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The thiol groups of the Folch-Pi protein from bovine white matter

Exposure, reactivity and significance

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The number and the reactivity of accessible thiol groups of the Folch-Pi apoprotein and proteolipid (50% of myelin proteins) were studied, by using a specific thiol–disulphide interchange reaction, in connection with the known solubility of this protein in organic and aqueous solvents. The high reactivity of 2,2'-dipyridyl disulphide towards thiol groups leads to the titration of 4.8 mol of SH groups/mol of protein (M_r 30000) in alkaline and acidic chloroform/methanol (2:1, v/v). Unlike previous findings, this value was consistently found from batch to batch and remained stable with time. In the proteolipid 1 mol of SH groups/mol was not accessible as compared with the apoprotein. In aqueous solvents, a similar number of 4.4 mol of SH groups/mol was also found. For the first time, kinetic studies carried out in chloroform/methanol discriminated between two classes of thiol groups. The reaction of 2 mol of SH groups/mol was characterized by apparent second-order rate constants whose values were 5–10-fold higher than those of the other class. Kinetic studies and cyanylation experiments in aqueous solvents also indicated the high reactivity of these thiol groups with Ellman's reagent. Together with kinetic results, studies on the stoichiometry of the interchange reaction of equimolar solutions of protein and disulphide indicate that these highly reactive thiol groups are near to each other in the amino acid sequence. The location of the thiol groups at the boundary between hydrophilic and hydrophobic domains of the Folch-Pi protein is suggested in connection with their possible structural and biological significance.

One major interest of investigations on the proteins of brain myelin is how these proteins interact with myelin constituents and what their biological function may be. The Folch-Pi protein, a major myelin protein, displays unusual structural properties in connection with its transmembrane disposition, the hydrophobic character of the protein, its disulphide-linked domains and its relatively high number of thiol groups (Lees *et al.*, 1979; Boggs *et al.*, 1982). Isolated from white matter as a proteolipid complex in chloroform/methanol, the Folch-Pi protein is also soluble in this solvent as a delipidated apoprotein, and can be transferred to water under appropriate solvent conditions (Sherman & Folch-Pi, 1970). This unusual solubility characteristic has been related to large changes in molecular shape (Lavialle *et al.*, 1979), involving thiol groups of the protein (de Foresta *et al.*, 1979). Nevertheless, the exposure and the reactivity of cysteine residues of a transmembrane protein may also be of importance for its state of aggregation, and therefore its interaction with other proteins and with lipids present within the myelin sheath, which can in turn affect the ion-channel formation described in the proteolipid by Lin & Lees (1982).

Because the number of the exposed thiol groups in the Folch-Pi protein has been the subject of inconsistent reports (Lees *et al.*, 1969; de Foresta *et al.*, 1979; Cockle *et al.*, 1980) and as a prerequisite for a better understanding of their biological function, we describe in the present paper a careful investigation of these residues in the apoprotein as well as in the proteolipid complex. Experimental conditions have been selected in order to ensure the specificity and the stability of all the reagents in organic and aqueous solvents. Kinetic measure-

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ments allowed, for the first time, discrimination between two classes of thiol groups with different reactivities. Also, the thiol groups have been tentatively located at known parts of the amino acid sequence, according to the model described by Stoffel *et al.* (1982). Their possible biological role is discussed in connection with the conformational properties of the Folch-Pi protein. A preliminary report of these results has been presented (Vacher *et al.*, 1982).

Experimental

Materials

Dithio-1,4-threitol (for biochemistry), 2-mercaptoethanol (for synthesis) and all the organic solvents (pro analysi) were purchased from E. Merck (Darmstadt, West Germany); 2,2'-dipyridyl disulphide (Aldrithiol-2) and bis-(4-nitrophenyl) disulphide (technical, recrystallized from ethyl acetate) were purchased from Aldrich (Janssen Pharmaceutica, Beerse, Belgium); 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was from Fluka (Buchs, Switzerland); 2-nitro-5-thiocyanobenzoic acid was purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.) and recrystallized from ethyl acetate/light petroleum. Sephadex G-25 (superfine grade) was obtained from Pharmacia (Uppsala, Sweden), and Bio-Beads S-X_1 (200–400 mesh) were from Bio-Rad (Richmond, CA, U.S.A.).

