Effects of Amino Group Modification in Discoidal Apolipoprotein A-I–Egg Phosphatidylcholine–Cholesterol Complexes on Their Reactions with Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: Discoidal complexes of human apolipoprotein A-I–egg phosphatidylcholine–cholesterol were prepared by the sodium cholate dialysis procedure and were reacted to varying extents with the amino group reagents citraconic anhydride, diketene, and formaldehyde in the presence of sodium borohydride. Modification of positive lysine residues with negative or neutral groups (citraconic anhydride and diketene, respectively) resulted, for extensively reacted complexes (90%), in structural alterations and in a marked decrease in reactivity with purified human lecithin:cholesterol acyltransferase. The structural and kinetic effects were partially reversible by removal of the modifying groups or by increased ionic strength. Similar extents of modification (84%) with retention of positive charge and introduction of two methyl groups (reductive methylation) had no effect on the structure or the reactivity of the complexes. These results, together with kinetic data at variable complex concentrations or at variable temperatures, indicate that specific lysine residues of apolipoprotein A-I are not involved in the lecithin:cholesterol acyltransferase activation process; instead, charge interactions and structural changes are responsible for the observed decrease in activating capacity. In terms of kinetic parameters, intrinsic K^*_m values and probably enzyme–substrate particle dissociation constants are affected, but the activation energies remain the same upon chemical modification.

It is generally recognized that high-density lipoproteins (HDL)[1] are the preferred native substrates of lecithin:cholesterol acyltransferase (LCAT) in plasma (Glomset, 1972; Marcel et al., 1980; Marcel, 1982) and that apolipoprotein A-I (apo A-I) is the main activator of this enzyme (Fielding et al., 1972; Soutar et al., 1975; Yokoyama et al., 1978; Albers et al., 1979). However, the activation is not completely specific for apo A-I since several synthetic peptides (Yokoyama et al., 1980; Pownall et al., 1980; Sparrow & Gotto, 1982), segments of apo A-I (Soutar et al., 1975), and other apolipoproteins (Soutar et al., 1975; Albers et al., 1979; Jonas et al., 1984) have been shown to partially activate LCAT in vitro. In addition, observations in patients with familial apo A-I deficiency have indicated that LCAT reaction can proceed, albeit less efficiently than in the presence of apo A-I (Schaefer, 1984).

The structural properties which determine the LCAT-activating role of apo A-I are not yet defined. The presence of amphipathic α-helical domains and the capacity to bind lipid are common properties of LCAT-activating polypeptides, but local structural features which may define an enzyme recognition site appear to be essential. For example, two variants of apo A-I have been shown to have a lower activating capacity for LCAT than native apo A-I (Rall et al., 1984). In addition, our laboratory recently demonstrated that apo A-I incorporated into discoidal complexes of dipalmitoylphosphatidylcholine and cholesterol can have a variable activating capacity for LCAT depending on the size of the complexes and the local structural environment of its tryptophan (Trp) residues (Jonas & McHugh, 1984).

Since studies of receptor recognition by apolipoproteins E and B have indicated the involvement of lysine (Lys) and arginine residues in the binding (Mahley & Innerarity, 1983), and the apo A-I variants defective in LCAT activation correspond to basic amino acid residue changes (Rall et al., 1984), we set out in this work to examine the effect of Lys residue modification on the ability of apo A-I to activate LCAT.

Experimental Procedures

Materials. The preparation and purity determination of human apo A-I, as well as the sources and purity checks of the lipids and [4-^{14}C]cholesterol used in this study, have been described, and the original references have been given in many of our recent publications on the properties of discoidal LCAT substrates (Jonas & Matz, 1982; Jonas & McHugh, 1983; Jonas et al., 1984). Our purification of LCAT (Matz & Jonas, 1982b), based on the procedures of Albers et al. (1976) and Chung et al. (1979), yields 15 000-fold-purified enzyme which is stable for several months when stored at 4 °C in 0.01 M Tris-HCl and 5 mM EDTA, pH 7.6, buffer under an atmosphere of N_2. Reagents for chemical modifications and for free amino group assays were the following: citraconic anhydride, sodium borohydride, and fluorescamine from Sigma Chemical Co.; sodium borate, hydroxylamine hydrochloride, and 37% aqueous formaldehyde (w/w) from Fisher Scientific Co.; trinitrobenzenesulfonic acid from Eastman Kodak Co. Distilled diketene was a gift from Dr. K. Weisgraber, Glad-

