Reactions of Fluorescent Probes with Normal and Chemically Modified Myelin Basic Protein and Proteolipid. Comparisons with Myelin†

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ABSTRACT: Basic (encephalitogenic) protein and water-soluble proteolipid apoprotein isolated from bovine brain myelin bind 8-anilino-1-naphthalenesulfonate and 2-*p*-toluidinylnaphthalene-6-sulfonate with resulting enhancement of dye fluorescence and a blue-shift of the emission spectrum. The dyes had a higher affinity and quantum yield, ϕ, when bound to the proteolipid ($K_{ANS} = 2.3 \times 10^{-6}$, $\phi = 0.67$) than to the basic protein ($K_{ANS} = 3.3 \times 10^{-5}$, $\phi = 0.40$). From the efficiency of radiationless energy transfer from tryptophan to bound ANS the intramolecular distances were calculated to be 17 and 27 Å for the proteolipid and basic protein, respectively. Unlike myelin, incubation with proteolytic enzymes (e.g., Pronase and trypsin) abolished fluorescence enhancement of ANS or TNS by the extracted proteins. In contrast to myelin, the fluorescence of solutions of fluorescent probes plus proteolipid was reduced by Ca^{2+}, not affected by La^{3+}, local anesthetics, or polymyxin B, and only slightly increased by low pH or blockade of free carboxyl groups. The reactions of the basic protein were similar under these conditions except for a two- to threefold increase in dye binding in the presence of La^{3+}, or after blockade of carboxyl groups. *N*-Bromosuccinimide oxidation of tryptophan groups nearly abolished native protein fluorescence, but did not affect dye binding. However, alkylation of tryptophan groups of both proteins by 2-hydroxy(or methoxy)-5-nitrobenzyl bromide reduced the ϕ of bound ANS (excited at 380 nm) to 0.15 normal. The same effect was observed with human serum albumin. The fluorescence emission of ANS bound to myelin was not affected by alkylation of membrane tryptophan groups with the Koshland reagents, except for abolition of energy transfer from tryptophan to bound dye molecules. This suggests that dye binding to protein is negligible in the intact membrane. Proteolipid incorporated into lipid vesicles containing phosphatidylserine did not bind ANS or TNS unless Ca^{2+}, La^{3+}, polymyxin B, or local anesthetics were added to reduce the net negative surface potential of the lipid membranes. However, binding to protein in the lipid–protein vesicles remained less than for soluble protein. Basic protein or bovine serum albumin dye binding sites remained accessible after equilibration of these proteins with the same lipid vesicles. It is proposed that in the intact myelin membrane the proteolipid is probably strongly associated with specific anionic membrane lipids (i.e., phosphatidylserine), and most likely deeply embedded within the lipid hydrocarbon matrix of the myelin membrane. Also, in the intact myelin membrane the fluorescent probes are associated primarily, if not solely with the membrane lipids as indicated by the binding data. This is particularly the case for TNS where the total number of myelin binding sites is three to four times the potential protein binding sites.

In this paper we report on the interactions of the fluorescent probes 8-anilino-1-naphthalenesulfonate (ANS)[1] and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) with two principal myelin proteins, the basic protein (A[1], encephalitogenic protein), and the myelin proteolipid first described by Folch and Lees (1951). These two proteins comprise 80–90% of the total myelin protein. They differ markedly in their physical properties and amino acid composition. The basic protein of molecular weight 18,400 is water soluble, possesses a high arginine–lysine content, and most of its carboxyl side chains are amidinated (Eylar et al., 1971). The protein is believed to have a highly ordered and folded structure, best described as a prolate ellipsoid with an axial ratio of about 10:1 (Epand et al., 1974). The protein's basic residues are randomly distributed and it is therefore unlike the bimodal or amphipathic proteins that have been postulated to be characteristic of *integral* membrane proteins (Singer, 1972). The ease with which the basic protein can be isolated from myelin also suggests that its location in the membrane is that of a so-called *peripheral* protein.

The myelin proteolipid on the other hand is insoluble in water, but soluble in chloroform–methanol solutions. The proteolipid apoprotein can be separated from associated membrane lipids and isolated in a water-soluble form (Folch-Pi and Stoffyn, 1972; Mokrasch, 1972). The proteolipid apoprotein, which is of as yet undetermined molecular weight, has a very high proportion of hydrophobic amino acid residues, and 2–4% covalently bound fatty acids. Under the proper conditions the proteolipid exhibits a readily reversible transformation of the hydrophilic water soluble form to the lipophilic chloroform–methanol soluble form accompanied by an increase in its high α-helix content (Sherman and Folch-Pi, 1970). The physical-chemical characteristics of this protein correspond to the properties of *integral* membrane proteins as described in the lipid–protein mosaic (Singer and Nicolson, 1972) or bimodal protein (Vanderkooi and Green, 1970) membrane models.

In this paper, we have described the interactions of the fluorescent probes 8-anilino-1-naphthalenesulfonate (ANS) and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) with the two water-soluble myelin proteins under various environ-

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

mental conditions (pH and salt concentration), in the presence of cationic membrane-active drugs (local anesthetics and polymyxin B), and after chemical modification of various protein functional groups. The properties of the isolated myelin proteins with respect to their interactions with the fluorescent probes were also compared with native myelin membranes, or reconstituted lipid–protein complexes.

Materials and Methods

Purification of Proteins. The preparation of myelin proteolipid was accomplished by a combination of procedures described by Mokrasch (1967) and Folch-Pi and Stoffyn (1972). The myelin basic protein was isolated from bovine brain white matter by the methods described by Eylar and Thompson (1969) and Oshiro and Eylar (1970). The amino acid analysis of both proteins was performed as described by Nobrega and Ozols (1971). The amino acid composition was essentially the same as that reported by Eylar (1972) and Folch-Pi and Stoffyn (1972). Analytical gel electrophoresis was carried out as described by Fairbanks et al. (1971).

Modification of Carboxyl Groups. The reaction of carbodiimide activated myelin proteins with amino acid esters was carried out in the same manner as described for myelin (Feinstein and Felsenfeld, 1975). After incubation of the proteins with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and the amino acid ester, the solution was dialyzed against water at 4° for 24 hr. The dialysis bath was changed twice.

