Cross-linking Studies of the Self-association Properties of Apo-A-I and Apo-A-II from Human High Density Lipoprotein*

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The state of self-association of the apoprotein components of human high density lipoprotein have been studied by use of the cross-linking reagent dimethylsuberimidate. Analysis of the cross-linked products was carried out by sodium dodecyl sulfate-gel electrophoresis and by agarose column chromatography in 6 M guanidine hydrochloride. Apo-A-I was found to exist as a monomer at low concentration, but associates to tetrameric and pentameric forms at concentrations of 0.5 mg/ml or higher. The self-association was found to be ionic strength-dependent, with association promoted by the presence of salt. Apo-A-II was also found to associate, but the major oligomeric form observed was dimeric (M₈ = 34,000), and the association was less dependent on ionic strength than for apo-A-I. Cross-linking in the presence of various concentrations of guanidine hydrochloride showed that apo-A-II self-association persisted at higher concentrations of the denaturant than for apo-A-I. Studies of the effect of temperature demonstrated that the self-association of both proteins was diminished at temperatures above 30°C. Recombination of apo-A-II with phospholipid resulted in the formation of particles which yielded primarily trimers upon cross-linking. This suggests that phospholipid binding causes major reorganization of the self-associated forms of apo-A-II.

The major polypeptide components of human high density lipoproteins, A-I and A-II, have been intensively studied because of their importance in maintaining the structure of the high density lipoprotein particle (1-13). Although the primary and, to a lesser degree, secondary structure of these proteins have been determined, little is known about higher orders of structure, and in fact, the quaternary structure of apo-A-I is the subject of controversy.

Although earlier reports indicated that apo-A-I exists solely as a monomer in solution (6, 12), more recent studies suggest that this protein self-associates (3, 10, 13). However, these reports are in disagreement as to whether this apoprotein exists as an equilibrium mixture of monomers and dimers (3), dimers and tetramers (10), or monomers, dimers, tetramers, and octamers (10). Studies on apo-A-II are in good agreement that this protein exists mainly as a dimer in solution (3, 5), although the presence of a trimer has also been reported (11).

It is of considerable importance to learn whether or not the quaternary structure of apo-A-I and apo-A-II influences the ability of these proteins to bind lipid; despite several studies on this problem, the answer remains unclear (13-15). In order to interpret lipid reconstitution experiments with these apo-proteins, it is necessary to establish the quaternary structure of these components, as well as to determine how this structure is perturbed under the conditions of reconstitution experiments.

We have recently reported preliminary studies of the quaternary structure of apo-A-I and apo-A-II by chemical cross-linking with the reagent dimethylsuberimidate (16), as contrasted to the physical methods used in other laboratories. Apo-A-I was observed to self-associate to tetrameric and pentameric forms, while apo-A-II was observed to exist as monomeric and dimeric species. In the present study, we report a detailed characterization of parameters affecting the self-association properties of these proteins by use of the cross-linking method.

EXPERIMENTAL PROCEDURES

Materials—Dimethylsuberimidate dihydrochloride was purchased from Pierce and triethanolamine was obtained from Fisher. Urea (ultrapure) was purchased from Merck/Mann. Ultrapure guanidine chloride was obtained from Schwartz/Mann and from Research Plus Laboratories, Inc. and was recrystallized from water. Rabbit muscle aldolase, human γ-globulin, and Coomassie blue G-250 were purchased from Sigma. Human serum albumin was obtained from A. B. Kabi (Stockholm).

Methods—HDL was isolated from human plasma by preparative ultracentrifugation using the procedure of Havel et al. (17). Lipoprotein fractions were lyophilized and delipidated with ethanol/chloroform (3:1) by the procedure of Brown et al. (18). Apo-A-I and apo-A-II were purified either by gel chromatography using Sphadex G-200 (19) or by ion exchange chromatography using DEAE-cellulose (Whatman DE52, Reeve Angel), or both (20). All urea solutions were passed over an ion exchange resin immediately prior to use and were kept at 4°C. Care was taken to remove the urea by dialysis (against H₂O or saline (0.9% NaCl)) as soon as possible after protein isolation. Prior to cross-linking, proteins were freshly renatured by the addition of a 6 M guanidine solution, followed by exhaustive dialysis against 0.15 M NaCl or water. Protein concentrations were determined by amino acid analysis on a Beckman 120C amino acid analyzer or by measurement of optical density (3).

The standard conditions for the cross-linking experiments were as follows: 20 mg of DMSO was dissolved in 1 ml of 1 M triethanolamine/HCl, pH 9.7, and 1 part of this solution was added to 10 parts of protein solution. Unless otherwise noted, the reaction was carried out at room temperature and allowed to proceed for 90 to 120 min before terminating the reaction by the addition of SDS or ammonium acetate. We have previously shown that under these circumstances

1 In the case of apo-A-II, which is composed of two identical M₈ = 8,600 polypeptide chains linked by covalent S-S bonds, the term monomer will be used to refer to the M₈ = 17,000 species.
2 The abbreviations used are: DMS, dimethylsuberimidate; SDS, sodium dodecyl sulfate; μ, ionic strength.
the cross-linking reaction proceeds to completion (16). In studies of the effect of varying ionic strength on self-association, the stock protein solution was mixed with an equal volume of 0 to 4 M NaCl. Co-linking was effected by the addition of 1 part DMS, 10 mg/ml in 0.3 M triethanolamine (to reduce the contribution to the ionic strength by the buffer), to 10 parts protein solution. Cross-linked samples were analyzed by SDS-polyacrylamide gel electrophoresis using a phosphate buffer and a 3 to 7% acrylamide gradient slab gel (21). Gels were stained in 0.1% Coomassie blue G-250, 50% methanol, 7% acetic acid and destained in 7% acetic acid, 5% methanol. In some experiments, gels were scanned at 550 nm on a Clifford Instruments densitometer (Corning Instruments).