Preparation of the Folch-Pi apoprotein

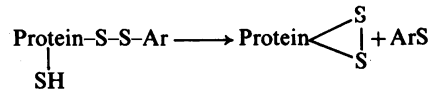
The protein was extracted with all myelin lipids from bovine white matter with chloroform/methanol (2:1, v/v) as described by Folch & Lees (1951) and partially separated from lipids by precipitation with light petroleum (b.p. 40–60°C) and dialysis at 4°C (Nicot *et al.*, 1973). The apoprotein was transferred from the chloroform/methanol mixture either to 2-chloroethanol after precipitation (Lavialle *et al.*, 1979) or to water (Sherman & Folch-Pi, 1970). In another set of experiments dithiothreitol was added to water during the transfer to a concentration of 0.15mM and then retained on a Sephadex G-25 column in 0.5% acetic acid before thiol titration. The M_r of the apoprotein was taken as 30000, in accordance with an amino acid sequence determination (Stoffel *et al.*, 1982). The absorption coefficient $E^{1\%}_{1cm}$ was taken as 13.5 at 278nm (Nicot *et al.*, 1973).

The phospholipids and cerebrosides were measured by phosphorus determination (Ames & Dubin, 1960) and by galactose determination (Devor *et al.*, 1958) respectively. The lipids of the proteolipid obtained by precipitation with light petroleum were cerebrosides (25 ± 5%) and phospholipids (12 ± 3%).

(1) Reaction of disulphide reagents with protein thiol groups:

$$\text{Protein–SH} + \text{Ar–S–S–Ar} \longrightarrow \text{Protein–S–S–Ar} + \text{ArS}^-$$

(2) Side reaction between protein thiol groups occurring after reaction (1) at low disulphide reagent concentration:



(3) Cyanylation of protein thiol group:

$$\text{Protein–SH} + \text{Ar–SCN} \longrightarrow \text{Protein–SCN} + \text{ArS}^-$$

(4) Equilibrium side reaction competing with reaction (3), giving rise to Ellman's reagent:

$$\text{ArS}^- + \text{Ar–SCN} \rightleftharpoons \text{CN}^- + \text{Ar–S–S–Ar}$$

reacts with protein SH as in (1)

Scheme 1. *Reactions of (i) thiol–disulphide interchange (reactions 1 and 2) and of (ii) cyanylation (reactions 3 and 4)*
Ar–S–S–Ar represents an aromatic disulphide reagent; in reaction (4) it is Ellman's reagent. Protein–S–S–Ar represents the chromophoric mixed disulphide. ArS^- represents the chromophore thiolate ion. Ar–SCN represents 2-nitro-5-thiocyanobenzoic acid.

Spectrophotometric determination of thiol groups by thiol–disulphide interchange reaction in various solvents (reaction 1)

All reactions were performed in spectrophotometric matched cuvettes, stoppered when organic solvents were used. The protein concentration was in the range 10–30μM and the disulphide reagent concentration 1.0–2.5mM. In aqueous solvents the concentration of Ellman's reagent was in the range 10–50μM. The reaction was monitored until completion on a Cary model 118C spectrophotometer. The kinetics of the reaction were followed at the maximum absorption wavelength and the absorption was recorded versus time. The molar absorption coefficients of the chromophoric ions were determined by reaction of the corresponding disulphide with solutions of acetyl-L-cysteine and dithiothreitol of known concentrations. The following molar absorption coefficients were determined at the maximum absorption wavelengths: at 422nm, in 2-chloroethanol, in the presence of 0.18M-triethylamine, 14.5 $mM^{-1} \cdot cm^{-1}$ for the reduced Ellman's reagent and 15.5 $mM^{-1} \cdot cm^{-1}$ for 4-nitrophenyl sulphide; at 432nm, in chloroform/methanol, in the presence of 50mM-HCl, 15.5 $mM^{-1} \cdot cm^{-1}$ for 4-nitrophenyl sulphide; at 360nm, in chloroform/methanol, in the presence of 50mM-HCl and 7mM-triethylamine, 6.9 $mM^{-1} \cdot cm^{-1}$ for 2-thiopyridone; at 343nm, in

aqueous solvents, $7.8\,mM^{-1}\cdot cm^{-1}$, independent of pH, for 2-thiopyridone. At 412nm, in aqueous solvents, a value of $13.6\,mM^{-1}\cdot cm^{-1}$ was taken for the reduced Ellman's reagent (Ellman, 1959).

