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Relationship between lipid saturation and lipid–protein interaction in liver mitochondria modified by catalytic hydrogenation with reference to cardiolipin molecular species

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Lipid acyl double bonds in isolated mitochondrial membranes were gradually reduced by palladium-complex-catalysed hydrogenation, and the resulting saturation was monitored by fatty acid analysis of phosphatidylcholine, phosphatidylethanolamine and cardiolipin. The courses of hydrogenation of these phospholipids suggested that cardiolipin is in a membrane compartment which is less accessible to the applied catalyst. Native cardiolipin and its hydrogenation products were further characterized by analysis of their molecular diacylglycerol species. A decrease in the double bond content was accompanied by an increased amount of motionally restricted lipids at the hydrophobic interface of proteins as measured by two different spin-labelled lipids (C-14 positional isomers of spin-labelled stearic acid and phosphatidylcholine analogues). The protein-immobilized fraction of spin-labelled stearic acid increased in parallel with the hydrogenation of cardiolipin rather than of phosphatidylcholine or phosphatidylethanolamine. These data are interpreted in terms of a tight association of cardiolipin with membrane proteins, which becomes looser upon double bond reduction leading to the replacement of cardiolipin by spin-labelled stearic acid in the solvation shell. Thus the hydrophobic moiety of cardiolipin, characterized by double-unsaturated C_{18}–C_{18} diacylglycerol species, seems to be an important structural requirement for the high protein affinity of this compound.

INTRODUCTION

The inner mitochondrial membrane can be regarded as a supramolecular lipoprotein complex owing to the strong functional interactions between its constituent proteins and phospholipids [which are essentially phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin (CL)]. Of these three major phospholipids, CL, although having the lowest abundance, is highly specific to the inner mitochondrial membrane (Daum, 1985). Various mitochondrial proteins such as complex I/III (Fry & Green, 1981), complex IV (Vik *et al.*, 1981), cytochrome P-450_{scc} (Pember *et al.*, 1983), the adenine nucleotide carrier (Beyer & Klingenberg, 1985), the phosphate carrier (Kadenbach *et al.*, 1982), carnitine acylcarnitine translocase (Noel & Pande, 1986) and the pyruvate carrier (Paradies & Ruggiero, 1988) were shown to co-purify with CL or to possess a functional requirement for this lipid. Furthermore, CL is believed to be involved in mitochondrial protein import (Ou *et al.*, 1988) and in the control of membrane structure order (Ellingson *et al.*, 1988).

^{31}P n.m.r. data suggested a strong binding of 6 mol of CL to the mitochondrial adenine nucleotide carrier, while its other phospholipids were in fast exchange (Beyer & Klingenberg, 1985). Cytochrome *c* oxidase and the adenine nucleotide carrier were shown by e.s.r. spectroscopy to be associated preferentially with spin-labelled phospholipids bearing a negative headgroup charge (for a review, see Marsh, 1985). Since CL displays the greatest effective association constant of acidic lipids and is the most effective of them in reconstituting enzymic activity, it is generally thought to display structural features, in addition to its charge, which facilitate interaction with intramembraneous proteins. Its hydrophobic core, in particular the number of acyl chains, their length and unsaturation, are the structural features which deserve special attention. In previous studies on protein interaction, attempts have already been made to vary the acyl chain number (Powell *et al.*, 1987) or the acyl chain composition (Dale & Robinson, 1988) of CL.

Much less is known about the relationship between lipid saturation and lipid–protein interactions. Hydrogenation, catalysed by the water-soluble palladium complex, has been successfully used to remove acyl double bonds from intact biological membranes (Vìgh & Joò, 1983). In the present work this approach has been extended to modify the acyl chain unsaturation of mitochondrial lipids in a controlled manner. Detailed structural data are provided on the hydrophobic core of CL by analysing the molecular species of the phosphatidyl moieties, and the unique characteristics are emphasized. Lipid–protein interaction was studied by spin-label e.s.r., and the presence of a protein-associated CL pool is postulated which is inaccessible to mild hydrogenation [double bond index (mean value of double bonds/acyl chain) > 1.0].

Abbreviations used: CL, 1,3-bis-(3-*sn*-phosphatidyl)-*sn*-glycerol (cardiolipin); PC, phosphatidylcholine; PE, phosphatidylethanolamine; 14-SASL, 14-(4,4-dimethyl-*N*-oxy-2-oxazolidinyl)stearic acid; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxy-2-oxazolidinyl)stearoyl]-*sn*-glycero-3-phosphocholine.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade. Phospholipase C (*Bacillus cereus*; 500 units/mg) as well as the buffers Tris, Hepes and Mops were purchased from Sigma Chemical Co. Precoated silica gel 60 plates were obtained from Merck (Darmstadt, Germany), phospholipase D (cabbage; 0.5 units/mg) was from Boehringer (Mannheim, Germany) and 3,5-dinitrobenzoyl chloride was from Serva (Heidelberg, Germany). Palladium–di(sodium alizarine monosulphonate) was supplied by Molecular Probes (Eugene, OR, U.S.A.). H.p.l.c. solvents were purified by double distillation and ultrafiltration.

