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Studies on Human Serum High-Density Lipoproteins. Self-Association of Human Serum Apolipoprotein A-II in Aqueous Solutions†

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ABSTRACT: Some of the solution properties of pure preparations of human serum high-density apolipoprotein A-II were studied by sedimentation equilibrium ultracentrifugation, conducted at different apoprotein concentrations and at several speeds. The concentration dependence of the apparent weight average molecular weight indicated that apolipoprotein A-II, when dissolved in 0.02 M EDTA (pH 8.6), undergoes self-association. Over a protein concentration range between 0.8 and 1.5 mg/ml, the self-association could best be described by a monomer–dimer–trimer step association, although indefinite self-association could not be ruled out. The equilibrium constants obtained were sufficient to describe the system over the concentration range investigated.

Apolipoprotein A-II (apo-A-II)[1] is one of the major components of the human serum high-density lipoprotein (HDL) class (Morrisett et al., 1975; Scanu et al., 1975). The chemical properties of this apoprotein are known, including its amino acid sequence (Brewer et al., 1972); it has a molecular weight of 17 400 and is made up of two identical polypeptide chains linked together at the sixth position from the amino terminal by a single disulfide bridge. It is now established that apo A-II retains binding capacity for lipids in vitro. Several investigations have been conducted on the definition of the molecular properties of this apoprotein with reference to its lipid binding (Assmann and Brewer, 1974; Makino et al., 1974; Morrisett et al., 1975; Reynolds and Simon, 1974; Stoffel et al., 1974; Vitello et al., 1975).

Even though a large amount of information is available on the properties of apo A-II, very little is known on the behavior of this polypeptide in aqueous solutions. In this report, we present data indicating that apo A-II tends to self-associate in aqueous solution.

Materials and Methods

Preparation of Apo A-II and Assessment of Purity. Human serum HDL (ρ 1.063–1.21 g/ml) were separated

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[1] Abbreviations used are: HDL, high-density lipoproteins; apo-HDL, apolipoprotein of HDL; apo A-II, one of the two major polypeptides of apo-HDL.

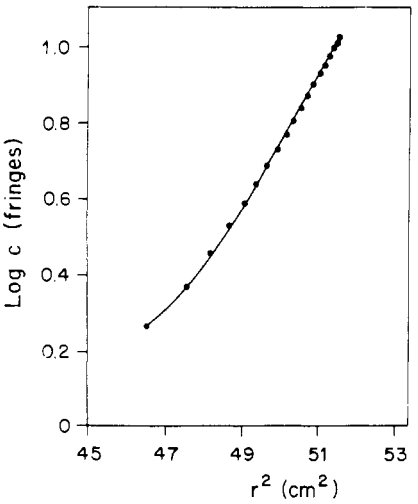

FIGURE 1: Typical plot of a sedimentation equilibrium experiment of apo A-II in 0.02 M EDTA (pH 8.6); initial concentration, 0.86 mg/ml; speed, 16 000 rpm; temperature, 20 °C. For details, see text.

and purified by preparative ultracentrifugation as previously described (Scanu, 1966), starting from fresh pooled blood. Apo-HDL was obtained by delipidation in 3:2 (v/v) ethanol–ethyl ether at −10 °C (Scanu and Edelstein, 1971). The resulting apo-HDL was solubilized in 0.01 M Tris (HCl) buffer (pH 8.2) and 8 M urea, and fractionated by Sephadex G-200 column chromatography in 8 M urea (Scanu et al., 1969). Peak IV eluted from this column was further resolved by DEAE-cellulose ion exchange column chromatography in 6 M urea under conditions described previously (Scanu et al., 1972). The fraction corresponding to apo A-II was dialyzed exhaustively against 0.01 M NH_4HCO_3, and was either used immediately after dialysis or lyophilized and stored under nitrogen at −10 °C. The purity of apo A-II was carefully controlled, since impurities would have affected the results. The preparations used migrated as a single band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) over a range of protein concentrations between 20 and 200 μg. They also gave a single line of immunoprecipitation with antibodies raised in the rabbit against human apo A-II, but did not react against anti-apo A-I antibodies. Occasionally, the amino acid composition of the preparation was checked and found to correspond to that in published reports (Scanu et al., 1975; Morrisett et al., 1975).

Studies in the Analytical Ultracentrifuge. The pure apo A-II was extensively dialyzed at 4 °C against 0.02 M EDTA (pH 8.6), $\mu = 0.12$, 10^{-3} M NaN_3. The final protein concentrations were between 0.8 and 1.5 mg/ml, as determined by the method of Lowry et al. (1951) with bovine serum albumin used as the standard and with an appropriate correction factor from amino acid analyses.

