Probing the conformation of a human apolipoprotein C-1 by amino acid substitutions and trimethylamine-N-oxide

OLGA GURSKY

Department of Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, Massachusetts 02118-2526

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Abstract

To test, at the level of individual amino acids, the conformation of an exchangeable apolipoprotein in aqueous solution and in the presence of an osmolyte trimethylamine-N-oxide (TMAO), six synthetic peptide analogues of human apolipoprotein C-1 (apoC-1, 57 residues) containing point mutations in the predicted α -helical regions were analyzed by circular dichroism (CD). The CD spectra and the melting curves of the monomeric wild-type and plasma apoC-1 in neutral low-salt solutions superimpose, indicating $31 \pm 4\%$ α -helical structure at 22°C that melts reversibly with $T_{m,\text{WT}} = 50 \pm 2^\circ\text{C}$ and van't Hoff enthalpy $\Delta H_{v,\text{WT}}(T_m) = 18 \pm 2 \text{ kcal/mol}$. G15A substitution leads to an increased α -helical content of $42 \pm 4\%$ and an increased $T_{m,\text{G15A}} = 57 \pm 2^\circ\text{C}$, which corresponds to stabilization by $\delta\Delta G_{\text{app}} = +0.4 \pm 1.5 \text{ kcal/mol}$. G15P mutant has $\sim 20\%$ α -helical content at 22°C and unfolds with low cooperativity upon heating to 90°C . R23P and T45P mutants are fully unfolded at 0 – 90°C . In contrast, Q31P mutation leads to no destabilization or unfolding. Consequently, the R23 and T45 locations are essential for the stability of the cooperative α -helical unit in apoC-1 monomer, G15 is peripheral to it, and Q31 is located in a nonhelical linker region. Our results suggest that Pro mutagenesis coupled with CD provides a tool for assigning the secondary structure to protein groups, which should be useful for other self-associating proteins that are not amenable to NMR structural analysis in aqueous solution. TMAO induces a reversible cooperative coil-to-helix transition in apoC-1, with the maximal α -helical content reaching 74%. Comparison with the maximal α -helical content of 73% observed in lipid-bound apoC-1 suggests that the TMAO-stabilized secondary structure resembles the functional lipid-bound apolipoprotein conformation.

Keywords: amphipathic α -helix; CD spectroscopy; plasma lipoproteins; protein folding; osmolyte

Exchangeable apolipoproteins are water-soluble protein components of plasma lipoproteins that solubilize lipids and control their metabolism by activating specific enzymes or binding to cell receptors (reviewed by Atkinson & Small, 1986; Eisenberg, 1990; Pownall & Gotto, 1992). Apolipoproteins are central in the pathogenesis of atherosclerosis, stroke, and coronary heart disease (Atkinson & Small, 1986; Pownall & Gotto, 1992). Structural adaptability of apolipoproteins to heterogeneous lipoproteins and to plasma is essential for their functions and transfer, and is attributed to conformational transitions of the constituent amphipathic α -helices (Breiter et al., 1991; Segrest et al., 1992; Rogers

et al., 1998). The molecular details of these transitions remain unclear. Physicochemical studies aimed at elucidating the energetic and structural basis for this structural adaptability have shown that apolipoprotein molecules in solution have characteristics of compact folding intermediates, with high α -helical content but lack of extensive tertiary contacts, broad low-cooperativity thermal unfolding, relatively low free energy of stability, substantial solvent accessibility of apolar groups, high tendency to aggregate, and affinity for a variety of small hydrophobic ligands (reviewed by Osborne et al., 1986; Gursky & Atkinson, 1996, 1998, and references therein), including anilino-naphthalene sulfonate binding to apoA-1 (Rogers et al., 1998).

The amino acid sequences of the exchangeable apolipoproteins are composed of 11-mer tandem repeats that have high propensity to form amphipathic α -helices with distinct radial distribution of charged groups and large (30–50%) well-demarcated apolar surface implicated in lipid binding (Segrest et al., 1990, 1992). Such α -helices have been observed in the X-ray crystal structures of lipid-free apolipoproteins (Breiter et al., 1991; Wilson et al., 1991; Borhani et al., 1997) and in the NMR structures of apolipoproteins

Reprint requests to: Olga Gursky, Department of Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, Massachusetts 02118-2526; e-mail: gursky@med-biophd.bu.edu.

Abbreviations: ApoC-1, apolipoprotein C-1; HDL, high density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; β -VLDL, β -migrating very low density lipoprotein; UV CD, ultraviolet circular dichroism; NMR, nuclear magnetic resonance; TMAO, trimethylamine-N-oxide dihydrate; SDS, sodium dodecyl sulfate; LEM, linear extrapolation model.

and their fragments in a "lipid-mimicking" environment such as sodium dodecyl sulfate (SDS) that is used to prevent apolipoprotein aggregation (Rozek et al., 1995, 1997, 1998). Since in neutral aqueous solutions apolipoproteins self-associate at 10–100 $\mu\text{g/mL}$ concentrations (Osborne et al., 1986), which is far below the useful range for NMR experiments, structural studies of the monomeric lipid-free apolipoproteins have been limited to low-resolution methods such as CD spectroscopy. CD data show that lipid binding or self-association of apolipoproteins and their peptide analogues leads to an increase in their α -helical content (Osborne et al., 1986). The largest increase is observed in apoC-1, from $\sim 30\%$ α -helix in lipid-free monomer in near-neutral aqueous solution (Osborne et al., 1977; Gursky & Atkinson, 1998) to the maximum of $\sim 73\%$ in the lipid-bound state (Jackson et al., 1974) and $\sim 82\%$ in the oligomeric state (Osborne et al., 1977). To elucidate the molecular mechanisms of functional apolipoprotein transitions, the groups forming the stable helical structure in the monomer must be distinguished from those in which the helical conformation is induced by lipid binding. To make this distinction, we use the smallest human apolipoprotein C-1 as an experimental system. The sequence homology, secondary structural, and functional similarity of apoC-1 and larger apolipoproteins make it an attractive model for the analysis of the energetics-structure-function relationship in this protein family.