Catalytic hydrogenation of rat liver mitochondria

Rat liver mitochondria were isolated in sucrose (0.25 mol/l)/EDTA (0.2 mmol/l)/Tris (0.02 mol/l; pH 7.4 at 4 °C)/2-mercaptoethanol (1 mmol/l) by a standard differential centrifugation procedure (Steinbrecht & Kunz, 1970). Mitochondria were diluted to a final concentration of 0.1 mg of protein/ml in sucrose (0.25 mol/l) containing Mops (0.03 mol/l) (pH 7.4) and EDTA (0.02 mmol/l). Hydrogenation was performed as described previously (Vìgh *et al.*, 1985, 1987) in rotating glass vessels, in which the gas phase was replaced by H_2 under atmospheric pressure. Preactivated palladium–di(sodium alizarine monosulphonate) (Vìgh *et al.*, 1987) was added through a rubber septum to give a final concentration of 0.05 mg/ml, and incubation was continued at 2 °C for up to 30 min.

Fatty acid analysis

Lipids were extracted from repelleted mitochondria according to Bligh & Dyer (1959), and phospholipid classes were separated on precoated silica gel 60 plates developed by chloroform/methanol/water (65:25:4, by vol.). Lipids were revealed as 8-anilinonaphthalene-sulphonate-stained spots which were scraped off and subjected to trans-esterification in HCl/methanol at 80 °C for 2 h under N_2 protection. Fatty acid methyl esters were separated isothermically (180 °C) on a Jeol JGC-20K gas chromatograph equipped with an SP-2330 capillary column (Vìgh *et al.*, 1987).

Analysis of CL diacylglycerol species

CL was isolated from mitochondrial lipid extracts by preparative t.l.c. on 0.75 mm silica gel H/Florisil (98:2, w/w) layers developed by chloroform/acetone/methanol/acetic acid/water (9:5:2:2:1, by vol.). All CL preparations were carefully checked for any trace of contaminating peroxidized species which may complicate further analyses (Teng & Smith, 1985). The reversed-phase h.p.l.c. method shown in Fig. 1 proved well suited to separate oxidized (peaks 1–3) from unoxidized (peak 4) CL. Peaks 1–3 could be identified as peroxidized CL species on the basis of t.l.c., fatty acid pattern, absorbance at 232 nm (see the lower trace of Fig. 1) and an increased tendency to hydrolyse. In agreement with the results of Parinandi *et al.* (1988), who assayed CL peroxidation by normal-phase h.p.l.c., the content of peroxidized species increased after ageing in chloroform. CL subjected to diacylglycerol analysis was essentially free of peroxidized species. To split off the diacylglycerol moiety, 300–500 nmol of CL was dissolved in 1 ml of diethyl ether/ethanol (98:2, v/v) and stirred for 12 h after addition of 0.2 ml of Hepes (0.25 mol/l; pH 5.5)/boric acid (0.03 mol/l)/zinc acetate (0.5 mmol/l)/2-mercaptoethanol (1 mmol/l)/10 units of phospholipase C. Released diacylglycerol was derivatized with 3,5-dinitrobenzoyl chloride and molecular species were analysed by h.p.l.c. separation exactly as described (Takamura *et al.*, 1986). Before being applied to h.p.l.c., the derivative was purified on silica gel plates developed by benzene/hexane/diethyl ether (50:45:4, by vol.). There are essentially two indications that the hydrolysis of CL by phospholipase was non-selective for molecular species, which is a prerequisite of such species analysis: (1) the fatty acid patterns of CL and the released diacylglycerols were identical and (2) an alternative CL hydrolysis by phospholipase D resulted in phosphatidylglycerol of identical species pattern. Phosphatidylglycerol was analysed as described previously (Schlame *et al.*, 1986). CL was also isolated from the crude mitochondrial fraction of bovine heart from freshly slaughtered animals, but its diacylglycerol species were analysed as naphthylurethane derivatives in accordance with Kruger *et al.* (1984) and Schlame *et al.* (1986, 1988).