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[1] Abbreviations: HDL, high-density lipoprotein(s); LCAT, lecithin:cholesterol acyltransferase; apo A-I, apolipoprotein A-I (major protein component of HDL); Trp, tryptophan; Lys, lysine; egg PC, egg phosphatidylcholine; apparent K_m, apparent Michaelis–Menten constant; apparent V_{max}, apparent maximal velocity; v_0, initial velocity; S_0, bulk substrate concentration; k_{cat}, catalytic rate constant; E_0, total enzyme concentration; S, interfacial substrate concentration (molecules per surface area); K^*_m, intrinsic Michaelis–Menten constant; K_s, enzyme–interface dissociation constant; I, ionic strength; ΔZ, change in charge; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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stone Foundation, University of California, San Francisco. All other salts and reagents, as well as the organic solvents, were high-purity chemical products from various commercial sources.

Complex Preparation. The sodium cholate dialysis method was used in the preparation of discoidal complexes from human apo A-I, egg phosphatidylcholine (egg PC), and cholesterol (Matz & Jonas, 1982a; Jonas & McHugh, 1983). Each reaction mixture contained egg PC/cholesterol/apo A-I/sodium cholate in a weight ratio of 11.1:0.55:4.0:6.2 (mg/mg), which is equivalent to a molar ratio of 100:10:1:100 (mol/mol). The specific activity of [4-^{14}C]cholesterol in the reaction mixtures was 1619 cpm/nmol. Lipid dispersions in detergent, subsequent additions of apo A-I, and incubations were performed in 0.01 M Tris-HCl, pH 8.0, buffer with 0.15 M NaCl, 1 mM NaN_3, and 0.01% EDTA. This buffer is referred to as the "standard Tris-HCl buffer" throughout this study. Prior to dialysis, three reaction tube contents (a total of 12 mg of apo A-I) were combined and were dialyzed against 0.1 M sodium borate, pH 8.5, buffer; the remaining reaction mixtures (a total of 8 mg of apo A-I) were dialyzed against 0.1 M sodium borate, pH 9.0, buffer. Complexes were prepared in five separate fractions because we had previously determined that reaction conditions are more easily adjusted, and yields appear higher, when small volumes with protein concentrations from 1 to 5 mg/mL are used in the reaction. Dialysis over 3 days with a total of six 1-L changes of buffer removed the sodium cholate completely and resulted in discoidal recombinant formation as reported previously (Matz & Jonas, 1982a; Jonas & McHugh, 1983).

Chemical Modification of Complexes. Modification of primary amino groups (i.e., mostly Lys residues) in complexes, with citraconic anhydride, was performed on solutions in 0.1 M sodium borate, pH 8.5, buffer containing 1 mg of apo A-I in 1-mL volumes. Cold citraconic anhydride, either neat or diluted 1:10 or 1:100 with absolute ethanol, was added in 2-μL aliquots to the complexes, vortexing and adjusting the pH to 8.5 with 1 N NaOH between additions. The equivalent of 0.1, 0.5, or 2 μL of neat reagent was added to achieve different levels of modification. After 30 min at room temperature and about 1 h at 5 °C, the reacted samples and the control were dialyzed against standard Tris-HCl buffer at 5 °C. A duplicate of the reaction mixture with 2 μL of neat citraconic anhydride was dialyzed first at room temperature against 1 L of a 0.2 M NaCl solution adjusted to pH 2–3 with HCl in order to remove the modifying groups (Means & Feeney, 1971). After about 12 h under the above conditions, this complex sample was transferred into the standard Tris-HCl buffer. Subsequent assays of the extent of amino group reaction showed that spontaneous hydrolysis of modifying groups did not take place for at least 5 days when the samples were stored cold for 6 h at room temperature or for 2 h at 40 °C. All our experiments with these modified complexes were completed in 4 days.

