The Intrinsic Fluorescence of Isolated Central-Nervous-System Myelin-Sheath Preparations

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The intrinsic fluorescence characteristics of tyrosine and tryptophan residues in the proteins of isolated central-nervous-system myelin were investigated to gain information concerning the location of these residues within the intact membrane system. Tryptophan fluorescence from isolated myelin has an emission maximum at 325 nm that appears to arise from at least two different populations of tryptophan residues. Further evidence for heterogeneity of tryptophan location in the membrane is obtained from quenching studies with chloroform and acrylamide. It is speculated that one tryptophan population is hydrophobically situated and may be derived from the proteolipid protein of myelin, whereas the other tryptophan population is located at the membrane surface and may arise from the extrinsic basic protein. A significant tyrosine fluorescence is detected from isolated myelin, indicating that some of these residues are not quenched by structural interactions within the lipid-protein membrane system. Studies with freeze-dried resuspended myelin suggest that the structural arrangement of protein components in the dried rehydrated membrane system differs significantly from that of the freshly isolated myelin membrane.

The u.v. fluorescence of proteins arises from the aromatic amino acid residues tyrosine and tryptophan (Teale, 1960). For the majority of proteins containing both aromatic residues fluorescence from tyrosine is of a low quantum yield and tryptophan fluorescence dominates the emission spectra (Teale, 1960). The detection of a significant tyrosine component in the emission spectra of native proteins can be taken as evidence of atypically situated tyrosine residues (Gerard *et al.*, 1975). The wavelength for maximum fluorescence, the bandwidth and the quantum yield for tryptophan fluorescence are each sensitive to the environment of the aromatic residue in a protein (Burststein *et al.*, 1973). The response of tyrosine and tryptophan fluorescence to their environment provides a sensitive tool that has found wide application for studying the conformation of proteins in solution (Hoggett, 1978).

The use of intrinsic fluorescence to study the environment of proteins in natural membrane systems has been limited (Sonenberg, 1971; Avruch *et al.*, 1972; Shinitzky & Rivnay, 1977). A major reason for this is that most biological membranes contain a complex variety of different protein species. The myelin sheath in central-nerve tissue, however, has a very low protein/lipid composition ratio, whereas some 80% of the total protein in myelin is accounted for by two protein species, the basic protein and the proteolipid protein (for review, see Rumsby & Crang, 1977; Rumsby, 1978). The

intrinsic-fluorescence properties of the myelin basic protein have already been studied in some depth (Jones & Rumsby, 1975) and give useful information about the conformation of the isolated protein and on its interaction with the anionic detergent sodium dodecyl sulphate (Jones & Rumsby, 1978). Cockle *et al.* (1978) have examined the intrinsic fluorescence of lipophilin, a proteolipid apoprotein from human myelin, in isolated forms and after interaction with zwitterionic complex lipid.

The present paper describes the intrinsic-fluorescence features of isolated central-nervous-system myelin-sheath preparations isolated from bovine brain tissue and how these characteristics respond to perturbation of the membrane system. Some preliminary data on this topic have been published previously (Crang *et al.*, 1974).

Materials and Methods

Central-nervous-system myelin was purified from bovine brain white matter by a routine method (Rumsby *et al.*, 1970). After washing several times with water to remove sucrose, final myelin preparations were resuspended in glass-distilled water, pH 7.2, for use. Some samples of myelin were freeze-dried and then resuspended to the original concentration in water. All reagents were of AnalaR standard unless specified. BaSO₄ (precipitated; Fisons, Loughborough, Leics., U.K.) was washed with dilute H₂SO₄ and with glass-distilled water and dried in an oven before use.

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Fluorescence spectra were recorded by using a Perkin-Elmer MPF3 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.), the ratio mode being employed to compensate for any variations in total lamp intensity during observations. Excitation and emission monochromator slit widths of 3 nm were used. The amplified signal from the emission photomultiplier was connected to a digital voltmeter (EEP, Los Angeles, CA, U.S.A.). Emission intensities were estimated at convenient wavelength intervals from the mean for ten observations. Optically clear samples (<0.05 *A* unit at excitation wavelength) were observed by using the standard cuvette arrangement. Isolated myelin in glass-distilled water, pH 7.2, and samples of freeze-dried and resuspended freeze-dried myelin were observed by front-surface illumination by using the solid sample attachment (Perkin-Elmer; cat. no. 018-0051). Spectra recorded by front-surface observation require correction for stray light-scattering due to monochromator inefficiency. This correction may be calculated from the following relationship: (Stray excitation scattering by sample)_λ =

