Eur Biophys J (1992) 21, 169–178

European Biophysics Journal

Thermal stability of bovine-brain myelin membrane

J. Ruiz-Sanz [1], J. Ruiz-Cabello [2], O. Lopez-Mayorga [1], M. Cortijo [2], and P. L. Mateo [1,*]

[1] Departamento de Química Física (Facultad de Ciencias) e Instituto de Biotecnología, Universidad de Granada, E-18071 Granada, Spain
[2] Departamento de Química Física II, Facultad de Farmacia, Universidad Complutense de Madrid, E-28039 Madrid, Spain

Received November 11, 1991/Accepted in revised form May 7, 1992

Abstract. The thermal behaviour of bovine-brain myelin membrane has been studied by high-sensitivity differential scanning calorimetry, Fourier-transform infrared spectroscopy and thermal gel analysis. Spectroscopic results indicate that protein transitions take place between 60 °C and 90 °C, while thermal gel analysis has provided the thermal denaturation profiles of myelin proteolipid, DM-20 protein and the Wolfgram Fraction. An irreversible calorimetric transition centred at 80.3 ± 0.2 °C with a specific enthalpy of 4.7 ± 0.6 J/g of total protein has been assigned to the thermal denaturation of myelin proteolipid and DM-20 protein. The effects of the myelin storage conditions, scan rate, ionic strength and pH on this calorimetric transition have also been investigated. The thermal transition of the proteolipid practically disappears after treatment of the myelin with different amounts of chloroform-methanol 2:1 (v/v), a treatment which is generally used in proteolipid purification. On the other hand, the addition of several detergents to myelin only causes minor modifications to this transition, which then occurs at about 70 °C, with a specific enthalpy of between 2.5 and 3.6 J/g of total protein. These results appear to show that detergents preserve the native conformation of the proteolipid far more than do organic solvents. Hence the use of detergents would seem to be the appropriate method for proteolipid purification.

Key words: Myelin – Proteolipid stability and denaturation – Differential scanning calorimetry – Thermal gel analysis – Fourier-transform infrared spectroscopy

Introduction

The thermal stability of proteins has become a subject of increasing interest over the last few years, both from an academic (Baldwin and Eisenberg 1987; Pace 1990; Jaenicke 1991; Creighton 1991) and from a technological point of view (Klibanov and Ahern 1987; Volkin and Klibanov 1989; King 1989; Fagain and O'Kennedy 1991). Differential scanning calorimetry (DSC) has been shown to be a particularly appropriate method for characterizing the energetics of the thermal stability of proteins by following their thermal denaturation (Privalov 1979, 1982; Mateo 1984; Sturtevant 1987; Freire et al. 1990). With membrane proteins this type of study is comparatively more complicated since the presence of several, often complex, protein systems makes it very difficult to assign the thermal data to individual components. Therefore, many fewer studies have been made on biomembranes than on water-soluble proteins (Sánchez-Ruiz and Mateo 1987). Nevertheless, the use of complementary techniques, especially that of thermal gel analysis (TGA), may overcome some of these difficulties (Rigell and Freire 1987; Morin et al. 1990).

Myelin membrane plays a crucial role in nervous transmission and its malfunction is related to human diseases such as multiple sclerosis and severe encephalomyelitis (Morell 1984). The protein content of this biomembrane is one of the lowest (about 30%) and it consists mainly of proteolipid (PLP) and basic protein (MBP) and, to a lesser extent, the protein DM-20 and the Wolfgram Fraction (WF). PLP is an intrinsic, very hydrophobic protein of 276 amino acid residues, while MBP is an extrinsic, very basic protein of 169 residues. The sequences of both are known (Lees and Brostoff 1984). DM-20 is merely a PLP molecule with an internal deletion of 35 residues (Trifilieff et al. 1986), while WF designates three protein components with a molecular weight between 46 000 and 60 000. The relative proportions of these proteins in our myelin samples are about 42% of both PLP and MBP, and 8% of both DM-20 and WF (Ruiz-Sanz et al. 1992). The exact biological function of

* *Correspondence to.* P. L. Mateo

Abbreviations: DSC, Differential scanning calorimetry; TGA, Thermal gel analysis; FTIR, Fourier-transform infrared spectroscopy; PLP, Proteolipid protein; MBP, Myelin basic protein; DM-20, Protein DM-20; WF, Wolfgram fraction; BSA, Bovine serum albumine; SDS, Sodium dodecyl sulfate; ANSA, 4-amino-3-hydroxynaphthalene-1-sulphonic acid; OG, β-D-glucopyranoside; PAGE, Polyacrylamide gel electrophoresis; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CNS, Central nervous system.

these proteins is still to be ascertained. Several conformational and protein-lipid interaction studies have already been carried out with PLP and MPB, purified using organic solvent and/or acidic media and reconstituted in natural or synthetic lipid systems (Boggs et al. 1982a, b; Mateo et al. 1986; Surewicz et al. 1987a, b; Goñi et al. 1988; Carmona et al. 1988; Sankaram et al. 1991). The few previous DSC studies of myelin membrane have shown thermal transitions that have been assigned by different authors to protein-lipid interactions, protein-protein interactions or lipid transitions (Moscarello et al. 1983; Johnston and Chapman 1988).

We have undertaken here a more systematic DSC investigation into the thermal stability of bovine-brain myelin proteins under different experimental conditions. Parallel Fourier-transform infrared spectroscopy (FTIR) and TGA experiments have allowed us to obtain the thermal profiles for PLP, DM-20 and WF, and to assign the irreversible DSC transition detected at $80.3 \pm 0.2\,^{\circ}C$ principally to PLP denaturation. This transition disappears if the myelin is pretreated with a small proportion of organic solvent, while treatment with detergent only causes a moderate decrease in the thermal stability of PLP. Finally, some comments are made on the use of detergents as the appropriate method for purifying myelin proteins.

Experimental

Materials

Fresh calf brains were obtained from a local slaughterhouse and were used immediately. Acrylamide, Coomassie Brilliant Blue R, Tris, the weight standard protein kit for electrophoresis, BSA sodium cholate, Triton X-100, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps), and n-octyl β-D-glucopyranoside (octylglucoside) were bought from Sigma. Temed and SDS came from Fluka, while Folin-Ciocalteou's reagent, ANSA, sucrose, sodium phosphate, glycine and sodium acetate were from Merck. All the other chemicals and organic solvents were of analytical grade. Distilled, deionized water was used throughout.

Myelin was isolated from calf-brain white matter as described by Toews et al. (1976), but using 20 mM phosphate buffer, pH 7.0, instead of water to wash the myelin membranes after the last sucrose centrifugation. X-ray diffraction and electron microscopy patterns of the myelin thus obtained were similar to those already published for this membrane (Morell 1984). Protein concentration was determined by Lowry's method (Lowry et al. 1951), modified according to Wang and Smith (1975) and using BSA as standard.

Methods

Calorimetric measurements were carried out using either a DASM-1M or a DASM-4 high-sensitivity differential scanning microcalorimeter with 0.96 and 0.47 mL cells respectively (Privalov et al. 1975; Privalov 1980), at a scan rate of 1 K/min unless otherwise stated. Both calorimeters were interfaced to microcomputers equipped with a DT 2801 single board analog and digital I/O systems from Data Translation for automatic data collection. DSC transitions were corrected for the instrumental and chemical base-lines as described by Galisteo et al. (1991). Reversibility of the transitions was checked by reheating the samples after the first run. The specific calorimetric enthalpy of the transitions, Δh, was obtained by a calibrated integration of the endotherms. Protein concentration in myelin samples for DSC runs was in the range 2.5 to 11 mg/mL.