Molecular weight determinations were carried out by means of a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and Rayleigh optics. The conventional sedimentation equilibrium method described by Richards et al. (1968) was followed. In order to reduce the time required to reach equilibrium, we used an initial overspeed interval of 2 to 3 h at 1.5 times the final speed. The range of final speeds was between 14 000 and 18 000 rpm. Details of this procedure have been described by Chervenka (1970). A synthetic boundary centerpiece was used in all experiments. We omitted the fluorocarbon oil layer, however, to avoid its possible interaction with apo A-II. The initial concentration in fringes, c_0, was determined from a diffusion experiment immediately following the completion of the sedimentation equilibrium run. The conversion factor between fringes and concentration was 2.39×10^{-4} g ml^{-1} fringes^{-1}. The partial specific volume of apo A-II, calculated from the amino acid composition, was 0.743 ml/g. Solvent densities were measured in a Mettler-Anton Paar magnetic density meter (Mettler, Hightstown, N.J.). All studies were carried out at 20 °C.

Results

The apparent weight average molecular weight, $M_{w_{app}}$, can be obtained from sedimentation equilibrium measurements by the basic equation:

$$M_{w_{app}} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \, \mathrm{d} \ln c / \mathrm{d}(r^2) \qquad (1)$$

where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume, ρ the density of the solution, ω the angular velocity, c the concentration of the solute, and r the distance from the center of rotation.

For apo A-II, the plots of log c vs. r^2 (Figure 1) were nonlinear over the concentration range studied (0.8 to 1.5 mg/ml) and for speeds between 14 000 and 18 000 rpm. The apparent weight average molecular weights determined from tangents to curves such as that shown in Figure 1 ranged from about 18 000 at the lowest to about 35 000 at the highest concentration. The actual molecular weight range in the individual runs depended upon the initial protein concentration and rotor speed. The observed increase of the apparent weight average molecular weight with the increase in protein concentration can be ascribed to nonideal effects and/or to higher molecular weight species present in solution. The first possibility may be ruled out by the fact that the plot of the reciprocal of the apparent weight average molecular weight as a function of protein concentration was nonlinear and had a large negative limiting slope. Also, the nonlinearity of the plot would require virial coefficients of higher orders to permit extrapolation to the monomer molecular weight. Alternatively, the effect may be attributed to charge fluctuations. This possibility seems improbable, however, due to the moderately high ionic strength of the medium employed, and due to the fact that the chosen pH of the solutions was far from the isoelectric point of the protein (pI = 4.65; Edelstein et al., 1973); both of these factors tend to minimize the effect of charge fluctuations (Tanford, 1967). Hence, the most likely interpretation for the increase in the apparent weight average molecular weight attending the increase in protein concentration appears to be the self-association of the apo A-II monomer to form higher molecular weight species. Based upon this conclusion, the concentration dependence of the apparent weight average molecular weight of apo A-II was analyzed according to standard procedures for self-associating systems (Adams, 1965; Rao and Kegeles, 1958; Steiner, 1952). The basic assumptions used in the analysis were that (a) the partial specific volumes of all species were equal, (b) the refractive index increments of all species were equal, and (c) the activity coefficient, y_i, of each species was represented by $\ln y_i = iBM_1c$, where B is the second virial coefficient and all terms higher than the first order in c are negligible.

For self-associating systems, the apparent weight average molecular weight, $M_{w_{app}}$, is defined by:

$$M_1/M_{w_{app}} = (M_1/M_w) + BM_1c \qquad (2)$$

where M_1 is the monomer molecular weight and M_w is the weight average molecular weight corrected for the nonideal effects (Adams and Williams, 1964; Goldberg, 1963) defined as:

$$M_w = \sum c_i M_i / c_0 \quad (3)$$

where M_i is the molecular weight of species i and c is the total protein concentration. The latter can be expressed (Adams and Williams, 1964; Adams, 1967) as:

$$c = \sum c_i \quad (4)$$

where c_i is the concentration of species i. Adams and Williams (1964) have shown that the concentration of a monomer is given by the equation:

$$c_1 = \alpha \exp(-BM_1 c) \quad (5)$$

in which

$$\alpha = c \exp \int_0^c [(M_1/M_{w_{app}}) - 1](dc/c) \quad (6)$$

The exponential term in eq 6 represents the apparent weight fraction of the monomer, f_a, which can be obtained from the experimental data by integration of a plot of $[(M_1/M_{w_{app}}) - 1]/c$ vs. c (Adams, 1967).

