Interaction of Apolipoprotein-Alanine and Apolipoprotein-Glutamine-I with Phosphatidylcholine. Effect on Solute Quenching of Intrinsic Fluorescence†

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ABSTRACT: The interaction of egg phosphatidylcholine with apoLP-alanine$_1$, apoLP-glutamine-I, and human serum albumin has been investigated by quenching of the intrinsic protein fluorescence in both the presence and absence of the phospholipid. The quenching by a negatively charged quencher, iodide ion, used in conjunction with a positively charged quencher, pyridinium ion, reflected the relative charge in the region of tryptophans in the protein and indicated the magnitude of binding of phospholipid. Expressions were derived to quantitate both protein accessibility and the phospholipid binding efficiency of these proteins. ApoLP-alanine$_1$ bound phospholipid more efficiently than apoLP-glutamine-I. Both apolipoproteins were more efficient than human serum albumin.

ApoLP-Ala, an apolipoprotein isolated from VLDL,[1] has been shown to interact with egg phosphatidylcholine (Morrisett *et al.*, 1973). Binding of phosphatidylcholine to apoLP-Ala is accompanied by an increase in protein helicity as determined by circular dichroic spectra and by a blue shift of the intrinsic protein fluorescence indicating that one or more of the three tryptophan residues is in a more hydrophobic environment. ApoLP-Gln-I, isolated from HDL, has been shown to interact with phospholipid by an increase in helical content (Lux *et al.*, 1972a). We now report the quenching of the intrinsic fluorescence of apoLP-Ala and apoLP-Gln-I by iodide and pyridinium ions in the presence and absence of phospholipid. For comparisons, similar studies were performed on human serum albumin and tryptophan. Tryptophan is used as a convenient reference for the accessibility of the fluorophore in the apolipoproteins. Human serum albumin, which functions in plasma as a carrier of fatty acids but not phospholipid, has been studied as a reference protein which does not interact strongly with phospholipids.

The fluorescence quenchers, iodide and pyridinium ions, have been selected as companion probes in a structural and lipid binding study of apoLP-Ala and apoLP-Gln-I since ions bear opposite charges and their relative quenching efficiencies should be related not only to the accessibility of the protein fluorophore but also to the net change in the vicinity of the fluorophore. An exposed tryptophan in a negatively charged region of the protein (*e.g.*, close to a glutamic acid residue) will be quenched more efficiently by the positively charged pyridinium ion than by a negatively charged iodide ion due to electrostatic attractions and repulsions, respectively. This conclusion is suggested from results in the accompanying paper (Pownall and Smith, 1974) where iodide and pyridinium ions quenched the fluorescence of anthracene-labeled micelles of cetyltrimethylammonium bromide and sodium dodecyl sulfate. If the quencher and micelle are oppositely charged, the quenching efficiency is about ten times that of diffusion controlled quenching. In contrast, no quenching is observed when micelle and quencher have similar charges. The differences in the relative efficiency of oppositely charged quenchers can be employed to analyze the structure and binding properties of proteins. We have specifically applied this technique to the lipoproteins, apoLP-Gln-I and apoLP-Ala$_1$.

Materials and Methods

Potassium iodide and sodium chloride (Fisher), Trizma (Sigma), tryptophan (Calbiochem), and human serum albumin (Schwarz/Mann) were used as received from the supplier. Pyridinium chloride was prepared by bubbling anhydrous HCl through an ether solution of pyridine and was purified by recrystallization from ethanol–ether (Taylor and Grant, 1955). Egg phosphatidylcholine (Singleton *et al.*, 1965) was purified to eliminate fluorescent impurities and migrated as a single spot on thin-layer chromatography on silica gel (Rouser *et al.*, 1963) after charring with sulfuric acid or after treatment with molybdate reagent (Dittmer and Lester, 1964). Dispersions of phosphatidylcholine were prepared by sonication as previously described (Morrisett *et al.*, 1973). The concentration of the phospholipid was determined by phosphate analysis (Bartlett, 1959). ApoLP-Ala$_1$ from human VLDL and apoLP-Gln-I from human HDL were purified by gel filtration and DEAE-cellulose chromatography as described previously (Shore and Shore, 1968a,b, 1969; Scanu *et al.*, 1969; Brown *et al.*, 1970). The apoproteins migrated as a single band on polyacrylamide gel electrophoresis in both 8 M urea and sodium dodecyl sulfate. These preparations gave a single precipitin line on immunoelectrophoresis against specific antisera and a characteristic amino