Prior to DSC experiments the myelin samples were either dialyzed against the appropriate buffer or underwent successive centrifugation and resuspension processes in the desired medium. No calorimetric difference was detected between samples prepared by these methods. To study the effect of organic solvents on myelin the required volume of the chloroform/methanol mixture (2:1, v/v) was added to the myelin suspension in phosphate buffer, pH 7.0. After mixing, the sample was lyophilized overnight and then resuspended in phosphate buffer for the DSC scanning. The effect of detergents on the thermal behaviour of myelin was investigated by adding the appropriate volume of detergent solution to the myelin suspension (both in phosphate buffer, pH 7.0) in order to obtain a final concentration of 3.5 to 4.5 mg of total protein/mL and the desired concentration of detergent. The effect was also studied after the removal of the detergents from the myelin. To this end the above mixture was incubated at 4 °C for 90 minutes with gentle agitation and then dialyzed against phosphate buffer; aliquots were taken at increasing dialysis times for the DSC runs. Radioactive detergent was used in parallel dialysis experiments to check detergent elimination. ^{14}C-OG (from New England Nuclear) radioactivity was measured in a Betamatic instrument from Beckman using a 22-Normascint from Scharlan.

Samples for FTIR experiments were placed in a thermostatically controlled Specac 20 500 cell for liquids, fitted with CaF_2 and with a spacer thickness of 6 μm. Myelin suspensions were centrifuged and resuspended in 20 mM phosphate buffer, pH 7.0, to obtain a total protein concentration of around 10 mg/mL (about 23 mg of lipid/mL). FTIR spectra were recorded using a 20 sxb Nicolet instrument equipped with a TGS detector. For each spectrum 400 interferograms were collected, co-added, apodized with a Happ-Genzel function and Fourier transformed to give a resolution of 2 cm^{-1}. Water contribution was subtracted by keeping a straight line between 1900–1800 cm^{-1} using the highest possible subtraction factor. To eliminate the spectral contribution from atmospheric water vapour, the instrument was continuously purged with air dried in an Ecodry adsorption desiccator. Some of the FTIR experiments were also carried out with a Nicolet 10 DX spectrometer at the Basque Country University (Bilbao). Fourier deconvolution and fourth derivative procedures were used to resolve overlapping infrared bands (Kauppinen et al. 1981; Surewicz and Mantsch 1988).

Thermal gel analysis experiments were based on the procedure described by Rigell and Freire (1987) and Morin et al. (1990). A similar procedure was initially used by Lysko et al. (1981) to study membrane proteins in red blood cells. This technique takes advantage of the fact that some integral membrane proteins or protein subunits may show different detergent solubility between their native and denaturated states (Rigell et al. 1985). We have been able to find two sets of experiment conditions under which the native state of some of the myelin proteins can be solubilized, but not their denaturated state (see Results). It has then been possible to follow the differential solubility of individual myelin proteins by a controlled heating of the sample, to check the native fraction as a function of temperature by electrophoresis, and thus obtain the thermal denaturation profiles of these proteins. Our general TGA method was as follows: 90 µL aliquots of myelin suspension were put into Eppendorf microfuge tubes and immersed in a thermally isolated water bath. The bath temperature was then linearly increased at a scan rate of 1 K/min and monitored by a bead thermistor in an additional tube filled with buffer solution (López-Mayorga 1983). At each desired temperature tubes were removed from the bath and immediately cooled in ice-water. 30 µL of buffer containing cholate or Triton X-100 at a determined concentration were then added to each tube, which was briefly shaken and bath sonicated for 45 to 90 s. The samples were centrifuged for 15 minutes in a Heraeus haemofuge at 4 °C. 50 µL of supernatant were removed from each tube and combined with 50 µL of electrophoresis sample buffer and also 10 µL of carbonic anhydrase (0.5 mg/mL) in buffer solution for use as an internal concentration standard. The mixture was incubated for 30 min at room temperature and 40 µL of the resulting samples were then loaded into each lane of the polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinous buffer system described by Laemmli (1970) with a 12% acrylamide separation gel and a 4% acrylamide stacking gel. Once the electrophoresis has been run the gels were stained in a 4:5.3:0.7 methanol/water/acetic-acid mixture containing 0.05% Coomassie Brilliant Blue R. Destaining took place overnight in 4:5.3:0.7 methanol/water/acetic-acid solution. Destained gels were dried with 4:5:1 methanol/water/glycerol and plastified for densitometry of the different lanes in a Beckman DU-70 spectrophotometer. The data was analysed with software developed in our laboratory (see Results).

Results

Calorimetry

DSC of myelin in 20 mM phosphate buffer, pH 7.0, gives rise to two main transitions centred at about 30 °C and 80 °C (Fig. 1 A). The former is only partially reversible on reheating the sample (Fig. 1 A) and the shape of this second-heating endotherm resembles that found with the pool of lipids extracted from myelin (results not shown). The latter transition is irreversible and has a small shoul-

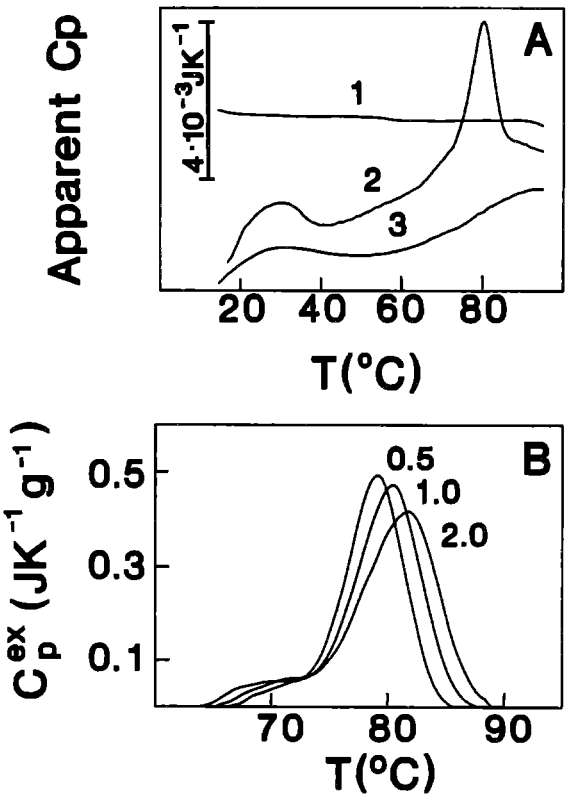

Fig. 1 A, B. (**A**) Original calorimetric recordings of (1) base line, (2) native myelin, 9.2 mg of protein/mL, in 20 mM phosphate buffer, pH 7.0, (3) reheating of (2). Scan rate 1 K/min. (**B**) Excess heat capacity of myelin at 2.0, 1.0 and 0.5 K/min

der at 70 °C. In somes cases a much smaller and broader irreversible transition has also been detected at around 55 °C. The temperature positions of these three transitions are similar to those reported by Moscarello et al. (1983). Since we are particularly interested at this point in characterizing the thermal stability of myelin protein we have focused our attention on the effects of several different conditions on the endotherm at 80 °C, which corresponds principally to myelin proteolipid thermal denaturation (see below). Thus, the transition temperature, Td, is actually 80.3 ± 0.2 °C and the specific enthalpy, Δh, 4.7 ± 0.6 J per g of total protein. This transition is not only calorimetrically irreversible on reheating the sample but also rate-limited, i.e. the Td value depends on the scan rate used (Fig. 1 B), although it does not quantitatively follow the predictions of the two-state kinetic model (Sánchez-Ruiz et al. 1988). When heating myelin in the DSC up to 70 °C and reheating the sample after cooling it to 20 °C, the shoulder at about 70 °C disappears while the main transition at 80 °C remains practically unchanged; so the transition at 80 °C and its low temperature shoulder seem to correspond to independent thermal transitions. In this case the single remaining 80 °C transition does not follow the two-state kinetic model either (results not shown).

