Effect of Pressure and Ionic Strength on the Self-association of Apo-A-I from the Human High Density Lipoprotein Complex*

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The self-association of apo-A-I isolated from the human high density lipoprotein complex has been investigated by gel permeation chromatography and sedimentation equilibrium. The apparent weight average molecular weight (MWave) versus Apo-A-I concentration profile was found to be sensitive to ionic strength and pressure; MWave increased with increasing ionic strength and decreasing rotor speed. The data were consistent with a monomer-dimer-tetramer-octamer association scheme over all conditions investigated if a change in the partial specific volume of apo-A-I upon association of \(5.5 \times 10^{-2}\) ml/g is postulated.

Several laboratories have recently reported data concerning the molecular properties of purified apo-A-I isolated from the human high density lipoprotein complex (1–3). Gwynne et al. (1) found that apo-A-I was monomeric below 0.53 mg/ml by sedimentation equilibrium, whereas Stone and Reynolds (2) and Vitello and Scanu (3) reported, also by sedimentation equilibrium measurements, that apo-A-I self-associates in this concentration range. In addition, the molecular weight versus protein concentration data from the latter two groups are not in agreement; for instance, at a concentration of 1 mg/ml, Stone and Reynolds reported a molecular weight of approximately 45,000, whereas Vitello and Scanu found a molecular weight greater than 100,000 at this concentration.

The self-association of apo-A-I may be sensitive to its degree of association (4, 5), a knowledge of the molecular species in solution is a prerequisite for the quantitative evaluation of most experimental data. In order to clarify the conflicting literature, and to characterize more fully the role of apo-A-I in the structure and function of lipoprotein particles, we have undertaken a systematic investigation of the molecular properties of apo-A-I in aqueous solution. In the present manuscript, we report the effects of pressure and ionic strength on the self-association of apo-A-I.

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MATERIALS AND METHODS

The isolation and purification scheme used for apo-A-I has been previously described (1). Protein concentrations were determined by absorbance using a molar extinction coefficient of 32,350 at 280 nm. The buffer used for apo-A-I was 0.01 M Tris (Ultrapure, Schwarz-Mann), 0.001 M sodium azide (Fisher Scientific), and 0.1 M potassium chloride, pH 7.4, buffer at room temperature (25°C). The sample used (Pharmacia K 15/90) was packed with the swollen gel to a height of 82 cm. A pressure differential of 50 cm was kept uniform during all runs by using a 500-ml Marriot flask. The flow rate was 10 mlh and 0.7-ml fractions were collected in an LKB fraction collector. Elution volumes were obtained by gravimetric analysis. Elution profiles were obtained by absorbance at 290, or 280 nm, or both, using a Beckman Acta III spectrophotometer and by the intensity of tryptophanyl fluorescence using a Perkin-Elmer model MPF-4 spectrofluorometer. The proteins used for column calibration, Ferritin (MW = 450,000), catalase (MW = 240,000), egg albumin (MW = 45,000), and chymotrypsinogen (MW = 25,000) were purchased from Boehringer Mannheim Biochemicals. The void and salt volumes of the column were determined with ferritin and acetyltryptophanilamide.

Sedimentation Equilibrium—Sedimentation equilibrium experiments were performed in a Spinco model E ultracentrifuge equipped with a photoelectric ultraviolet scanner and a temperature control system. Double sector cells with charcoal-filled epon centerpieces and quartz windows were centrifuged in an Ang-Ti rotor. The temperature was maintained at 21°C. Each cell contained 100 μl of sample and 110 μl of buffer and the layering fluid was omitted. Initial protein concentrations were obtained by absorbance measurements at 280 nm prior to each run. An initial scan (280 to 287) of each cell at time zero when the rotor reached the desired speed allowed the calculation of an extinction coefficient for each run. Equilibrium was monitored by comparing the concentration profiles at 4-h intervals after a period of 42 to 72 h. After equilibrium had been obtained, the rotor speed was increased to 22,000 rpm for baseline determinations. For each run concentration profiles were obtained at equilibrium at several rotor speeds. The effect of rotor speed was reversible in that profiles obtained at equilibrium with increasing rotor speed from 10,000 to 22,000 rpm were the same as those obtained at equilibrium with decreasing rotor speed from 32,000 to 10,000 rpm. The relative radius squared (r²) data were fitted to the following equation using a nonlinear least squares technique to obtain the least squares values of A, B, C, and D.

\[ Y(x) = \frac{A + BX + CX^2}{1 + DX} \]  

(1)
The Self-association of Apo-A-I

![Graphs and plots representing elution profiles and absorbance data.](image)