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[1] Abbreviations used are: HDL, high density lipoprotein; VLDL, very low density lipoprotein. The system of nomenclature for the apoproteins from human plasma lipoproteins is based on the COOH-terminal amino acids (Lux *et al.*, 1972b). ApoLP-Gln-I and apoLP-Gln-II are the major protein components of HDL present in a molar ratio of 3:1. ApoLP-Ser, apoLP-Glu, apoLP-Ala, and apoLP-Ala$_2$ are the protein species of VLDL which also occur as minor components of HDL. The subscripts for apoLP-Ala designate the number of sialic acid residues present.

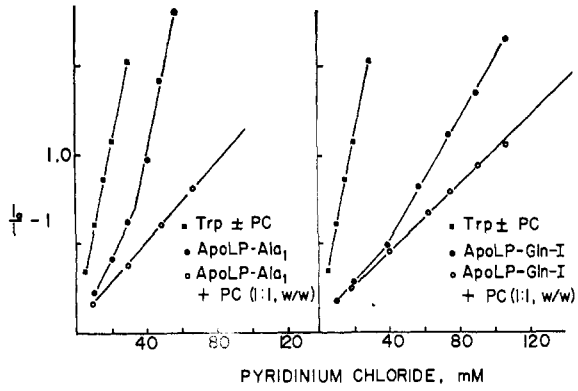

FIGURE 1: Fluorescence quenching by pyridinium chloride. Experimental details are described in Materials and Methods and in the legend to Table I.

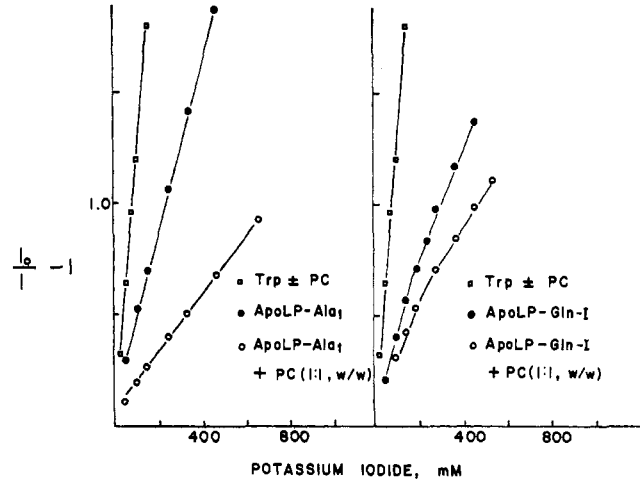

FIGURE 2: Fluorescence quenching by potassium iodide. Experimental details are described in Materials and Methods and in the legend to Table I.

TABLE I: Stern–Volmer Quenching Constants for ApoLP-Alanine, ApoLP-Glutamine-I, and Human Serum Albumin.[a]

	K_{sv}	
	Iodide	Pyridinium
Tryptophan[b]	13	50
ApoLP-alanine	3.9	20.6
ApoLP-glutamine-I	3.7	10.8
Human serum albumin	6.5	45

[a] The protein concentration varied from 1.25 to 5 × 10^{-6} M. The values for the apolipoproteins are the average of four determinations measured with quencher concentrations less than 40 mM pyridinium and 200 mM iodide ion. Experiments with albumin were done in duplicate. The excitation wavelength was 285 nm with a bandpass of 10 nm. The excitation and analyzing slit widths were 2.0 and 0.5 mm, respectively. [b] Using iodide ion, Lehrer (1971) has determined essentially identical quenching constants for *N*-acetyltryptophanamide and tryptophan.

acid analysis consistent with published reports, including missing specific amino acid residues.[2] ApoLP-Ala containing one residue of sialic acid was used throughout these studies. Other details of the fluorescence methods are found in the accompanying paper (Pownall and Smith, 1974).