Modification of Protein Tryptophan Residues with Koshland Reagents I and II. BASIC PROTEIN. Lyophilized basic protein was reacted with Koshland reagent I (2-hydroxy-5-nitrobenzyl bromide) as previously described (Feinstein and Felsenfeld, 1975). The solution was dialyzed and then passed through a Bio-Gel P-10 column equilibrated with 0.01 *M* Tris-HCl (pH 7.4) to separate the protein from the untreated Koshland reagent and its hydrolysis product, 2-hydroxy-5-nitrobenzyl alcohol. A major yellow band was eluted in the void volume. This material (KR-I protein) exhibited absorbance at 280 nm and an additional absorbance peak at 320 nm, at pH 3.5, which shifted to 415 nm at pH 7.4. The KR-I protein was dialyzed against water at 4° and then lyophilized to yield a yellow powder which, when subjected to gel electrophoresis, migrated exactly as the unreacted basic protein. The reaction with Koshland reagent II (2-methoxy-5-nitrobenzyl bromide) was carried out as described previously (Feinstein and Felsenfeld, 1975). The modified protein was dialyzed at 4° for 24 hr, with two changes of the dialysis bath.

PROTEOLIPID. The proteolipid was modified by reaction with KR-I, dialyzed, and then passed through a small Bio-Gel P-2 column equilibrated with 0.01 *N* acetic acid. The major band obtained from the column exhibited absorbance at 320 nm. It was dialyzed against 1 l. of 0.01 *N* Tris-HCl (pH 7.4). When chromatographed on Eastman Kodak silica gel chromatoplates with acetone as solvent, the dialyzed protein solution showed only a single yellow spot which remained at the origin. A sample of 2-hydroxy-5-nitrobenzyl alcohol ran nearly to the solvent front (R_f 0.83).

Results

The amino acid composition of the isolated proteins agreed very well with analyses in other laboratories. The phosphorus content of the proteolipid preparation was 0.07%. The basic protein migrated in sodium dodecyl sulfate polyacrylamide gel electrophoresis as a single band with molecular weight of 18,000–19,000. Presumably because of its tendency to aggregate, the proteolipid migrated only 1–3 mm into the gel. The proteolipid is known to aggregate readily in aqueous solution even in the presence of dodecyl sulfate (Mokrasch, 1972) and exhibited an apparent molecular weight of about 200,000 when run on Sephadex G-200 with 1 *M* acetic acid and 0.5% dodecyl sulfate. Gonzalez-Sastre (1970) and Eng et al. (1971) also observed that the protein migrated with low mobility in polyacrylamide gels. Moscarello et al. (1973) isolated a water-insoluble protein, similar to the proteolipid described by Stoffyn and Folch-Pi, which could be prepared in either a predominantly β conformation of molecular weight greater than 500,000 or predominantly α helical with a molecular weight of 86,000. In 98% formic acid or 0.5% dodecyl sulfate the molecular weights were reduced to 24,000 and 28,000, respectively. The α-helical form was presumably a trimer of 28,000 molecular weight subunits. Chan and Lees (1974) prepared a proteolipid fraction from bovine white matter which was not as highly purified as the Stoffyn and Folch-Pi preparation and found two major bands on dodecyl sulfate polyacrylamide gels, one the principal band of 30,000 molecular weight and another band termed the DM-20 protein of molecular weight 25,000. It was reported that the proteolipid had a higher mobility in dodecyl sulfate gels than standard proteins. On the basis of various considerations it was suggested that the proteolipid may actually be a hexamer of 5000 molecular weight subunits. Nicot et al. (1973) reported molecular weights of 25,000 and 20,000 for two major bands of proteolipid apoprotein on dodecyl sulfate polyacrylamide gels.

Intrinsic Fluorescence of Myelin Proteins. The fluorescence of the protein chromophore groups (i.e., tryptophan, tyrosine, and phenylalanine) was typical of that of most proteins, in that the emission spectrum was predominantly that of the tryptophan groups. The emission peak for tryptophan in water is 350 nm, but it is often lower in proteins depending upon the polarity of its surroundings (Edelhoch and Steiner, 1964), or the degree of restriction of vibrational degrees of freedom of the amino acid residue (Vladimirov and Chin-ho, 1962). The fluorescence emission maximum at room temperature for the basic protein was 345 nm, very close to that of the amino acid tryptophan in solution and displayed a prominent shoulder in the region of tyrosine emission. Myelin and the myelin proteolipid had fluorescence emission peaks at 330 and 325 nm, respectively, indicating that their tryptophan residues were in a hydrophobic environment, or had more limited vibrational freedom.

The efficiency of collisional iodide quenching of tryptophan fluorescence is found to be well correlated with the tertiary structure of proteins. Tryptophan groups "hidden" in hydrophobic regions of a protein are usually poorly quenched unless the tertiary structure is disturbed by urea or guanidine hydrochloride (Vladimirov and Konev, 1957). The ratio of the initial fluorescence of the myelin proteins F^0 (excitation at 280 nm, emission maximum recorded at 330–350 nm) to the fluorescence F in the presence of potassium iodide was plotted against the iodide concentration [Q] according to the Stern-Volmer equation: $F^0/F = 1 + K[Q]$. The quenching constant K (M^{-1}) is given by the slopes of the lines. The fluorescence of the basic protein was quenched more effectively by iodide ion ($K = 5.4$) than that of globular proteins, such as serum albumin ($K = 2.6$), but significantly less so than that of tryptophan ($K = 12$). This

