Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 466-474

# HUMAN PLASMA HIGH DENSITY APOLIPOPROTEIN A-I: EFFECT OF PROTEIN-PROTEIN INTERACTIONS ON THE SPONTANEOUS FORMATION OF A LIPID-PROTEIN RECOMBINANT

John B. Massey, Antonio M. Gotto, Jr., and Henry J. Pownall

Department of Medicine
Baylor College of Medicine
and
The Methodist Hospital
Houston, Texas 77030

Received February 6,1981

## SUMMARY

The effect of the self-association of apolipoprotein A-I on the dynamics of lipid-protein complex formation was studied. Treatment of self-associated apolipoprotein A-I with guanidine hydrochloride initially resulted in dissociation of the oligomers into monomers and subsequent denaturation of the monomers. The association of monomeric and oligomeric apolipoprotein A-I with dimyristoylphosphatidylcholine resulted in identical lipid-protein recombinants as determined by chemical analysis and gel-filtration column elution profiles. Denaturation of a recombinant with guanidine hydrochloride indicated that the protein is more stable in a lipid-protein recombinant than as an oligomer; however, self-association does decrease the rate of lipid-protein recombinant formation. Because apolipoprotein A-I is more stable when it is associated with lipid, we conclude that the association of this protein with a variety of lipids is subject to kinetic control.

## INTRODUCTION

ApoA-I,[1] the major protein of the human plasma high density lipoproteins, has been the subject of numerous structural studies in several laboratories (1-6). ApoA-I readily self-associates in solution (3,4,5) and also interacts with alkanes, lysophosphatides, sodium dodecyl sulfate, and certain phosphatidylcholines (7) such as DMPC (8-10). However, with physiologic lecithins, such as egg PC and those found in HDL, the interaction is negligible (11). This anomaly may be rationalized, in part, by studies demonstrating that the interaction of apoA-I with DMPC is kinetically controlled (8). The rate of association occurs preferentially at the transition temperature of DMPC where lattice defects or "holes" exist due to

[1] Abbreviations: apoA-I, major protein of plasma high density lipoproteins; HDL, plasma high density lipoproteins; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; $T_c$, gel to liquid crystalline transition temperature; CD, circular dichroism; Gdm·Cl, guanidine hydrochloride.

0006-291X/81/060466-09$01.00/0

the imperfect packing of coexisting gel and liquid-crystalline phases (12,13). The poor association of apoA-I with other phosphatidylycholines may also be kinetically controlled. Lipid-protein recombinants of apoA-I and HDL lipids or several synthetic saturated and unsaturated PC's have been formed by sonication (14) or by cholate dialysis (15). Although apoA-I forms stable lipid-protein complexes, the rate of spontaneous formation is very slow.

Although apolipoproteins self-associate, the relationship between this process and the dynamics of lipid-protein association remains obscure. Ritter and Scanu have suggested that self-associated apoA-I does not interact with certain PCs (14). The CD and fluorescence spectra of apoA-I oligomers and its complexes with DMPC are similar but distinct from those of monomeric apoA-I. This finding suggests that a similar protein structure and mechanism may be involved in its self-association and its association with lipids. Thus, apoA-I can presumably exist in a thermodynamic equilibrium between those species which are self-associated and those which are associated with lipid. Under conditions where these two processes can compete, the distribution of apoA-I would be under thermodynamic control.

We now report the effect of self-association of apoA-I on protein stability as assessed from its denaturation by guanidinium hydrochloride and on the formation of lipid-protein recombinants.

## Materials and Methods:

The isolation of apoA-I from human plasma high density lipoproteins and the preparation of [$^{125}$I]apoA-I and [$^{3}$H]DMPC has been previously described (8,13). All experiments were performed at 25$^{o}$ in a buffer consisting of 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, and 0.001 M Na Azide, pH 7.4. Solutions containing Gdm·Cl (BRL, Bethesda, Md.) were made by dilution from a stock solution of 8M Gdm·Cl.

Spectra were recorded on a Cary 61 spectropolarimeter. Mean residue ellipticities were calculated using the following equation:

$$[\theta]_{\lambda} = 116\ [\theta]_{obsd}/10\ lc$$

where $[\theta]_{\lambda}$ is the mean residue ellipticity at wavelength $\lambda$; $[\theta]_{obsd}$, the observed ellipticity; 116, the mean residue molecular weight of apoA-I; 1, the path length of the cell in centimeters; and c, the concentration of protein in mg/ml. All samples were dialyzed against the above buffer without sodium azide before spectra were measured.

