The Protein HC Chromophore Is Linked to the Cysteine Residue at Position 34 of the Polypeptide Chain by a Reduction-resistant Bond and Causes the Charge Heterogeneity of Protein HC*

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Protein HC, an extremely charge-heterogeneous lipocalin, carries a yellow-brown fluorescent chromophore of unknown structure covalently bound at an unidentified site of its polypeptide chain. Two chromophore-carrying peptides with 60% of the chromophore material (defined as material absorbing light at 330 nm) were isolated from pepsin-digested native human protein HC by reversed-phase high performance liquid chromatography. Sequence analysis of these peptides indicated that the chromophore was bound to the cysteine residue at position 34 of the protein HC polypeptide chain. Sequence analysis of a native chromophore-tripeptide complex, isolated by pronase digests of the pepsin-produced peptides, identified the sequence Thr33-X34-Pro35, corroborating the position of the chromophore linkage. Quantitative amino acid analysis of the hydrolyzed, performic acid-oxidized, chromophore-tripeptide complex demonstrated approximately equal amounts of threonine, cysteic acid, and proline in the complex. Reduction and carboxymethylation of the native chromophore-tripeptide complex did not remove the chromophore from the peptide. The absorption spectrum of the chromophore-tripeptide complex was similar to that of native protein HC, implying that all of the heterogeneity of protein HC resides in its chromophore.

Protein HC, or α1-microglobulin, is a charge-heterogeneous glycoprotein widely distributed in body fluids as free monomers associated with a fluorescent yellow-brown chromophore and as HC-IgA complexes (1–13). The monomer contains a single polypeptide chain with 3 cysteine residues, 2 of which form a disulfide bridge, while the status of the 3rd one at position 34 remains unknown (14). The free protein displays immunoregulatory properties in vitro (15, 16), and HC-IgA carries antibody activity (17). Protein HC is synthesized in the liver as the N-terminal part of a proprotein with the serine protease inhibitor HI-30 forming the C-terminal part of the proprotein (18, 19). The diprotein is completely cleaved into its two mature proteins before its secretion from the liver cells. The amino acid sequence of the polypeptide chain of protein HC is unique (20, 21), and homology studies have demonstrated that the protein belongs to a recently defined family of proteins, called the lipocalin superfamily, as all proteins of it carry lipophilic substances (22, 23). Presently, about 20 proteins, among them the extensively studied retinol-binding protein and β-lactoglobulin, are known to belong to this superfamily (24, 25), being the human complement component C8-γ, the lipocalin member which displays the highest degree of identity with protein HC (26, 27). It is conspicuous, however, that protein HC differs in several significant respects from these three proteins and all other lipocalins so far described. For example, while the other lipocalins generally are homogeneous in charge and carry substances which are easily dissociated from the apoproteins by procedures disrupting secondary and tertiary protein structures, this is not the case for protein HC. Furthermore, protein HC is so heterogeneous in charge that it resembles the immunoglobulin family in this respect. As the amino acid sequence of the polypeptide chain of protein HC and the structures of its two N-linked and single O-linked carbohydrate prosthetic groups (28) do not vary between different protein HC populations, the charge heterogeneity of protein HC is probably due to variations in structure and/or amount of its chromophore. Although several biological properties have been assigned to protein HC (15, 16), its crucial biological role as a lipocalin is currently unknown. Knowledge of the structure of the chromophore and its binding site(s) to the protein HC polypeptide chain will most probably define the central role of protein HC as a lipocalin, and considerable efforts have therefore been made to elucidate the structure and binding of the protein HC chromophore. These efforts have, unfortunately, so far been unsuccessful. The present work was undertaken to localize the binding site(s) between the chromophore and the polypeptide chain of protein HC. The results strongly indicate that virtually all of the chromophore is linked to the cysteine residue at position 34 of the polypeptide chain by a reduction-resistant bond, and that the extensive charge heterogeneity of protein HC is ascribable to the heterogeneity of its chromophore.

