Nature of the Cysteinyl Residues in Lipophilin from Human Myelin*

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Ellman's reagent was used to investigate the status and exposure of the cysteinyl residues in lipophilin, a proteolipid apoprotein from human myelin. The hydrolyzed protein contained 3.5 to 4.5 cysteines per molecule, which increased to 11 after complete reduction. The native protein was thought to contain three disulfide bonds and five free sulfhydryl groups, which undergo partial oxidation during purification. Exposure of —SH groups in the aqueous protein was minimal, even in the presence of 6 M guanidinium chloride, suggesting a location in hydrophobic domains not disrupted by this reagent. In the helicogenic solvent 2-chloroethanol, the full complement of —SH groups could not be revealed, even with the addition of sodium dodecyl sulfate; a difference of two sulfhydryls between intact and hydrolyzed protein was consistently observed. Similar sulfhydryl reactivity toward iodoacetamide was also established in this solvent. Sulfhydryl assays on whole myelin in 2-chloroethanol indicated that the occurrence of —SH groups in the proteolipid component was at least as high as in the purified apoprotein. Lipophilin was reduced and alkylated with 4-vinylpyridine at 10 of its cysteinyl residues. The modified protein adopted a β structure under conditions where lipophilin is normally highly α-helical, and was also less helical than usual in 2-chloroethanol; however, it was still abnormally resistant to denaturation by guanidinium chloride. Modified lipophilin contained as many ester groups as the intact protein; thus, it appeared unlikely that the long chain fatty acids associated with the protein were attached to cysteine residues.

The proteolipid protein fraction of white brain matter is comparatively rich in cysteine residues (1), of which a significant proportion is present in the reduced form (2, 3). However, no complete analysis has been carried out on a homogenous delipidated apoprotein. In previous publications (4–8) we have investigated the conformational properties of such a protein, termed lipophilin, isolated from human central nervous system myelin (9). This protein may be solubilized in aqueous and nonaqueous solvents and can exist in a variety of structural forms (4–6); in aqueous solution it appears to contain a richly hydrophobic core which cannot be disrupted by guanidinium salts (8). In view of the importance of both oxidized and reduced cysteine to the structure and biological activity of proteins, we have now determined the nature and exposure of the cysteinyl residues of lipophilin using the specific sulfhydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and both hydrolyzed and intact protein in aqueous media and in 2-chloroethanol. For comparative purposes we have also looked at reactivity toward iodoacetamide and at the availability of sulfhydryl groups in whole myelin dissolved in 2-chloroethanol. Finally, to assess the effects of disulfide bond cleavage, lipophilin was alkylated with 4-vinylpyridine at over 90% of its cysteiny1 residues, and the product was studied by circular dichroism. Our results are discussed in terms of the possible roles of disulfide and sulfhydryl groups in lipophilin, in relation to the function of the protein itself in the myelin membrane. The attachment of the 2% to 3% of long chain fatty acid always associated with proteolipid protein is also considered as a potential mode of involvement of cysteine residues.

MATERIALS AND METHODS

Myelin and Lipophilin—Myelin was prepared from the white matter of normal human brains and of brains from multiple sclerosis patients by the method of Lowén et al. (10). Lipophilin was isolated and purified from the chloroform/methanol-soluble proteolipid fraction as described by Gagnon et al. (9). This preparation has previously been shown to be homogeneous by polyacrylamide gel electrophoresis and ultracentrifugation (5, 6). Aqueous solutions of the protein were obtained by dialysis from 2-chloroethanol solution (7.5 mg/ml) into distilled water at 5°C (6).

Other Reagents—2-Chloroethanol (BDH) was purified by vacuum distillation over Tris, Girard's reagent 7 (Fisher), and Norit charcoal, and stored at −20°C. Iodoacetamide (Aldrich) was recrystallized twice from benzene, and a tracer amount of [1-14C]iodoacetamide was added to a final activity of about 60,000 cpm/μmol. 4-Vinylpyridine (Aldrich) was redistilled under vacuum immediately prior to use. Sodium dodecyl sulfate (Fisher sodium lauryl sulfate) was recrystallized from 95% ethanol and was then completely soluble in 2-chloroethanol.

