Self-Association and Phospholipid Binding Properties of Iodinated Apolipoprotein A-I†

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ABSTRACT: Kinetic turnover studies of apolipoprotein metabolism often utilize radioiodinated tracers. These studies rely on the "tracer assumption" that the modified tracer is physiologically and metabolically identical with the native unmodified tracer. This paper addresses the validity of this assumption on the basis of the examination of the state of self-association and binding properties with egg yolk phosphatidylcholine small unilamellar vesicles of native and iodinated apolipoprotein A-I (apoA-I). Human apoA-I was iodinated to the extent of 1.0 and 3.7 mol of nonradioactive iodine/mol of protein. At concentrations from 0.013 to 0.8 mg/mL, iodinated apoA-I underwent concentration-dependent self-association similar to that of native apoA-I as evidenced by circular dichroism and gel filtration. At all concentrations, however, the iodinated preparations were more highly self-associated as judged by gel filtration in relation to the extent of iodination. Scatchard analysis of fluorometric titrations of apoA-I/vesicle interactions demonstrated that the binding capacity of vesicles for apoA-I increased and apoA-I binding affinity decreased upon iodination. In addition, the kinetics of apoA-I binding to vesicles was enhanced by iodination. The affinity, capacity, and kinetics of apoA-I binding were each altered 2–3-fold dependent on the extent of iodination. Since the dynamic interactions of apoA-I are perturbed by iodination, one may legitimately question whether the "tracer assumption" is valid for ^{125}I-apoA-I under all experimental conditions.

The plasma lipoprotein system serves to transport exogenous and endogenous lipids to the liver and peripheral tissues where the lipid moieties are used for cellular maintenance functions or are eliminated from the body. The metabolism of lipoproteins has been an active area of research for many years. In order to better understand lipoprotein metabolism, it is essential to study the metabolic turnover kinetics of the apolipoprotein moieties, which are responsible for lipoprotein structure, metabolic conversions, and cellular uptake via specific cell membrane receptors [for reviews, see Morrisett et al. (1977), Schaefer et al. (1978), and Mahley et al. (1984)]. With a few notable exceptions, these studies have utilized exogenous radioiodination to study apolipoprotein catabolism. The validity of these studies ultimately relies on the validity of the often unstated "tracer assumption": the chemically modified tracer is physiologically and hence metabolically identical with the unmodified biological tracee.

Generally, two approaches have been taken to address the metabolic kinetics of radioiodinated apolipoproteins. The first is to radioiodinate and reinject intact lipoprotein particles (Blum et al., 1977; Shepherd et al., 1978a). Because of the compositional heterogeneity of lipoproteins, simultaneous labeling of many different apolipoproteins results. Thus, studying the metabolic kinetics of individual apolipoproteins by this method requires fractionation of each time point sample into its individual protein components. The second approach is to radioiodinate a purified apolipoprotein; this protein is then reinjected into a subject with (Shepherd et al., 1978a; Gregg et al., 1984) or without (Schaefer et al., 1982) an initial equilibration of the tracer with the subject's plasma or an isolated lipoprotein fraction. Thus, selectivity is greatly enhanced. This approach may be suspect, however, if the iodination has perturbed the manner in which apolipoproteins associate with intact lipoproteins in vitro or in vivo.

Recently, Osborne et al. (1984) observed that the secondary structure and state of self-association of radioiodinated apolipoprotein A-I (apoA-I)[1] were perturbed compared to the native protein. ApoA-I is the principal structural and lipid binding protein in HDL (Mahley et al., 1984) and exists in a state of dynamic equilibrium between lipoprotein-bound and -free forms. ApoA-I hence may freely exchange between lipoprotein subclasses (Grow & Fried, 1978; Pownall et al., 1978; Shepherd et al., 1978b). In this report we examine the effect of iodination on the lipid binding properties of apoA-I. The results question the validity of the tracer assumption, particularly with respect to the in vitro or in vivo association of radioiodinated apoA-I with intact HDL.

