ELSEVIER

FEBS Letters 343 (1994) 22–26

FEBS 13894

FEBS LETTERS

Fatty acid binding site of the mitochondrial uncoupling protein

Demonstration of its existence by EPR spectroscopy of 5-DOXYL-stearic acid

Petr Ježek[a,*], Hans-Joachim Freisleben[b]

[a]*Institute of Physiology, Department of Membrane Transport Biophysics, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague, Czech Republic*

[b]*Gustav-Embden-Zentrum der Biologischen Chemie, J.W. Goethe-Universität, Theodor-Stern-Kai 7, D-60 596 Frankfurt am Main, Germany*

Received 1 March 1994

Abstract

Fatty acid binding site on isolated mitochondrial uncoupling protein (UcP) is demonstrated using EPR spectroscopy of 5-DOXYL-stearic acid (5-SASL), which also activated H^+ transport in proteoliposomes containing UcP. In the presence of UcP the EPR spectrum showed reproducible broadening of the low field peak as well as an increase in h_{+1I}/h_{+1M} ratio, rotational correlation time and in order parameter. The half-height width of the low field peak was even doubled in the presence of another UcP ligand, GDP. Palmitic acid reversed the effect of 5-SASL and non-ionizable 5-DOXYL-decane did not exhibit it.

Key words: Uncoupling protein; Brown fat mitochondrion; H^+ transport; Reconstitution; Fatty acid binding site; 5-DOXYL-stearic acid

1. Introduction

Fatty acids have recently been recognized as also playing a role as allosteric regulators. In mitochondria, fatty acids are believed to be uncouplers (decouplers) of oxidative phosphorylation [1]. In the specialized brown adipose tissue (BAT) [2], a specific mitochondrial protein, the uncoupling protein (UcP) [3] is activated by fatty acids to short-circuit the protonmotive force and generate heat. The fatty acid binding site has not yet been identified. Nevertheless, specific effects of fatty acids related to the UcP are well documented in mitochondria [4–6] and with reconstituted UcP in liposomes [7,8]. Activation of GDP-sensitive uncoupling in mitochondria has a characteristic pattern requiring long chain (C > 10) unesterified fatty acids with optimum response at 14 carbons (myristic acid) and increasing with degree of unsaturation [4]. In proteoliposomes, palmitic acid activates the apparent H^+ transport which is inhibited by GDP [7,8].

The lack of an appropriate methodology has resulted in the inability to detect a putative fatty acid binding site on UcP. A classic radioligand binding protocol was useless because the membrane, representing an unlimited reservoir for fatty acids, produced extremely high non-specific binding [6]. We now have the first evidence of binding of fatty acids to the isolated UcP. We employed 5-DOXYL-stearic acid [9–12] and showed reproducible broadening of the low field peak and increase in h_{+1I}/h_{+1M} ratio in its EPR spectrum which was enhanced even further (the half-height width was doubled) by the presence of GDP. Palmitic acid readily reversed the effect of 5-SASL and non-ionizable 5-DOXYL-decane did not exhibit it.

* Corresponding author. Fax: (42) (2) 471 9517.

Abbreviations: BAT, brown adipose tissue; h_{+1}, low field peak; h_{-1}, high field peak; h_0/h^{-1}, the ratio of heights of the middle- and high-field peak; Octyl-POE, octyl-pentaoxyethylene; PBFI, potassium-binding benzofuran phtalate; 16-SASL, 16-DOXYL-stearic acid; 5-SASL, 5-DOXYL-stearic acid, where DOXYL is the 4,4-dimethyl-3-oxazolinyloxy residue; τ_R, rotational correlation time; TEA, tetraethylammonium; TES, *N*-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid; UcP, uncoupling protein; $W(½)_{h+1}$), half height peak width.

2. Material and methods

BAT mitochondria were isolated from Syrian hamsters by a standard method in a medium containing 5 mg BSA/mg, and swelling in potassium acetate was measured as described previously [4].