The extent of cross-linking was determined by acid hydrolysis and amino acid analysis of the cross-linked protein. Since some lysine is a residual of partial hydrolysis of the amidinated lysines, a time course was carried out with cross-linked apo-A-I to provide a correction factor. By linear extrapolation back to zero time, it was found that after 18 h of hydrolysis, approximately 15% of the amidinated lysines were hydrolyzed to yield lysine residues.

Phospholipid vesicles were prepared by sonication of egg yolk phosphatidylcholine (Sigma) as described by Morrisett et al. (22). Sedimentation equilibrium studies were carried out in a Beckman model E analytical ultracentrifuge, using the high speed technique of Yphantis (23). Circular dichroism measurements were performed using a Cary model 60 Spectrophotometer, with a 6001 CD attachment, which was calibrated at 280 nm with (+)-camphorsulfonic acid. Fluorescence measurements were carried out with a Perkin-Elmer MPF-3 fluorescence spectrophotometer.

Gel permeation chromatography in 6 M guanidine hydrochloride (ultrapure, Schwarz/Mann) was carried out essentially as described by Fisk et al. (24), with a few modifications. A column (1.6 x 90 cm) containing Sepharose CL-4B (Pharmacia) was used in these studies; the greater porosity permitted resolution in the 50,000- to 300,000-dalton range and the cross-linked gel gave better stability and reproducibility than its uncross-linked counterpart. Molecular weight standards (γ-globulin, human serum albumin) were tagged with a small amount of [14C]succinic anhydride and cross-linked with dimethyl-suberimidate (2 mg/ml) at a protein concentration of 10 mg/ml using the method already described. One or the other of these cross-linked proteins was added to cross-linked apo-A-I in order to serve as an internal standard in any given experiment. The void volume (Vo) was determined by the elution of blue dextran and the total accessible volume (Va) was determined with [14C]succinic acid. The volume Va represents the total bed volume less the volume occupied by the polymer matrix.

The identity of oligomeric peaks was verified by dialysis of individual fractions, followed by SDS-gel electrophoresis. The elution position of marker proteins was determined by counting radioactivity. The distribution coefficient Kd was determined from the relationship

\[ K_d = \frac{(V_e - V_o) / (V_a - V_o)}{V_e} \]

where Ve is the elution volume for a given peak.

RESULTS

Cross-linking of Apo-A-I (Control Experiments)—We have previously shown (16) that when apo-A-I is cross-linked with DMS and subjected to SDS-gel electrophoresis, five prominent bands can be seen (Fig. 1F). A cross-linked sample of the tetrameric protein, aldolase (Mr = 160,000), was co-electrophoresed (Fig. 1G) and used as a molecular weight standard (25). Above the main band can be seen a small amount of α,β-dimer, tetramer, hexamer, and octamer forms. However, since cross-linking at high protein concentrations were obtained, thus extending the range of molecular weight calibration. The apparent molecular weights for the apo-A-I oligomers are quite close to those obtained by SDS-gel electrophoresis. Since apo-A-I has been reported to associate to octamers (10), we were particularly interested in evaluating whether the four main oligomeric species we observed might not represent dimer, tetramer, hexamer, and octamer forms. However, since...
the largest major oligomer nearly co-elutes with the $\gamma$-globulin monomer ($M_r = 150,000$), this species is most likely a pentamer ($M_r = 140,000$) rather than an octamer ($M_r = 224,000$) of apo-A-I. It should be noted that the nonlinearity observed at values of $K_d$ above 0.65 is consistent with the results of Fish et al. (24).

Because the apoproteins are isolated under denaturing conditions, it was necessary to establish whether the cross-linking pattern observed might be dependent upon the method used for refolding the protein. To test this, solutions of apo-A-I were dialyzed against water, 0.15 M NaCl, 0.1% SDS, 6 M guanidine hydrochloride, or 8 M urea. Following this, the solutions were dialyzed against several changes of 0.15 M NaCl, cross-linked, and electrophoresed on SDS-gels. The patterns obtained were virtually indistinguishable for each treatment. However, for all subsequent studies the apoprotein was carefully "renatured" by dissolution in 6 M guanidine, followed by slow removal of the denaturant by dialysis, in order to guarantee reproducibility in refolding. This refolding was checked by measurement of the CD spectrum in the 200- to 230-nm region (4, 6). To evaluate the effect of the reagent-to-protein ratio used, experiments were carried out in which the DMS concentration ranged from 0.1 to 9 mg/ml, while the apo-A-I concentration was maintained at 1 mg/ml.