The transition at 80 °C is not appreciably affected after keeping myelin for two days at 4 °C, but the transition enthalpy clearly begins to decrease after one week to reach about half its initial value after two weeks at this

temperature. Lyophilization of myelin leads to a DSC transition at 70 °C with an enthalpy of 1.6±0.3 J/g (Fig. 2A). Freezing myelin to −20 °C even for only three hours causes a decrease in the transition at 80 °C and an increase in the size of the shoulder at 70 °C; this effect increases with the freezing time and after about one month only a lower enthalpy transition, at about 71 °C remains (Fig. 2B). Therefore, all the subsequent experiments reported here with myelin were carried out with samples stored in the cold room at 4 °C for less than 48 hours.

The DSC transition is not noticeably affected by an increase in ionic strength up to 0.5 M in KCl but it is clearly modified by acidic media. Thus, at both 20 mM glycine, pH 3.0, and 20 mM acetate, pH 5.0, the transition becomes much smaller (about 0.5 J/g) with Td values of 70 °C and 80 °C respectively. The pH 5.0 effect can be reversed, i.e. a transition similar to that of native myelin is recovered by returning the sample to phosphate buffer, pH 7.0. The pH 3.0 effect is not reversible, however, and the very small 70 °C transition remains on returning to pH 7.0.

Since myelin proteolipid has usually been purified using organic solvents (Folch-Pi and Lees 1951; Monreal 1975; Brophy 1977) we have studied the effect on the 80 °C transition of different quantities of chloroform-methanol 2:1 (v/v) added to myelin phosphate buffer, pH 7.0. Figure 3 shows that the organic mixture almost eliminates the transition even at a phosphate buffer/organic mixture ratio of 10:1 (v/v) after removing the organic solvents before the DSC experiment. Note that the reference transition in Fig. 3 is now at 70 °C since all the samples were lyophilized and suspended in phosphate buffer for the DSC experiments.

The use of detergents is also a common procedure to purify membrane proteins (Tanford and Reynolds 1976), so we have looked into the effect of some detergents on the 80 °C DSC transition. The effect of different amounts of sodium cholate is shown in Fig. 4A, where both the Td and enthalpy values of the thermal transition decrease concomitantly with an increase in the detergent content. Extensive dialysis to eliminate the detergent leads once more to an 80 °C transition for initial cholate concentrations of 0.2% (w/v) or below, while at initial detergent concentrations of 0.4% (w/v) or above the remaining DSC transition after dialysis appears at about 70 °C (results not shown). We also obtained several myelin DSC endotherms with 2% (w/v) octylglucoside (OG) following the increasing removal of detergent by dialysis against phosphate buffer for different dialysis times (Fig. 4B). The elimination of detergent during dialysis was checked by using radioactive ^{14}C-OG (see Experimental), 37.2%, 3.4% and <0.1% values being obtained for the remaining radioactivity after 4.5, 18.0 and 41.0 hours respectively, changing the dialysis bath at the two former times (Fig. 4B). The Td value of the transition after detergent elimination was about 71 °C. The same result was also obtained in a parallel experiment with one other detergent, Chaps (results not shown). The specific enthalpy corresponding to the myelin transitions at 70–71 °C, obtained after the removal of OG or Chaps, ranged from 2.5

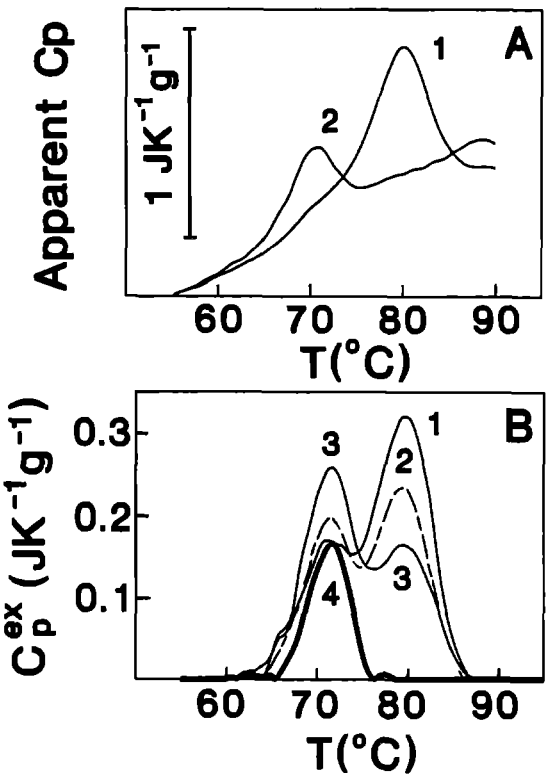

Fig. 2 A, B. (A) Normalized calorimetric recordings of (1) myelin stored in the cold room for 1 day and (2) lyophilized myelin. (B) Excess heat capacity of myelin after being frozen at −20 °C for (1) 2 days, (2) 7 days, (3) 22 days and (4) 36 days. Conditions: 20 mM phosphate buffer, pH 7.0, scan rate 1 K/min

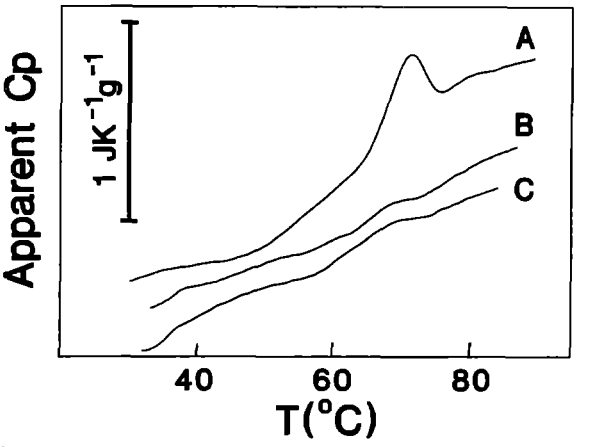

Fig. 3. Effect of the chloroform-methanol 2:1 (v/v) mixture on the thermal stability of myelin. Normalized DSC recordings of myelin after being pretreated with different proportions (v/v) of phosphate buffer/organic mixture: (A) 1:0, (B) 10.1 and (C) 5:1. Before the calorimetric recordings the samples were lyophilized and then resuspended in phosphate buffer, pH 7.0. Scan rate 1 K/min

to 3.6 J per g of protein. Whenever we obtained this transition after detergent elimination the left-side shoulder, which is present with native myelin (Fig. 1), was absent.