Uncorrected fluorescence spectra were recorded on a SLM 8000 or Amino-Bowman spectrofluorometers. Viscosity measurements were obtained with a Cannon-Manning semi-micro viscometer (Cannon Inst. Co., P.O. Box 16, State College, PA, 16801) having a calibration constant of 0.002017 centistokes/seconds. The specific viscosity ($[\eta_{sp} = (\eta - \eta_o/\eta_o)]$ where $\eta$ and $\eta_o$ are the viscosity of the solution and that of the solvent respectively) was measured. The temperature of the viscometer was controlled by immersion in an externally regulated thermostatted bath.

Chromatography of apoA-I, DMPC, and apoA-I/DMPC complexes was conducted over Sepharose CL-4B on a 1.6 cm x 40 cm thermostatted column. The column effluent was monitored by scintillation counting of [$^3$H]DMPC; apoA-I concentrations were measured by $^{125}$I-gamma counting.

The kinetics of lipid-protein complex formation were followed by centrifugation. ApoA-I and DMPC were incubated at 25° and then mixed. At various times, 0.1 ml of this sample was mixed with 0.9 ml of ice cold buffer (4°C) to rapidly cool the reaction mixture. Since the reaction of apoA-I and DMPC is extremely slow below 20° (8), this procedure essentially stops the reaction. The aliquot was placed in a 1.5 ml tube and spun at 15,000 rpm in a Beckman microfuge for 5 minutes. The top 0.5 ml supernatant was then removed for scintillation and gamma counting.

## RESULTS

The self-association of apoA-I was studied by gel filtration chromatography and circular dichroism. The elution profile of apoA-I on a column of Sephacryl S-200 at 25° with initial protein concentrations of 0.25, 0.5, 1.6, 5, and 35 mg/ml Sephacryl has the expected shape for rapid equilibrium, reversible, protomer-oligomer association (4). The ellipticity, as assessed from CD spectra of apoA-I increases with increasing protein concentration from 0.01 mg/ml apoA-I ($[\theta]_{222}$ = -17,500±300 deg cm$^2$/decimole) to 0.4 mg/ml ($[\theta]_{222}$ = -20,900±300 deg cm$^2$/decimole) where it levels off; no additional change is seen even at 23.4 mg/ml apoA-I ($[\theta]_{222}$ = -20,900±300 deg cm$^2$/decimole). These results (not shown) indicate that our preparation of apoA-I self-associated in a manner that was similar to that previously described (4,16).

The denaturation of apoA-I (2.5 mg/ml) with Gdm·Cl was monitored by several methods. The intrinsic viscosity (Figure 1) shows a decrease in viscosity going from 0 M Gdm·Cl to approximately 0.5 M Gdm·Cl. Above 0.5 M Gdm·Cl the intrinsic viscosity increases. We assign the initial decrease to dissociation of multimeric apoA-I into monomers; this is succeeded by unfolding of the monomers into a randomly coiled protein. At 0.5 M Gdm·Cl,

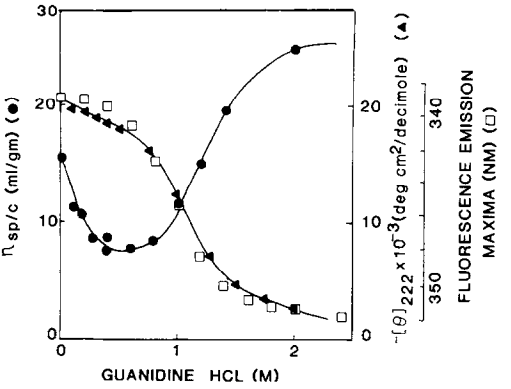


Figure 1. The denaturation of apoA-I (2.5 mg/ml) with Gdm·Cl was followed by measuring the intrinsic viscosity, circular dichroic spectra, and fluorescence emission maxima. Uncorrected fluorescence emission spectra were recorded on a SLM 8000 photon counting spectrofluorometer. To minimize inner filter effects a 0.1 cm by 0.1 cuvette was used. Intrinsic viscosity and circular dichroic spectra were analyzed as described in the methods section.