$$\left(\frac{\text{Sample scatter}}{\text{BaSO}_4 \text{ scatter}} \right)_\lambda \times \left(\frac{\text{BaSO}_4 \text{ stray excitation}}{\text{scatter}} \right)_\lambda$$

where (stray excitation scatter by sample)_λ is the stray excitation light-scatter by the sample at wavelength λ, (sample scatter/BaSO₄ scatter)_λ is the ratio of photomultiplier signals recorded from the sample and the BaSO₄ reference standard with excitation and emission monochromator set at wavelength λ and with monochromator slits of 1 nm, and (BaSO₄ stray excitation scatter)_λ is the photomultiplier signal recorded from the BaSO₄ standard at wavelength λ with monochromator slits of 3 nm. This correction is derived for stray light-scattering from excitation at both 280 nm and 295 nm (at 5 nm intervals from 285 nm to 440 nm). The scatter spectra are normalized to the recorded fluorescence spectra from the sample at 440 nm, where fluorescence emission from tryptophan is essentially zero and the emission signal recorded is due to stray excitation light-scattering.

Samples were maintained at room temperature (22°C) and were stirred during observation. Emission spectra are corrected for the wavelength dependence of the quantum efficiency of the photomultiplier (Hamamatsu R106 photomultiplier tube; Hamamatsu TV Company, Hamamatsu, Japan). Excitation spectra are corrected for the xenon arc spectrum. This correction is derived by using a Rhodamine B quantum counter (Fisons SLR; 3 mg/ml of ethylene glycol) as described by Parker & Rees (1960). Quantum yields for tryptophan fluorescence were calculated by the method of Parker & Rees (1960) by assuming a quantum yield for free tryptophan (chromatographically pure; BDH, Poole, Dorset,

U.K.) of 0.14 (Gerard *et al.*, 1975) essentially as described by Jones & Rumsby (1975).

The molar ratio of tyrosine to tryptophan in isolated myelin was determined with myelin dispersed in 100 mM-NaOH by the method of Beaven & Holiday (1952) by using the equation:

$$\frac{M_{\text{Tyr}}}{M_{\text{Trp}}} = \frac{0.592A_{294} - 0.263A_{280}}{0.263A_{280} - 0.170A_{294}}$$

where $M_{\text{Tyr}}/M_{\text{Trp}}$ is the molar ratio of tyrosine to tryptophan. Apparent absorbancies were corrected for turbidity from plots of log *A* against log λ as described by Winder & Gent (1971). The contribution of tyrosine fluorescence to the total protein fluorescence was determined by normalizing spectra excited at 280 nm and 295 nm to give equal emission at 370 nm. Tyrosine fluorescence is obtained from the 280 nm–295 nm difference spectra (Longworth, 1971).

For quenching experiments a stock solution of 8 M-acrylamide (especially purified for electrophoresis; BDH) was prepared. Chloroform was redistilled in a glass apparatus. Quenching results are plotted by using the Stern-Volmer relationship (Stern & Volmer, 1919):

$$\frac{F_0}{F} = 1 + K[Q]$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of the quencher, Q , and K is the overall quenching constant.

Results

The fluorescence emission spectrum for isolated myelin excited at 280 and 295 nm is shown in Fig. 1. Excitation at 295 nm reveals tryptophan emission with a λ_{max} at 328 nm and a bandwidth of 58 nm (spectrum A). With excitation at 280 nm a tyrosine fluorescence component is apparent (spectrum B). A difference spectrum (280–295 nm), (C), reveals the tyrosine component with a λ_{max} at 305 nm and an emission intensity of 24% of that of tryptophan at 328 nm. The fluorescence excitation spectrum for emission at 325 nm confirms the composite nature of the intrinsic fluorescence from myelin (Fig. 2, spectrum A). A peak at 295 nm that corresponds to the long-wavelength absorption maximum for tryptophan, where absorption due to tyrosine is less than 10% (Teale, 1960), is observed. A shoulder in the region 270–280 nm corresponds to absorption by both tyrosine and tryptophan. The fluorescence excitation spectrum for emission at 360 nm demonstrates the excitation spectrum for the tryptophan component alone (Fig. 2, spectrum B). The prominence of the 295 nm component in both excitation spectra in Fig. 2 indicates that for isolated myelin,

Fig. 1. Intrinsic-fluorescence emission spectra for isolated central-nervous-system myelin

Purified myelin was suspended in double-distilled water, pH 7.2, at a concentration of 41 mg/ml. Fluorescence emission was measured as described in the Materials and Methods section. Spectrum A, excitation at 295 nm; spectrum B, excitation at 280 nm, normalized to spectrum A at 370 nm; spectrum C, difference spectrum (B-A).

