 0.15 *M* NaCl and was centrifuged. The supernatant was mixed with heme-agarose (100 ml) at room temperature for 1–2 h. Except for a 12-fold increase in scale, the heme-agarose was synthesized exactly as described by Tsutsui and Mueller[5]. The incubated mixture of heme-agarose and Cohn Fraction IV-4 supernatant was then packed in a glass column (15 × 5 cm, I.D.) and the resin was washed with the Tris buffer until the absorbance at 280 nm of the eluent went

down to 0.02. Then apohemopexin was eluted with 0.2 *M* sodium citrate buffer (pH 4.0). Next, the column was eluted with 0.2 *M* sodium citrate buffer (pH 2.0), and was finally washed with 8 *M* urea. The purity of the human apohemopexin was determined by SDS–PAGE, by immunoelectrophoresis, and by N-terminal sequence analysis.

Whole rabbit serum (250 ml) was mixed with the heme-agarose (100 ml) for 2 h at room temperature and packed in the glass column. The resin was washed with 0.01 *M* sodium phosphate buffer (pH 7.5), containing 0.5 *M* sodium chloride until the absorbance at 280 nm approached zero and the column was then eluted with 0.2 *M* sodium citrate buffer (pH 4.0). The column was washed with 8 *M* urea as the final step to elute any proteins still bound to the heme-agarose. Double immunodiffusion was also performed with antihuman hemopexin antiserum that was found to cross-react with the rabbit hemopexin.

The acidic sodium citrate buffer eluted two major proteins, hemopexin (66 kDa) and histidine-rich glycoprotein (95 kDa), in a single peak. The fractions in this peak were pooled, concentrated, and then dialyzed against 0.02 *M* Tris–acetic acid buffer (pH 8.0). These two proteins were separated by ion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column with a linear gradient from 0 to 0.3 *M* sodium acetate in 0.02 *M* Tris–acetic acid buffer (pH 8.0), during 60 min at a flow-rate of 1.0 ml/min. Hemopexin was identified by double immunodiffusion and histidine-rich glycoprotein by N-terminal sequence analysis.

Limited enzymatic digestions and separation of the fragments by HPLC

Limited tryptic digestion of human apohemopexin was carried out with TPCK-treated trypsin (enzyme-to-substrate weight ratio of 1:140) in 0.1 *M* ammonium bicarbonate at room temperature for 60 min. In all cases, the reaction was stopped by addition of soybean trypsin inhibitor. The limited digest was separated by ion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column with a linear gradient from 0 to 0.15 *M* sodium acetate in 0.02 *M* Tris–acetic acid buffer (pH 8.0), during 75 min at a flow-rate of 1.0 ml/min.

Limited tryptic digestion of human hemopexin, saturated with hemin, was done with TPCK-treated trypsin (enzyme-to-substrate ratio of 1:40) in 0.2 *M* Tris–HCl (pH 8.0), at 37°C for 6 h. After reduction and aminoethylation, the limited digest was separated by gel HPLC on a TSK-2000SW column, connected in tandem with a TSK-3000SW column. The columns were eluted with 0.1 *M* Tris–HCl (pH 8.0), containing 8 *M* urea and 0.15 *M* sodium chloride at a flow-rate of 0.5 ml/min.

Limited tryptic digestion of rabbit apohemopexin was carried out with TPCK-treated trypsin (enzyme-to-substrate ratio of 1:100) in 0.1 *M* ammonium bicarbonate at room temperature for 60 min. The limited digest was separated on a Spherogel-TSK IEX-540 DEAE column with a linear gradient from 0 to 0.25 *M* sodium acetate in 0.02 *M* Tris–acetic acid buffer (pH 8.0), during 100 min at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Purification of apohemopexin from Cohn Fraction IV-4 of human serum by heme-agarose chromatography

Although several methods for the synthesis of affinity resins for heme-binding proteins have been published[5–9], the method of Tsutsui and Mueller[5] was used to prepare the affinity column because of its simplicity and the high binding capacity and selectivity of the synthesized resin. The affinity chromatography of Cohn Fraction IV-4 of human serum was carried out with this heme-agarose resin (Fig. 1); apohemopexin, which did not exhibit significant absorbance at 413 nm, was identified by immunoelectrophoresis (in Peak 2), which gave a single component in SDS–PAGE. As shown in Fig. 2, the protein in Peak 2 gave a single arc against anti-whole

