Deletion of Central α-Helices in Human Apolipoprotein A-I: Effect on Phospholipid Association†

Philippe G. Frank,‡ Jean Bergeron,‡,§ Florence Emmanuel,|| Jean-Pierre Lavigne,⊥ Daniel L. Sparks,‡ Patrice Denèfle,|| Eric Rassart,⊥ and Yves L. Marcel*,‡

Lipoproteins & Atherosclerosis Group, University of Ottawa Heart Institute, 1053 Carling Avenue, Ottawa, Ontario, Canada K1Y 4E9, Département de Sciences Biologiques, Université du Québec à Montréal, Québec, Canada H3C 3P8, and Atherosclerosis Department, Biotechnology Institute, Rhône-Poulenc Rorer, 94403 Vitry sur Seine, France

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ABSTRACT: In order to better understand the structure−function properties of apolipoprotein (apo) A-I, we have constructed and expressed three apoA-I mutants using a system previously described for the expression of human apolipoprotein A-I (Rec.-apoA-I). These mutants (corresponding to deletion of apoA-I residues 100−143, 122−165, 144−186) have been studied for their ability to form reconstituted apoA-I-containing lipoproteins (LpA-I) with POPC and DMPC, and for their structural and physical properties. Rec.- and native apoA-I can form homogeneous discoidal Lp2A-I over a wide range of POPC/apoA-I ratios [(20−130)/1] and exhibit sizes ranging from 9.5 to 10.5 nm. When recombined with varying POPC content [(20−130)/1, POPC/A-I)], the three mutants produce homogeneous discoidal Lp2A-I that contain a low POPC/A-I molar ratio [(20− 40)/1 for all mutants] and exhibit a nearly constant size [7.5−7.6 nm for Δ(100−143) and 7.9−8.0 nm for the other two mutants]. Kinetics of association of these proteins with DMPC are similar for Δ(100−143) and Rec.-apoA-I ($t_{1/2}$ of 4.0 and 4.4 min, respectively) but appear significantly reduced for Δ(122−165) and Δ(144−186) ($t_{1/2}$ of 7.5 and 6.9 min, respectively). While in the lipid-free form, all proteins have a similar thermodynamic stability with a very comparable free energy of unfolding ($\Delta G_D°$) for the α-helical structure, as determined by isothermal denaturation studies, Δ-(100−143) has a significantly lower α-helical content (33%) as compared to the other proteins [40, 41, and 45% for Rec.-apoA-I, Δ(122−165), and Δ(144−186), respectively]. When associated to POPC, Δ(122−165) and Δ(144−186) have a higher α-helicity (63 and 63%) and an enhanced stability (2.5 and 2.3 kcal/mol, respectively) as compared to Δ(100−143) (49% and 1.8 kcal/mol) and Rec.-apoA-I (52% and 1.9 kcal/mol). These results suggest that the amphipathic α-helices within residues 100−186 are directly involved in interactions with phospholipids. The helical region 100−121 appears to be more important to the stabilization of the lipid−apoprotein complex formed whereas helices within residues 122−186 appear to be critical to the initial rates of association of the apoprotein with DMPC. These data suggest that an important role of the central domain 100−186 may be to maintain the plasticity of apoA-I and its ability to form different classes of HDL particles. Therefore, it is likely that this region may also play an important role in the functional properties of this apoprotein.

Apolipoprotein A-I (apoA-I)[1] is the major protein of HDL. It has been implicated in the removal of cellular cholesterol (Castro & Fielding, 1988; Lagrost *et al.*, 1995). ApoA-I is also the most efficient activator of lecithin:cholesterol acyltransferase (LCAT; Dolphin, 1992). Despite a number of important functions that have been ascribed to apoA-I (Brouillette et *al.*, 1995), little is known about apoA-I structure−functions relationships. Although mutagenesis studies have been done (Minnich *et al.*, 1992; Sorci-Thomas *et al.*, 1993), the lipid binding properties of the mutants were not fully analyzed. Yet the lipid binding properties are central to the other functional properties of apoA-I since the amount and the nature of the lipids associated as well as the type of complex formed can affect both apoA-I structural (Jonas *et al.*, 1989; Sparks *et al.*, 1992; Dalton & Swaney, 1993; Calabresi *et al.*, 1993; Bergeron *et al.*, 1995) and functional properties (Lagrost *et al.*, 1995; Sparks *et al.*, 1995).

ApoA-I gene is a member of the exchangeable apolipoprotein superfamily that have arisen by gene duplication and/

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* Address correspondence to this author at the University of Ottawa Heart Institute, 1053 Carling Ave., Ottawa, Ontario, Canada, K1Y 4E9. Telephone: (613) 761-5254. Fax: (613) 761-5281. E-mail: ymarcel@heartinst.on.ca.

‡ University of Ottawa Heart Institute.

§ Present address: Centre de recherche de l'Hôtel-Dieu de Québec, 11, Côte du Palais, Québec, Canada, G1R 2J6.

|| Biotechnology Institute, Rhône-Poulenc Rorer.

⊥ Universite du Québec à Montréal.

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[1] Abbreviations: apoA-I, apolipoprotein A-I, DMS, dimethyl suberimidate; DMPC, dimyristoylphosphatidylcholine; GGE, gradient gel electrophoresis; GdnHCl, guanidine hydrochloride; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LpA-I, apoA-I-containing lipoproteins; POPC, 1-palmitoyl-2-oleylphosphatidylcholine; Rec.-apoA-I, recombinant wild-type apoA-I expressed in *E. coli* with an N-terminal extension of 11 residues; SDS, sodium dodecyl sulfate.

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or rearrangement. Its mature product is a 243 aa protein formed by a repetitive motif of 11 residues found in other exchangeable apolipoproteins (Li *et al.*, 1988). The use of several algorithms for the prediction of protein secondary structure has revealed the presence of eight consecutive amphipathic antiparallel 22-mer α-helices interrupted by β-turns at Pro or Gly residues. These helices are thought to be important mediators of the association with phospholipids (Segrest *et al.*, 1994), and synthetic peptides corresponding to this region as well as model peptides have been shown to associate with phospholipids (Chung *et al.*, 1985; Ponsin *et al.*, 1986; Palgunachari *et al.*, 1996). Through this interaction, apoA-I could form discoidal as well as spherical complexes. In discoidal complexes, apoA-I can associate with varying amounts of phospholipids and produces complexes with increasing size when the POPC/A-I molar ratio increases (Jonas *et al.*, 1989; Sparks *et al.*, 1992), a property that may be associated with the presence of a hinge domain (Brouillette *et al.*, 1984; Calabresi *et al.*, 1993). Proteolysis and site-directed mutagenesis of apoA-I have shown that N-terminal fragments have a lower ability to bind phospholipid (Ji & Jonas, 1995; Schmidt *et al.*, 1995). However, very little is known about the structural requirements necessary for the binding of apoA-I to phospholipids.