In order to determine the amount of protein–chromophore mixed disulphide formed (reaction 1), the protein was separated from other reaction products by gel filtration with gels appropriate for the solvent used. With chloroform/methanol (2:1, v/v) the gel was Bio-Beads S-X_1 (200–400 mesh), and with water Sephadex G-25 equilibrated in 0.1mM-Tris/glycine buffer, pH7.2, containing 0.1% sodium dodecyl sulphate. The number of mol of chromophoric mixed disulphide/mol of protein was then calculated by taking the measured molar absorption coefficient of the chromophore as half that of the corresponding disulphide reagent [$\varepsilon_{325} = 21.8\,mM^{-1}\cdot cm^{-1}$ for bis-(4-nitrophenyl) disulphide and $18.0\,mM^{-1}\cdot cm^{-1}$ for Ellman's reagent]. The corresponding protein concentration was then evaluated by assuming that the bound chromophore contributed an absorption at 280nm identical with that contributed by the same amount of the corresponding disulphide reagent. The $\varepsilon_{325}/\varepsilon_{280}$ ratio determined for bis-(4-nitrophenyl) disulphide in chloroform/methanol and for Ellman's reagent in aqueous solvents was 2.0:1 for both.

Cyanylation of thiol groups

The cyanylation reaction shown in reaction (3) was performed by the procedure of Degani & Patchornik (1974) with protein concentrations in the range 10–50μM. The pH was adjusted to 7.5 with a solution of 2.0mM-Tris. A solution of 2-nitro-5-thiocyanobenzoic acid in ethanol was added in increments, with stirring, to a final concentration of 2–10-fold the protein concentration, and the pH was kept constant by addition of 2.0mM-Tris. The absorbance at 412nm was quickly read against the corresponding blank in order to determine the number of cyanylated thiol residues. The reaction was stopped by addition of acetic acid to a final concentration of 20% (v/v). Dialyses against the same solvent were then performed in order to eliminate the excess reagent. The absorption spectrum of the cyanylated protein was recorded, and the number of mol of chromophoric mixed disulphide/mol of protein arising from reaction (4) was calculated as described above.

Results and discussion

Exposure of thiol groups in the Folch-Pi apoprotein and proteolipid

In order to test the accessibility of thiol groups of the protein in appropriate solvents, several disulphide reagents were tried and selected in relation to their solubility and stability in each solvent. The aromatic disulphides used were bis-(4-nitrophenyl) disulphide in organic solvents, and its carboxylated derivative (Ellman's reagent) in chloroethanol and aqueous solvents; 2,2'-dipyridyl disulphide was used in all the above-mentioned solvents (Brocklehurst, 1979).

The results of thiol titrations are presented in Table 1. In our hands a total number of 4.8mol of SH groups/mol of protein (M_r 30000) was found in chloroform/methanol with the use of 2,2'-dipyridyl disulphide. The thiol titre was remarkably stable as long as the protein solution was kept in this solvent, at 4°C. Unlike the results reported by Cockle *et al.* (1980), this value was consistently titrated from batch to batch. A very similar value (4.4mol of SH groups/mol) was found for the apoprotein in aqueous solvents as long as the aqueous transfer was carried out in the presence of 0.15mM-dithiothreitol, this value falling to 3.4mol

Table 1. *Titration of the thiol groups of the Folch-Pi proteolipid and apoprotein*

The results expressed in number of mol of free SH groups/mol of protein (M_r 30000) are means ± S.E.M. for at least five determinations. The reactions were performed as described in the Experimental section in the presence of: (*a*) 7mM-triethylamine; (*b*) 50mM-HCl; (*c*) 0.18M-triethylamine (Cockle *et al.*, 1980); (*d*) 0.15mM-Tris/glycine buffer, pH7.2; (*e*) 1mM-Tris/maleate buffer, pH7.2; (*f*) 20mM-formic acid or 80mM-acetic acid, pH3.0.