Results

The fluorescence spectra of apoLP-Ala$_1$ and apoLP-Gln-I consist of one broad band with maxima at about 345 nm which may be assigned to the three and four tryptophan residues, respectively, contained in these proteins. The decrease in the fluorescence intensity, effected by iodide ion (Lehrer, 1971) or pyridinium ion (Pownall and Smith, 1974), can be treated quantitatively by the Stern–Volmer theory (Stern and Volmer, 1919) in which the decrease in fluorescence intensity, $((I_0/I) - 1)$, is a linear function of the quencher concentration. The slope of this plot is the Stern–Volmer quenching constant, K_{sv}. The values of K_{sv} for tryptophan, apoLP-Ala, apoLP-Gln-I with and without phospholipid, and human serum albumin have been obtained from the slopes of the plots in Figures 1 and 2. The K_{sv} has been calculated at low concentrations of quenching ions, less than 40 mM pyridinium ion and less than 200 mM iodide ion. The absorbance of 40 mM pyridinium ion between 280 and 290 nm is less than 0.02. The increase in slope of the plot at higher concentrations of quenching ion may cause some unfolding of the proteins so that accessibility of the tryptophans is changed. The values of K_{sv} for the protein in all cases were lower than the values of K_{sv} for tryptophan, indicating the protein-bound fluorophore is less accessible to quenching ions and solvent. A further decrease in the K_{sv} of each of the proteins is affected by the addition of a 1:1 (w/w) ratio of egg phosphatidylcholine, whereas addition of the phospholipid did not affect the K_{sv} values of free tryptophan.

The quenching of tryptophan and protein-bound tryptophan can be related by the expression, $R = [K_{sv}(\text{protein})/K_{sv}(\text{tryptophan})]100$. K_{sv}(protein) is the quenching constant for the protein, and K_{sv}(tryptophan) is the quenching constant of free tryptophan using the same quencher. The steric factor, R, represents the per cent accessibility of the tryptophan residues of a protein relative to that of free tryptophan. The R values of apoLP-Ala, apoLP-Gln-I, and human serum albumin with and without phospholipid (1:1 w/w) are presented in Table II. Additional phosphatidyl-

TABLE II: Effect of Phosphatidylcholine on Quenching of ApoLP-Ala, Apo-LP-Gln-I, and Human Serum Albumin.

	Steric Factor R[a]		
Compound	Iodide	Pyridinium	Av
ApoLP-Ala	30	41	36
ApoLP-Ala + PC	10	23	17
ApoLP-Gln-I	29	22	26
ApoLP-Gln-I + PC	20	19	20
Human serum albumin	50	100	75
Human serum albumin + PC	40	90	65

[a] Calculated using the values of K_{sv} given in Table I in the expression: $R = (K_{sv}(\text{protein})/K_{sv}(\text{tryptophan}))100$. The phospholipid–protein ratio was 1:1 w/w. Quenching of tryptophan fluorescence was not affected by phospholipid.

[2] We are indebted to Drs. Richard L. Jackson and Joel D. Morrisett, Department of Medicine, Baylor College of Medicine, for preparation and characterization of these proteins.

TABLE III: Binding Parameters of ApoLP-Ala, ApoLP-Gln-I, and Human Serum Albumin.

	B (Iodide)	B (Pyridinium)	Av[a]
ApoLP-alanine + phosphatidylcholine (1:1)	2.2	0.76	1.2
ApoLP-glutamine-I + phosphatidylcholine (1:1)	0.6	0.10	0.35
Human serum albumin + phosphatidylcholine (1:1)	0.25	0.10	0.15

[a] Calculated from: $\bar{B} = [(R(I^-) + R(\text{PHCl})\text{ for protein})/(R(I^-) + R(\text{PHCl})\text{ for protein + lipid})]^{-1}$.

choline (2:1 w/w) further decreases K_{sv} and the steric value by only about 10%. The use of higher concentrations of phosphatidylcholine was precluded by optical interference arising from turbid solutions. Addition of phosphatidylcholine to tryptophan (1:1 w/w) does not alter the rate of quenching by either iodide ion or pyridinium ion. Table II includes data on the quenching of the apoproteins by iodide ion and pyridinium ion in the presence of phosphatidylcholine.

The effect of phosphatidylcholine on the quenching of the intrinsic fluorescence of apoLP-Ala and apoLP-Gln-I is strikingly different. With both iodide ion and pyridinium ion as quencher, interaction of apoLP-Ala with phosphatidylcholine greatly reduced the efficiency of quenching. In contrast, phosphatidylcholine does not affect the quenching of apoLP-Gln-I with pyridinium ion and reduces the accessibility of tryptophan only slightly with iodide ion as the quenching agent. The interaction of both apolipoproteins with phosphatidylcholine prevents the change in accessibility caused by high concentrations of pyridinium ion (Figures 1 and 2). The plot of iodide quenching of apoLP-Gln-I has a change in slope in the presence and absence of phosphatidylcholine, at about 200 mM. Because of the high concentration of quenching ion, the significance of this break is not clear. The binding parameters of the apolipoproteins are recorded in Table III.