To analyze the role and importance of the central helices in apoA-I association with lipids, we have produce three mutants corresponding to deletions of central α-helices Δ(100−143), Δ(122−165), and Δ(144−186) using a previously characterized system (Bergeron *et al.*, 1997). These deletions have been designed to characterize the lipid binding properties of the central helices and to test the hypothesis of a possible hinge domain in human apoA-I. In the resulting mutants, the periodicity of the helices and the overall secondary structure found in native human apoA-I were maintained.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl 2-oleylphosphatidylcholine and 1, 2-dimyristoylphosphatidylcholine were obtained from Avanti Polar Lipids (Birmingham, AL). Sodium oleate was purchased from Sigma Chemical Co. (St. Louis, MO).

Construction, Expression, and Purification of the Mutant Protein. Wild-type apoA-I with an N-terminal Met-Arg-Gly-Ser-$(His)_6$ extension (Rec.-apoA-I) has been produced using an expression vector under the control of the T7 promoter as already described (clone pXL2116; Bergeron *et al.*, 1997). Three mutants each lacking two contiguous 22-mer α-helices (100−143, 122−165, and 144−186) were constructed by site-specific deletion on the cDNA. Briefly, the cDNA encoding the entire apoA-I sequence was cloned in the *Pst*I site of pBluescript SK^+ vector. Three 36-mer oligonucleotides were synthesized (Gene assembler II, Pharmacia) so that each oligo was complementary to two portions of 18 nucleotides each located upstream and downstream, respectively, of the DNA sequence to be deleted. The sequences are as follows: for oligo 100−143, 5′-GCGCATCTCCTCGCCCAGGGGCTGCACCTTGGCCTT-3′; for oligo 122−165, 5′-GCGCAGCTCGTCGCTGTACGGCTCCACCTTCTGGCG-3′; and for oligo 144−186: 5′-GTACTCGGCCAGTCTGGCTGGGCTCAGCTTCTCTTG-3′. Each oligo was annealed to the denatured plasmid and served as a primer in the repair reaction that was performed according to the method of Kunkel (Kunkel *et al.*, 1987). The DNA of each mutant was completely sequenced prior to its transfer into the expression vector.

Expression was performed with some modifications of the initial procedures (Bergeron *et al.*, 1997). Briefly, an overnight culture (LB medium) of each mutant or recombinant apoA-I (Rec.-apoA-I) was used to inoculate 1 L of culture in M9 (supplemented with ampicillin, 100 μg/mL, and chloramphenicol, 50 μg/mL). The culture was grown at 37 °C until it reached an OD at 600 nm of 0.5, and expression was induced by adding IPTG (1 mM final). The expression was continued for 90 min, and the cells were then harvested by centrifugation and frozen at −20 °C. The pellet was then resuspended in 5 mL/g cells of buffer A (100 mM Na_2HPO_4, 1 mM PMSF, and 6 M GdnHCl, pH 8). Each step of the purification was performed in 6 M GdnHCl. The solution obtained was then sonicated on ice (5 min cycles × 3, 250 W mode pulsed, 50% duty cycle, 60 output control). Bacterial debris were removed by centrifugation (1 h at 11000*g*). Nucleic acids were removed by incubation of the supernatant with 10 mL/g protein of streptomycin sulfate (10%), at +4°C for 1 h. After centrifugation, the supernatant was applied on a nitrilotriacetic acid−agarose (NTA, Qiagen) column equilibrated with buffer A. The column was washed with the same buffer. Weakly bound proteins were eluted by washing the column with 100 mM phosphate/citrate, 6 M GdnHCl, pH 6. Finally, the recombinant protein was eluted with the latter buffer at pH 5. Fractions collected were immediately neutralized with 1 M NaOH (30 μL/mL) and supplemented with 0.1 M EDTA (20 μL/mL) and 0.2 M PMSF (5 μL/mL). They were analyzed on 14% SDS−PAGE, and the most concentrated fractions were pooled and incubated with His powder (50 mM final), for 1 h at +4 °C. The sample was then dialyzed against TBS, pH 8. Each sample was analyzed on 4−20% SDS−PAGE and transferred onto nitrocellulose to test for the presence of possible degradation with well-characterized monoclonal antibodies (Marcel *et al.*, 1991).

Preparation and Characterization of Reconstituted Lipoproteins. Discoidal LpA-I were produced using the cholate dispersion/Biobeads removal method originally described by Sparks *et al.* (1992). The reconstituted lipoproteins were generated with either native Rec.-apoA-I or the mutant proteins and POPC (POPC/protein molar ratios are indicated for each experiment). LpA-I complexes were filtered through a 0.22 μm syringe tip filter. For analysis, all complexes were reisolated by gel filtration on a Superose 6 column (Pharmacia).

LpA-I were sized by using gradient gel electrophoresis under nondenaturing conditions using a Pharmacia precast 8−25% polyacrylamide gel (Pharmacia Biotech Inc., Phast-Gel), after protein staining and densitometric scanning, using molecular markers as already described (Bergeron *et al.*, 1995). Protein concentration was determined by the method of Lowry as modified by Markwell *et al.* (1978). Phospholipid and cholesterol compositions were determined with enzymatic kits (Boehringer Mannheim kits). The number of proteins per complex was determined by cross-linking the proteins with dimethyl suberimidate as described by Swaney *et al.* (1978).

