Circular dichroic, infrared and other studies on the protein component of pig brain thromboplastin

Roy M. HOWELL and Houri REZVAN*

Department of Biochemistry, Queen Elizabeth College, University of London, Campden Hill Road, London W8 7AH, U.K.

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1. A four-step procedure used to isolate the protein component (apoprotein III) of pig brain thromboplastin yielded approximately 25 mg from 500 g of brain. 2. In the absence of detergent, apoprotein III had an apparent mol.wt. of 360 000 by gel-filtration, and, after electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate, it appeared as a major protein band of mol.wt. 59 000, suggesting the existence of polymeric and monomeric forms. 3. Chemical analyses of apoprotein III revealed that hydrophilic and hydrophobic amino acids were present in a ratio of 3:2, together with approx. 9% (w/w) of carbohydrate. 4. The far-u.v.c.d. and i.r. spectral data indicated that, like other membrane proteins, apoprotein III has a high percentage of unordered structure with lesser amounts of α - and β -forms. 5. Relipidation of apoprotein III to restore clotting activity caused no extensive alteration in the c.d. and i.r. spectra, indicating that the phospholipid associates with a comparatively small hydrophobic segment. The constrained unordered conformation, which makes the major contribution to the c.d. spectrum, probably forms a separate domain in the aqueous phase. The absence of any increase in the amplitude of both negative c.d. extrema, following relipidation, contrasted with the substantial increase observed in a helix-forming solvent and raises the possibility that the more stable polymeric form of apoprotein III is retained as the active form in the lipid phase. 6. We suggest that as a consequence of cell membrane damage, the recognition and activation of factor VII may involve minor changes of conformation that are dependent upon the flexibility inherent in an unordered secondary structure.

The specific binding and activation of factor VII from blood by tissue thromboplastin (factor III), a lipoprotein released from damaged cell membranes initiates the extrinsic pathway of coagulation (Nemerson & Pitlick, 1972). The glycoprotein component of factor III (apoprotein III) is similar to several other functional proteins associated with cell membranes in displaying a total dependence on lipid for its activity, as well as a high level of specificity for aminophospholipids such as phosphatidylserine and phosphatidylethanolamine (Nemerson & Pitlick, 1970). These lipids are mostly confined to the cytoplasmic half of the bilayer in intact membranes (Bretscher, 1973), whereas immunological studies (Zeldis *et al.*, 1971) have shown that apoprotein III protrudes from the external surface of blood vessel endothelium. Thus the separation of the components of thromboplastin

on opposite sides of the intact cell membrane may serve an important function in maintaining it in a latent state, so preventing continuous coagulation on blood vessel endothelium (Zwaal *et al.*, 1977).

Little is known of the mechanism whereby a loss of lipid asymmetry after injury to cell membranes releases thromboplastin from a latent state, so that it acquires an enzyme-like specificity for the smaller factor VII molecule. One aim of our investigation was to determine whether the non-enzymic activation of this substrate may be interpreted in terms of a sequence of conformational changes initiated when procoagulant phospholipids impose a specific secondary structure, or even quaternary configuration, upon apoprotein III. Since changes in protein conformation resulting from lipid interaction are frequently difficult to interpret from the application of a single physical technique, c.d. and i.r. spectroscopy were used in combination.

In the present paper we report that, although the

* Present address: Iranian National Blood Transfusion Service, 138 Villa Avenue, Tehran, Iran.

c.d. of apoprotein III alone was suggestive of an α -helix, closer inspection revealed a spectrum dominated by unordered structure, a conclusion supported by the i.r. data. In the presence of activity-restoring lipids, the significance of the aggregated form of apoprotein III is discussed as a quaternary state within which a flexible structure can adopt a stable configuration for the recognition of factor VII.

A homogeneous preparation of apoprotein III from pig brain was obtained by the procedure of Nemerson & Pitlick (1970) in sufficient yield to allow the configuration of the apoprotein from this source and other characteristics, such as thermal stability, molecular weight, amino acid and carbohydrate composition, to be defined for the first time. Where a preliminary account of some aspects of the work has already been given, it is referred to in the text.