2.1. Isolation of UcP

UcP was isolated by the step-wise elution method (cf. [13]) as follows: BAT mitochondria (117 mg protein) were extracted without lipid protection by 5 ml of 4.5% octylpentaoxyethylene (Octyl-POE) in 25 mM TEA-TES, 50 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2. The extract

0014-5793/94/$7.00 © 1994 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)00279-5

was centrifuged at 100,000 × g for 30 min and loaded on to two 10 ml spin-columns, each containing 3 g of dry hydroxylapatite. UcP was eluted in five subsequent steps by centrifugation. The elution medium contained 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2. The first 'pass-through' fraction contained approx. 50% of the total UcP in diluted form. Two subsequent 1 ml elutions yielded all the remaining UcP concentrated in the detergent. These fractions were stored at −80°C and used for reconstitution. Fractions 1–3 showed a single band of M_r 32,000, fraction 4 contained some contaminants above M_r 40,000, and the fifth eluate had virtually no UcP, as revealed by the electrophoresis on 12% acrylamide silver-stained minigels. For EPR measurement the protein from several fractions 2 and 3 was collected and pressure concentrated.

2.2. PBFI Detection of H^+ efflux as the K^+ counterflux in liposomes containing UcP

Isolated UcP was reconstituted by essentially the same method and using the same materials as described previously [14–16]. H^+ efflux was followed as the counterflux (uptake) of K^+ in the presence of valinomycin [15]. Internal [K^+] was detected by the fluorescent indicator, PBFI [16] (Molecular Probes, Eugene, OR). Measurements were performed on a Shimadzu RF 5001PC (Shimadzu Europa GmBH, Germany). Vesicle interior contained 75 mM TEA_2SO_4, 75 mM TEA-TES, pH 7.2, 0.05 mM K_2SO_4 and 50 μM PBFI. For the assay, 100 μl of vesicles were mixed with 1.9 ml of 75 mM K_2SO_4, 75 mM TEA-TES, pH 7.2, and after 20 s 1 μM valinomycin was added*. The fluorescence traces were transformed into 'K^+ traces' using calibrations by aliquots of KCl (cf. [16]) in the presence of 0.5 μM nigericin and 5 μM tributyltin. Transport rates were calculated using known (added) protein content and evaluations of the vesicle volume from size distribution estimated with a Malvern laser particle sizer. The size was 230–250 nm giving a single vesicle volume of $0.6–0.75 \cdot 10^{-11}$ μl.

2.3. EPR spectroscopy

EPR spectroscopy was accomplished in 50 μl capillaries with a Bruker B-R 70 Digital EPR spectrometer with a B-E 25 magnet. For detection of binding of the spin-probe several parameters were used. Splitting of the low field peak h_{+1} into the two peaks, h_{+1M} and h_{+1I}, corresponding to 'mobile' and immobilized fractions, respectively, reflects binding of the probe [18,19]. If their separation is low, binding can be described simply by an increase in the half-height width of the h_{+1} peak ($W(^1/_2)_{h_{+1}}$). Another measure of binding is the ratio of heights of the middle- and high-field peak, h_0/h^{-1} [19,20], a term necessary for calculation of the rotational correlation time [11,12], given as:

$$\tau_R = 6.5 \cdot 10^{-10} \cdot W_0 \cdot [(h_0/h_{-1})^{1/2} - 1] \quad (1)$$

where W_0 is the width of the middle peak. The other parameter used, the order parameter, S, also describes immobilization of a probe, namely its anisotropic motion in lipid systems [11,12,21]:

$$S = 1.723 \cdot [A_{||} - (A_\perp + C)]/[A_{||} + 2 \cdot (A_\perp + C)] \quad (2)$$

where

$$C = 1.4–0.053 \cdot (A_{||} - A_\perp) \quad (3)$$

and $A_{||}$ and $A_\perp$ is outer and inner hyperfine splitting due to parallel and antiparallel coupling, respectively.