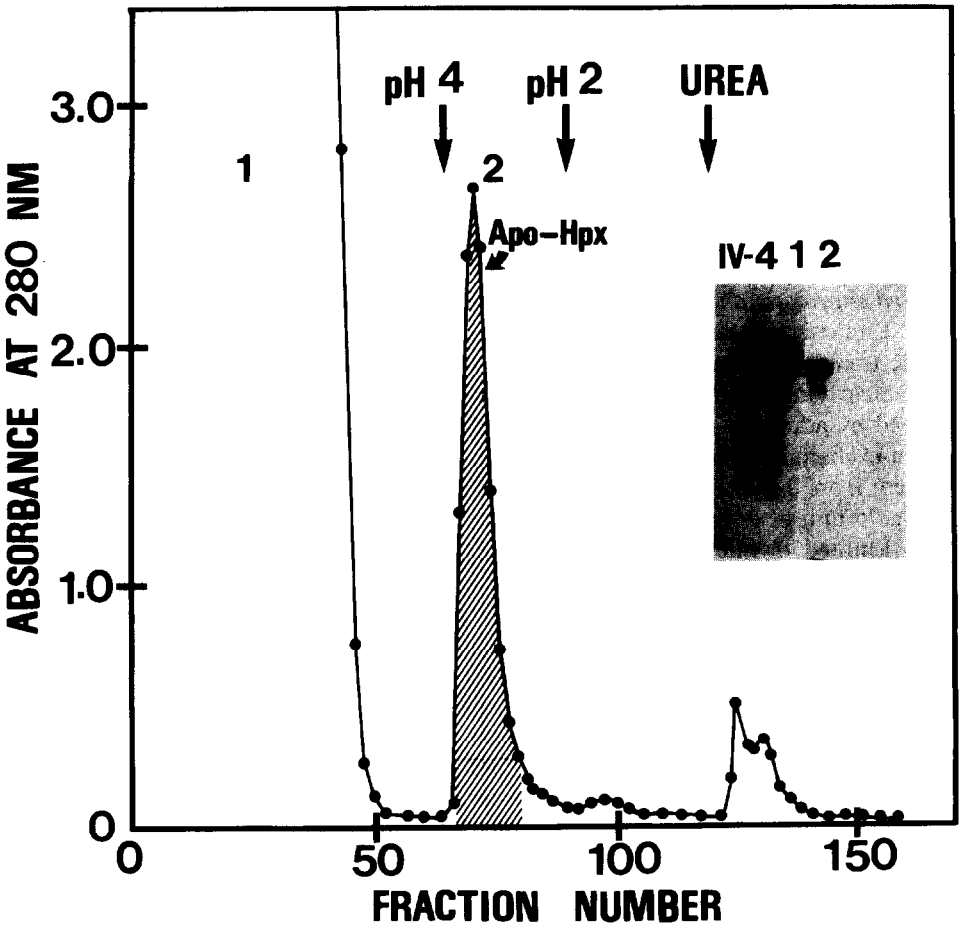

Fig. 1. Heme-agarose affinity chromatography of apohemopexin from Cohn Fraction IV-4 of human serum. Fractions of 12 ml were collected. Apohemopexin was eluted with 0.2 *M* sodium citrate buffer (pH 4). Pooled fractions containing apohemopexin are shaded. Inset shows SDS–PAGE (15–30% acrylamide gradient) of Cohn Fraction IV-4 (first lane), Peak 1 (second lane), and Peak 2 (third lane) of the chromatogram. Peak 2 also contains some degraded hemopexin.

