Cyst(e)ine residues of bovine white-matter proteolipid proteins

Role of disulphides in proteolipid conformation

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Cyst(e)ine residues of bovine white-matter proteolipid proteins were characterized in a highly purified preparation. From a total of 10.6 cyst(e)ine residues/molecule of protein, as determined by performic acid oxidation, 2.5–3 thiol groups were freely accessible to iodoacetamide, iodoacetic acid and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), when the proteins were solubilized in chloroform/methanol (C/M) (2:1, v/v). The presence of lipids had no effect on thiol-group exposure. One thiol group available to DTNB in C/M could not be detected when proteolipids were solubilized in the more polar solvent n-butanol. In a C/M solution of purified proteolipid proteins, SDS did not increase the number of reactive thiol groups, but the cleavage of one disulphide bridge made it possible to alkylate six more groups. C.d. and fluorescence studies showed that rupture of this disulphide bond changed the protein conformation, which was reflected in partial loss of helical structure and in a greater exposure to the solvent of at least one tryptophan residue. Cyst(e)ine residues were also characterized in the different components [PLP (principal proteolipid protein), DM₂₀ and LMW (low-*M_r* proteins)] of the proteolipid preparation. Although the numbers of cyst(e)ine residues in PLP and DM₂₀ were similar, in LMW fewer residues were alkylated under four different experimental conditions. The differences, however, are not simply related to differences in *M_r*.

INTRODUCTION

Proteolipid proteins are the principal protein components of myelin in the central nervous system. They are highly hydrophobic and can be extracted from bovine white matter with chloroform/methanol (C/M). They are also soluble in aqueous solutions containing SDS. Electrophoresis of proteolipids by SDS/PAGE shows two main bands, PLP and DM₂₀, and secondary bands composed of high-*M_r* proteins (HMW) and low-*M_r* proteins (LMW). The amino acid sequence of the principal proteolipid protein (PLP) has been described by Lees *et al.* (1983), Jolles *et al.* (1983) and Stoffel *et al.* (1983). Two models have been proposed for its organization in myelin (Laursen *et al.*, 1984; Stoffel *et al.*, 1984). The functional role of proteolipids in myelin is not known, although some evidence supports their ionophoric function (Ting Beall *et al.*, 1979; Lin & Lees, 1982; Helynek *et al.*, 1983). A characteristic feature of myelin proteolipids is their high content of cyst(e)ine (Folch-Pi & Stoffyn, 1972). These groups have been partially characterized in bovine white-matter proteolipid proteins (Lees *et al.*, 1969; Nicot *et al.*, 1973; De Foresta *et al.*, 1979; Vacher *et al.*, 1984) and in lipophilin, a homogeneous proteolipid protein isolated from human central nervous system (Cockle *et al.*, 1980). There are some discrepancies among investigators about the number, exposure, and reactivity of the thiol (SH) and disulphide (S–S) groups in proteolipids. Such disagree-

ments may be ascribed in part to the different methodologies employed to purify the proteins. A better characterization of the number and reactivity of SH and S–S groups is important in order to develop a possible model of the arrangements of the proteolipids in the myelin membrane. Characterization of SH and S–S groups would also advance understanding of their biological roles, either in their interaction with other myelin components or in their participation in yet unknown functions.

The development in our laboratories of a rapid technique to delipidate proteolipids (Bizzozero *et al.*, 1982) made it possible to obtain a highly purified proteolipid preparation. With this preparation a study of SH and S–S groups was carried out to compare SH reactivity towards different thiol reagents and their exposure in different solvents in the presence and absence of lipids. C.d. and fluorescence studies revealed that at least one S–S bridge appears to be fundamental for maintenance of the conformation of proteolipids.

The cysteine residues in the different bands (PLP, DM₂₀ and LMW) were characterized in order to elucidate their nature. Cyst(e)ine residues are highly conserved during evolution (Thornton, 1981), and they are therefore a characteristic of each protein. In PLP and DM₂₀, the number of carboxymethylated groups was similar. In LMW the number of residues was significantly lower; however, the results indicated a close structural relationship among the three fractions.