Solvent	Reagent ...	Titration (mol of SH groups/mol): Ellman's reagent	Bis-(4-nitrophenyl) disulphide	2,2'-Dipyridyl disulphide
Chloroform/methanol (2:1, v/v)	Apoprotein	–	4.0 ± 0.2 (*a*)	4.8 ± 0.2 (*a*, *b*)
	Proteolipid	–	–	3.7 ± 0.1 (*b*)
2-Chloroethanol	Apoprotein	3.8 ± 0.2 (*c*)	3.8 ± 0.2 (*c*)	–
Water	Apoprotein	2.9 ± 0.3 (*d*)	–	3.2 ± 0.3 (*e*) 3.0 ± 0.3 (*f*)
Transferred to water in the presence of dithiothreitol	Apoprotein	–	–	4.4 ± 0.2 (*f*)

of SH groups/mol in the absence of dithiothreitol owing to oxidation.

In this respect our results differ again from those obtained by Cockle *et al.* (1980), who reported only 0.3 mol of SH groups/mol in aqueous solvents. In alkaline 2-chloroethanol these authors titrated 1.9 mol of SH groups/mol, whereas under identical experimental conditions our value was higher, namely 3.8 mol of SH groups/mol. It is worthwhile remarking that the total number of thiol groups titrated in an acid hydrolysate of the protein by Cockle *et al.* (1980) was 5.2 mol/mol (for M_r 30000), in good agreement with our number of exposed thiol groups.

Since Cockle *et al.* (1980) seem to rule out any oxidation mechanism responsible for the low accessibility of thiol groups, differences in preparation procedures leading to different protein conformations may explain these discrepancies. The results summarized in Table 1 also demonstrate the higher reactivity of 2,2'-dipyridyl disulphide even towards thiol groups located in relatively inaccessible sites of the protein, as pointed out by Norris & Brocklehurst (1976). We believe therefore that in the proteolipid the missing 1 mol of SH groups/mol (compared with the apoprotein) is masked by the bound lipids.

Kinetics of thiol groups

As shown in the preceding subsection, all the thiol groups of the Folch-Pi apoprotein are accessible to 2,2'-dipyridyl disulphide in aqueous and organic solvents. The kinetics of the disulphide exchange reaction, studied under pseudo-first-order conditions, allow discrimination between classes of thiol groups of different reactivities. The plotted curves of absorption versus time were resolved into two logarithmic plots by the method given in Frost & Pearson (1961). These curves, characteristic of two parallel first-order reactions, gave the stoichiometries and the pseudo-first-order rate constants of each of the phases (Fig. 1), providing second-order rate constants designated as K_1 for the higher rate and K_2 for the lower rate. In chloroform/methanol or in aqueous acetic acid the reaction was performed in excess disulphide reagent; in neutral aqueous solution the reaction too fast to be recorded on a spectrophotometer was therefore slowed down to measurable rates by using dilute reagent solutions (down to 5.0 μM) and by using the protein at a concentration in 8-fold excess of thiol groups over disulphide reagent as described by Degani & Patchornik (1974).

The values of the second-order rate constants K_1 and K_2 are presented in Table 2. In excess disulphide reagent the reaction is characterized by approx. 45% of thiol groups having an apparent