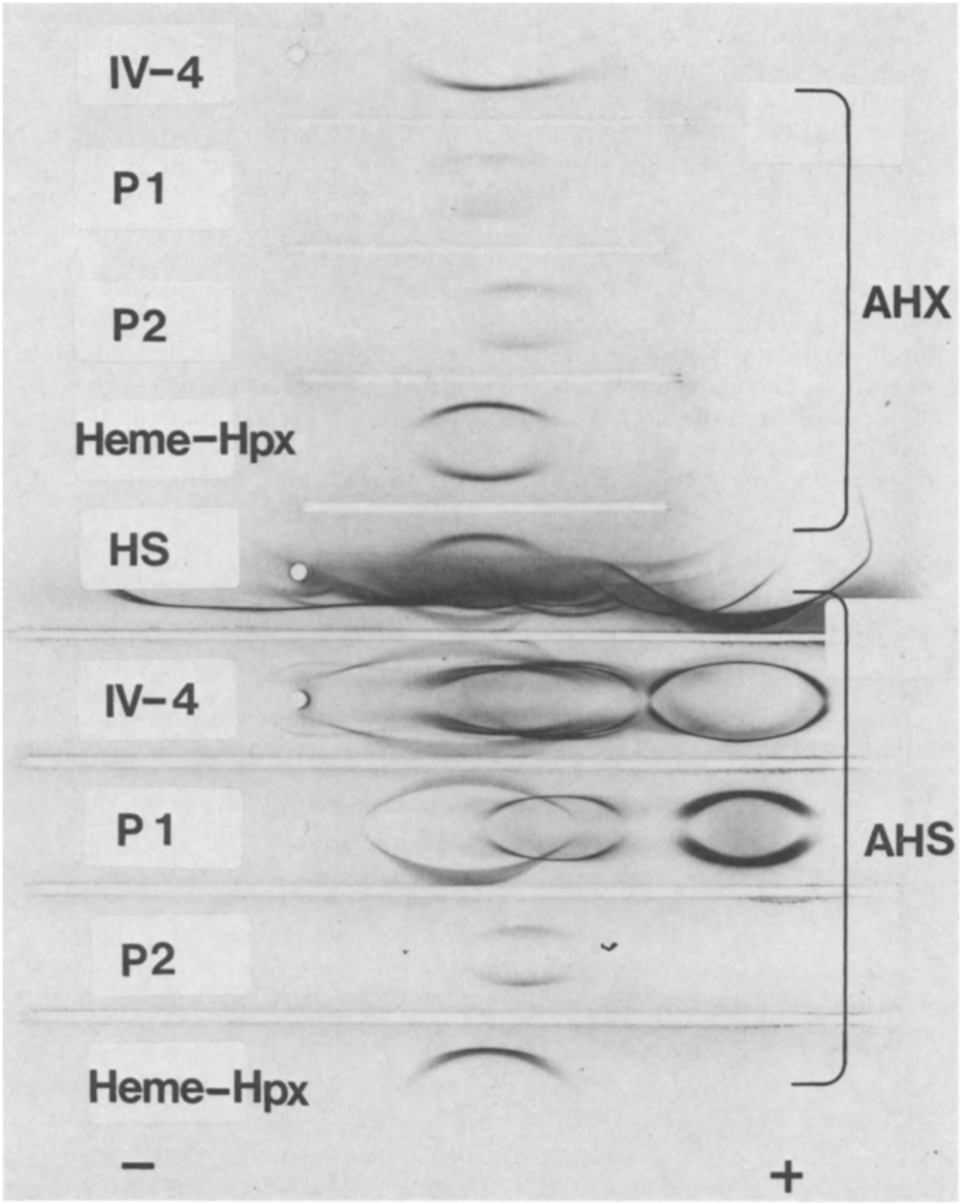

Fig. 2. Immunoelectrophoresis of Cohn Fraction IV-4, heme-saturated hemopexin, and Peaks 1 and 2 of the affinity chromatography in Fig. 1. The upper four lanes are obtained with anti-human hemopexin, and the remaining lanes are with anti-whole human serum. Abbreviations used are as follows: IV-4, Cohn Fraction IV-4; P1, Peak 1 in Fig. 1; P2, Peak 2 in Fig. 1; Heme-Hpx, hemopexin, saturated with hemin; HS, whole human serum; AHX, anti-human hemopexin; AHS, anti-whole human serum.

human serum and against anti-hemopexin. Although a component in Peak 1 reacted with anti-hemopexin by immunoelectrophoresis, the component had a lower mobility than that of apohemopexin in Peak 2. Because the mobility was consistent with that

of hemopexin, saturated with hemin, the component reacting with anti-hemopexin in Peak 1 is probably heme-saturated hemopexin which could not bind to the heme-agarose column (Fig. 2).

The recovery of hemopexin by this method was estimated by single radial immunodiffusion. The amount of hemopexin in the supernatant of dissolved Cohn Fraction IV-4 that was applied to the column was 207 mg, and the yield of apohemopexin purified by the affinity chromatography was 130 mg (63%). The amino acid composition of the purified protein (Table I) is in good agreement with that calculated from the complete amino acid sequence of human hemopexin[3]. Amino-terminal sequence analysis of the purified apohemopexin gave the expected sequence and no minor sequences were detected. When the heme-binding ability of the purified apohemopexin was tested by repeating the affinity chromatography, the protein bound to the column and eluted as a single major component. Furthermore, after addition of hemin to the purified hemopexin, the absorption spectrum exhibited maxima at 282 nm, 413 nm, and ill-defined bands in the visible region at 560 nm and 535 nm, which are typical absorption maxima for heme-saturated hemopexin[1].

TABLE I

AMINO ACID COMPOSITION OF HUMAN HEMOPEXIN AND THE DOMAIN FRAGMENTS PRODUCED BY LIMITED TRYPTIC DIGESTION

The amino acid composition determined by analysis is compared to that calculated from the amino acid sequence of the corresponding region of hemopexin (in parenthesis). One asterisk (*) indicates obtained as carboxymethylcysteine; two, as half cystine; three, as aminoethylcysteine. Other symbols used are as follows: +, detected by amino acid analysis but not quantitated; −, not detected by amino acid analysis.

Amino acid	*Hpx*	*Apo-Hpx 29-kDa fragment*	*Heme-Hpx*	
			15-kDa fragment	*45-kDa fragment*
Asp	39.0 (39)	17.0 (17)	11.4 (13)	26.0 (26)
Thr	21.6 (23)	13.3 (14)	5.9 (7)	15.5 (16)
Ser	26.9 (30)	16.6 (19)	7.3 (7)	23.0 (23)
Glu	38.4 (37)	17.4 (16)	8.7 (8)	30.2 (29)
Pro	35.3 (35)	17.8 (15)	8.8 (9)	29.4 (26)
Gly	42.0 (43)	23.0 (23)	8.5 (7)	38.1 (36)
Ala	25.7 (28)	15.1 (17)	5.0 (5)	22.5 (23)
Cys	13.8* (12)	4.1** (6)	0.8*** (1)	9.5*** (11)
Val	23.2 (24)	10.2 (11)	5.7 (7)	16.8 (17)
Met	5.1 (6)	2.6 (3)	1.2 (1)	4.8 (5)
Ile	6.6 (9)	4.7 (6)	2.0 (2)	5.9 (7)
Leu	37.8 (37)	22.1 (24)	5.1 (5)	32.0 (32)
Tyr	14.5 (16)	10.7 (10)	1.3 (1)	14.2 (15)
Phe	18.0 (19)	4.4 (4)	6.3 (7)	12.6 (12)
Lys	20.3 (21)	9.0 (9)	9.4 (9)	12.4 (12)
His	18.3 (19)	16.3 (13)	3.6 (3)	16.6 (16)
Arg	22.1 (23)	7.0 (7)	4.2 (4)	17.4 (19)
Trp	+ (18)	+ (9)	+ (5)	+ (13)
GlcN	+	+	+	+
GalN	+	−	+	−