Human apoC-1 (57 residues of known sequence) in plasma rapidly transfers among very low density lipoproteins (VLDL), high density lipoproteins (HDL), and chylomicrons, thereby altering the metabolic properties of these lipoproteins (Windler & Havel, 1985; Eisenberg, 1990; Weisgraber et al., 1990). ApoC-1 on HDL acts as a secondary activator of lecithin:cholesterol acyltransferase, an enzyme that is central in the reverse cholesterol transport (Steyrer & Kostner, 1988). In addition, apoC-1 delays the clearance of potentially atherogenic triglyceride-rich β -migrating VLDL remnants (Windler & Havel, 1985) by displacing apoE or altering its conformation, thereby inhibiting β -VLDL uptake via the LDL receptor-related pathway (Weisgraber et al., 1990; Sehaye & Eisenberg, 1991). To date, apoC-1 is the only full-size plasma apolipoprotein that has been obtained by solid state synthesis in its folded functional form (Harding et al., 1976; Sigler et al., 1976) and that has been crystallized (Weisgraber et al., 1994), although the X-ray crystal structure has not been reported. Thermodynamic parameters of human plasma apoC-1 monomer in near-neutral solution have been determined, and the monomer unfolding has been dissected from the temperature-dependent self-association (Gursky & Atkinson, 1998). The amino acid sequence of apoC-1 contains four 11-mer repeats encompassing residues 7–50 and is predicted to form two class-A amphipathic helices (7–32) and (33–53) that comprise 82% of the apoC-1 residues (Segrest et al., 1992). The agreement with the maximal α -helical content observed in the lipid bound and in self-associated states suggests that the predicted secondary structure closely approximates the apoC-1 conformation in these states. However, it remains unclear what part(s) of the apoC-1 backbone (an average of $\sim 31\%$ or ~ 18 amino acids) form the α -helical structure in the monomer in solution.

Here, we use the predicted secondary structure as a target model for probing the solution conformation of the apoC-1 monomer by Pro substitutions. Pro scanning mutagenesis was originally used by Schulman and Kim (1996) to probe the cooperativity of the molten globular state by unfolding individual α -helices in α -lactalbumin. We use Pro because the destabilizing effects of Pro substitutions in the middle of the secondary structures of globular proteins and

peptides, 2.7–5.6 kcal/mol (Kallenbach et al., 1996; Schulman & Kim, 1996, and references therein) are comparable to the apparent free energy of the apoC-1 monomer stability in solution, $\Delta G_{app}(0^\circ) \sim 2.8$ kcal/mol and $\Delta G_{app}(25^\circ) \sim 1.8$ kcal/mol (Gursky & Atkinson, 1998). Consequently, Pro inserted in the middle of the α -helical structure in apoC-1 monomer should lead to its unfolding, provided that this structure comprises a single cooperative unit. In contrast, Pro substituted at the periphery or outside of this helical structure should lead to little or no unfolding, respectively. We test this hypothesis by the CD analysis of three apoC-1 mutants, G15P, R23P, and T45P, containing Pro substitutions in the predicted helices (7–32) and (33–53).

Another objective is to obtain apoC-1 mutants of increased secondary structure and stability that provide useful candidates for crystallographic studies. To do so, we utilize the "entropic stabilization" that provides a universal approach to stabilization of engineered proteins (Shortle, 1989; Sturtevant, 1993) by substituting the "helix-breaking" Gly15 in the predicted apoC-1 α -helix (4–32) with the "helix-making" Ala (Chakrabarty et al., 1991; Myers et al., 1997).

Furthermore, we test the effect of a naturally occurring organic osmolyte trimethylamine-N-oxide dihydrate (TMAO) on the solution conformation of apoC-1. TMAO has been shown to induce the folding of enzymes destabilized by urea, Pro substitutions, or reduction of disulfide bonds into a native-like functional conformation (Yancey et al., 1982; Wang & Bolen, 1997; Baskakov & Bolen, 1998). Since the stabilizing effect of TMAO is linked to its unfavorable interactions with the polypeptide backbone (Wang & Bolen, 1997), TMAO has been proposed as a universal protein folding agent that may stabilize a variety of marginally stable proteins, such as apolipoproteins. The α -helical content in apolipoproteins, as well as in certain other proteins and peptides, is known to increase in the presence of halogenated alcohols or detergents such as SDS. However, alcohols and SDS may drive formation of nonnative helical species, which results, in part, from their direct interactions with the protein side chains (Wang et al., 1996; Hirota et al., 1998). In contrast, TMAO has little or no significant interactions with the protein side chains (Wang & Bolen, 1997); consequently, TMAO is thought to cause protein folding in its native-like functional conformation. Here, we test whether TMAO induces apoC-1 to fold in a highly helical conformation similar to the functional lipid bound apolipoprotein conformation.

Results

Secondary structure and thermal unfolding of the synthetic wild-type and plasma apoC-1

Figure 1 shows the far-UV CD spectra of the synthetic wild-type apoC-1 measured at several temperatures from 22–80 $^\circ\text{C}$. Unless otherwise stated, the CD data in this work were recorded from 6–10 $\mu\text{g/mL}$ peptide solution in 2–5 mM phosphate buffer at pH 7.5. Plasma apoC-1 has been shown to be fully monomeric under these conditions (Osborne et al., 1977; Gursky & Atkinson, 1998). The normalized CD spectra (Fig. 1) and the melting curves (Fig. 2) recorded at these solvent conditions from 6 and 10 $\mu\text{g/mL}$ solutions of synthetic apoC-1 completely superimpose, confirming the monomeric peptide state. Comparison of the data in Figure 1 and the insert shows that the CD spectra of the monomeric synthetic wild-type and plasma apoC-1 recorded under nearly identi-

Fig. 1. CD spectra of synthetic wild-type apoC-1 monomer recorded at several constant temperatures from 22–80 °C. The temperatures are indicated on the lines. The isochromatic point is shown by arrow. The spectra were recorded from 10 $\mu\text{g/mL}$ peptide solution in 4 mM sodium phosphate buffer at pH 7.5. **Insert:** The spectra recorded from the plasma apoC-1 monomer under similar conditions (Gursky & Atkinson, 1998) are shown for comparison.

cal solvent conditions and temperatures are in close agreement. The isochromatic points in the two families of spectra agree within 1 nm, and the difference CD between the spectra of the plasma and synthetic apoC-1 at 22 °C is comparable to the noise level in the data. The α -helical content in synthetic wild-type apoC-1 monomer estimated from the molar residue ellipticity $[\theta_{222}]$ at 222 nm (see Materials and methods) is $31 \pm 4\%$ at 22 °C, identical to the value determined for the plasma apoC-1 monomer under similar conditions (Gursky & Atkinson, 1998).