there is essentially no change in the environment of the tryptophanyl residues when compared to 0 M Gdm·Cl as measured by the fluorescence wavelength maxima and the ellipticity at 222 nm ($[\theta]_{222}$ = -17,500±300deg cm$^2$/decimole) is identical to that of monomeric apoA-I. The increase in the viscosity above 0.5 M Gdm·Cl is associated with a loss of secondary structure as shown by a decrease in the negative ellipticity at 222 nm and by a fluorescence wavelength shift which indicates the transfer of tryptophanyl residues from an hydrophobic to an aqueous environment. This behavior is consistent with dissociation of apoA-I into monomers at low Gdm·Cl concentration, i.e., below 0.5 M, and denaturation of the apoA-I monomers into a randomly coil at higher Gdm·Cl concentrations.

The association of apoA-I and DMPC was followed by column chromatography on Sepharose CL-4B (Figure 2). A change in the relative content of monomer and oligomer is expected for the range of protein concentrations used (0.05 to 4.7 mg/ml). The concentration of DMPC was adjusted to maintain a constant lipid to protein molar ratio of 150 to 1. At all concentrations of apoA-I, there is complete incorporation of DMPC and apoA-I into a lipid-protein

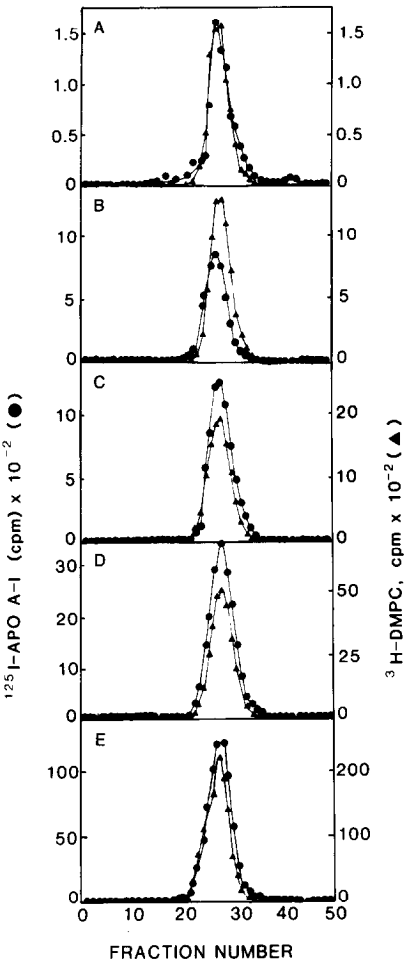


Figure 2. Recombinants of apoA-I and DMPC formed at various initial concentration of apoA-I in the initial incubation mixture. Panels A,B,C,D, and E contain original protein concentrations of 0.10, 0.250, 1.0, 2.5, and 9.2 mg/ml. One ml of apoA-I was added to one ml of DMPC at an appropriate concentration to keep a lipid to protein molar ratio of 150 to 1. The sample was incubated for 24 hrs at 25° and then chromatographed. Fraction volumes were 1.6 ml. In all cases, lipid and protein eluted as a single peak. Lipid to protein stoichiometries were determined by analysis of the peak fractions. In panels B,C,D, and E, the molar stoichiometries were 147±10, 145±5, 145±5, and 154±5. Because of the low number of counts in panel A, lipid to protein molar stoichiometries was not determined.

recombinant. The identical elution profiles and stoichiometries indicate that similar complexes are formed.

The stability of an apoA-I/DMPC recombinant was studied by denaturation with Gdm·Cl. The extent of denaturation is followed by CD and

