On the Subunit Structure of the Protein of Human Serum High Density Lipoprotein

II. A STUDY OF SEPHADEX FRACTION IV*

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SUMMARY

Fraction IV, separated and purified from delipidated human serum high density lipoprotein of d 1.063 to 1.125 g per ml (HDL) by gel filtration in 8 M urea, was further studied before and after reduction with β-mercaptoethanol (β-ME), or reduction and carboxymethylation (iodoacetamide). Both the reduced (R-IV) and the reduced and S-carboxymethylated (SC-IV) preparations exhibited a single band by polyacrylamide gel electrophoresis with a molecular weight of about 8,500, a figure corroborated by Sephadex G-200-8 M urea and agarose-guanidine HCl column chromatography. In the absence of the reducing agent, Fraction IV had a molecular weight of about 17,000. Treatment of R-IV (in the presence of β-ME) or of SC-IV by the bifunctional reagent dimethylsuberimidate led to the formation of four components separable by analytical sodium dodecyl sulfate (SDS)-polyacrylamide gel, with an apparent molecular weight of approximately 8,500; 17,000; 25,000; and 33,000. In turn, unreduced and suberimidate-treated IV, exhibited two bands (SDS-polyacrylamide) with an apparent molecular weight of 17,000 and 33,000, respectively. In initial studies, where partially carbamylated preparations were used, fractionation of SC-IV by DEAE-cellulose column chromatography yielded two major and three minor fractions which all reacted with antiserum raised in rabbit against whole Fraction IV, and differed mainly from each other in lysine and homocitrullin content.

In subsequent studies, which used noncarbamylated products, SC-IV gave a single peak by both Sephadex and DEAE-chromatography, a major component by isoelectric focusing, and exhibited a single band by 8 M urea SDS-polyacrylamide gel electrophoresis. This preparation had no histidine, arginine, or tryptophan, and had glutamine as COOH-terminal (carboxypeptidase digestion and hydrazinolysis). It contained no sialic acid. showed no NH-terminal by either dinsylation or by the Edman's procedures, and gave a single precipitin line against specific antisera.

It is concluded that Fraction IV, as separated and purified from apo HDL by Sephadex chromatography, is made up of chemically very similar, and possibly identical protomers each having the same molecular weight (about 8,500). The data also indicate that in HDL these protomers are paired by a single disulfide linkage into dimers of equivalent weight.

Previous studies from this laboratory showed that Fraction IV, as isolated from apo HDL by Sephadex chromatography in 8 M urea, gives multiple bands by analytical isoelectric focusing polyacrylamide gel, this suggesting, although not proving, that it may have heterogeneous composition (1). More recently, we provided evidence for the important structural role of disulfides in Fraction IV (2, 3). Such observation proved important for the progress of our work on apo HDL structure. We here report on studies dealing with the isolation of pure Fraction IV and on its partial characterization by chemical, physical, and immunological methods. The relevance of these results as well as those on Fraction III, presented in the accompanying paper (4), will be discussed in the context of the over-all problem of HDL structure.

EXPERIMENTAL PROCEDURE

The sera used for the separation of IV were those employed in the preparation of III (4). Although the separation of IV by Sephadex G-200 chromatography has been described (1), there were significant departures from the published methods, so that a detailed description of the procedure employed in the current work is warranted.

Isolation and Purification of IV—Fraction IV was first obtained by Sephadex G-200 chromatography at 10°, using columns (100 × 5 cm) with ascending flow rate of the buffer (0.01 M Tris-HCl, pH 8.6, 10−4 M EDTA, 8 M urea) at 10 ml per hour. The location of the peaks was continuously monitored (ultraviolet recorder, Instrumentation Specialties Co., Lincoln, Neb.) at 280 nm and rechecked by manual readings at the same wave length.