Figure 2 shows the heat unfolding curve of the synthetic monomeric wild-type apoC-1 (squares) monitored by the ellipticity at 222 nm upon heating from 0–98 °C. Reversibility of the heat unfolding of this and other apoC-1 mimicking peptides analyzed in this work was verified by a superposition of the melting curves recorded at different heating rates from 2–30 °C/h, and by a full restoration of the CD spectra upon brief heating to 70 °C followed by re-equilibration at 22 °C. The melting curve $[\theta_{222}](T)$ recorded from the synthetic wild-type apoC-1 is in close agreement with the melting curve recorded under similar conditions from monomeric plasma apoC-1 (Gursky & Atkinson, 1998). Fitting the data in Figure 2 by a modified van't Hoff Equation 1 (see Materials and methods) gives the transition temperature $T_{m,WT} = 50 \pm 2$ °C and the effective enthalpy $\Delta H_{v,WT}(T_m) = 18 \pm 2$ kcal/mol K for the synthetic apoC-1; conventional van't Hoff analysis gives similar values (Fig. 2, insert). The uncertainty in these estimates, which incorporates the fitting errors by Equation 1 (± 0.9 °C for T_m , ± 1 kcal/mol for ΔH_v) and the deviations between different data sets,

Fig. 2. Thermal unfolding of synthetic wild-type and G15A apoC-1. The melting of the synthetic monomeric wild-type (■) and G15A peptides (●) upon heating from 0–98 °C was monitored by ellipticity at 222 nm. The peptide concentrations and solvent conditions are the same as in Figure 1. The data are plotted as a fraction of the unfolded species determined from the observed ellipticity as $([\theta_{obs}(T)] - [\theta_F(T)]) / ([\theta_U(T)] - [\theta_F(T)])$, where $[\theta_F(T)]$ and $[\theta_U(T)]$ are the baselines for the folded *F* and unfolded *U* states determined by fitting to Equation 1 (see Materials and methods). Solid lines show fitting of the data to Equation 1 (fitting errors are ± 1 K for T_m , ± 1.1 kcal/mol for ΔH_v , $\chi^2 = 0.013$); arrows show the midpoint temperatures T_m . **Insert:** Van't Hoff plots ($\ln K$ vs. $1/T$) of the wild-type and G15A apoC-1. The apparent equilibrium constant *K* between the *U* and *F* states is defined as $K = [U]/[F] = ([\theta_{obs}(T)] - [\theta_F(T)]) / ([\theta_U(T)] - [\theta_{obs}(T)])$. Nearly parallel shift in the van't Hoff plot of G15A compared to that of the wild-type apoC-1 indicates an increase in T_m , from $T_{m,WT} \sim 50$ °C to $T_{m,G15A} \sim 57$ °C, without any significant changes in $\Delta H_v(T_m)$.

is lower than the uncertainty in $T_m = 50 \pm 3$ °C and $\Delta H_v(T_m) = 19 \pm 3$ kcal/mol K determined for human plasma apoC-1 by a conventional van't Hoff analysis (Gursky & Atkinson, 1998). Importantly, the values of T_m and $\Delta H_v(T_m)$ determined for the plasma apoC-1 and for synthetic wild-type apoC-1 monomers are identical.

Increasing the concentration of synthetic wild-type apoC-1 from 10 to 50 $\mu\text{g/mL}$ in near-neutral low salt solutions, 22 °C, leads to no major CD changes. This contrasts with large concentration-dependent CD changes observed under similar conditions in plasma apoC-1 and linked to the formation of additional α -helices upon apoC-1 self-association (Osborne et al., 1977; Gursky & Atkinson, 1998). The reason for the absence of comparable concentration-dependent changes in synthetic apoC-1 is not clear; a possible factor may be Met38 oxidation (Anantharamaiah et al., 1988). Regardless of these differences, the CD measurements from solutions of 6–10 $\mu\text{g/mL}$ protein concentrations show that the secondary structures and thermodynamic parameters of the synthetic wild-type and plasma apoC-1 monomers are identical.

G15A mutant

Figure 2 shows the reversible melting curves of the synthetic monomeric wild-type (squares) and G15A apoC-1 (circles) recorded at 222 nm. The melting curve of G15A is shifted to higher temperatures compared to that of the wild-type peptide; the van't Hoff plots (Fig. 2, insert) and fitting by Equation 1 indicate an increase in the transition temperature by $\delta T_m = +7 \pm 1.5^\circ\text{C}$, from $T_{m,\text{WT}} = 50 \pm 2^\circ\text{C}$ to $T_{m,\text{G15A}} = 57 \pm 2^\circ\text{C}$. The nearly parallel van't Hoff plots for the two peptides suggest similar effective enthalpies of unfolding (Fig. 2, insert); fitting by Equation 1 gives the value $\Delta H_{v,\text{G15A}}(T_m) = 19 \pm 2 \text{ kcal/mol}$.

The far-UV CD spectra of G15A apoC-1 recorded at several constant temperatures from 22–80°C (data not shown), similar to those of the wild-type peptide (Fig. 1), show an isochromatic point at 203.5 nm, suggesting significant population of only two alternative conformations (α -helix and random coil). Figure 3 shows far-UV CD spectra of the wild-type and G15A peptides at 22°C. The increased intensities of the CD bands at 222, 208, and 193 nm indicate an increased α -helical content in G15A compared to the wild-type apoC-1. The difference spectrum $[\Theta_{\text{G15A}}] - [\Theta_{\text{WT}}]$ between G15A and wild-type apoC-1 at 22°C (insert in Fig. 3, solid line) shows the negative CD bands at 222 and 208 nm and a positive band below 200 nm, which indicates the presence of an additional α -helical structure in G15A peptide. The ellipticity ratio $[\Theta_{222}]/[\Theta_{208}]$ that has been proposed as a concentration-independent measure of the protein α -helical content (Bruch et al., 1991) in-

Fig. 3. Effects of the amino acid substitutions in position 15 on the apoC-1 conformation. CD spectra were recorded from synthetic wild-type (solid line), G15A (dashed line), and G15P apoC-1 monomers (broken line) at 22°C, using the peptide concentrations and solvent conditions specified in Figure 1. **Insert:** Difference spectra between G15A and the wild-type peptide (solid line) and between the wild-type and G15P peptide (broken line) show the loss of the α -helical structure upon Ala15 $\rightarrow$ Gly and Gly15 $\rightarrow$ Pro substitutions.

creases from 0.71 for the wild-type to 0.93 for G15A apoC-1. Furthermore, the average α -helical content in G15A peptide estimated from the value of $[\Theta_{222}]$ and confirmed by the CD spectral deconvolution (see Materials and methods) is $42 \pm 5\%$, or 24 ± 2 groups, significantly larger than 18 ± 2 groups in the wild-type apoC-1. In summary, the CD data in Figures 2 and 3 show that G15A substitution leads to $\sim 11\%$ increase in the α -helical content at 22°C and to $\sim 7^\circ\text{C}$ increase in the melting temperature, but has no large effect on the van't Hoff enthalpy.