Limited tryptic digestion of human apohemopexin and its separation by HPLC

When the purified human apohemopexin was subjected to limited tryptic digestion, a stable 29-kDa fragment was produced; however the other fragments formed were rapidly degraded. Small quantities of uncleaved hemopexin (60 kDa) and a 33-kDa fragment were observed to remain after limited tryptic digestion for 60 min (Fig. 3). Although the 33-kDa fragment is not easily distinguished in the inset of Fig. 3, it is present in lane A as a faint band just above the 29-kDa band. The digest was separated by ion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column (Fig. 3). The main peak contained the 29-kDa fragment. On a molar basis, the yield was about 40%. The N-terminal sequence of this fragment was ( )-Gly-Thr-Gly-His-Gly-( )-Ser-Thr-His-His-Gly-Pro-Glu-Tyr-Met-. This corresponds to the sequence from residue 217 to 232 in hemopexin, but two glucosamine oligosaccharides attached at Asn-217 and Asn-223 prevent the identification of these residues by Edman degradation[3]. Thus, the 29-kDa fragment was produced by cleavage after Arg-216

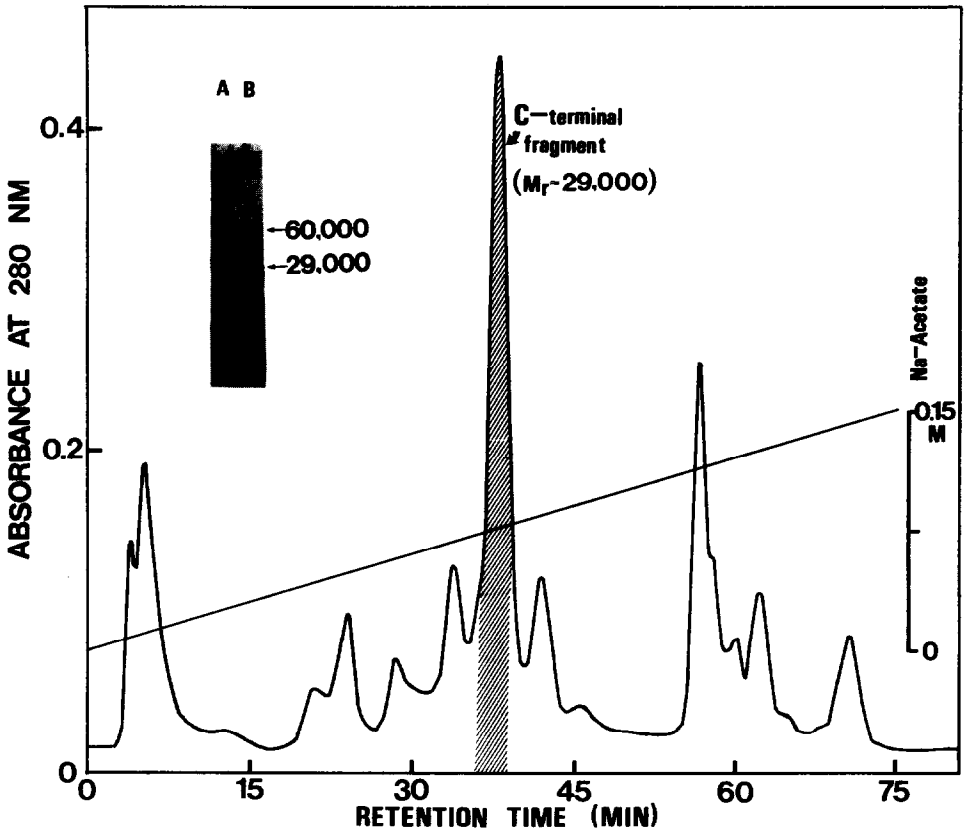

Fig. 3. Separation of C-terminal fragment from the limited tryptic digest of human apohemopexin by anion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column. A peak containing the C-terminal fragment is shaded. SDS–PAGE of the limited tryptic digest (A) and the separated C-terminal fragment (B) is shown as an inset of the figure. This fragment was estimated by SDS–PAGE to have a molecular mass of 29 kDa.

in the middle of the molecule and is the C-terminal half of the protein (Fig. 4). Although the C-terminus was not determined, the amino acid composition of the 29-kDa fragment (Table I) is reasonably consistent with that of the region from residue 217 to the C-terminus of the protein sequence[3]. The other peaks in Fig. 3 contained small peptides, derived from N-terminal half of the hemopexin molecule, indicating that the N-terminal half is degraded by trypsin. These results support the proposal that hemopexin is composed of two domains that are connected by a histidine-rich hinge-like region that is exposed by a series of β-turns and by the hydrophilic character of this region[3] (Fig. 4).