Within the interval of 0.5 to 5 mg of DMS/ml the number of cross-links per molecule of protein increased approximately linearly with the DMS concentration. However, the reaction with lysine was complete, with only 0.9 unmodified lysines remaining. With such extensive modification, we were concerned that the cross-linking reaction might perturb the structure of the protein being studied. To evaluate this possibility, the circular dichroic spectra of samples of cross-linked and untreated apo-A-I were compared in the peptide bond region (200 to 230 nm) and were found to be identical (Fig. 3), indicating that no alteration in secondary structure accompanies the cross-linking reaction. This is most likely accounted for by the fact that the amidination reaction does not alter the net charge on the protein (26). It can also be seen in Fig. 3 that the circular dichroic spectrum of apo-A-I shows concentration-dependent changes, as has already been observed by Stone and Reynolds (3).

The fluorescence emission spectra were also studied and were found to be identical for treated and untreated apo-A-I when the samples were excited at 280 nm ($\lambda_{em} = 338$ nm) or at 296 nm ($\lambda_{em} = 339$ nm). This suggests that the tertiary structure, as reflected in the environment of the tryptophan, is not modified by cross-linking.

Since a major feature of cross-linked apo-A-I was the predominance of tetrameric and pentameric forms, it was necessary to evaluate the possibility that these forms were the result of transient collisions between monomeric species. Such artificial results are ruled out by the fact that oligomers of apo-A-I are observed at low protein concentrations (<1 mg/ml) where cross-linking due to transient collisions is regarded as being negligible (27). This is shown in Fig. 1 (A to C) where the proteins myoglobin, cytochrome $c$, and ovalbumin yield monomers on cross-linking at concentrations where apo-A-I yields tetramers and pentamers (Fig. 1F). Inclusion of SDS in the cross-linking mixture eliminates the oligomeric forms of the protein, presumably through dissociation (Fig. 1E); two bands can be seen in these samples which may correspond to conformers which differ in the amount of detergent bound. SDS does not interfere with cross-linking per se as shown by the equal modification of lysines in samples with and without the detergent (Table II). Also, cross-linking at higher temperatures, which does not significantly affect the extent of cross-linking, eliminates the higher oligomeric forms in apo-A-I. Since higher temperatures would favor increased collision frequencies, we believe that cross-links resulting from collision do not contribute significantly to the observed results.

**Concentration Dependence of Apo A-I Self association**

The extent of the cross-linking reaction with apo-A-I, when carried out under our standard conditions (see "Methods"), was determined by amino acid analysis (Table I). It can be seen that the only amino acid reduced in amount is lysine, thus verifying the specificity of the reaction for primary amino groups (26); contrary to expectations, no reduction in aspartic acid (corresponding to the NH$_2$ terminal group) was observed. However, the reaction with lysine was complete, with only 0 to 1 unmodified lysines remaining.

With such extensive modification, we were concerned that the cross-linking reaction might perturb the structure of the protein being studied. To evaluate this possibility, the circular dichroic spectra of samples of cross-linked and untreated apo-A-I were compared in the peptide bond region (200 to 230 nm).

**Table I**

| Amino acid | Apo-A-I | Apo-A-II |
|------------|---------|---------|
|            | Unreacted | Cross-linked | Unreacted | Cross-linked |
| Lysine     | 21       | 0.1 $\pm$ 0.06 | 9         | 0.0 $\pm$ 0.3 |
| Histidine  | 5        | 0.7 $\pm$ 0.1  | 0         | 0.5 $\pm$ 0.3 |
| Arginine   | 16       | 16.0 $\pm$ 0.4 | 0         | 0.6 $\pm$ 0.2 |
| Aspartic   | 21       | 21.5 $\pm$ 0.2 | 3         | 3.6 $\pm$ 0.1 |
| Threonine  | 10       | 9.6 $\pm$ 0.2  | 6         | 5.9 $\pm$ 0.2 |
| Serine     | 14       | 14.1 $\pm$ 0.1 | 6         | 5.5 $\pm$ 0.2 |
| Glutamic   | 47       | 48.2 $\pm$ 0.4 | 16        | 16.2 $\pm$ 0.3 |
| Proline    | 10       | 8.8 $\pm$ 0.3  | 4         | 3.3 $\pm$ 0.9 |
| Glycine    | 10       | 10.2 $\pm$ 0.1 | 3         | 2.9 $\pm$ 0.1 |
| Alanine    | 19       | 18.8 $\pm$ 0.3 | 5         | 4.8 $\pm$ 0.1 |
| Valine     | 13       | 13.0 $\pm$ 0.2 | 6         | 5.5 $\pm$ 0.7 |
| Methionine | 3        | 3.8 $\pm$ 0.1  | 1         | 0.9 $\pm$ 0.1 |
| Isoleucine | 0        | 0           | 1         | 0.1 $\pm$ 0.1 |
| Leucine    | 39       | 38.9 $\pm$ 0.6 | 8         | 8.2 $\pm$ 0.2 |
| Tyrosine   | 7        | 6.6 $\pm$ 0.3  | 4         | 3.6 $\pm$ 0.4 |
| Phenylalanine | 6       | 6.1 $\pm$ 0.2 | 4         | 3.7 $\pm$ 0.4 |

* Corrected for 15% conversion of modified lysine to free lysine.
Cross-linking of Apo-A-I and Apo-A-II

![Circular dichroic spectra of cross-linked and untreated apo-A-I](image)

**FIG. 3.** Circular dichroic spectra of cross-linked and untreated apo-A-I. Samples of apo-A-I in 0.1 M NaCl at either high (open symbols) or low (filled symbols) concentration were treated with DMS (1.8 mg/ml in 0.091 M triethanolamine-HCl, pH 9.5) or with buffer alone and allowed to react for 2 h. These samples were then dialyzed against 10 mM NaHPO₄, pH 6.9, for 2 days prior to circular dichroism measurements. Final concentrations of untreated apo-A-I were 0.458 mg/ml (M) and 0.0364 mg/ml (U); for cross-linked apo-A-I these were 0.459 mg/ml (■) and 0.0364 mg/ml (□).