The number average molecular weight, M_n, can also be obtained from sedimentation equilibrium studies (Adams, 1965) by eq 7 where $M_{n_{app}}$ is the apparent number average molecular weight and M_n is defined in eq 8.

$$\int_0^c (M_1/M_{w_{app}})dc = (cM_1/M_n) + (BM_1c^2/2) = (cM_1/M_{n_{app}}) \quad (7)$$

$$M_n = c/\sum c_i M_i \quad (8)$$

Additional expressions of the type $M_1{}^n \Sigma c_i M_i{}^n$ can be developed as needed for the analysis (Adams, 1967; Adams and Lewis, 1968).

Monomer–Dimer Self-Association. The initial model used in an attempt to fit the data was the monomer–dimer equilibrium as described by the relation:

$$2P_1 \rightleftharpoons P_2$$

where P is the molecule undergoing association and P_2 is its associated form.

The equilibrium constant, K, for this process is given by eq 9. By expressing c_2 as $K_2(c_1)^2$, substituting this value into both eq 2 and 3, and rearranging the terms, one obtains eq 10. Equation 10 contains two unknowns, B and K_2. By using eq 4 and 9 and expressing c_1 in terms of the second virial coefficient (eq 5), one can eliminate K_2, and eq 11 now only contains one unknown, B.

$$K_2 = [c_2/(c_1)^2] \quad (9)$$

$$\frac{1}{(M_1/cM_{w_{app}}) - BM_1} = c_1 + 2K_2c_1{}^2 \quad (10)$$

$$2c = \alpha \exp(-BM_1c) + \frac{1}{(M_1/cM_{w_{app}}) - BM_1} \quad (11)$$

Solution of eq 11 for BM_1 by successive approximations at each protein concentration (solutions of apo A-II in 0.02 M EDTA, pH 8.6) gives values of BM_1 which were found to increase systematically from -1.67×10^2 ml/g at low concentrations to -0.584×10^2 ml/g at the highest concentration investigated. This indicates that the monomer–dimer model is not adequate to explain the experimental data.

Monomer–Dimer–Trimer Model. The next model included the possibility of trimer formation, which may be represented by:

$$2P_1 \rightleftharpoons P_2$$

$$3P_1 \rightleftharpoons P_3$$

The constant K_2 has been defined by eq 9, and

$$K_3 = c_3/(c_1)^3 \quad (12)$$

Expressing c_2 as $K_2(c_1)^2$ and c_3 as $K_3(c_1)^3$ and substituting the values into eq 2 and 3, one obtains:

$$\frac{1}{(M_1/cM_{w_{app}}) - BM_1} = c_1 + 2K_2c_1{}^2 + 3K_3c_1{}^3 \quad (13)$$

which contains three unknowns: BM_1, K_2, and K_3. K_2 and K_3 may be eliminated by use of eq 4, 5, 8, 9, and 12:

$$\frac{6cM_1}{M_{n_{app}}} - 5c = 2\alpha \exp(-BM_1c) + 3BM_1c^2 - \frac{1}{(M_1/cM_{w_{app}}) - BM_1} \quad (14)$$

The solution of eq 14 by successive approximations at each concentration gave values of BM_1 which varied randomly between 5.84×10^2 and -2.29×10^2 ml/g; the average value was $(-0.3 \pm 2.1) \times 10^2$ ml/g. Since BM_1 was zero within experimental error, the system was assumed to be ideal in the subsequent calculations. By rearranging eq 13, one can show that a plot of:

$$\left[\frac{1}{(M_1/cM_{w_{app}}) - BM_1} - c_1\right]\left[\frac{1}{2c_1{}^2}\right]$$

vs. c_1 should yield a straight line with a slope of $3K_3/2$ and an intercept of K_2. The results for apo A-II follow a linear relationship with the derived values of K_2 and K_3 being 8.87×10^3 M^{-1} and 2.05×10^8 M^{-2}, respectively. These results indicate that the experimental data may be explained on the basis of the monomer–dimer–trimer model.

In addition to the two models discussed above, alternatives were examined, namely, the monomer–trimer and monomer–dimer–tetramer models. Neither of these fitted the experimental data.

Finally, we considered the indefinite self-associating model.