FTIR spectroscopy

Figure 5 shows the original FTIR spectrum after solvent subtraction, the fourth derivative and the deconvolved

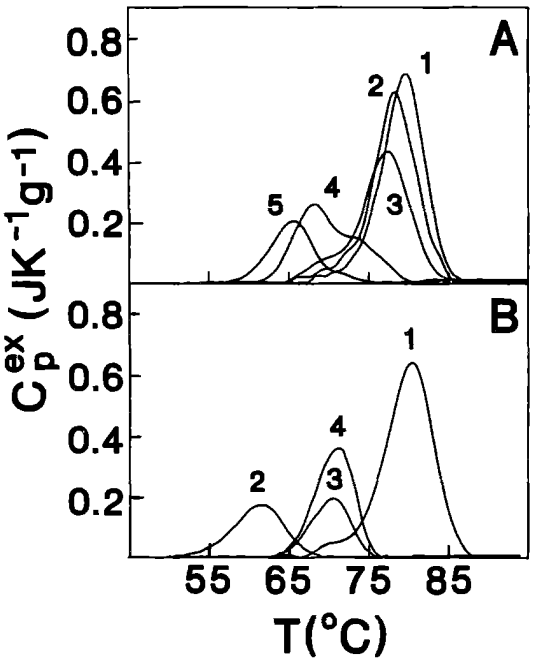

Fig. 4 A, B. Effect of detergents on the thermal stability of myelin in 20 mM phosphate buffer, pH 7.0. (**A**) Excess heat capacity of myelin containing different concentrations (w/v) of sodium cholate: (1) 0%, (2) 0.1%, (3) 0.2%, (4) 0.4% and (5) 0.6%. (**B**) Excess heat capacity of (1) native myelin, and of myelin with 2% (w/v) of octylglucoside after different dialysis times: (2) 4 hours, (3) 18 hours and (4) 41 hours. Scan rate 1 K/min

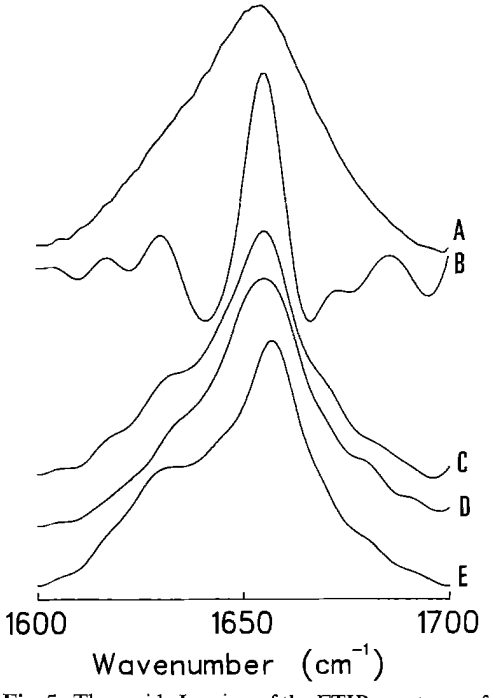

Fig. 5. The amide I region of the FTIR spectrum of myelin in 20 mM phosphate, pH 7.0. (A) Original spectrum at 20 °C after solvent subtraction. (B) Fourth derivative of (A). (C) Band narrowing by Fourier deconvolution of (A) at 20 °C. (D) The same as (C) at 60 °C. (E) The same as (C) at 20 °C after heating the sample to 90 °C

amide I region of myelin suspension in 20 mM phosphate buffer, pH 7,0 at 20 °C (Curve C). The characteristic α-helix and β-structure components can be seen at 1655 and 1629 cm^{-1} (Dong et al. 1990). The minor contribution at 1686 cm^{-1} is ascribed to the β-turn structure (Dong et al. 1990), or together with that at 1629 cm^{-1} to an antiparallel β-structure (Rial et al. 1990) (Fig. 5). Similar results have been reported by Ayala et al. (1987) using FTIR and Raman spectroscopy with myelin in the presence of OG at pH 7.4. In our case the α-helix is the main component, altough with a large β-structure contribution, probably due to MBP, which is known to have more than 50% β-structure in a lipid medium (Surewicz et al. 1987b). Further quantitation of the FTIR spectrum was not attempted owing to the cerebroside absorption in this region.

There were no significant temperature-dependent changes in the FTIR spectrum of the amide I region of myelin up to 60 °C. Heating to 90 °C and cooling to 20 °C, however, generates pronounced changes with a large increase in the 1629 cm^{-1} component. This has been assigned to the denaturation of some membrane proteins (Arrondo et al. 1988, Prado et al. 1990). Hence, it seems that the thermal transition of myelin protein take place mainly between 60 °C and 90 °C, with minor protein modifications at temperatures below 60 °C. This can be correlated with the DSC transition centred at 80 °C.

The spectral region between 2800 cm^{-1} and 3000 cm^{-1} has also been investigated by FTIR as a function of temperature (Fig. 6). The symmetric (2850 cm^{-1}) and asymmetric (2920 cm^{-1}) stretching vibrations of the C–H bonds, related to the static order of the hydrophobic chains in the membrane lipids (Cameron and Mantsch 1978; Cortijo et al. 1982; Brauner and Mendelson 1986; Jackson et al. 1989), shift to higher wavenumbers in a continuous manner between 20 °C and 70 °C (Fig. 6). The shift in the vibration wavenumbers is reversible, since its initial value at 20 °C is recovered after cooling the myelin sample heated to 90 °C back down to 20 °C (Fig. 6). These wavenumbers values indicate that myelin lipids are, at 20 °C, already in a more fluid state than that of the gel phase and that they attain a typical liquid crystal phase at 70 °C (Casal and Mantsch 1984, Jackson et al. 1989). The fact that there is no noticeable change between 70 and 90 °C confirms that the 80 °C DSC endotherm in myelin corresponds to a protein transition.

Thermal gel analysis

Thermal gel analysis (TGA) is a technique based on the differing detergent solubility of native and denaturated membrane proteins and is able, in principle, to distinguish between the thermal behaviour of the several proteins or protein subunits in a given biomembrane (Rigell and Freire 1987; Morin et al. 1990). The technique is thus a most appropriate complement for the DSC results of such a system. In order, therefore, to assign the DSC endotherms of myelin to particular myelin proteins different experimental conditions for carrying out thermal gel

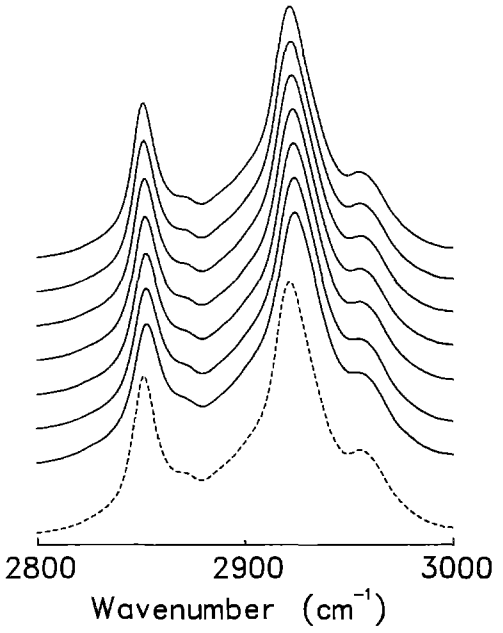

Fig. 6. FTIR spectra between 2800 and 3000 cm^{-1} of myelin in 20 mM phosphate, pH 7.0, at different temperatures. Temperature values from top to bottom are 20, 30, 40, 50, 60, 70 and 80 °C. Dashed line corresponds to the spectrum at 20 °C of myelin previously heated to 90 °C