**Fig. 1.** Elution profile of apo-A-I at several different concentrations, from a Sephadex G-150 superfine column. Each protein solution was clarified by centrifugation and concentrations were determined by absorbance at 280 nm. The buffer used and the experimental conditions are described under "Materials and Methods." All experiments were performed at room temperature. The elution volumes of standards were: ferritin ($M_r = 540,000$), 45 ml; catalase ($M_r = 280,000$), 51.3 ml; egg albumin ($M_r = 45,000$), 71.8 ml; chymotrypsinogen ($M_r = 25,000$), 75.2 ml; acetyl tryptophanamide, 126 ml. Initial apo-A-I volumes and concentrations were: Panel A, 1.5 ml of apo-A-I at 0.05 mg/ml; Panel B, 1.5 ml of apo-A-I at 0.2 mg/ml; Panel C, 1.5 ml of apo-A-I at 0.5 mg/ml; Panel D, 1.5 ml of apo-A-I at 2.0 mg/ml.

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**RESULTS**

The elution profile of apo-A-I from a Sephadex G-150 column at 25°C, equilibrated in 0.01 M Tris, 0.1 M potassium chloride, 0.001 M sodium azide, pH 7.4, buffer, at several different initial protein concentrations is illustrated in Fig. 1. At each concentration investigated the gradient profiles have the bimodal shape expected for protomer-oligomer systems with $N$ greater than two (6–8). At the intermediate concentrations the profiles are complex and indicate the presence of several oligomeric species.

With increasing concentration, the elution volume of the fast moving component decreases, reaching a plateau above 0.9 mg/ml. Thus, the minimum molecular weight of the fast moving component is approximately 240,000 by gel chromatography. The elution volume of the slowest moving component in these studies corresponded to a molecular weight of approximately 50,000, which would seem to suggest that the protomer in this association scheme is a dimer of apo-A-I (dimeric...
molecular weight of 56,028). However, since the elution volume of a protein is sensitive to its molecular shape, it is difficult to predict the molecular weight of the protomeric species from gel filtration studies alone. Of particular importance to the chromatographic behavior of apo-A-I is the reported frictional ratio of 1.4 (1).

Since the elution volume of oligomers undergoing a reversible association with protomers is quite sensitive to the initial protein concentration, it is difficult to estimate the molecular size of the oligomeric species in solution by gel chromatography alone (6). In addition, although the conditions used in these experiments (i.e., the solute concentration is low so that no plateau with the initial protein concentration is observed) are useful for detecting dissociation, they cannot be easily used for the determination of equilibrium constants (9). We have, therefore, used sedimentation equilibrium studies to assess the association scheme of apo-A-I.

Sedimentation Equilibrium—The molecular weight versus concentration profile obtained at equilibrium (14,000 rpm) for apo-A-I at several different ionic strengths is illustrated in Fig. 2. With increasing ionic strength Mw,app increases, reaching a plateau between 0.1 and 0.5 M in added salt. The majority of the effect of ionic strength occurred below 0.1 M in added salt. This same trend in molecular weight as a function of ionic strength was observed at rotor speeds of 10,000, 14,000, 15,000, 18,000, 20,000, and 22,000 rpm (data not shown).

The effect of rotor speed on the apparent weight average molecular weight of apo-A-I at ionic strength 0.11 is illustrated in Fig. 3. With decreasing rotor speed, the Mw,app of apo-A-I increased dramatically. We have analyzed these data using the nonlinear least squares technique described under "Materials and Methods" and the appendix for the monomer-dimer-tetramer-octamer association scheme (M-D-Tet-Oct) suggested by Vitello and Scanu (9). The least squares values (± standard deviation) obtained by simultaneously fitting data obtained at different rotor speeds (see "Appendix" for details) were: K12(m) = 0.37 ± 0.42 ml/g, K13(m) = 165 ± 146 (ml/g)2; K1,m(m) = 621,624 ± 214,746; (ml/g)3 and V2 = 0.792 ± 0.008 ml/g. Thus the data were consistent with monomer-dimer-tetramer-octamer association scheme over all conditions investigated if a change in the partial specific volume of apo-A-I upon association of 5.5 × 10⁻² ml/g is postulated. For illustration, the theoretical fit of the concentration versus radius data (see "Appendix" for details) for the monomer-dimer-tetramer-octamer scheme with K12 = 0.372 ml/g, K13 = 165 (ml/g)², K1,m = 621,624 (ml/g)³, a monomeric molecular weight of 28,014 and Δν of 5.5 × 10⁻² ml/g is given by the solid lines in Fig. 4.