G15P mutant

The CD spectrum of G15P mutant at 22°C, compared to that of the wild-type apoC-1, shows a reduction in the CD amplitudes at 193 and 222 nm and a shift in the ellipticity minimum from 208 to ~ 204 nm (Fig. 3). This indicates an increase in the random coil content at the expense of the α -helical content. Comparison of the difference spectrum between the wild-type and G15P with that between G15A and the wild-type (Fig. 3, insert) suggests a similar reduction in the α -helical content upon Ala15 $\rightarrow$ G and Gly15 $\rightarrow$ P substitutions. On the basis of these difference spectra and the results of the CD spectral deconvolution (see Materials and methods), the helical content in G15P at 22°C is estimated to be $\sim 20\%$, or ~ 11 residues.

The CD spectra of G15P recorded at 22 and 90°C (Fig. 4, broken lines), their difference spectrum $[\Theta_{T=22^\circ}] - [\Theta_{T=90^\circ}]$ (Fig. 4, left insert), and the melting curve recorded at 222 nm from 0–95°C (Fig. 4, right insert, open diamonds) indicate the loss of the α -helical structure in G15P upon heating to 90°C. The shape of the melting curve suggests the low-cooperativity unfolding; the broad extent of this unfolding transition (from about 0–95°C) precludes accurate baseline determination from the pre- and post-transitional regions, thereby precluding the van't Hoff analysis. This is consistent with the reduced number of groups in the α -helical conformation that may form a cooperatively unfolding unit in the G15P mutant. The estimated α -helical content in G15P peptide is also consistent with the difference spectrum $[\Theta_{22^\circ}] - [\Theta_{90^\circ}]$ that shows a negative band near 208 nm (Fig. 4, left insert). This band corresponds to the $\pi \rightarrow \pi^*$ transition and is detected in the CD spectra of α -helices that are at least 10 residues long (Kallenbach et al., 1996, and references therein). In summary, our data indicate that G15P substitution leads to a reduction in the α -helical content from 31% to $\sim 20\%$ accompanied by a significant reduction in the unfolding cooperativity.

R23P and T45P mutants

Figure 4 shows the CD spectra of the R23P mutant recorded at 22 and 90°C (black solid line) and similar spectra of the T45P mutant (dashed line). The overlap of the spectrum of the heat-unfolded G15P peptide at 90°C (broken line) suggests that the R23P and T45P mutants are unfolded at 22–90°C. Furthermore, the molar residue ellipticity of R23P and T45P peptides at 222 nm (Fig. 4, right insert, triangles) and other characteristic wavelengths is nearly invariant upon heating from 0–95°C. In addition, the value of $[\Theta_{222}] \sim -4,500 \text{ deg cm}^2 \text{ dmol}^{-1}$ observed for R23P and T45P at 0–95°C (Fig. 4, insert) is characteristic of heat-unfolded helical proteins that are devoid of stable hydrogen-bonded helical structure (Privalov et al., 1989; Robertson & Baldwin, 1991, and references therein). Consequently, the CD data in Figure 4 show that, in near-neutral, low salt aqueous solution at 0–95°C, R23P and T45P peptides are thermodynamically unfolded.

Fig. 4. Effects of Pro substitutions in positions 15, 23, and 45 on the apoC-1 conformation. CD spectra of synthetic monomeric G15P (broken lines), R23P apoC-1 (solid line), and T45P (dashed line) were recorded at 22 and 90 °C. The temperatures are as indicated; the spectra of R23P and T45P are invariant at these temperatures. The peptide concentrations and solvent conditions are as in Figure 1. **Left insert:** Difference spectrum $[\Theta_{T=22^\circ}] - [\Theta_{T=90^\circ}]$ represents the α -helical structure that is lost upon heating the G15P peptide from 22–90 °C. **Right insert:** Melting curves of G15P ($\diamond$), R23P ($\blacktriangle$), and T45P (∇) apoC-1 monomers monitored by CD at 222 nm upon heating from 0–95 °C. The G15P peptide shows temperature-induced ellipticity changes that indicate a loss of the α -helical structure upon heating, while R23P and T45P peptides show no such changes.

Effects of TMAO on the apoC-1 conformation

Figure 5 shows that addition of TMAO to solutions of the synthetic peptide analogues of apoC-1 leads to large changes in their far-UV CD. The largest TMAO-induced spectral changes are observed in the R23P mutant, with the ellipticity $[\Theta_{222}]$ at 22 °C changing from $-4,500 \text{ deg cm}^2 \text{ dmol}^{-1}$ in the absence of TMAO to $-24,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ in $\geq 2.7 \text{ M}$ TMAO (Fig. 5, solid and broken lines compared; Fig. 5, insert, triangles). The information content in the spectra recorded from solutions of high TMAO concentrations is limited by a short-wavelength cutoff near 220 nm that is a consequence of the UV absorption by TMAO. Nevertheless, the TMAO-induced spectral changes observed at 220–250 nm can be unambiguously linked to an increase in the α -helical structure that has a predominant contribution to the protein CD at these wavelengths (Chen et al., 1972; Hennessey & Johnson, 1981). The α -helical content in R23P mutant, as monitored by $[\Theta_{222}]$, increases from about zero to 70% upon increasing TMAO concentration from 0 to 2.7 M, with the midpoint ellipticity observed at about 1.4 M TMAO (Fig. 5, insert, triangles).