Materials and Methods

Apolipoprotein A-I was isolated from normal human plasma as previously described (Jonas et al., 1980) utilizing gel filtration chromatography over Sephadex G-200 equilibrated with 7 M urea. The purified protein migrated as a single band during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Egg yolk phosphatidylcholine (EYPC, type V-EA) was purchased from Sigma Chemical Co. (St. Louis, MO), stored at −10 °C, and used without further purification. A standard buffer of 0.1 M $NaHCO_3$ and 0.01% EDTA, pH 8.0, was used for all studies. Reagent-grade chemicals and water purified by a Milli-Q water system (Millipore) were used throughout. Protein was quantitated by both the method of Lowry et al. (1951) and by amino acid quantitation (performed in the laboratory of Dr. Ben Dunn, Department of Biochemistry and Molecular Biology, Univ-

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[1] Abbreviations: apoA-I, apolipoprotein A-I isolated from HDL; HDL, high-density lipoprotein; EYPC, egg yolk phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

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ersity of Florida). EYPC was quantitated by measuring organic phosphorus by the method of Chen et al. (1965) and multiplying by 25 to adjust for the molecular weight of EYPC.

Iodination. Purified apoA-I was iodinated by the iodine monochloride method of McFarlane (1958). Different iodine incorporations into protein were provided by minor alterations of the concentration of protein used, the ICl to protein ratio, and the incubation time. After extensive dialysis, the extent of iodination was assessed by the rate-sensing spectrophotometric method of Palumbo et al. (1982).

Spectroscopic Studies. Intrinsic tryptophan fluorescence was monitored on an SLM-Aminco SPF-500c spectrofluorometer at ambient temperature. Protein emission spectra from 300 to 400 nm (excitation at 280 nm) were obtained by using excitation and emission bandwidths of 2 nm. All other fluorometric studies utilized excitation and emission wavelengths of 280 and 340 nm, with bandwidths of 0.5 and 20 nm, respectively, to minimize tryptophan photobleaching. Circular dichroism spectra between 200 and 240 nm were obtained on a Jasco J-500c spectropolarimeter at ambient temperature. Cell path lengths were 0.05 cm for samples at 0.8 and 0.2 mg/mL and 0.5 cm for samples at 0.05 and 0.013 mg/mL. Molar ellipticity values at 222 nm, indicative of α-helical content and reported in units of deg cm^2 dmol^{-1}, were obtained from the relationship:

$$[\theta]_{222} = \frac{115\theta^\circ_{222}}{10lc}$$

where θ° = measured ellipticity (degrees), l = path length (cm), and c = protein concentration (g/mL).

Chromatography. Gel filtration chromatography was performed using a 1.6 cm × 60 cm column of Sephadex G-100 superfine (Pharmacia Fine Chemicals). The column was equilibrated with standard buffer and eluted at 6 mL/h at 4 °C. Fractions were monitored for intrinsic protein fluorescence.

Vesicle Preparation. Small unilamellar vesicles of EYPC were prepared by sonication (Huang, 1969) in an ice bath under nitrogen using a Heat Systems Ultrasonics Model W-220F sonicator equipped with a microtip probe. Essentially homogeneous vesicles were isolated by ultracentrifugation (Barenholz et al., 1977).

Fluorometric Titrations. Fluorescence cuvettes initially containing 0.3 μM protein were titrated with aliquots of 12.4 mM EYPC vesicles to a maximum lipid:protein molar ratio of 3700:1. Alternately, cuvettes initially containing 0.33 mM EYPC were titrated with aliquots of 3 μM protein. Data from the latter titrations were analyzed by the method of Scatchard (1949), where the EYPC is considered to be the matrix to which the protein is binding as a ligand. Cuvettes of protein and lipid alone at equivalent concentrations were monitored throughout all titrations, so that the fluorescence enhancement of protein in the presence of lipid relative to free protein were determined after the subtraction of appropriate buffer and lipid backgrounds. Fluorescence was measured 5 min after mixing of cuvette contents to allow reactions to reach completion.

Fluorometric Kinetics. An aliquot of protein was rapidly mixed into a cuvette containing EYPC vesicles so that final protein and lipid concentrations were 4×10^{-8} and 7×10^{-4} M, respectively. Tryptophan fluorescence was monitored continuously for 8 min. Two kinetic acquisitions were averaged for each experiment. Data were expressed as the lipid background corrected fluorescence relative to a cuvette of free protein at equivalent concentration and were normalized to the maximum relative fluorescence enhancement observed for each sample.