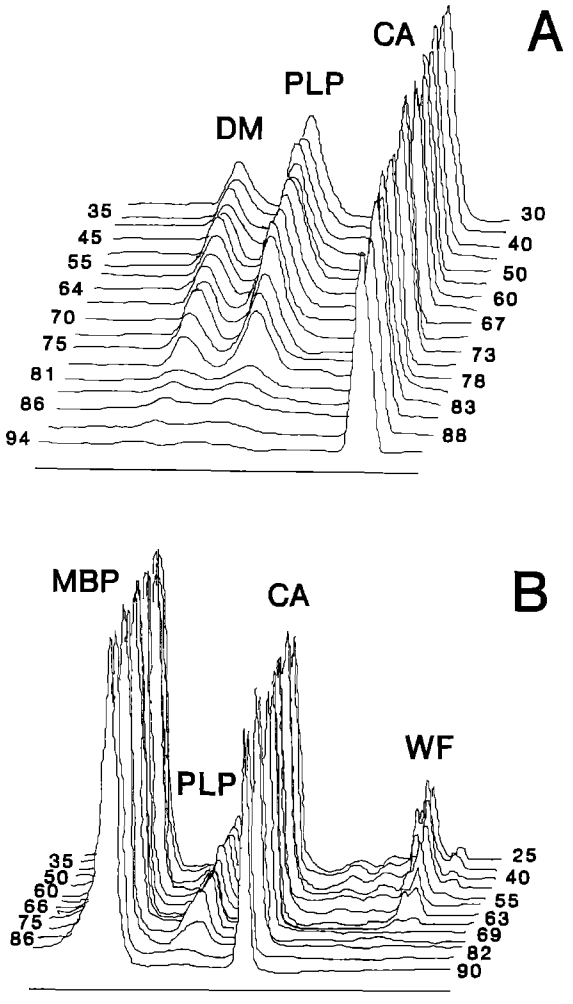

Fig. 7 A, B. Densitometry traces of the thermal gel analysis of myelin at different temperatures using either (**A**) Triton X-100 or (**B**) sodium cholate for the differential solubility of myelin proteins as explained in the text. Temperature values (°C) are included in the Figure. DM, protein DM-20; PLP, myelin proteolipid; MBP, myelin basic protein; WF, Wolfgram Fraction; CA, carbonic anhydrase added as an internal concentration standard

analysis of the membrane were tried. Several detergents, detergent concentrations, sonication times and other modifications were used to obtain the appropriate solubilization conditions (see Experimental). In this way Triton X-100 and sodium cholate were chosen as suitable detergents to characterize the thermal denaturation profiles of PLP, DM-20 and WF. We were unable, however, to find conditions under which the detergent solubility of native and denaturated MBP differed. Triton X-100 has been shown to provide suitable conditions for the differential solubilization of both PLP and DM-20, as does sodium cholate for PLP and the WF. In the former case 30 µL of a 3% (w/v) Triton X-100 solution in phosphate buffer were added at 4 °C to 90 µL of a myelin sample which had been heated previously at 1 K/min to a given temperature. After sonication for 45 s and subsequent centrifugation for 15 min, the detergent-solubilized proteins were subjected to SDS-PAGE and the gels to densitometry (see Experimental). Figure 7A shows a set of densitometry profiles obtained from SDS-PAGE of the detergent-soluble proteins after incubation of myelin at the desired temperature. Carbonic anhydrase, used as a concentration standard to normalize the densitometry data (Rigell and Freire 1987), was added after the centrifugation of the detergent-solubilized samples. Under these experimental conditions it is clear that there is a decrease in both the PLP and DM-20 peak areas between 70 °C and 90 °C, while there is no noticeable solubilization of MBP or WF (Fig. 7A).

In the case of sodium cholate 30 µL of a 12% (w/v) detergent solution in phosphate buffer was added to 90 µL of the previously heated myelin solution. The mixture was sonicated for 1 min and then centrifuged for 15 min. SDS-PAGE was again carried our with the detergent-solubilized proteins, to which carbonic anhydrase had also been added, and densitometry of the gels led to the results shown in Fig. 7B. Under these new conditions the PLP peak area decreases in the same temperature range as with the Triton X-100 method, that of WF decreases between 45 °C and 70 °C, there is no evidence for solubilization of the DM-20 protein, and MBP is apparently always solubilized (Fig. 7B).

It is now quite straightforward to get the population fraction of the native protein state at each temperature from the normalized peak areas (Rigell and Freire 1987). Thus Fig. 8A shows plots of the fraction of the native states of PLP, DM-20 and WF as a function of temperature. The curve shapes are characteristic of temperature-induced transitions. The melting temperature, T_m, at which the native fraction equals 1/2 can be obtained from the plots in Fig. 8A; they are 80.6 °C, 80.5 °C and 62.2 °C for PLP, DM-20 and WF respectively. According to these

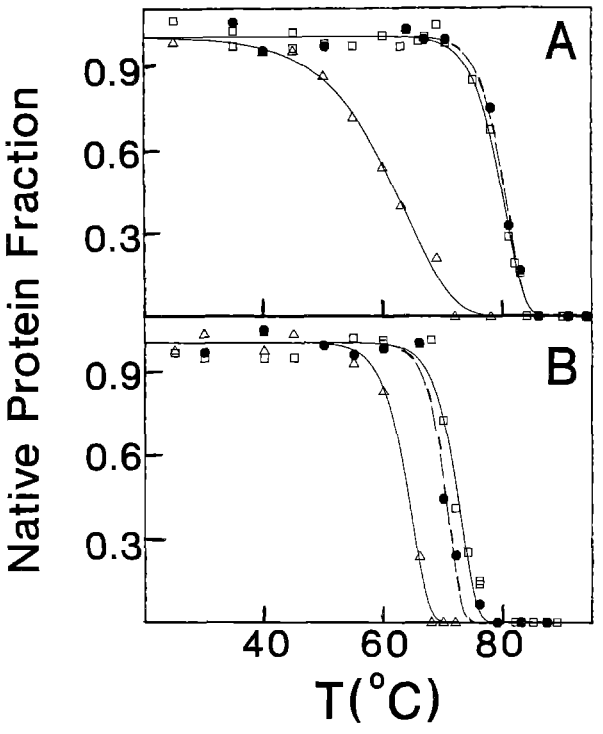

Fig. 8 A, B. Temperature denaturation profiles of myelin proteins obtained from thermal-gel-analysis experiments. Data values are obtained from the normalized peak areas of the densitometry traces. **(A)** Values obtained for native myelin from the densitometry results of Fig. 7. **(B)** Corresponding values obtained for myelin pretreated with 2% (w/w) octylglucoside (see text). Fittings were carried out according to the equation in the text. (□) Myelin proteolipid; (●) protein DM-20; (△) Wolfgram Fraction. Solid lines: myelin proteolipid and Wolfgram Fraction; dashed line: protein DM-20

results both PLP and DM-20 undergo thermal denaturation at about 80 °C, which can be related to the 80 °C DSC endotherm, since no observable lipid transition has been detected around that temperature in the FTIR results set out above.