Materials and methods

Purification of apoprotein III from porcine brain

The procedure described by Nemerson & Pitlick (1970) was used. This involved the further extraction of lipids from an acetone-dried powder of pig brain using butan-1-ol and sodium deoxycholate, followed by precipitation of the apoprotein III with $(\text{NH}_4)_2\text{SO}_4$, then chromatography on DEAE-Sephadex and Sepharose 6B. Those fractions containing factor-III activity were pooled and stored at -20°C .

Electrophoresis

Disc-gel electrophoresis was carried out in 4.5% (w/v) polyacrylamide gels at pH 9.0, using the discontinuous buffer system of Davis (1964). Electrophoresis in sodium dodecyl sulphate/5% (w/v) polyacrylamide gels was performed at pH 7.0 by the procedure of Dunker & Rueckhart (1969).

Reconstitution of factor III and its coagulation activity

A mixture of phospholipids from soya beans available as commercial 'lecithin' (Sigma Chemical Co., Poole, Dorset, U.K.) was used for the relipidation of purified apoprotein III. Analysis by t.l.c. was in agreement with the conclusions of Erdahl *et al.* (1973) and revealed that the main components were phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine), phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine) and phosphatidylinositol (1,2-diacyl-*sn*-glycero-3-phosphoinositol). A mixed preparation of soya bean phospholipids has been used successfully for the restoration of activity to several functional proteins solubilized from their membrane-bound state (Lenaz *et al.*, 1975; Knowles *et al.*, 1976).

Individual phospholipids, chromatographically pure, were obtained from Lipid Products, Surrey, U.K. The commercial phospholipids were dispersed in 0.25% (w/v) sodium deoxycholate (Calbiochem) in 0.025 M-Tris/HCl buffer, pH 7.0, containing 0.25 M-NaCl. The interaction of apoprotein III with phospholipid was studied by mixing equal volumes of each component in the proportions desired. Protein/lipid ratios ranged from 1:1 to 1:20 (w/w) and, after the mixture had been incubated at 37°C for 1 h, the recombinants remained optically clear in all cases.

The procoagulant activity was determined by measuring the coagulation time in duplicate in a one-stage prothrombin assay (Hvatum & Prydz, 1966); reference curves were obtained by serial dilution of a standard thromboplastin preparation from rabbit brain. All clotting-test reagents were supplied by Diagnostic Reagents, Thame, Oxon., U.K. Where the results of assays are presented as arbitrary units, they were obtained by comparison with the clotting time of standard thromboplastin. A clotting time of 14 s for this undiluted thromboplastin is taken as representing 100 arbitrary units of activity in 0.1 ml.

Amino acid analysis

Purified apoprotein III was dialysed free of buffer and the procedures described by Hauschka *et al.* (1975) were used for acid and alkaline hydrolyses, which were carried out in duplicate in sealed tubes under an atmosphere of N_2 . Acid hydrolysis was in 6 M-HCl at 105°C for 28 h, whereas alkaline hydrolysis was in 2 M-KOH at 105°C for 22 h. Both methods of hydrolysis were used so as to compensate for the destruction of certain amino acids, particularly serine during alkaline hydrolysis and tryptophan during acid hydrolysis. The hydrolysates were dialysed against water and then analysed in a Locarte amino acid analyser.

Carbohydrate analysis

Factor-III apoprotein was dialysed free of buffer and inositol was added as internal standard. After drying *in vacuo* the preparation was treated for 2.5 h with 1 ml of methanolic 0.5 M-HCl at 80°C . Silylation was carried out as described by Chamber & Clamp (1971). The silylated samples were dissolved in heptane and analysed in a Pye Unicam 104 gas chromatograph. The column (0.35 cm $\times$ 150 cm) contained SE-30 as stationary phase deposited on diatomite (80–100 mesh) supplied by J.J. Chromatographic Equipment, Norfolk, U.K. The oven temperature was programmed to increase from 130°C to 240°C at $2^\circ\text{C}/\text{min}$ and the N_2 carrier gas was set at a flow rate of 40 ml/min.

Thermal stability

This was investigated for apoprotein III alone

and in the presence of phospholipid by preincubating the sample for various time intervals up to 1 h at 55°C before assaying for clotting activity at 37°C.

Measurement of c.d.