### Table II

| Cross-linking conditions | Lysines modified |
|--------------------------|-----------------|
| 0.091 M TEA (pH 9.5), RT | 20.1            |
| 0.091 M TEA (pH 8.5), 35°C | 18.9           |
| 0.091 M TEA (pH 8.5) + 0.1% SDS, RT | 20.7        |
| 0.027 M TEA (pH 8.5), μ = 0.150, RT | 13.3        |
| 0.027 M TEA (pH 8.0), μ = 0.029, RT | 11.8        |
| 0.091 M TEA (pH 9.0), 4.0 M guanidine, RT | 12.5        |

*TEA, triethanolamine; RT, room temperature.*

The effects of parameters such as concentration, ionic strength, temperature, etc., on the self-association equilibrium.

**Ionic Strength Dependence of Apo-A-I Self-association—**
The ionic strength dependence of the self-association of apo-A-I was investigated as described under "Methods." It was observed that increasing the ionic strength at a constant apo-A-I concentration caused enhanced self-association (Fig. 5A). At an ionic strength of 0.025, no tetramer or pentamer was observed. However, 32% of the protein was found in the tetrameric and pentameric forms when the ionic strength was increased 10-fold. At lower concentrations of apo-A-I, the general features of the curve were the same, except that a lower level of association was observed.

To determine if the cross-linking reaction was being altered by changes in ionic strength, aldolase was also reacted with DMS over the range of ionic strength used for A-I (0.027 to 0.245); the cross-linking pattern was found to be independent of the ionic strength. In addition, samples of A-I cross-linked at high and low ionic strength were subjected to amino acid analysis. The same number of lysine residues were modified under both conditions (Table II). These experiments indicate

![Concentration dependence of the self-association of apo-A-I and apo-A-II](image)

**FIG. 4.** Concentration dependence of the self-association of apo-A-I (A) and apo-A-II (B). Apoproteins were cross-linked at various concentrations and electrophoresed on SDS slab gels, the stained gels were scanned for optical density and the per cent of total area in each peak was calculated. For apo-A-I, the results are expressed as summation of the areas for n-mers, n = 2 to 5, (Σs > 1) as a per cent of total area (n = 1 to 5). Apo-A-II data are presented as the percentage of dimer as a function of concentration. Data for apo-A-II are averages of three values for each concentration. Data for apo-A-I are averages of four values, except at concentration ≥2.5 mg/ml which are averages of two values. Since apo-A-I was readily available at higher concentrations, the study of apo-A-I was extended to higher concentrations than were used for apo-A-II. The vertical lines indicate the standard deviation for each point. Points for apo-A-I concentrations of 2.5, 7.5, and 10.7 mg/ml had a range of ±0.3% or less. At the higher apo-A-I concentrations, a higher concentration of reagent was found to be required. Experiments where the final DMS concentration was 1.8 mg/ml; experiments where the DMS concentration was 9.1 mg/ml. The ionic strength in the reaction mixture was 0.226.

**Table III**

| Concentration (mg/ml) | % Dimer |
|-----------------------|--------|
| 2.5                   | 50 ± 1 |
| 7.5                   | 60 ± 2 |
| 10.0                  | 70 ± 3 |

**TABLE III**

| Distribution of oligomers as a function of protein concentration during cross-linking |
|-----------------------------------------------|
| Per cent of total area ± S.D.                 |
| Protein Concentration (mg/ml)                 |
| 0.01  | 0.10  | 0.5   | 5.0   | 10.7  |
| Monomer | 85 ± 15 | 60 ± 3 | 44 ± 3 | 14 ± 1 | 13 ± 0 |
| Dimer   | 15 ± 15 | 20 ± 1 | 15 ± 0 | 9 ± 1  | 10 ± 1 |
| Trimer  | 0 ± 1  | 11 ± 1 | 10 ± 2 | 16 ± 4 | 16 ± 1 |
| Tetramer| 0 ± 1  | 6 ± 0  | 21 ± 1 | 41 ± 5 | 36 ± 2 |
| Pentamer| 0 ± 1  | 4 ± 1  | 11 ± 1 | 21 ± 7 | 25 ± 2 |
Cross-linking of APO-A-I and APO-A-II

![Graph A](image1)

**Fig. 5.** Ionic strength dependence of the self-association of apo-A-I (A) and apo-A-II (B) as evaluated by cross-linking with DMS. Samples of apo-A-I (0.42 mg/ml) or apo-A-II (0.5 mg/ml) were cross-linked in the presence of various concentrations of NaCl at a final buffer concentration of 0.027 M triethanolamine HCl (pH 8.5). Data for apo-A-I are averages of four values; that for apo-A-II are averages of two values; the vertical lines indicate the standard deviation for each point.

that at low ionic strength the self-association of apo-A-I is diminished. It should be noted that since a lower concentration of buffer was used in these experiments to minimize the contribution to ionic strength, cross-linking occurred at a lower pH than under our standard conditions, resulting in decreased lysine modification.