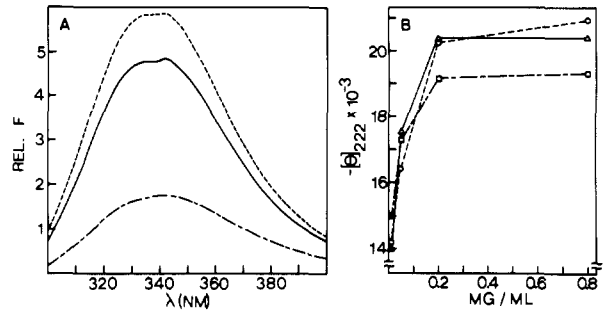

FIGURE 1: Spectroscopic properties of native and iodinated apoA-I. (A) Fluorescence emission spectra. Samples were adjusted to an absorbance at 280 nm of 0.050. Excitation wavelength = 280 nm. Excitation and emission bandwidths = 2 nm. (- - -) Native apoA-I; (—) 1.0 mol of iodine/mol of apoA-I; (– - –) 3.7 mol of iodine/mol of apoA-I. (B) Molar ellipticity at 222 nm as a function of concentration. Samples at 0.8, 0.2, 0.05, and 0.013 mg/mL were scanned from 240 to 200 nm. Points represent the average of two scans. (○) Native apoA-I; (△) 1.0 mol of iodine/mol of apoA-I; (□) 3.7 mol of iodine/mol of apoA-I.

RESULTS

ApoA-I was iodinated to different extents by modifying the protein concentration, ICl to protein ratio, and incubation time of the iodination reaction. This resulted in preparations that contained 1.0 and 3.7 mol of iodine/mol of apoA-I by assay. These samples will be referred to as 1.0 I and 3.7 I, respectively. A control aliquot of native apoA-I was exposed to identical iodination conditions except for the presence of iodine. The absorbance at 280 nm of these preparations yielded an extinction coefficient $E^{1\%} = 1.25$. The Lowry assay overestimated protein concentrations by 6% relative to amino acid quantitation. The presence of iodine was not found to alter protein extinction coefficients or Lowry reactivities.

Spectroscopic properties of native and iodinated apoA-I are illustrated in Figure 1. Fluorescence emission spectra are shown in Figure 1A. Each sample exhibited a broad fluorescence spectrum with a maximum in the vicinity of 340 nm. The wavelength at which maximum fluorescence occurred was not altered by the presence of iodine. However, the fluorescence yield decreased in relation to the extent of iodination as expected for quenching of tryptophan fluorescence by iodine.

Circular dichroism spectra for native and iodinated apoA-I were similar to published spectra (Osborne et al., 1984) and will not be presented here. Figure 1B summarizes the molar ellipticity values at 222 nm as a function of concentration. The constant ellipticity observed for each sample above 0.2 mg/mL markedly decreased as the samples were diluted. These results parallel literature reports whereby self-association of apoA-I is manifested by a concentration-dependent increase in molar ellipticity (Osborne et al., 1984). Both iodinated preparations underwent concentration-dependent alterations in secondary structure in a manner similar to that of native apoA-I.

Gel filtration chromatography was used to characterize the extent of aggregation at various concentrations. Figure 2 illustrates gel filtration profiles for native and iodinated samples applied at concentrations of 0.8 mg/mL in separate runs. Each sample eluted in two peaks, aggregated and monomeric apoA-I, respectively. The 3.7 I sample consistently eluted one fraction later than the native apoA-I and 1.0 I preparations; this may reflect an interaction between iodine and the gel matrix, since the circular dichroism spectra (Figure 1B) did not reveal a striking difference in secondary structure between these proteins. The relative size of the aggregate peak increased in relation to the extent of iodination. This is illustrated in the inset to Figure 2, where the ratio of the aggregate

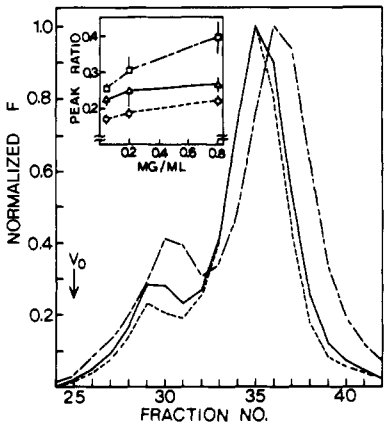

FIGURE 2: Gel filtration chromatography of native and iodinated apoA-I. Samples at 0.8 mg/mL were applied separately to a Sephadex G-100 superfine column (1.6 × 60 cm) and eluted in 0.1 M $NaHCO_3$ and 0.01% EDTA at 6 mL/h. Tryptophan fluorescence of eluted fractions was normalized to peak values. Arrow marks column void volume; total volume was at fraction 62. (Inset) The ratio of the aggregate to monomer peak is plotted as a function of concentration for all three preparations. Error bars represent the range of duplicates. (---) Native apoA-I; (—) 1.0 mol of iodine/mol of apoA-I; (– – –) 3.7 mol of iodine/mol of apoA-I.