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on a Gilford (Oberlin, Ohio) recording spectrophotometer. Fraction IV was freed of urea, concentrated in an Amicon Diaflo Ultrafiltration Cell, model 52, using UM2 membranes and then lyophilized. All of these operations were carried out at 4°C. Identification of Fraction IV was made by its position of migration in 8 M urea- or SDS-polyacrylamide gel electrophoresis (1) and by its reactivity against anti-rabbit human Fraction IV antisera. Whenever contamination by Fraction III was encountered, it was resolved by rechromatography of the impure fraction in Sephadex G-200 columns (100 x 2.5 cm) at 10° with a linear flow rate of the eluting buffer (0.01 M Tris, pH 8.2, 10° M EDTA, 8 M urea) at 10 ml per hour. A more effective separation of IV from III was achieved by adding, to the eluting buffer, β-ME (concentration of 0.1 g/100 ml); this agent has been shown to significantly retard the elution of Fraction IV from Sephadex columns (3). Alternatively, the impure preparations of IV were reduced and carbosytmethylated (see below) before column fractionation. The purity of Fraction IV, R-IV, or SC-IV, after rechromatography, was tested by SDS-polyacrylamide electrophoresis and reactivity against antisera prepared in the rabbit against unreduced IV, according to the scheme described for Fraction III (4).

**Conditions for Preparing Fraction IV Protomers—** In a previous report (3) we have shown that disulfide cleavage promotes the conversion of IV into its monomeric form. To effect such conversion, IV was either reduced by β-ME, or reduced by β-ME and then carbosytmethylated with iodoacetamide. The conditions for reduction or reduction and carbosytmethylatiori, utilized in the present report, were as described in the accompanying paper (4). The final product was extensively dialyzed against 0.01 M Tris, pH 8.2, 10°3 M EDTA before fractionation by DEAE-chromatography. The preparations directed for amino acid analysis were extensively dialyzed against distilled water.

Fractionation of R-IV or SC-IV by DEAE-chromatography—In the initial experiments, R-IV was fractionated with buffer gradients containing 0.01% β-ME. Later, however, because of high absorbance of β-ME at 280 nm, fractionation of SC-IV without the reducing agent was commonly adopted. Whatman DE 52 microgranular anion exchange cellulose was precelyed, equilibrated, packed, and degassed under the conditions described for Fraction III (4). Before application, SC-IV was dialyzed against the starting buffer, 0.01 M Tris HCl, pH 8.2, 10°3 M EDTA, 8 M urea. Up to 4 mg of protein were fractionated at 10° in columns (1 x 25 cm). In early studies a two-step Tris linear gradient of pH 8.2 was used; the first, 100 ml total volume, from 0.04 to 0.06 M Tris, pH 8.2; and the second, 500 ml total volume, between 0.06 and 0.1 M Tris, pH 8.2. Subsequently a single linear gradient, 0.01 to 0.1 M Tris, pH 8.2, was used. All buffers contained 10°3 M EDTA and 8 M urea and were pumped at a flow rate of 10 ml per hour. The effluents, collected in 2-ml fractions, were continuously monitored for absorbance at 280 nm and conductivity as described for Fraction III (4). The fraction obtained was freed of urea, concentrated, and then used for analysis either immediately or after storage in the lyophilized state.

Analytical Methods Employed in the Characterization of R-IV or SC-IV Subfractions Obtained by DEAE-chromatography—The methods of 8 M urea- and SDS-polyacrylamide gel electrophoresis and isoelectric focusing have been reported previously (1) as have the immunological analyses (1) that were carried out with anti-IV antisera prepared in the rabbit. Conditions for amino acid, NH2- and COOH-terminal and sialic acid analyses were those described for Fraction III (4).

**Cross-linking Experiments Using Unreduced IV, R-IV, or SC-IV—** The studies were carried out using the agent dimethyl suberimidate as described by Davies and Stark (5). After the reaction, each fraction was separated by SDS-polyacrylamide (1), and the apparent molecular weight was determined according to Weber and Osborn (6). Cleavage of the cross links was obtained by treatment with ammonia-acetic acid, 30:2 (4).

**Total Protein Determination—** This was carried out by the Lowry method (7) or by absorbance at 290 nm using the extinction coefficient E280 = 9.2, determined in the laboratory, taking into account the amino acid composition of Fraction IV. The value was not affected by reduction and carbosytmethylation.

Chemicals were all reagent grade or distilled as described for Fraction III. The precautions in utilizing urea solutions were detailed in the accompanying paper (4) and their significance outlined in the section on "Results."