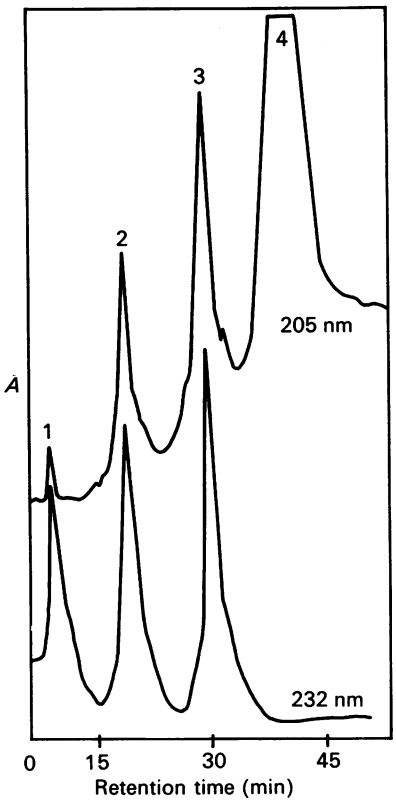

Fig. 1. H.p.l.c. separation of peroxidized CL species (peaks 1–3) from native CL (peak 4)

Bovine heart CL, stored in chloroform for several months, was separated on an RP-18 column (Nucleosil, 5 μm, 200 mm × 4.6 mm) which was eluted by an acetonitrile/methanol/water linear gradient changing from 4:80:16 (by vol.) at 0 min to 19:80:1 (by vol.) at 45 min (Hewlett–Packard chromatograph model 1084 B; flow rate 0.6 ml/min). Portions (2 × 200 nmol) of the same CL preparation were separated consecutively and the variable wavelength detector was set at 205 or 232 nm.

E.s.r. measurements

Rotenone (25 nmol) and 10 μmol of $K_3Fe(CN)_6$ were added to 1 ml of mitochondrial suspension containing 3 mg of protein and incubated at 4 °C for 1 min in order to inhibit spin-label reduction (Quintanilha & Packer, 1977; Ligeti & Horvàth, 1980). Then, a 10 μl aliquot of a solution of spin probe in ethanol (1 mg/ml) was added while vigorously vortexing the sample and incorporation of 14-(4,4-dimethyl-*N*-oxy-2-oxazolidinyl)stearic acid (14-SASL) and 1-acyl-2-[14-(4,4-dimethyl-*N*-oxy-2-oxazolidinyl)stearoyl]-*sn*-glycero-3-phosphocholine (14-PCSL) was allowed for 5 and 60 min respectively. The mitochondrial suspension was centrifuged and the pellets were transferred into 1 mm-diam. glass capillaries. E.s.r. spectra were recorded with an X-band spectrometer (JEOL JES-PE-1X, Tokyo, Japan) using a 100 kHz modulation technique. In the e.s.r. spectra of 14-SASL, weak 'aqueous peaks' due to incomplete label incorporation were observed, and the intensity of the aqueous peaks depended on the level of hydrogenation. Thus 14-SASL was added before lipid hydrogenation to minimize the aqueous peaks; the integrated intensity of this spectrum component was < 5%. These overlapping aqueous peaks were first digitally subtracted and subsequent analyses of the two-component spectra were done as described in Marsh (1982) by using a high resolution (1280 × 800 pixel points) graphics monitor and our e.s.r. software package.

RESULTS

Catalytic hydrogenation of major mitochondrial phospholipids

Isolated rat liver mitochondria were subjected to catalytic hydrogenation and the course of double bond reduction was monitored by fatty acid analysis in PC, PE and CL. The resulting double bond indices are plotted in Fig. 2 as correlation diagrams of PE versus PC and CL versus PC. The double bond content of PE has a linear relationship with that of PC, whereas the CL versus PC plot is biphasic with significant CL hydrogenation starting only after half-reduction of double bonds in PC. This feature is maintained during the hydrogenation of extracted mitochondrial lipids (insets of Fig. 2), hence it does not depend on the intact membrane but, most likely, reflects the different fatty acid compositions of CL and PC (or PE). In Fig. 3 the double bond indices of C_{18} chains were plotted separately since it was these fatty acids which were found in all phospholipid species. Again, a linear relationship was obtained between PE and PC, whereas a biphasic relationship characterized PC and CL. However, after extraction from the membrane, CL hydrogenation became linearly related to PC hydrogenation (○, Fig. 3) indicating that, unlike in the native mitochondrial membrane, both phospholipids became accessible to the catalyst to the same extent after lipid extraction.

Molecular diacylglycerol species of CL in the course of hydrogenation

Diacylglycerol moieties of native rat liver CL could be resolved into 10 molecular species (Fig. 4) and their percentage composition is given in Table 1. For comparison, an analysis of bovine heart CL was included. Both rat liver and bovine heart CL have an unusually high abundance of unsaturated C_{18}–C_{18} configurations, with $C_{18:2}$–$C_{18:2}$ being the principal species.

