The possible proton translocating activity of the mitochondrial uncoupling protein of brown adipose tissue

Reconstitution studies in liposomes

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Received 1 October 1983

Loose coupling of thermogenic mitochondria of brown adipose tissue is related to a high proton (or hydroxyl) conductance of the inner membrane and to the presence of a unique 32 kDa uncoupling protein. Reconstitution experiments of the purified protein in liposomes are reported which suggest that this component could form proton channels in the membrane.

Uncoupling protein *Reconstitution* *Proton translocation* *Phospholipid vesicle* *Freeze-fracture microscopy* *Brown adipose tissue* *Mitochondria*

1. INTRODUCTION

The thermogenic mechanism for uncoupling respiration from ADP phosphorylation in brown adipose tissue mitochondria relies on an increase in the normally low proton conductance of the inner membrane; an efficient proton uniport or an indistinguishable hydroxyl uniport is inserted in the membrane [1]. Experiments using whole mitochondria have shown that the activity of such a channel is inhibited by some purine nucleotides and activated by free fatty acids [1-3]. Moreover, it is known that the recoupling nucleotides bind to a characteristic 32 kDa polypeptide of the inner membrane [1,4] and that the amount of this unique component (uncoupling protein) is related to the thermogenic capacity of the brown adipocyte [1,5,6,]. Thus the hypothesis can be made that this protein is the proton channel.

Here, we report on reconstitution experiments of the purified uncoupling protein in liposomes. The proton conductance of liposomes containing detergent but no protein was low. The proteoliposomes containing the protein exhibited a proton conductance of 20 nM $H^+ \cdot min^{-1}$ mg $protein^{-1} \cdot mV^{-1}$ which could be sufficient to allow uncoupling of respiration by mitochondria. In some experiments (but not systematically) the monitored proton conductance of the proteoliposomes was inhibited by added recoupling nucleotides. This variability might be explained by an activation of the protein due to bond free fatty acids. In such a case, these data could favor the hypothesis of a free fatty acid-activated proton translocation activity of the uncoupling protein of brown fat mitochondria [3].

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Pure lecithin was prepared from egg yolk phospholipids using high-pressure liquid

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chromatography. GDP, GTP, ADP, ATP, valinomycin and FCCP were purchased from Sigma; Biobeads SM2 were from Biorad Laboratories. Dialysis membrane were obtained from Spectrapor.

2.2. *Isolation of the uncoupling protein*

The 32 kDa uncoupling protein was isolated from brown adipose tissue mitochondria of cold-adapted hamsters as in [7,8]. The protein was obtained in 20 mM Mops (pH 6.7), 20 mM Na_2SO_4 and 5% Triton-X 100; its purity was checked using SDS-polyacrylamide gel electrophoresis and double-immunodiffusion using antibodies raised against the rat uncoupling protein [9].

2.3. *Reconstitution experiments*

Lecithin (40 mg) was dried under nitrogen, dispersed and sonicated at 2–4°C in 1 ml of 150 mM Na-phosphate buffer (pH 8.0) for 10–12 min using a Biosonik sonifier. The purified protein was then added to liposomes (320 μg protein in 0.25 ml medium for 1 ml liposomes) and allowed to incubate for 5 min at 0°C and then submitted to a second sonication for 10–12 s in ice. Liposomes were then treated with Biobeads SM2 for 1–2 h in ice in order to eliminate Triton excess brought by the protein as described in [10]. In parallel, control liposomes were prepared using the same procedure except that the protein was omitted, although protein medium containing the same amount of detergent as for proteoliposomes was added. In order to eliminate most of phosphate buffer external to liposomes, they were then dialysed overnight at 4°C against isotonic Na_2SO_4 (150 mM), 2 mM Na-phosphate buffer (pH 8.0) using a Spectrapor membrane.

2.4. *Measurement of proton translocating activity*

Liposomes (200 μl) containing 150 mM Na-phosphate buffer (pH 8.0) were added to 3–4 ml dialysis medium of which the pH was lowered to 6.7 by H_2SO_4 addition to generate a pH gradient between liposomes and the external medium. The proton translocation through liposomes was measured using an acid-base titration technique and a Radiometer pH-stat apparatus [11]. Incubation was carried out under a constant stream of N_2 at room temperature.

2.5. *Electron microscopy*

The samples were incubated with glycerol (25%, w/v final concentration) before being rapidly frozen in liquid propane. Platinium-carbon replicas were obtained using a Balzers 301 freeze-etching unit and examined in a Philips 301 electron microscope [12].