In order to obtain supporting evidence for the idea that apo-A-I self-association is ionic strength-dependent, sedimentation equilibrium studies were carried out on apo-A-I at three values of ionic strength (μ): 0.010, 0.045, and 0.245 (these values having been used for such experiments by others (see Refs. 6, 3, and 10, respectively)). Fig. 6 shows the results of this experiment. At a single value of μ, the molecular weight is observed to increase with increasing protein concentration. This result is consistent with the concentration dependence already described. Additionally, at a given apo-A-I concentration molecular weight values were observed to increase with increasing ionic strength. At a concentration of 0.42 mg/ml (the concentration at which the analogous cross-linking experiment was performed), the observed molecular weight is about 50,000 at μ = 0.010. A shift of the observed molecular weight to 78,000 could be obtained simply by raising the value of μ to 0.245.

Sedimentation velocity experiments on apo-A-I samples (1 mg/ml) dialyzed against high (μ = 0.160) or low (μ = 0.045) values of ionic strength gave corroborating results. While the major peak in each sample sedimented with a value of 4.5 S, the higher ionic strength samples evidence a poorly resolved minor peak (not apparent in the low ionic strength sample) with a higher sedimentation coefficient.

**Effect of Guanidine on A-I Self-association—**Apo-A-I at a final concentration of 0.52 mg/ml was incubated with various concentrations of guanidine (ultrapure, recrystallized) in order to obtain an estimate of the strength of the self-association of this protein. The cross-linking reaction was carried out in the usual manner, except that the initial pH of the 1 M triethanolamine HCl was 9.0 (rather than 9.7). That the cross-linking reaction could be carried out in the presence of this denaturant is demonstrated in Table II, which gives the amino acid analysis of apo-A-I reacted with DMS in the presence of 4 M guanidine. The number of lysine residues is decreased from 21 in the native protein to 8.5 in the reacted protein. Electrophoresis and scanning of cross-linked samples demonstrates the dissociating effect of the denaturant (Fig. 7). Most of the effect was observed to occur between guanidine concentrations of 0.2 and 1.0 M, with a midpoint occurring at 0.5 M. At concentrations as high as 4.0 M guanidine, a residual 10% of the stained material was found to be in dimeric or trimeric species.

**Effect of Temperature on APO-A-I Self-association—**The quaternary structure of apo-A-I was observed by cross-linking at various temperatures. At a concentration of 0.45 mg/ml,
apo-A-II was incubated at temperatures between 4 and 55°C for 3 h before addition of DMS, with the cross-linking reaction allowed to proceed for 2 h. We have carried out time course studies at 25°C (16) and at 4°C and have found that the cross-linking reaction is essentially complete within 90 min. Fig. 8 shows the results of cross-linking experiments on apo-A-II under the standard conditions all of the lysines in apo-A-II are modified by the cross-linking reaction. When apo-A-II was incubated with guanidine hydrochloride at concentrations of guanidine for 1 h prior to cross-linking. Each point represents the average of duplicate determinations. %Dimer > 1 is defined in the legend to Fig. 2.

Studies on Apo-A-II Self-association—Studies similar to those performed on apo-A-I were also carried out with apo-A-II. A sample of untreated apo-A-II, with a single band of Mr = 17,000, can be seen in Fig. 1J. Fig. 1H shows a cross-linking profile for apo-A-II reacted with DMS in 0.15 M NaCl. The forms of apo-A-II observed on SDS-gels are the monomeric (Mr = 17,000) and dimeric (Mr = 34,000) species. Due to partial reduction of the interchain disulfide bonds, low levels of a triple chain (25,000) species can also be seen in these gels. Subsequent experiments have shown that this partial reduction can be decreased or eliminated by the use of other buffer systems, such as the bicarbonate system. Table I shows that under the standard conditions all of the lysines in apo-A-II are modified by the cross-linking reaction. When apo-A-II was cross-linked at several concentrations, ranging from 0.01 to 1.8 mg/ml (Fig. 4B), the self-association showed a dependence similar to that observed with apo-A-I, except that for apo-A-II the plateau is reached at a lower concentration.

In a study of the ionic strength dependence of apo-A-II self-association, the equilibrium was found to shift slightly towards the dimeric species at higher ionic strengths (Fig. 5B). At this concentration of apo-A-II (0.5 mg/ml), the effect of ionic strength was found to be less pronounced than for apo-A-I.

Effect of Guanidine on Apo-A-II Self-association—Fig. 7 shows the results of cross-linking experiments on apo-A-II which was incubated with guanidine hydrochloride. The dimer of apo-A-II appears to be more resistant to guanidine dissociation than the oligomers of apo-A-I since the midpoint of this dissociation curve occurs at higher guanidine concentration than that observed for apo-A-I.