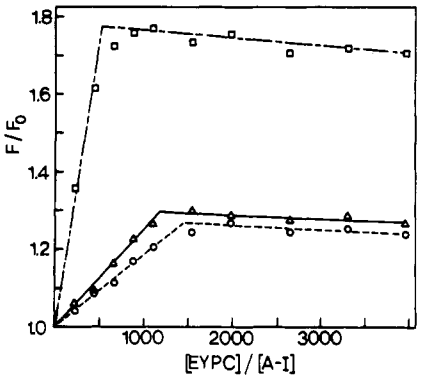

FIGURE 3: Fluorometric titration of apoA-I with EYPC vesicles. Tryptophan fluorescence of apoA-I was monitored as aliquots of EYPC were added. Excitation and emission wavelengths (bandwidths) were 280 (0.5) and 340 (20) nm, respectively. Ratio of fluorescence in the presence vs. absence of lipid is plotted vs. lipid:protein molar ratio. (○) Native apoA-I; (△) 1.0 mol of iodine/mol of apoA-I; (□) 3.7 mol of iodine/mol of apoA-I.

to monomer peaks from column profiles is shown at various apoA-I loading concentrations. Each preparation exhibited an increase in aggregation with increased concentration, indicative of concentration-dependent self-association. Furthermore, at each concentration studied, the degree of aggregation increased in relation to the extent of iodination.

Fluorometric titrations of native and iodinated apoA-I with aliquots of EYPC are shown in Figure 3. Upon addition of EYPC, the relative fluorescence of each preparation increased to a maximum value and thereafter exhibited evidence of photobleaching. The degree of fluorescence enhancement was different for each sample, reflecting the different microenvironments of tryptophan and iodine within each protein in the presence as compared to the absence of EYPC. In particular, the 3.7 I sample which showed the largest iodine quenching effect (Figure 1A) exhibited the largest relative fluorescence enhancement in the presence of lipid. The stoichiometries of lipid binding estimated from the abscissa values at the break points of the titrations were approximately 1500:1, 1200:1, and 550:1 for the native, 1.0 I, and 3.7 I preparations, respectively. Thus, the binding capacity of lipid vesicles for apoA-I (the inverse of the abscissa) increased approximately 2–3-fold in relation to the extent of iodination of apoA-I.

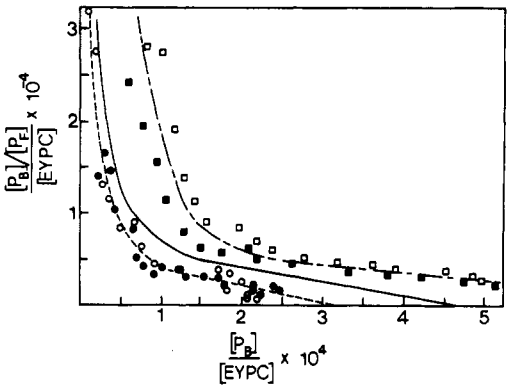

FIGURE 4: Scatchard analysis of apoA-I/EYPC vesicle interaction. Cuvettes containing EYPC in buffer were titrated with aliquots of apoA-I. Bound/free protein concentrations (P_B/P_F) were determined by relating the observed relative fluorescence enhancement in the presence vs. absence of lipid to the maximum fluorescence enhancement observed for each protein. Open and filled data points represent two independent titrations of the native and 3.7 mol of iodine/apoA-I samples. Data points (also for two independent titrations) are not illustrated for the 1.0 mol of iodine/apoA-I sample for clarity. Lines are included for all three samples, however. Same fluorescence parameters as for Figure 3. (---) (○) Native apoA-I; (—) 1.0 mol of iodine/mol of apoA-I; (– – –) (□) 3.7 mol of iodine/mol of apoA-I.