3. Results

3.1. 5-DOXYL- and 16-DOXYL-stearic acid activate H^+ transport in proteoliposomes containing reconstituted UcP

In order to demonstrate that spin-labeled derivatives of stearic acid (SASL) can functionally replace natural fatty acids, we have evaluated the activation of apparent H^+ transport by SASL in proteoliposomes with reconstituted UcP. When added either prior to reconstitution (cf. [8]) or added to the assay medium, both 16-DOXYL-(16-SASL) and 5-DOXYL-stearic acid (5-SASL) (Fig. 1) were found to powerfully activate H^+ flux which was very low in their absence or in the presence of BSA. Transport was inhibited by approx. 50% by 500 μM GDP. (Note that full inhibition can be observed only when GDP is present on both sides of the membrane, cf. [14].) The position of the spin label at C5 or C16 had no significant effect on the apparent strength of activation in proteoliposomes. K_a for 5-SASL, estimated from a Hill plot (not shown), was about 18 μM when GDP-sensitive rates were used and maximum activation was set at values obtained prior to lysis which predominate above 200 μM. Activation by 16-SASL with a K_a of 11.3 μM is also demonstrated with intact BAT mitochondria when H^+ transport was assayed as valinomycin-induced swelling in potassium acetate (Fig. 2). The apparent K_a is lower due to the lower lipid-to-protein ratio in mitochondria.

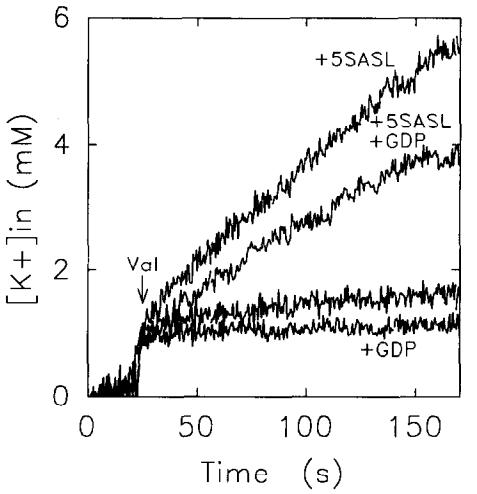

Fig. 1. Activation of H^+ transport by 5-DOXYL-stearic acid in vesicles with the reconstituted UcP. Typical K^+ traces detected by PBFI in response to valinomycin-induced H^+ efflux were measured with and without 100 μM 5-SASL in the presence and absence of external 0.5 mM GDP. Rates reached 1,018 and 500 nmol H^+/min·mg protein in controls without and with GDP, respectively, and 6,350 nmol H^+/min·mg protein with 5-SASL or 4330 with 5-SASL plus GDP.

3.2. EPR spectra of 5-DOXYL stearic acid bound to UcP

EPR spectroscopy can be suitable for detection of binding only in cases where the nitroxide group in the bound ligand is satisfactorily immobilized [9,10]. In the case of membrane proteins such as UcP, which, if not inserted into membranes must be preserved in detergent micelles, the immobilization of the probe in the micellar

*The media must be free of UcP substrates otherwise K^+ uptake reflects the sum of anion uptake and H^+ efflux. However, accepting the Skulachev hypothesis [17] that fatty acid are substrates, the method then actually detects the efflux of anionic fatty acid balancing the K^+ uptake.

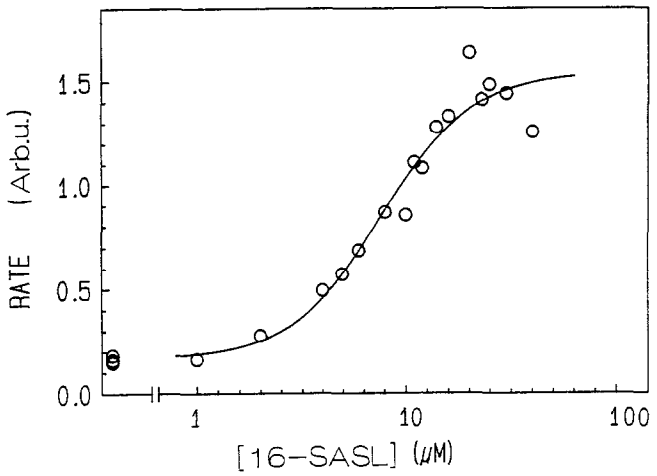

Fig. 2. Activation of H^+ transport in BAT mitochondria by 16-DOXYL stearic acid. The dose–response curve was constructed using absolute rates of swelling in potassium acetate induced by valinomycin at given concentrations of 16-SASL. The solid line represents the best fit using the Hill equation, with a Hill coefficient of 1.3 and a K_a of 11.3 μM.