Abbreviations used: APLs, total purified proteolipid apoproteins; C/M, chloroform/methanol; CBB, Coomassie Brilliant Blue R-250; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; IAm, iodoacetamide; IAA, iodoacetic acid; PAGE, polyacrylamide-gel electrophoresis; PLP, principal proteolipid protein; LMW, low-*M_r* proteins; HMW, high-*M_r* proteins.

EXPERIMENTAL

Chemicals

DTT, SDS, CBB and IAM were purchased from Sigma, St. Louis, MO, U.S.A.; IAA from Fluka, Buchs, Switzerland; Sephadex LH-60 from Pharmacia Fine Chemicals, Uppsala, Sweden; iodo[^{14}C]acetic acid (11.1 mCi/mmol) and iodo[^{14}C]acetamide (53 mCi/mmol) from New England Nuclear, Boston, MA, U.S.A.

Extraction of proteolipids

Lipids and proteolipids were extracted from bovine white matter as described by Folch-Pi *et al.* (1957). The washed total lipid extract was used as such or delipidated by column chromatography on Sephadex LH-60 (Bizzozero *et al.*, 1982). After delipidation, the peak containing the total purified proteolipid apoproteins (APLs) [10 mg of APLs in 10 ml of C/M (1:1, v/v)/5 mM-HCl] was dialysed against 1 litre of C/M (2:1, v/v) for 24 h with two changes of the external medium.

Extraction of proteolipid proteins with n-butanol/water (1:1, v/v) was performed as described by Pasquini & Soto (1972); 8.3 mg of protein/g of fresh tissue was recovered.

Performic acid oxidation

Performic acid oxidation of APLs and bovine growth hormone (used as standard) was carried out as described by Hirs (1956). To prepare the performic acid reagent, 5 vol. of 30% (v/v) H_2O_2 and 95 vol. of 99% formic acid were left in a closed container at 25 °C for 2 h. Samples of the different solutions of proteins, containing 50 μg of protein, were dried under vacuum and then dissolved in 50 μl of 99% formic acid and 10 μl of methanol. The performic acid reagent and the various protein solutions were cooled separately at -5 °C for 30 min. Aliquots (100 μl) of the performic acid reagent were added to each protein preparation, and the reaction was allowed to proceed at -5 °C for 150 min. The mixture was then dried under vacuum, and the residue was hydrolysed by adding 100 μl of 6 M-HCl/0.01 M-phenol and incubating for 24 h at 106 °C. The cysteine content of the hydrolysate was determined with a Beckman 119L automatic amino acid analyser.

Measurement of cysteine and disulphide bridges with DTNB

The content of reduced and oxidized cysteine was determined spectrophotometrically by using DTNB as described by Cockle *et al.* (1980) for lipophilin, but adapting the technique to use it with C/M (2:1, v/v) or n-butanol solutions. A 0.9 ml standard solution of dithiothreitol or protein (0.4 mg/ml), with or without SDS, was incubated at room temperature with 0.1 ml of DTNB (40 mg/ml) and 5 μl of triethylamine. The maximum absorbance at 412 nm was reached after 10 min of incubation and was stable for 50 min, both in C/M (2:1, v/v) and in n-butanol. The molar absorption coefficient at 412 nm was 16900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in n-butanol and 17900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in C/M (2:1, v/v).

In the experiments in which disulphide bridges were cleaved, proteolipid proteins were reduced as described in the following subsection. The excess DTT was removed by gel filtration through Sephadex LH-60, C/M (2:1, v/v) being used as elution solvent.

Alkylation of reduced proteolipid proteins with iodoacetic acid and iodoacetamide

Reduction and alkylation of the preparation in C/M (2:1, v/v) were carried out basically as described by Lees *et al.* (1969), except that DTT was used as reducing agent. A 10 ml portion of protein solution (1 mg/ml), 10 mg of EDTA and, in some experiments, 100 mg of SDS, were placed in screw-capped vials and sonicated in a Bransonic 220 instrument at 60 Hz for 10 min, followed by the addition of 100 μl of triethylamine. In those experiments where proteolipid proteins were reduced, 1.54 mg of DTT was added under N_2 . After 18 h at room temperature in the dark, 10 mg of iodo[^{14}C]acetic acid or iodo[^{14}C]acetamide (0.2–0.4 mCi/mmol) and 0.4 ml of triethylamine were added with continuous bubbling of N_2 . Alkylation was allowed to proceed for 6 h at room temperature, in the dark, with periodic shaking. The reaction was halted by acidification of the mixture, and the excess reagents were removed by gel filtration through Sephadex LH-60, C/M (1:1, v/v)/5 mM-HCl being used as eluent. The amounts of DTT and IAA used were adjusted to give the maximum yield of carboxymethylcysteine in the presence of SDS.