Samples were submitted to electrophoresis on a precast 0.5% agarose gel (LipoGel, Beckman) under a constant electric field for 30 min at 25 °C. After protein staining,

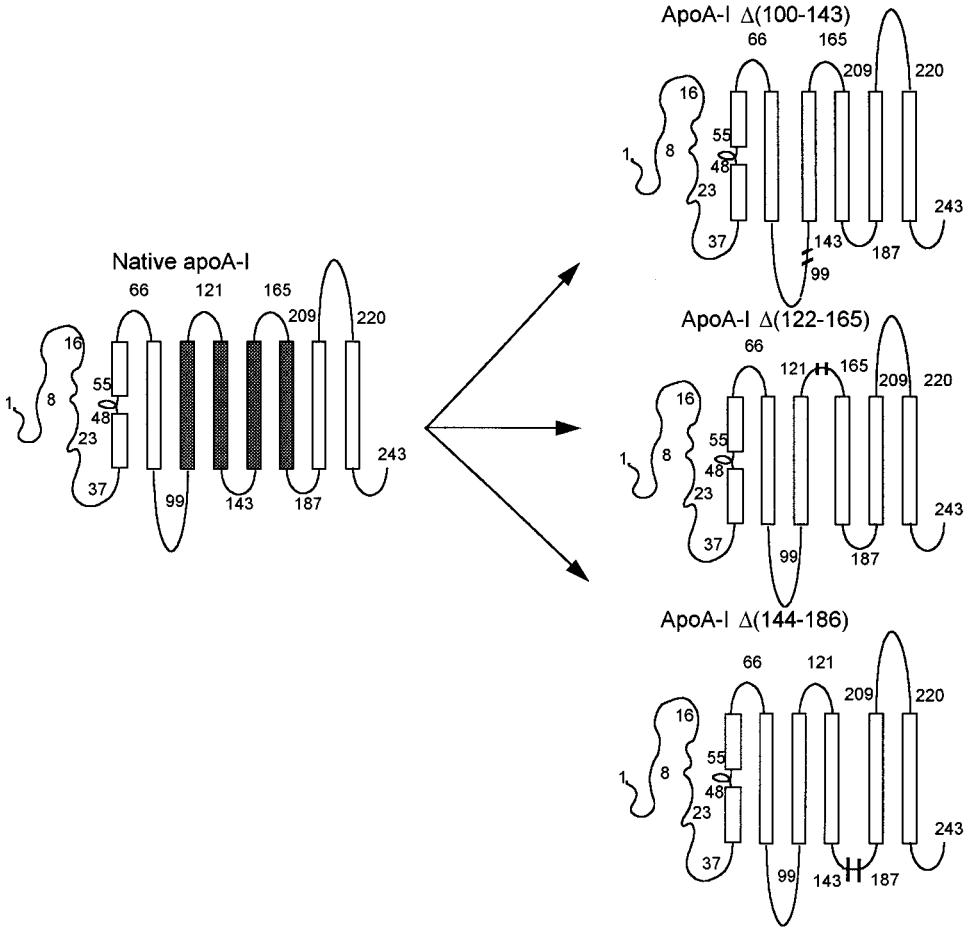

FIGURE 1: Rationale for the construction of the three deletion mutants. The predicted secondary structure of apoA-I illustrates the central α-helical region studied and the predicted maintained periodicity in each of the three constructed mutants. Amphipathic α-helices, *β*-turns, and random coils are respectively represented by boxes, curvilinear sections, and curves.

electrophoretic mobility, surface potential, density of surface charge, and valence per protein were determined with the method described by Sparks and Phillips (1992).

Electron Microscopy. Electron microscopy was performed as described by Forte *et al.* (1986). Briefly, samples to be analyzed were dialyzed against $(NH_4)OAc$ before each experiment. The samples were mixed with sodium phosphotungstate 2% (1/2 dilution) and then observed on a Hitachi H-7100 electron microscope.

DMPC Kinetic Analysis. The ability of the different proteins to clear a DMPC solution was determined as previously described by Pownall et al. (1978). Briefly, DMPC in a chloroform solution was dried out under nitrogen and then solubilized above its transition temperature in TBS, pH 8 (1 mg/mL final). DMPC/protein molar ratios of 50/1 were used, and the reaction was followed at 24 °C by monitoring the decrease in absorbance at 325 nm.

Circular Dichroic Measurements. Circular dichroic spectroscopy was performed using a Jasco J41A spectropolarimeter. The percentage of α-helix content was calculated from the molar ellipticity at 222 nm and using a mean residue weight of 115.2 for native apoA-I, 116 for Rec.-apoA-I, 114.4 for Δ(100−143), 116.2 for Δ(122−165), and 116.6 for Δ(144−186). The change in molar ellipticity was followed to determine the effect of GdnHCl concentration on the secondary structure of apoA-I and recombinant proteins. In this experiment, the free energy of unfolding ($\Delta G_D°$) of theprotein on the surface of reconstituted lipoproteins was calculated as described by Sparks *et al.* (1992).

RESULTS

Secondary Structure Analysis of Rec.-ApoA-I and the Mutants. Three apoA-I mutants corresponding to the elimination of residues (100−143), (122−165), and (144−186) have been constructed (Figure 1). These mutants were selected to avoid any disruption of the secondary structure and keep the periodicity of the resulting protein. The periodicity marked by the Pro or Gly residues was previously shown to be an important feature that allows apoA-I to associate with phospholipids (Mishra *et al.*, 1995). To demonstrate the absence of any effect, the secondary structure of the protein has been determined using the algorithm proposed by Garnier *et al.* (1978). The results showed no significant difference in the overall secondary structure in the deleted mutants as compared to native apoA-I, especially around the deleted regions.