**RESULTS**

**Separation and Purification of Fraction IV by Sephadex G-200 Chromatography—** As described previously (1) and shown in Fig. 1A, Sephadex G-200 chromatography in 8 M urea affords a separation of Fraction IV from the other apo HDL polypeptides. If contamination by Fraction V was suspected, this was readily resolved by rechromatography of the impure fraction under the same elution conditions (Fig. 1B). Contamination by Fraction III was more commonly observed principally by SDS-polyacrylamide. The most effective way to obtain a pure Fraction IV was by rechromatography (Sephadex G-200, columns (100 x 2.5 cm)) under reducing conditions (eluting buffer, 0.01 M Tris, 10° M β-ME). For experimental conditions, see text.

![Fig. 1. Steps of isolation and purification of IV from apo HDL2 by Sephadex G-200 chromatography. A, fractions from apo HDL2, B, rechromatography of IV in the absence of reducing agent; C, re-chromatography of IV (from A) in the presence of β-ME. For experimental conditions, see text.](http://www.jbc.org/content/jbc/144/1/5851/F1.large.jpg)
pH 8.6, 0.01% β-ME, 10⁻³ M EDTA) of the impure mixture. As shown previously (3) and also indicated in Fig. 1C, the reducing agent produces a marked retardation of the elution of Fraction IV, while not affecting III. We found it more practical to prepare SC-IV and purify it by Sephadex G-200 (100 × 2.5 cm column) equilibrated and eluted with 0.01 M Tris, pH 8.6 buffer, 10⁻³ M EDTA in the absence of β-ME. Under such conditions SC-IV is eluted as a single peak.

Electrophoretic Properties of IV, R-IV, and SC-IV—In initial studies, when urea-induced carbamylation was not completely controlled, unreduced or SC-IV exhibited four to six closely migrating bands by urea-polyacrylamide gel electrophoresis (Fig. 2). The fractions were readily separated by ion exchange chromatography (Fig. 3) and each gave a single line of precipitation against anti-IV antisera and exhibited a very similar amino acid composition (Table I) mainly characterized by the inverse relationship between lysine and homocitrullin content. In all subsequent studies where the precautions for preparing urea solutions, outlined in the accompanying paper (4) were used, no carbamylation was observed. SC-IV exhibited a single band by both 8 M urea (Fig. 4) and SDS-polyacrylamide gel electrophoresis.

![Fig. 2. Eight m urea-polyacrylamide electrophoresis of SC-IV and its DEAE-subfractions. For experimental conditions, see text (carbamylated preparation).](image)

![Fig. 3. DEAE-column chromatography of SC-IV. For details, see text (see Fig. 2).](image)

![Fig. 4. Polyacrylamide gel electrophoresis in 8 m urea of a purified preparation of SC-IV (uncarbamylated).](image)

| Amino acids | Whole protein | a | b | c | d | e |
|-------------|--------------|---|---|---|---|---|
| Aspartic...  | 33.0         | 30.4 | 31.0 | 31.0 | 30.8 | 33.0 |
| Threonine... | 61.0         | 62.5 | 62.0 | 56.2 | 52.6 | 45.0 |
| Serine...... | 50.7         | 50.8 | 52.2 | 50.2 | 50.8 | 56.2 |
| Proline..... | 40.1         | 38.2 | 39.1 | 39.0 | 41.2 | 38.4 |
| Glutamic...  | 155.2        | 156.0 | 159.0 | 156.0 | 146.2 | 130.2 |
| Glycinne..  | 30.6         | 32.2 | 32.6 | 32.1 | 33.0 | 45.5 |
| Alanine..... | 50.9         | 51.7 | 51.7 | 50.6 | 47.3 | 46.7 |
| Valine...... | 60.1         | 60.2 | 62.0 | 62.3 | 54.2 | 50.2 |
| Half-cystine | 10.0         | 9.8  | 9.8  | 9.9  | 7.9  | 7.5  |
| Methionine  | 8.9          | 8.8  | 8.7  | 8.1  | 6.3  |       |
| Isoleucine  | 9.8          | 9.1  | 9.0  | 8.8  | 7.9  | 7.0  |
| Leucine..   | 90.2         | 91.3 | 90.5 | 85.4 | 77.6 | 60.9 |
| Tyrosine... | 40.7         | 42.1 | 40.8 | 38.2 | 38.4 | 30.4 |
| β-Alanine.. | 40.7         | 41.4 | 40.3 | 39.2 | 37.9 | 30.2 |
| Lysine..... | 94.3         | 90.0 | 91.0 | 76.8 | 59.6 | 56.8 |
| Histidine...| 0            | 0    | 0    | Trace | 1.1 |
| Arginine... | 0            | 0    | 0    | Trace | 0.9 |
| Tryptophan..| 0            | 0    | 0    | 0    | 0    |
| Homocitrullin| 0            | 0    | 7.5  | 15.7 | 17.0 | 18.6 |

Average of two determinations.