The preparations used in c.d. and fluorescence experiments were dialysed against 1 litre of C/M (2:1, v/v) for 24 h and then evaporated to dryness under N_2 . Proteins were solubilized in 2-chloroethanol.

Carboxymethylation of proteolipid proteins in SDS/buffer was carried out by incubation of the proteins (1 mg/ml) dissolved in 20 mM-Tris/HCl buffer, pH 7.0, containing 1% SDS and 154 μg of DTT/mg of protein. After 24 h, 1 mg of [^{14}C]IAA/mg of protein was added, and the mixture was incubated at room temperature, in the dark, with periodic shaking for 6 h. Finally, the reagents were removed by dialysis against the Tris/HCl buffer.

The amount of radioactivity incorporated was measured in a Beckman LS 7000 liquid-scintillation spectrometer. Samples in C/M (2:1, v/v) were first evaporated to dryness and dissolved in 10 ml of a scintillation solution containing 5.5 g of PPO (2,5-diphenyloxazole) and 0.1 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] per litre of Triton X-100/toluene (1:4, v/v). Vials were counted for radioactivity until the measurements had a maximum error of 2% or less. The efficiency was greater than 75%.

C.d.

C.d. spectra were obtained in a Jasco J-20 spectropolarimeter. Cells of 0.1 cm path length were used, and spectra were recorded at 25 °C over the wavelength range 250–190 nm. APLs in C/M (2:1, v/v), alkylated under different conditions with IAM, were evaporated to dryness under a stream of N_2 and then solubilized in 2-chloroethanol at a final protein concentration of 0.5–0.6 mg/ml. The spectrum was determined twice for each experimental condition on three different APL preparations, and data were averaged and expressed as the mean residue ellipticity ($[\theta]_{\text{m.r.w.}}$, expressed in degrees $\cdot\text{cm}^2\cdot\text{dmol}^{-1}$), 108 being taken as the mean residue weight.

Fluorescence

Fluorescence experiments were performed in an SLM spectrofluorimeter interfaced with a Hewlett-Packard computer, which allowed the storage of spectral data

digitally on a magnetic tape. This characteristic allowed accumulation of successive spectra to decrease noise and to facilitate data processing. Fluorescence spectra were recorded at 25 °C using 4 nm slit widths. APL preparations were the same as used in the c.d. experiments, but were diluted with 2-chloroethanol to a protein concentration that gave an A_{280} of 0.1. The solvent spectrum was subtracted from the emission spectrum after each experiment. Spectra were also corrected for the instrument response by using a series of correction factors provided by the manufacturer. The instrument was operated in the ratio mode, using rhodamine 6G as a quantum counter to monitor the lamp excitation intensity. A Corning 4-74 filter was placed in the excitation-beam path to decrease the monochromator second-order contribution.

Isolation of PLP, DM₂₀ and LMW components

After delipidation of the crude preparation by column chromatography on Sephadex LH-60, the lipid-free protein peak, in C/M (1:1, v/v)/5 mM-HCl, was dialysed against 5 mM-sodium phosphate buffer, pH 7.2, containing 0.1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol, with two daily changes until the proteins were completely dissolved in the aqueous SDS medium. The APLs were separated by preparative SDS/PAGE (Agrawal *et al.*, 1977). To localize the different bands, a slice was cut along the gel and stained with a 0.25% (w/v) solution of CBB in methanol/acetic acid/water (45:7:48, by vol.). After destaining, the slice was placed on top of the unstained gel and bands were cut out according to the pattern appearing in the stained gel. During the staining procedure, the preparative gel was kept refrigerated at 4 °C. The unstained gel slices containing PLP, DM₂₀ or LMW proteins were placed in individual tubes, crushed, and eluted by incubation for 24 h at 37 °C in the same phosphate buffer mentioned above. The samples were filtered and concentrated by dialysis against a concentrated solution of polyvinylpyrrolidone.