Table I: Summary of ApoA-I Binding Capacity and Affinity for EYPC by Scatchard Analysis

sample	mol of I/mol apoA-I	K_D (M)	N (mol of apoA-I/mol of EYPC)
native	0	6.3×10^{-8}	3.1×10^{-4}
1.0 I	1.0	7.8×10^{-8}	4.7×10^{-4}
3.7 I	3.7	14.5×10^{-8}	8.4×10^{-4}

Although vesicles do not contain unique and specific "sites" for protein binding, Scatchard plots of apoA-I binding to vesicles give insight into average binding affinity and capacity. Such plots are illustrated in Figure 4. The fraction of protein bound at any point in the titration was determined by relating the relative fluorescence enhancement in the presence of lipid to the maximum fluorescence enhancement observed under saturated binding conditions for each protein. Each protein preparation exhibited a curvilinear titration pattern. After the initial curvature, all three titrations approached the abscissa with linearity. Table I summarizes the dissociation constants and binding capacities obtained from these linear segments. As iodination increased from 0 to 3.7 mol of iodine/mol of protein, the dissociation constant increased from 6.3×10^{-8} to 14.5×10^{-8} M. The molar ratio of protein to lipid at saturation of binding capacity (abscissa intercept, N; Table I) increased nearly 3-fold, dependent upon the extent of iodination, in a manner similar to the titration of protein with lipid (Figure 3).

The kinetics of approach to equilibrium for apoA-I binding to EYPC vesicles is shown in Figure 5. Each protein reached a maximum fluorescence and then showed evidence of photobleaching. The binding of native apoA-I was rather slow, requiring 3–4 min to reach completion. The kinetics of binding was increased in relation to the extent of iodination, such that the 3.7 I sample reached a maximum in less than 2 min. The initial rate of change of fluorescence enhancement was markedly increased in relation to the extent of iodination.

DISCUSSION

The self-association properties of apoA-I have been actively studied by a number of investigators primarily using techniques

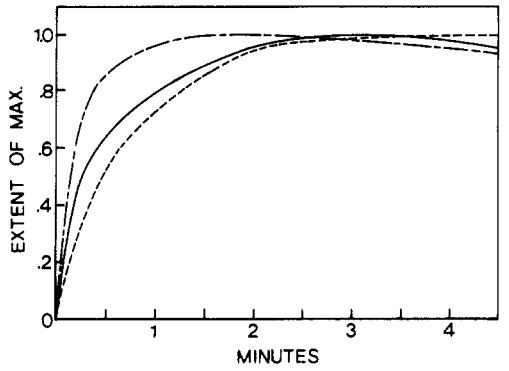

FIGURE 5: Kinetics of apoA-I binding to EYPC vesicles. Aliquots of protein were rapidly mixed into cuvettes containing EYPC vesicles and buffer at time zero. The first 4 min of an 8-min acquisition are illustrated. Data were normalized to the maximum relative fluorescence enhancement attained for each sample. (---) Native apoA-I; (—) 1.0 mol of iodine/mol of apoA-I; (– – –) 3.7 mol of iodine/mol of apoA-I.

of sedimentation equilibrium, gel filtration, circular dichroism, fluorescence polarization of dansylated protein, and viscometry (Barbeau et al., 1979; Vitello & Scanu, 1976; Formisano et al., 1978). These studies demonstrate that apoA-I self-associates in a monomer–dimer–tetramer–octamer manner and that the monomer has a greater affinity for lipid than do associated forms (Massey et al., 1981). Osborne et al. (1984) recently examined the solution properties of radioiodinated apoA-I. A mixture of labeled protein with unlabeled carrier eluted from a gel filtration column in two peaks, an aggregate and monomer peak, respectively. The distribution of radioactivity between the peaks was altered by the concentration of carrier, demonstrating that the radioiodinated protein associated with the carrier. However, the specific activity of the monomer peak was always higher than the aggregate, suggesting the iodinated protein had a greater propensity to remain a monomer. By sedimentation equilibrium and circular dichroism criteria, the aggregate peak was "competent" to undergo concentration-dependent self-association, whereas the monomer peak was "incompetent".