If the above analysis is valid, then one would also expect to observe a decrease in Mw,app, at any given concentration of protein, with increasing hydrostatic pressure at constant angular velocity. Josephs and Harrington (10) have investigated the effects of pressure on the self-association of myosin, at constant angular velocity, by layering mineral oil over the solution column in order to increase the hydrostatic pressure at any given position in the cell. Since apo-A-I has a high affinity for nonpolar compounds, and hence for oil-water interfaces, mineral oil could not be used in the present study. Instead, the effect of pressure at constant angular velocity was investigated by comparing the concentration versus Mw,app profiles of apo-A-I (initial concentration 0.43 mg/ml) obtained at equilibrium from two cells differing only in column height, 11.45 versus 2.86 mm. The results, illustrated in the inset of Fig. 5, are consistent with the above model, in that at any given protein concentration Mw,app decreases with increasing pressure.

![Fig. 2. Results from sedimentation equilibrium measurements plotted as the apparent weight average molecular weight versus apo-A-I concentration. The rotor speed was 14,000 rpm and the temperature was maintained at 21°C. The buffers used were: O, 0.01 M Tris, 0.001 M sodium azide, pH 7.4 (T = 0.01); ▲, 0.01 M Tris, 0.001 M sodium azide, 0.003 M potassium chloride, pH 7.4 (T = 0.075); ●, 0.01 M Tris, 0.01 M sodium azide, 0.13 M potassium chloride, pH 7.4 (T = 0.14); △, 0.01 M Tris, 0.001 M sodium azide, 0.5 M potassium chloride, pH 7.4 (T = 0.81).

![Fig. 3. Results from sedimentation equilibrium measurements plotted as the apparent weight average molecular weight versus apo-A-I concentration at different rotor speeds. The buffer used was 0.01 M Tris, 0.001 M sodium azide, 0.1 M potassium chloride (T = 0.11), and the temperature was maintained at 21°C. The rotor speeds were □, 10,000 rpm; O, 15,000 rpm; △, 20,000 rpm; ●, 22,000 rpm. Initial protein concentrations were: 0.42 mg/ml, □, O, △; 0.54 mg/ml, ●.
The self-association of apo-A-I

The most striking finding in the present studies is the effect of rotor speed on the apparent weight average molecular weight of apo-A-I obtained by sedimentation equilibrium. Pressure effects on interacting systems, especially proteins, have received an increasing amount of attention over the past few years (10-15). The volume changes found for associating protein systems are usually quite small, on the order of $10^{-4}$ to $10^{-5}$ ml/g; however, these changes can produce quite large effects at the pressures generated in the ultracentrifuge (10-15). The effects of pressure on interacting systems where volume changes occur can be related to the equilibrium constant through the following equation:

$$
\frac{\delta \ln K}{(\delta P)_T} = -\frac{\Delta V}{RT}
$$

where $P$ is the pressure, $R$ is the gas constant, $K$ is the equilibrium constant, $T$ is the absolute temperature, and $\Delta V$ is the change in volume upon interaction. For systems that have a positive volume change upon interaction, the apparent equilibrium constant at the bottom of the cell in the ultracentrifuge, where pressures can exceed 150 atm, can be several orders of magnitude smaller than the apparent equilibrium constant at the meniscus (10-15).

The hydrostatic pressure at the meniscus is approximately 1 atm, and therefore, pressure effects should be minimal at this position in the cell. The data in Fig. 3 conform to this in that $M_w^{app}$ obtained at different rotor speeds superimpose near the meniscus of the cell. Near the bottom of the cell, however, the $M_w^{app}$ data obtained with different angular velocities or different column heights do not overlap one another (Figs. 3 and 5). Thus when the partial specific volume of apo-A-I is assumed to be independent of concentration (i.e. when $\delta \ln K$ is constant at the meniscus; (10-15)).

For purposes of illustration, we have calculated the theoretical concentration of monomer, dimer, tetramer, and octamer using the equilibrium constants given in Fig. 4, which should correspond to atmospheric pressure at neutral pH in the presence of 0.1 M potassium chloride (Fig. 5). At low concentrations, less than 0.1 mg/ml, monomers represent greater than 90% of the species in solution. The concentration of tetramers does not exceed 20% of the total and dimers represent less than 3% of the total material at all concentrations. Octamers represent greater than 80% of the species in solution at concentrations above 2.5 mg/ml.