Protein Determinations—Protein concentrations were estimated from the optical absorbance at 280 nm, using absorption coefficients 1.89 and 1.64 liters g−1 cm−1 for lipophilin in 2-chloroethanol and water respectively (7); the value in 2-chloroethanol containing 1% SDS was found to be 1.60 liters g−1 cm−1. All measurements were carried out on a Cary 118 spectrophotometer at 25°C; samples were centrifuged beforehand for 30 min at 30,000 × g, and corrections were applied for residual light scattering as previously (7). The estimation of protein concentrations in solutions of whole myelin in 2-chloroethanol is described under "Results.

Determination of Reduced and Oxidized Cysteine Content of Lipophilin—A solution of lipophilin in deoxygenated 2-chloroethanol,

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid; CD, circular dichroism; GdmCl, guanidinium chloride.
containing 2 to 3 mg of protein from the absorbance at 280 nm, was evaporated to dryness in a Pierce hydrolysis tube. The residue was dissolved in 2 ml of 6 M HCl, deoxygenated, heated under vacuum for 24 h at 110°C, and again evaporated to dryness. The amino acid mixture was taken up in 3 ml of deoxygenated 0.01 M HCl and immediately assayed for cysteine spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma). Aliquots (0.45 ml) were added to a mixture of 0.46 ml of 3 M Tris- HCl, pH 7.5, and 0.10 ml of 10 mM DTNB in 0.1 M phosphate, 10 mM EDTA, pH 7.0: the absorption coefficient of the released 2-nitro-5-thiobenzoate diion was taken as 14,350 M⁻¹ cm⁻¹ at 412 nm (11).

The total half-cystine content of lipophilin was determined in a second assay after reduction of cystine to cysteine. A portion of the hydrolysate was diluted with an equal volume of 0.01 M HCl, and the whole treated with an equal volume of 3 M Tris- HCl, pH 7.5, then reduced with nascent hydrogen generated by continuous dropwise addition of a 0.5% sodium-mercury amalgam to maintain vigorous effervescence. After 15 min, 0.9-mi aliquots were removed and added to 0.1 ml of 10 mM DTNB solution. This technique yielded quantitative results with authentic cysteine (Sigma), but did not reduce methionine (Sigma). A comparable method employing dithiothreitol as reducing agent has been described by Zahler and Cleland (12).

Determination of Sulfhydryl Groups in Intact Lipophilin—Approximately 2 mg of lipophilin in 0.1 M EDTA, pH 8.0, at a final concentration 0.4 to 0.5 mg/ml. Aliquots of 0.9 ml were treated with 0.1 ml of 10 mM DTNB, prepared in water, and carefully adjusted to pH 7.5 with NaOH, and the absorbance at 412 nm was recorded after 1 h. Very low ionic strength was required in this assay to avoid precipitation of the protein. A similar method was employed for lipophilin in 8 M urea and 6 M guanidinium chloride (Heico products), except that solutions were deoxygenated before introduction of the denaturant. The molar absorption coefficient of the TNB anion in these media was taken as 13,700 M⁻¹ cm⁻¹ at 412 nm (11).

The DTNB method was adapted for sulfhydryl determinations on lipophilin in 2-chloroethanol. Solutions of lipophilin (0.4 to 0.5 mg/ml) were prepared in deoxygenated 2-chloroethanol, with or without 1% SDS. Aliquots of 0.9 ml were treated with 0.1 ml of 10 mM DTNB and then 0.1 ml of 3 M triethylamine (Fisher), both in 2-chloroethanol, and the absorbance at 412 nm was followed over a period of 1 h, during which time a maximum was reached, followed by a slow linear decrease. The true reading was obtained by extrapolating the decay curve back to zero time. The assay was standardized using dithiothreitol (Sigma), and a molar absorption coefficient of 14,500 M⁻¹ cm⁻¹ was calculated for the TNB anion. The same method was employed with whole myelin; a concentration of 2 mg/ml in 2-chloroethanol gave a satisfactory response.

Alkylation of Lipophilin with Iodoacetamide—Lipophilin (10 mg) was dissolved in 1.8 ml of 2-chloroethanol, then treated with 0.2 ml of 2 M triethylamine in 2-chloroethanol and 1 ml of 0.01 M "C-labeled iodoacetamide, and allowed to stand 4 h in the dark. The solution was then dialyzed against distilled water and the protein recovered by lyophilization. Incorporation of carboxamidomethyl groups was monitored by scintillation counting and by analysis for S-carboxamidomethylcysteine after hydrolysis with 6 M HCl under vacuum.