The major diacylglycerol species produced by catalytic hydrogenation were $C_{18:1}$–$C_{18:1}$, $C_{18:0}$–$C_{18:1}$ and $C_{18:0}$–$C_{18:0}$ (Fig. 4). With the exception of $C_{18:0}$–$C_{18:0}$, the hydrogenated C_{18}–C_{18} species appeared as double shaped peaks due to a second, non-native component. It is known that the applied palladium catalyst produces different $C_{18:1}$ isomers from *cis*-$\Delta^{9,12}$-$C_{18:2}$, namely *trans*-Δ^9-$C_{18:1}$ *cis*-Δ^9-$C_{18:1}$, *trans*-Δ^{12}-$C_{18:1}$ and *cis*-Δ^{12}-$C_{18:1}$ (Vigh *et al.*, 1987), which could all be identified in hydrogenated mitochondria (results not shown). Considering two *sn*-1,2 positional isomers of every diacylglycerol species, the number of isomeric compounds is eight for $C_{18:0}$–$C_{18:1}$ and $C_{18:2}$–$C_{18:1}$, and 16 for $C_{18:1}$–$C_{18:1}$. This is the most likely cause of the asymmetric peak components found after hydrogenation.

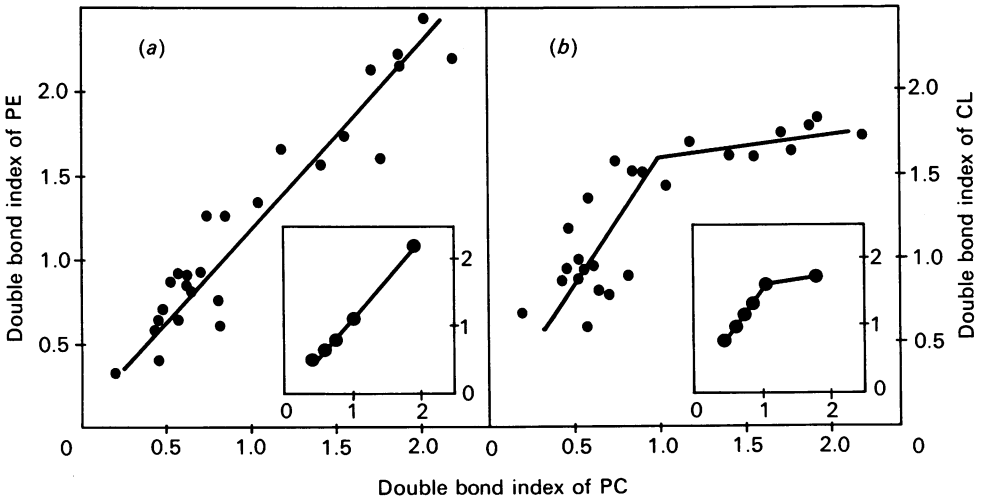

Fig. 2. Relationships between double bond indices of mitochondrial phospholipids

Mitochondrial membranes (main Figures.) or extracted mitochondrial lipids (insets) were subjected to catalytic hydrogenation for various lengths of time to achieve different degrees of unsaturation. The double bond indices were plotted as PC versus PE (*a*) and PC versus CL (*b*).

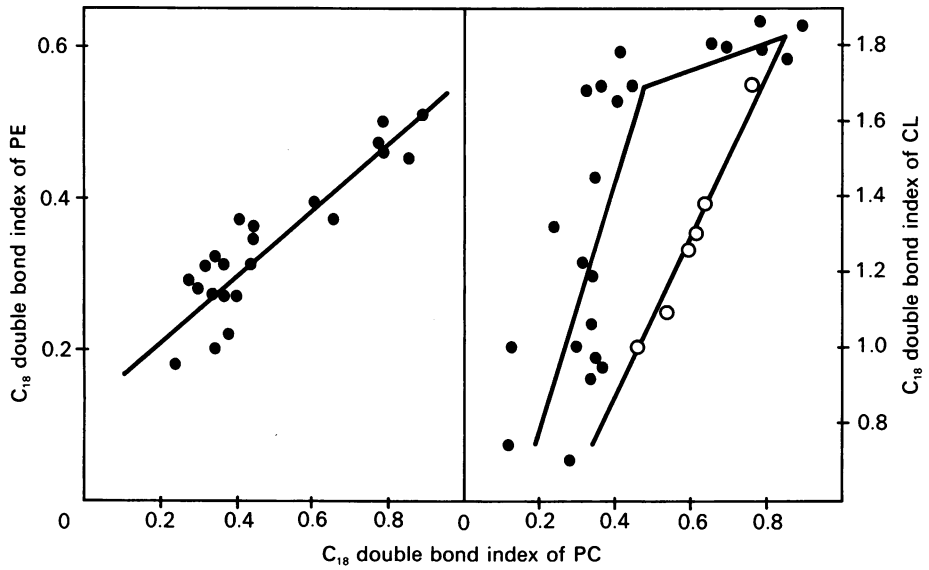

Fig. 3. Relationship between C_{18} double bond indices of mitochondrial phospholipids