Reactions with diketene to give acetoacetylated derivatives of apo A-I in complexes were performed according to the method of Marzotto et al. (1968). Neat or 1:100 diluted diketene (in 0.1 M sodium borate buffer, pH 8.5) was added in 2–4-μL aliquots to complex solutions containing 1 mg of apo A-I in 1 mL of the borate buffer. Between additions of diketene, the samples were vortexed, and the pH was adjusted to 8.5 with 1 N NaOH. Reaction mixtures had total additions of diketene equivalent to 0.03, 0.08, or 2 μL of neat reagent. They were allowed to stand at room temperature for 30 min prior to dialysis against 2 L of 0.2 M sodium carbonate/bicarbonate, pH 9.5, buffer at room temperature for 7 h. This dialysis step was intended to remove modifying groups from tyrosine residues. Subsequently, all the samples were transferred into standard Tris-HCl buffer for dialysis at 5 °C; one exception was a duplicate of the sample with 2 μL of diketene which was dialyzed first against 1 L of 0.1 M hydroxylamine hydrochloride adjusted to pH 7 with NaOH. Dialysis at room temperature for about 12 h under these conditions is reported by Marzotto et al. (1968) to cause significant reversal of acetoacetylation.

Reductive methylation of apo A-I in complexes was carried out essentially by the method of Means & Feeney (1968). Complexes in 0.1 M sodium borate buffer, pH 9.0, containing 1 mg of apo A-I in 1-mL volumes were placed on ice, and 1 mg of $NaBH_4$ was added to each sample. After the samples were vortexed, they were allowed to stand for 5 min prior to the addition of 37% aqueous formaldehyde (w/w). The formaldehyde solution was added in 2-, 5-, 10-, or 20-μL volumes. Following a 30-min incubation over ice, the reaction mixtures and a control exposed to $NaBH_4$ were dialyzed against the standard Tris-HCl buffer at 5 °C.

Analysis of the free (i.e., unmodified) amino groups following all three reactions was performed by the Habeeb (1966) and the fluorescamine (Böhlen et al., 1973) methods. For these analyses, the complex samples (1 mg/mL apo A-I) were transferred into 0.1 M sodium borate buffer, pH 8.5, by dialysis; duplicate 20-μL aliquots were taken for the fluorescamine assay (Böhlen et al., 1978), and duplicate 50-μL aliquots were used in the Habeeb (1966) procedure. For the latter method, all the reagent volumes were reduced by half.

Complex Characterization. The chemically modified complexes and the corresponding controls were analyzed by chemical, spectroscopic, and chromatographic methods. Complex compositions, in terms of protein, phospholipid, and cholesterol content, were analyzed by the standard methods employed in our laboratory (Matz & Jonas, 1982a; Jonas & McHugh, 1983; Jonas et al., 1984). Intrinsic protein fluorescence spectra, fluorescence polarization, and circular dichroism spectra were used to detect protein structural changes, and elution through a calibrated Bio-Gel A-5m column was employed to estimate the size of the complexes. All these experiments were performed in the standard Tris-HCl buffer at apo A-I concentrations around 0.25 mg/mL. Electron microscopy was carried out with a JOEL-100C electron microscope on negatively stained samples, as described previously (Matz & Jonas, 1982a). Uncorrected fluorescence spectra were recorded with a Perkin-Elmer MPF III spectrofluorometer, using 5-nm slit widths and 280-nm exciting wavelength over a 290–380-nm emission band in the ratio recording mode. Fluorescence polarization was measured at 25 °C with an SLM Model 400 polarization instrument using 4-nm slits, a 280-nm exciting wavelength, a 0-74 Corning glass filter in the exciting beam, and 0-54 Corning glass filters in the paths of the emitted light. Circular dichroism spectra were recorded between 260 and 205 nm, at room temperature, with a JOEL J40-A recording spectropolarimeter. The empirical approach of Greenfield & Fasman (1969) was used in estimating the percent α-helix content, together with a mean residue weight of 115 for apo A-I. A Bio-Gel A-5m column (1.8 × 50 cm), equilibrated with the standard Tris-HCl buffer and calibrated with the size markers described previously (Jonas & McHugh, 1984), was used to estimate the Stokes radii of complexes. A 0.2-mL aliquot of each sample (1 mg/mL apo A-I) was eluted through the column, and [4-^{14}C]cholesterol cpm in 0.5-mL aliquots of the 2-mL fractions

Table I: Chemical Modification of Complexes

reagent (charge modification)[a]	volume of neat reagent[b] (μL)	amino group modification[c] (%)
citraconic anhydride (+ → −)	0	0
	0.1	38 ± 5
	0.5	78 ± 9
	2	90 ± 6
	2 (reversed)[d]	13 ± 2
diketene (+ → 0)	0	0
	0.03	28 ± 3
	0.08	52 ± 9
	2	90 ± 6
	2 (reversed)[d]	36 ± 6
$NaBH_4$ + formaldehyde (+ → +)	0	0
	2	7 ± 1
	5	38 ± 2
	10	80 ± 8
	20	84 ± 8