Human serum albumin is the serum component which binds fatty acids, but is not a lipoprotein. The quenching by both iodide and pyridinium ions is decreased slightly by phosphatidylcholine. The magnitude of this change with albumin is not as large as that for the apolipoproteins (Table III).

The rate of quenching of the apolipoproteins was dependent on protein concentration. A decrease in protein concentration in the range from 5 to 1.25 μM increased the magnitude of K_{sv} and R about 20%. It is possible that the values of K_{sv} and R would increase further if the protein concentration was reduced further. The protein concentrations in these experiments represent the limit of sensitivity of the spectrofluorimeter for this type of experiment.

Discussion

Theoretical Relationship of Quenching and Structure. The theoretical basis for fluorescence quenching observed in the gas phase systems was first described by Stern and Volmer (1919). The equations developed for these systems were extended with minor modification to the fluorescence quenching in solution (Parker, 1968). In macromolecular systems such as proteins, the accessibility of the fluorophore is restricted. This fact requires a modified form of Stern–Volmer equations which will be developed here. The system of fluorescent molecules, F, can undergo the following processes involving the excited state F*. The intensity of absorbed light is I_a;

	Rate	
$F + h\nu \longrightarrow F^*$	I_a	(1)
$F^* \longrightarrow F + \Delta$	$k_{rl}[F^*]$	(2)
$F^* \longrightarrow F + h\nu'$	$k_f[F^*]$	(3)
$F^* + Q \longrightarrow F + Q + \Delta$	$k_q[Q][F^*]$	(4)

k_{rl}, k_f, and k_q are the specific rate constants for radiationless transitions, fluorescence, and fluorescence quenching, respectively. The Stern–Volmer equation relates fluorescence intensity to the amount of quencher, Q.

$$((\varphi_0/\varphi) - 1) = k_q P \tau_f[Q] = K_{sv}[Q] \quad (5)$$

Substitution gives

$$K_{sv} = k_d P \tau \quad (6)$$

where k_d is the rate constant for the diffusion controlled interaction; τ_0 is the fluorescence decay time; and P is a steric factor. The steric factor, P, is introduced to account for possible specific orientation requirements and inaccessibility of the fluorophore to the quencher. Consideration of the factors in eq 6 indicates that Stern–Volmer constants for tryptophan would be less when the fluorophore occurs in the protein. Fluorescence lifetime of tryptophan is fairly insensitive to environment and does not change appreciably when the fluorophore is incorporated into a protein (Longworth, 1971). In these systems the rate of interaction is diffusion controlled. The concentration of the quencher is a known independent variable. Since the rate of fluorescence quenching of free tryptophan is essentially diffusion controlled, P has a value of 1. Therefore, the ratio of the Stern–Volmer constant of tryptophan to that of a protein using the same quencher is the steric factor. In the expression

$$R = (K_{sv}(\text{protein})/K_{sv}(\text{tryptophan}))100\% \quad (7)$$

it can be seen by comparison with eq 6 that R is the steric factor of the protein expressed as the per cent of the average number of accessible tryptophan residues.

Thus, the value of R is a function of the steric interference to quenching which, in the absence of electrostatic interactions, may arise from the partial or complete inaccessibility of the fluorophore. Since iodide and pyridinium ions are oppositely charged, it is appropriate to define

$$\bar{R} = (R(\text{iodide}) + R(\text{pyridinium}))/2 \quad (8)$$

as a measure of fluorophore accessibility. The deviation of R(iodide) and/or R(pyridinium) from that of $\bar{R}$ depends on the magnitude of the charge around the fluorophore, *e.g.*, a positive charge in the microenvironment of a particular tryptophan residue in a protein will attract iodide ions and repel pyridinium ions through electrostatic interaction to produce values for R(iodide) and R(pyridinium) which differ significantly from those predicted by collision theory. This effect is compensated for by $\bar{R}$ and provides a parameter related to accessibility and not charge.