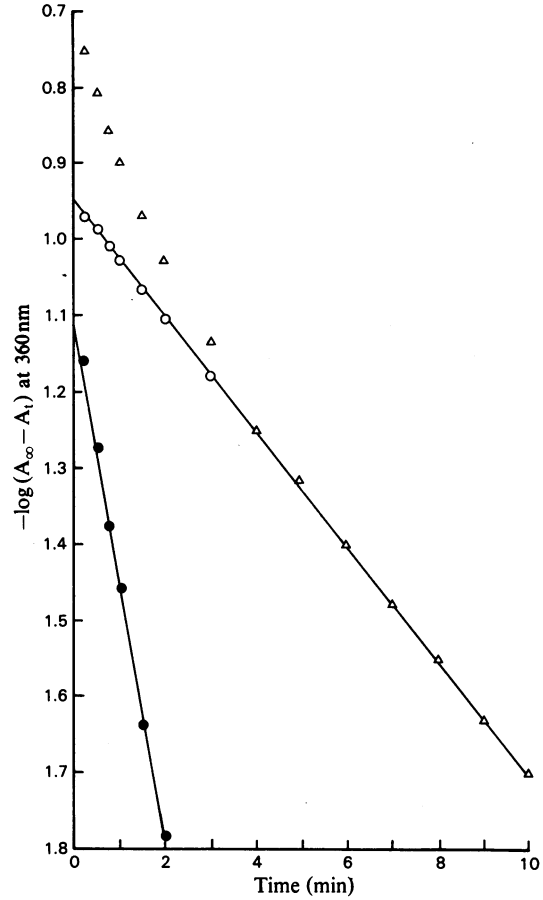


Fig. 1. *Pseudo-first-order rate plots of the reaction of the Folch-Pi protein with 2,2'-dipyridyl disulphide in chloroform/methanol*

The reaction was carried out at a protein concentration of 10 μM and a disulphide reagent concentration of 1.4 mM in the presence of 50 mM-HCl at 20°C. The absorption as a function of time (A_t) was read at 360nm. A_∞ was the absorption at the end of the reaction. Data were treated as described by Frost & Pearson (1961). △, Plot corresponding to the experimental data; ○, calculated plot of the reaction of the slower-reacting thiol groups; ●, calculated plot of the reaction of the faster-reacting thiol groups.

second-order rate constant 5–10-fold higher than that of the 55% remaining thiol groups. This would account for 2 and 3 mol of SH groups/mol of each class respectively.

In the presence of excess protein thiol groups over disulphide reagent and in neutral aqueous solvent a very high value was obtained for the apparent second-order rate constant. Comparison with the values obtained by Degani & Patchornik (1974) under similar experimental conditions with

Table 2. *Reactivity of the thiol groups of the Folch-Pi apoprotein*

The second-order rate constants were calculated by using the pseudo-first-order rate constants and the concentration of the reagent in excess, i.e. either the disulphide or the protein thiol groups. Experimental conditions (*a*), (*b*), (*d*), (*e*) and (*f*) are the same as indicated in Table 1 legend; (*d'*) same solvent as in (*d*), but at pH 6.2. In excess protein thiol groups, K_2 is not given, as it corresponds to the rate of the side reaction (2) occurring at low disulphide reagent concentration.

		Second-order rate constant ($M^{-1} \cdot s^{-1}$)		
	Solvent	In excess disulphide reagent		In excess protein thiol groups
Reagent	Chloroform/methanol (2:1, v/v)	K_1	K_2	K_1
Bis-(4-nitrophenyl) disulphide	(*a*)	14	2.6	–
2,2'-Dipyridyl disulphide	(*b*)	12	2.5	–
2,2'-Dipyridyl disulphide	(*a*)	1.6	0.16	–
2,2'-Dipyridyl disulphide (proteolipid)	(*b*)	27	5	–
	Water			
2,2'-Dipyridyl disulphide	(*f*)	37	3.8	–
2,2'-Dipyridyl disulphide	(*e*)	–	–	400
Ellman's reagent	(*d*)	–	–	1500
Ellman's reagent	(*d'*)	–	–	600

reduced glutathione and in the presence of Ellman's reagent ($K_1 = 149 M^{-1} \cdot s^{-1}$) leads to the conclusion that the most reactive thiol group of the Folch-Pi protein in aqueous solvent is displaying an extremely high reactivity ($K_2 = 600 M^{-1} \cdot s^{-1}$).