Mitochondrial membranes (●) or extracted mitochondrial lipids (○) were subjected to catalytic hydrogenation for various lengths of time, and the double bond indices of C_{18} fatty acids were calculated from fatty acid patterns of PC, PE and CL.

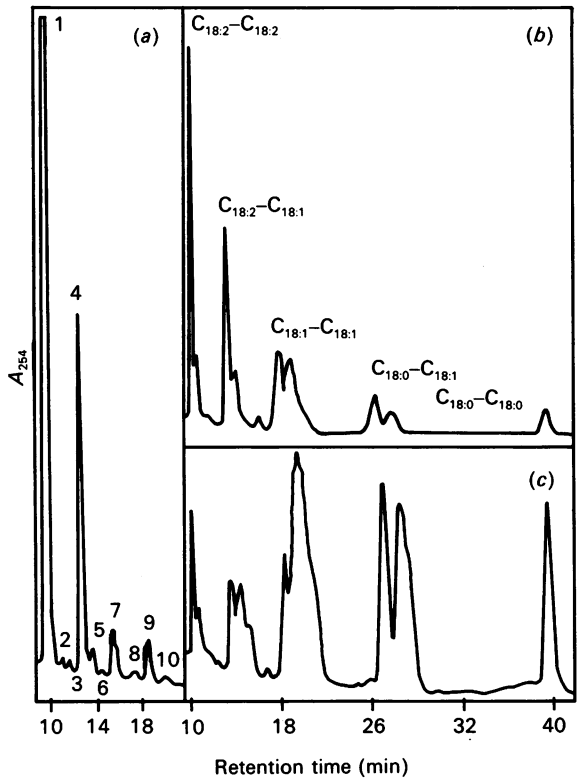

Fig. 4. H.p.l.c. separation of dinitrobenzoyldiacylglycerol molecular species derived from native (*a*) and hydrogenated (*b*, *c*) CL

Molecular species were separated in accordance with Takamura *et al.* (1986) on an RP-18 column (Lichrosorb, 5 μm, 250 mm × 4 mm) eluted by acetonitrile/propan-2-ol (4:1, v/v). A Gilson pump (model 302, set at 1 ml/min), a 254 nm absorbance detector (Waters Associates model 440) and a Hitachi integrator (model 263-80) were used. (*a*) Native CL; peak numbers are explained in Table 1. (*b*), (*c*) CL from mitochondria which were hydrogenated for 3 (*b*) or 10 (*c*) min.

Table 1. Molecular diacylglycerol species of mitochondrial CL

CL was isolated from mitochondrial membranes of bovine heart or rat liver and the molecular species of its diacylglycerol moieties were analysed as naphthylurethane derivatives (bovine heart) according to Kruger *et al.* (1984), or as dinitrobenzoyl derivatives (rat liver) according to Takamura *et al.* (1986). n.d., not detected; tr., traces. Values for rat liver are means ± S.E.M. ($n = 3$). Peak nos. refer to Fig. 4(*a*).

Molecular species	Bovine heart (%)	Rat liver (%)	Peak number
$C_{18:2}$–$C_{18:3}$	18.2	n.d.	–
$C_{18:2}$–$C_{16:1}$	4.3	–	–
$C_{18:2}$–$C_{18:2}$	48.1	58.5±6.6	1*
$C_{16:0}$–$C_{20:4}$	tr.	0.4±0.2	3
$C_{18:2}$–$C_{18:1}$	5.2	30.7±1.6	4
$C_{16:0}$–$C_{18:2}$	tr.	1.0±0.6	5
$C_{18:0}$–$C_{20:4}$	n.d.	2.1±2.0	7
$C_{18:1}$–$C_{18:1}$	5.1	1.2±0.4	8
$C_{16:0}$–$C_{18:1}$	6.1	4.0±2.4	9
$C_{18:0}$–$C_{18:2}$	13.0	n.d.	–
Unknown	–	1.3±0.4	2,6,10

* This peak contained traces of $C_{18:2}$–$C_{16:1}$.