As we have already said, the 80 °C DSC endotherm becomes a 70 °C endotherm under a variety of conditions. Therefore, to find out whether this 70 °C transition can also be related to the PLP and/or DM-20 thermal denaturation, additional TGA experiments were carried out (using either Triton X-100 or sodium cholate) with myelin samples that had been pretreated with 2% (w/v) OG, which was then removed by extensive dialysis under similar conditions to those described in Fig. 4 B. In this case, however, the sonication time was 90 s when using Triton X-100, while the sodium cholate concentration was 6% (w/v); the other experimental conditions were the same as those described for Fig. 7. These TGA results prove that while the WF is thermally denatured between 50 °C and 70 °C, both PLP and DM-20 undergo thermal denaturation at around 70 °C. Thus, Fig. 8 B shows the population fractions of the native state of these three proteins as a function of temperature, which lead to T_m values of 72.3 °C, 71.5 °C and 64.0 °C for PLP, DM-20 and WF respectively. The former two values agree with that of the irreversible DSC transition, 71 °C, detected in myelin pretreated with OG (Fig. 4 B). Thus, both DSC transitions at about 80 °C and 70 °C found with native myelin and with detergent-treated myelin (and probably the one at 70 °C which we have also reported here for native myelin after freezing or lyophilization (Fig. 2)) seem to correspond to the thermal denaturation of PLP and DM-20. The thermal stability of these two proteins would similarly depend then on certain of the storage conditions and detergent effects. This equivalent thermal behaviour of PLP and DM-20 is to be expected given their amino-acid sequence analogy (Nave et al. 1987), similar physical-chemical properties (Lees and Brostoff 1984; Trifilieff et al. 1986) and the consequent difficulty in purifying them separately (Helynck et al. 1983, Ross and Braun 1988; Ruiz-Sanz et al. 1991).

The curve fitting in Fig. 8 has been made according to

$$X\,n = \exp(-\exp[E(T - T_m)/R\,T_m^2])$$

where X n stands for the native protein fraction and E for the activation energy of the transition process. This equation is easily deduced from the two-state kinetic model reported by Sánchez-Ruiz et al. (1988). Although the irreversible DSC endotherm for myelin does not follow this model exactly (see above), the equation used is the closest one for an irreversible, scan-rate-dependent transition. Nevertheless, it should also be pointed out that non-linear least-square fittings of the set of data in Fig. 8 B to the two-state reversible model lead to similar sigmoidal curves and the same T_m values (results not shown).

Discussion

Investigation into the thermal behaviour of myelin membrane have generally been directed towards the phase transitions of the lipids or lipid fractions present in the membrane (Ladbrooke et al. 1968; Johnston and Chapman 1988), as is usual with the majority of biomembranes (Sánchez-Ruiz and Mateo 1987). Our high-sensitivity DSC results, complemented by those of FTIR and, particularly, TGA, have allowed us to characterize the thermal stability of myelin proteins, except for MBP.

A few papers have been published on DSC studies into myelin, for example that by Moscarello et al. (1983) with human CNS myelin, where no enthalpy data were reported, and those of Chapman and co-workers (Ladbrooke et al. 1968; Johnston and Chapman 1988) with myelin from ox brain and from guinea pig CNS, where no transition was assigned to the myelin proteins. The general features of our DSC results for native myelin (Fig. 1 A) agree on the whole with those of Moscarello et al. (1983) and Johnston and Chapman (1988), i.e. there are two main endothermic transitions, at 30 °C and 80 °C, and a much smaller and broader one at about 55 °C, which can hardly be seen in our experiments and that is not reported by Johnston and Chapman (1988). Moscarello et al. (1983) assigned the low-temperature transition to proteolipid simply on the basis that they had previously assigned the high-temperature transition to MBP, and assumed that the two main DSC endotherms should correspond to the two major myelin proteins, PLP and MBP. Our FTIR experiments, however, show that modifications in the amide I region occur mainly between 60 °C

and 90 °C (Fig. 5), while the TGA results clearly prove that PLP and DM-20 undergo thermal denaturation at around 80 °C (Fig. 7 A and 8 A). Thus the DSC transition at this temperature must now be assigned principally to the denaturation of PLP, together with that of DM-20 (Fig. 7 A and 8 A). Moscarello et al. (1983) concluded that the high temperature DSC transition involved MBP because extraction of MBP at pH 3.2 abolished that transition but not the 30 °C one. We have also shown here, however, that both at pH 3.0 and pH 5.0 the 80 °C transition becomes an almost negligible one with an enthalpy of about 0.5 J/g; the effect of pH 5.0 is reversible on bringing the myelin sample back to pH 7.0, while that at pH 3.0 is completely irreversible (see Results).

A similar effect of pH on this transition was also reported by Moscarello et al. (1983). To check whether our acid treatment might also involve the loss of MBP from the membrane, quantitative SDS-PAGE of these samples showed that the MBP/PLP ratio did not change at pH 5.0 and decreased only about 10% at pH 3.0. When the samples at pH 5.0 and 3.0 were brought back to pH 7.0, these ratios had in fact decreased by about 30% and 45%. Nevertheless, the DSC transition of the former was equal to that of native myelin at this pH, while in the latter case there was no recovery of the transition. Thus it seems that it is precicely the acid treatment which is mainly responsible for the disappearance of the 80 °C transition by reversibly (pH 5.0) or irreversibly (pH 3.0) affecting the native proteolipid conformation. On the other hand, according to Moscarello et al. (1983) the elimination of MBP does not affect the 30 °C transition, while we have seen in our TGA experiments that the thermal transitions of PLP, DM-20 and WF (the other major protein components of myelin) take place at higher temperatures (Fig. 8). The 30 °C endotherm, therefore, ought to be assigned principally to a lipid transition. This is in accord with Johnston and Chapman (1988), who propose that this endotherm corresponds to the melting of a galactocerebroside gel phase. In fact, their enthalpy value for this transition, 1.1 J/g of myelin, is similar to our own, 0.8 ± 0.2 J/g of myelin.

Finally, the very small, broad DSC transition we have sometimes seen at around 55 °C has also been reported as a very small one at 61–62 °C by Moscarello et al. (1983), although it was not mentioned by Johnston and Chapman (1988), perhaps owing to the lower sensitivity of their DSC. The former authors tentatively related this endotherm to a lipid phase transition. Our FTIR results, however, show only a smooth change in the wavenumbers of the symmetric and asymmetric stretching vibrations of the C–H bonds between 20 °C and 70 °C (Fig. 6). On the other hand, this DSC transition temperature compares with that of the WF transition detected here by TGA (Fig. 8).

The overall FTIR data in the amide I region show α-helix structure as the main component, as well as a large β-structure contribution, which may well be due to MBP, a protein known to have more than 50% of this structure in lipid media with only 15% of α-helix (Surewicz et al. 1987 b). The modifications of the FTIR spectra between 60 °C and 90 °C (Fig. 5), which give rise to a higher β-structure content, are similar to those found in the denaturation of several membrane proteins (Arrondo et al. 1988; Prado et al. 1990). This temperature effect agrees with the TGA results, which show that the T_m values for WF, DM-20 and PLP are 62.2 °C, 80.5 °C and 80.6 °C respectively (Fig. 8 A). The two latter values correlate very well with the high-temperature DSC endotherm (where both TGA and DSC measurements were carried out at the same scan rate), particularly on bearing in mind that there is no evidence of lipid transition from the FTIR results in the 2800–3000 cm^{-1} region (Fig. 6).

The similarity in thermal behaviour of DM-20 and PLP was to be expected, given that the former is merely a PLP molecule with an internal deletion of 35 (from residue 116 to 150) out of 276 amino-acid residues (Nave et al. 1987). Since the proportion of PLP to DM-20 in myelin is about 5/1 (Ruiz-Sanz et al. 1991), the DSC transition at 80 °C must be assigned mainly to thermal denaturation of PLP. On the other hand, we have been unable to find suitable TGA conditions to follow the possible thermal denaturation of MBP, the major protein in myelin together with PLP. This fact might be related to the partially hydrophilic character of this water-soluble, peripheral membrane protein (Boggs et al. 1982 b). A similar TGA situation has been found with the V_a and V_b subunits of mitochondrial cytochrome c oxidase from bovine heart (Rigell and Freire 1987) and with the IV and VI subunits of cytochrome c oxidase from *Saccharomyces cerevisiae* (Morin et al. 1990). In addition MBP does not show any DSC transition in water solution (results not shown) in agreement with the previous results of Moscarello et al. (1983) and the unstructured organization for MBP in water proposed by Surewicz et al. (1987 b) and Gow and Smith (1989).