Cyanylation of thiol groups

Out of the 3.2 mol of SH groups/mol titrated (see Table 1) only an average number of 1.3 mol of SH groups/mol could be cyanylated (reaction 3) at protein concentrations similar to those used by Degani & Patchornik (1974). Since the yield of the reaction is higher at low protein concentrations, the protein concentration was subsequently lowered to 10 μM, and still only 1.3 mol of SH groups/mol could be cyanylated. At the same time, the spectrum of the cyanylated protein shows the presence of 2.0 ± 0.3 mol of chromophoric mixed disulphide/mol of protein. Degani & Patchornik (1974) have described the formation of Ellman's reagent by an equilibrium reaction of 2-nitro-5-thiocyanobenzoic acid with the excess reagent, as shown in reaction (4). The released Ellman's reagent reacts quickly with protein thiol groups as in reaction (1), leading to the formation of a chromophoric disulphide. The large amount of the disulphide present in the above-mentioned experiments accounts for the importance of the side reaction (4). It must be emphasized that the more reactive the thiol groups of the Folch-Pi protein in reaction (1), the more reaction (4) is competing with the cyanylation process. These results are in good agreement with kinetics pointing to the presence of at least two highly reactive cysteine residues/molecule.

The accessibility and the high reactivity of the free thiol groups of the Folch-Pi protein in aqueous solvent towards Ellman's reagent might appear to be in contradiction with previous observations by de Foresta *et al.* (1979). These authors studied a fluorescent label, *N*-(1-anilinonapth-4-yl)maleimide, covalently bound to free thiol groups, displaying fluorescent characteristics consistent with an environment of low polarity and/or of a highly ordered structure around thiol groups. This apparent discrepancy can be explained in connection with the distribution of the half-cystine residues of the Folch-Pi protein between hydrophobic and hydrophilic domains, proposed by Stoffel *et al.* (1982). The cysteine residues bound to the fluorescent probe and located at a boundary between these two domains could be freely accessible in the hydrophilic domain, while the hydrophobic fluorescent probe itself might be buried in the hydrophobic core of the Folch-Pi protein.

Stoichiometry of the reaction between disulphide reagents and the Folch-Pi protein

When the protein in alkaline solution of chloroform/methanol or of water reacts with an approximatively equimolar amount of nitrophenyl disulphide reagents (molar ratio 1.0–1.2 : 1), the number of mol of the chromophore released/mol was found to be higher than the amount of the reagent used could account for: about 2 mol/mol of protein. At the same time a low value (0.5) of the number of mol of the bound chromophore/mol was found.

Such a lack of stoichiometry has been previously reported in different proteins (Wassarman & Major, 1969; Carlson *et al.*, 1978; Wells & Yount, 1980). According to these authors, a side reaction

between the mixed disulphide arising from the titration reaction (reaction 1) and a free thiol group of the protein leads to the formation of a new disulphide bond (reaction 2); at the same time, an excess of chromophoric anion is released, explaining the lack of stoichiometry observed. According to Wells & Yount (1980), the displacement of the bound chromophore to form an intramolecular cystine disulphide bond (reaction 2) implies the existence of two thiol groups within 0.2 nm (2 Å) of each other. It is also to be noted that the above-mentioned side reaction occurs both in chloroform/methanol and in water. Therefore the two neighbouring thiol groups are not brought to this short distance apart by a solvent effect on the protein conformation, and must rather be close together in the amino acid sequence.

Concluding remarks

Finally, we wish to emphasize the following points. (i) The Folch-Pi protein exhibits the maximum number of accessible thiol groups in both organic and aqueous solvents when prepared from white matter by the procedures described by Nicot *et al.* (1973). Although these results disagree with those reported by Cockle *et al.* (1980), they are in good agreement with the transmembrane model proposed by Stoffel *et al.* (1982). According to these authors, all the half-cystine residues sequenced (except for two) are located in hydrophilic loops outside the lipid bilayer and are therefore accessible to reagents. (ii) One has to be exceedingly cautious when studying protein–lipid interactions in model membranes and using the Folch-Pi protein, since the conformation seems to depend strongly on the history of the protein. The accessibility and reactivity of thiol groups constitute an excellent structural probe in this respect. (iii) Although it is uncertain whether the thiol groups are in some way involved in ionophoric functions of the protein, it is quite reasonable to assume that the most reactive will be the most likely to react with heavy metals, for example, or with neurotoxic drugs. This possibility remains to be investigated.

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