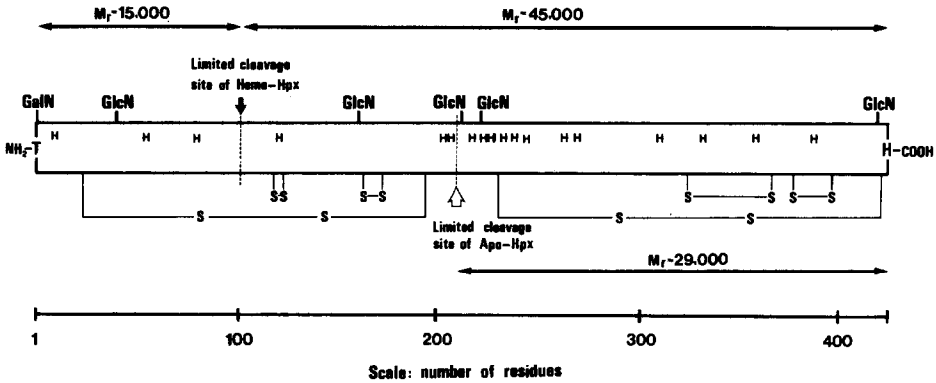

Fig. 4. Schematic model of the structure of human hemopexin, based on sequence analysis and the limited tryptic cleavage sites. The main features are diagrammed to the scale shown. The sites of attachment for galactosamine (GalN) and glucosamine (GlcN) oligosaccharides are given. Disulfide bridges are indicated by –S–S–. Each half of the molecule has three intrachain disulfide bonds; however, the N- and C-terminal halves are not linked by a disulfide bond. The N- and C-terminal residues of the protein are indicated by T (threonine) and H (histidine). The location of all identified histidine residues is also shown. A histidine- and glucosamine-rich region is located in the middle of the molecule between two homologous disulfide bridges. Presumably exposed by a series of β-turns and its hydrophilic nature, this region is tentatively described as being hinge-like. Specific tryptic cleavage of apohemopexin, indicated by the open arrow, occurs after Arg-216 in this hinge-like region and produces a major stable C-terminal fragment of 29 kDa, as shown under the structural model. In heme-saturated hemopexin specific tryptic cleavage, indicated by the solid arrow, occurs after Lys-101 and produces two disulfide-connected fragments, as shown over the structural model. Heme-Hpx and Apo-Hpx indicate heme-saturated hemopexin and apohemopexin, respectively.

In order to determine whether the heme-binding site is located in the C-terminal domain, the limited digest of apohemopexin was first incubated with the heme-agarose resin and chromatographed. However, the stable C-terminal domain fragment did not bind to the column. Although uncleaved hemopexin and a minor 33-kDa component bound to the heme-agarose column, the yield of the component was very low; thus, limited tryptic digestion of human apohemopexin was not an effective method for obtaining a heme-binding domain.

Limited tryptic digestion of hemopexin, saturated with hemin, and its separation by HPLC

Human hemopexin, saturated with hemin, was also subjected to limited tryptic

digestion. When the digest was analyzed by SDS–PAGE without reduction, a single band of 60 kDa was observed on the gel. However, this was split into two bands of 45 and 15 kDa after reduction and aminoethylation (Fig. 5), indicating that the two fragments were connected by a disulfide bridge. Therefore, the digest was reduced and aminoethylated before being separated by gel HPLC (Fig. 5). Peak 2 and Peak 4 in Fig. 5 contained the 45 kDa and 15-kDa fragments, respectively. On a molar basis, the yield of both fragments was about 60%. The amino acid compositions of the separated fragments are shown in Table I; the sum of their compositions is in good agreement with that of the whole protein. The N-terminal sequence of the 45-kDa fragment was Glu-Lys-Gly-Tyr-Pro-Lys-Leu-Leu-Gln-Asp-, which corresponds to that of the region from residue 102 to 111 in human hemopexin[3]. The 15-kDa fragment contained galactosamine which had been shown to be attached to Thr-1, the N-terminal residue of hemopexin; the first five residues of the 15-kDa fragment were consistent with those of the N-terminal sequence of intact hemopexin. Thus, the 15-kDa fragment is derived from the N-terminal portion of intact hemopexin, and the 45-kDa fragment is from the C-terminal portion of hemopexin. The site of limited cleavage of heme-hemopexin with trypsin is between Lys-101 and Glu-102, as shown in the structural model of human hemopexin (Fig. 4). These results suggest that different peptide bonds are scissile in apo- and heme-hemopexin and