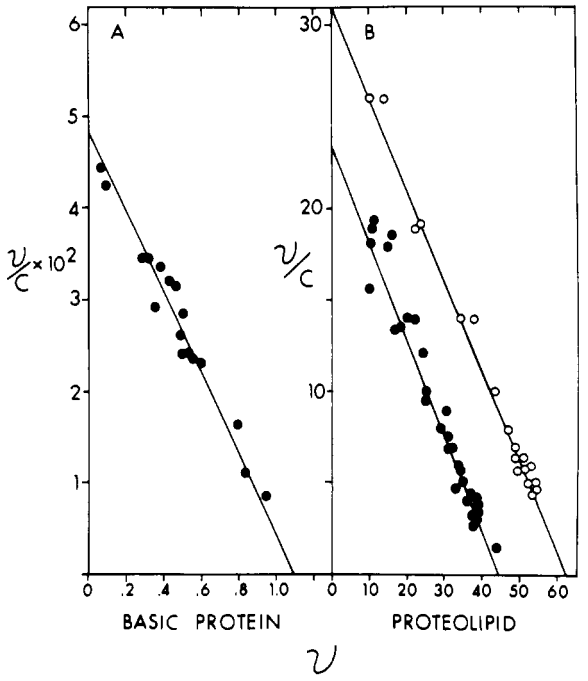

FIGURE 1: Scatchard plot of the binding of ANS (●) and TNS (○) to myelin basic protein (A) and proteolipid (B) and ν is the average ratio of nanomoles of bound ligand to nanomoles of basic protein or to milligrams of proteolipid and c is the equilibrium free-dye concentration. The fluorescence titrations were carried out in 0.01 *M* Tris-HCl buffer (pH 7.4) and binding calculations were made as described by Feinstein and Felsenfeld (1975).

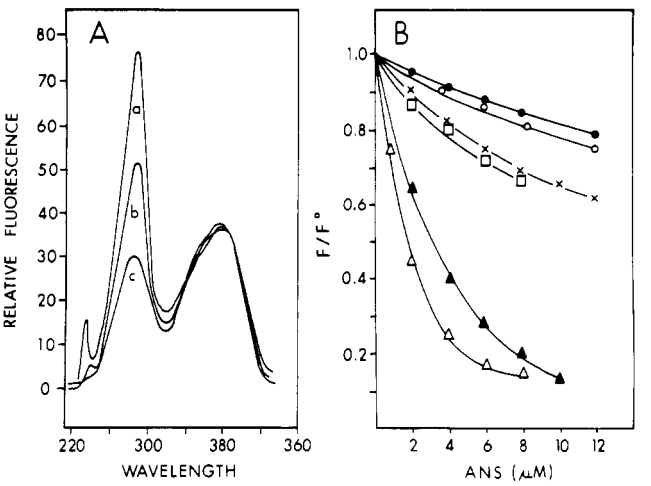

FIGURE 2: (A) Excitation spectra (uncorrected) of ANS bound to myelin proteolipid (A), myelin basic protein (B), and dissolved in ethanol (C). The ANS concentration was 4 μM. (B) Quenching of intrinsic protein fluorescence by ANS. Protein, myelin, and tryptophan solutions yielding approximately equal fluorescence emission when excited at 280 nm were titrated with ANS in 0.01 *M* Tris-HCl at pH 7.4. Quenching is the ratio of fluorescence emission in the presence of ANS (F) to the original fluorescence in the absence of ANS (F_0). Quenching of tryptophan (●) is due to the "inner-filter" effect. The difference between the latter and the other quenching curves is due to nonradiative energy transfer from tryptophan groups of the proteins to bound ANS molecules: myelin (○), basic protein (X), bovine serum albumin (▲), proteolipid (△), proteolipid incorporated into phosphatidylserine membranes (□).

is consistent with the physical determinations previously referred to which showed that the basic protein has a highly ordered and folded configuration. A greater relative inaccessibility of the tryptophan residues of myelin and the proteolipid to the solvent (water) was evident from the very weak quenching by iodide (K_{myelin} = 1.3, $K_{proteolipid}$ = 0.2). The value for myelin is comparable to that observed in erythrocyte membranes.

Binding of Fluorescent Probes to Myelin Basic Protein and Proteolipid. ANS and TNS bound to both myelin proteins with a resultant enhancement of the dye fluorescence. The *apparent* statistical dissociation constants differed by an order of magnitude. K_{app}ANS-basic protein = 3.3×10^{-5} *M*, K_{app}ANS-proteolipid = 2.3×10^{-6} *M*, K_{app}TNS-proteolipid = 1.1×10^{-6} *M*, K_{app}TNS-basic protein = 1.1×10^{-5} *M*. The affinity of ANS or TNS for the proteolipid was the highest for any protein yet studied. Only apomyoglobin ($K = 3.4 \times 10^{-6}$ *M*, Stryer, 1965) approaches the proteolipid in its affinity for ANS. All other proteins studied have apparent ANS dissociation constants in the range of 3×10^{-5} to 2×10^{-4} *M*.

The basic protein–ANS titration curves indicated the presence of one dye binding site per molecule of protein (Figure 1A). At low concentrations of TNS a similar result was calculated, but at higher TNS concentrations (>85 μM) a sudden large increase in fluorescence occurred accompanied by an increase in light scattering and optical density and the subsequent precipitation of the protein from the solution. Under these conditions it was not possible to determine the total number of TNS binding sites from the fluorescence data.

A Scatchard plot of the data for dye binding to the proteolipid showed that one class of binding sites was present, over the concentration range of the dye employed, with 43 nmol of ANS and 63 nmol of TNS bound per mg of protein (Figure 1B). Because of the uncertainty as to the molecular weight of the proteolipid, one cannot state the number of binding sites per molecule, but the data indicate that 1.2 and 1.8 mol of ANS and TNS, respectively, would bind to a polypeptide of 28,000.