system itself provides a 'background' over which the signal of bound spin label must be distinguished. Fig. 3, trace a, demonstrates a typical EPR spectrum of 5-SASL in 2% OctylPOE. The shape of such a spectrum with a strongly diminished high field peak h_{-1} is already different from the isotropic spectrum of freely tumbling 5-SASL in ethanol, where the parameters were $W(½)_{h+1}) = 2.4$ gauss, $\tau_R = 0.34 \cdot 10^{-9}$ s and $S = 0.090$ (compare to Table 1). As expected, addition of palmitic acid or GDP or other substances to the detergent solution did not influence the spectrum of 5-SASL (Table 1).

In contrast, the EPR spectrum of 5-SASL in the presence of UcP exhibits remarkable broadening of h_{+1} (Fig. 3, trace b). At 2.6:1 or 1.3:1 molar ratios of 5-SASL-to-UcP, $W(½)_{h+1})$ was 1.3- or 1.6 times broader, respectively (Table 1). Moreover, correlated with increased $W(½)_{h+1})$, the h_{+1} peak signal was extended to the lower field. This indicates the existence of a new peak corresponding to the immobilized 5-SASL population, however without clear peak separation. It was possible to calculate the h_{+1I}/h_{+1M} ratio which increased four times in the presence of UcP (Table 1). Among other EPR parameters, τ_R, increased by 30% or 35% and the order parameter, S, by 20% or 45% at 2.6:1 or 1.3:1 molar ratios, respectively. The reason for the less dramatic changes in these cases lies in the fact that τ_R and S reflect the averaged bound and unbound 5-SASL populations.

When an excess of palmitic acid was added after 5-DOXYL-stearic acid, h_{+1} retained the original shape of the spectrum of 5-SASL in the detergent (Fig. 3, trace c). Similarly, all parameters reached the values of the 'background' spectrum (Table 1).