The c.d. spectra of apoprotein III were recorded in duplicate on a Cary 61 spectropolarimeter at 25°C; cells of 2 mm path length were used. The solvents used in the measurements, which were done under an atmosphere of nitrogen, were 0.025 M-Tris adjusted to pH 7.0 with HCl and containing 0.25 M-NaCl, or a mixture of trifluoro-ethanol and Tris/HCl (90:10, v/v). C.d. measurements were made from 200 to 250 nm; in common with other workers (Cockle *et al.*, 1978), we excluded the region of positive ellipticity below 200 nm in the far-u.v. region, since the high signal-to-noise ratio complicated the curve-fitting analysis. All c.d. magnitudes are given as mean residue ellipticities, $[\theta]_{m.r.w.}$, (degree·cm²·dmol⁻¹) and were calculated by using the average residue mol.wt. of 127, obtained from the amino acid analysis. A semi-quantitative estimation of the relative amounts of α -helical, β - and random-coil conformations of the apoprotein was made by the method of Greenfield & Fasman (1969).

I.r. spectroscopy

I.r. spectra of dried films of apoprotein III were recorded with a Perkin-Elmer 325 double-beam grating i.r. spectrophotometer operating at room temperature (15–20°C). Instrument accuracy and precision was within ± 0.5 cm⁻¹ based on the 1601.8 cm⁻¹ peak of a standard polystyrene film. The dried films of apoprotein III alone, and after reconstitution with soya bean phospholipids, were prepared by placing 0.1 ml of the preparation, containing about 1 mg of protein, on an AgCl disc (20 mm × 2 mm; supplied by Beckman Instruments, Glenrothes, Scotland, U.K.) and drying it first in a stream of cool air and then in a desiccator. Alternatively, samples were prepared by reconstituting a freeze-dried preparation of apoprotein III in ²H₂O.

Thin films of this preparation were prepared by sandwiching one drop of the sample between two AgCl discs.

I.r. analysis allows β -structure to be distinguished as a separate band at 1630 cm⁻¹ (Timasheff & Gorbunoff, 1967), whereas the maximum ellipticities due to α - and β -configurations merge near 223 nm in the c.d. spectra.

Results

Isolation of apoprotein III

The isolation of apoprotein III from porcine brain in relatively high yield was achieved by using the procedures devised by Nemerson & Pitlick (1970) for obtaining it from bovine lung. Starting with 500 g of pig brain, it was possible to isolate an average of 25 mg of apoprotein III (Table 1) and this gave a 10 000-fold purification overall. The only problem encountered with this scheme was the failure to sediment of the amorphous precipitate obtained between 30% and 60% saturation with (NH₄)₂SO₄, unless the highly dispersed material precipitated at 30% saturation coalesced, thereby ensuring its complete removal. The retention of sodium deoxycholate from the previous stage improved the sedimentation of the (NH₄)₂SO₄ fractions and assisted the removal of any residual lipid (Markino *et al.*, 1977).

Final purification of apoprotein III was achieved by molecular-sieve chromatography on a column of Sepharose 6B to separate it from various proteins of similar molecular weight. After relipidation of each fraction with the soya-bean-phospholipid mixture, the second peak was the only one with identifiable clotting potential. A typical elution profile is shown in Fig. 1; protein peaks devoid of potential clotting activity are apparent on either side of apoprotein III. One of these was eluted near the void volume of the column; the presence of a similar high-molecular-weight protein co-purifying with apoprotein III from porcine lung was reported by Wijngaards *et al.* (1977). The fractions compris-

Table 1. Purification of thromboplastin (factor III) from 500 g of pig brain

Relipidation experiments were carried out at each step using different weight ratios of lipid to protein. The reconstituted clotting activity at the optimal weight ratio is expressed as units in the next column. The experiments were performed in duplicate with mixed soya bean phospholipids dispersed in 0.25% (w/v) sodium deoxycholate. —, No addition of phospholipid. For further details see the text.