The quantum yield (ϕ) of ANS bound to proteolipid (ϕ = 0.67) exceeded that for the dye bound to the basic protein (ϕ = 0.40), and it was comparable to that for ANS bound to serum albumin (ϕ = 0.68). The excitation spectra of ANS bound to each protein showed enhancement of the low-wavelength peak especially evident in the case of the proteolipid (Figure 2A), indicating that energy transfer from protein tryptophan to bound dye was occurring. The efficiency of quenching of the protein tryptophan fluorescence by bound dye was also much stronger in the case of the proteolipid than the basic protein (Figure 2B). From the efficiency of energy transfer (T) we calculated (Feinstein and Felsenfeld, 1975) the dye–tryptophan intramolecular distances to be 27 Å for the basic protein, and 17 Å in the proteolipid. In the case of myelin the dye binding site(s) were calculated to be an average of about 32 Å from the membrane tryptophan. These values are, of course, subject to several uncertainties imposed by the lack of exact knowledge about the mutual orientation of the absorption and emission vectors involved, the relative quantum yields of donor residues, the ratio of donor–acceptor sites, and energy transfer among donor molecules. In the basic protein there is one ANS binding site and one tryptophan residue per molecule; however, distance calculations according to the Förster equation are also somewhat uncertain because the protein is a prolate ellipsoid of about 15 × 150 Å (Epand et al., 1974). Because of the uncertainty about the molecular weight of the proteolipid we do not know the exact number of donor and acceptor molecules per mole-

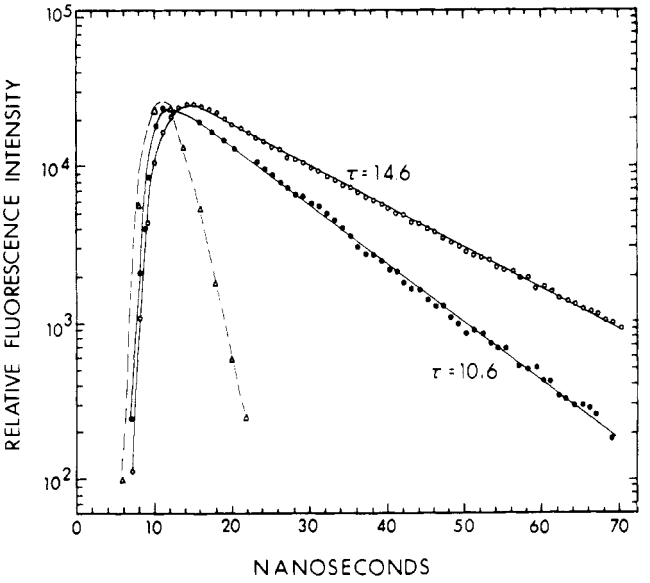

FIGURE 3: Fluorescence decay of ANS in myelin membrane (●) and bound to proteolipid apoprotein (○). Membranes (50 μg of protein/ml) were incubated with 80 μ*M* ANS in 0.01 *M* Tris-HCl buffer (pH 7.4). Proteolipid (30 μg of protein/ml) was incubated with 10 μ*M* ANS in the same buffer. Lamp flash decay (--△--) is shown. Symbols are actual experimental points. Lines with computed decay times (τ) were calculated from the method of moments computer program. The data were normalized and corrected for light scattering. Temperature, 25°.

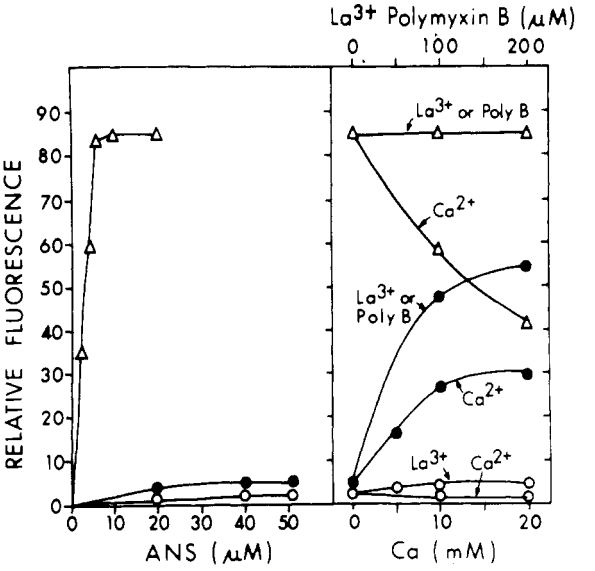

FIGURE 5: (A) Comparisons of the fluorescence of ANS bound to myelin, basic protein, or proteolipid. Myelin (●) was present at a concentration of 47 μg of protein/ml. Proteolipid (△) and basic protein (○) were present at concentrations equivalent to that present in the sample of myelin used, i.e., 28 and 12.5 μg/ml, respectively. All titrations were carried out in 0.01 *M* Tris-HCl buffer (pH 7.4). Fluorescence excitation was at 280 nm, with slits at 8 nm bandwidth. (B) Effects of Ca^{2+}, La^{3+}, and polymyxin B on ANS fluorescence with isolated myelin proteins as compared to myelin membranes.

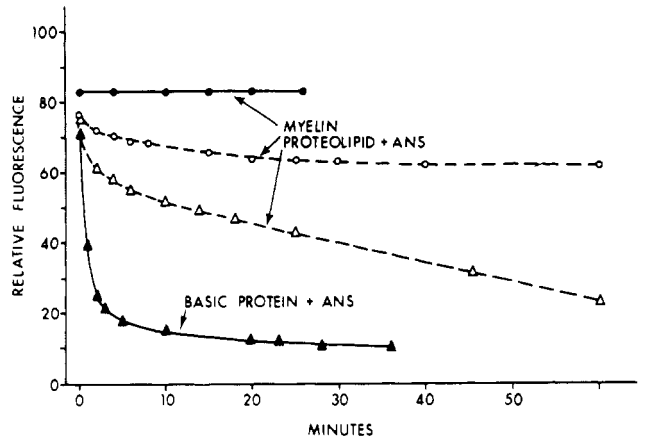

FIGURE 4: Effect of proteolytic enzymes on the binding of ANS to myelin basic protein and proteolipid. Proteolipid (180 μg/ml) in 0.01 *M* Tris-HCl buffer (pH 7.4) was incubated at 25° with trypsin (●), 125 μg/ml, chymotrypsin (○), 50 μg/ml, and Pronase (△), 50 μg/ml. Basic protein (0.5 mg/ml) was incubated with trypsin (▲). ANS concentration was 80 μ*M*. Fluorescence intensity (excitation at 380 nm) was measured at 470 nm.

cule. Calculating from a reported tryptophan content of 1.3 mol/100 mol of amino acids (Gagnon et al., 1971) we estimate that there are 2.7 tryptophan residues per 28,000 molecular weight. Thus, the Trp/dye ratio is about 3:1 for ANS and 3:2 for TNS. The high rate of energy transfer demonstrated by sensitized fluorescence emission and quenching of Trp fluorescence strongly indicates that fluorescent probe molecules bind very close to the proteolipid's tryptophan residues. Calculations of average Trp–dye distances in myelin are the least certain because of the factors mentioned above as well as a further complication, the intermolecular distances between proteins within the membrane. However, the rate of energy transfer is significantly less than in red cell membranes suggesting less direct binding to membrane proteins. Furthermore, if dye molecules did bind to the proteolipid in intact myelin we would expect a more highly efficient quenching of myelin intrinsic fluorescence since most of the membrane's tryptophan content is contributed by this protein.