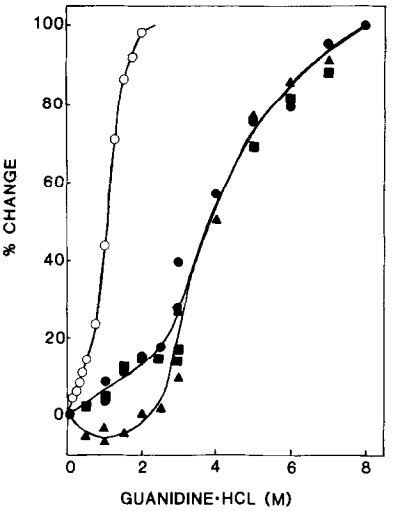


Figure 3. An apoA-I and DMPC recombinant (0.29 mg/ml apoA-I) formed in Panel E of Figure 2 was denatured with Gdm·Cl. The denaturation was monitored by circular dichroic maxima at 222 nm (●), fluorescence emission maxima (■) and fluorescence intensity (▲). The % change is defined as [(Io-Ix)/(Io-$I_\infty$) x 100%] where Io is the spectroscopic measurement at 0 M Gdm·Cl, $I_\infty$ is at 8M Gdm·Cl, and $Ix_2$ is of other concentrations. The values of $(\theta)_{222}$ went from -23,000 deg $cm^2$/decimole at 0 Gdm·Cl to -200 deg $cm^2$/decimole at 8M Gdm·Cl. Fluorescence data was measured in a Amino-Bowman spectrofluorometer. Fluorescence emission maxima of the trytophyl residues changed from 334 nm at 0 Gdm·Cl to 352 nm at 8 M Gdm·Cl. The intensity of the fluorescence from the trytophyl residues decreased by one half in going from 0 to 8 M Gdm·Cl. For comparison, the denaturation of apoA-I as measured by CD values in Figure 1, have been expressed as % change (0).

spectrofluorimetry. As seen in Figure 3, DMPC increases the stability of apoA-I to denaturation by Gdm·Cl. The denaturation of apoA-I in the complexes occurs over a range from 0-8 M Gdm·Cl with midpoint at 4 M Gdm·Cl; with apoA-I in the absence of DMPC, the midpoint based upon CD data, is at 1.1 M Gdm·Cl. The denaturation curve of the recombinant by this method is complex and appears to contain at least two transitions. Since much higher denaturant concentrations are required to produce these spectral changes in the complexes, we conclude that apoA-I is much more stable in a lipid-protein recombinant than as an oligomer.

The effect of self-association on the kinetics of lipid-protein complex formation was evaluated from a kinetic analysis in the absence of Gdm·Cl, where apoA-I is oligomeric, and at 0.5M where it is monomeric. As seen in

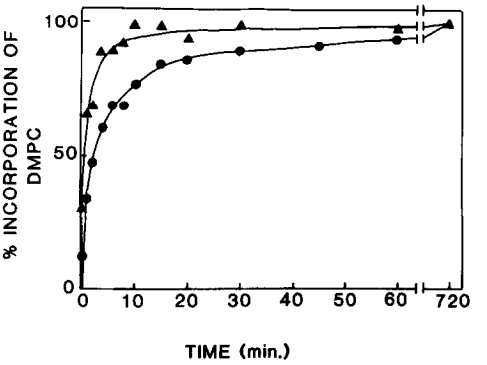


Figure 4. The kinetics of apoA-I/DMPC complex formation was followed by the % incorporation of DMPC into a complex as compared to DMPC in the incubation mixture. ApoA-I (2.5 mg/ml) and DMPC (10 mg/ml) were incubated for various times and the product isolated as described in the methods section. The curves are for a buffer of 10 mM tris, and 100 M NaCl at pH 7.4 (●) and for the above buffer plus 0.5 M Gdm•Cl (▲). There was no structural effect of Gdm•Cl upon DMPC as measured by the temperature or enthalpy of the gel to liquid crystalline phase transition by differential scanning calorimetry on a Perkin-Elmer DSC-2.

Fig. 4, apoA-I in 0.5 M Gdm•Cl associates much more rapidly with DMPC than does apoA-I in buffer. Column chromatography of the apoA-I/DMPC recombinants formed in buffer and 0.5 M Gdm•Cl after 12 hrs incubation shows identical elution profiles and stoichiometries. This finding corroborates the results from the experiment shown in Fig. 2, in that there is no discernable effect of apoA-I self-association on the final product formed. The self-association of apoA-I appears to inhibit the rate of lipid-protein complex formation.

## DISCUSSION

This study was undertaken to determine whether self-association of apoA-I has any kinetic or thermodynamic effect on the formation of apoA-I/DMPC recombinants. Our results show that low concentrations of Gdm•Cl (0-0.5 M Gdm•Cl) dissociate apoA-I into monomers, a process which is consistent with the cross-linking results of Swaney and O'Brien (17) and sedimentation and viscosity results of Edelstein and Scanu (1). A Gdm•Cl concentration of approximately 0.15 M is required to decrease the intrinsic viscosity of pure apoA-I in the oligomeric state (2.5 mg/ml) by 50%; concentrations of 1.1 and 4.0 Gdm•Cl are needed to obtain 50% denaturation of