Fraction e was found to be contaminated by Fraction V, a minor fraction of apo HDL2 (1), as assessed by its reactivity against anti-V antisera. All of the other fractions reacted only against anti-IV antisera.

As carboxymethylecysteine.
sis (Fig. 11A). Unreduced IV, treated with $[^{14}C]$iodoacetamide, exhibited no changes in mobility in either of the two supporting media. Such a preparation, after extensive dialysis, was free of radioactivity indicating the absence of $-SH$ groups. In turn, when IV was either reduced with β-ME or the step or reduction was followed by carboxymethylation (iodoacetamide), there was a significant increase of its electrophoretic mobility by SDS-polyacrylamide as shown previously (3) with apparent molecular weight of unreduced IV and SC-IV of 17,000 and 8,500, respectively (Fig. 5). Identical results were obtained when HDLβ, first incubated with 1% SDS, was analyzed by SDS-polyacrylamide in the presence and absence of β-ME.

**DEAE-column Chromatography Studies**—Under the experimental conditions followed, either IV or SC-IV exhibited a single component (Fig. 6) which again gave a single band by either 8 M urea or SDS-polyacrylamide electrophoresis. A major component, although with a minor undefined shoulder was observed by isoelectric focusing (Fig. 7). Unreduced or SC-IV gave a single line of precipitation against rabbit anti-IV antiserum (Fig. 8).

**Chemical Studies on DEAE-fraction**—The amino acid composition of IV is given in Table I. Characteristically, this protein had 1 mole of carboxymethylcysteine per mole of protomer (mol wt ~8500) and had virtually no histidine, arginine, or tryptophan.

The time course of hydrolysis by carboxypeptidase A suggested glutamine as the COOH-terminal with threonine as the penultimate (Fig. 9). This conclusion was supported by the results with carboxypeptidase B and by the hydrazinolysis studies which failed to detect any COOH-terminal residue. No NH$_2$-terminal amino acid was detected in either IV or SC-IV by either dansylation or by the Edman’s procedure, suggesting a blocked terminal.

Fraction IV contained no sialic acid as assessed by both enzymatic (neuraminidase) or acid hydrolysis.

**Cross-linking Experiments**—After reaction with the bifunctional reagent suberimidate, both SC-IV and R-IV gave, on
FIG. 9. Hydrolysis of SC-IV by carboxypeptidase A. Protein, 0.025 μmole, was used in these studies.

SDS-polyacrylamide electrophoresis, four bands (Fig. 10) with approximate molecular weights of 8,500; 17,000; 26,000; and 34,000 (Fig. 11) indicating a protomer, dimer, trimer, tetramer relationship and suggesting, according to Davies and Stark (5), that Fraction IV contains four protomeric units. This conclusion was also supported by the observation that unreduced IV (dimer) treated with suberimidate gave two bands on SDS-polyacrylamide with molecular weights of 18,000 and 32,000, respectively.

Studies on Single Donors—These studies showed no significant differences in results among the individuals examined. Only in one case COOH-terminal threonine was found and this was reported in a preliminary communication (8). In subsequent studies this proved to be an exception as glutamine was the common C-terminal among the individuals studied. It may be stressed that the present work relates to a rather homogeneous class of donors. Whether the same results will extend to a more heterogeneous group of subjects remains to be established.