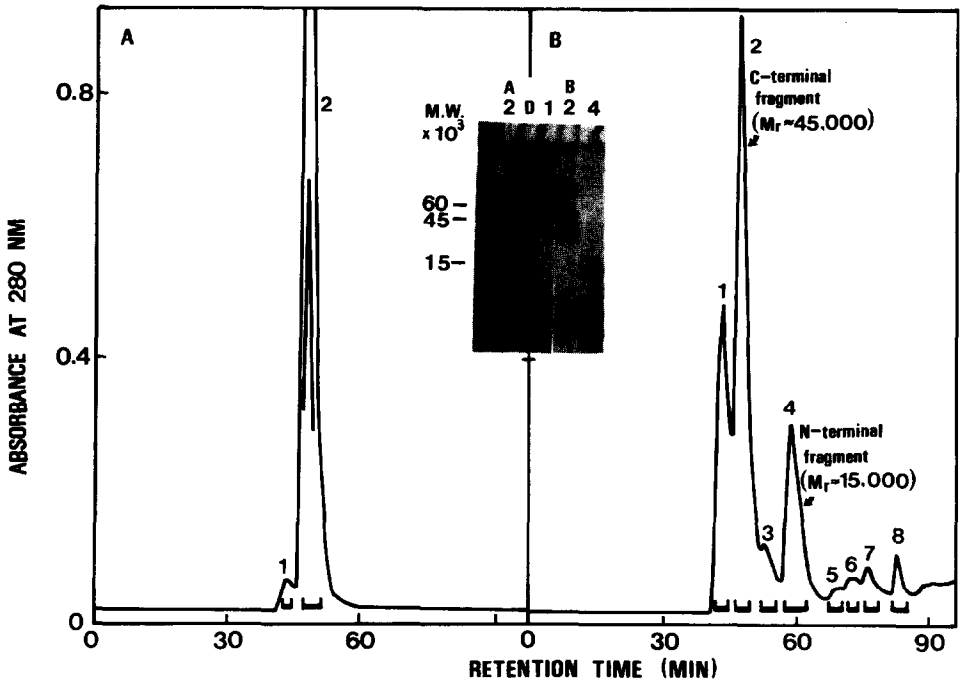

Fig. 5. Gel HPLC of the limited tryptic digest of human hemopexin, saturated with hemin. The elution was performed with 0.1 *M* Tris–HCl buffer (pH 8.0), containing 8 *M* urea. (A) Before reduction; (B) after reduction and aminoethylation of the digest. SDS–PAGE patterns of the limited tryptic digest and of the separated fragment are shown in the inset. From left to right: first lane, Peak 2 from chromatography A; second lane, the aminoethylated digest; third to fifth lanes, Peaks 1, 2, and 4 from chromatography B.

that the binding of heme induces conformational changes in the hemopexin molecule that result in a different cleavage pattern.

Purification of rabbit apohemopexin from whole rabbit serum by heme-agarose chromatography and ion-exchange HPLC

When heme-agarose affinity chromatography was applied to whole rabbit serum, the same basic chromatogram as in Fig. 1 was obtained. The only difference is that two major proteins are eluted by the switch to an acidic buffer instead of one protein, as was the case with Cohn Fraction IV-4 of human serum. These two rabbit proteins were identified as hemopexin and histidine-rich glycoprotein and were recovered as approximately 1.5% of the total starting absorbance at 280 nm.

The hemopexin (66 kDa) and histidine-rich glycoprotein (95 kDa) can then be

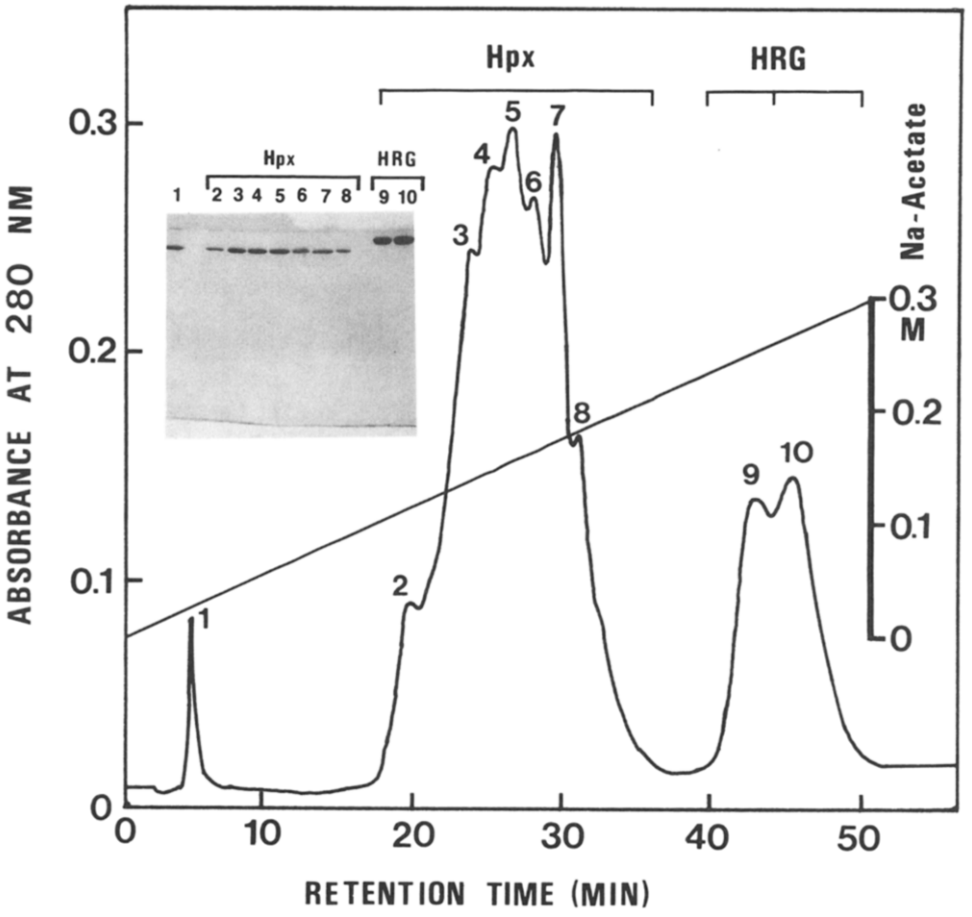

Fig. 6. Purification of the rabbit apohemopexin, eluted from heme-agarose with 0.2 *M* sodium citrate buffer (pH 4.0), by anion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column. SDS–PAGE of each peak is shown in the inset. The number above each lane corresponds to the peak number in the chromatogram. No difference in migration was noted for the seven hemopexin (Hpx) peaks or for the two histidine-rich glycoprotein (HRG) peaks. Peak 1 probably contains aggregated Hpx. Extinction coefficients at 280 nm for Hpx and HRG are 19.7 and 5.7, respectively[1,10].

purified by ion-exchange HPLC (Fig. 6). Hemopexin was eluted as a series of seven closely spaced peaks (numbered 2–8 in Fig. 6) and histidine-rich glycoprotein as a double peak. No difference in migration in SDS–PAGE was noted for any of the seven hemopexin peaks or for either of the histidine-rich glycoprotein peaks (see insert of Fig. 6). The microheterogeneity of both proteins could be due either to variations in carbohydrate structure or to genetic polymorphism, and such minor differences would not be detected by SDS–PAGE under the conditions used. Using absorption coefficients of 19.7 and 5.7 at 280 nm for hemopexin and histidine-rich glycoprotein[1,10], respectively, the two proteins were found to be present in approximately equivalent amounts by weight.