[a] The Lys residue and terminal amino group charges are 1+ at pH 8.0; thus, the introduction of a carboxyl group in the citraconylation reaction represents a change from a positively to a negatively charged group; acetoacetylation with diketene represents a change from a positive charge to zero charge; reductive methylation to dimethylamino groups preserves the positive charge. [b] Less than 2 μL of reagent was added in dilute form, using 1:100 or 1:10 dilutions of citraconic anhydride in ethanol or 1:100 diketene in borate buffer. [c] Two independent assays of amino group modification were performed on duplicate complex samples. The Habeeb (1966) procedure gave lower degrees of modification than the fluorescamine method (Böhlen et al., 1973). The average of the two procedures is given here, with an error representing the variability between the two methods. Within each method, the experimental error was on the order of 5%. [d] These samples were chemically modified in a manner identical with that used for the preceding complexes in each series but were subsequently subjected to reversal reactions as described under Experimental Procedures.

were determined. In a few cases, the protein elution pattern was analyzed by measuring the fluorescence intensity at 330 nm while exciting at 280 nm.

Enzymatic Reactions. Each reaction mixture contained from 6.2 to 50 μg of apo A-I (in complexes), 2 mg of defatted bovine serum albumin, 4 mM β-mercaptoethanol, and buffer up to 0.45-mL total volume. The buffer in the experiments of LCAT activation as a function of apo A-I modification, concentration dependence, and temperature dependence was the standard Tris-HCl buffer with the additives including 0.15 M NaCl. For the experiments on ionic strength effects, the buffer was 0.01 M Tris-HCl, 0.01% EDTA, and 1 mM NaN_3, pH 8.0, with appropriate NaCl additions to give 0, 0.2, 1, or 2 M NaCl concentrations and final ionic strengths (I) of 0.04, 0.17, 0.70, or 1.36, respectively. Prior to LCAT addition, the reaction mixtures were equilibrated at 37 °C (or the appropriate temperature for the temperature dependence experiments) for about 15 min. The enzymatic reactions were initiated by the addition of 50 μL of LCAT stock solution or of stock solution diluted with the appropriate buffer down to one-fifth of its original concentration in order to obtain initial reaction velocities in 15- or 30-min periods. Linear reaction rates, linearly related to enzyme concentration, were observed under our reaction conditions which precluded a cholesterol transformation greater than 20–30%. Reactions were stopped by addition of 5 mL of chloroform/methanol (2:1 v/v); the organic phase with added cold carrier cholesterol and cholesteryl oleate was dried under N_2 and redissolved in 0.2 mL of heptane for TLC analysis. Lipid separation by TLC and quantitation by scintillation counting were previously described (Matz & Jonas, 1982b; Jonas & Matz, 1982).

Results

The results of chemical modification of complexes are summarized in Table I. Up to 84–90% amino group modifications were achieved with all three reagents; furthermore, substantial reversals of the citraconic anhydride (from 90% to 13%) and diketene (from 90% to 36%) reactions were effected. The average values from two amino group determination methods (Habeeb, 1966; Böhlen et al., 1973) are reported in order to account for possible differences in reagent accessibility and selectivity for free amino groups.

Spectral properties and sizes of chemically modified complexes are compared to the corresponding properties of control samples in Table II. Small increases in Stokes radii (10–15%) were observed after extensive reaction with citraconic anhydride or diketene upon elution of samples from the gel filtration column. Similar changes in diameters were observed by electron microscopy for the diketene-modified complexes.

For the citraconylated samples, the relative Trp fluorescence intensity decreases with increasing chemical modification, and there is a slight red shift in the wavelength of maximum fluorescence, indicating a modest change in the average Trp environment, probably to a more polar state. Concomitantly, the α-helix content of apo A-I decreases by about 10%, and the overall size of the particles increases by a similar percentage. Fluorescence polarization remains essentially constant. Upon reversal of the citraconic anhydride chemical modification, the properties of these complexes become in-