Reduction and Alkylation of Lipophilin with 4-Vinylpyridine—Lipophilized protein (50 mg) was dissolved in 1.5 ml of lipified phenol (Baker) and 12 ml of 2-chloroethanol; this solution was treated with 125 ml of 0.5 M EDTA, 125 ml of 1.0 M NaOH, and 280 ml of 2-mercaptoethanol (BDH) (200 times the molar quantity of cysteine residue), then flushed overnight with nitrogen. To the reduced protein was added 50 ml of 1.0 M NaOH and 870 ml of 4-vinylpyridine (twice the molar quantity of thiol). After incubation for 4 h in the dark, the reaction was stopped by acidification with HCl; reagents were removed by dialysis against distilled water and the protein recovered by lyophilization. The extent of alkylation was determined spectrophotometrically (described under "Results") and by amino acid analysis after hydrolysis with 6 M HCl under vacuum, using authentic S-(4-pyridyl)ethyl-1-cysteine (BDH) as standard.

In some preparations, covalently bound fatty acyl groups were removed from lipophilin by hydroxylamine and the change behavior toward reduction and alkylation. Lyophilized protein (50 mg), dissolved in phenol/2-chloroethanol as above, was incubated 4 h with 12 ml of 2.0 M NH₄OH-HCl (Aldrich) adjusted to pH 8.5. The mixture was then acidified, reagents were removed by dialysis against distilled water, and the solution was lyophilized. To ensure elimination of free fatty acids, the dried protein was dissolved in 98% formic acid (BDH), precipitated with 5 volumes of ether, then washed twice with isopropanol alcohol and once with water, and recovered by lyophilization.

Determination of Fatty Acid Bound to Lipophilin—Lyophilized protein (2 mg) was hydrolyzed with 2 ml of 6 M HCl for 5 h under vacuum. KCl was removed by evaporation and the residue was washed several times with water, then finally dissolved in 1.5 ml of water. Heptadecanoic acid (Applied Sciences) (50 nmol) was added as internal standard, and fatty acids were extracted into chloroform by shaking with 9 ml of chloroform/methanol (21: v/v). The organic (lower) phase was evaporated to dryness, and the residue was treated with 0.5 M HCl in methanol, made by adding 75 ml of acetyl chloride to 5 ml of dry methanol. The mixture was heated in a sealed tube under vacuum at 80°C for 30 min, then analyzed on a Varian Aerograph 2100 gas chromatograph, using a column of 3% OV-210 on Gas Crom Q at 110-190°C.

Determination of Ester Groups by Reaction with Hydroxylamine—The protein sample (0.5 to 2.5 mg) was suspended in 0.1 ml of isopropanol alcohol, then treated with 0.2 ml of 1.0 M NH₄OH-HCl previously adjusted to pH 7 or pH 11. The mixture was blended on a Vortex mixer and allowed to react at room temperature for periods of 10 min to 3 h. The reaction was terminated by adding 0.1 ml of a 1:2 (v/v) solution of isopropyl alcohol and 0.1 M glycylglycine, 0.1 M EDTA, pH 8.0, to quantitate the resulting hydroxamic acids. 0.1 ml of 0.37 M FeCl₃ in a 1:1 (v/v) mixture of 0.1 M HCl and isopropanol alcohol was added, the suspension was centrifuged, and the absorbance was read at 540 nm. With control samples, the order of addition of NH₄OH and HCl was reversed to check for nonspecific color development (none detected). The procedure was standardized using acetyldihydroxamic acid (Sigma), which gave a molar absorption coefficient of 1000 M⁻¹ cm⁻¹ at 540 nm in the presence of Fe(III). Color yield was assumed to be independent of hydroxamic acid alkyl chain length. Acetylcholine bromide and ace-tylthiocholine bromide (Sigma) were used to compare the reactivity of authentic alcohol and thiol esters at pH 7 and pH 11.

Circular Dichroism—CD spectra in the "peptide region" (250 to 200 nm) were recorded on a Cary 61 instrument at 25°C. Protein concentrations were in the range of 0.2 to 1.0 mg/ml in cells of pathlength 1 mm. Mean residue ellipticity was calculated using a mean residue weight of 109.0. The dichrometer was calibrated at 220 nm with a standard solution of (-)-pantolactone (Fluka) (13).