The molecular species distribution in the course of hydrogenation (Table 2) suggests the hydrogenation path: $C_{18:2}$–$C_{18:2} \rightarrow C_{18:1}$–$C_{18:2} \rightarrow C_{18:1}$–$C_{18:1} \rightarrow C_{18:0}$–$C_{18:1} \rightarrow C_{18:0}$–$C_{18:0}$; hence both chains are consecutively affected without obvious positional specificity. $C_{18:1}$–$C_{18:1}$ and $C_{18:0}$–$C_{18:1}$ were the major accumulating species, indicating that conversion of $C_{18:2}$ into $C_{18:1}$ was faster than the subsequent hydrogenation of $C_{18:1}$. The isomerization ratio (for explanation, see Table 2) correlated with the degree of hydrogenation and was always highest in the $C_{18:1}$–$C_{18:1}$ fraction.

Table 2. Major CL species during the course of hydrogenation

Mitochondria were hydrogenated by a water-soluble palladium complex catalyst for 3, 10 or 20 min (for description see the Experimental section) denoted as low, medium and high hydrogenation respectively, and CL was analysed for molecular diacylglycerol species.

Molecular species	Composition (%)				Isomerization ratio*			
		Hydrogenated				Hydrogenated		
	Control	Low	Medium	High	Control	Low	Medium	High
$C_{18:2}$–$C_{18:2}$	54.0	27.7	3.5	1.2	0.0	0.03	0.17	0.40
$C_{18:2}$–$C_{18:1}$	32.7	25.3	6.1	3.8	0.0	0.05	0.10	0.76
$C_{18:1}$–$C_{18:1}$	1.6	21.3	35.0	32.5	0.0	0.99	7.14	4.19
$C_{18:0}$–$C_{18:1}$	0.0	16.8	35.7	32.8	–	0.83	1.40	1.36
$C_{18:0}$–$C_{18:0}$	0.0	5.5	19.0	27.6	–	0.0	0.0	0.0

* Isomerization ratio = (peak area of non-native isomer)/(peak area of native isomer).

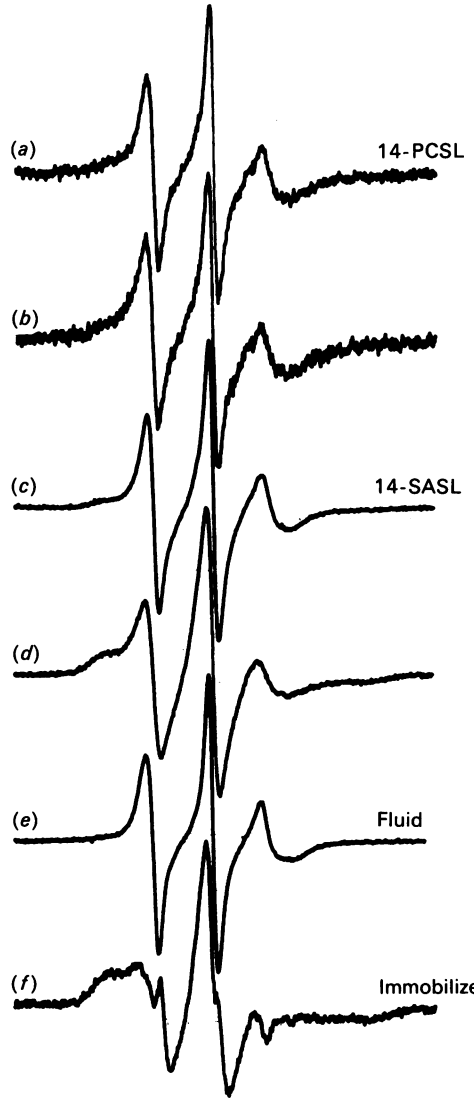

Fig. 5. E.s.r. spectra of C-14 isomers of spin-labelled phosphatidylcholine (14-PCSL) and stearic acid (14-SASL) in native mitochondrial membrane

(*a*) and (*b*) 14-PCSL in native mitochondria before and after catalytic hydrogenation; (*c*) and (*d*) 14-SASL spectra before and after hydrogenation; all of these spectra were recorded at 25 °C. (*e*) E.s.r. spectrum of fluid lipid component recorded in dispersions of extracted lipids. (*f*) E.s.r. difference spectrum obtained by subtraction of the fluid component (spectrum *e*) from the e.s.r. spectrum of 14-SASL in hydrogenated mitochondria (spectrum *d*); motionally restricted component end point. Total scan width = 10 mT.