3. RESULTS

3.1. *Freeze-fracture electron microscopy*

Sonicated pure liposomes containing the same amount of detergent as reconstituted proteoliposomes showed only the presence of small vesicles displaying smooth fracture faces (fig. 1A). The reconstituted uncoupling protein-lipid proteoliposomes showed the presence of larger vesicles, some of which were highly particulate (fig. 1B).

The number of fractured vesicles showing particles was close to 4% of the total number of fractured vesicles. About 50% of vesicles contained one particle, 30% two particles, 20% three particles or more. The distribution of the protein particles between convex and concave fracture faces was roughly equal.

The size distribution of the particles in reconstituted proteoliposomes is given in fig. 2, the mean value of the diameters being close to 100 Å.

3.2. *Proton-translocation through liposomes*

Control vesicles containing no protein but the same amount of detergent as reconstituted proteoliposomes could not translocate measurable amounts of protons; in comparison, the proteoliposomes were able to translocate protons (fig. 3). In this case the monitored proton conductance was generally 20 nM $H^+ \cdot min^{-1} \cdot mg\ protein^{-1} \cdot mV^{-1}$ in lecithin vesicles (table 1). Similar data were obtained from 10–15 separate reconstitution experiments.

3.3. *Nucleotide control of the proton conductance of reconstituted proteoliposomes*

As illustrated in fig. 3 and table 1, in a few experiments the monitored proton conductance of proteoliposomes was sensitive to nucleotides. In lecithin vesicles the proton conductance was lowered by GDP (exp. a, b, table 1; exp. a, fig. 3).