Effect of Temperature on Apo-A-II Self-association—Apo-A-II (0.7 mg/ml in 0.15 M NaCl) was incubated for 3 h at temperatures ranging from 4 to 55°C. After this preincubation, the protein was reacted with DMS for 2 h; the results are shown in Fig. 8. Apo-A-II self-association was found to be similar to apo-A-I in that dissociation begins to occur at temperatures above 25°C. However, the curve for apo-A-II is slightly broader than for apo-A-I, suggesting greater thermal stability for the oligomers of the apo-A-II protein.

Effect of Phospholipid Binding on Apo-A-II Self-association—Apo-A-II (0.4 mg/ml) in 0.15 M NaCl was incubated with various concentrations of phospholipid vesicles which were prepared from egg yolk phosphatidylcholine by the sonication method of Morrisett et al. (22) and which were applied to a calibrated Sepharose 6B column (29). After incubation at 37°C for 1 h, the protein samples were cross-linked with DMS as described. Samples were dialyzed with ethanol-ether (3:1) and applied to SDS-polyacrylamide gels. It was observed that the presence of the phospholipid vesicles caused an alteration in the cross-linking profile (Fig. 9). When the molar ratio of lipid to protein used ranged between 50 and 100, the amounts of monomer and dimer were greatly reduced and a trimmer (Mr = 51,000) species became predominant. Very little apo-A-II trimer is observed in the absence of phospholipid.

Apo-A-II was also incubated with phospholipid vesicles (molar ratio of lipid to protein = 150) in 0.15 M NaCl at 37°C for 24 h and the resulting mixture was fractionated into three density ranges. In the d < 1.085 fraction 40% of the added protein was found, 15% was recovered in the fraction d = 1.085 to 1.21, and 46% was in the d > 1.21 fraction. These protein fractions were dialyzed to remove KBr and were reacted with DMS. The fraction of d < 1.085 yielded a profile in which the trimer was the predominant species and some dimer was present. Trimer was the only species observed in the d = 1.085 to 1.21 preparation. The fraction d > 1.21 yielded a mixture of monomers, dimers, and trimers, although the trimer represented the largest single component.

Fig. 8. Temperature effect on the self-association of apo-A-I (A) and apo-A-II (B). Samples of apo-A-I (0.45 mg/ml) and apo-A-II (0.7 mg/ml) were preincubated at the indicated temperature for 2 to 3 h prior to cross-linking at that temperature. Individual points represent average of two to seven values and the vertical lines indicate the standard duration.
molar ratios of egg yolk phosphatidylcholine to protein used were: 0, 1, 5, 10, 50, 100, 285 (Positions 1 to 7, respectively). Position 8 contains a cross-linked sample of apo-A-I, for reference.

FIG. 9. Effect of phospholipid binding on apo-A-II self-association.


discussion

The cross-linking approach to studies of apo-A-I and apo-A-II self-association represents an alternative to the physical techniques which have been applied to this problem by others (3, 5, 6, 10, 11). Because there is a great deal of controversy among the various workers in the field regarding apoprotein self-association, we have attempted to carry out various types of control experiments in order to preclude misinterpretation of the cross-linking data. Specifically, the generation of cross-linked species by transient collisions was shown to be negligible by studies with monomeric proteins and by cross-linking in the presence of detergent (Fig. 1). The possibility that cross-linking perturbs the protein structure was also considered. Circular dichroism studies on apo-A-I indicated that cross-linking does not perturb its secondary structure, and fluorescence measurements suggested that tertiary structure is not significantly modified. We have not obtained direct evidence as to whether the modification reaction perturbs the self-association equilibria. However, the t1/2 of equilibration of A-I was found to be on the order of several hours at room temperature (3) and at 4°C (14), although this latter group found a faster rate at room temperature (10). Since the cross-linking reaction is essentially complete by 60 min and the same results are obtained at 4 and 25°C, we presume that no redistribution of oligomeric forms occurs during the cross-linking reaction. Also, the fact that the fluorescence emission spectra for cross-linked and untreated apo-A-I were identical would suggest that cross-linking has not altered the proportion of oligomers, assuming fluorescence to be sensitive to changes in quaternary structure.

Cross-linking of apo-A-I shows the presence of oligomers from monomer through pentamer. The identity of these oligomeric species has been verified by both SDS-gel electrophoresis (16) and by gel permeation chromatography in 6 M guanidine hydrochloride (Fig. 2). We should point out, in passing, that this is the first instance, to our knowledge, that the reliability of SDS-gel electrophoresis for the estimation of molecular weight of cross-linked proteins has been verified by another methodology. The validity for SDS-gel electrophoresis has also been substantiated in a recent report by Steele and Nielsen (29).

Under the appropriate conditions of protein concentration and ionic strength, the predominant A-I species observed through cross-linking are tetramers and pentamers. This pattern is unlike any cross-linking pattern reported in the literature for proteins and is not entirely consistent with any models proposed for the self-association of apo-A-I. The prominence of a tetramer form is most consistent with the results of Vitello and Scanu (10), although the finding of a pentamer form is unexpected. We have discounted the possibility that this band represents a higher oligomer, such as octamer, since: 1) the observed molecular weight by both SDS-gel electrophoresis and gel filtration is close to that expected for pentamer and 2) no bands intermediate between putative tetramer and pentamer are seen on SDS-gels, as would be expected for incompletely cross-linked octamer (e.g. hexamer and heptamer).