Fluorescence Lifetime of ANS Bound to Proteolipid and Myelin. The decay of ANS fluorescence with time was measured with an Ortec nanosecond fluorometer. The decay curves (Figure 3) were corrected for the lamp emission and light scattering, and the fluorescence lifetime was calculated utilizing a computer program based on the method of moments analysis developed by Dyson and Isenberg (1969, 1971). The fluorescence lifetime of ANS bound to myelin proteolipid was 14.5 nsec (±0.05 SE). The fluorescence of ANS bound to myelin was fitted by a single decay curve significantly different from that of the proteolipid with lifetimes ranging between 10.4 and 12.9 nsec. La^{3+} addition did not significantly alter the decay time. It is not possible to conclude whether or not dye molecules are bound to protein in the membrane solely on the basis of these lifetime measurements since certain lipids (e.g., sphingomyelin) also bind ANS with lifetimes in the range of 12–13 nsec (Loeser et al., 1972).

Effects of Proteolytic Enzymes on Fluorescent Probe Binding to Myelin Proteins. The basic protein was rapidly hydrolyzed by trypsin, pepsin, Pronase, and α-chymotrypsin. The fluorescence of bound probe molecules was rapidly lost as the proteolysis proceeded (Figure 4). Myelin proteolipid was not attacked by trypsin as previously reported by Folch-Pi and Stoffyn (1972), and this enzyme had no effect on fluorescent dye binding. Pronase did attack the protein and it completely abolished the probe fluorescence as the digestion proceeded, but much more slowly than in the case of the basic protein. α-Chymotrypsin also produced a slow but only partial loss of probe fluorescence. In the case of myelin neither trypsin nor Pronase digestion affected the

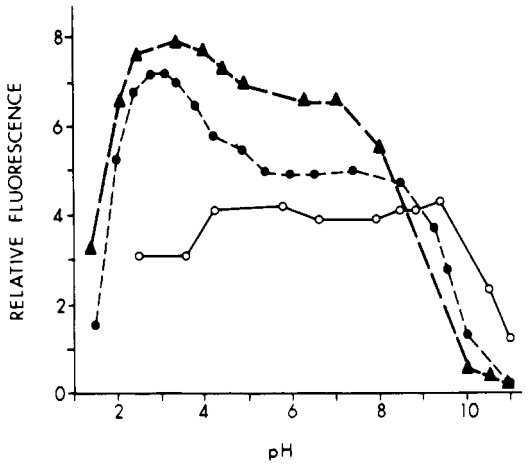

FIGURE 6: Effect of pH on the enhancement of fluorescent probe emission by basic protein and proteolipid. Protein concentrations were 20–50 μg/ml, dye concentrations were 10–20 μM. Fluorescence was excited at 366 or 380 nm and the spectra were recorded for peak emission. Basic protein + TNS (●) or ANS (○), proteolipid + TNS (▲).

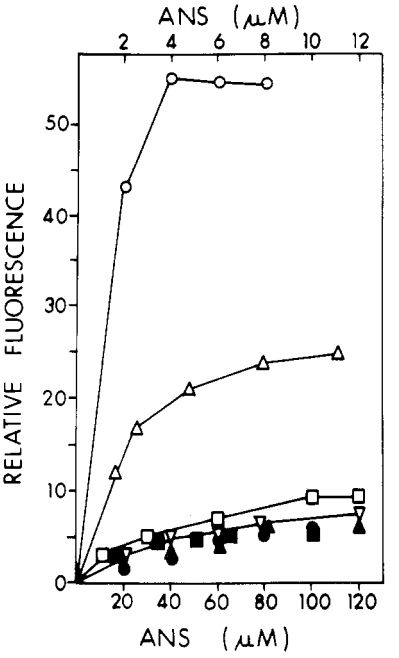

FIGURE 7: Effect of blockade of carboxyl groups on interactions with ANS. Normal myelin (▲), basic protein (■), and proteolipid (▲) data normalized. ANS titrations of myelin-arginine methyl ester (○), myelin-glycine methyl ester (△), basic protein-glycine methyl ester (□), and proteolipid-arginine methyl ester (▽).

fluorescence enhancement of ANS or TNS, even in the presence of La^{3+} which allowed maximal binding of the dyes to the membrane (Feinstein and Felsenfeld, 1975).

Effects of Cations, H^+, Local Anesthetics and Polymyxin B on Fluorescent Probe Interactions with Myelin Proteins. In Figure 5 are shown ANS titrations of the basic protein and proteolipid as compared to myelin. The soluble proteins were present at concentrations equivalent to their content in the myelin sample. Note especially the much greater dye fluorescence in the presence of the soluble proteolipid as compared to intact myelin or basic protein. Addition of Ca^{2+} reduced the ANS fluorescence in proteolipid and basic protein solutions. The reactions of fluorescent probes with proteolipid were not affected by La^{3+}, but the fluorescence of ANS–basic protein solutions was increased. Local anesthetics (butacaine, procaine, and tetracaine) and polymyxin B, all of which increase ANS binding to myelin (Feinstein and Felsenfeld, 1975), had no effect on probe reactions with either protein.

The effects of the pH on the dye–protein interactions were rather small in the pH range 7–3 in sharp contrast quantitatively to those on dye–myelin fluorescence. The greatest effect of H^+ on dye fluorescence in the presence of protein was an increase of about 40% in the case of TNS plus basic protein (Figure 6). The enhancement of dye fluorescence by H^+ in the presence of myelin (Feinstein and Felsenfeld, 1975) was about 30 times greater than the effect upon either protein.