DISCUSSION

The present studies have shown, in accord with a recent report by this laboratory (3), that Fraction IV, isolated and purified from apo HDL, by Sephadex chromatography in 8 m urea (1), exists as dimers (mol wt of about 18,000) which are converted into protomers by disulfide cleavage. The protomers were shown to have the same molecular weight (about 8,500) and to contain one half-cystine per mole of protein, thus indicating that these protomers are linked as dimers by a single disulfide bridge. The reasons for the chemical selectivity of such a pairing process are not apparent and reoxidation experiments presently underway in this laboratory are expected to help in resolving this problem. In this context, it is important to establish whether the dimers are composed of identical or nonidentical monomers, a question which was not conclusively answered in the present studies. Our data showed that the SC-IV protomers were indistinguishable from each other when reacted against antisera raised in the rabbit against unreduced IV. On the other hand, their reactivity against anti-SC-IV was not tested. When care was taken to prevent carbamylation, SC-IV separated as a single component by either analytical polyacrylamide gel electrophoresis or preparative DEAE-column chromatography. Yet the partially carbamylated fractions exhibited, besides the expected changes in lysine content, slight but possibly signifi-
dant differences in amino acid composition suggesting, but far from proving, polypeptide heterogeneity. Furthermore, no sialic acid was detected in either SC-IV or its partially carbamyalted DEAE-subfractions; yet it cannot be ruled out that differences among protomers may exist regarding other sugars whose structural significance is under investigation. This information and the results of the NH₂-terminal and COOH-terminal analysis may be taken to conclude that the dimers of Fraction IV are made of very similar polypeptide chains. However, establishment of their identity is in need of further work, now in progress in this laboratory.

The finding that Fraction IV has COOH-terminal glutamine is in accord with the data of Shore and Shore (9) and Kostner and Alaupovic (10). The lack of detection of NH₂-terminal by two common methodologies (dansylation and Edman’s procedure), an observation in agreement with the report of Kostner and Alaupovic (10), suggests that Fraction IV has a blocked terminus, whose nature is presently under investigation.

The cross-linking studies with dimethylsuberimidate may be taken to suggest that in the Fraction IV studied there were four protomers totalling to a mass of 32,000 daltons. On the other hand there are reservations against this conclusion since the investigation was not directed at the intact particle where this question is difficult to resolve because of the presence of other polypeptides of different chemical nature. Thus, it may be simply concluded on the basis of these the results in the accompanying paper (2) that HDL₄ contains heavy and light chains, the latter linked together as dimers. This conclusion does not account for Fraction V, which represents about 2 to 5% of the apo HDL₄ mass (1), and may not be considered as a true constituent of HDL₄. The latter statement is based both on reassembly studies (12) and on our recent work on HDL₄ (d 1.125 to 1.21 g per ml) and on VHDL of d 1.21 to 1.25 g per ml which prove the existence of high density lipoprotein species having both III and IV but little (HDL₃) and no (VHDL) Fraction V. The above conclusions open several questions of structural relevance. It is possible that HDL₄ as well as the HDL₃ class, as isolated by conventional cumulative flotation techniques may represent mixtures of subclasses varying in molecular weight, size, lipid content, and relative distribution of polypeptide chains. In terms of molecular weight, various figures for both HDL₃ and HDL₄ have been reported (14) probably reflecting a difference in source of material and method of preparation. Size heterogeneity for both HDL₃ and HDL₄ has been noted by electron microscopy (14) and data on the lipid distribution and protein-lipid ratio, although scanty, indicate significant variations from laboratory to laboratory (14). In addition, variations in III-IV ratios (R-Thr:R-Glu) within the HDL₃ and HDL₄ classes have been noted by immunochemical methods (15). Thus, it is possible as also suggested by the data on the reassembly studies (12), that in each molecular species of HDL, size and lipid content are determined by the ratio of III-IV and the relative affinity of those polypeptides for lipids. While this hypothesis is approachable experimentally, a more difficult question to resolve is whether or not ultracentrifugal species produced in vitro have their counterpart in circulation as a part of the physiological role of HDL in fat transport. At the moment, it is our hope that the resolution of the molecular structure of HDL may aid in the understanding of its function.

Finally, from the technical standpoint, we wish to comment on the fact that by employing high concentrations of urea under appropriate conditions of liquid chromatography, it is possible to effectively fractionate the various components of the protein moiety of serum HDL. However, the data in this and in the accompanying paper (4) have clearly shown that urea should be used cautiously because of its known capacity of inducing chemical modification in proteins (16) a phenomenon now documented for human serum high density lipoproteins. These precautionary steps have been outlined and should prevent the difficulties which may be encountered in the subfractionation of apo HDL₄ polypeptides.

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