Other synthetic apoC-1 mimicking peptides analyzed in this work show TMAO-induced spectral changes similar to those de-

Fig. 5. TMAO-induced folding of apoC-1. CD spectra of R23P were recorded in the absence (solid line) and in the presence (broken lines) of different TMAO concentrations as indicated. Peptide concentrations and buffer conditions are as in Figure 1. **Insert:** Reversible TMAO-induced folding of R23P ($\blacktriangle$) and wild-type apoC-1 ($\blacksquare$) monitored by CD at 222 nm. Solid lines show least-squares fitting of the data by the linear extrapolation model (Pace, 1986) using the method of Santoro and Bolen (1988) (Equation 2 in Materials and methods, $\chi^2 = 0.86$).

picted in Figure 5, with $[\Theta_{222}]$ approaching $-24,000$ to $-26,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ at high TMAO concentrations. This corresponds to a maximal α -helical content of about 70–74%. This increase in the α -helical content may not result from the peptide self-association, as evidenced by a superposition of the normalized CD spectra recorded from solutions of identical TMAO concentrations (1 to 2.6 M) but different peptide concentrations (6 to 20 $\mu\text{g/mL}$; Fig. 5 shows the data recorded from solutions of 10 $\mu\text{g/mL}$ peptide concentrations). Furthermore, the concentrations of TMAO that induce the maximal folding of the wild-type and mutant apoC-1 are different, as is illustrated in the insert in Figure 5 showing the TMAO titration curves of R23P (triangles) and the wild-type peptide (squares). The titration curve of R23P is shifted by about +1.1 M compared to that of the wild-type peptide. Similarly, the titration curve of G15P is shifted by about +0.5 M TMAO, and the titration curve of G15A by about -0.5 M TMAO compared to that of the wild-type apoC-1 (data not shown). Thus, the peptides showing higher α -helical content in the osmolyte-free solution adopt a highly helical conformation at lower TMAO concentrations, i.e., have greater apparent stability of this conformation in TMAO-free buffer.

The TMAO titration curves were fitted by Equation 2 (see Materials and methods), which combines the linear extrapolation model (LEM) (Pace, 1986) analyzed by the method of Santoro and Bolen (1988) with the two-state model (Fig. 5, insert, thin solid curves). In the LEM, the free energy of protein stability ΔG in the presence

of a specific solute is assumed to depend linearly on the solute concentration $[c]$, $\Delta G = \Delta G^\circ - m \cdot [c]$, where ΔG° is the stability in the absence of the specific solute. The m -values for the TMAO-induced folding of the wild-type and R23P apoC-1 are about -1.1 ± 0.2 kcal/mol(peptide)·M(TMAO), and the apparent free energy of stability of the TMAO-induced highly helical conformation in the TMAO-free buffer is $\Delta G_{app,R23P} = \Delta G_{app,T45P} = -1.5 \pm 0.2$ kcal/mol and $\Delta G_{app,WT} = -0.4 \pm 0.2$ kcal/mol. For a two-state model, this translates to ~ 7 and $\sim 30\%$ population of the TMAO-induced conformation in TMAO free solution of the mutant and the wild-type apoC-1, respectively. These numbers should be interpreted with caution, since the phenomenological two-state model may oversimplify the TMAO-induced apoC-1 folding. Nonetheless, the two-state model adequately approximates the alcohol induced α -helix formation in mellitin (Hirota et al., 1998) and should be useful for at least a qualitative description of TMAO-induced α -helix formation in apoC-1. Qualitatively, the presence of a small plateau in the titration curve of R23P mutant at low (0–0.3 M) TMAO concentrations (Fig. 5, triangles) suggests that the TMAO-induced helical conformation of this mutant is not significantly populated in TMAO-free solution. This is consistent with the unfolded conformation inferred for the R23P mutant in TMAO-free buffer (Fig. 4). In contrast, the absence of such a plateau in the titration curve of the wild-type peptide (Fig. 5, insert, squares) suggests that the TMAO-stabilized highly α -helical conformation in this peptide may be significantly populated in the TMAO-free solution.

Discussion

One objective of this work is to test the applicability of synthetic full-size apoC-1 for the structural thermodynamic analysis of the

human plasma apolipoprotein. Comparison of the CD data in Figures 1 and 2 with similar data recorded of plasma apoC-1 (Gursky & Atkinson, 1998) shows that secondary structural and thermodynamic parameters of the synthetic wild-type and plasma apoC-1 monomers, such as the α -helical content, melting temperature T_m , and van't Hoff enthalpy $\Delta H_v(T_m)$, are identical within the errors of their experimental estimates (Table 1). This suggests strongly that the free energies of stability of the synthetic and plasma apoC-1 monomers are also identical. Consequently, synthetic apoC-1 provides a suitable experimental system for probing the energetics and structure of the monomeric human plasma apolipoprotein in aqueous solution.

Furthermore, we test the utility of Pro mutagenesis coupled with far-UV CD for the identification of the parts of apoC-1 backbone forming the stable helical structure in the monomer in solution. The CD data in Figure 4 show that the destabilizing effect of Pro substitutions in positions 23, 45, and 15 are significantly different: R23P and T45P mutants are completely unfolded at 0–90 °C, whereas the G15P mutant retains $\sim 20\%$ α -helical content at 22 °C (Table 1). Observation of the complete unfolding by the R23P and T45P substitutions contrasts with partial unfolding of the molten globular α -lactalbumin by single Pro substitutions (Schulman & Kim, 1996). It implies that the helical structure in apoC-1 monomer comprises a single cooperative unit, with R23 and T45 located in the key positions within this unit. Observation of an incomplete unfolding by G15P substitution points to G15 location peripheral to this structure, possibly in the fluctuating helical conformation. The latter is consistent with the increase in the α -helical content observed upon G15A substitution (Table 1, Fig. 3) that can be interpreted as entropic stabilization of the fluctuating helical conformation near residue 15. Furthermore, the absence of a plateau in the TMAO titration curve of the wild-type apoC-1 (Fig. 5, insert)

Table 1. Secondary structural and thermodynamic parameters of monomeric human plasma apoC-1 and its synthetic peptide analogues

Peptide	$-\Theta_{222}$ at 22 °C (deg cm ² dmol ⁻¹)	α -helix (%)	T_m (°C)	$\Delta H_v(T_m)$ (kcal/mol)	$\delta\Delta G_{app}^a$ (kcal/mol)	$[\text{TMAO}]_{\min}^b$ (M)
Plasma apoC-1 ^c	-9,500	$31 \pm 4^{d,e}$	50 ± 3^f	19 ± 3^f		
Synthetic apoC-1						
Wild-type	-9,500	$31 \pm 4^{d,e}$	50 ± 2^g	18 ± 2^g	0 ^h	1.5 ⁱ
G15A	-13,200	$42 \pm 4^{d,e}$	57 ± 2^g	19 ± 2^g	$+0.4 \pm 0.1^h$	1.0 ⁱ
G15P	-7,200	$\sim 20^j$	— ^k	$<19^k$	$<0^l$	2.0 ⁱ
R23P, T45P	-4,500	Unfolded ^m	— ^m	$\sim 0^m$	$<-2.8^n$	2.6 ⁱ

^aChange in the apparent free energy of stability compared to plasma apoC-1 monomer.