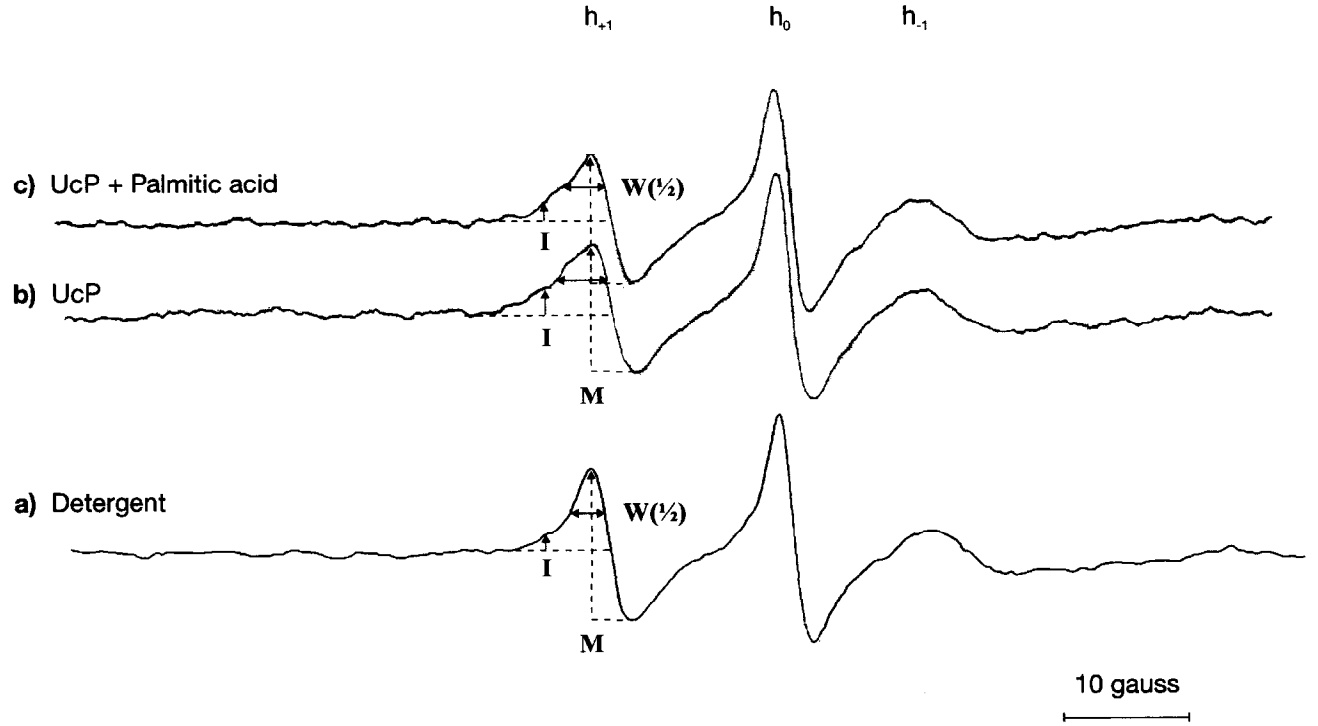

Fig. 3. Typical EPR spectra of 5-DOXYL-stearic acid in the presence and absence of the UcP. (Trace a) 20 μM 5-SASL in 2% OctylPOE dispersed in 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2. (Trace b) 20 μM 5-SASL in the presence of 520 μg/ml UcP in the same solution. 60 μl contained 1.25 nmol of 5-SASL and 470 pmol of UcP, hence the stoichiometry was 2.6:1. (Trace c) Same as b but with 37.5 μM palmitic acid. In the figure, the low-, middle- and high-field peak (h_{+1}, h_0, h_{-1}), the half-height width of the low field peak ($W(½)_{h+1}$) are indicated, as well as the heights of the low field peak of the immobilized (I) and mobile (M) spin label populations, h_{+1M} and h_{+1I}, respectively.

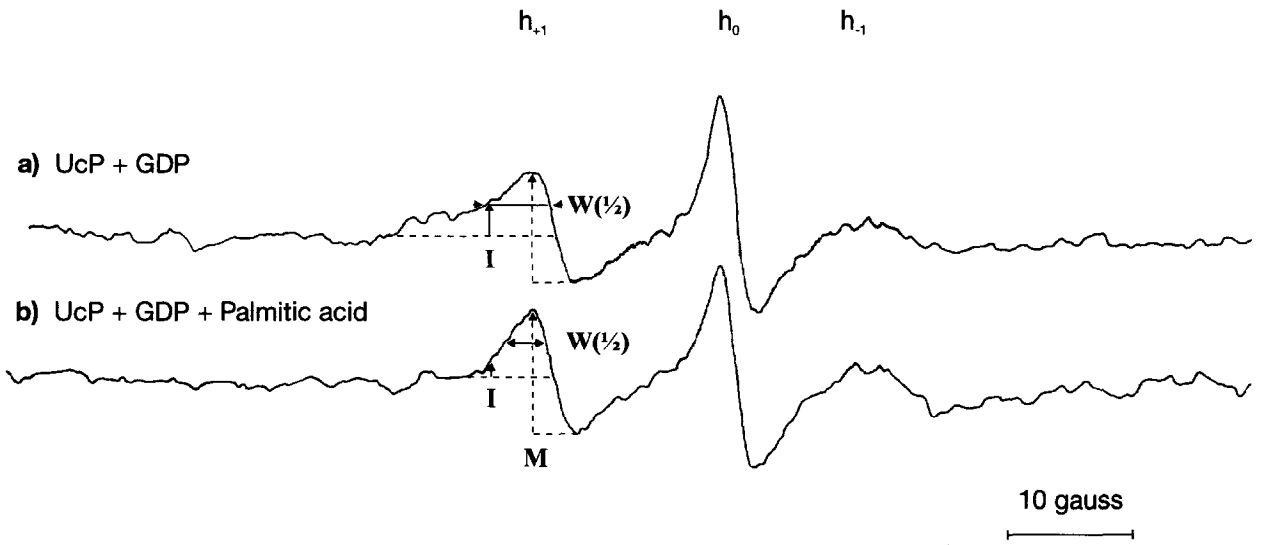

Fig. 4. Effect of GDP on EPR spectra of 5-DOXYL-stearic acid in the presence of UcP. (Trace a) 10 μM 5-SASL in the presence of 520 μg/ml UcP and 2 mM GDP in 2% OctylPOE, 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2 (stoichiometry 1.3:1). (Trace b) Same as a but with 37.5 μM palmitic acid.