We have considered the possibility that this cross-linking profile might be a special result of the conditions employed. However, identical results were obtained when apo-A-I was renatured in a variety of ways. Likewise, the general pattern of monomer through pentamer was observed over a range of cross-linker-to-protein ratios, protein concentrations, ionic strengths, and temperatures. On this basis, we feel that the cross-linking patterns realistically represent the species present in solution. We also note that identical results are obtained with freshly renatured A-I and with material left standing several weeks at 4°C. This suggests to us that the oligomers observed are not aggregates, which would be expected to increase in amount as a function of time, but are reversibly self-associated forms. It should be noted that because of the pH limitations of the cross-linking reaction our data was obtained only over the pH interval of 8.5 to 9.5.

Our results with apo-A-II are in good agreement with the hydrodynamic studies reported in the literature for this protein (3, 5, 11). It is clear that this protein associates mainly to a dimeric form, although a small amount of trimer is also present under certain conditions.

The concentration dependence studies (Fig. 4) show that, while the self-association of apo-A-I and apo-A-II are both dependent upon concentration, complete association of apo-A-II appears to occur at lower concentrations. This would suggest that the apo-A-II self-association is more energetically favored than apo-A-I self-association. This is supported by studies on the effect of guanidine hydrochloride (Fig. 7), which show that higher concentrations of the denaturant are required to dissociate apo-A-II than to dissociate apo-A-I. Likewise, the self-association of apo-A-II appears to be slightly less affected by elevated temperatures than that of apo-A-I (Fig. 8).

The data in Fig. 7 indicate that complete dissociation of apo-A-I or apo-A-II requires higher concentrations of guanidine hydrochloride than might be expected from measurements of fluorescence or circular dichroism (4, 5). However, these latter techniques are used to monitor changes in secondary and tertiary structure and may be relatively insensitive to changes in quaternary structure. The finding of 10% residual oligomers even at 4 M guanidine HCl indicates some resistance to complete dissociation; similar residual associated forms of apo-A-I have also been reported in the presence of 8 M urea (30).

A significant aspect of these studies has been the finding of a strong ionic strength dependence for the apo-A-I self-association. We believe that this finding constitutes at least a
partial explanation for the discrepancies reported by several groups working on this problem. Gwynne et al. found no self-association in a low ionic strength ($\mu = 0.010$) buffer (6). Stone and Reynolds, using a buffer of $\mu = 0.045$, found that apo-A-I associated mainly to dimers (3). Vitello and Scanu reported data for apo-A-I in higher ionic strength buffers ($\mu = 0.12$ to 0.16) for which apo-A-I fitted a monomer-dimer-tetramer-octamer model (10). We have carried out sedimentation equilibrium experiments (Fig. 7) at comparable ionic strengths and have demonstrated an ionic strength effect similar to that which we find in cross-linking experiments. Sedimentation velocity experiments have also confirmed this finding. We are unable to reconcile these data with the finding by Vitello and Scanu of equivalent sedimentation equilibrium profiles at both high and low values of ionic strength (10). Our ultracentrifugal data are in good agreement with that reported by Formisano et al. (31), who also report a strong ionic strength dependence of the self-association equilibrium. In addition, these authors report a dependence of molecular weight on the angular velocity of the rotor, although contrary results have been reported by Stone and Reynolds (3).

It is possible to calculate apparent weight average molecular weights from the percentages of the different cross-linked oligomeric forms of the protein as a function of concentration (Table III). Despite the error inherent in such a treatment due to incomplete cross-linking, the data so calculated for apo-A-I are in suprisingly good agreement with data obtained by sedimentation equilibrium experiments (Fig. 6). This suggests that the cross-linking results are consistent with the results from ultracentrifugation.

The finding of dimers and tetramers in apo-A-I solutions by Rosseneu et al. (13) is not consistent with our studies or with other proposed association models. We have carried out gel filtration studies on apo-A-I and have found highly variable elution volumes for this protein, depending on the elution buffer used. We believe that these results may be due to shape changes, and have found gel filtration unreliable for estimation of A-I oligomer sizes.

The observation that self-association is promoted by increased ionic strength is suggestive that hydrophobic interactions might be instrumental in the self-association, although charge shielding effects cannot be ruled out. Self-association might result from the high degree of exposure of hydrophobic groups in apo-A-I and apo-A-II that has been reported by Reynolds (4) and Tall et al. (9). This mechanism is consistent with the dissociation of apo-A-II at low temperatures reported by Gwynne et al. (5). Our data also suggest that a slight decrease in association may be occurring at lower temperatures (Fig. 8).

When we asked the question whether reconstitution of apoproteins with phospholipid would affect the distribution of associated species, we were interested to observe reconstituted particles of fairly defined quaternary structure. Although apo-A-I failed to reconstitute with our phospholipid preparation to any significant extent, the addition of egg yolk phosphatidylcholine to apo-A-II in the range of 50 to 100 mol of phospholipid/mol of protein drastically alters the cross-linked profile from one of monomers and dimers to one where trimers constitute the predominant species. This underscores the utility of using cross-linking reagents to aid in the characterization of reconstituted lipoproteins. These results also indicate that substantial molecular reorganization occurs when apo-A-II interacts with lipids. The simple addition of lipid molecules to existent apoprotein structures would not be sufficient to account for the observed results. While these data indicate that 3 A-II molecules are situated on the same particle, it is not possible to specify whether they enter into stable interactions with each other or "float" over the surface of the particle, making random and intermittent contacts.