Table II: Physical Properties of Control and Chemically Modified Complexes[a]

sample						
reagent	% amino groups modified	% rel fluorescence intensity	wavelength of max fluorescence (nm)	fluorescence polarization at 25 °C	α-helix content (%)	av Stokes radius[b] (Å)
citraconic anhydride	0	100 ± 4	332 ± 2	0.117 ± 0.002	72 ± 4	76 ± 4
	38	96	332	0.120	72	74
	78	81	333	0.119	64	74
	90	72	334	0.116	62	83
	13 (reversed)	102	332	0.126	73	74
diketene	0	100	332	0.118	72	76
	28	94	332	0.119	68	78
	52	101	332	0.123	68	
	90	47	335	0.131	55	86
	36 (reversed)	68	334	0.124	62	89
$NaBH_4$ and formaldehyde	0	100	332	0.119	72	76
	7	101	332	0.119		
	38	103	332	0.118	70	77
	80	100	332	0.117		
	84	98	332	0.117	68	80

[a] The errors indicated are representative experimental errors for each physical measurement. The errors in percent amino group modification are indicated in Table I. [b] Stokes radii were determined from peak elution positions of each complex as assayed by [4-^{14}C]cholesterol cpm. The protein peaks were displaced about three fractions toward larger elution volumes (53-Å Stokes radii for controls), indicating complex heterogeneity.

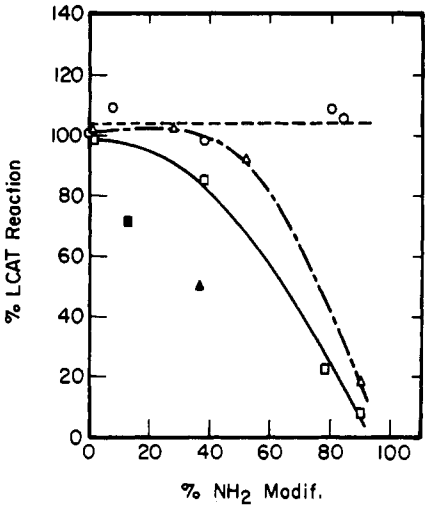

FIGURE 1: Reactivity of complexes with LCAT as a function of the chemical modification of amino groups: (□) citraconylated, (△) acetoacetylated, and (○) reductively methylated complexes. The closed symbols represent maximally reacted samples (90% modification) after reversal of the chemical modifications. Initial enzyme velocities were measured for each complex and were compared to the corresponding control. All three controls had very similar reaction rates with the enzyme, and all samples contained equal apo A-I concentrations.

distinguishable from the controls, indicating that the observed structural changes are reversible. The slight increase in fluorescence polarization cannot be easily explained but does not seem to have significant structural or functional correlates.

Samples extensively modified with diketene also exhibit structural alterations. At 90% modification, the fluorescence intensity of apo A-I is reduced markedly; there is a slight red shift in the wavelength of maximum fluorescence, together with an increase in fluorescence polarization. In addition, α-helix content drops by about 17%, and the complexes increase in size. Reversal of the reaction, to 36% modification, returns the structural properties only partially toward those of the control or a similarly modified sample (28% modification). Evidently, some irreversible structural changes, also reflected in the size of the complexes, have occurred during extensive diketene modification.

Reductive methylation with $NaBH_4$ and formaldehyde, up to 84% modification of amino groups in complexes, does not have any significant effects on the structural properties listed in Table II.

Figure 1 illustrates the effect of the three chemical modification series on the reactivity of LCAT with the complexes. Reaction with citraconic anhydride results in a progressive decrease in complex reaction with LCAT, which is already significant when 38% of the amino groups are modified. With acetoacetylation, 50% or greater modification of the complexes must be achieved before a significant decrease in LCAT activation is observed. In contrast, reductive methylation does not reduce LCAT activation with up to 84% modification of amino groups; rather, it appears to increase modestly the reactivity of some of the complexes. Since the experiments shown in Figure 1 suggested general charge effects in the activation process, we examined the effects of ionic strength on the LCAT reaction with the various complexes. Figure 2 shows the initial reaction velocities for the citraconic anhydride modified and diketene-modified complexes as a function of the solvent ionic strength. Clearly, increasing ionic strength restores much of the activating capacity of citraconylated and acetoacetylated complexes relative to the controls. For example, the complexes with 90% modification of amino groups had 40–50% of the control activity at $I = 1.36$, but only 8%