When 2 mM GDP was added to the micellar solution of UcP containing 5-SASL at a 1.3:1 molar ratio, $W(½)_{h+1}$) and τ_R even doubled in comparison to the protein-free solution (Fig. 4, Table 1). Without GDP, the $W(½)_{h+1}$) increased by 50%. The h_{+1I}/h_{+1M} ratio increased five times and order parameter S by 50% (Table 1). Palmitic acid again reversed this effect and the parameters went back to the values found without protein (Table 1).

The above described effects of UcP on EPR spectra were not observed in the case of 5-DOXYL-decane as a probe (Table 1), confirming that the carboxyl of the amphiphiles is required for binding. SL-fatty acids with DOXYL in positions further from the carboxyl, such as 7-SASL, exhibited similar effects as 5-SASL, although the changes in parameters were less pronounced in parallel with increasing distance. The parameters of the 7-SASL spectra in the presence of UcP were $W(½)_{h+1}) = 3.4$ gauss, $\tau_R = 2.1 \cdot 10^{-9}$ s and $S = 0.167$, whereas without UcP in 2% OctylPOE the values were $W(½)_{h+1}) = 2.8$ gauss, $\tau_R = 1.9 \cdot 10^{-9}$ s and $S = 0.142$. The spectra of 16-SASL showed virtually no effects, confirming the well-established fact, that nitroxide in the positions 16 is very mobile [10–12].

4. Discussion

Our results demonstrate for the first time the existence of a binding site for fatty acids on the UcP. The 5-

Table 1
Parameters of spectra of 5-DOXYL-stearic acid in the absence and presence of the uncoupling protein, in OctylPOE micelles

Conditions	Half-height peak width, $W(½)_{h_{+1}}$ (gauss)	Ratio of immobilized/mobile fraction, h_{+1I}/h_{+1M}	Rotational correlation time, τ_R (10^{-9} S)	Order parameter S
5-SASL:UcP, 2.6:1				
2% OctylPOE	3.0	0.05	2.03	0.162
2% OctylPOE + palmitic acid	3.0	0.07	1.90	0.160
UcP	3.8	0.19	2.52	0.192
UcP + palmitic acid	3.5	0.14	2.17	0.166
5-SASL:UcP, 1.3:1				
2% OctylPOE	2.7	0.07	1.70	0.140
2% OctylPOE + GDP	2.7	0.07	1.60	0.150
UcP	4.2	0.24	2.15	0.202
UcP + GDP	5.3	0.34	3.21	0.208
UcP + GDP + palmitic acid	3.3	0.08	1.56	0.172
5-DOXYL-decane				
2% OctylPOE	1.27	–	0.32	0.050
Ucp	1.32	–	0.34	0.054

DOXYL-stearic acid exhibited substantial immobilization which was not found in the protein-free micellar solution. One might still argue that the observed immobilization of 5-DOXYL-stearic acid is due to SASL entrapped in the protein/detergent interface and not to a specific binding site. However, this should occur with any related hydrophobic spin label and should not exhibit competitive replacement by palmitic acid. Since 5-DOXYL-decane does not exhibit any immobilization in the presence of UcP it supports the conclusion that 5-DOXYL-stearic acid binds to a specific site on the UcP. In lipid bilayers, fatty acids with chains shorter than the chains of the lipid were immobilized more than those with chains longer than the lipid chains [10]. Similarly, one would expect that 5-DOXYL-decane would cause a strong background, i.e. immobilized-type of EPR spectrum, if the effects were non-specific rather than UcP-related.

Moreover, the enhanced immobilization of 5-SASL by the natural UcP ligand, GDP, independently proves that the immobilization was specific due to binding to UcP. In addition, this phenomenon might indicate the conformational changes related to GDP binding. This is furthermore important in the light of the recent hypothesis proposing that fatty acid binds inside the anion channel of UcP [22]. Since the transport of various monovalent unipolar anions is inhibited by GDP [22] and other purine nucleotides, one may speculate that the observed additional immobilization reflects, in fact, the fatty acid 'entrapped' in the channel. However, addition of palmitic acid in this case should not remove bound probe.