Effect of Modification of Protein Carboxyl or Amino Groups on Fluorescent Probe Interactions with Myelin Proteins. Blocking carboxyl groups of the basic protein by reaction with amino acid ester after carbodiimide activation increased the binding of ANS to 2 mol/mol of protein. The effect on the proteolipid was to increase fluorescence probe quantum yield but not binding, by about 20–25%. The effect was quantitatively the same whether carboxyl groups were blocked with arginine methyl ester or glycine methyl ester (Figure 7) in contrast to the results with myelin (Feinstein and Felsenfeld, 1975). About half of the total of 10.2 mol % protein dicarboxylic acids in the proteolipid are believed to be amidinated (Reynolds and Green, 1971). The degree to which the remaining free proteolipid carboxyl groups were blocked by arginine methyl ester was assessed by amino acid analysis of the incorporation of extra arginine groups into the protein. This was found to be 4.2 mol/100 mol of amino acids.

Succinylation of the amino groups of the proteolipid resulted in a fall in maximum fluorescence of bound ANS due to a reduction in quantum yield and a sixfold decrease in affinity. A decrease in the efficiency of energy transfer (about 50%) from tryptophan to ANS was also observed as a result of succinylation. All of these effects may occur as a result of expansion of the protein molecules or dissociation into subunits (Hass, 1964).

Reaction of Protein Tryptophan Groups of Basic Protein and Proteolipid with Koshland Reagents. Treatment of the myelin proteins with the Koshland reagents resulted in a nearly complete loss of the native tryptophan fluorescence and the appearance of new yellow chromophore groups (Figure 8) which in the case of KR-I were sensitive to pH. The absorbance spectrum of the 2-hydroxy-5-nitrobenzyl bromide treated basic protein closely resembles that of BNPS-skatole oxidized basic protein (Burnett and Eylar, 1971) and the small residual fluorescence emission had a peak at 303 nm, characteristic of tyrosine. Both of the modified proteins when titrated with ANS or TNS produced very weak enhancement of dye fluorescence (Figure 9). This may have been due to the alteration of the dye binding site region, or a change in the protein conformation, by introduction of the alkylating agent onto the indole side chains. It was also possible that the fluorescent probes were bound, but their fluorescence emission was weak due to the absorbance of exciting light by the new chromophore. The absorbance spectra of the KR-modified proteins (especially KR-I treated proteins at pH 7.4) suggested this as a distinct possibility. However, at the concentrations of protein usually employed in the fluorescent dye titrations the absorbance at 380 nm was less than 0.1. Furthermore the presence of KR-modified basic protein in the front compartment of a

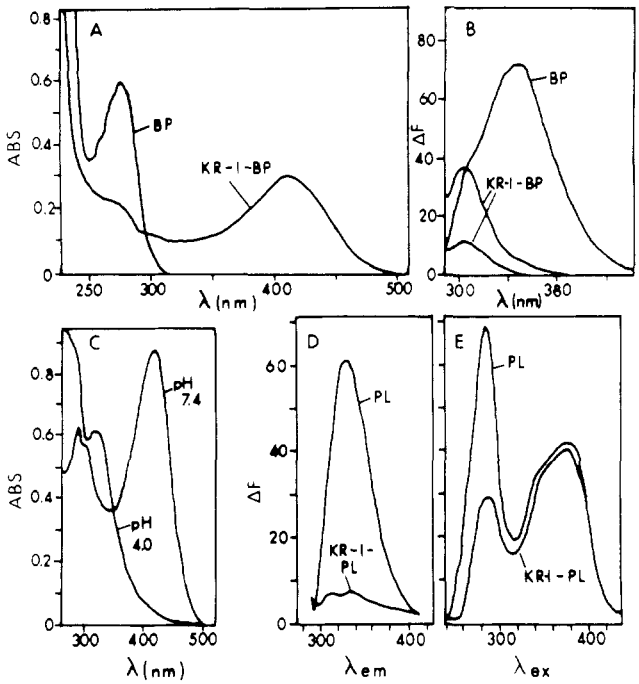

FIGURE 8: (A) Absorbance spectra of normal (1.0 mg/ml) and KR-I-modified basic protein (BP) (0.5 mg/ml) in Tris-HCl (pH 7.4). (B) Fluorescence emission of normal BP and KR-I-BP (2.4 mg/ml). Two curves are shown for the latter. The lower one is recorded at the same sensitivity as normal BP. (C) The effect of pH on the absorbance spectra of 200 μg/ml of normal and KR-I modified proteolipid (PL). (D) Fluorescence emission of normal PL and KR-I-modified PL. (E) Excitation spectra of normal and KR-I-modified PL. The latter was recorded at greater amplifier sensitivity because of the lower quantum yield, but the excitation peaks at 380 nm are normalized for comparison.

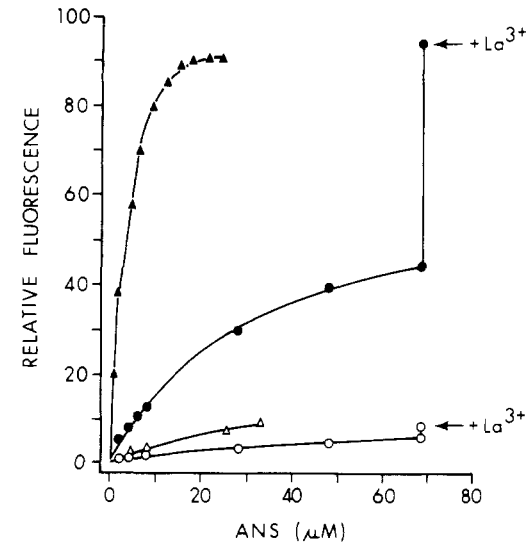

FIGURE 9: Fluorescence of ANS in presence of normal proteolipid (▲), KR-I-treated proteolipid (△), normal basic protein (●), and Kr-I-treated basic protein (○). Excitation at 380 nm.

split fluorometer cell did not significantly affect the fluorescence emission of the normal basic protein–dye complexes in the rear compartment of the cell. The optical arrangement was such that all exciting light had to pass through the front compartment to reach the rear, and only fluorescence emission from the rear compartment was measured. Also, titration of unmodified protein into a solution of KR-treated protein plus ANS resulted in a normal enhancement of dye fluorescence. By carrying out ANS titrations of KR-I modified proteins at pH 4.0 the absorbance of the proteins at 380 nm was much reduced. Even under these conditions fluorescence of bound ANS (excitation at 380 nm) was very weak in the presence of the modified proteins. These results all indicate that absorbance of exciting photons by the new chromophore cannot account for the results.