^bMinimal TMAO concentration that induces peptide folding to its full helical potential.

^cFrom the CD analysis of Gursky and Atkinson (1998).

^dEstimated from the measured value of $[\Theta_{222}]$ as described in Materials and methods.

^eEstimated by CD spectral deconvolution using the program LINEQ (Mao & Wallace, 1982) with the reference spectra from Chen et al. (1972).

^fDetermined by conventional van't Hoff analysis.

^gDetermined by fitting to Equation 1 (see Materials and methods).

^hEstimated from the thermal unfolding data in Figure 2 as $\delta\Delta G_{app}(T_m) = \delta T_m \cdot \Delta H_v / T_m$.

ⁱEstimated from the TMAO titration curves (Fig. 5) by fitting to Equation 2 (see Materials and methods).

^jEstimated based on the difference spectra $[\Theta_{WT}] - [\Theta_{G15P}]$ of the wild-type and G15P peptides at 22 °C (Fig. 3, insert) and $[\Theta_{T=22^\circ}] - [\Theta_{T=90^\circ}]$ of G15P peptide at 22 and 90 °C (Fig. 4, left insert).

^kLow unfolding cooperativity suggests that $\Delta H_{v,G15P} < \Delta H_{v,WT}$ but precludes accurate determination of T_m and $\Delta H_v(T_m)$.

^lInferred from the reduction in the α -helical content of apoC-1 upon G15P substitution.

^mInferred from the absence of the temperature-dependent CD changes at 0–90 °C (Fig. 4).

ⁿInferred from the unfolded state of R23P and T45P observed at 0–90 °C and from the comparison with the free energy of stability of plasma apoC-1 monomer measured by chemical unfolding, $\Delta G(0^\circ) = 2.8$ kcal/mol (Gursky & Atkinson, 1998).

suggests that the highly helical TMAO-stabilized conformation is significantly populated in TMAO-free solution, thereby suggesting the presence of a fluctuating helical structure in apoC-1 monomer.

Comparison of the size of the R23-T45 segment with the average size of the α -helical structure in apoC-1 (~18 residues) suggests that this helical structure is discontinuous between residues R23 and T45 (also see Note added in proof). Thus, the preliminary model of the apoC-1 monomer structure in solution can be envisioned as two helical segments, one containing R23 and the other containing T45, flanked by fluctuating helical structure that includes G15. This model is consistent with the predicted discontinuity in the apoC-1 helical structure at residues 32–33 (Segrest et al., 1990) and with NMR structure of apoC-1 in SDS that comprises two α -helices (4–30) and (34–52) linked by an unstructured segment (31–33) (Rozek et al., 1998). The assignment of the fluctuating helical conformation to G15 is also consistent with ^1H NMR analysis of apoC-1(1–38) fragment in SDS, which indicated α -helical conformation for the central portion of the peptide, with high mobility of the K12–G15 segment (Rozek et al., 1997). The CD analysis of an extensive series of Pro-containing apoC-1 mutants will test the solution conformation of apoC-1 monomer in more detail, potentially leading to a complete secondary structural assignment to individual apolipoprotein groups (work in progress). A similar approach combining Pro scanning mutagenesis with low-resolution spectroscopic techniques such as CD may be applicable for the structural analyses of other small proteins and peptides, such as other class-C apolipoproteins, whose self-association hampers their high-resolution NMR analysis in the monomeric detergent-free state in solution.

Another objective, which is obtaining mutant apoC-1 of increased secondary structure and/or stability, was achieved by G15A substitution. The CD spectra in Figure 3 indicate that G15A substitution leads to an increase in the α -helical content by ~11%, or 6–7 groups (Table 1). This is expected to lead to about 6–9 kcal/mol increase in calorimetric enthalpy of the unfolding, based on the enthalpy of 0.9–1.3 kcal/mol per residue measured for the unfolding of α -helical peptides in water (Scholtz et al., 1991). However, no significant increase in the van't Hoff enthalpy of apoC-1 unfolding is observed upon G15A substitution (Table 1; Fig. 2), suggesting that the ~25-residue helical structure in G15A does not comprise a single cooperative unit. This is consistent with the <20 residue size of the cooperativity unit characteristic of the unfolding of the peptide α -helices in water (Privalov, 1982). Furthermore, G15A substitution leads to an increase in the melting temperature by $\delta T_m = +7 \pm 1.5^\circ\text{C}$ (Table 1; Fig. 2); comparable increases in T_m resulting from G $\rightarrow$ A substitutions have been reported for other proteins (Sturtevant, 1993, and references therein). The increase in the apparent free energy of stability resulting from G15A mutation can be estimated according to Becktel and Schellman (1987) as $\delta\Delta G_{app,G15A}(T_m) = \delta T_m \cdot \Delta H_v / T_{m,WT} = +0.4 \pm 0.1$ kcal/mol. This is only a fraction of the stabilizing effects of G $\rightarrow$ A substitutions (0.7–1 kcal/mol) reported for other proteins and peptides (Kallenbach et al., 1996; Myers et al., 1997). The entropic contribution to such stabilization is $-T\delta\Delta S_{G\rightarrow A} \sim +0.78$ kcal/mol at $T = T_m = 323$ K, where $\delta\Delta S_{G\rightarrow A} = -2.4$ cal/mol K has been estimated based on the reduction in the conformational space available to the unfolded backbone containing Ala as compared to Gly (D'Aquino et al., 1996). The enthalpic contribution due to interactions formed by the additional methyl group of Ala may also be favorable and significant, leading to a total stabilization of ~1 kcal/mol. Comparison with the value of $\delta\Delta G_{app,G15A} \sim 0.4$ kcal/

mol inferred for apoC-1 is consistent with the presence of more than one cooperatively unfolding units in G15A peptide. A possible interpretation is that the "original" helical structure in apoC-1 monomer and the "additional" structure stabilized by G15A substitution do not integrate in a single cooperative unit. This interpretation will be further tested by mutagenesis. Furthermore, the increased α -helical content and increased thermal stability of G15A apoC-1 make it an attractive candidate for the X-ray crystallographic analysis, which should complement the thermodynamic and low-resolution CD structural analysis (work in progress).