monomeric apoA-I and for apoA-I recombined with phospholipid, respectively. There is no effect of protein concentration on the formation of a lipid-protein recombinant as measured by the resulting stoichiometry and elution profile of a gel filtration column (Fig. 2) because the protein is thermodynamically more stable when associated with phospholipid. However, the self-association of apoA-I does effect the rate of complex formation. Pownall et al. (8) have shown that the rate of apoA-I/DMPC complex formation is accelerated by the formation of defects or "holes" in the DMPC matrix at the phase transition. Theoretical analysis of lipid phase transitions (12) have indicated that the size distribution of these "holes" is such that the rate of complex formation depends on the size of the apolipoprotein. The smaller the molecular weight of the protein, the faster the rate of association because of a larger number of suitably sized "holes" into which the protein can insert. As a consequence of that theory, it is predicted that the association of oligomeric apoA-I with DMPC should be much slower than that of monomeric apoA-I. In light of our results and those of Formisano et al. (4) and Vitello and Scanu (3), we conclude that apoA-I is in a rapidly reversible equilibrium of protomer to oligomer association. We propose the following scheme:


The higher stability of apoA-I associated with DMPC drives the reaction in the direction of lipid-protein complex formation. Monomer apoA-I reacts much more rapidly with DMPC than multimeric apoA-I (Figure 4). According to this scheme, a reaction of monomeric apoA-I and DMPC should give similar lipid-protein complexes independent of the initial concentration of protein (Figure 2). All complexes of apoA-I with lipid, including native HDL, are expected to be relatively stable. Thus, the in vitro association of apoA-I with a variety of lipids is subject to kinetic control.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Sarah Myers and Mrs. Sharon Bonnot for assistance in the preparation of the manuscript and Ms. Susan McNeely for providing the line drawings. This Research is supported by a grant from the American Heart Association, Texas Affiliate, the National Institutes of Health (HL 26250), and was developed by the Atherosclerosis, Lipids and Lipoprotein Section of the National Heart and Blood Vessel Research and Demonstration Center, Baylor College of Medicine, a grant supported research project of the National Institutes of Health, Grant HL-17269. HJP is an Established Investigator of the American Heart Association.

REFERENCES

1. Edelstein, C. and Scanu, A.M. (1980) J. Biol. Chem. 255, 5747-5754.
2. Tall, A.R., Shipley, F.F., and Small, D.M. (1976) J. Biol. Chem. 251, 3749-3755.
3. Vitello, L.B. and Scanu, A.M. (1976) J. Biol. Chem. 251, 1131-1136.
4. Formisano, S., Brewer, H.B., Jr., and Osborne, J.C. (1978) J. Biol. Chem. 253, 354-360.
5. Stone, W.L., and Reynolds, J.A. (1975) J. Biol. Chem. 250, 8045-8048.
6. Barbeau, D.L., Jonas, A., Teng, T., and Scanu, A.M. (1979) Biochemistry 18, 362-368.
7. Morrisett, J.D., Jackson, R.L., and Gotto, A.M., Jr., (1977) Biochim. Biophys. Acta 422, 93-133.
8. Pownall, H.J., Massey, J.B., Kusserow, S.K., and Gotto, A.M., Jr. (1978) Biochemistry 17, 1183-1188.
9. Jonas, A., Dengler, S.M., and Patterson, B.W. (1980) J. Biol. Chem. 255, 2183-2189.
10. Swaney, J.B. (1980) J. Biol. Chem. 255, 8798-8803.
11. Assman, G., and Brewer, J.B., Jr. (1974) Proc. Nat. Acad. Sci. USA 71, 984-993.
12. Kanechisa, M.I., and Tsong, T.Y. (1978) J. Am. Chem. Soc. 100, 424-432.
13. Pownall, H.J., Massey, J.B., Kusserow, S.K. and Gotto, A.M., Jr. (1979) Biochemistry 18, 574-579.
14. Ritter, M., and Scanu, A.M. (1977) J. Biol. Chem. 252, 1208-1216.
15. Pownall, H.J., Pao, Q., Rohde, M., and Gotto, A.M., Jr., manuscript in preparation.
16. Osborn, J.C. and Brewer, J.B., Jr. (1977) Adv. Protein Chem. 31, 253-337.
17. Swaney, J.B. and O'Brien, K. (1978) J. Biol. Chem. 253, 7069-7077.