Quantification of proteolipids was determined by amino acid analysis after hydrolysis of the proteins with 6 M-HCl/10 μ M-phenol for 24 h at 106 °C. Protein concentration was also determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. As the results obtained with both methods agreed, the Lowry method was used to standardize the amounts of protein in the quantitative study of dye-binding capacity.

Determination of CBB-binding capacity of PLP, DM₂₀, and LMW

Since the CBB-binding capacity varies among the different proteins (Fishbein, 1972), the dye-binding capacity of PLP, DM₂₀ and LMW were estimated quantitatively. Portions of the different eluted bands containing 1–50 μ g of protein were subjected to analytical SDS/PAGE {T = 12.5%, C = 4% [12.5% (w/v) acrylamide; 4% bisacrylamide]; Neville 1971}. After electrophoresis, gels were removed from the tubes, fixed with 10% (w/v) trichloroacetic acid for 24 h, and individually stained for 12 h with CBB as described above. After destaining by diffusion, the stained bands were cut out and the dye eluted with aq. 25% (v/v) pyridine for 12 h with continuous shaking at room temperature. Absorbance was measured at 605 nm, and

total absorbance was calculated by multiplying the absorbance of a 1 ml sample by the total sample volume. Blanks were obtained from unstained areas of the gels treated in a similar way. The absorbance of CBB was linear between 0.05 and 1.5 absorbance units (Fenner *et al.*, 1975).

Measurement of the CBB-binding capacity/ μ g of protein for PLP, DM₂₀ and LMW gave similar results. The data fit a straight line within a range of 1–40 μ g of protein, with a 95% confidence limit (statistical analysis was by the method of Ostle, 1974).

Determination of cyst(e)ine residues in PLP, DM₂₀ and LMW

Reduction and carboxymethylation of the APLs in C/M (2:1, v/v) were carried out as described above. After separation of excess reagents, the protein peak was dialysed against 40 mM-Tris/borate buffer, pH 8.2, containing 0.1% SDS, with two daily changes until the proteins were in aqueous SDS solution. PLP, DM₂₀ and LMW were separated by analytical SDS/PAGE; electrophoresis, staining and destaining of the gels, and elution of the dye were carried out as described above. After measuring the A_{605} of the eluate, it was discarded, since it contained negligible amounts of radioactivity. Gel slices were dried at 60 °C in separate vials and dissolved in 0.4 ml of 88% formic acid, the preparations being heated at 60 °C for 12 h. Control experiments were carried out to determine whether [¹⁴C]IAA, either free or non-specifically adsorbed, was present in the bands. Results indicated that, after alkylation of the APLs with [¹⁴C]IAA and electrophoretic separation, all the radioactivity was covalently associated with the proteins.

Performic acid oxidation of the isolated PLP, DM₂₀ and LMW was carried out as described above.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) as modified for proteolipids by Lees & Paxman (1972).

RESULTS

The total proteolipid preparation contains three protein components: PLP, DM₂₀, and LMW, with M_r values of 29 500, 24 600 and 20 000 respectively (Chan & Lees, 1974). The present study determined that HMW components disappeared when the preparation was completely reduced, indicating that they are oxidation products of the proteins of lower M_r , at least in freshly prepared samples. For calculation of the number of cyst(e)ine residues per molecule of protein in the total proteolipid protein preparation, it was necessary to determine an average M_r , and for that purpose the contribution of each component (PLP, 51%, DM₂₀, 24%, LMW, 25%, as determined by SDS/PAGE) was taken into consideration. From these experiments the calculated average M_r was 26 000.