Purification step	Total protein (mg)	Lipid/protein ratio in recombinant (w/w)	10 ⁻⁴ × Total factor III activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Acetone-extracted powder	50 000	—	3.5	0.7	100	1
(NH ₄) ₂ SO ₄ fraction (30–60%)	348	1.5:1	2.45	70	70	100
DEAE-Sephadex eluate	90	6:1	2.40	267	68.5	382
Sepharose 6B chromatography	24.5	10:1	2.0	816	57.1	1182

Fig. 1. Gel filtration of the pooled apoprotein III fractions of the DEAE-Sephadex eluate

These fractions contained 90 mg of protein with procoagulant activity, which was applied to the Sepharose 6B column (2.6 cm $\times$ 40 cm) equilibrated at 4°C with the eluant [0.05 M-imidazole/HCl buffer (pH 7.0) containing 0.5 M-NaCl]. The upward flow rate was 11 ml/h and the effluent was collected in 3.5 ml fractions. Activity was located after relipidation of individual fractions with 10 mg of mixed soya bean phospholipid/mg of protein. Fractions between the arrows were pooled, rechromatographed on a calibrated column and stored at -20°C for further characterization. —, A_{280} ; O, thromboplastin activity.

ing apoprotein III in the central peak of Fig. 1 were combined, excluding the shoulder which formed part of the third peak, and were concentrated by vacuum dialysis. When apoprotein III was rechromatographed on the calibrated Sepharose 6B column, the elution volume for the single peak was unchanged, being midway between those for standard apoferritin (mol.wt. 480 000) and catalase (mol.wt. 240 000) but slightly less (10 ml) than that for fibrinogen (mol.wt. 340 000). Its close proximity to fibrinogen corresponded to an apparent mol.wt. of $360\,000 \pm 40\,000$, and was fairly near to the value of approx. 300 000 determined for apoprotein III from porcine lung by Wijngaards *et al.* (1977).

When porcine brain apoprotein III was electrophoresed in 4.5% (w/v) polyacrylamide gels at pH 9.0, using the discontinuous buffer system of Davis (1964), a homogeneous band was observed. Electrophoresis of the reduced apoprotein III on an analytical polyacrylamide gel in buffer containing sodium dodecyl sulphate and with reduced globular marker proteins as standards revealed (Fig. 2) a major protein band corresponding to a mol.wt. of 59 000.

Fig. 2. Densitometric scan of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of apoprotein III

The following proteins were used as reference standards (●) to estimate the subunit mass. 1, Bovine serum albumin (mol.wt. 6.8×10^4); 2, ovalbumin (mol.wt. 4.3×10^4); 3, pepsin (mol.wt. 3.6×10^4); 4, γ -globulin light chain (mol.wt. 2.35×10^4); 5, cytochrome *c* (mol.wt. 1.24×10^4). The 4.5% (w/v) acrylamide gels were calibrated from a plot of the molecular weight for each protein against their relative mobilities obtained by dividing each migration distance by that for Bromophenol blue. —, A_{560} .

Amino acid and carbohydrate analysis

The quantitative amino acid analysis of isolated porcine apoprotein III is given in Table 2 and reveals the most abundant amino acids to be glutamic acid and proline. The content of hydrophobic amino acids was estimated to be 40% of the total, [based on those amino acids more hydrophobic than serine (Nozaki & Tanford, 1971)]. The mol.wt. of 59 000 determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2) was used for the calculations. A notable feature of the carbohydrate composition of porcine apoprotein III (Table 3) is the high amount of mannose as the major sugar component. The total carbohydrate content was calculated to be 9.6 g/100 g of protein.

Phospholipid requirement to restore the activity of apoprotein III

Porcine apoprotein III was found to contain less

Table 2. *Amino-acid composition of purified apoprotein III*

Samples of protein (1–5 mg) were hydrolysed for 28 h in 6M-HCl, except that tryptophan was determined after alkaline hydrolysis. Other experimental details are given in the text. The results are the means of duplicates. Mean residue weight = 127.

Amino acid	Composition (residues/molecule)
Aspartic acid	41
Threonine	20
Serine	25
Glutamic acid	52
Proline	50
Glycine	35
Alanine	32
Cysteine	3
Valine	19
Methionine	7
Isoleucine	19
Leucine	33
Tyrosine	11
Phenylalanine	14
Histidine	11
Lysine	38
Arginine	23
Tryptophan	6
Total residues	439

Table 3. *Carbohydrate composition of purified apoprotein III*

Sugars were determined by g.l.c. with inositol as the internal standard. The results are means of duplicates. For further details see the text.