Limited tryptic digestion of rabbit apohemopexin and its separation by HPLC

The purified rabbit apohemopexin (66 kDa) was subjected to limited tryptic digestion on an analytical scale (Fig. 7) and was quickly cleaved into two relatively stable fragments (40 and 26 kDa). Densitometric calculations indicated that both fragments were produced in approximately equivalent amounts, but that the yield of the 26-kDa fragment decreased slightly with time. The two fragments migrated separately in SDS–PAGE under non-reducing conditions and were therefore not connected by a disulfide bridge. According to Morgan and Smith[11], plasmin digestion of rabbit apohemopexin produces a 35-kDa fragment that is derived from the N-terminal half of the molecule and was judged by spectrophotometric methods to bind

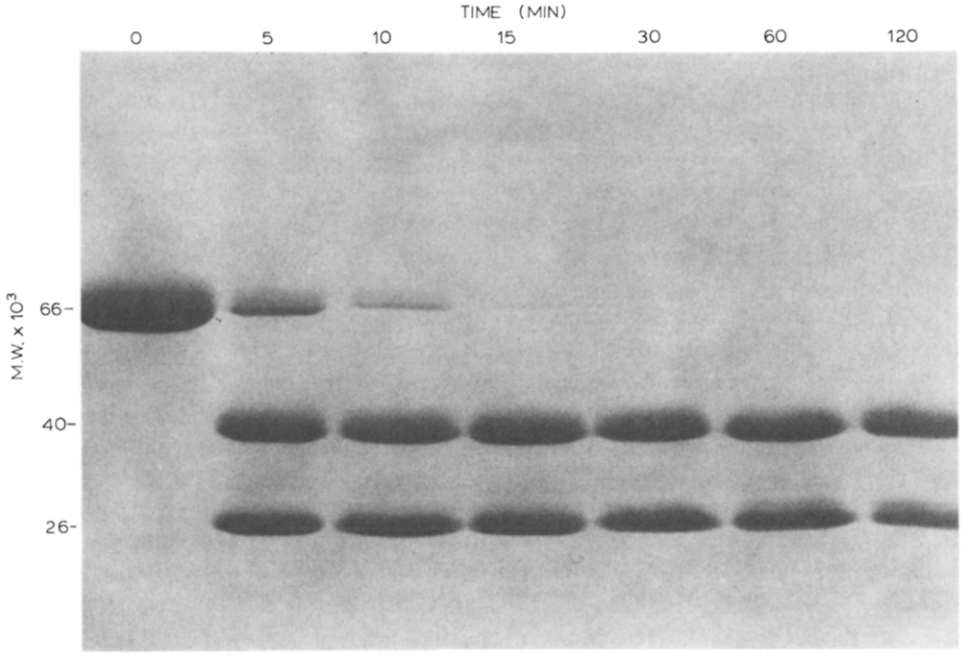

Fig. 7. SDS–PAGE of a limited tryptic digestion of rabbit apohemopexin. The amount of time the digestion was allowed to proceed before the addition of soybean trypsin inhibitor is shown above each lane. The 66-kDa band is intact rabbit apohemopexin, and the 40- and 26-kDa bands correspond to the N- and C-terminal fragments, respectively. Densitometric analysis revealed that the yield of the 26-kDa C-terminal fragment decreased with increased time of digestion.

heme in an equimolar ratio. A 25-kDa fragment was also produced that is probably derived from the C-terminal half of the molecule and that may also be cleaved at secondary sites[11].

When the limited tryptic digestion of rabbit apohemopexin was scaled up to preparative conditions, a different pattern was observed (see lane D, inset of Fig. 8). The only change in reaction conditions from the previous digest was that the apohemopexin solution was about ten times more concentrated. Both the 40- and the 26-kDa fragments were degraded further, and the 26-kDa fragment was quite unstable. The digest was first incubated with the heme-agarose resin and chromatographed to determine whether one of the fragments still contained a heme-binding site. Only a small amount of uncleaved hemopexin was bound to the column, and all of the tryptic fragments were recovered unchanged in the eluate. The 20-kDa band and the lighter band above it probably contain degraded fragments of hemopexin as well as trypsin inhibitor (20 kDa) and trypsin (24 kDa). When the digest was separated by ion-exchange HPLC (Fig. 8), a stable major fragment of 40 kDa was obtained, which appears to correspond to the N-terminal domain, described by Morgan and Smith[11].