The effect of the Koshland reagents on dye interactions with myelin proteins was duplicated by similar treatment of human serum albumin. Human serum albumin was reacted with 2-hydroxy-5-nitrobenzyl bromide and the modified protein product isolated by gel filtration. The alteration of the single tryptophan residue of this protein essentially abolished its native fluorescence emission, and that of added ANS. Titration of a constant amount of ANS with increasing amounts of normal human serum albumin or KR-HSA yielded linear plots of $1/F$ vs. $1/[\text{protein}]$. The intercepts on the ordinate measured the relative quantum yields of the bound dye in each case. The relative quantum yield of KR–human serum albumin was only about 0.15 of that of normal human serum albumin. This indicates that the small residual fluorescence of KR–human serum albumin–ANS is not due to a fraction of unmodified protein molecules. The same result was obtained with KR-I modified proteolipid and basic protein. KR-modified human serum albumin and basic protein migrated in 5.6% polyacrylamide–1% dodecyl sulfate gels exactly the same as the native proteins. Myelin treated with Koshland reagents exhibited very much reduced native fluorescence, but except for the loss of energy transfer reacted essentially normally with ANS (Feinstein and Felsenfeld, 1974).

Reactions of Proteolipid–Phospholipid Complexes with Fluorescent Probes. The interaction of the proteolipid with sonicated dispersions of lipids in aqueous media at pH 7.4 resulted in certain cases in a very significant alteration of the reactivity toward fluorescent probes. When incorporated into phosphatidylserine vesicles, the proteolipid exhibited little or no interaction with ANS (Figure 10) as was evident from the lack of ANS fluorescence emission, and the weak quenching of proteolipid fluorescence by the added probe (see Figure 2B). When Ca^{2+} or polymyxin B was added to reduce the negative surface potential of the lipid micelles, a marked increase in ANS fluorescence and an increase in quenching of the protein fluorescence occurred. The fluorescence enhancing effect of Ca^{2+} is apparently not due to a dissociation of the lipid–protein complexes, which could thereby lead to a binding of the dye to free protein molecules. Braun and Radin (1969) found that Ca^{2+} actually prevented dispersion or disaggregation of the protein–anionic lipid complexes by detergent-like lipids. The increased fluorescence intensity after addition of Ca^{2+} or polymyxin B was in part due to ANS binding to proteolipid, and not solely to increased ANS binding to the phospholipid, since it was reduced about 50–60% by digestion with Pronase. When proteolipid was incorporated into lecithin micelles, the effect was qualitatively the same, but the extent to which the dye was accessible to the protein was somewhat greater than with phosphatidylserine. Cerebroside dispersions on the other hand had no effect and the proteolipid was fully accessible to ANS. Bovine serum albumin, unlike the proteolipid, does not form complexes with acidic lipids separable by density gradient centrifugation (Braun and Radin, 1969) and remained fully accessible to ANS when added to phosphatidylserine vesicles.

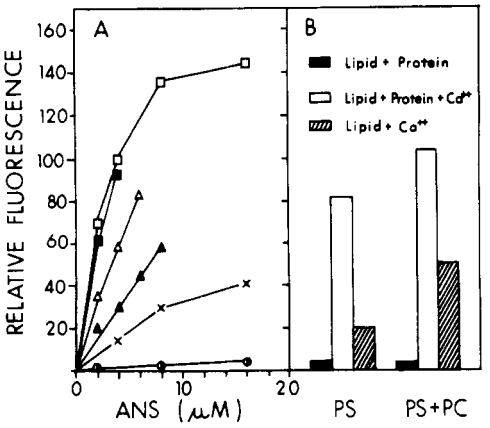

FIGURE 10: Proteolipid incorporation into lipid membranes. Effect on interaction with ANS. (A) Proteolipid (43 μg/ml) alone in 0.01 *M* Tris-HCl. (pH 7.4) (□); + brain cerebrosides (■); + cerebrosides + lecithin 1:1 (△); + lecithin (▲); + artificial myelin lipids (x); + phosphatidylserine (○) or phosphatidylserine + lecithin, 1:1 (●). (B) Effect of Ca^{2+} (10 m*M*) on ANS fluorescence of: PL in phosphatidylserine (PS) or PS + lecithin (PC) vesicles (□); and lipid vesicles alone (▨).

Mixed lipid dispersions (equal amounts of phosphatidylserine and lecithin) displayed properties typical of phosphatidylserine alone when the proteolipid was incorporated and then titrated with ANS, i.e., little or no fluorescence enhancement. The addition of Ca^{2+} or polymyxin B greatly increased dye binding. In this case, independent titrations of lipid vesicles showed that a significant proportion of the binding of ANS was probably directly due to the lipid (Figure 10B). In purely lipid membranes the inclusion of phosphatidylserine into a lecithin micelle imparted a net negativity to the micelle surfaces and nearly abolished the binding of ANS to lecithin. The subsequent addition of cations, local anesthetics, or polymyxin B, all of which can reduce the surface negativity, markedly increased dye binding. A sonicated lipid dispersion prepared from purified lipids at concentrations about equivalent to those in the intact membrane,[2] along with incorporated proteolipid (at a concentration equivalent to that in myelin) enhanced the fluorescence of added ANS to about 30% that produced by soluble proteolipid. Subsequent addition of Ca^{2+} increased the fluorescence very weakly. These effects suggest that the composition of membrane lipid strongly influences interactions with the proteolipid as well as with the fluorescent probes.