Expression of Recombinant ApoA-I and Mutants. The recombinant wild-type protein (Rec.-apoA-I) and the site directed mutants have been produced in *Escherichia coli* using the pET expression system already described (Bergeron *et al.*, 1997). The expression of the T7 RNA polymerase was induced by adding IPTG (1 mM final) to a growing culture ($Abs_{600} = 0.5$). In this system, we were able to produce Rec.-apoA-I (10.5 mg of pure protein/L of culture) and three mutants [Δ(100−143), 2.6 mg/L; Δ(122−165), 14

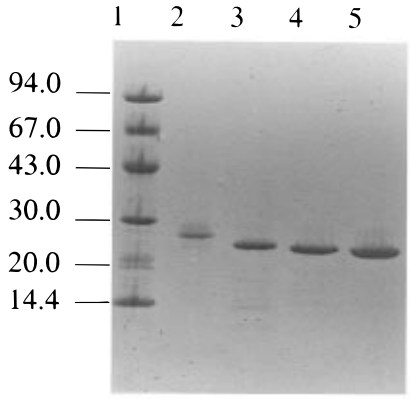

FIGURE 2: SDS gradient gel electrophoresis of the recombinant proteins. Lane 1, Molecular weight markers; 2, Rec.-apoA-I; 3, Δ(100−143); 4, Δ(122−165); 5, Δ(144−186)). The purity of each protein was shown to be greater than 90% as demonstrated by densitometric scan analysis.

mg/L; Δ(144−186), 9.9 mg/L]. However, this expression system was slightly modified in that it did not include rifampicin (after the induction of the T7 RNA polymerase) that would have specifically directed the expression of genes under the control of the T7 promoter. This modification resulted in a highly improved expression especially for one mutant [Δ(100−143)] that was hardly expressed in the classical system (not shown). The purified proteins have been shown to be pure at greater than 95% as observed on SDS−polyacrylamide gel (Figure 2).

Determination of the Ability To Form Discoidal Reconstituted Lipoproteins (Lp2A-I). The first study was performed to test the ability of each mutant to associate with phospholipid. The objective was first to evaluate the optimal POPC/A-I molar ratio for each mutant that would produce homogeneous complexes. In a first attempt, 20, 30, 40, 50, 60, 80, 100, and 130/1 ratios were tested. These complexes were prepared with the cholate dispersion/Biobeads removal method described by Sparks *et al.* (1992). The resulting complexes were analyzed directly by nondenaturing gradient gel electrophoresis (8−25%) to determine their size and homogeneity (Figure 3). Whereas Rec.-apoA-I could form stable complexes containing two proteins (Lp2A-I) with POPC over a wide ratio range [(20−130)/1] and with a size varying between 9.5 and 10.5 nm (Figure 3A), the three mutants formed stable and homogeneous Lp2A-I complexes over a lower and narrower POPC/apoA-I ratio range [(20−40)/1; Figure 3B,C,D]. The size of the corresponding Lp2A-I was also reduced [7.5−7.6 nm for Δ(100−143) and 7.9−8.0 nm for the other two mutants]. Above 40/1, each of the three mutants had a tendency to form large and heterogeneous complexes that contain three or more molecules of the protein per complex. Overall, these results suggest that the mutant apoproteins have a reduced capacity to bind phospholipids. At the highest ratios, Δ(100−143) formed the largest and the most heterogeneous complexes, even larger than those formed by Rec.-apoA-I. It is also noteworthy that this mutant formed a diffuse band, while the other two mutants produced narrower bands. These results suggest that Δ(100−143) is the most affected in its lipid binding properties.

The reduced size of the complexes formed by the mutants did not appear to be due to a change in the morphology of the complex. All the mutants and Rec.-apoA-I (with POPC/A-I 30/1) were shown to form discoidal complexes as observed by electron microscopy (Figure 4).

DMPC Kinetic Assays. The kinetics of association with DMPC were performed at 24 °C and followed by the decrease in the turbidity at 325 nm, which reflects the formation of discoidal complexes. The results of the kinetics of association with DMPC are represented on Figure 5. Rec.-apoA-I and mutant Δ(100−143) presented very similar kinetic properties. However, mutants Δ(122−165) and Δ(144−186) displayed very similar properties, but their kinetics of association were reduced when compared to the other two proteins. All the complexes obtained exhibited a discoidal shape as demonstrated by the presence of rouleaux of stacked disks by electron microscopy (not illustrated). When submitted to a GGE, at the ratio tested (50/1), the mutants produced slightly larger complexes as compared to Rec.-apoA-I. However, whereas the control protein produced Lp2A-I, the mutants formed predominantly Lp3A-I, as shown by cross-linking experiments (data not shown), therefore explaining the difference in the size of the complexes formed. The results are therefore similar to those obtained with the POPC experiments. The decreased phospholipid/apoA-I ratio, which is indicative of a reduced phospholipid capacity, results in the formation of multi-apoA-I complexes (Lp3A-I with DMPC, in some cases Lp4A-I at the highest POPC/A-I ratios).

Circular Dichroic Analysis and Stability of the Mutants in Lipid-Free and Lipid-Associated Forms. The mutants were analyzed for their α-helical content and stability in discoidal Lp2A-I formed with POPC. The composition of the complexes is presented in Table 1. As expected by the preliminary experiments presented in Figure 3, the complexes obtained with the mutants were smaller than those obtained with Rec.-apoA-I complexes.

Complexes incubated with increasing GdnHCl concentrations were analyzed by circular dichroism at 222 nm (Figure 6). The stability of the α-helices was estimated according to the method developed by Sparks *et al.* (1992). The results are presented in Table 2. For the lipid-free form, no significant difference in the stability was evident for any of the mutants when compared to Rec.-apoA-I. However, when the stabilities of the Lp2A-I were compared, the mutant Δ(100−143) was significantly less stable than the other two mutants which were significantly more stable than Rec.-apoA-I. When the α-helical content of the proteins was determined, similar trends were obtained (Table 4). These results show that the stability is not necessarily correlated to the α-helix content of a protein, but may be affected by other parameters such as its charge properties. In both the lipid-free and lipid-associated forms, Δ(100−143) has a significantly lower α-helical content than the other two mutants.