EXPERIMENTAL PROCEDURES

Materials

Urinary protein HC was purified from the urine of patients with tubular proteinuria by ion exchange chromatography, followed by gel filtration as earlier described (1). High performance liquid chroma-
tography (HPLC) grade acetoniitrile was purchased from Scharlau (Barcelona, Spain); pepsin, guanidinium chloride, iodoacetic acid, and diithiothreitol from Sigma; 4-vinylpyridine from Aldrich; and iodo[1-14C]acetic acid (17.9 mCi/mmol) and [14C]methylammonium chloride (40 Ci/mmol) from Du Pont-New England Nuclear. Pronase E (Serva, Heidelberg, Germany) and chymotrypsin from Worthington, and Staphylococcus aureus V8 protease from Miles Scientific (Naperville, IL). Ultrapure water for HPLC, generated by a Milli-PQ4, connected to a Milli-Q water purification system (Millipore), was used in the preparation of all buffers. All other chemicals used were reagent grade or of highest available quality.

Methods

**Pepsin Digestion**—Native protein HC (6.5 mg) in 0.276 ml of 5% (v/v) formic acid was digested with pepsin for 2 h at 37°C using an enzyme to substrate ratio of 1:50 (w/w). After digestion, the material was lyophilized and redissolved in 0.1% (v/v) trifluoroacetic acid immediately before chromatography.

**Pronase E Digestion**—Peptides (1.5 mg) isolated from pepsindigested native protein HC were incubated with pronase E in 0.15 ml of 0.1 M Tris-HCl, pH 8.5, for 24 h at 37°C using an enzyme to substrate ratio of 1:100 (w/w). The incubation period was extended up to a total of 48 h after a second addition of the same amount of pronase E. The material was then lyophilized and redissolved in 0.1% (v/v) trifluoroacetic acid immediately before chromatography.

**Chymotryptic Digestion**—Native protein HC (3.8 mg) in 0.48 ml of 0.2 M N-methylmorpholine acetate buffer, pH 8.2, was incubated with a-chymotrypsin for 23 h at 37°C using an enzyme to substrate ratio of 1:50 (w/w). The incubation period was extended up to a total of 48 h after a second addition of the same amount of a-chymotrypsin. The material was then lyophilized and redissolved in 0.1% (v/v) trifluoroacetic acid immediately before chromatography.

**V8 Protease Digestion**—Completely reduced and alkylated protein HC (4.0 mg) in 900 µl of 0.2 M N-methylmorpholine acetate buffer, pH 8.2, was incubated with V8 protease for 48 h at 37°C using an enzyme to substrate ratio of 1:50 (w/w). The material was then lyophilized and redissolved in 0.1% (v/v) trifluoroacetic acid immediately before chromatography.

**Reduction and Alkylation**—A chromophore-tripeptide complex (0.5 mg), isolated from native protein HC digested with pepsin and pronase, was dissolved in 0.2 ml of 1 M Tris-HCl buffer, pH 8.5, containing 5 mM EDTA and 6 M guanidinium chloride, and treated with 55 mM dithiothreitol for 120 min at 37°C. Reduction was accomplished by the addition of 27 µCi of iodo[1-14C]acetic acid and incubation for 15 min at room temperature in the absence of light. Unlabeled iodoacetic acid was then added to a final concentration of 20 µM. After incubation for 15 min at room temperature in the absence of light, the chromophore-tripeptide complex was separated from low molecular weight reagents by reversed-phase HPLC of the mixture. Complete reduction and carboxymethylation of native protein HC was performed by an identical procedure. S-Pyridylethylation of native protein HC was achieved by adding 5 µl of 4-vinylpyridine to a solution of 2.0 mg of protein HC in 0.1 M Tris-HCl buffer, pH 8.5, containing 2 mM EDTA and 6 M guanidinium chloride, and incubating the mixture for 15 min at room temperature. Protein HC was then separated from low molecular weight reagents by size-exclusion HPLC.