RESULTS

Reduced and Oxidized Cysteine Content of Lipophilin—Complete denaturation of the polypeptide chain of lipophilin is extremely difficult to achieve (8). The number of reduced cysteine residues was therefore determined most reliably by sulfhydryl analysis after acid hydrolysis; the total half-cystine content was then obtained following reduction and removal of excess reducing agent. Table 1 summarizes the results from four separate batches of lyophilized protein.

The molecular weight of the lipophilin subunit is around 26,000 according to analytical ultracentrifugation (5, 6). On this basis, the total number of cysteyl residues was consistently close to 11 per molecule. The half-cystine content from amino acid analysis (Ref. 9 and more recent determinations)

| TABLE I |
|----------|
| Cysteine and cystine content of lipophilin |
| Determinations were made on protein hydrolysates using DTNB. The molecular weight of lipophilin was assumed to be 26,000. |

| Protein batch | No. of determinations | Total half-cystine* | Reduced cystine* | Oxidized cystine* |
|---------------|----------------------|---------------------|------------------|------------------|
| A             | 10                   | 11.4 ± 0.2          | 3.6 ± 0.1        | 7.8              |
| B             | 8                    | 11.1 ± 0.2          | 3.7 ± 0.1        | 7.4              |
| B'            | 3                    | 3.6 ± 0.1           | 4.4 ± 0.1        | 6.5              |
| C             | 8                    | 10.9 ± 0.5          | 4.5 ± 0.1        | 6.4              |
| D             | 8                    | 10.9 ± 0.1          | -                | -                |

* Mean values with their standard deviations.

+ By difference from Columns 3 and 4.

+ By relyophilization from aqueous solution.
is about 4 per 100 residues, equivalent to 9.5 per molecule. This discrepancy, although not unreasonable, was investigated by reduction and alkylation of the protein, as described later. Comparable results have been reported for other proteolipid apoproteins: preparations from bovine brain (1) and rat brain (14), as well as the human brain protein P7 (15), each contain 4 to 5 half-cysteines per 100 residues; Nicot et al. (3) found 9 to 11 cysteinyl residues per molecule in bovine proteolipid apoprotein, after reduction with dithiothreitol in aqueous SDS.

The reduced cysteine content of lipophilin varied appreciably from batch to batch, the fractional values suggesting heterogeneity. The possibility that variable amounts of oxidation occurred during acid hydrolysis appears unlikely in view of the consistency of the results for each individual protein preparation. Since an even number of cysteinyl residues must participate in disulfide bonds, we conclude that lipophilin as purified contains minimally three disulfide bridges and maximally five free sulfhydryl groups, of which two may become oxidized. To test the effect of protracted handling on the reduced cysteine content, a sample of lipophilin was dissolved in 2-chloroethanol, transferred to water by dialysis, reisolated by lyophilization, then redissolved in 2-chloroethanol, dried down, and subjected to acid hydrolysis as before. Although no precautions were taken to exclude oxygen until the hydrolysis stage, there was little or no change in sulfhydryl activity (Table I). Thus, susceptibility to partial oxidation presumably arises during the course of isolation of the apoprotein from myelin. Removal of associated lipid is likely to cause substantial structural reorganization of the protein, perhaps involving transient conformations in which two sulfhydryl groups are in close contact; once delipidation is complete, formation of new disulfide bridges becomes an unlikely event. Other authors have observed a decrease in the number of sulfhydryl groups available for carboxymethylation (2) or for reaction with DTNB (3) during purification of apoprotein from crude bovine proteolipid. However, under these circumstances, a drop in sulfhydryl activity may reflect not only oxidation but also a decrease in accessibility caused by conformational changes.

Availability of Sulfhydryl Groups in Intact Lipophilin—The reactivity with DTNB of the reduced cysteine residues in intact lipophilin was investigated using various solvent media previously employed in conformational studies on the protein (6-8). These results are presented in Table II.