Fig. 2. Intrinsic-fluorescence excitation spectra from isolated central-nervous-system myelin

Myelin was suspended in double-distilled water, pH 7.2, at a concentration of 41 mg/ml. Fluorescence excitation spectra were measured for emission at 325 nm (spectrum A) and for emission at 360 nm (spectrum B). Other details are as described in the Materials and Methods section.

resonance-energy transfer from tyrosine to tryptophan in the proteins of the membrane is insignificant.

Emission spectra for freeze-dried myelin and for resuspended freeze-dried myelin are essentially identical with that for isolated myelin in terms of λ_{max} and bandwidth for tryptophan emission and of the contribution that tyrosine fluorescence makes to the overall fluorescence observed for excitation at 280 nm.

The effect of sodium dodecyl sulphate and urea, both membrane and protein denaturants, on the intrinsic fluorescence of myelin proteins was investigated to gain more information on the nature of the tryptophan environment in the isolated membrane preparation. Solubilization of myelin by a 7-fold weight excess of sodium dodecyl sulphate (Crang & Rumsby, 1978) gives a fluorescence emission from tryptophan with λ_{max} 335 nm and a bandwidth of 68 nm (Fig. 3, spectrum A). The 280–295 nm difference spectrum reveals a tyrosine component with an emission maximum at 305 nm and a maximum fluorescence intensity of 40% of that of tryptophan at 335 nm. The action of 8 M-urea

on the fluorescence features of isolated myelin is shown in Fig. 4. A tryptophan fluorescence with an emission maximum at 340 nm and no significant tyrosine component is observed with excitation at 280 nm. The quantum yields for tryptophan fluorescence from myelin in the two denaturant systems were estimated to be 0.14 for sodium dodecyl sulphate and 0.03 for 8 M-urea.

The quenching of the intrinsic myelin fluorescence by two uncharged molecules, acrylamide and chloroform, was studied in an attempt to resolve any heterogeneity in the tryptophan fluorescence. Ionic quenchers such as caesium and bromate cause precipitation of isolated-myelin preparations (Crang, 1976). Data on the quenching of tryptophan fluorescence in myelin by chloroform and acrylamide were

Fig. 3. Intrinsic-fluorescence emission spectra from isolated myelin solubilized in sodium dodecyl sulphate. Samples were prepared as described in the Materials and Methods section. Spectrum A, fluorescence emission for excitation at 295 nm; spectrum B, fluorescence emission for excitation at 280 nm; spectrum C, difference spectrum (B-A).

analysed by Stern-Volmer plots as shown in Fig. 5 for acrylamide and as difference spectra in Fig. 6. For chloroform a quench constant of 6.10 mol^{-1} is estimated, whereas the value for acrylamide is 0.37 mol^{-1} (Fig. 5). Difference spectra calculated for tryptophan fluorescence in the absence of quencher minus tryptophan fluorescence in the presence of quencher resolve a quenched tryptophan fluorescence by chloroform (Fig. 6a) with λ_{max} 320 nm and bandwidth 51 nm in the presence of both 35 and 60 mM-chloroform. Similar difference spectra for quenching by acrylamide (Fig. 6b) resolve a quenched tryptophan fluorescence with λ_{max} 335 nm and bandwidth 64 nm in the presence of 718 mM-acrylamide. At a lower acrylamide concentration (304 mM) the λ_{max} for quenched tryptophan fluorescence is at 340 nm with a shoulder at 320 nm (Fig. 6b).

For freeze-dried resuspended myelin Stern-Volmer plots give values for the tryptophan quench constant of 0.26 mol^{-1} for acrylamide. Quenched tryptophan difference spectra reveal a tryptophan emission with a maximum at 328 nm and a bandwidth of 58 nm (Fig. 7a) for quenching by chloroform at a concen-

Fig. 4. Intrinsic-fluorescence emission spectra from isolated myelin in 8M-urea

Samples were prepared as described in the Materials and Methods section. The fluorescence emission spectrum is for excitation at 280 nm.