The data in Fig. 6 can be used to predict the molecular species in solution at atmospheric pressure. However, it should be noted that these data correspond to the specific conditions employed in this study, i.e. solvent, pH, temperature.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Results from sedimentation equilibrium experiments plotted as the concentration of apo-A-I (milligrams per ml) versus the distance from the center of rotation (centimeters) obtained at equilibrium at 21°. The buffer used was 0.01 M Tris, 0.001 M sodium azide, 0.1 M potassium chloride, pH 7.4. Initial protein concentrations were: filled symbols, 0.42 mg/ml; open symbols, 0.88 mg/ml. Rotor speeds were: 0, 10,000 rpm; ▲, 15,000 rpm; 0, 20,000 rpm. The solid lines represent the theoretical fit of the data to a monomer-dimer-tetramer-octamer association scheme with a monomer molecular weight of 28,014, $K_{mm} = 0.372$ ml/g, $K_{mm} = 165$ (ml/g)$^2$, $K_{m} = 621,624$ (ml/g)$^3$, a partial specific volume of protomer = 0.737 ml/g and a partial specific volume of oligomer = 0.792 ml/g (see "Appendix" for details).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Results from sedimentation equilibrium experiments plotted as the concentration of apo-A-I (milligrams per ml) versus the distance from the center of rotation (centimeters) obtained at equilibrium at 15,000 rpm at 21°. The buffer used was 0.01 M Tris, 0.001 M sodium azide, 0.1 M potassium chloride, pH 7.4. The initial protein concentration was 0.45 mg/ml and the column heights were: ▲, 11.45 mm; 0, 2.8 mm. The solid lines represent the theoretical fit of the data to a monomer-dimer-tetramer-octamer association scheme (see "Appendix" for details) with a monomer molecular weight of 28,014, $K_{mm} = 0.372$ ml/g, $K_{mm} = 165$ (ml/g)$^2$, $K_{m} = 621,624$ (ml/g)$^3$, a partial specific volume of protomer = 0.737 ml/g and a partial specific volume of oligomer = 0.792 ml/g (see "Appendix" for details). Inset, results of the sedimentation equilibrium data presented in Fig. 5 plotted as the $M_w^{app}$ versus protein concentration.}
\end{figure}
The volume change found for apo-A-I is larger than that reported for other associating proteins. It should be noted, however, that apo-A-I is, when compared to other globular proteins, relatively unstable in aqueous solutions (1, 16). In addition, it has been shown that the secondary structure of apo-A-I increases upon association (2). Increases in the secondary and tertiary structure of two other apolipoproteins, apo-A-II and apo-C-I have also been reported (17-20). These latter two apolipoproteins have little structure in their monomeric forms and are also easily denatured.

Thus, with the apolipoproteins which have been well characterized, the exposure of amino acid side chains to solvent decreases dramatically with increasing protein concentration. Studies with model compounds have shown small positive volume changes upon shielding from solvent, which have been attributed to a decrease in the amount of "ordered water" around these solutes (21-24). Thus, in systems where large conformational changes accompany interaction, one would predict changes in the partial specific volume of the solute. In the ultracentrifuge, volume changes can cause large changes in the apparent equilibrium constant of associating systems (10-15). The magnitude of this effect would depend upon the change in partial specific volume and the molecular weight of the oligomeric species in solution. With apo-A-I, the oligomeric species has a molecular weight of approximately 240,000, and the effect of pressure on \( M_{\text{app}} \) is easily demonstrated by varying the rotor speed in sedimentation equilibrium experiments. Since apo-A-II and apo-C-I also undergo dramatic conformational changes upon association, one would predict a volume change for these systems also. However, since the molecular weight of the oligomeric species of these two proteins is lower, 34,000 and approximately 20,000, respectively, the magnitude of the effect would be expected to be much smaller. These systems are currently being investigated in our laboratory.

REFERENCES

14. TenEyck, L., and Kaummann, W. (1967) Proc. Natl. Acad. Sci. relatively independent of ionic strength. The reasons for the difference between their findings and those presented in Fig. 2 above are not known.
APPENDIX

EVALUATION OF VOLUME CHANGES IN ASSOCIATING SYSTEMS BY SEDIMENTATION EQUILIBRIUM

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Following the analysis presented by Pedersen in 1940 (25, also see Refs. 11 to 14) the concentration versus radius profile for each component of an ideal associating system, at equilibrium in the ultracentrifuge, corresponds to the following equation:

\[ C_i(r) = C_i(C) e^{\Delta M \rho (r^2 - m^2)} \]  

(1)

where: \( i \) corresponds to the number of protomers comprising each oligomer; \( C_i \) is the concentration of species \( i \) (activity coefficients are assumed to be unity); \( r \) corresponds to the radial distance from the center of rotation; \( m \) is the distance from the center of rotation to the meniscus; \( M_0 \) is the protomer molecular weight; \( A_i = \omega^2 (1 - \nu \rho)/2RT \) where \( \omega \) is the angular velocity; \( R \) is the gas constant; \( T \) is the absolute temperature; \( \rho \) is the solvent density, which is assumed to be independent of pressure; and \( V_i \) is the partial specific volume of Species \( i \), which is also assumed to be independent of pressure.