In aqueous solutions of lipophilin, the sulfhydryl groups were almost unreactive. This was not due simply to oxidation during dialysis from 2-chloroethanol into water since greater numbers of sulfhydryl groups were detected in 2-chloroethanol solution (below) and in acid hydrolysates of the reisolated protein. Neither 8 M urea nor 6 M GdmCl appreciably increased —SH group exposure, in accord with our earlier findings that these reagents can only partially denature lipophilin (8). The obvious explanation is that the reduced cysteine residues are physically inaccessible to DTNB, being buried in the hydrophobic interior. In this respect, our results differ from those of de Foresta et al. (16), who have recently reported that bovine proteolipid apoprotein in aqueous solution contains 1.7 —SH groups reactive with DTNB in the presence or absence of detergent. While this protein is undoubtedly similar to lipophilin, our previous experience has indicated that differences in methods of preparation and solubilization can cause large variations in structural properties (5, 6).

Spectroscopic studies on lipophilin in 2-chloroethanol have shown that the protein exists as long helical chains in which the aromatic residues are exposed to the medium (6-8); some residual tertiary structure might be expected to arise from the presence of disulfide bridges. Surprisingly, only about 35% of the free —SH groups were detected by DTNB, and protein batch variations were noted as before. Further assays were performed in the presence of 1% SDS, which is known to disrupt tertiary structure (17), and which was shown to improve the extent of carboxymethylation of bovine proteolipid apoprotein in chloroform/methanol solution (2). The increased exposure amounted to about 0.4 —SH groups per molecule, leaving close to two —SH groups undetected (compare Tables I and II). This discrepancy might conceivably be due to oxidation despite our efforts to maintain anaerobic conditions, but we regard this as unlikely in view of the consistent results for each protein batch and the consistent differences with the preceding results on protein hydrolysates. In the absence of any precautions to exclude oxygen, sulfhydryl yields decreased by only 0.1 to 0.3 groups per molecule. We therefore conclude either that two —SH groups were inaccessible to DTNB on account of steric or polar factors, or that these groups do not occur at all in the intact protein but are generated during acid hydrolysis. Both possibilities were investigated further.

Alkylation of Lipophilin with Iodoacetamide—A sample of lipophilin in 2-chloroethanol was alkylated with 14C-labeled iodoacetamide, which is somewhat smaller than DTNB and carries no charge. The yield of S-carboxymethylcysteine after acid hydrolysis was 1.8 residues per molecule of protein; thus, the availability of sulfhydryl groups was the same as with DTNB, suggesting the presence of a tight fold of tertiary structure produced by disulfide bridging.

The first stage in the purification of lipophilin from myelin, following the original procedure of Gagnon et al. (9), is extraction of a minor component with 2-mercaptoethanol. It has recently been found that this step may be eliminated with no loss of product purity. In view of the possibility that 2-mercaptoethanol might cause some disulfide bond reduction in lipophilin, protein isolated by the modified procedure was also alkylated with 14C-labeled iodoacetamide. The incorporation of radioactivity was only 10% lower than before and the S-carboxymethylcysteine content after hydrolysis was 1.6 residues per molecule. It therefore appeared unlikely that appreciable new sulfhydryl activity was generated in lipophilin by 2-mercaptoethanol treatment of myelin.

Sulfhydryl Groups in Whole Myelin—Since myelin has a rather simple protein composition, we were able to compare

### Table II

| Medium         | Protein batch label | No. of determinations | No. of sulfhydryl groups
|----------------|---------------------|-----------------------|--------------------------|
| Water          | A                   | 0.15 ± 0.05           |                          |
|                | E                   | 0.25 ± 0.1            |                          |
| 8 M urea       | A                   | 0.20 ± 0.05           |                          |
|                | E                   | 0.30 ± 0.05           |                          |
| 6 M GdmCl      | A                   | 0.25 ± 0.10           |                          |
|                | E                   | 0.30 ± 0.05           |                          |
| 2-Chloroethanol| A                   | 1.20 ± 0.05           | 45.5 ± 1                 |
|                | C                   | 1.65 ± 0.05           | 63.5 ± 1                 |
|                | E                   | 1.55 ± 0.05           | 60.2 ± 2                 |
|                | F                   | 1.25 ± 0.05           | 49.1 ± 1                 |
| 2-Chloroethanol-1% SDS | B   | 1.85 ± 0.15           | 70 ± 5                   |
|                | C                   | 2.10 ± 0.05           | 80.5 ± 1                 |
|                | D                   | 2.45 ± 0.10           | 94 ± 3                   |
|                | F                   | 1.65 ± 0.05           | 63 ± 1                   |

*Mean values with their standard deviations.
For comparison with Table III.
its sulfhydryl content with that of isolated lipophilin. Two groups of proteins account for 80% by weight of total myelin protein (18, 19); the proteolipid protein (53%) contains 4 to 5% cysteine, while the basic protein (27%) is devoid of cysteinyl residues. The remaining proteins must also be low in cysteine since amino acid analyses of total myelin protein show no more than 2% cysteine (18, 20, 21). Thus, most of the sulfhydryl activity detected in whole myelin is likely to arise from proteolipid proteins, of which lipophilin comprises at least 50% (9).