Electrophoretic Properties of the Mutants. The electrokinetic analysis of the different proteins was done on both the lipid-free and lipid-associated proteins by electrophoresis on an agarose gel. The mobility, surface charge, charge density, and valence per protein were calculated and are presented in Table 3. This type of analysis allows for the determination of surface charge properties related to very fine conformational changes of a protein such as apoA-I in association with various lipids (Sparks & Phillips, 1992). These results need to be compared to the calculated p*I* of each protein [Rec.-apoA-I, 5.76; Δ(100−143), 5.91; Δ(122−

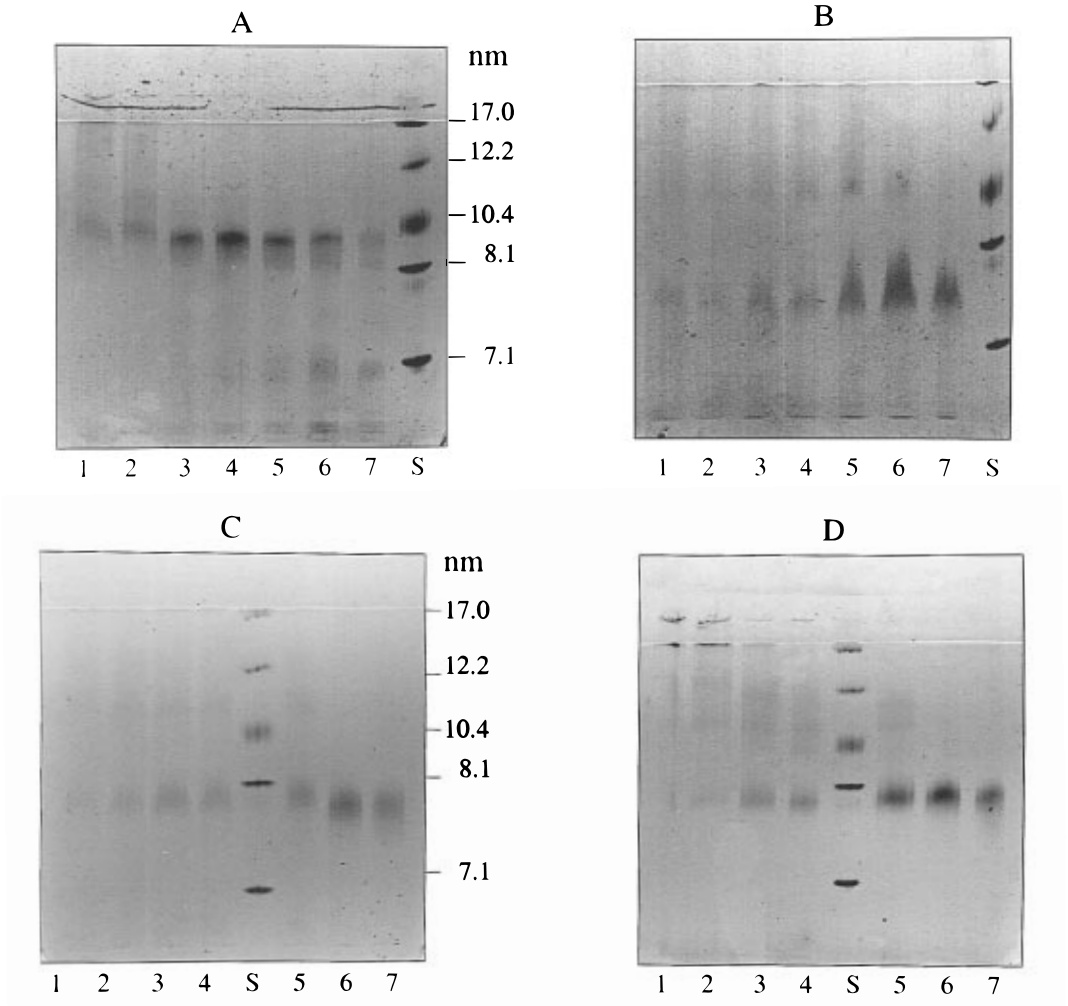

FIGURE 3: Gradient gel electrophoresis (8−25%) of the LpA-I complexes obtained with the different proteins and with varying POPC content. Molecular weight markers were used to calculate the size of the resulting complexes [complexes obtained with panel A, Rec.-apoA-I, Panel B, Δ(100−143); panel C, Δ(122−165); panel D: Δ(144−186)]. Complexes with varying POPC/A-I molar ratios were loaded on the GGE (1, 130/1; 2, 100/1; 3, 60/1; 4, 50/1; 5, 40/1; 6, 30/1; 7. 20/1).

165), 5.43; and Δ(144−186), 5.54]. Rec.-apoA-I exhibited an increased molar valence (−2.9/mol) as expected with the additional six His as already reported (Bergeron *et al.*, 1997). For all proteins, we also observed a decreased charge density when the proteins are associated to POPC. The mutant Δ(100−143) presents different properties compared to those of Rec.-apoA-I, with, as expected, a lower valence. Like Rec.-apoA-I, its charge density decreases in association with POPC, but contrary to Rec.-apoA-I, its valence decreases. Compared to Rec.-apoA-I, Δ(122−165) and Δ(144−186) have greater surface potentials and charge densities in both lipid-free and lipid-associated forms. The predicted p*I* would also suggest an increased negative charge at pH 8.6 for the last two mutants. In the lipid-free form, they are, as expected, less charged than Rec.-apoA-I, but in the lipid-associated form they have a similar charge. We also observed that the p*I* values observed for the mutants, are not always well correlated with the charge properties determined in both the lipid-free and lipid-associated forms. Thus, p*I* is not the only factor that influences the electrokinetic properties; also, the conformation of the protein that is affected by the lipidated state is another factor. Whereas Rec.-apoA-I formed complexes of ∼10.7 nm in size, the other proteins form much smaller complexes. This size difference could explain the decreased charge density of the complexes formed with Rec.-apoA-I compared to the complexes with Δ(122−165) and Δ(144−186). However, lipidation of Δ(100−143) did not significantly decrease its charge density, suggesting a different conformation in keeping with a reduced stability compared to the other mutants.

DISCUSSION

The apoA-I sequence was first determined by Brewer *et al.* (1978). It is formed by repetitive segments of 11 residues (Li *et al.*, 1988), with six 22-mers and two 33-mers that form α-helices as proposed by several models of secondary structure (Andrews *et al.*, 1976; Brasseur *et al.*, 1990; Marcel *et al.*, 1991; Nolte & Atkinson, 1992; Sparks *et al.*, 1992). These helices would interact with different affinities with the phospholipids (Segrest *et al.*, 1994). The different models proposed agree well on the secondary structure of apoA-I between residues 100 and 186 (represented in Figure 1). This domain would be formed by six amphipathic antiparallel α-helices interrupted by Pro and Gly residues. The orientation of these helices would be parallel to the phospholipid acyl chain (Brasseur *et al.*, 1990; Wald *et al.*, 1990). Among these helices, domain 100−121 was proposed by several groups to be important in the formation of a hinge domain and also found to be sensitive to limited proteolysis (Brouillette *et al.*, 1984; Calabresi *et al.*, 1993; Dalton & Swaney, 1993).