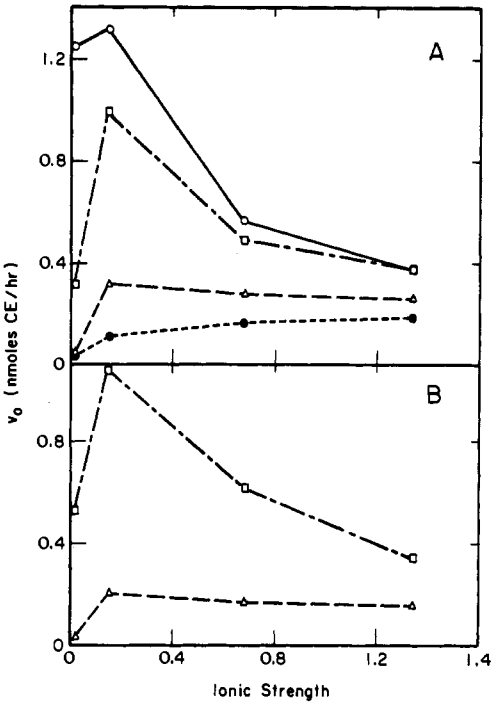

FIGURE 2: Effects of ionic strength on the initial velocity of the LCAT reaction with complexes. (A) Citraconylated samples with (○) 0%, (□) 38%, (△) 78%, and (●) 90% reacted amino groups. (B) Acetoacetylated complexes with (□) 28% and (△) 90% reacted amino groups. The ionic strength of the 0.01 M Tris-HCl, pH 8.0, 1 mM NaN_3, and 0.01% EDTA buffer was adjusted with NaCl. The enzymatic reactions were performed at 37 °C by using a one-third dilution of the stock enzyme preparation.

activity at $I = 0.17$. As expected, the charge effects of the chemical modifications are most marked at the lowest ionic strengths; yet for a single complex, particularly the controls and the samples with low and intermediate chemical modifications, the activation of LCAT does not change monotonically with ionic charge. There is maximal reactivity around $I = 0.17$ and declining reactivities at higher ionic strengths. The reductively methylated samples behaved similarly to controls in having high reactivities at low ionic strengths and decreasing ones at high ionic strengths (data not shown).

The temperature dependence results for selected complexes are shown in Figure 3 as Arrhenius plots. Evidently, complexes with substantial chemical modification (e.g., 78% citraconylated complexes) have very similar activation energies to controls and to moderately modified complexes, as indicated by the slopes of the Arrhenius plots and the activation energy values listed in Table III.

Apparent kinetic constants for the LCAT reactions with complexes were obtained from the double-reciprocal plots of initial velocity against concentration shown in Figure 4. In these experiments, selected complexes, with measurable reaction rates, were diluted over an 8-fold concentration range. The results were then expressed in terms of apo A-I concentrations. For the control complexes, the apparent K_m values are consistent with previously determined values (Jonas et al., 1984). Partially reacted samples with similar reactivities to the controls (e.g., the 52% diketene- and 84% formaldehyde-modified complexes) have very similar apparent kinetic parameters to those of controls. Samples with intermediate degrees of modification have curved plots, but an extensively modified sample (90% diketene modification) gives a linear plot which yields considerably different apparent K_m and V_{max} values from those of controls. The apparent kinetic constants from linear plots are listed in Table III.

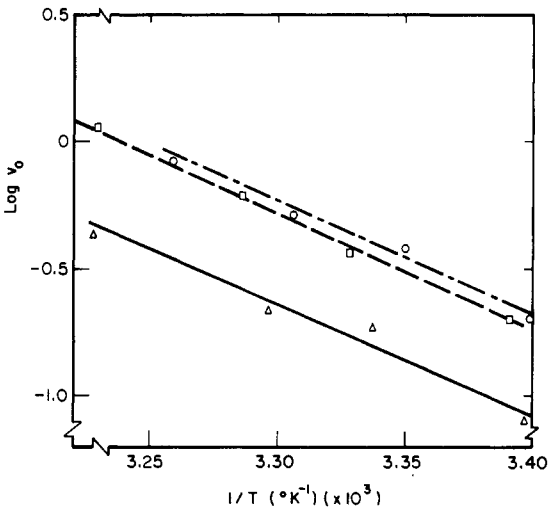

FIGURE 3: Temperature dependence of the enzymatic reactions of complexes expressed as Arrhenius plots: (△) 78% citraconylated complexes, (□) 52% diketene-modified complexes; (○) 84% reductively methylated complexes. The enzymatic reactions were performed between 21 and 37 °C in standard Tris-HCl buffer by using a one-third dilution of the stock enzyme preparation.