**Tryptic Digest of Protein HC with [14C]Methylammonium Chloride**—Native protein HC (2.0 mg) in 1.0 ml of phosphate-buffered saline, pH 7.3, was incubated with 0.1 M [14C]methylammonium chloride for 1 h at 37°C. Protein HC was then separated from low molecular weight reagents by size-exclusion HPLC.

**High Performance Liquid Chromatography and Photodiode Array Monitoring**—A Waters HPLC system connected on line with a Waters 900 photodiode array detector with a dynamic range from the ultraviolet to the visible region (190-800 nm) was used. A NEC APC III personal computer was used to control the chromatographic system and analyze the resulting chromatograms. A Nova Pak C-18 column (3.9 x 150 mm) protected by a guard column packed with µBondapak C-18/Corsair was used for reversed-phase HPLC. The column was equilibrated with 0.1% (v/v) trifluoroacetic acid and eluted with the following acetonitrile gradient: from 0% to 23% in 270 min, from 23% to 32% in 30 min, and from 32% to 80% in 60 min (Fig. 1A). The column was run at room temperature at a flow rate of 0.5 ml/min. A TSK-3000 SWG column (2.5 x 30 cm), fitted with a TSK-3000 SWG guard column, was used for size-exclusion HPLC. The column was eluted with 0.1 M ammonium acetate buffer, pH 6.9, at room temperature and at a flow rate of 1 ml/min. All the photodiode array spectra were run from 200 to 600 nm during reversed phase or size-exclusion HPLC and were analyzed using several different postrun data programs. The “spectrum index” plot was used to determine automatically all absorption spectra either at the peak maximum or at any point of the peak. The “spectrum analysis” plot was used to provide another view of the spectra to discern easily any similarities or differences (Figs. 1 and 2). Chromatograms produced by monitoring a separation for absorption at 230 and 330 nm were compared by using the “multichromatogram” mode of analysis. Peak areas were calculated by use of the “integrator” program.

**Amino Acid Sequence Analysis**—The method of Edman and Begg (29) was used for automated sequence analysis in a Knauer modular liquid-phase protein sequencer model 810 equipped with a Knauer phenylthiophenylpentin (PTH) amino acid derivative analyzer. Samples were analyzed in the presence of Polybrene employing the wet filter technique in a flow-through reactor. The PTH-derivatives were identified and quantified using a reversed-phase HPLC system based upon a Lichrospher RP 18 column (0.2 x 25 cm) eluted with 6.5 mM sodium acetate, pH 4.77, and a 15–100% acetonitrile gradient.

**Amino Acid Analysis**—Samples were hydrolyzed at 110°C for 20 h in 0.1 ml of 5.7 M HCl, containing 0.05% (v/v) 2-mercaptoethanol, in evacuated and sealed tubes. The quantitative analyses were performed using a Beckman system 6000 high performance amino acid analyzer. Half-cystine was determined either as carboxymethylcysteine or as cysteic acid after performic acid oxidation (30).

RESULTS

Protein HC displays a characteristic absorption spectrum with appreciable absorbance above 300 nm and with an inflection point at 330 nm (Fig. 1A). As the amino acid residues of polypeptide chains do not show any absorbance above 320 nm, it was decided to use the absorbance at 330 nm as an indicator for the chromophore of protein HC upon fractionation of proteolytic digests of the protein.