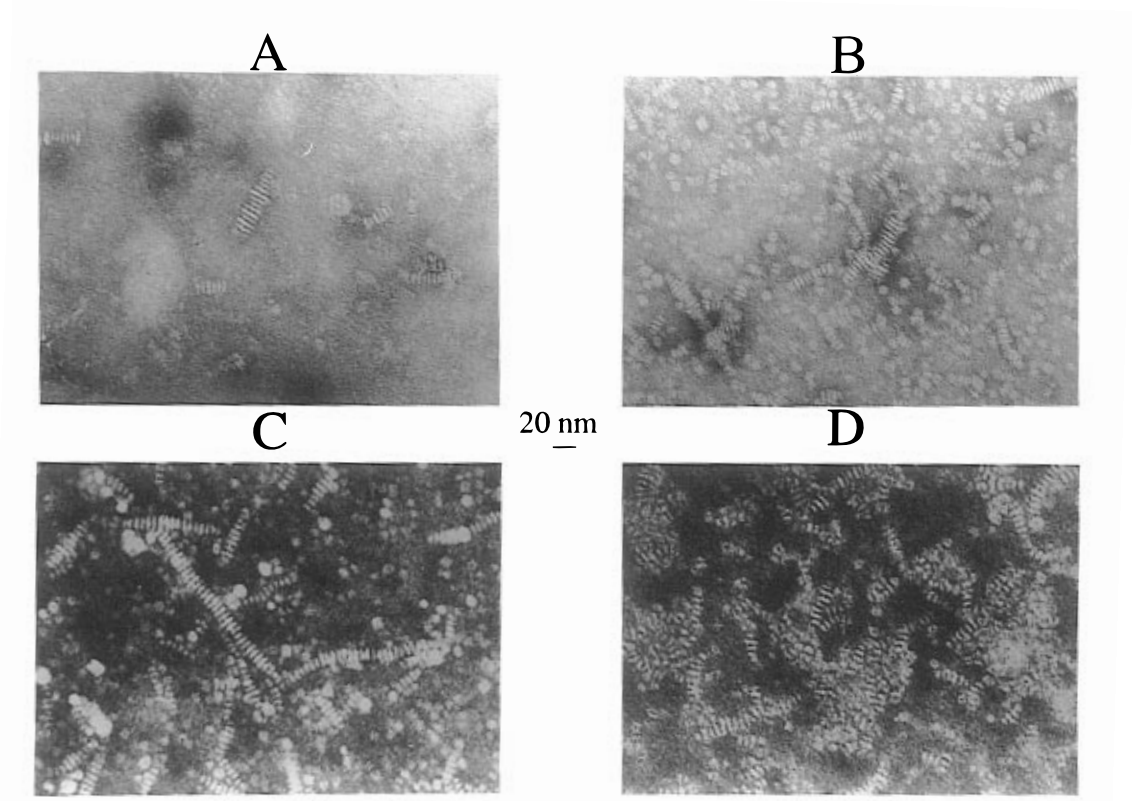

FIGURE 4: Electron microscopy of the negatively stained POPC complexes obtained with the different proteins. All complexes were prepared using the cholate dispersion/Biobeads removal method. Immediately before the experiment, they were dialyzed against 0.125 M ammonium acetate, 2.6 mM ammonium carbonate, and 0.26 mM EDTA, then diluted at ~0.2 mg/mL and then diluted with sodium phosphotungstate (1% final w/v). The samples were applied on Formvar carbon-coated grids and visualized on the microscope. Panel A, Rec.-apoA-I; panel B, Δ(100−143); panel C, Δ(122−165), panel D, Δ(144−186).

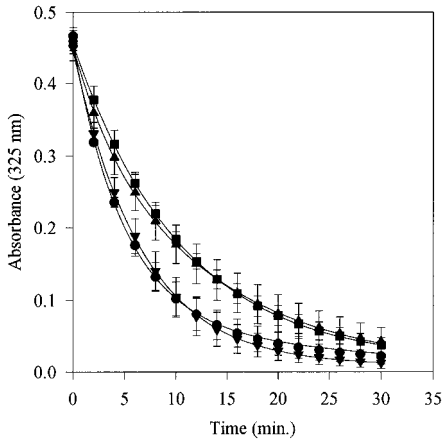

FIGURE 5: DMPC kinetic association with the different recombinant apoproteins. DMPC was solubilized in TBS, pH 8, and the proper amount diluted in the same buffer (DMPC/A-I, 50/1) was added to the protein after a 10 min preincubation at 24 °C. The reaction was followed at 24 °C for 30 min, at 325 nm in a thermo-controlled cell [▼, Rec.-apoA-I; ●, Δ(100−143); ■, Δ(122−165); ▲: Δ(144−186)].

Table 1: Composition and Size of Lp2A-I Complexes

protein	POPC/A-I[a] ratio (mol/mol)	size (nm)
Rec.-apoA-I	42 (10)	10.7 (0.6)
Δ(100−143)	24 (2)	7.9 (0.3)
Δ(122−165)	45 (6)	8.3 (0.5)
Δ(144−186)	35 (5)	8.0 (0.1)

[a] Average of three different preparations (±SD); Lp2A-I complexes as determined by cross-linking experiments.

In this study, we clearly showed that the region between residues 100 and 186 is implicated in the interaction with phospholipids. We also present evidence that these helices have different structural and physical properties. When the pair of helices 100−143 is eliminated, there is no effect on the initial association with phospholipids. In contrast, when other pairs of helices (122−165 and 144−186) are deleted, the initial rate of association with phospholipids is reduced. This suggests that helix 100−121 is not important for that step, in keeping with the results of Palnugachari *et al.* (1996) which indicate that the terminal amphipathic helices 44−65 and 220−241 are the most important for the initial binding of apoA-I to lipids. However, these authors also showed that helices 99−120 and 143−164 have a higher hydrophobic moment and affinity for phospholipids than helices 121−142 and 165−186 (Table 5). This study and ours are not directly comparable, and the results may reflect the intrinsic difference between studies based on single helices versus our own approach with a pair of adjacent helices which are intended to minimize disruption of interhelix salt bridges that play a role in the interaction with phospholipids and/or the stabilization of the secondary structure as also suggested by others (Brouillette *et al.*, 1984; Brasseur *et al.*, 1991; Lins *et al.*, 1995). In support of this concept, Vanloo *et al.* (1995) were able to obtain complexes with synthetic peptides formed by two adjacent helices of apoA-I, even with certain helices that according to Palgunachari *et al.* (1996) do not form by themselves any complex with lipids. Our observations on the charge density showing an increased charge density for Δ(122−165) and Δ(144−186) (Table 3) are compatible with a more compact and more stable structure for Δ(122−165) and Δ(144−186), possibly by stabilization with interhelix salt bridges or hydrogen bonds. We therefore conclude that