Fig. 5. Stern-Volmer plot for the quenching of myelin tryptophan fluorescence by acrylamide

Tryptophan fluorescence emission was monitored at 325 nm. Other details are as described in the Materials and Methods section. Error bars indicate $\pm 1 \text{ s.d.}$

Fig. 6. Quenching of tryptophan fluorescence in isolated myelin by chloroform (a) and by acrylamide (b)

These difference spectra are derived from fluorescence emission spectra in the absence of quencher minus the fluorescence emission spectra in the presence of quencher. Chloroform quenching (a) was at a concentration of 60 mM (spectrum A) and 35 mM (spectrum B). Acrylamide quenching (b) was at a concentration of 718 mM (spectrum A) and 304 mM (spectrum B).

tration of 31 mM. For quenching by acrylamide at a concentration of 674 mM a tryptophan emission with a maximum at 340 nm and a bandwidth of 61 nm is recorded (Fig. 7b).

The tyrosine/tryptophan ratio for isolated bovine central-nervous-system myelin is calculated to be 2.2:1, by using the procedure described in the Materials and Methods section.

Discussion

The intrinsic-fluorescence features of isolated-myelin preparations can be considered to derive largely, if not exclusively, from aromatic residues in the proteolipid protein and basic protein, which account for some 80% of the total protein of central-nervous-system myelin (for review see Rumsby & Crang, 1977; Rumsby, 1978). Taking data from Jones & Rumsby (1975) for the myelin basic protein and from Cockle *et al.* (1978) for a proteolipid apoprotein, lipophilin, an overall tyrosine/tryptophan ratio for these two major proteins in myelin is 3.2:1, in contrast with the 2.2:1 ratio calculated for the total proteins of isolated bovine central-nervous-system myelin.

The tryptophan emission maximum at 328 nm for isolated myelin in water is considerably 'blue-shifted' from the emission maximum of free tryptophan in aqueous solution (λ_{max} , 352 nm). It is also significantly more blue-shifted than the maximum blue

shift to 330–332 nm proposed for tryptophan fluorescence in a model for the intrinsic fluorescence of isolated water-soluble proteins (Burstein *et al.*, 1973). The maximum blue shift so far reported for tryptophan fluorescence in proteins is for azurin, where the λ_{max} is 308 nm (Burstein *et al.*, 1977). This atypical tryptophan fluorescence is considered to arise from an aromatic residue situated in a very rigid environment in the protein. From Burstein *et al.* (1973) a single tryptophan residue with a λ_{max} of 328 nm should be expected to have a bandwidth of about 45 nm. The 58 nm bandwidth for tryptophan fluorescence in isolated myelin (Fig. 1) suggests that the observed tryptophan fluorescence arises as a composite peak from at least two different populations of tryptophan residues, one with λ_{max} < 328 nm, whereas the other has λ_{max} > 328 nm.

Two uncharged quenching molecules, chloroform and acrylamide, give information about the differential situation of tryptophan residues within isolated myelin. Acrylamide will preferentially quench tryptophan residues at the surface of the myelin lamellae in contact with the aqueous phase (Eftink & Ghiron, 1976), whereas chloroform with its lipophilic nature will partition into the lipid phase of myelin to quench preferentially hydrophobically situated tryptophan residues (Hoss & Abood, 1974). For isolated myelin quenched by chloroform the difference spectra resolve a quenched tryptophan component with an emission maximum at 320 nm and a

Fig. 7. Quenching of tryptophan fluorescence in freeze-dried resuspended myelin by chloroform and acrylamide. Myelin samples were freeze-dried and resuspended in double-distilled water, pH 7.2, as described in the Materials and Methods section. Difference spectra were recorded as described in the legend to Fig. 6. Quenching by chloroform (a) was at a concentration of 31 mM and by acrylamide (b) was at 674 mM. In (a) spectrum A is the normal intrinsic-fluorescence emission of freeze-dried resuspended myelin, and spectrum B is the quenched difference spectrum.

bandwidth of 51 nm. This blue-shifted tryptophan fluorescence and decreased bandwidth indicate the preferential quenching of a tryptophan population with a λ_{max} of about 320 nm. Quenching by acrylamide at 304 nm resolves quenched tryptophan with an emission maximum of 340 nm and a shoulder at 320 nm. At higher acrylamide concentrations the proportion of this 320 nm component increases, shifting the λ_{max} for overall quenched tryptophan to 335 nm. This observation is interpreted as revealing that acrylamide at low concentrations preferentially quenches surface tryptophan residues, whereas at the higher concentration the proportion of buried tryptophan residues in the quenched spectrum is increased. These quenching data support the view that the observed fluorescence from isolated myelin arises from at least two different populations of tryptophan residues, one located primarily in the hydrophobic phase and the other at the hydrophilic surface. It is tempting to speculate that these arise respectively from the proteolipid protein, an intrinsic protein of known lipophilic nature and the myelin basic protein, which is an extrinsic membrane protein (for review see Rumsby & Crang, 1977; Rumsby, 1978).