Determination of the total cyst(e)ine content of APLs by performic acid oxidation

The efficiency of the performic acid oxidation reaction was calculated by oxidizing simultaneously with APLs two proteins with a known content of cyst(e)ine: bovine growth hormone and bovine white-matter PLP (M_r 29 500). The efficiency of the oxidation was 88% and 92%

respectively. The total cyst(e)ine content determined in the APLs was 9.5 mol/mol of protein, a value that when corrected for the calculated efficiency (90%) increases to 10.6 mol/mol of protein.

Quantification of SH and S-S groups with DTNB

The number of free SH groups detected in the proteolipid preparation before delipidation (Table 1) was higher in the C/M extract than in the butanol extract. In n-butanol one SH group was not accessible to the DTNB reagent. After delipidation of the C/M extract (APLs), the same number of thiol groups (3 mol of SH/mol of protein) was found. The presence of 1% SDS did not increase the number of thiol groups, using either DTNB or carboxymethylation (Table 2). On the other hand, after addition of a reducing agent, an additional cyst(e)ine residue was found (Table 1). However, the

presence of 1% SDS did not expose additional cyst(e)ine residues.

Alkylation of the proteolipid proteins with IAA and IAM

Under certain conditions, histidine, lysine and methionine can react with IAA or IAM (Gurd, 1967). A control experiment, carried out to study the amino acid composition ($\mu\text{mol}/100 \mu\text{mol}$) of APLs and alkylated APLs, showed similar values for histidine, lysine and methionine in both preparations [histidine, 2.4 and 2.1; lysine, 5.1 and 4.6; methionine, 1.8 and 2.3 (respectively)]. Similar results were obtained previously by Lees *et al.* (1969). In view of these results, it is reasonable to equate the amount (mol) of ^{14}C -labelled reagent incorporated with the amount (mol) of alkylated cysteine formed.

Table 2 shows the number of thiol groups detected with $[^{14}\text{C}]\text{IAA}$ in the C/M extract and in the APLs in C/M under different experimental conditions. Lipids did not seem to interfere with the exposure of thiol groups, since the results obtained in these experiments showed again that the delipidation did not change the number of thiol groups detected. The possibility that the method used to delipidate proteolipid proteins could oxidize the thiol groups can also be ruled out.

The kinetic study of the carboxymethylation of SH groups in the APLs, using a 3 M excess of IAA over thiol groups, showed that data fit a pseudo-first-order rate plot, indicating that all the 2.5 SH groups detected are homogeneously reactive toward IAA.

The addition of 1% SDS to the crude or delipidized preparation did not change the number of reactive SH groups, whereas the reducing agent increased by two the number of groups detected, indicating the breakage of a S-S bridge. This cleavage appeared to be essential for the denaturing action of SDS, since another six cyst(e)ine residues were detected in the presence of DTT and SDS (Table 2).

Data obtained after alkylation of the APLs with IAM can be expressed as the $[^{14}\text{C}]\text{carboxamidomethylcysteine}/[^{14}\text{C}]\text{carboxymethylcysteine}$ ratio in order to compare the relative reactivities of SH groups towards IAM and IAA respectively. For the APLs in C/M (2:1, v/v) the ratio

Table 1. Measurement of SH and S-S groups in proteolipid proteins from bovine white matter using DTNB

The number of SH groups was determined as described in the Experimental section. The APLs dissolved in C/M (2:1, v/v) were treated with 1% SDS, with 154 μg of DTT/mg of protein or with both reagents. Results are means $\pm$ S.E.M. ^aSignificantly different from ^b ($P < 0.02$); differences ^{a-c} and ^{c-d} were not significant (Student's *t* test). The numbers of independent experiments are shown in parentheses.

Preparation	Thiol-group content (mol of SH/mol of protein)
Total lipid extract	
C/M (2:1, v/v)	3.29 ± 0.17 (3) ^a
n-Butanol	2.05 ± 0.02 (3) ^b
Delipidized proteo- lipid proteins	
C/M (2:1, v/v)	2.73 ± 0.26 (9) ^c
+ SDS	2.71 ± 0.06 (4) ^d
+ DTT	3.71 ± 0.52 (3)
+ SDS + DTT	3.21 ± 0.24 (7)

Table 2. Quantification of cyst(e)ine residues in proteolipid proteins from bovine white matter by carboxymethylation with iodo $[^{14}\text{C}]$ acetic acid