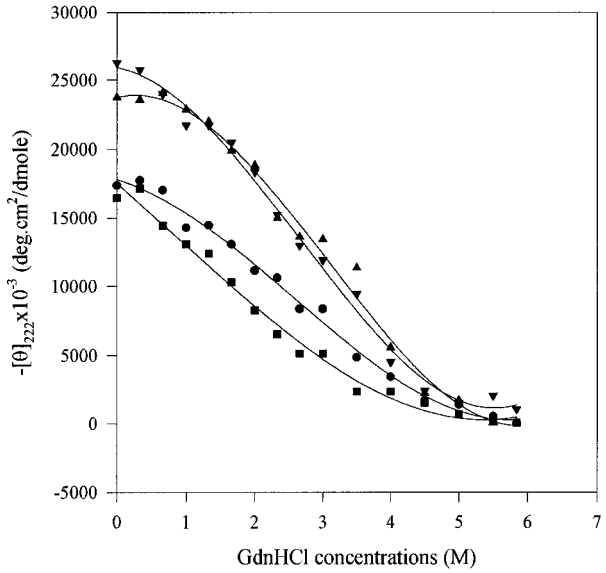

FIGURE 6: Effect of GdnHCl on the α-helix stability of lipid-associated wild-type and mutant apoA-I (●, Rec.-apoA-I; ■, Δ(100−143); ▲, Δ(122−165); ▼, Δ(144−186)]. Aliquots of each protein (33 μg/mL) were preincubated with 0−6 M GdnHCl in phosphate buffer, pH 7.4, for 72 h, at 4 °C. The molar ellipticity at 222 nm was then recorded on a Jasco J41A spectropolarimeter. Values are the average of duplicate determination.

Table 2: Denaturation Characteristics of Lipid-Free and -Bound Apoproteins

complex	$D_{1/2}$ (M GdnHCl)[b]	$\Delta G_D°$ (kcal/mol of apoprotein)[c]	Δn (mol of GdnHCl/mol of apoprotein)[d]
Lipid-Free Apoproteins			
Rec.-apoA-I	0.83 (0.1)	2.0 (0.3)	13.5 (1.9)
Δ(100−143)	0.74 (0.1)	2.1 (0.3)	15.9 (1.9)
Δ(122−165)	0.82 (0.1)	2.3 (0.1)	16.7 (0.9)
Δ(144−186)	0.79 (0.1)	2.2 (0.2)	15.9 (1.0)
Lipid-Associated Apoproteins[a]			
Rec.-apoA-I	1.6 (0.5)	1.9 (0.2)	8.5 (3.8)
Δ(100−143)	2.1 (0.1)	1.8 (0.6)	6.7 (1.9)
Δ(122−165)	3.0 (0.1)	2.5 (0.2)[e]	6.5 (0.4)
Δ(144−186)	2.7 (0.1)	2.3 (0.2)[e]	6.7 (0.6)

[a] POPC complexes (composition indicated in Table 1). [b] $D_{1/2}$: midpoints of GdnHCl denaturation (±SD). [c] $\Delta G_D°$: free energy of denaturation at zero GdnHCl concentration (±SD). [d] Δn: GdnHCl bound during denaturation (±SD). [e] $p < 0.05$, comparison vs Rec.-apoA-I.

the decreased stability of mutant Δ(100−143) is due to the loss of helix 100−121 that may not be important for initial phospholipid association but for interaction with adjacent helices. The association of apoA-I with lipids usually enhances the stability of the protein. However, this is not the case with the small discoidal complexes described here for either Rec.-apoA-I or any of the mutants. However the association of Δ(122−165) and Δ(144−186) with lipids increases the stability compared to Rec.-apoA-I. We propose that the deletion of two helices in this case generates a mutant protein more adaptable to the small discoidal complexes formed.

The central α-helices of apoA-I have been proposed by several groups to be important in the formation of discoidal complexes with varying phospholipid content (Brouillette *et al.*, 1984; Jonas *et al.*, 1989; Calabresi *et al.*, 1993). Some of these helices may interact with the phospholipids only in larger LpA-I, therefore allowing apoA-I to associate varying amounts of phospholipid. In this study, we have observed

Table 3: Electrokinetic Analysis of Lipid-Free and Lipid-Associated Apoproteins

complex	mobility[a] ($-\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$)	surface potential[b] (−mV)	charge density[c] ($\times 10^3$ esu/cm^2)	valence[a] (per mol of protein)
Lipid-Free Apoproteins				
Rec.-apoA-I	0.36	7.0	1.30	2.9
Δ(100−143)	0.27	5.2	1.05	1.6
Δ(122−165)	0.39	7.6	1.55	2.3
Δ(144−186)	0.38	7.3	1.48	2.2
Lipid-Associated Apoproteins[e]				
Rec.-apoA-I	0.32	6.2	1.07	2.9
Δ(100−143)	0.31	6.1	0.95	1.9
Δ(122−165)	0.41	7.9	1.25	2.7
Δ(144−186)	0.42	8.1	1.28	2.8

[a] Corrected electrophoretic mobility (agarose 0.5%, pH 8.6) ±0.04 (SD). [b] Potential at the complex surface ±0.6 (SD). [c] Density of surface charge ±0.1 (assuming a globular morphology for the lipid-free proteins). [d] Number of negative charges in electronic unit ±0.2 (SD). [e] POPC complexes as described in Table 1.