The protein content of dry myelin is around 25% by weight (19); for the present purposes, the protein concentration in solutions of myelin in 2-chloroethanol was estimated from the optical absorbance at 280 nm, assuming the tyrosine and tryptophan residues to be normalized and exposed in this medium (7). From the amino acid composition of whole myelin (20) and molar absorbance coefficients previously determined for N-acetyl-L-tyrosinamide and N-acetyl-L-tryptophanamide (7), the 280 nm absorbance of total myelin protein was estimated to be 1.1 liters g⁻¹ cm⁻¹. Determinations were made on several samples of both normal and multiple sclerotic myelin, and results were expressed in terms of —SH groups per g of protein and hence per g of proteolipid protein (Table III). Comparison with the final column of Table II shows that the number of sulfhydryl groups assignable to the proteolipid fraction in this way is at least as great as the number actually detected in the purified apoprotein. This confirms that our results on lipophilin are a valid reflection of the situation in whole myelin.

**TABLE III**

**Sulfhydryl group analysis of whole myelin**

Determinations were carried out on myelin solutions in 2-chloroethanol-1% SDS using DTNB. The method of quantitation is described in the text.

| Sample | Batch label | No. of determinations | No. of sulfhydryl groups* | µmol/g protein | µmol/g PLP* |
|--------|-------------|------------------------|--------------------------|----------------|-------------|
| Normal | 1           | 4                      | 60 ± 1                   | 113 ± 1        |
| myelin | 2           | 7                      | 56 ± 3                   | 106 ± 5        |
|        | 3           | 4                      | 55 ± 1                   | 103 ± 1        |
| Multiple | 1          | 4                      | 56 ± 1                   | 105 ± 1        |
| sclerotic myelin | 2 | 4 | 58 ± 1 | 108 ± 2 |

* Mean values with their standard deviations.
* PLP, proteolipid protein.

**TABLE IV**

**Cysteine analysis of (4-pyridyl)ethyl-lipophilin**

The extent of cysteine modification was determined by amino acid analysis and by spectrophotometry. The number of residues per molecule was taken as 239 (mean residue weight, 109.0).

| Fatty acid removal | PE-cysteine* | Pyridyl groups* | Free —SH groups* |
|--------------------|--------------|-----------------|------------------|
| mol %               | mol/mol protein |                  |                  |
| No                 | 4.0          | 9.7             | 0.5              |
| Yes                | 4.4          | 10.5            | 0.8              |

* PE-cysteine, S-(4-pyridyl)ethyl-L-cysteine, by amino acid analysis.
* From absorption at 258 nm in 2-chloroethanol.
* From DTNB assay in 2-chloroethanol.

**FIG. 1. Absorption spectra.** Left panel: absorption spectra at 25°C of lipophilin (—) and (4-pyridyl)ethyl-lipophilin (— ) (1 mg/ml) in 2-chloroethanol. Right panel: difference between these spectra (— — ) and spectrum of S-(4-pyridyl)ethyl-L-cysteine (0.5 mM) in 2-chloroethanol (—).
Circular Dichroism of (4-Pyridyl)ethyl-lipophilin—In previous circular dichroism studies (4–6), lipophilin was shown to exhibit considerable conformational variability in aqueous solution, according to the procedure chosen to solubilize it. It was therefore of interest to examine the effect of disulfide bond cleavage on the structure of the protein in different media. CD spectra of unmodified and defatted, reduced and alkylated lipophilin in 2-chloroethanol, and in water after dialysis from 2-chloroethanol, are compared in Fig. 2. The ellipticity of the protein in 2-chloroethanol was surprisingly decreased after modification, representing a loss of helical character. The effect was the same in protein alkylated with the less bulky iodoacetamide, but no such change was found in protein that was defatted but not modified further (spectra not shown). Thus, the relaxation of tertiary structure expected from cleavage of disulfide bridges, possibly in combination with removal of fatty acids, is sufficient to favor alternative conformations in certain regions of the polypeptide chain, thereby fragmenting the helical coil. The proportion of α helix was estimated to be about 75% from the ellipticity at 224 nm, assuming an average helical chain length of 15 residues (23).