Monosaccharide	Total carbohydrate (residues/molecule)
Fucose	2.4
Mannose	7.1
Galactose	4.2
Glucose	5.6
N-Acetylglucosamine	3.5
N-Acetylgalactosamine	1.7
N-Acetylneuraminate	1.4

than 5% (w/w) of phospholipid after elution from Sepharose 6B and was devoid of clotting activity unless relipidated with a suitable mixture of soya bean phospholipids dispersed in 0.25% (w/v) sodium deoxycholate (Fig. 3). The increasing amount of soya bean phospholipids (1.5, 6.0 and 10.0 mg/mg of apoprotein III) required to restore optimum clotting activity at each stage of the purification (Table 1) represents the deficit arising from the extraction of lipid (Nemerson & Pitlick, 1970). For porcine brain apoprotein III this is shown clearly in Fig. 3,

Fig. 3. *Coagulant activity of thromboplastin reconstituted from chromatographic fractions*

Pooled fractions of apoprotein III eluted from DEAE-Sephadex (O) or Sepharose 6B (●) were used. The factor-III activity reconstituted from apoprotein III (fixed amount) and a mixture of soya bean phospholipids (variable amount) is expressed as a percentage of the activity of the most active recombinant. For further details see the Materials and methods section.

where the progressive establishment of optimum activity for relipidated protein eluted from DEAE-Sephadex in the penultimate stage occurred at a lower amount of lipid than from a Sepharose 6B column in the final stage. The presence of excess lipid inhibited the activity of the recombinant from each step.

The observations of Nemerson & Pitlick (1970), Wijngaards *et al.* (1977) and Bjørklid & Storm (1977) that purified lipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were not as effective as mixed lipids in restoring activity to apoprotein III were also supported by us in a separate series of experiments. The maximum activity-restoring capacity of individual pure phospholipids was only 5–15% of that achieved with soya bean phospholipids, or 30–40% when mixed in combinations of two or three components.

Effect of heat treatment on porcine apoprotein III

The isolated apoprotein remained stable for several hours at room temperature, up to 3 days at 4°C and several months at –20°C. In contrast, over 80% of the clotting activity was lost after heat treatment at 70°C for 30 min. For a similar period at 55°C, only 30% of the activity disappeared and this loss could be further decreased to a negligible figure (<2%) when the apoprotein III was

present with soya bean phospholipids in a ratio of 1:10 (w/w). However, the protection against denaturation afforded by phospholipid was lost in the presence of 0.25% (w/v) sodium deoxycholate, since only 35% of activity remained after 30 min at 55°C. There was a loss of 80% of the activity in the presence of sodium taurodeoxycholate.

C.d. spectra of pig brain apoprotein III in the far u.v.

The c.d. spectra of an aqueous solution of apoprotein III (0.04 mg/ml) at pH 7.0, either alone or in the presence of varying amounts of soya bean phospholipids dispersed in sodium deoxycholate, are shown in Fig. 4. Apoprotein III alone displays the overlapping negative bands between 200 and 230 nm, with amplitudes characteristic of a protein with a low α -helical content of 15–25%, the rest of the secondary structure being divided in the proportion of 33–40% β and 35–52% unordered conformations (Greenfield & Fasman, 1969). A comparison was made also with published c.d. spectra of similar shape from a number of well-investigated native polypeptides (Tiffany & Krimm, 1969; Choules & Bjørklund, 1970; Pittz & Timasheff, 1978) which have a predominance of

unordered structure and consequently illustrate similar blue-shifted bands near 218 nm.

Recombination of apoprotein III with soya bean phospholipids in ratios of 1:10 and 1:16 (w/w) can be seen from Fig. 3(b) to induce high and low clotting activities respectively; Fig. 4 illustrates how the ellipticity values of apoprotein III at 218 and 208 nm vary with the weight ratio of phospholipid to protein. It is evident from curves (b) and (c) in Fig. 4 that, whereas the intensity of the negative dichroic bands near 218 nm were decreased to the same extent, those at 208 nm behaved differently at each value of the protein/phospholipid ratio. When the weight ratio of protein to lipid was raised to 1:16, which is above the optimum for the re-establishment of clotting activity, the decrease at 208 and 218 nm in curve (c) suggests a limited decrease of 5–10% in the α -helix content. However, when the protein/lipid ratio was at an optimum for the restoration of the highest clotting activity (1:10, w/w), the decrease in ellipticity near 218 nm in curve (b) was not accompanied by an equivalent decrease at 208 nm.