A proteolipid apoprotein, lipophilin, in aqueous solution shows tryptophan fluorescence with a λ_{max} of 331–333 nm (Cockle *et al.*, 1978), which is considered to arise from the residues in a hydrophobic environment within the protein. Interaction of this

apoprotein with zwitterionic phospholipid does not result in a significant blue shift of the tryptophan emission (Cockle *et al.*, 1978). The tryptophan fluorescence component resolved in isolated myelin by quenching with chloroform (λ_{max} 320 nm), which might be expected to arise from the proteolipid protein, is significantly blue shifted from the tryptophan λ_{max} reported for the isolated proteolipid apoprotein (Cockle *et al.*, 1978). This suggests that this hydrophobic protein from myelin has a significantly different overall conformation in its isolated form as monitored by tryptophan fluorescence. The isolated myelin basic protein shows a tryptophan emission maximum at 348 nm (Jones & Rumsby, 1975) that is very close to the emission maximum of free tryptophan in aqueous solution. In isolated myelin the tryptophan population with a λ_{max} at 340 nm resolved by quenching with low concentrations of acrylamide could arise from the myelin basic protein that *in situ* in the membrane system has tryptophan residues in a surface location and accessible to the quencher.

Detection of significant tyrosine emission from isolated myelin suggests that those structural features that are responsible for quenching tyrosine in the majority of proteins are absent around a significant population of tyrosine residues in the membrane. Tyrosine fluorescence can only be considered in terms of emission intensity relative to that of trypto-

phan, and for isolated myelin tyrosine emission at 305 nm is 25% of that of the tryptophan emission at 328 nm. Solubilization of myelin in sodium dodecyl sulphate increases the tyrosine contribution to 40%, indicating that disruption of the membrane releases tyrosine residues from structural quenching effects. For myelin in urea significant tyrosine fluorescence is not detected. As urea does not quench tyrosine fluorescence (Teale, 1960), this finding suggests that denaturation of myelin proteins in urea places tyrosine residues in situations where their fluorescence is entirely quenched. The observed tyrosine emission from isolated myelin is consistent with the findings of significant tyrosine contributions in the intrinsic-fluorescence spectra of the isolated myelin basic protein (Jones & Rumsby, 1975) and lipophilin (Cockle *et al.*, 1978).

The effect of sodium dodecyl sulphate and 8 M-urea on tryptophan fluorescence from isolated myelin gives λ_{max} values of 335 and 340 nm with bandwidths of 68 and 72 nm respectively. Whereas this tryptophan emission for myelin in sodium dodecyl sulphate is in keeping with the effect of this detergent on protein tryptophan fluorescence (Burstein *et al.*, 1973), the bandwidth of 68 nm suggests that a significant population of tryptophan residues that do not respond normally to sodium dodecyl sulphate still exists in the solubilized membrane. Tryptophan emission from proteins in 8 M-urea usually has a λ_{max} of 350 nm (Burstein *et al.*, 1973). Our findings for myelin in urea, where the tryptophan emission maximum of the normal membrane at 328 nm alters to 340 nm, suggest that although a population of tryptophan residues in the denatured membrane may respond normally to urea there is a significant population of residues that do not. It may thus be that in both these denaturants some lipid-protein interactions still exist.

Although the tryptophan and tyrosine emission features of freeze-dried resuspended myelin are similar to those in the freshly isolated membrane, quenching by chloroform and by acrylamide reveal quenched components that have significantly different characteristics from those detected with the fresh system. Quenching by chloroform resolves a component in freeze-dried resuspended myelin that has an emission maximum at 328 nm, compared with 320 nm for chloroform-quenched fresh myelin. Acrylamide resolves a quenched component in freeze-dried resuspended myelin (tryptophan emission maximum at 340 nm) that is altered from the value of 335 nm for quenched tryptophan emission from fresh myelin. These findings suggest that in freeze-dried resuspended myelin the majority of tryptophan residues are in a significantly different structural situation in the lamellae compared with the freshly isolated system. When myelin is dehydrated a free lipid and a residual lipoprotein phase separate out (Finean, 1961). The

differences detected in the quenched fluorescence features of freshly isolated and freeze-dried resuspended myelin may be due to the fact that the dried membrane system does not rehydrate with its major proteins in their original structural environments. This observation must be taken into account in the use of freeze-dried resuspended myelin for work relating to the structural organization of lipids and proteins in the natural myelin membrane (Rumsby & Crang, 1977).

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