Carboxymethylation of proteolipid proteins was carried out in the presence of lipids (C/M extract) or after delipidation (APLs). Proteins in C/M (2:1, v/v) were treated with 1% SDS, with 154 μg of DTT/mg of protein or with both reagents. Separation of PLP, DM₂₀, and LMW was carried out as described in the Experimental section. The buffer was 20 mM-Tris/HCl, pH 7.9, containing 1% SDS and 154 μg of DTT/ml. Results are means $\pm$ S.E.M., and the numbers of independent experiments are shown in parentheses. The differences ^{a-b} and ^{a-c} were not significant; ^d was significantly different from ^e ($P < 0.02$), and ^f from ^g ($P < 0.0002$) (Student's *t* test). A-G are different proteolipid apoprotein preparations.

Treatment	Content of cyst(e)ine residues (mol of carboxymethylcysteine/mol of protein)					
	APLs	C/M extract	PLP	DM ₂₀	LMW	
C/M	2.5 ± 0.1 (6) ^a	2.3 ± 0.1 (4) ^c	A (8)	2.5 ± 0.2	2.5 ± 0.1	1.2 ± 0.1
			B (3)	2.7 ± 0.2	2.4 ± 0.4	0.9 ± 0.1
+ SDS	2.3 ± 0.2 (7) ^b	2.6 ± 0.2 (3)	C (3)	3.3 ± 0.3	3.2 ± 0.1	1.8 ± 0.2
			D (5)	2.9 ± 0.1	2.8 ± 0.1	1.0 ± 0.0
+ DTT	4.2 ± 0.1 (4)		E (11)	5.6 ± 0.1 ^d	5.2 ± 0.2 ^e	2.4 ± 0.2
			F (6)	5.8 ± 0.2 ^f	4.8 ± 0.2 ^g	2.1 ± 0.2
+ SDS + DTT	9.9 ± 0.5 (5)		G (8)	10.2 ± 0.4	9.9 ± 0.4	5.0 ± 0.3
SDS/buffer	0.4 ± 0.1 (5)					

was 1.1; in the presence of 1% SDS, 1.1; in the presence of DTT, 1.1; and after the addition of both reducing and denaturing agents, 1.0. These results indicate that the APL preparations, studied under four different experimental conditions, did not show significant differences in the number of cyst(e)ine residues detected with IAA or IAM. Carboxymethylation of the APLs solubilized in SDS/buffer showed that SH and S-S groups were practically unreactive to IAA.

Determination of SH and S-S groups of PLP, DM₂₀ and LMW components

Cysteine residues were determined only in PLP, DM₂₀ and LMW components, since, as explained above, after reduction and carboxymethylation the HMW bands disappeared.

In C/M (2:1, v/v), PLP showed 2.5 thiol groups accessible to IAA. The exposure of these groups improved slightly treating the preparation with SDS, whereas the presence of the reducing agent cleaved at least one extra disulphide bridge. After reduction in the presence of 1% SDS, however, only 71% of the cyst(e)ine residues were carboxymethylated. This percentage was calculated by considering 100% to be the number of total cyst(e)ine residues determined by performic acid oxidation, a result that agrees with the sequence data published by Lees *et al.* (1983) and Stoffel *et al.* (1983). No significant differences were found between the numbers of SH and S-S groups determined in PLP and DM₂₀, except that, in the presence of reducing agent, between 0.5 and 1 residue less was detected in DM₂₀ than in PLP. The number of groups alkylated in the LMW fraction was considerably lower than that determined in PLP or DM₂₀. However, changes in the number of residues detected after the four treatments were similar for the three proteins.

The total numbers of cyst(e)ine residues in PLP, DM₂₀, and LMW, as determined by performic acid oxidation, were 14.3 ± 0.3 , 10.1 ± 0.2 and 6.8 ± 0.2 respectively. Results are averages for three independent experiments and are corrected for the efficiency of the reaction.