When native myelin has been previously lyophilized or frozen, the initial 80 °C DSC transition shifts to about 70 °C, though with freezing this shift is gradual and increases with the freezing time (Fig. 2 B). This explains why Moscarello et al. (1983) obtained their transition at 72 °C, since they lyophilized their myelin samples before the calorimetric runs. Similar DSC behaviour can be seen after the removal of various detergents added to native myelin (see Results). To find out whether this new endotherm at 70 °C might also correspond to PLP denaturation, TGA experiments were carried out with detergent-free myelin pretreated with 2% (w/v) OG. The results showed that both PLP and DM-20 undergo thermal denaturation at about 70 °C, while WF does so at 62 °C. Hence, the DSC transition at 70 °C found in detergent-treated myelin, as well as possibly those found in previously lyophilized or frozen myelin samples, do correlate with the thermal denaturation of PLP, and to a lesser extent with that of DM-20. The enthalpy variation between the two transitions, at 80 °C (4.7 J/g of protein) and 70 °C (about 3 J/g of protein), cannot be explained here in terms of a heat capacity effect since in all cases the DSC samples are under the same experimental conditions. Thus, the difference in the samples must lie in their "history" or previous manipulation. A speculative interpretation would be that under certain conditions the native PLP might give rise to another conformation with a

somewhat lower thermal stability (see Fig. 2B). Both denaturation enthalpy values are similar to those found for membrane proteins (Sánchez-Ruiz and Mateo 1987) and lower than those of globular proteins (Privalov 1979), as might be expected.

Since PLP purification procedures normally involve the use of chloroform-methanol mixtures (Folch-Pi and Lees 1951; Monreal 1975; Brophy 1977; Vacher et al. 1984; Bizzozero et al. 1987), we have also checked the effect of these solvents on myelin thermograms after the removal of the organic solvent. The DSC transition at 80 °C irreversibly disappears after treatment with chloroform-methanol (2:1 v/v), even at an organic-mixture/buffer ratio as low as 1/10 (v/v) (Fig. 3). It therefore seems that these organic solvents affect the native conformation of PLP in such a way that after their removal the protein is unable to undergo the co-operative thermal denaturation process that we have found in myelin. On the other hand, crude PLP, i.e. PLP purified using this organic mixture, has ben reported to have a thermal transition at about 60 °C, which Mateo et al. (1986) initially assigned to PLP denaturation. We showed in a later work using DSC and FTIR studies, however, that this transition actually corresponds to a thermal phase transition of the lipids included in the crude PLP (Ruiz-Sanz et al. 1991). Therefore, the absence of any thermal transition in PLP purified by organic solvents casts some doubts on the "quality" of the conformation of this PLP. As a matter of fact, PLP is a protein that has no enzymatic activity or any other known biological function which can be monitored and used as a test of its correct native conformation. We have shown here that PLP undergoes a distinct DSC transition which could be used as an appropriate "pointer" to its characteristic conformation. Furthermore, since we have seen that detergents do not eliminate the DSC transition it would seem to us more suitable to purifiy PLP using an appropriate detergent.

Acknowledgements. This research was supported by Grants PB87-0871 (P.L.M.) and PB87-0083 (M.C.) from the CICYT, Ministerio de Educación y Ciencia (MEC), Spain. J.R-S and J.R-C acknowledge predoctoral fellowships from the MEC, Spain. We also thank Dr. J. L. R. Arrondo and Dr. J. Castresana for helping us in the use of their FTIR equipment at the Basque Country University (Bilbao).

References

Arrondo JLR, Young NM, Mantsch HH (1988) The solution structure of concanavalin A probed by FTIR spetroscopy. Biochim Biophys Acta 952:261–268

Ayala G, Carmona P, Cózar M, Monreal J (1987) Vibrational spectra and structure of myelin membranes. Eur Biophys J 14:219–225

Baldwin RL, Eisenberg D (1987) Protein stability. In: Oxender DL, Fox CF (eds) Protein engineering. Alan R Liss, New York, pp 128–148

Bizzozero OA, McGarry JF, Lees MB (1987) Autoacylation of myelin proteolipid protein with acyl coenzyme A. J Biol Chem 262:13550–13557

Boggs JM, Moscarello MA, Papahadjopoulos D (1982a) Structural organization of myelin. Role of lipid-protein interactions determined in model systems. In: Jost PC, Griffith DM (eds) Lipid-protein interactions. John Wiley & Sons, New York, pp 1–51

Boggs JM, Stamp D, Moscarello MA (1982b) Effect of pH and fatty acid chain length on the interaction of myelin basic protein with phosphatidylglycerol. Biochemistry 21:1208–1214

Brauner JW, Mendelson R (1986) A comparison of differential scanning calorimetric and Fourier transform infrared spectroscopic determination of mixing behavior in binary phospholipid systems. Biochim Biophys Acta 861:16–24

Brophy PJ (1977) Association of proteolipid apoproteins from bovine myelin with phospholipid in bilayer vesicles. FEBS Lett 84:92–96

Cameron DG, Mantsch HH (1978) The phase transition of 1,2-dipalmitoyl-sn-glycero-3-phosphocoline as seen by Fourier transform infrared difference spectroscopy. Biochim Biophys Res Commun 83:886–892

Carmona P, Cózar M, García-Segura LM, Monreal J (1988) Conformation of brain proteolipid apoprotein. Eur Biophys J 16:169–176

Casal HL, Mantsch HH (1984) Polymorphic phase behaviour of phospholipid membranes studies by infrared spectroscopy. Biochim Biophys Acta 779:381–401

Cortijo M, Alonso A, Gómez-Fernández JC, Chapman D (1982) Intrinsic protein-lipid interactions. Infrared spectroscopic studies of gramicidin A, bacteriorhodopsin and Ca^{2+}-ATPase in biomembranes and reconstituted systems. J Mol Biol 157:597–618

Creighton TE (1991) Stability of protein conformations. Cur Opin Str Biol 1:5–16

Dong A, Huang P, Caughey WS (1990) Protein secondary structures in water from second-derivative amide I infrared spectra. Biochemistry 29:3303–3308

Fagain C, O'Kennedy R (1991) Functionally-stabilized proteins. A review. Biotech Adv (in press)

Folch-Pi J, Lees MB (1951) Proteolipid, a new type of tissue lipoproteins, their isolation from brain. J Biol Chem 191:807–817

Freire E, van Osdol WW, Mayorga OL, Sánchez-Ruiz JM (1990) Calorimatrically determined dynamics of complex unfolding transitions in proteins. Ann Rev Biochim Biophys Chem 19.159–188

Galisteo ML, Mateo PL, Sánchez-Ruiz (1991) Kinetic study on the irreversible thermal denaturation of yeast phosphoglycerate kinase. Biochemistry 30:2061–2066

Goñi FM, Cózar M, Alonso A, Durrani AA, García-Segura LM, Lee DC, Monreal J, Chapman D (1988) Lipid-protein interaction. The incorporation of myelin proteolipid into phosphatidylcholine bilayers. Eur J Biochem 174: 641–646