In the present studies, solution properties of iodinated apoA-I have been studied without recourse to mixing a radioactive tracer with unlabeled carrier protein. These preparations underwent concentration-dependent self-association as evidenced by circular dichroism (Figure 1B) and gel filtration (Figure 2). Solution properties of the aggregate and monomer fractions (Figure 2) as performed by Osborne et al. (1984) were not compared. The present gel filtration results contrast with those of Osborne et al. in that iodinated apoA-I was more aggregated at all concentrations. Our studies furthermore demonstrate that the extent of aggregation is increased in relation to the extent of iodination. Whether iodination alters the association constants or results in an increased proportion of irreversible aggregation, or a combination of these two processes, is not evident from our data.

In order to more directly assess the metabolic significance of the iodination of apoA-I, phospholipid binding properties of our preparations were examined. ApoA-I causes the spontaneous disruption of saturated fatty acid phosphatidylcholine vesicles into micellar disks at their gel to liquid-crystalline transition temperature (Pownall et al., 1979; Jonas et al., 1980; Swaney, 1980; Wetterau & Jonas, 1983). Due to greater bilayer stability, however, vesicles of unsaturated phosphatidylcholine are not disrupted by apoA-I (Matz & Jonas, 1982; Yokoyama et al., 1980; Verdery & Nichols, 1974). Since equilibrium binding of protein with lipid vesicles can be measured only when the lipid bilayer remains stable, and since its heterogeneous composition more accurately reflects physiologic phospholipid compositions, EYPC was chosen for these studies. Upon the addition of EYPC vesicles, native and iodinated apoA-I underwent enhancements in tryptophan fluorescence (Figure 3). The apparent binding stoichiometries obtained from these titrations suggested that the vesicles had a higher capacity for iodinated than native apoA-I in relation to the extent of iodination. This conclusion was strikingly evident by a Scatchard analysis of binding (Figure 4), which also demonstrated that iodinated apoA-I bound to EYPC vesicles with lower affinity, again in relation to the extent of iodination. Furthermore, the kinetics of apoA-I binding to EYPC vesicles is enhanced by iodination (Figure 5). Thus, by all techniques examined, modification of apoA-I by iodination perturbs phospholipid binding properties dependent on the degree of modification.

ApoA-I exchanges dynamically between HDL density subclasses in vitro (Grow & Fried, 1978) and in vivo (Shepherd et al., 1978b). The current model of apolipoprotein exchange envisions free protein monomers associating and dissociating with lipoprotein surfaces by an equilibrium process. Free apolipoproteins in plasma may undergo homologous or heterologous associations and may associate with lipid monomers (Osborne & Brewer, 1980). The present studies demonstrate that the presence of iodine on apoA-I perturbs both the state of association and phospholipid binding properties. This may account in part for the observation of Shepherd et al. (1977) that not all of the endogenous apoA-I of HDL can be displaced by ^{125}I-apoA-I. Furthermore, Shepherd et al. (1978a) observed that ^{125}I-apoA-I reconstituted with HDL exhibited a more rapid catabolic clearance in vivo than did apoA-I radioiodinated in situ within HDL. On the basis of our results, the difference in catabolic rates observed by Shepherd et al. (1978a) may reflect altered protein/lipid interactions induced by the differing extents or sites of apoA-I iodination that accompany these extrinsic labeling methods. Direct testing of the hypothesis that altered protein/lipid interactions result in altered metabolic kinetics must await further studies.

Some experimental protocols have recognized that iodination may perturb physical properties of proteins and have attempted to circumvent this problem by minimally modifying the apolipoprotein with less than one radioiodine per protein molecule (Schaefer et al., 1982; Gregg et al., 1984). However, by definition, a metabolic tracer must have a minimum of one exogenous label attached. Even if only a small fraction of protein molecules within an injected sample contains one ^{125}I label, metabolic kinetic data will be obtained only from that fraction of protein on which the label resides and whose physiologic properties may have been perturbed. The present studies demonstrate that self-association and the binding affinity, capacity, and kinetics of apoA-I with lipid are perturbed 2–3-fold by iodination. One may therefore speculate that since the dynamic interactions of apoA-I are perturbed by iodination, the tracer assumption may not be valid for ^{125}I-apoA-I under all experimental conditions. Comparative metabolic kinetics studies of apoA-I tracers labeled by iodine and by an endogenous amino acid label would help to clarify the metabolic consequences of the iodine perturbation to apoA-I.

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