We have clearly shown that, whereas both 16-SASL and 5-SASL activate apparent H^+ transport, only DOXYL in position 5 provides good EPR detection of the immobilization. Similarly as in bilayers, the part of the stearic acid molecule close to the carboxyl is immobilized when bound to UcP.

Our finding justifies ongoing recent re-evalution of the uncoupling mechanism mediated by UcP [17,22]. This mechanism has been traditionally considered as protonophoric. However, Skulachev [17] suggested a fatty acid cycling mechanism: anionic fatty acid passes through the UcP while its return in protonated form via the membrane causes the H^+ flux. Alternatively, fatty acid might allosterically regulate H^+ transport from the 'docking site' in the anion channel of UcP [22]. Both hypotheses inherently assume a specific fatty acid binding site, the existence of which we just confirmed.

Acknowledgements: This work was supported by Deutsche Forschungsgemeinshaft Grant 436 TSE 17/15/93 (S) to both P.J. and H.-J.F. and by Academy Sciences of the Czech Republic Grant 51151 to P.J.

References

[1] Luvisetto, S., Pietrobon, D. and Azzone, G.F. (1987) Biochemistry 26, 7332–7338.
[2] Nicholls, D.G. and Locke, R.M. (1984) Physiol. Rev. 64, 1–64.
[3] Klingenberg, M. (1990) Trends Biochem Sci. 15, 108–112.
[4] Rial, E., Poustie, A. and Nicholls, D.G. (1983) Eur. J. Biochem. 137, 197–203.
[5] Ježek, P., Krasinskaya, I.P., Smirnova, I. and Drahota, Z. (1989) FEBS Lett. 243, 37–40.
[6] Cunningham, S.A., Weisinger, H. and Nicholls, D.G. (1986) Eur. J. Biochem. 157, 415–420.
[7] Strieleman, P., Schalinske, K.L. and Shrago, E. (1985) J. Biol. Chem. 260, 13402–13405.
[8] Ježek, P., Drahota, Z. and Ring, K. (1989) J. Lipid Mediators 2, 85–94.
[9] Gantchev, T.G. and Shipova, M.B. (1990) Biochim. Biophys. Acta 1037, 422–434.
[10] Reboiras, M.D. and Marsh, D. (1991) Biochim. Biophys. Acta 1063, 259–264.
[11] Gordon, L.M. and Curtain, C.C. (1988) in: Methods for Studying Membrane Fluidity (Aloia, R.C., Curtain, C.C. and Gordon L.M., eds.) pp. 25–88, Liss, NY.
[12] Freisleben, H.-J. and Zimmer, G. (1988) in: Methods for Studying Membrane Fluidity (Aloia, R.C., Curtain, C.C. and Gordon L.M., eds.) pp. 25–88, Liss, NY.
[13] Winkler, E. and Klingenberg, M. (1992) Eur. J. Biochem. 207, 135–145.
[14] Ježek, P., Orosz, D.E. and Garlid, K.D. (1990) J. Biol. Chem. 265, 19296–19302.
[15] Murdza-Inglis, D.L., Patel, H.V., Freeman, K., Ježek, P., Orosz, D.E. and Garlid, K.D. (1991) J. Biol. Chem. 266, 11871–11875.
[16] Ježek, P. Mahdi, F. and Garlid, K.D. (1990) J. Biol. Chem. 265, 10522–10526.
[17] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.
[18] Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkoi, G. (1973) Proc. Natl. Acad. Sci. USA 70, 480–484.
[19] Freisleben, H.-J., Fuchs, J., Mainka, L. and Zimmer, G. (1988) Arch. Biochem. Biophys. 266, 89–97.
[20] Kivelsen, D. (1960) J. Chem. Phys. 33, 1094–1106.
[21] Freisleben, H.-J., Ruckert, S., Wiernsperger, N. and Zimmer, G. (1992) Biochem. Pharmacol. 43, 1185–1194.
[22] Ježek, P. and Garlid, K.D. (1990) J. Biol. Chem. 265, 19303–19311.