The c.d. spectrum of apoprotein III in deoxycholate (fig. 5) was intended to examine the

Fig. 4. Far-u.v. c.d. spectra of apoprotein III

Mean residue ellipticities of: —, thromboplastin apoprotein (0.04 mg/ml) alone in 0.025 M-Tris/HCl buffer (pH 7.0) containing 0.25 M-NaCl at 25°C; ----, apoprotein in same solvent+soya bean phospholipids (0.4 mg/ml)+sodium deoxycholate (2.5 mg/ml); ····, apoprotein in same solvent+soya bean phospholipids (0.8 mg/ml)+sodium deoxycholate (2.5 mg/ml). See the text for details.

Fig. 5. Far-u.v. c.d. spectra of apoprotein III. Mean residue ellipticities of: —, thromboplastin apoprotein (0.07 mg/ml) alone in 0.025 M-Tris/HCl buffer (pH 7.0) containing 0.05 M-NaCl at 25°C; ----, apoprotein in same solvent with sodium deoxycholate (2.5 mg/ml); ····, apoprotein in the same solvent with 90% (v/v) trifluoroethanol.

possibility that its use for dispersing phospholipid during the relipidation experiments recorded in figs. 3 and 4 may of itself introduce conformational alterations in the protein. Whereas the intensity of the c.d. band at 208 nm was almost identical with that of apoprotein III alone, the negative peak from 218 to 216 nm underwent a change that corresponded to a minor decrease of 5–6% in the helical content and this was also accompanied by a limited blue shift. It is notable from the spectrum in Fig. 5 that, although the positions of the negative peaks at 208 and 218 nm were generally unchanged, their amplitudes in trifluoroethanol were greatly increased. Since the ability to realize the full helical potential was seen only in a halogenated solvent, which is able to penetrate the protein surface and make contact with hydrophobic 'patches' (Pittz & Timasheff, 1978), it seems unlikely that the low helical content of apoprotein III in aqueous solution has been underestimated, nor a major contribution from unordered segments exaggerated. Although the c.d. spectra display deceptive α -helical characteristics, these would have greater amplitude if apoprotein III were predominantly in this form. From a comparison with the spectra of other natural polypeptides, it also seems improbable that

Fig. 6. I.r. spectra of thromboplastin apoprotein as a dried film

Absorbances of films cast on AgCl discs from an aqueous solution of (a) 1 mg of apoprotein alone/ml; (b) 1 mg of apoprotein/ml in 0.25% (w/v) sodium taurodeoxycholate; (c) 1 mg of apoprotein/ml relipidated with soya bean phospholipids (10 mg/ml) dispersed in 0.25% sodium deoxycholate. Films were dried at prevailing room humidity and temperature. See the text for other details.

the low amplitude signals are due to the presence of short helical segments (Chen *et al.*, 1974; Cockle *et al.*, 1978).

I.r. spectra of apoprotein III from 1300 to 1800 cm^{-1}

I.r. spectra were measured as a second method for evaluating the dominant unordered structure suggested by c.d. measurements for apoprotein III, both alone and after insertion into phospholipid.

It can be seen from Figs. 6 and 7 that all spectra of apoprotein III, whether alone or relipidated in the dry state and in $^2\text{H}_2\text{O}$, display an amide-I band ($\text{C}=\text{O}$ stretching). This is dominated by unordered conformation near 1640 cm^{-1} , rather than by α -helix, since a band nearer 1650 cm^{-1} is not apparent. The other prominent amide-I band at 1630 cm^{-1} points to the presence of β -structure, although the absence of a shoulder at 1695 cm^{-1} indicates that the chains are not in an antiparallel arrangement (Fraser & Suzuki, 1970). The distinctive carbonyl band at 1740 cm^{-1} , which is due to $\text{C}=\text{O}$ stretching vibrations of the fatty acyl ester groups in the polar region of the lipid bilayer, is also well displayed in Figs. 6(c) and 7(b), both in the dry state and in $^2\text{H}_2\text{O}$.

Fig. 7. I.r. spectra of thromboplastin apoprotein in $^2\text{H}_2\text{O}$. Absorbances of (a) 1 mg of apoprotein/ml and (b) 1 mg of apoprotein/ml in the presence of soya-bean phospholipids (10 mg/ml) dispersed in 0.25% (w/v) sodium deoxycholate. Measurements were carried out after dissolving freeze-dried apoprotein in 0.25 M-NaCl in $^2\text{H}_2\text{O}$ buffered with 0.025 M-Tris/HCl, pH 7.0.