Gow A, Smith R (1989) The thermodynamically stable state of myelin basic protein in aqueous solution is a flexible coil. Biochem J 257:535–540

Helynck G, Luu B, Nussbaum JL, Picken D, Skalidis G, Trifilieff E, Dorsselaer AV, Seta P, Sandeaux R, Gavach C, Heitz F, Simon D, Spach G (1983) Brain proteolipids. Isolation, purification and effect on ionic permeability of membranes. Eur J Biochem 133:689–695

Jaenicke R (1991) Protein folding: local structures, domains, subunits and assemblies. Biochemistry 30:3147–3161

Jackson M, Haris PI, Chapman D (1989) Fourier transform infrared spectroscopic studies of lipids, polypeptides and proteins. J Mol Struct 214:329–355

Johnston DS, Chapman D (1988) A calorimetric study of the thermotropic behaviour of mixtures of brain cerebrosides with other brain lipids. Biochim Biophys Acta 939:603–614

Kauppinen JK, Moffat DJ, Mantsch HH, Cameron DG (1981) Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. Appl Spectrosc 35:271–276

King J (1989) Deciphering the rules of protein folding. Chem Eng News 67:32–54

Klibanov AM, Ahern TJ (1987) Thermal stability of proteins. In: Oxender DL, Fox CF (eds) Protein engineering. Alan R Liss, New York, pp 213–218

Ladbrooke BD, Jenkinson TJ, Kamat VB, Chapman D (1968) Physical studies of myelin. I. Thermal analysis. Biochim Biophys Acta 164:101–109

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lees MB, Brostoff SW (1984) Proteins of myelin. In: Morell P (ed) Myelin. Plenum Press, New York, pp 197–224

López-Mayorga O (1983) Development, characterization and applications of electronic control instruments for measuring flow microcalorimetry. Ph D Thesis, University of Granada, Spain

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lysko KA, Carlson R, Taverna R, Snow J, Brandts JF (1981) Protein involvement in structural transitions of erythrocyte ghosts. Use of thermal gel analysis to detect protein aggregation. Biochemistry 20:5570–5576

Mateo PL (1984) Differential scanning calorimetry applications to protein solutions. In: Silva MR (ed) Thermochemistry and its applications to chemical and biochemical systems. Reidel, Holland, pp 541–568

Mateo PL, López-Lacomba JL, Moreno MC, Cózar M, Cortijo M, Monreal J (1986) Reversible thermal transition of brain myelin proteolipid. A preliminary report on a high-sensitivity differential scanning calorimetry study. FEBS Lett 197:221–224

Monreal J (1975) Chromatographic fractionation of brain white matter proteolipid. J Neurochem 25:539–541

Morell P (ed) (1984) Myelin. Plenum Press, New York

Morin PE, Diggs D, Freire E (1990) Thermal stability of membrane-reconstituted yeast cytochrome c oxidase. Biochemistry 29:781–788

Moscarello MA, Neumann AW, Wood DD (1983) Thermotropic phase transitions in normal human myelin as observed in a sensitive microcalorimeter. Biochim Biophys Acta 728:201–205

Nave KA, Lai C, Bloom FE, Milner RJ (1987) Splice site selection in the PLP protein (PLP) gene transcript and primary structure of the DM-20 protein of central nervous system myelin. Proc Natl Acad Sci USA 84:5665–5669

Pace CN (1990) Measuring and increasing protein stability. Trends Biotechnol 8: 93–98

Prado A, Muga A, Castresana J, Goñi FM, Arrondo JLR (1990) Solubilization of sarcoplasmic reticulum membranes by sodium dodecylsulphate. A Fourier-transform infrared spectroscopic study. FEBS Lett 269:324–327

Privalov PL, Plotnikov VV, Filimonov VV (1975) Precision scanning microcalorimeter for the study of liquids. J Chem Thermodyn 7:41–47

Privalov PL (1979) Stability of proteins. Small globular proteins. Adv Prot Chem 33:167–241

Privalov PL (1980) Scanning microcalorimeters for studying macromolecules. Pure Appl Chem 52:479–497

Privalov PL (1982) Stability of proteins. Proteins which do not present a single cooperative system. Adv Prot Chem 35:1–104

Rial E, Muga A, Valpuesta JM, Arrondo JLR, Goñi FM (1990) Infrared spectroscopic studies of detergent-solubilized uncoupling protein from brown-adipose-tissue mitochondria. Eur J Biochem 188:83–89

Rigell CW, Saussure C, Freire E (1985) Protein and lipid structural transitions in cytochrome c oxidase-dimyristoylphosphatidylcholine reconstitutions. Biochemistry 24:5638–5646

Rigell CW, Freire E (1987) Differential detergent solubility investigation of thermally induced transitions in cytochrome c oxidase. Biochemistry 26:4366–4371

Ross NW, Braun PE (1988) Acylation in vitro of the myelin proteolipid protein and comparison with acylation in vivo: acylation of a cystein occurs nonenzymatically. J Neurosc Res 21:35–44

Ruiz-Sanz J, Ruiz-Cabello J, Mateo PL, Cortijo M (1992) The thermal transition in crude myelin proteolipid has a lipid rather than protein origin. Eur Biophys J 21:71–76

Sánchez-Ruiz JM, Mateo PL (1987) Differential scanning calorimetry of membrane proteins. Cell Biol Rev 11:15–45

Sánchez-Ruiz JM, López-Lacomba JL, Cortijo M, Mateo PL (1988) Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. Biochemistry 27:1648–1652

Sankaram MB, Brophy PJ, Marsh D (1991) Lipid-protein and protein-protein interactions in double recombinants of myelin proteolipid apoprotein and myelin basic protein with dimyristoylphosphatidylglycerol. Biochemistry 30:5866–5873

Sturtevant JM (1987) Biochemical applications of differential scanning calorimetry. Ann Rev Phys Chem 38:463–488

Surewicz WK, Moscarello MA, Mantsch HH (1987a) Secondary structure of the hydrophobic myelin protein in a lipid environment as determined by Fourier-transform infrared spectrometry. J Biol Chem 262:8598–8602

Surewicz WK, Moscarello MA, Mantsch HH (1987b) Fourier transform infrared spectroscopic investigation of the interaction between myelin basic protein and dimyristoylphosphatidylglycerol bilayers. Biochemistry 26:3881–3886

Surewicz WK, Mantsch HH (1988) New insight into protein secondary structure from resolution-enhance infrared spectra. Biochim Biophys Acta 952:115–130

Tanford C, Reynolds JA (1976) Characterization of membrane proteins in detergent solutions. Biochim Biophys Acta 457:133–170

Toews AD, Horrocks LA, King JS (1976) Simultaneous isolation of purified microsomal and myelin fractions from rat spinal cord. J Neurochem 27:25–31

Trifilieff E, Luu B, Nussbaum JL, Roussel G, Espinosa de los Monteros A, Sabatier JM, Van Rietschoten J (1986) A specific immunological probe for the major myelin proteolipid. Confirmation of a deletion in DM-20. FEBS Lett 198:235–239

Vacher M, Waks M, Nicot C (1984) The thiol groups of the Folch-Pi protein from bovine white matter. Exposure, reactivity and significance. Biochem J 218:197–202

Volkin DB, Klibanov AM (1989) Minimizing protein inactivation. In: Creighton TE (ed) Protein function. A practical approach. IRL Press, Oxford, pp 1–24

Wang CS, Smith RL (1975) Lowry determination of protein in the presence of Triton X-100. Anal Biochem 63:414–417