**Pepsin Digestion of Native Protein HC**—When native human protein HC was analyzed by a reversed-phase HPLC system using a C-18 column, elution with an acetonitrile gradient, and monitoring the light absorption of the effluent at 230 and 330 nm, the resulting chromatogram showed a single peak of coinciding 230 and 330 nm absorption near the
end of the gradient at about 60% acetonitrile (Fig. 1A). The native protein HC preparation was then digested with pepsin, and the resulting fragments were separated by the reversed-phase HPLC system. The resulting chromatogram showed that virtually no undigested protein HC was present in the mixture, that 60% of the material absorbing light at 330 nm was represented by a wide irregularly shaped peak at an acetonitrile concentration of about 30%, and that this peak only represented 23% of the material absorbing light at 230 nm (Fig. 1B). The overall yields of material with 230 or 330 nm light absorption were calculated from the areas below the corresponding absorption chromatograms for undigested and digested protein HC and were found to be 60 and 65%, respectively. The material represented by the main peak of 330 nm absorption was pooled as indicated (pool A of Fig. 1B). Each fraction constituting pool A was oxidized by performic acid and analyzed for amino acid composition. The results showed that all fractions had nearly identical amino acid compositions and contained significant amounts of cysteic acid. The absorption spectrum of this pool was compared to that of undigested native protein HC and was found to be similar, except that the absorption at 250 nm was lower than that of native protein HC (Fig. 2A). Amino acid sequence analysis of the material of pool A identified two sequences corresponding to the amino acid residues 26-48 of the protein HC polypeptide chain (Fig. 3). No derivative identifying the cysteine residue at position 34 could be detected. Quantitative amino acid analysis of all fractions of the whole separation of pepsin-digested protein HC was also performed and showed that only the fractions of the above-mentioned pool A contained peptides including the cysteine residue at position 34 of the protein HC polypeptide chain. Peptides including the 2 additional cysteine residues at positions 72 and 169 of the polypeptide chain could be identified in other fractions.

Pronase Digestion of the Pepsin-produced Chromophore-Peptide Mixture—In order to more clearly define the position of the bond between the chromophore and the polypeptide chain, the material of pool A was further digested with pronase, and the resulting mixture was fractionated by the reversed-phase HPLC system described above. The resulting chromatogram showed that a major portion of the material absorbing light at 230 and 330 nm (pool B of Fig. 2B) still co-eluted at the same acetonitrile concentration as the material of pool A (Fig. 1B). The absorption spectrum of the material of pool B was compared to that of the material of pool A and was found to be essentially identical, except that the relative absorbance at 280 nm was considerably less (Fig. 2A). The material of pool B was then subjected to automated Edman degradation. The chromatograms for identification of the PTH-derivatives of the first six degradation cycles are shown in Fig. 4 and indicate the presence in the analyzed material of a tripeptide with the sequence Thr-X-Pro, representing the amino acid residues 33-35 of the protein HC polypeptide chain (Fig. 3).

An aliquot of pool B was oxidized with performic acid and then subjected to quantitative amino acid analysis after hydrolysis with 5.7 M hydrochloric acid. Approximately equal amounts of threonine, cysteic acid, and proline could be identified in the hydrolyzed material.

Reduction and Carboxymethylation of the Pronase-produced Chromophore-Tripeptide Complex—The material of pool B was reduced by 35 mM dithiothreitol in 6 M guanidinium chloride and then 14C-carboxymethylated with a molar excess of iodoacetic acid. The mixture was thereafter fractionated by the reversed-phase HPLC system. Eighty-five percent of the reduced and carboxymethylated material with light absorption at 330 nm eluted at essentially the same position in the acetonitrile gradient as the unreduced material and was labeled with radioactivity as shown in Fig. 2C. The absorption spectrum of the reduced material (pool C in Fig. 2C) was virtually identical with that of the material before reduction (Fig. 2A). The reduced material of pool C was then subjected to six Edman degradation cycles. The chromatograms for identification of the PTH-derivatives of these cycles were almost identical with those shown in Fig. 4 for the unreduced material and thus indicate the presence in the analyzed material of a tripeptide with the sequence Thr-X-Pro. An aliquot of the reduced and carboxymethylated material of pool C was hydrolyzed with 5.7 M hydrochloric acid and then subjected to quantitative amino acid analysis. The amino acids threonine, carboxymethylcysteine, and proline were found in the analyzed material in approximately equal amounts.