In accordance with the c.d. data, sodium deoxycholate, at the concentration (0.25%, w/v) used to disperse phospholipid, had no effect on the conformation of apoprotein III as shown by the i.r. spectrum at 22°C. However, exposure to the same concentration of sodium taurodeoxycholate, which inhibited clotting and decreased c.d. ellipticities (Howell & Rezvan, 1977) broadened the amide-I band near 1640 cm^{-1} , so that the split due to β -structure at 1630 cm^{-1} was no longer differentiated (Fig. 6b). The possibility that a limited amount of β -structure is required for normal clotting, in addition to an unordered state, was also suggested when a similar broadening of the amide-I band near 1630 cm^{-1} on heating the apoprotein III at 70°C for 30 min was associated with a loss of 80% of the clotting activity.

Support for the presence of some α -helix in the relipidated preparations, as revealed by c.d., is evident from the strong amide-II absorption at 1540 cm^{-1} in the dried film (Fig. 6c). A distinct shoulder also appeared in the region around 1535 cm^{-1} , where the amide-II band for the unordered conformation is expected.

When apoprotein III prepared in H_2O was transferred to $^2\text{H}_2\text{O}$, an important feature was the shift of the amide-II doublet, with peaks at 1540 and 1535 cm^{-1} (Fig. 7a), to an enhanced broad band at 1450 cm^{-1} , indicating that most of its protons are accessible for exchange with $^2\text{H}^+$ from $^2\text{H}_2\text{O}$. The high degree of deuteration implies the presence of a large proportion of unordered structure, comprising a hydrophilic domain, and upholds the conclusion from the c.d. study that a hydrophobic helix interacting with lipid and deoxycholate would be separated in a bimodal structure. However, the retention of a weak amide II doublet at 1540 and 1553 cm^{-1} in the i.r. spectrum of apoprotein III reactivated with an optimum amount of soya bean phospholipids (1:10, w/w) in $^2\text{H}_2\text{O}$ indicated (Fig. 7b) that some of the protons in the limited amount of ordered helical structure were no longer available for exchange (Cherry *et al.*, 1971). This suggests a zone of lipid held more tightly by apoprotein III in the recombinant and is supported by the data from the integrated intensities of the protein and lipid bands at 1640 cm^{-1} and 1740 cm^{-1} (Fig. 7b). In the immediate microenvironment of apoprotein III these gave a weight ratio of approx. 1:1, despite an overall ratio of protein to lipid of 1:10 (Brazhnikov *et al.*, 1978).

Discussion

The apoprotein III eluted from the Sepharose 6B column in the final stage of the preparation was homogeneous by several criteria. For instance, it migrated as a single protein by polyacrylamide-gel electrophoresis, and c.d., as well as i.r., spectra contained a characteristic set of wavelength bands for this protein. The requirement for a low weight ratio of apoprotein III to phospholipid (1:10) to restore clotting activity in the recombinant was also considered to be indicative of a pure apoprotein by Nemerson & Pitlick (1970).

Our finding that the residual phospholipid (less than 5%, w/w) in isolated apoprotein III is tightly bound supports that of Nemerson & Pitlick (1970). During the isolation it is possible that the peroxidation of phospholipid, with which apoprotein III is in close contact, could lead to some covalently-linked protein polymer (Roubal & Tappel, 1966). Since the product is unaffected by reducing conditions, this reaction might explain the presence of the low amount of lipid and the higher molecular weight chains (95 000 and 130 000) observed as minor peaks alongside the main band (mol.wt. 59 000) after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of apoprotein III. The minor species accounted for 5–10% of the total; a procedure that controls the air-oxidation of lipids during the purifi-

cation may be necessary to yield a homogeneous protein under reducing conditions.

The mol.wt of 59000 obtained by us for porcine brain apoprotein III on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis compares favourably with the values of 52000 and 59000 reported for that from human brain by Bjørklid *et al.* (1973) and bovine brain by Liu & McCoy (1975). However, in agreement with the observations of Bjørklid & Storm (1977) on the human apoprotein, gel-filtration chromatography revealed that porcine apoprotein III readily undergoes self-association in the absence of detergent, to give a polymeric form with an apparent mol.wt. of 360000.