Assuming that the laws of mass action are valid,

\[ K_{eq,i} = C_i(m) e^{\Delta V_i (r^2 - m^2)} \]  

(2a)

\[ K_u(r) = C_u(r) C_i(m)^{i-1} \]  

(2b)

where \( K_u \) is the \( i^{th} \) equilibrium constant and \( C_i \) is the concentration of protomer.

Combining Equations 1 and 2, the total concentration of solute, \( C \), at any position \( r \) in the cell obeys the following equation:

\[ C(r) = \sum_{i=1}^{n} K_u(m) C_i(m)^{i-1} e^{\Delta M \rho (r^2 - m^2)} \]  

(3)

where: \( K_u(m) = 1 \) Since the hydrostatic pressure at the meniscus is approximately 1 atm, the equilibrium constants, \( K_u(m) \), in Equation 3 correspond to those at atmospheric pressure. Combining Equations 2a, 2b, and 3, one obtains the following relationship between the equilibrium constants at position \( r \) in the cell, \( K_u(r) \), and those at the meniscus, \( K_u(m) \):

\[ K_u(r) = K_u(m) e^{\Delta V_1 (r^2 - m^2)} \]  

(4a)

Thus if the partial specific volumes of the species in solution are equal, the equilibrium constants are independent of pressure, and thus radial position, and

\[ K_u(r) = K_u(m) \]  

(4b)

In the more general case where the partial specific volumes are not equal, \( K_u(r) \) is not equal to \( K_u(m) \), and will vary with radial position in the cell according to Equation 4.

Equations 3 and 4 are general and can be used to describe any associating protein system, given the above basic assumptions, with a knowledge of the partial specific volumes of the species in solution and the corresponding equilibrium constants at atmospheric pressure.

For a monomer-dimer-tetramer-octamer association scheme, Equation 3 reduces to:

\[ C(r) = C_1(m)^{2} e^{\Delta V_1 (r^2 - m^2)} + C_2 C_1(m) e^{\Delta V_2 (r^2 - m^2)} + C_3 C_2 C_1(m) e^{\Delta V_3 (r^2 - m^2)} + C_4 C_3 C_2 C_1(m) e^{\Delta V_4 (r^2 - m^2)} \]  

(5)

Since this equation contains both equilibrium constants and partial specific volumes as unknowns, a unique solution cannot be obtained from a least squares fit to data from a single cell, i.e. at any given initial solute concentration, column height, and angular velocity. We have chosen, therefore, to simultaneously fit Equation 5 to sets of data obtained, at equilibrium, at different angular velocities. Since the radial distance (and hence the hydrostatic pressure) corresponding to equal concentrations of solute for two equilibrium runs at different angular velocities is not the same, a unique solution to Equation 5 can theoretically be obtained with this procedure.

The apo-A-I system is quite complex, and in order to reduce the number of unknowns in Equation 5 two additional assumptions have been made in the data analysis.

1. The partial specific volume of the protomer was taken to be 0.737 ml/g.

\[ K_u(r) = K_u(m) e^{\Delta V_1 (r^2 - m^2)} \]  

(6)

Thus if the partial specific volumes of the species in solution are equal, the equilibrium constants are independent of pressure, and thus radial position, and

\[ K_u(r) = K_u(m) \]  

(7b)

In the more general case where the partial specific volumes are not equal, \( K_u(r) \) is not equal to \( K_u(m) \), and will vary with radial position in the cell according to Equation 4.

Equations 3 and 4 are general and can be used to describe any associating protein system, given the above basic assumptions, with a knowledge of the partial specific volumes of the species in solution and the corresponding equilibrium constants at atmospheric pressure.

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(8)

Since this equation contains both equilibrium constants and partial specific volumes as unknowns, a unique solution cannot be obtained from a least squares fit to data from a single cell, i.e. at any given initial solute concentration, column height, and angular velocity. We have chosen, therefore, to simultaneously fit Equation 5 to sets of data obtained, at equilibrium, at different angular velocities. Since the radial distance (and hence the hydrostatic pressure) corresponding to equal concentrations of solute for two equilibrium runs at different angular velocities is not the same, a unique solution to Equation 5 can theoretically be obtained with this procedure.

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