Table III: Apparent Kinetic Constants and E_a Values of Complexes

sample reagent	% amino groups modified	app K_m[a] (M)	app V_{max}[a] (nmol of CE/h)	E_a[b] (kcal/mol)
citraconic anhydride	0	2.2×10^{-7}	5.55	17.3
	38	c	c	15.5
	78	c	c	18.7
	13 (reversed)	c	c	
diketene	28	3.2×10^{-7}	5.67	
	52			19.8
	90	6.4×10^{-7}	1.08	
	36 (reversed)	c	c	16.6
$NaBH_4$ and formaldehyde	0	1.5×10^{-7}	4.92	
	7			17.8
	84	2.5×10^{-7}	5.76	16.0

[a] The apparent kinetic constants were calculated from the linear double-reciprocal plots in Figure 4. The very shallow slope of the plot for the controls introduces a large error in the apparent K_m values, estimated on the order of 50–100%. [b] Activation energies were calculated from the slopes of the Arrhenius plots shown in Figure 3. The estimated error is ±2 kcal/mol. [c] Curved plots in Figure 4. CE, cholesteryl ester.

DISCUSSION

The structural consequences of amino group modification of discoidal complexes of apo A-I–egg-PC–cholesterol are summarized in Table II. Citraconic anhydride modification of up to 40% amino groups has no effect on the size or on the spectral properties of these particles. More extensive modification, to the extent of 78% and 90% of amino groups, causes minor changes in the Trp residue environment, a decrease in the α-helix content, and an increase in the Stokes radius. Since all these effects are reversed upon removal of most of the modifying groups, the size increase with 90% amino group modification is probably due to a reversible apolipoprotein expansion as seen with citraconic anhydride modified serum albumin (Jonas & Weber, 1970). Aggregation–disaggregation or partial fusion events are not plausible explanations for the size changes because particles with a large net negative charge would tend to repel each other. If fusion somehow occurred, in spite of the electrostatic repulsion, fusion products would not spontaneously reverse into smaller particles.

Diketene modification results in significant structural changes only after more than 50% of the amino groups have been reacted. At 90% modification, there are slight changes

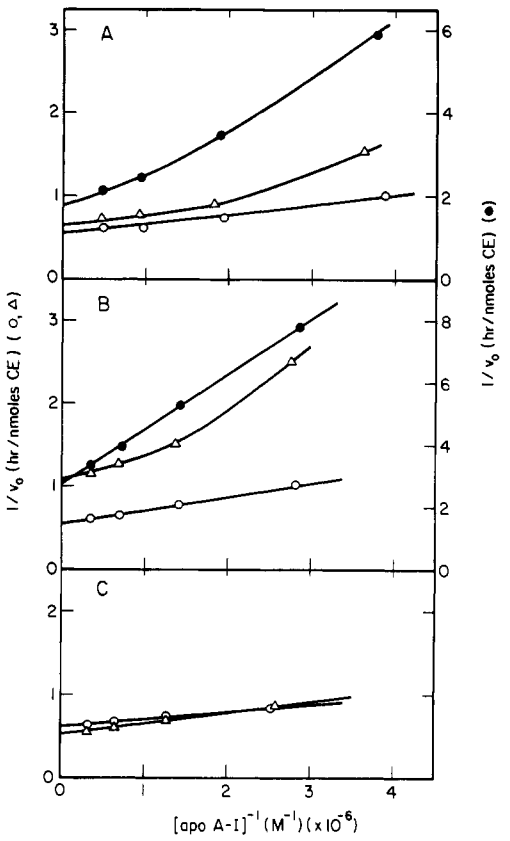

FIGURE 4: Double-reciprocal plots of initial velocities (v_0) against bulk substrate concentrations, expressed in terms of apo A-I concentrations. (A) Citraconylated complexes with (○) 0%, (△) 38%, and (●) 78% amino groups reacted; (B) acetoacetylated complexes with (○) 7%, (△) 36% (reversed), and (●) 90% reacted amino groups; (C) reductively methylated complexes with (○) 0% and (△) 84% reacted amino groups. The enzymatic reactions were performed at 37 °C in standard Tris-HCl buffer by using a one-third dilution of the stock enzyme. Note that the open and closed data points are plotted by using different $1/v_0$ scales.

in the Trp residue environment as reflected in the fluorescence intensity and polarization changes; there is a 17% decrease in α-helix content and an expansion of the particles. Removal of 54% of the modifying groups results in only partial return of the properties toward those of a similarly modified complex, and the size change remains. There is no firm explanation for these irreversible structural effects, but contributing factors could be selectivity in the reversal reaction or the persistence of tyrosine residue modification.