Table 4: Calculated and Predicted Secondary Structures of Wild-Type and Mutant Apoproteins

complex	α-helix content[a] (%)	no. of helices predicted[b]	no. of helices calculated[c]
Lipid-Free Proteins			
Rec.-apoA-I	40 (1)	nd[f]	nd[f]
Δ(100−143)	33 (1)[e]		
Δ(122−165)	41 (3)		
Δ(144−186)	45 (5)		
Lipid-Associated Apoproteins[e]			
Rec.-apoA-I	52 (4)	8	8
Δ(100−143)	49 (1)	6	6
Δ(122−165)	63 (2)[e]	6	8
Δ(144−186)	63 (2)[e]	6	8

[a] α-Helicity determined by the measurement of the ellipticity at 222 nm. [b] According to the model described (Calabresi *et al.*, 1993). [c] Assuming that 17 residues participate in the formation of an α-helix. [d] POPC complexes as described in Table 1. [e] $p < 0.05$, comparison vs Rec.-apoA-I. [f] nd: not determined.

Table 5: Hydrophobic Moment and Hydrophobicity Properties of the Four Helical Segments of ApoA-I

helix	hydrophobic moment (per residue)[a]	lipid affinity (kcal/mol)[b]
99−120	0.42	3.5
121−142	0.23	1.4
143−164	0.39	3.5
165−186	0.23	1.0

[a] As reported by Brouillette and Anantharamaiah (1995). [b] As reported by Palgunachari *et al.* (1996).

that variation in the initial POPC/apoA-I ratio was associated with a 1 nm size variation of the complexes formed with Rec.-apoA-I. In contrast, no change in size was evident for any of the three (∼0.1 nm, a value that is below the accuracy of the method). Thus, deletion of any pair of central helices in apoA-I abolishes the ability to bind varied amounts of phospholipids, implying that the process of LpA-I size variation involves more than one pair of helices and maybe also cooperativity between these helices. The denaturation study demonstrated that elimination of helix 100−121 results in a reduced stability of the α-helices. This property may be explained either by the interaction with adjacent helices by salt bridge formation (as explained above) or by a specific conformation of this domain that would allow the helix to

form a hinge between two domains. Helix 100−121 is also a class Y amphipathic α-helix as opposed to the class A amphipathic α-helix formed by the other helices examined in this study (Segrest *et al.*, 1994). It is characterized by the presence of positively charged residues at the polar−nonpolar interface as well as at the center of the polar face, a charge distribution particularly adapted to interhelix salt bridges and in keeping with the abundance of predicted salt bridges (Lins *et al*., 1995). More recently, we suggested (Bergeron *et al.*, 1995) the existence of an interaction between the central and N-terminal domains of apoA-I that may be important in the stabilization of the LpA-I complex formed. The N-terminal domain 8−22 was classified as a class G* amphipathic α-helix that is characterized by a random distribution of positively and negatively charge residues on the polar face (Segrest *et al*., 1994). Therefore, the elimination of helix 100−121 may abolish an important tertiary motif in apoA-I that stabilizes the conformation and the association to phospholipids due to the presence of salt bridges and hydrogen bonds. Possible interactions between pairs of helices via putative salt bridges may include, for example, Lys_{107}−Lys_{108} and acidic residues such as Glu_{91}−Glu_{92}, or Lys_{94}−Lys_{96} and Asp_{103}−Asp_{104}. Another possible ionic interaction could be between Lys_{107}−Lys_{108} and the acidic residues located in the N-terminal domain (Asp_1−Glu_2): These two domains have been shown to exhibit similar changes of immunoreactivity when exposed to different lipid environment (Bergeron *et al*., 1995). It is also noteworthy that the absence of Lys_{107} has been associated with significantly altered lipid binding properties compared to wild-type apoA-I (Jonas *et al*., 1991).

Another interesting result of this study is presented in Table 4 where we compared the predicted (according to the model) and the calculated number of helices (based on the α-helix content and assuming an average helix length of 17 residues; Lins *et al.*, 1995) for the different mutants. The results obtained for Δ(122−165) and Δ(144−186) are unexpected and show the presence of two supplementary helices as compared to the model whereas Δ(100−143) behaves according to the model. When domain 122−165 or 144−186 is deleted, it appears that new helices are formed, probably in the N-terminal domain. These newly formed α-helices contribute to the increase in the overall stability of the protein associated to phospholipids. However, these helices neither would directly interact with phospholipids nor have an important role in the association with phospholipids since these mutants, Δ(122−165) or Δ(144−186), do not produce significantly larger or phospholipid-enriched Lp2A-I as compared to Δ(100−143). This is even more clear when the number of helices associated to the phospholipid acyl chains for each complex was calculated. Based on the size of the lipoprotein formed, we calculated [according to Jonas *et al*. (1989)] that all mutants have a very similar number of associated helices (7), as compared to 9.5 helices for Rec.-apoA-I. These results suggest that the new helices observed with the last two mutants do not directly interact with phospholipids. The slight differences with the calculations obtained for the α-helix content may be explained by an expanded structure of the helices in association with phospholipids.

The ability of these mutants to activate LCAT, one of the major functions of apoA-I, will also be of special interest since several authors have assigned the domain responsible for the activation of LCAT to the central region of apoA-I. Well-characterized and defined (homogeneous) preparations of complexes including cholesterol will be required. However, obtaining homogeneous particle preparations with these mutants is proving difficult, possibly because they cannot bind high levels of POPC, and, therefore, addition of only a few molecules of cholesterol per molecules of mutants may destabilize the complexes formed. A similar result has also been observed for wild-type apoA-I at high POPC/cholesterol molar ratio where the complexes formed were more heterogeneous. Preliminary experiments with the mutants and Rec.-apoA-I show that addition of as little as 5 molecules of cholesterol with 30 POPC results in the formation of heterogeneous preparations for all apoA-I mutants with central deletions (P. G. Frank and Y. L. Marcel, unpublished results), suggesting a critical role for the POPC/cholesterol ratio in the formation of these discoidal complexes.

In conclusion, this work is consistent with the existence of a phospholipid-binding domain located between residues 100 and 186, which has an important role in the ability of apoA-I to associate with varying phospholipid contents. Within this domain, the sequence 122−186 appears more important in the initial association with phospholipid whereas helix 100−121 is more important in the stabilization of the complex formed possibly through interhelix interactions. Future experiments with these interesting mutants will give us more information on the functional properties of the central domain in LCAT activation and the ability to promote cholesterol efflux.

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