The present work showed that apoprotein III from pig brain contains about 9% (w/w) of carbohydrate, whereas Bjørklid & Storm (1977) reported 6% (w/w) for apoprotein III from human brain. By comparison, the figure of 14–20% (w/w) carbohydrate for apoprotein III from bovine brain (Pitlick, 1976) indicates a wide variation based on the species of origin.

The high content (40%) of hydrophobic amino acid residues is in reasonable agreement with the value of 42% reported by Bjørklid & Storm (1977) for a preparation of apoprotein III from human brain and is average for membrane-associated glycoproteins (Sturgess *et al.*, 1978). A proportion of the hydrophobic residues present in apoprotein III could be expected to constitute a helical segment that interacts with lipid alkyl chains in the membrane core (Segrest *et al.*, 1972; Rezvan & Howell, 1977) and the lack of any major effect of phospholipid or sodium deoxycholate on the secondary conformation of apoprotein III may be due to their mode of association with a comparatively small hydrophobic segment (Helenius & Simons, 1975). The procoagulant effect of deoxycholate is probably the result of its contribution to the co-micellization of the hydrophobic domain with the amphipathic phospholipid. In contrast, sodium taurodeoxycholate may prevent protein–lipid and protein–protein interactions (Donner *et al.*, 1976).

Although no major changes of ellipticity occurred when apoprotein III was relipidated to optimum activity, it was no longer possible to fit the experimental c.d. spectrum exactly with the spectra computed for synthetic polypeptides in solution by Greenfield & Fasman (1969). The term 'constrained unordered' was used by Fasman *et al.* (1970) to describe a similar anomaly and to emphasize that reliance on the three conventional secondary states may be an oversimplification. The relative loss of amplitude at 218nm in the c.d. spectrum may, according to our i.r. results, be due to apoprotein III interacting closely with its own weight of specifically selected lipids, whilst retaining an unordered but more constrained structure.

In view of the requirement for charged aminophospholipids for optimum activity, rather than neutral ones containing choline, a limited charge-induced conformational transition may be responsible for a more reactive structure of apoprotein III being maintained in the presence of the most effective phospholipids (Pitlick & Nemerson, 1970; Wojtczak & Nalecz, 1979). A role for the polar groups of procoagulant phospholipids in imparting a specific structure was also suggested by Bjørklid *et al.* (1973) when they reported that cleavage of these groups completely inactivated thromboplastin. Although an understanding of the lipid requirements of membrane-associated proteins is still obscure it was postulated by Moscarello *et al.* (1973) that, as in the case of plasma lipoproteins (Scanu *et al.*, 1969), phospholipids may act to stabilize flexible apoprotein structures. However, Nemerson & Pitlick (1970) concluded that lipid may serve as a nidus for the aggregation of thromboplastin into forms with different activities, whereas excessive aggregation leads to denaturation and inactivation.

If a functional significance can be attributed to the observations from the present work, it is possible that, by virtue of a specific conformation (Maynard *et al.*, 1975) of apoprotein III, locked by aminophospholipids, factor VII is bound and is thereby enabled to undergo self-activation (Neurath & Walsh, 1976). The type of constrained–unordered structure proposed here for relipidated apoprotein III may be stabilized further as a result of the aggregation of apoprotein III molecules in the presence of cations (Nemerson, 1969; Howell & Rezvan, 1979). The absence of any increase in the amplitude of both negative c.d. extrema after relipidation suggests that the high-molecular-weight aggregate obtained by gel-filtration chromatography is retained, since it was demonstrated by Ji & Urry (1969) that disaggregation of polypeptides is accompanied by the dispersal of α -helices and a much higher negative ellipticity. An aggregate as the most reactive form of apoprotein III *in vivo* would have the advantage of being less vulnerable to degradation by proteolytic clotting factors and could account for its thermodynamic stability *in vitro*, especially in the presence of phospholipid.

Previous attempts to resolve apoprotein III as a low-molecular-weight form, especially in the presence of detergents, have underplayed the tendency to self-associate. More detailed studies on the secondary and quaternary configuration of apoprotein III are necessary to achieve an understanding of its binding to factor VII, as well as the role played by membrane phospholipids following their redistribution during cell injury (Zwaal *et al.*, 1977).

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