Reductive methylation, in contrast to the previous two reactions which alter the charge of Lys residues, does not affect at all the overall structure of the complexes even at an 84% level of amino group modification.

In view of these structural results, the reactions of the various complexes with LCAT (Figure 1) indicate that 84% modification of Lys residues of apo A-I without changes in the charge or structure of the complexes does not affect the activation of LCAT by apo A-I. Charge modification, with concomitant structural changes, is reflected in decreased activation of the enzyme. The greater effects of citraconylation (+ → −, charge change) relative to acetoacetylation (+ → 0, charge change), and the absence of effects by reductive methylation (+ → +, charge retention with introduction of two methyl groups), suggest that general ionic effects are directly or indirectly responsible for the observed decline in LCAT activation by chemically modified apo A-I. The participation of specific Lys residues in the activation process is not supported by these observations.

That ionic effects are present is confirmed by the results shown in Figure 2. The reactivity of modified complexes increases, relative to that of controls, with increasing ionic strength of the medium, probably as a result of charge shielding. For example, the 78% citraconylated complex has 3, 24, 49, and 68% of the activity of controls at ionic strengths of 0.04, 0.17, 0.70, and 1.36, respectively. For the unmodified or partially modified complexes, the pattern of increasing activity, up to ionic strength $I = 0.17$, and then a decline at higher values is probably due to optimal enzyme stability and/or enzyme–complex interaction effects.

In a recent study (Jonas et al., 1985), we demonstrated that the LCAT reaction with discoidal substrates follows closely the Verger (Verger et al., 1973; Verger & de Haas, 1976) kinetic model of an interfacial enzyme where two enzyme binding steps occur: one to the interface and another to lipids in the active site. The apparent kinetic parameters experimentally obtained from plots of $1/v_0$ vs. $1/S_0$ (v_0, initial velocity; S_0, bulk substrate concentration), and derived from the Verger model, are

$$\text{apparent } V_{max} = (k_{cat}E_0S)/(S + K^*_m)$$

and

$$\text{apparent } K_m = K_sK^*_m/(S + K^*_m)$$

where k_{cat} is the catalytic rate constant, E_0 is the total enzyme concentration, S is the interfacial substrate concentration (i.e., molecules per surface area), K^*_m is the intrinsic Michaelis–Menten constant, and K_s is the dissociation constant of the enzyme from the substrate particle.

The concentration dependence experiments on the modified complexes and controls, shown in Figure 4 and summarized in terms of apparent kinetic constants in Table III, indicate that controls, and modified complexes without structural and kinetic changes, give linear plots with very similar kinetic constants. Complexes with intermediate modification show curved plots which suggest the presence of a population of complexes with variable kinetic constants, and not simply a decrease in fully active substrate concentrations. This observation supports the conclusion that the effects on activity are general charge–structure effects. Finally, a very extensively modified complex (90% acetoacetylated complex) exhibits a linear plot with decreased apparent V_{max} and increased apparent K_m values. This suggests the presence of uniform substrates with increased K^*_m and probably altered K_s values, since k_{cat}, S, and E_0 are constant. The assumption that k_{cat} is constant in these systems is based on the observation of constant activation energies (E_a) with increasing chemical modification (Table III).

In summary, we have shown that chemical modification of apo A-I Lys residues in discoidal LCAT substrates leads to observable structural changes when the net change in charge (ΔZ) is of the order of −15. Enzymatic effects appear to be more sensitive to charge and start occurring when $\Delta Z \sim -9$. The charge and structural effects, but not specific Lys residue modifications, are responsible for decreasing LCAT activation. The activation process is apparently affected by changes in the K^*_m and possibly K_s constants, without significant changes in activation energy. The decrease in LCAT activation can be partially reversed by removal of the modifying groups or by shielding of charges at high ionic strength. These results suggest that activation of LCAT by apo A-I in vivo depends on the structure and charge of the apolipoprotein, which may be altered by mutations, by HDL size and composition changes, or by interactions with macromolecules or ions.

Registry No. LCAT, 9031-14-5; citraconic anhydride, 616-02-4; diketene, 674-82-8; formaldehyde, 50-00-0.

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