Effect of hydrogenation on lipid–protein interactions

The e.s.r. spectra of two different spin probes (14-PCSL and 14-SASL) in native and hydrogenated mitochondrial suspensions are shown in Fig. 5 (spectra *a*–*d*). In all of these spectra, a second spectral component typical of motionally restricted lipids could be resolved in addition to the fluid component. The line shape of the fluid component (spectrum *e*) was subject to change on hydrogenation of the mitochondrial lipids, and thus all hydrogenated mitochondrial suspensions were measured at higher temperatures. The amount of temperature correction was estimated from preliminary measurements on untreated and hydrogenated mitochondrial lipid extracts (results not shown). This temperature correction was 7 °C and ensured approximately homeoviscous conditions in which the line shapes, and hence the integrated intensities of the two components, remained identical at various levels of hydrogenation.

The quantitative evaluation of the two-component spectra was done by spectral subtraction, as illustrated in Fig. 5. The spectrum of 14-SASL in vesicles of extracted lipids (spectrum *e* in Fig. 5) was subtracted from the above two-component spectra to obtain slow motion line shapes; a typical immobilized endpoint is shown together with the fluid component for comparison (spectrum *f*). All of the spectra consisted of these two components, and the fractions of motionally restricted component at various levels of hydrogenation are plotted as a function of the double bond index (total fatty acids) in Fig. 6. As judged by qualitative (spectra *a* versus *b*, and spectra *c* versus *d* of Fig. 5) and quantitative comparisons (Fig. 6), the fractions increased when using both 14-PCSL or 14-SASL, indicating significantly more protein-associated spin labels on hydrogenation of the mitochondrial suspension. However, a striking biphasic dependence was

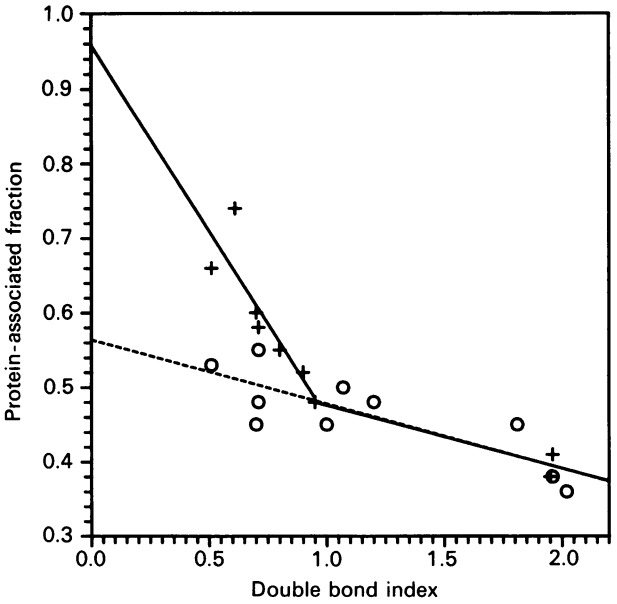

Fig. 6. Unsaturation-dependence of fractions of the motionally restricted component of spin-labelled phosphatidylcholine (14-PCSL) and stearic acid (14-SASL) in native mitochondria after various degrees of hydrogenation at pH 7.4

The e.s.r spectra of 14-PCSL (○) and 14-SASL (+) were analysed by spectra subtractions as described in the Results section. Each point corresponds to the result of a separate experiment; the estimated uncertainty of these data is smaller than the symbol size. Least-squares fits were done by assuming a linear relationship.

observed in the case of 14-SASL: in the double bond index range between 1.0 and 2.2, 14-SASL displayed rather similar behaviour to that of 14-PCSL, whereas at lower values (i.e. more hydrogenated) 14-SASL became associated with steeply increasing specificity.

DISCUSSION

Catalytic hydrogenation of isolated membranes has become a widely accepted tool for the selective modification of the hydrophobic moieties of lipids (Vìgh & Joò, 1983; Vìgh *et al.*, 1985, 1987; Szalontai *et al.*, 1986; Thomas *et al.*, 1986; Quinn *et al.*, 1989). Applying this method for the first time to mitochondria, we found the expected shift towards more saturated chains, mainly at the expense of the native acyls $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$. Neither the lipid/protein ratio nor the enzymic activities of cytochrome oxidase or phospholipase A_2 were significantly altered (results not shown), whereas both PC and PE underwent an effective time-dependent saturation. The plot of their double bond indices indicated an identical susceptibility of these two phospholipids to catalytic hydrogenation. In contrast, the correlation diagram between PC and CL revealed two phases of hydrogenation. Since this course was paralleled in extracted mitochondrial lipids, it did not depend on intramembranous lipid compartmentation, but rather reflected two populations of hydrogenation targets, one solely associated with PC and the other being present in both PC and CL. The former group probably consisted of $C_{20:4}$- and $C_{22:6}$-containing species which were the major components of PC (Schlame *et al.*, 1988) but were found in CL only in trace amounts. According to the fatty acid analysis, $C_{20:4}$ and $C_{22:6}$ displayed by far the highest susceptibility to hydrogenation. As discussed below, the two-phase progress of hydrogenation is useful in relating hydrogenation effects to either CL or PC and PE.