One important result of these studies is to show the need for specification of the conditions under which recombination with lipid is affected. Since the state of association of these proteins is affected by concentration, temperature, and ionic strength, we are currently investigating the effects of these parameters on the ability of apoproteins to combine with lipid.

Our observation that recombination of apo-A-II with lipid yields particles with a fairly homogeneous quaternary structure is noteworthy. While it is possible that the number of protein chains is dictated by the lipid surface to be covered, it would be expected that sonicated dispersions of egg yolk lecithin would possess a spectrum of particles with a wide range of surface areas. An attractive alternative is that specific protein-protein interactions are major forces in determining the quaternary structure of reconstituted particles, as well as of intact lipoproteins. Preliminary studies have suggested that such protein-protein associations occur in mixtures of apo-A-I and apo-A-II (16), and we are currently studying this in detail in order to understand the relative importance of protein-protein and protein-lipid interactions in intact lipoproteins.

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**REFERENCES**

1. Baker, H. N., Gotto, A. M., Jr., and Jackson, R. L. (1975) *J. Biol. Chem.* 250, 2725-2730
2. Lux, S. E., John, K. M., Honan, K., and Brewer, H. B. (1972) *J. Biol. Chem.* 247, 7519-7527
3. Stone, W. L., and Reynolds, J. A. (1975) *J. Biol. Chem.* 250, 8045-8049
4. Reynolds, J. A. (1976) *J. Biol. Chem.* 251, 6013-6015
5. Gwynne, J., Palumbo, G., Osborne, J. C., Brewer, H. B., and Edelhoch, H. (1975) *Arch. Biochem. Biophys.* 176, 204-212
6. Gwynne, J., Brewer, B., Jr., and Edelhoch, H. (1974) *J. Biol. Chem.* 249, 2411-2474
7. Gwynne, J., Brewer, H. B., Jr., and Edelhoch, H. (1975) *J. Biol. Chem.* 250, 2929-2934
8. Tall, A. R., Small, D. M., Shipley, G. G., and Lees, R. S. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 4940-4942
9. Tall, A. R., Shipley, G. G., and Small, D. M. (1975) *J. Biol. Chem.* 251, 3749-3755
10. Vitello, L. B., and Scanu, A. M. (1976) *J. Biol. Chem.* 251, 1131-1136
11. Vitello, L. B., and Scanu, A. M. (1976) *Biochemistry* 15, 1161-1165
12. Reynolds, J. A., and Simon, R. H. (1974) *J. Biol. Chem.* 249, 3937-3940
13. Rosseneu, M., Blaton, V., Vercaemst, R., Soetewey, F., and Peeters, H. (1977) *Eur. J. Biochem.* 74, 83-88
14. Ritter, M. C., and Scanu, A. M. (1977) *J. Biol. Chem.* 252, 1208-1216
15. Jonas, A., Krajnovich, D. J., and Patterson, B. W. (1977) *J. Biol. Chem.* 252, 2200-2205
16. Swanyne, J. B., and O’Brien, K. J. (1976) *Biochem. Biophys. Res. Commun.* 71, 636-643
17. Haefl, R. J., Eder, H. A., and Bradgon, J. H. (1955) *J. Clin. Invest.* 34, 1345-1350
18. Brown, W. V., Levy, R. I., and Fredrickson, D. S. (1969) *J. Biol. Chem.* 244, 5687-5694
19. Swanyne, J. B., Braithwaite, F., and Eder, H. A. (1977) *Biochemistry* 16, 271-278
20. Scanu, A. M., Lim, C. T., and Edelstein, C. (1972) *J. Biol. Chem.* 247, 5500-5505
21. Swanyne, J. B., and Kuehl, K. S. (1976) *Biochem. Biophys. Acta* 446, 461-465
22. Morrisett, J. B., David, J., Pownall, H. J., and Gotto, A. M. (1973) *Biochemistry* 12, 1290-1299
23. Yphantis, D. A. (1964) *Biochemistry* 3, 297-317

*J. B. Swaney and K. O’Brien, unpublished data.*
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24. Fish, W. W., Mann, K. G., and Tanford, C. (1969) J. Biol. Chem. 244, 4989–4994
25. Davies, G. E., and Stark, G. R. (1970) Proc. Natl. Acad. Sci. U. S. A. 66, 651–656
26. Ludwig, M. L., and Hunter, M. J. (1967) Methods Enzymol. 11, 595–604
27. Lad, P. M., and Hammes, G. G. (1974) Biochemistry 13, 4530–4537
28. Middelhoff, G., Rosseneu, M., Peeters, H., and Brown, W. V. (1976) Biochim. Biophys. Acta 441, 47–57
29. Steele, J. C., and Nielsen, T. B. (1970) Anal. Biochem. 84, 218–224
30. Scanci, A. M., Toth, J., Edelstein, C., and Stiller, E. (1969) Biochemistry 8, 3309–3316
31. Formisano, S., Brewer, H. B., Jr., and Osborne, J. C., Jr. (1978) J. Biol. Chem. 253, 354–360
Cross-linking studies of the self-association properties of apo-A-I and apo-A-II from human high density lipoprotein.  
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