The CD spectrum of (4-pyridyl)ethyl-lipophilin in water was markedly affected by the modification, the single extremum at 218 nm indicating a preponderence of β rather than α structure. Evidently disulfide bonds are important in establishing the secondary structure of lipophilin; therefore, they might also contribute to its remarkable resistance to denaturation by urea and guanidinium salts (8). When the modified protein was exposed to 6 M GdmCl, the loss in ellipticity was 40% after 7 days at room temperature, but the spectrum retained the features of a β structure (Fig. 2), and the observed drop in [θ] at 218 to 220 nm was no more than for unmodified lipophilin. Thus, the ability to withstand strong protein denaturants is a consequence solely of the apolar character of the protein (8).

Attachment of Fatty Acids to Lipophilin—An unusual feature of lipophilin and other brain proteolipid proteins is the presence of 2% to 3% of covalently bound long chain fatty acids, equivalent to 2 to 3 molecules per molecule of protein (1, 9, 24). Although these fatty acids (mainly palmitic, oleic, and stearic) appear to be bound through ester linkages, the sites of attachment are unknown. To account for our observations that hydrolyzed lipophilin contained two more —SH groups than could be detected in the intact protein, we investigated the possibility that cysteinyl side chains might be involved. Thioureas are not uncommon but are normally associated with "active" acyl groups, as in acyl coenzyme A species and intermediates in the action of sulfhydryl proteases (25, 26).

The fatty acid content of lipophilin was checked before and after reduction and alkylation of the protein with 4-vinylpyridine, by gas chromatography of the methyl esters produced by acid hydrolysis, organic solvent extraction, and methylation. Decreases of up to two fatty acid groups per molecule accompanied protein modification (Table V). Since reduction and alkylation were carried out under mildly alkaline conditions, these results were consistent with the presence of two particularly reactive ester bonds in the protein. On the other hand, when lipophilin in 2-chloroethanol was treated with 1 M ammonia, followed by organic extraction, methanolysis, and gas chromatography as before, no evidence was obtained for the existence of any labile ester. Possibly, side reactions during acid hydrolysis and lack of quantitative extraction of fatty acids from reaction mixtures might account for this inconsistency.

To resolve this problem, ester linkages in lipophilin were determined in situ by hydroxylamine cleavage and conversion of the resulting hydroxamic acids to the characteristic purple complex with ferric ion (27). The reaction with hydroxylamine was carried out at pH 7 and pH 11 to distinguish between thiol and alcohol esters: both should react quantitatively at alkaline pH, but only the former at neutral pH. Preliminary experiments with acetycholine and acetyltiocholine verified this behavior.

At pH 11, the half-time of reaction of lipophilin was less than 10 min, and the yield of hydroxamic acid was steady between 75 and 195 min. Seven batches of protein extracted from both normal and diseased (multiple sclerosis) myelin all produced close to two hydroxamic acids per molecule (Table V). (4-Pyridyl)ethyl-lipophilin, containing almost 10 S-alkyl groups plus residual free sulfhydryl activity, reacted somewhat more slowly but gave the same final yield. Since in the latter case more than 10 of 11 cysteinyl residues were accounted for,

**Table V**

| Material               | Fatty acids | Ester groups |
|------------------------|-------------|--------------|
|                        | pH 11 (90 min) | pH 7 (15 min) | pH 7 (90 min) |
| Lipophilin*             | 2.7         | 2.0 ± 0.1    | 0.5 ± 0.2    | 0.8 ± 0.1 |
| Reduced lipophilin      | 1.2         | 2.1 ± 0.3    | 0.2 ± 0.1    |            |
| (4-Pyridyl)ethyl-lipophilin | 0.7         | 2.1 ± 0.3    | 0.2 ± 0.1    |            |
| Acetylcholine bromide   | ~1*         | 0.1 ± 0.03   | 0.4 ± 0.1    |            |
| Acetyltiocholine bromide| ~1*         | ~1*          |              |            |

*Palmitic, oleic, and stearic acids accounted for 95% of the recovered fatty acids.