Data shown in Fig. 3 suggested that the C_{18} chains of CL were not as accessible to the catalyst as were the C_{18} chains of PC and PE. Since this phenomenon disappeared after lipid extraction, it seemed to be caused by intramembranous compartmentation rather than headgroup specificity of hydrogenation (see also Vìgh *et al.*, 1987). Of the several possibilities, the existence of a CL compartment, which is partly protected against the catalyst (the lipid annulus of membrane proteins) seems to be the most attractive. If the majority of CL molecules is protein-associated, this lipid must display structural features which guarantee tight association to a variety of different membrane proteins.

To obtain a more detailed insight into the hydrophobic interactions between CL and membrane proteins, we analysed the lipid hydrophobic core on the level of molecular diacylglycerol species. The analysis considerably extends previous structural information about CL; CL diacylglycerol moieties were resolved only according to their acyl length (Wood & Harlow, 1969) or double bond content (Keenan *et al.*, 1970). An h.p.l.c. approach to non-derivatized CL has already illustrated the molecular complexity of this lipid, but the presence of four acyl positions has complicated the assignment of well-defined molecular species (Teng & Smith, 1985). The present analysis revealed 10 molecular diacylglycerol species of CL, the majority being C_{18}–C_{18} species with 3–5 double bonds (Table 1). Very little is known about the role of this specific acyl configuration in the association of CL with integral membrane proteins. Powell *et al.* (1987) concluded that the specificity of CL association with cytochrome oxidase is not solely due to ionic interactions. However, removal of one acyl chain had little effect on the association of CL with cytochrome oxidase (Powell *et al.*, 1987), and removal of one double bond did not change the effectiveness in reconstituting delipidated cytochrome oxidase (Dale & Robinson, 1988). In the present experiments, we could further decrease the number of double bonds, accumulating species like $C_{18:1}$–$C_{18:1}$, $C_{18:0}$–$C_{18:1}$ or $C_{18:0}$–$C_{18:0}$, and could follow the consequences for lipid–protein interaction by e.s.r. spectroscopy.

Spin-label e.s.r. spectroscopy, due to its optimal time scale, can sensitively resolve motionally restricted lipids at the protein–lipid interface from fluid-unassociated lipids (for a review, see Marsh & Horvàth, 1989). Of the various mitochondrial intramembrane proteins, cytochrome *c* oxidase and the adenine nucleotide carrier were studied in some detail in reconstitution experiments and a pronounced specificity for CL and other negatively charged lipids was reported (Knowles *et al.*, 1979; Drees & Beyer, 1988; Horvàth *et al.*, 1988). Since the adenine nucleotide carrier is very abundant in mitochondria, a similar lipid specificity pattern can be anticipated there. Spin-label e.s.r. results based on lipid/protein titration experiments of reconstituted systems (Brotherus *et al.*, 1981) can quantitatively be described by competition

between unlabelled and spin-labelled lipids for solvation sites. Within the framework of this model, the amount of motionally restricted lipids depends on the lipid/protein ratio, the number of binding sites and the relative association constant of the lipid–protein interaction. The first two factors did not seem to make any significant contribution in this case since (1) hydrogenation did not significantly affect the lipid/protein ratio and (2) the number of solvation sites was found to be independent of lipid specificity in several reconstituted lipid–protein systems (for a review, see Marsh, 1985). Hence we postulated that it is the relative association constant which is modulated by hydrogenation; implicit to this assumption is that the lipid competition for solvation sites depends sensitively on the unsaturation of acyl chains. Previous experiments were conducted primarily to understand the role of the headgroup charge (Esmann & Marsh, 1985; Horvàth *et al.*, 1988), and a similar conclusion was reached as to the origin of changes in the bound/fluid ratio in pH and salt titration experiments. Analogously, a decreasing average specificity can be assigned to less unsaturated lipid species in the present experiments, and hence the apparent specificity of spin-labelled PC and stearic acid should increase with respect to the endogenous lipids which are subject to hydrogenation. The striking correlation between the increasing motional restriction of 14-SASL and the hydrogenation of CL suggests that both lipids compete for a specific class of protein-binding sites and that CL becomes less competitive upon hydrogenation.

In summary, the present data demonstrate that the association of mitochondrial membrane lipids with proteins is influenced by the double bond content of the lipid acyl chains. In particular, some fraction of CL seems to be accommodated preferentially in the protein solvation shell, and the specificity of the CL–protein interaction partly depends on double bonds within its hydrophobic moiety.

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