The third objective is to test the effect of TMAO on the apolipoprotein conformation. The apoC-1 mimicking peptides analyzed in this work show a large increase in their α -helical content upon addition of TMAO, from 0–42% α -helix in TMAO free solution (Table 1) up to 70–74% α -helix at high (1–2.7 M) TMAO concentrations. The stabilizing effect of TMAO on the α -helical conformation in apoC-1 can be assessed by considering a shift in the conformational equilibria of R23P peptide, from <10% folded conformation in TMAO free buffer to >90% folded 72%-helical conformation in 2.7 M TMAO (Fig. 5, insert). This translates to an increase in the apparent free energy of stability of the 72%-helical conformation from ≤ -1.5 kcal/mol in the buffer to ≥ 1.5 kcal/mol in 2.7 M TMAO. Similarly, T62P mutant of staphylococcal nuclease, which is fully unfolded by Pro substitution in the center of an α -helix, adopts a near-native functional fold in 2.7 M TMAO and shows a 6.9 kcal/mol increase in the stability upon increasing TMAO concentration from 0 to 2.7 M (Baskakov & Bolen, 1998). Thus, the destabilizing effects of Pro substitutions in these protein systems are comparable to the stabilizing effects of 2.7 M TMAO.

The TMAO-induced apoC-1 folding observed in this work may result from the peptide self-association or from solvophobic effect of TMAO on the solvent-exposed polypeptide backbone. The following observations are consistent with, but do not necessarily prove, the latter mechanism. First, self-association observed in near-neutral TMAO-free solutions of >10 $\mu\text{g/mL}$ plasma apoC-1 (Osborne et al., 1977; Gursky & Atkinson, 1996) has no apparent effect on the secondary structure of synthetic apoC-1. Second, the CD data recorded from the TMAO-containing apoC-1 solutions are independent of the peptide concentration in the range explored (6–20 $\mu\text{g/mL}$). It, therefore, appears likely that the TMAO-induced folding of synthetic apoC-1 is not coupled to the peptide self-association. If this is the case, then TMAO induced apoC-1 folding is not driven by hydrophobic interactions, since TMAO has been shown to have no significant interactions with solvent-exposed apolar protein groups (Wang & Bolen, 1997). This contrasts with the central role of hydrophobic interactions involving the apolar surfaces of amphipathic α -helices in stabilizing the lipid-bound, detergent-bound, or self-associated apolipoprotein conformations. Nevertheless, the maximal α -helical content observed in TMAO-containing apoC-1 solutions (70–74%) is comparable to that reported for lipid-bound (73%, Jackson et al., 1974) and self-associated states of human plasma apoC-1 (82%, Osborne et al., 1977). This agreement suggests that, despite the difference in their stabilization mechanisms (hydrophobic interactions involving the side chains vs. solvophobic interactions involving the main chain), the TMAO-induced secondary structure in apoC-1 may resemble the fully folded functional lipid-bound apolipoprotein conformation. This supports the proposed role of TMAO as a universal protein folding agent and suggests that TMAO may provide a useful model solvent for the structural analysis of functional apolipoprotein conformations.

Materials and methods

Peptide synthesis, purification, and sample preparation

Five 57-residue peptide analogues of human apoC-1, including wild-type, G15A, G15P, R23P, and T45P mutants, were custom-synthesized and purified at Quality Control Biochemicals, Inc. (Hopkinton, Massachusetts) using the solid phase methodology utilizing the 9-fluorenylmethoxy-carbonyl chemistry and taking advantage of the published protocols for the solid state synthesis and purification of full-size human apoC-1 (Harding et al., 1976; Sigler et al., 1976). The amino- and carboxy-termini of the peptides were not chemically blocked. The peptide purification to 97% purity was performed by reverse-phased HPLC using Gilson automated HPLC system. The identity and purity of the peptides were confirmed by amino acid analysis and by electrospray mass-spectrometry carried out at Quality Control Biochemicals. The mean residue weights measured by mass spectrometry (116.3 for the wild-type, 116.6 for G15A, 117.3 for G15P, 115.3 for R23P, and 118.0 for T45P) agreed with the values calculated from the peptide amino acid composition and were used for the concentration normalization of the CD spectra.

The peptides were lyophilized in ~1 mg aliquots and stored at -20°C. Stock solutions prepared by dissolving the peptides to ~0.5 mg/mL concentrations in 5 mM phosphate buffer at pH 8 were diluted with 2–5 mM phosphate buffer at pH 7.5 to a final peptide concentration of 6–50 µg/mL and were used in the CD experiments within two weeks. No time-dependent changes in CD spectra were detected during this period. Buffer solutions were prepared using double-deionized water, filtered by 0.2 µm Millipore filter, and degassed prior to use; sodium phosphate salts (from Fisher, Springfield, New Jersey), trimethylamine-N-oxide dihydrate (from Sigma, St. Louis, Missouri), and other chemicals were of highest purity analytical grade. Peptide concentrations were determined by a modified chromatogenic Lowry assay (Markwell et al., 1985) and by UV absorbance at 280 nm, which was facilitated by the presence of the single Trp41 residue in the apoC-1 sequence. Peptide concentrations measured before and after the CD experiments agreed within 10%.

Circular dichroism spectroscopy

Far-UV CD spectra were recorded from 190–250 nm on an AVIV 62DS spectropolarimeter (Aviv Associates, Inc., Lakewood, New York) equipped with thermoelectric control and calibrated with d₁₀-camphorsulfonic acid. The spectra were recorded from peptide solutions of 6–50 µg/mL concentration using 10–1 mm path-length quartz cuvettes, with 1 nm bandwidth, 0.5–1 nm step size, 15–30 s accumulation per data point, and averaged over 5–8 runs. Buffer baselines were subtracted from the spectra that were subsequently subjected to a noise reduction by using AVIV software. The CD data were normalized on the peptide concentration and expressed as mean residue ellipticity [Θ].