Chymotryptic and V8 Protease Digestion of Protein HC—The reversed-phase HPLC system was also used to fractionate native protein HC digested with chymotrypsin or reduced and carboxymethylated protein HC digested with S. aureus V8 protease. The chromatograms (not shown) demonstrated the presence in both digests of material with absorbance at 330 nm, eluting at the same acetonitrile concentration as the major portion of the 330 nm absorbing material produced by pepsin digestion of protein HC. However, the yields of the corresponding materials were lower. Sequence analysis of the chymotrypsin-produced material showed the presence of a peptide representing residues 27-35 of the protein HC polypeptide chain. No PTH-derivative demonstrating the presence of a cysteine residue at position 34 could be found (Fig. 3). Sequence analysis of the V8 protease-produced material identified a sequence corresponding to residues 15-37 of the protein HC.
protein HC polypeptide chain. No PTH-derivative identifying the carbohydrate prosthetic group carrying asparagine residue at position 17 could be found. Neither could any derivative identifying the cysteine residue at position 34 of the protein HC polypeptide chain (Fig. 3).

Treatment of Native Protein HC with $^{14}$C Methylammonium Chloride or 4-Vinylpyridine—In order to investigate the presence of thioester bonds between the protein HC polypeptide chain and the chromophore, native protein HC was incubated with $^{14}$C methylammonium chloride and thereafter analyzed by the reversed-phase HPLC system described above. No radioactivity was incorporated into the protein and the chromatograms recorded at 230 and 330 nm for treated and untreated protein HC were identical, showing the absence of thioester bonds in protein HC. To investigate the presence of free sulfhydryl groups in native protein HC, the native molecule was incubated with 4-vinylpyridine in the absence of reducing agents and then analyzed by the reversed-phase HPLC system. Treated and untreated protein HC eluted at identical positions and displayed identical ultraviolet absorption spectra, demonstrating the absence of free sulfhydryl groups in the protein.

**DISCUSSION**

The fractionation of pepsin-digested native protein HC by a reversed-phase HPLC system was monitored by measuring the light absorption of the effluent at 230 and 330 nm. The resulting recordings demonstrated that most of the peaks of 230 nm absorption did not coincide with peaks of 330 nm absorption. Only the wide and irregularly shaped peak of 230 nm absorption at the end of the chromatogram coincided with a peak of 330 nm absorption. The sequence analysis and amino acid composition of the material of all fractions of this peak gave essentially the same results and demonstrated that the same peptide fragments were present in all of the fractions. The same type of analyses were used to establish the homogeneity of the major chromophore-carrying peptide material produced by chymotryptic and V8 protease digestion of protein HC. The reason that the chromophore-carrying peptides are eluted as wide, irregularly shaped peaks might be that the chromophores bound at specific residues of the polypeptide chain are heterogeneous, and that the separation system used, developed for high resolution of peptides, is unsuitable for efficient separation of the heterogeneous chromophores. Although the pepsin-digested protein HC material subjected to automated Edman degradation was represented by such a wide, irregularly shaped peak of 230 nm light absorption, the released amino acid derivatives strongly indicated that the material contained only two major peptides released from the polypeptide chain. Both peptides correspond to segments comprising amino acid residues 26–48 of the protein HC polypeptide chain. Significantly, no amino acid derivative could be identified corresponding to the cysteine residue at position 34. Since the peptide material analyzed by automated Edman degradation carried 60% of the material with light absorption at 330 nm, these results strongly indicate that a major part of the chromophore material is linked to the cysteine residue at this position. The isolation of a chromophore-carrying tripeptide from the longer pepsin-produced
chromophore-carrying peptides by pronase digestion gives further support to this conclusion, in particular since the automated Edman degradation of this tripeptide indicated its sequence to be Thr-X-Pro, which corresponds to residues 33–35 of the protein HC polypeptide chain. Reduction and $^{14}C$-carboxymethylation of the isolated chromophore-carrying tripeptide in 6 M guanidinium chloride does not cleave the chromophore-tripeptide bond, thus demonstrating that it is resistant to reduction. This result agrees with the repeated earlier observation that not even complete reduction and carboxymethylation of native protein HC will disrupt its chromophore linkage (1, 3).