C.d. and fluorescence measurements

C.d. and fluorescence studies were carried out to investigate the apparent susceptibility of APLs to the denaturing action of SDS only after the cleavage of one disulphide bridge. Fig. 1 shows the c.d. spectra in 2-chloroethanol of the unmodified APLs after their reduction and alkylation with IAM. The spectrum of the unmodified proteins is that characteristic of brain proteolipids in 2-chloroethanol (Cockle *et al.*, 1978a; Sherman & Folch-Pi, 1970), with double minima at 222 and 209 nm. The reduction of the S-S bridge produced a marked decrease in ellipticity, indicating a partial loss of α -helix structure. When APLs were alkylated with or without SDS in the absence of the reducing agent, the value of $[\theta]_{222}$ decreased only slightly (Table 3). These results indicate that changes measured after reduction were not artefacts attributable to the action of the reagents used in the alkylating procedure. The $[\theta]_{222}$ values (Table 3) show that, after the cleavage of a disulphide, ellipticity fell by about 37%. The alkylation of another six groups did not produce additional changes. Identical results were obtained with either IAA or IAM as alkylating agents.

Fig. 1. C.d. spectra of unmodified and reduced APLs

APLs in C/M (2:1, v/v) were reduced (in the absence of SDS) and alkylated with IAM as described in the Experimental section. The C/M solutions of unmodified and reduced APLs were evaporated under a stream of N₂ and dissolved in 2-chloroethanol to a final concentration of 0.6 mg/ml. —, Unmodified APLs; ----, reduced and alkylated APLs.

Table 3. Changes in $[\theta]_{222}$ associated with the cleavage of disulphide bridges

APLs in C/M (2:1, v/v) were alkylated with IAM, without additions, with 1% SDS, with 154 μ g of DTT/mg protein or with both reagents. APL solutions in C/M (2:1, v/v) were transferred to 2-chloroethanol (see Fig. 1). ^aSignificantly different from ^c ($P < 0.05$), but not from ^b (Student's *t* test).

Preparation	$10^{-3} \times [\theta]_{222}$ (degrees $\cdot$ cm ² $\cdot$ dmol ⁻¹)
Unmodified APLs	-30 ^a
+ IAM	-26 ^b
+ SDS + IAM	-27 ^b
+ DTT + IAM	-19 ^c
+ SDS + DTT + IAM	-22 ^c

The fluorescence spectra of APLs in 2-chloroethanol, excited at 275 nm (Fig. 2a), presented two maxima, corresponding to the emission of tyrosine and tryptophan at 303 and 340 nm respectively. In comparison, the spectra of the reduced and alkylated APLs showed an identical maximum at 303 nm, and a red shift of the maximum corresponding to tryptophan emission. When both APLs preparations, unmodified and reduced, were excited at 295 nm (Fig. 2b), where tryptophan fluor-

Fig. 2. Changes in the fluorescence emission spectra of APLs generated after their partial reduction

—, Unmodified APLs; ---, reduced and alkylated APLs. APL preparations were those used in the c.d. studies (see Fig. 1), but were diluted with 2-chloroethanol to a final absorbance of 0.1. Emission spectra were normalized to the maximum intensity of fluorescence. (a) $\lambda_{\text{excitation}}$ 275 nm; (b) $\lambda_{\text{excitation}}$ 295 nm.

escence can be selectively excited, the maximum of emission of the unmodified APLs appeared at 340 nm, whereas the reduction of one S–S bridge produced a shift in the maximum of 2–3 nm. Alkylation of the other six groups after reduction in the presence of 1% SDS did not produce any additional shift. The changes in quantum yields were within the error of the determinations, but in the three experiments, the quantum yield was 10% lower in the reduced APLs. These changes cannot be seen in Figs. 2(a) and 2(b) because both spectra were normalized to the maximum value of emission.