A second steric factor, the binding parameter (B), to estimate the additional steric hindrance introduced by phospholipid–protein interactions, is introduced by the expression

$$B = \frac{R(\text{protein})}{R(\text{protein + phospholipid})} - 1 \quad (9)$$

In the absence of interaction between phospholipid and protein that partially covers the protein fluorophore, the R values of protein with and without phospholipid are equal and $B = 0$. Values greater than zero indicate a decreased accessibility of the fluorophore which can be attributed to (1) a modification of the tertiary structure due to phospholipid binding, (2) a simple covering of the fluorophore with phospholipid, or (3) a combination of 1 and 2.

Binding Properties of Lipoproteins. In Table III the binding parameters, B, for apoLP-Ala, apoLP-Gln-I, and human serum albumin with phosphatidylcholine (1:1 w/w) are summarized. The interaction of phospholipid and protein is greatest with apoLP-Ala, and less for apoLP-Gln-I and human serum albumin. In each case (Table II), the steric values, R, obtained with iodide ion are not identical with those obtained with pyridinium ion.

ApoLP-Ala. The steric values, R, for apoLP-Ala are 32 with iodide ion and 44 with pyridinium ion. The average steric value is 38, which indicates that 38% of the three tryptophans in the apoprotein are exposed to solvent and quencher. The larger steric value obtained with pyridinium ion as quencher suggests that the tryptophans are located in a relatively negatively charged environment. This conclusion is supported by the preferential fluorescence quenching of aromatic hydrocarbons contained in micelles which bear a charge opposite to that of the quencher (Pownall and Smith, 1974). The addition of lipid reduces the steric value, R, of the protein with iodide ion as quencher to about one-third its former value. The steric value obtained with pyridinium ion as a quencher is reduced by a factor of one-half. This difference indicates preferential phospholipid interaction with a positively charged region of the protein containing tryptophan residues accessible to solvent. This conclusion is further illustrated by comparison of the binding parameters, B (eq 9). A value of 2.2 for the binding parameters was obtained with iodide ion as the quencher in contrast to a value of 0.76 with pyridinium ion as quencher.

ApoLP-Gln-I. Inspection of the steric values for apoLP-Gln-I indicates that approximately 25% of the tryptophan in the protein is exposed to the solvent, and that the greater proportion of the four tryptophan residues is in a positively charged region of the protein. The binding parameters, B values, indicate that the phospholipid bound to apoLP-Gln-I is much less than that bound to apoLP-Ala. The small degree of phospholipid binding occurs largely in the positively charged region of the protein since the binding parameter, B, for iodide ion is greater than the binding parameter for pyridinium ion.

Human Serum Albumin. The value, $\bar{R} = 0.5$, suggests that the tryptophan in human serum albumin is quite accessible to solvent and quenchers. The relatively large value for R(pyridinium) and the small value for R(iodide) compared to $\bar{R}$ suggest that the tryptophan residue resides in a negatively charged region of this protein. The addition of phospholipid to human serum albumin does not alter appreciably the values obtained with iodide and pyridinium ions, suggesting that little binding occurs. This is indicated by the small values of B(iodide) = 0.25 and B(pyridinium) = 0.10.

The decrease in the quenching constant, K_{sv}, and the steric value, R, of the apolipoproteins with an increase in protein concentration is a probable consequence of protein aggregation.[3] In the aggregated state, which occurs in more concentrated solutions, less protein surface and therefore fewer tryptophan residues are accessible to the solvent and the quencher. The fluorescence quenching should not be as efficient as it is when the apoprotein is in the monomeric state.

[3] J. D. Morrisett, personal communication.

Conclusions

Quenching of tryptophan fluorescence in proteins can provide useful information about the microenvironment of the fluorophore when certain restrictions in interpretation are observed. The fluorescence quenching behavior of tryptophan reflects only those regions of the protein containing the fluorophore and may not reflect changes that occur in the entire protein. In addition, the values of the binding parameters, B, may reflect only phospholipid–protein interactions in the vicinity of the tryptophans. If the two tryptophans are in close proximity in the tertiary structure, one of which is accessible to solvent and the other buried in the more hydrophobic regions, both tryptophans will be quenched through a molecular exciton mechanism which occurs because the exposed residue can accept energy from the buried tryptophan residue. The phospholipid dispersions used for binding studies are highly variable and difficult to reproduce. Comparisons of the binding parameters of several proteins are valid only on the same lipid dispersion.

The advantages of this quenching technique with oppositely charged quenching ions are the speed and simplicity, the high sensitivity which requires very small amounts of protein (equivalent to 1 nM of tryptophan), and the easily derived steric values and binding parameters which facilitate interpretation of the data.

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