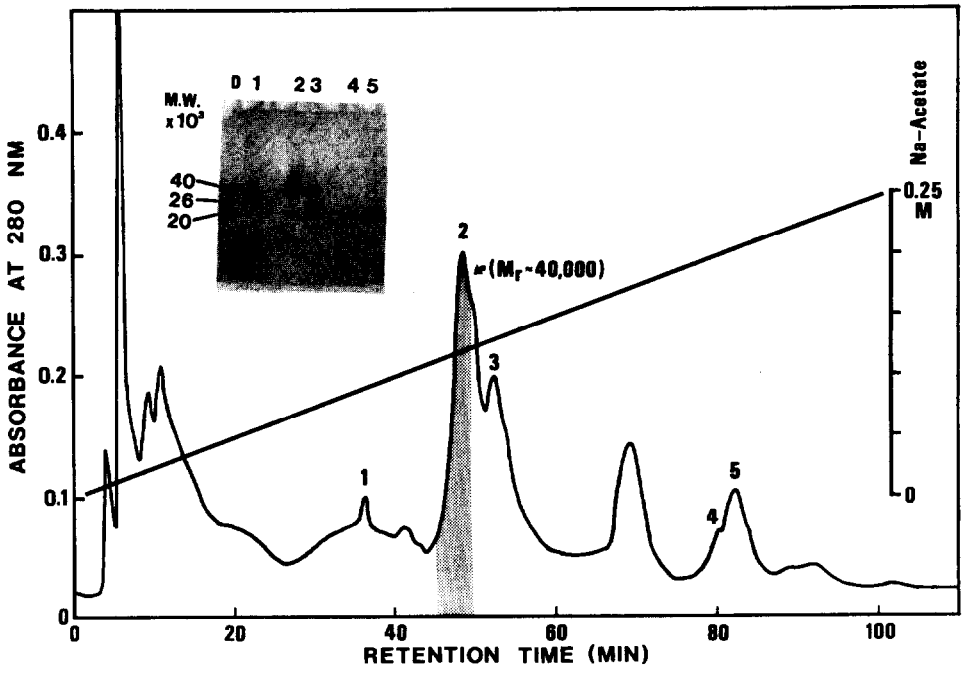

Fig. 8. Separation of the N-terminal fragment from the limited tryptic digest of rabbit apohemopexin by anion-exchange HPLC on a Spherogel-TSK IEX-540 DEAE column. A peak containing the N-terminal fragment is shaded. SDS–PAGE patterns of the limited tryptic digest and of separated fragments are shown in the inset. Lane D is the tryptic digest of rabbit apohemopexin after passage through a heme-agarose column. Lanes labeled 1 through 5 correspond to the peak labeled with the same number in the chromatogram.

CONCLUSIONS

We have applied heme-agarose affinity column chromatography to the purification of apohemopexin from Cohn Fraction IV-4 of human serum. Human apohemopexin was purified in one step by this method. Affinity chromatography was also applicable to the isolation of heme-binding proteins from rabbit serum. Two major heme-binding proteins of the rabbit, histidine-rich glycoprotein and apohemopexin were isolated by affinity chromatography, and they could subsequently be separated by anion-exchange HPLC. The purified apohemopexins of both species were subjected to limited proteolytic digestion in order to obtain domain fragments. The digests were analyzed by HPLC, which enabled the rapid isolation of domain-size fragments. A stable C-terminal fragment (29 kDa) was purified from the limited tryptic digest of human apohemopexin; however, the N-terminal half was not isolated because the fragment was degraded into smaller peptides. The C-terminal domain failed to bind to the heme-agarose column. Although human hemopexin, saturated with hemin, was relatively resistant to tryptic digestion, cleavage occurred in the middle of the N-terminal half. Two fragments (15 and 45 kDa) were obtained, which were connected by a disulfide bridge. The finding that different peptide bonds of apohemopexin and heme-saturated hemopexin are scissile is attributed to a conformational change induced by the binding of the heme. We could also apply HPLC to the isolation of the N-terminal fragment from a limited tryptic digest of rabbit apohemopexin. Because the primary structure of human hemopexin has been determined, further study of the isolated fragments should facilitate identification of the heme-binding site and the site for uptake of hemopexin by the hepatocyte receptor.

REFERENCES

1 U. Muller-Eberhard and H. H. Liem, in A. C. Allison (Editor), *Structure and Function of Plasma Proteins,* Plenum, London, Vol. 1, 1974, p. 35.
2 N. Takahashi, Y. Takahashi and F. W. Putnam, *Proc. Nat. Acad. Sci. U.S.,* 81 (1984) 2021.
3 N. Takahashi, Y. Takahashi and F. W. Putnam, *Proc. Nat. Acad. Sci. U.S.,* 82 (1985) 73.
4 P. Lambin, D. Rochu and J. M. Fine, *Anal. Biochem.,* 74 (1976) 567.
5 K. Tsutsui and G. C. Mueller, *Anal. Biochem.,* 121 (1982) 244.
6 T. P. Conway and U. Muller-Eberhard, *Fed. Proc.,* 32 (1973) 469.
7 J. Suttner, Z. Hrkal, Z. Vodrazka and J. Rejnkova, *J. Chromatogr.,* 169 (1979) 500.
8 K. W. Olsen, *Anal. Biochem.,* 109 (1980) 250.
9 R. Majuri, *Biochem. Biophys. Acta,* 719 (1982) 53.
10 F. W. Putnam (Editor), *The Plasma Proteins,* Vol. 4, Academic Press, Orlando, FL, 1984, 2nd ed., p. 1.
11 W. T. Morgan and A. Smith, *J. Biol. Chem.,* 259 (1984) 12 001.