*Extracted from both normal and multiple sclerotic myelin.

*Precise quantitation was difficult owing to the hygroscopic character of the material.
it is unlikely that any of the acyl groups detected as hydroxamic acid could have arisen from thiolester linkages. Nevertheless, there was a detectable rapid reaction of both modified and alkylated lipophilin with hydroxylamine at pH 7, although the conversion to hydroxamic acid represented less than one ester group per molecule (Table V). The subsequent continuing slow phase was comparable with the slow transamination observed in the model compound acetylocline at pH 7. Therefore, at least some of the alcohol ester linkages in lipophilin were more reactive than normal.

The pH 11 results are again difficult to reconcile with our earlier finding that only low yields of fatty acid are recoverable from lipophilin after reduction and alkylation. However, it is not certain that quantitative yields of hydroxamic acid were obtained in the present test, particularly as the fatty acid content of lipophilin is frequently greater than two groups per molecule (cf. Table V). A particularly labile acyl group might be cleaved by hydroxylamine-catalyzed hydrolysis rather than by hydroxaminolysis, or else be already lost from the protein by hydrolysis during reduction-alkylation. For this reason, our combined results do not allow a conclusive statement regarding the presence of S-acylcysteine residues in lipophilin, although it is clear that at least two fatty acids are covalently linked as alcohol esters, presumably to serine and/or threonine side chains. In view of the biphasic, partial reactivity with hydroxylamine at pH 7, it is possible that the sites of attachment are not always the same in different protein molecules.

**DISCUSSION**

Proteins may be broadly classified into those, mainly extracellular, that contain disulfides, and those, mainly intracellular, that contain sulphydryls (25, 26). As a membrane component, lipophilin belongs to neither class, and it is interesting that it contains almost equal numbers of cysteinyl residues in disulfide and sulphydryl form. Since lipophilin has no measurable biological activity, while myelin itself has an essentially passive function, it is difficult to be certain of the roles of the protein or its \( \textit{SH} \) groups. Lipophilin behaves as an intrinsic membrane protein, although partially exposed on both sides of the lipid bilayer (19, 28), and might therefore have a structural or organizational role. Thus, when introduced into phospholipid vesicles, it exerts both short and long range ordering effects on the surrounding lipid and can cause phase separation in mixed lipid systems through its preference for binding acidic phospholipids (29). In such complexes, lipophilin adopts highly \( \alpha \)-helical conformations (6), and there is evidence that the same is true in myelin (29). The present studies show that, for purified lipophilin at least, \( \alpha \)-helical forms are very dependent on the presence of disulfide bonds; therefore, it is reasonable to infer that these groups have some importance in stabilizing or maintaining the native structure of the protein.

The high chemical reactivity of thiol groups suggests that they must play a central role in the biological activity of proteins. However, this is frequently not the case, even in sulphydryl-containing enzymes (26). The unionized thiol group is very dependent on the presence of disulfide bonds; therefore, it is reasonable to infer that these groups have some importance in stabilizing or maintaining the native structure of the protein. Nevertheless, it is interesting to consider a possible active role for lipophilin to which the sulfhydryl groups might contribute in two ways by virtue of their particular chemical reactivity. In previous publications (4–6), we have demonstrated that lipophilin can exhibit a range of secondary structures in aqueous solution which may or may not involve changes in aggregation. Moscarello (30) has suggested that conformation flexibility in lipophilin as it occurs in myelin might provide a mechanism for dissipation of heat generated by the passage of impulses along the underlying nerve. Owing to its remarkably equal content of sulfhydryl and disulfide groups, lipophilin is potentially capable of undergoing complex sequences of sulfhydryl-disulfide interchanges. Such exchange reactions can occur under very mild conditions; they are well known from studies on protein denaturation and refolding and are also likely to occur \textit{in vivo} during protein biosynthesis (26, 31). A mechanism is therefore available in lipophilin for the rapid dissemination of structural changes at the tertiary and quaternary levels as would be required in an effective heat sink. This process could be extended to involve the lipids of myelin through a second reactivity of thiol groups, namely, their tendency to form adducts with olefinic compounds (cf. the alkylation of lipophilin with 4-vinylpyridine). A labile interaction between membrane proteins and unsaturated lipids through this reaction was originally proposed by Robinson (32) and, if it exists, would clearly have many interesting consequences.

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