Several results obtained in the present investigation relate to the status of the cysteine residue at position 34 of the native protein HC polypeptide chain. 1) Native protein HC does not contain any free sulfhydryl groups. 2) Amino acid sequence analysis of the native and of the reduced and carboxymethylated chromophore-tripeptide complex does not identify any amino acid derivative at the position corresponding to the cysteine residue. 3) Hydrolysis of the performic acid-treated native chromophore-tripeptide complex and of the reduced and carboxymethylated chromophore-tripeptide complex releases cysteic acid and carboxymethylcysteine, respectively. 4) No thioester bonds are present in protein HC. Two models for the status of the cysteine residue at position 34, compatible with all these observations, can be conceived.

In the first model, the sulfhydryl group of the cysteine residue is involved in a reduction-sensitive bond to the chromophore and an additional, reduction-resistant, bond is also involved in linking the chromophore to the cysteine residue. In the second model, the sulfhydryl group of the cysteine residue is involved in a reduction-sensitive bond to an unidentified ligand, different from the yellow-brown chromophore, while an additional, reduction-resistant bond is involved in linking the chromophore to the cysteine residue. Obviously, the models exclude that the cysteine residue is involved in thioester bonding.

It should be emphasized that the results of the present study do not allow a final conclusion that 100% of the chromophore of protein HC is bound via the cysteine residue at position 34 of the polypeptide chain, since not all material absorbing light at 330 nm in different types of proteolytic digest could be demonstrated to co-elute with peptides including the cysteine residue at position 34. Whether this is due to experimental difficulties or signals that a minor part of the chromophore is bound via other residues remains to be established.

The present results may explain earlier difficulties in elucidating the primary structure of the protein HC polypeptide chain around the cysteine residue at position 34. For example, during our studies to define the disulfide bridges of protein HC by the diagonal map technique, no peptides containing the cysteine residue at position 34 were found to move off the diagonal, presumably because they all carried the heterogeneous protein HC chromophore (14). A study concerning the complete amino acid sequence of the protein HC polypeptide chain failed to identify a 10-residue long segment of the chain including the cysteine residue at position 34 (31). In another study of the primary structure of protein HC (21), the cysteine residue at position 34 was identified, although the yields of the isolated peptides carrying this cysteine residue were always extremely low in comparison to the yields of the peptides carrying the other 2 cysteine residues of the protein HC polypeptide chain. All these difficulties are probably caused by the fact that the presence of the heterogeneous chromophore in peptides renders them chromatographically heterogeneous. Two more recent studies of cDNA for human protein HC confirm the presence of a cysteine residue at position 34 of the polypeptide chain (18, 32).

The uncharacteristic absorption spectrum of the chromophore-tripeptide complex is almost identical with that of native protein HC, except that the absorption at 280 nm is considerably less. This demonstrates that the chromophore material linked at only one specific site of the protein HC polypeptide chain is very heterogeneous and that it significantly contributes to the overall heterogeneity of the molecule. Indeed, the heterogeneity of the chromophore linked to the cysteine residue at position 34 probably accounts for all of the heterogeneity of protein HC, since no other sites for chromophore linkage have been identified and since no variation has been described for the sequence of the protein HC polypeptide chain, nor for the structure of the carbohydrate prosthetic groups of the molecule. The absorption at 280 nm by the chromophore-tripeptide complex is higher than its absorption at 330 nm, despite the absence of aromatic amino acid residues in it, demonstrating that the chromophore of protein HC significantly contributes to the high absorbance at 280 nm displayed by the protein. It also explains the early observation that the molar extinction coefficient of protein HC, as calculated from its content of aromatic amino acid residues, is much lower than that experimentally determined (3).

Although the present study does not allow a final determination of the chemical nature of the protein HC chromophore and its bond to the polypeptide chain, it strongly indicates that virtually all of the heterogeneous chromophore is linked to the cysteine residue at position 34 of the polypeptide chain via a reduction-resistant bond and that the heterogeneity of the chromophore is responsible for a major portion, if not all, of the protein HC charge heterogeneity. The results can be used to design further studies of the chromophore and its bond to the polypeptide chain and thus aid in the final elucidation of the crucial biological role of protein HC as a lipocalin.

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