DISCUSSION

The conformation of myelin proteolipid proteins varies when they are solubilized in different solvents (Sherman & Folch-Pi, 1970). De Foresta *et al.* (1979) demonstrated by fluorescence studies that SH groups were freely accessible in C/M (2:1, v/v), but in aqueous solution they were located in constrained areas of the molecule. In accordance with these results, the present study showed that one SH group was available to DTNB in C/M (2:1, v/v), but that it could not be detected in n-butanol, a solvent of higher polarity. A change in the conformation of proteolipids with an increase in the

polarity of the solvent may explain the burying of one SH group. The polarity of n-butanol is similar to that of 2-chloroethanol, and it is interesting to point out that the number of thiols detected in n-butanol is similar to that detected with DTNB in lipophilin solubilized in 2-chloroethanol (Cockle *et al.*, 1980). In aqueous solution containing DTT and SDS, thiol groups were almost unavailable to IAA. The strong differences in thiol accessibility found by different investigators may depend on the method employed to dissolve proteolipids in aqueous media. In the C/M (2:1, v/v) solutions of APLs, there were no significant differences in the number of SH groups determined with IAA, IAm or DTNB, although these thiol reagents differ in charge and size. This fact, together with the homogeneous reactivity of these thiols towards IAA, indicate that these 2.5–3.0 thiol groups are highly exposed in C/M (2:1), and it suggests that they are probably located on hydrophobic areas of the molecule. Vacher *et al.* (1984) detected 3.5 mol of SH/mol of protein (recalculated for M_r 26000), using bis-(4-nitrophenyl) disulphide, whereas with the carboxylated derivative of the latter, DTNB, only 2.7 groups were detected in the present study. This difference may be due to the presence of negative charges in DTNB that could determine electrostatic repulsion with other negative charges sterically near to thiol-groups, but that this difference could be due to the different methods used to purify the proteins cannot be disregarded. Some disagreements with data reported by Lees *et al.* (1969) could also be attributed to the application of different methods for the preparation of proteolipid proteins.

The reduction of proteins with DTT cleaved one disulphide bridge, since two additional SH groups were alkylated. However, only one additional SH group was accessible to DTNB, even in the presence of 1% of SDS. It is possible that, after treatment of the proteins with DTT, the newly formed SH groups remain unavailable to DTNB, a reagent of higher charge and size than IAA or IAm.

The total number of cyst(e)ine residues that were detected by performic acid oxidation (10.6 mol/mol of protein) agrees with that found by Cockle *et al.* (1980). When APLs were reduced in the presence of 1% SDS, 9.9 cyst(e)ine residues per molecule of protein, 93% of the total oxidized groups, could be alkylated.

C.d. spectra showed that the cleavage of one disulphide produced a considerable decrease in $[\theta]_{222}$, indicating that this S–S bridge may be critical for the preservation of protein conformation, and that its breakage could determine the disorganization of a limited α -helix region. Cockle *et al.* (1980) also found an important loss of the helical character of lipophilin after the complete reduction of its disulphide bridges. The average reduction of one disulphide produced a red shift in the maximum of emission of tryptophan. Tryptophan is highly sensitive to solvent polarity, and this red shift may reflect a higher exposure of at least one tryptophan residue to the solvent. After the rupture of this critical disulphide bridge, the alkylation of another six cyst(e)ine groups did not produce further changes in c.d. or fluorescence spectra. These results suggest that this S–S bridge may be critical in preserving the conformation of proteolipids, and it is possible that, through a change in its tertiary structure, the protein would become more susceptible to denaturation by SDS. In connection with these results, it will be interesting to determine the

localization of this critical S-S bridge to define further its role in proteolipid proteins.

Characterization of the cyst(e)ine residues of PLP, DM₂₀ and LMW showed that the number of groups alkylated in LMW, in the different preparations, was a constant percentage of those determined in PLP and DM₂₀, suggesting that the three fractions are closely related. This would be in accordance with the hypothesis that the different bands may originate by internal sequence deletions from a common protein (Laursen *et al.*, 1983; Lin & Lees, 1985; Nussbaum *et al.*, 1985; Trifilieff *et al.*, 1985). The number of groups detected in LMW was not simply related to differences in M_r ; in LMW, the numbers of cysteine residues detected were 42% and 46% of that detected in PLP and DM₂₀ respectively, whereas the M_r value for LMW represents 68% and 80% of that of PLP and DM₂₀ respectively.

Since cyst(e)ine residues are highly conserved and characteristic for each protein, the reported characterization of SH and S-S groups in PLP, DM₂₀ and LMW proteolipid proteins could be a tool in the determination of their interrelationships.

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