The near-UV CD measurements were hampered by the high aggregating propensity of apoC-1 in near-neutral aqueous solutions, and by the presence of only one Trp and no Tyr in the apoC-1 sequence. Since apoC-1 self-associates at >10 µg/mL concentrations (Osborne et al., 1977), which is below the useful range for the near-UV CD experiments, no tertiary structure analysis of apoC-1 by near-UV CD could be carried out.

Thermal unfolding of the peptides upon heating from 0–98°C was monitored by ellipticity at 222 nm and/or other characteristic

wavelengths (193, 208, and 230 nm). To verify the equilibrium character of the heat unfolding, the melting curves were recorded from identical samples at different heating rates from 2 to 30°C/h, with the time for recording a data point ranging from 15 to 99 s and the temperature step size from 1 to 0.5°C, respectively. Buffer baselines that were recorded prior and following the CD measurements of the peptide solutions were averaged and subtracted from the peptide data to minimize the effects of UV lamp drift.

TMAO-induced apoC-1 folding was carried out by using a series of solutions containing identical peptide and buffer concentrations (6–20 µg/mL peptide, 4 mM phosphate buffer, pH 7.6) but different TMAO concentrations (0–4 M). These solutions maintained a stable pH of 7.6–7.8 for several days and were typically used within 1 day. The solutions were equilibrated at room temperature overnight, and the CD data at 222 nm were taken at 22°C, with 1 nm bandwidth and 5 min for recording a data point. The reversibility of the TMAO-induced folding was verified by the CD measurements upon dilution of 10–20 µg/mL peptide solutions containing 1–2.6 M TMAO.

Estimates of the α-helical content

The α-helical content in the wild-type and G15A peptides in the buffer, and in all peptides in the presence of high TMAO concentrations, was estimated from the measured mean residue ellipticity at 222 nm by using the empirical formula % α-helix = $(-[\Theta_{222}] + 3,000)/39,000 \times 100$ (Chen et al., 1972; Pownall et al., 1977). This formula is based on the assumption that the helical structure provides the only significant contribution to [Θ₂₂₂]. Since this assumption does not hold for the largely unfolded G15P, R23P, and T45P peptides, the helical content in these peptides was assessed as follows. The α-helical content in G15P was estimated based on the comparison of the CD difference spectra [Θ_{WT}]–[Θ_{G15P}] and [Θ_{G15A}]–[Θ_{WT}] at 22°C (Fig. 3, insert), and the difference spectrum [Θ₂₂₂]–[Θ_{90°}] (Fig. 4, left insert) as described in Results. The R23P and T45P mutants were inferred to have no stable α-helical structure, on the basis of the overlap of their spectra with the spectrum of the heat-unfolded G15P, and the absence of large temperature-dependent CD changes from 0–95°C (Fig. 4). The α-helical content in the wild-type, G15A, and G15P peptides in the buffer was also estimated by spectral deconvolution using unconstrained least-squares fitting (program LINEQ, Mao & Wallace, 1984) with the database from Chen et al. (1972). The results agreed within 4% with those estimated from the value of [Θ₂₂₂].

Thermodynamic analysis

Equilibrium thermodynamic analysis of the cooperative heat unfolding of the wild-type and G15A peptides was done by fitting the observed ellipticity Θ_{obs}(*T*) and the baselines for the folded and unfolded states, Θ_F(*T*) and Θ_U(*T*), to Equation 1, which is an extension of van't Hoff equation (Pace et al., 1989):

$$\Theta_{obs}(T) = \frac{\Theta_F(T) + \Theta_U(T) \cdot \exp(-\Delta H_v/RT + \Delta H_v/RT_m)}{1 + \exp(-\Delta H_v/RT + \Delta H_v/RT_m)} \quad (1)$$

Here, *R* = 1.98 cal/mol K is the universal gas constant, *T* is absolute temperature, the baselines Θ_F(*T*) = *a_F* + *b_F*·*T* and Θ_U(*T*) = *a_U* + *b_U*·*T* are assumed to be linear functions of *T*. The fitting parameters are the effective enthalpy Δ*H_v*, melting temper-

ature T_m , and the baseline coefficients a_F , b_F , a_U , and b_U . These parameters and the associated errors were determined by least-squares fitting using ORIGIN software (MicroCal, Inc., Northampton, Massachusetts). The values of ΔH_0 and T_m determined for apoC-1 by fitting to Equation 1 (Fig. 2, solid curves) and by conventional van't Hoff analysis (Fig. 2, insert) are identical within the errors of their estimates.

The TMAO-induced cooperative folding of apoC-1 peptides was analyzed by fitting the observed ellipticity $\Theta_{obs}([c])$ and the baselines $\Theta_F([c])$ and $\Theta_U([c])$ for the folded and unfolded states to Equation 2 (Santoro & Bolen, 1988), which combines the linear extrapolation model (Pace, 1986) with the two-state model:

$$\Theta_{obs}([c]) = \frac{\Theta_F([c]) + \Theta_U([c]) \cdot \exp((m \cdot [c] - \Delta G^\circ)/RT)}{1 + \exp((m \cdot [c] - \Delta G^\circ)/RT)}. \quad (2)$$

Here, $[c]$ is the molarity of TMAO, T is temperature in Kelvin, R is the gas constant, $\Theta_F([c]) = A_F + B_F \cdot [c]$ and $\Theta_U([c]) = A_U + B_U \cdot [c]$ are the linear baselines. The fitting parameters are ΔG° (free energy of TMAO-stabilized conformation in TMAO-free buffer), m , A_F , B_F , A_U , B_U ; their values and the associated statistical errors were determined by least-squares fitting using ORIGIN.

Note added in proof

The CD analysis of Q31P mutant showed that, in contrast to Pro substitutions in positions 15, 23, and 45, a similar substitution in position 31 leads to no significant destabilization or unfolding of the apoC-1 monomer in solution. This points to Q31 location in a nonhelical region linking the two apoC-1 α -helices, in agreement with the NMR structure of apoC-1 in SDS (Rozek et al., 1998).

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