Discussion

Both of the major structural proteins of myelin bind the fluorescent probes ANS and TNS with a resultant enhancement of their fluorescence emission intensity. The proteolipid was particularly noteworthy in this respect, both because of the large quantum yield of the bound dyes and their high affinity for the protein. Since the proteolipid is the major protein constituent of myelin (50–60%) it is an important potential site for dye binding in the intact membrane. However, from the data presented it is clear that the effects which H^+, cations, local anesthetics, and polymyxin B exert on fluorescent probe binding to native myelin (Feinstein and Felsenfeld, 1975) cannot be due to their direct effects on myelin basic protein or proteolipid. In contrast to their effects on the major myelin proteins, those agents which so substantially increase dye binding to myelin also markedly increase ANS and TNS binding to lipids (Vanderkooi and Martonosi, 1969; Feinstein et al., 1970). Similarly, the modification of the carboxyl groups of isolated myelin proteins had a negligible effect upon dye binding compared to that observed (Feinstein and Felsenfeld, 1975) in the intact myelin membrane. Presumably the contribution of exposed protein carboxyl groups to the overall negative surface charge density of the membrane strongly affects binding of ANS and TNS to membrane lipids. The role of the other minor protein constituents of the membrane, particularly the acidic Wolfgram fraction, is not known, but may be of significance because about 50% of the carboxyl groups of the basic protein and the proteolipid are naturally in the amide form.

We can now take into account the measured stoichiometry of dye–protein and dye–myelin interactions in an attempt to assign a possible distribution of sites for fluorescent probe binding in myelin. Based on our calculations that myelin can bind 251 nmol of TNS/mg of myelin protein, the proteolipid 63 nmol/mg, and the basic protein about 50–150 nmol/mg,[3] it is possible to estimate the total amount of dye which could actually be bound to membrane protein binding sites based upon the actual amounts of each protein species in myelin. In this way about 52–75 nmol of TNS could be assigned per mg of myelin protein. Thus, about 175–200 nmol of TNS is probably bound to the lipids, assuming that the other membrane proteins (10–20% of the total protein) do not bind large amounts of dye per mole. There are about 470 nmol of phospholipid/mg of myelin dry weight (>1800 nmol/mg of myelin protein). Therefore 10 mol of lipid is present for every mol of TNS bound (the cerebroside and cholesterol content is not included in this calculation).

In the case of ANS a similar calculation indicates as much as 42 nmol out of a total of 72 nmol bound per mg of myelin protein (or per 4 mg of myelin dry weight) might be accounted for by protein binding sites. These calculations indicate the potential distribution of dye molecule binding, but they do not answer the critical question of what amounts of the dyes are *actually* bound to protein as opposed to lipid sites in the intact myelin membrane. It is not obvious why the maximum amount of TNS that can be bound to myelin is several times that for ANS. However, molecular models of the two dyes with their polar sulfonate groups oriented toward the polar head group region of the phospholipids indicate that ANS must produce more lateral expansion of the membrane than TNS in order to penetrate into the polar–hydrocarbon interface.

H^+, cations, local anesthetics, and polymyxin B directly increase fluorescent probe binding to lipids, but not to myelin proteins. The effects on lipids may be the basis for their ability to increase binding of ANS and TNS to intact myelin. On the other hand, a reduction in membrane surface potential, as a result of direct interaction with lipid charged groups, might allow access of dye molecules to the proteolipid binding sites. The latter mechanism could be deduced from the behavior of the artificial lipid–proteolipid apoprotein complexes. However, if the proteolipid in myelin was predominantly placed outside the lipid bilayer and arranged tangentially to the polar head group region of the lipid bilayer surface we would *not* expect: (1) interactions with the lipid fixed negative charges to strongly influence dye bind-

[2] Concentrations in mg/ml: cholesterol 1.4, cerebrosides 1.4, sphingomyelin 0.35, lecithin 0.66, phosphatidylserine 0.25, phosphatidylethanolamine 0.8, phosphatidylinositide 0.5.

[3] The higher value may be attained in the presence of La^{3+} but not with polymyxin B which has no effect on this protein, yet increases dye binding to myelin to as great an extent as La^{3+}.

ing to protein sites located outside the lipid domain, and (2) immunity of the probe binding sites to proteolytic attack. The data taken all together argue rather for an *integral* location of the proteolipid deep within the lipid bilayer ordinarly inaccessible to ANS and TNS. Consistent with this view is the fact that the proteolipid can be extracted from the membrane into aqueous media only after exhaustive removal of the acidic lipids to which it binds avidly (Stoffyn and Folch-Pi, 1972). By contrast the basic protein is easily extracted by acidic solutions, Triton X-100 or 0.5 *M* ammonium acetate.

The best evidence that ANS and TNS binding sites on the proteolipid (even in the presence of La^{3+} or polymyxin B) are inaccessible in the native state of the membrane, as compared to reconstituted membranes, is: (1) the efficiency of energy transfer from tryptophan to ANS is much lower in myelin than in the solubilized proteolipid, and (2) treatment with 2-hydroxy-5-nitrobenzyl bromide or 2-methoxy-5-nitrobenzyl bromide very substantially reduced the quantum yield of proteolipid bound dye but had little effect on ANS–myelin fluorescence, except for the marked reduction of intrinsic membrane fluorescence and energy transfer from membrane tryptophan groups to bound dye.

Though some recent X-ray diffraction work supports the concept that proteins in myelin are located exclusively external to the lipid bilayer (Kirschner and Caspar, 1972), other interpretations of the data hold that globular proteins are partly submerged in a lipid bilayer (Blaurock, 1972). To account for the fact that freeze-etching experiments do not show the particulate structure of a typical membrane, Singer (1971) has proposed that myelin protein molecules might be predominantly globular and embedded in the lipid–protein mosaic, but unable to span the thickness of a single myelin layer. Our fluorescence data are consistent with this interpretation for the structure of myelin, insofar as the location of the proteolipid is concerned. These fluorescent probe studies have also revealed subtle differences which exist in the organization of natural as opposed to artifically reconstituted membranes.

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