Indefinite Self-Association. If the self-association of a given solute proceeds without limit, the system represents an indefinite self-association (Adams, 1967; Adams and Lewis, 1968) which may be described by expressions of the type: $2P_1 \rightleftharpoons P_2$, $K_{12} = [P_2][P_1]^2$; $P_1 + P_2 = P_3$, $K_{123} = [P_3]/[P_1][P_2]$, etc. Following the convention described by Adams (1967), the concentrations of the reacting species are expressed in grams per milliliter. If it is assumed that all the equilibrium constants, K, are equal, the intrinsic association constant k may be defined as $k = 1000K/M_1$. In terms of k, the total concentration will be represented by the series:

$$c = c_1(1 + 2kc_1{}^2 + 3k^2c_1{}^2 + 4k^4c_1{}^4 + \ldots) \quad (15)$$

which, when $kc_1 < 1$, becomes:

$$c = c_1/(1 - kc_1)^2 \quad (16)$$

It has also been shown that the relationship between the

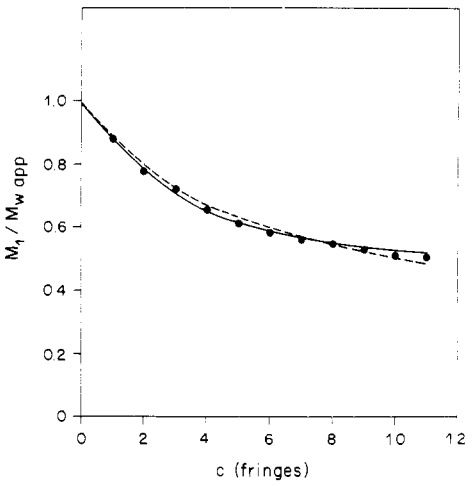

FIGURE 2: Comparison of the experimental values of $M_1/M_{w_{app}}$ for apo A-II with two theoretical models: (●) experimental data; (—) monomer-dimer-trimer self-association; (- -) indefinite self-association. (See text for details.)

experimentally obtained quantities $M_1/M_{w_{app}}$ and $M_1/M_{n_{app}}$ and the nonideal term BM_1 is given by eq 17 and 18.

$$\frac{M}{M_{w_{app}}} = \frac{1}{\dfrac{2}{M_1/M_{n_{app}} - BM_1c/2} - 1} + BM_1c \qquad (17)$$

$$\frac{M_1}{M_{n_{app}}} = (1 - kc_1) + \frac{BM_1c}{2} \qquad (18)$$

By solving eq 17 for BM_1 at each protein concentration, we obtained the values of BM_1. These values were found to vary randomly between 0.584×10^2 ml/g and -1.42×10^2 ml/g, with an average of -0.08 ± 0.71 ml/g. Since BM_1 is again zero within experimental error, the system can be assumed to be ideal. Therefore, the second term on the right side of eq 18 becomes zero. A plot of $M_1/M_{n_{app}}$ vs. c_1 should be linear, with an intercept of 1 and a slope equal to k. This relationship holds and yields a value of $k = 3.09 \times 10^2$ ml/g which, in units of moles per volume, is $K_{ISA} = (5.4 \pm 0.1) \times 10^3$ M^{-1}. Hence, indefinite self-association also fits the data within experimental error.

According to our analyses, both the monomer-dimer-trimer and the indefinite self-associations have been shown to describe the dependence of the $M_{w_{app}}$ of apo A-II as a function of concentration. A plot of $M_1/M_{w_{app}}$ vs. concentration is shown in Figure 2. The solid and dashed lines were calculated for the monomer-dimer-trimer and indefinite self-associations, respectively. A detailed analysis of the fit indicates that the sum of the squares of the deviations between experimental and theoretical points for the monomer-dimer-trimer model is 0.0011 and that for the indefinite self-association is 0.0017.

The association constants for the models considered as well as the ratios of $M_1/M_{w_{app}}$ obtained experimentally and calculated at several concentrations for the monomer-dimer-trimer and indefinite self-associations are summarized in Table I.

Table I: Comparison of Experimental and Calculated $M_1/M_{w_{app}}$ for Apo A-II.

	$M_1/M_{w_{app}}$		
c (Fringes)	Exp	n = 1, 2, 3[a]	ISA[b,c]
2	0.784	0.778	0.792
4	0.662	0.653	0.678
6	0.590	0.591	0.604
9	0.530	0.541	0.527
11	0.510	0.521	0.488

[a] $K_2 = (8.87 \pm 0.02) \times 10^3$ M^{-1}; $K_3 = (2.05 \pm 0.06) \times 10^8$ M^{-2}. [b] ISA = indefinite self-association. [c] $K_{ISA} = (5.4 \pm 0.1) \times 10^3$ M^{-1}.

Discussion

By using established procedures to study the behavior of self-associating systems, we have shown that aqueous solutions of pure human apo A-II follow self-associating patterns which may be described by a discrete self-association of monomer-dimer-trimer or by an indefinite self-association system. In the protein concentration range studied, and under the chosen experimental conditions, it is not possible to distinguish unequivocally between these two modes of association. However, a detailed numerical analysis of the data shows that the sum of the squares of the deviations is about 25% lower for the monomer-dimer-trimer system than for the indefinite self-association mode. This would tend to support the former model. In a recent report, which appeared after this work was completed, Osborne et al. (1975) make reference to unpublished results indicating that native apo A-II undergoes dimerization in aqueous solutions. In the absence of technical details we are unable to make a direct comparison between those results and the ones presented in this paper. It is possible, however, that conditions of analyses may account for the different modes of apo A-II association observed. According to Osborne et al. (1975), the tendency to dimerize is retained by the single reduced and carboxymethylated chain of apo A-II although the potential influence of the chemical modification on this process was not determined. In this context it is of interest to note that the naturally occurring single chain of apo A-II of the Rhesus monkey (*M. mulatta*) self-associates in aqueous solutions (Barbeau and Scanu, 1975).

The definition of the mode of association of apo A-II in solution is also important for the understanding of the mechanism whereby this apoprotein interacts with lipids. Work conducted in this laboratory (M. C. Ritter and A. M. Scanu, manuscript in preparation) has shown that lipid binding by apo A-II is markedly influenced by its state of aggregation. Similar observations were made with apolipoprotein A-I, the major apoprotein of HDL (Vitello et al., 1975; Vitello and Scanu, 1976). It follows that the solution properties of the HDL apolipoproteins, and probably of any other apoprotein, must be established before studies on their interaction with lipids or any other ligand are carried out and interpreted.

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A Kinetic Study of Protein–Protein Interactions†

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ABSTRACT: Kinetic studies have been carried out of the monomer–dimer interaction of insulin, β-lactoglobulin, and α-chymotrypsin using stopped-flow and temperature-jump techniques. The pH indicators bromothymol blue, bromophenol blue, and phenol red were used to monitor pH changes associated with the monomer–dimer interaction. In all three cases a kinetic process was observed which could be attributed to a simple monomer–dimer equilibrium, and association (k_1) and dissociation (k_{-1}) rate constants were determined. The results obtained are as follows: for insulin at 23 °C, pH 6.8, 0.125 M KNO_3, $k_1 = 1.14 \times 10^8$ M^{-1} s^{-1}, $k_{-1} = 1.48 \times 10^4$ s^{-1}; for β-lactoglobulin AB at 35 °C, pH 3.7, 0.025 M KNO_3, $k_1 = 4.7 \times 10^4$ M^{-1} s^{-1}, $k_{-1} = 2.1$ s^{-1}; for α-chymotrypsin at 25 °C, pH 4.3, 0.05 M KNO_3, $k_1 = 3.7 \times 10^3$ M^{-1} s^{-1}, $k_{-1} = 0.68$ s^{-1}. The kinetic behavior of the separated β-lactoglobulin A and B was similar to that of the mixture. In the case of chymotrypsin, bromophenol blue was found to activate the enzyme catalyzed hydrolysis of *p*-nitrophenyl acetate, and a rate process was observed with the temperature jump which could be attributed to a conformational change of the indicator–protein complex. The association rate constant for dimer formation of insulin approaches the value expected for a diffusion-controlled process, while the values obtained for the other two proteins are below those expected for a diffusion-controlled reaction unless unusually large steric and electrostatic effects are present.

A number of studies have been directed toward characterizing the equilibrium properties of protein–protein interactions (cf. McKenzie, 1967; Blundell et al., 1972; Aune et al., 1971; Horbett and Teller, 1974). However, relatively little information is available concerning the dynamics of protein–protein interactions. In the work reported here, kinetic studies have been carried out of the self-association of insulin, β-lactoglobulin, and α-chymotrypsin using stopped-flow and temperature-jump techniques. Changes in pH accompanying the self-association have been used to monitor the course of the reaction.

Ultracentrifuge studies of insulin (Zn free) aggregation have been made at pH 2 (Jeffrey and Coates, 1966) and at neutral pH (Pekar and Frank, 1972). Monomer, dimer, tetramer, and higher aggregates have been used to account for the data at pH 2, while at neutral pH values monomer, dimer, hexamer and higher aggregates were found to accommodate the data better. Although a detailed study of the pH dependence of the aggregation equilibria is not available, the amount of polymerization appears to decrease with decreasing pH (Blundell et al., 1972). In the kinetic studies, attention was confined to a range of protein concentrations in which the monomer and dimer are the prevalent species.

The aggregation properties of β-lactoglobulin have been studied over a wide pH range (McKenzie, 1967; Albright

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