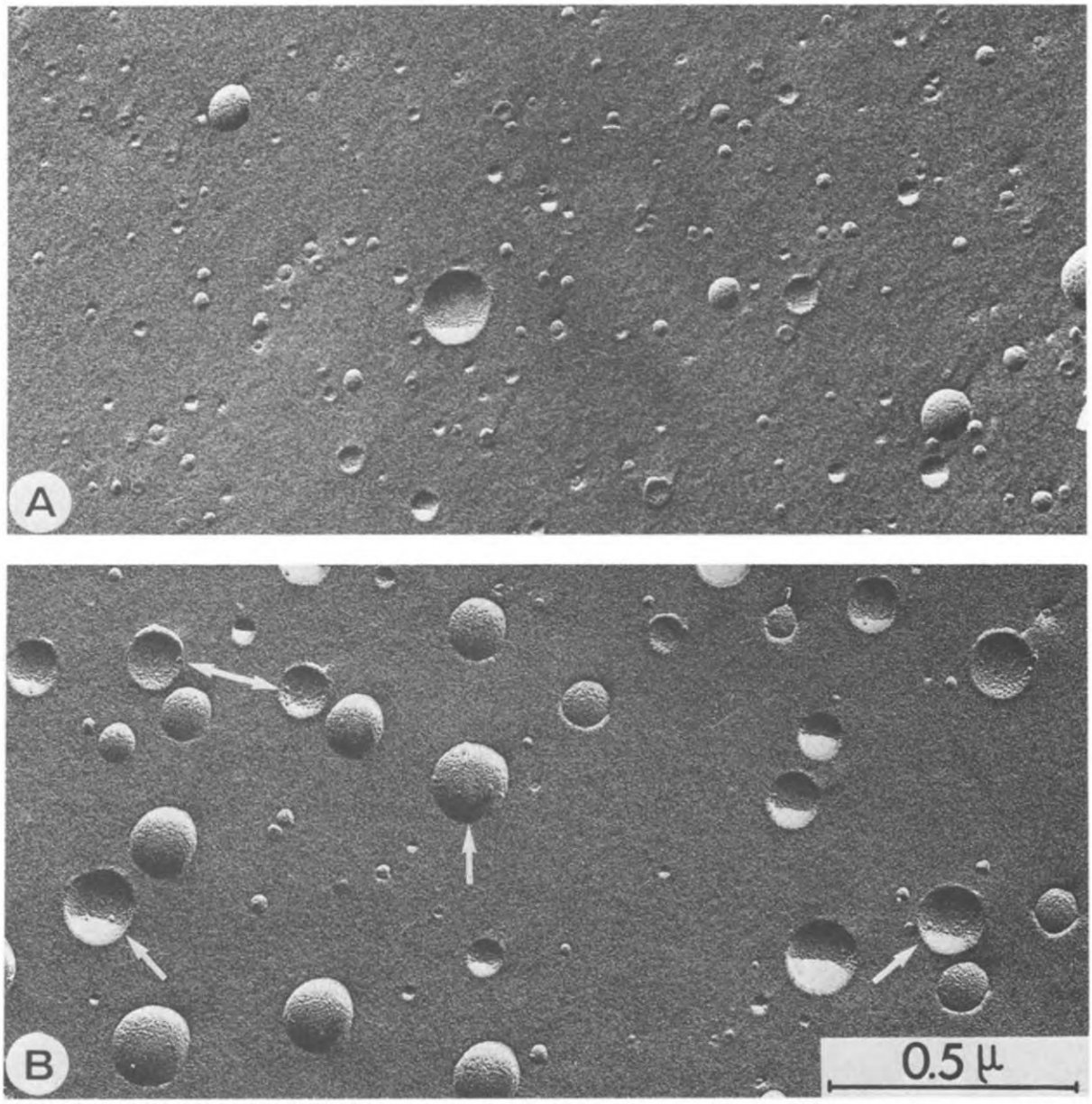

Fig. 1. Freeze-fracture electron micrographs. (A) Sonicated egg lecithin liposomes incubated with the same amount of detergent (Triton X-100) as that used for the reconstitution of uncoupling protein proteoliposomes. Note the presence of smooth concave and convex fracture faces, typical of fractured pure lipid vesicles. (B) Uncoupling protein-lecithin proteoliposome preparation (see section 2). Note the presence of a few highly particulate fractured vesicles (arrows).

In one experiment (exp. a, table 1) the conductance was also lowered by guanylyl imidodiphosphate but not by AMP or CDP. Moreover GDP was not able to induce proton conductance in control vesicles. Nevertheless these data could not be systematically reproduced and in numerous cases, GDP was not able to reduce the proton translocation.

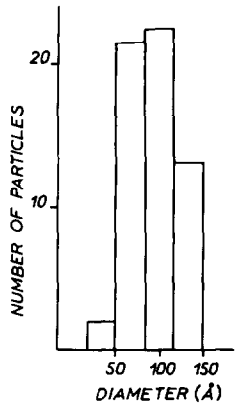

Fig. 2. Size distribution of the particles, such as those shown in fig. 1B, measured perpendicularly to the direction of their shadows.

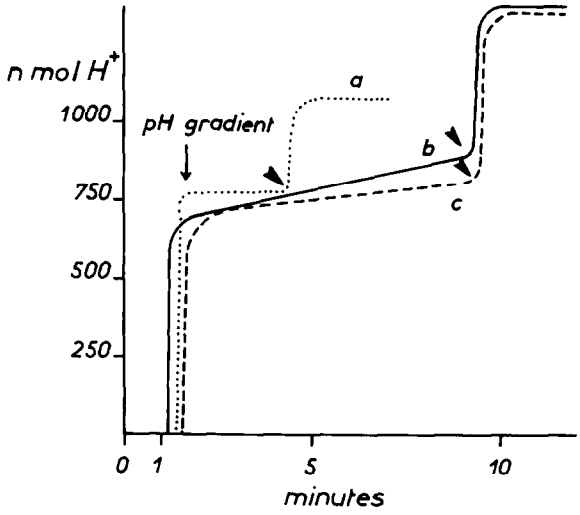

Fig. 3. The proton translocation through liposomes was monitored as described in section 2. At the time 1 min, a pH gradient was generated by addition of H_2SO_4. (a) Control vesicles, containing no uncoupling protein; (b and c) vesicles reconstituted with purified uncoupling protein, in the presence (c) and absence (b) of 0.05 mM GDP. The arrow indicates the addition of 50 μl 10% Triton X-100.

4. DISCUSSION

4.1. *Morphological aspect of reconstitution*

Freeze-fracture electron microscopy of uncoupling protein-lecithin proteoliposome preparations reveals the presence of a small proportion, about 4%, of vesicles displaying mostly one or two particles per fracture face. The distribution of sizes (mean particle diameter about 100 Å, fig. 2) and number of particles between convex and concave fracture faces are roughly the same. All these observations indicate that the uncoupling protein is inserted into the lipid bilayer in a symmetric way, probably as small oligomers of protein, since the observed diameters of the particles are much too large to reflect individual monomers of 32 kDa.

Table 1

Effects of various nucleotides on the rate of H^+ transport in liposomes reconstituted with the uncoupling protein

Exp.	No nucleotide	GDP	GMP-PNP*	AMP	CDP
a	20	11	10	20	25
b	20	10	–	–	–
c	6.2	6.2	–	–	–

Liposomes containing the uncoupling protein were prepared as described in section 2 (a and b, lecithin vesicles; c, lecithin/phosphatidylinositol vesicle). A pH gradient was generated and H^+ movement monitored. The nucleotide concentration was 0.05 mM. Values are expressed as nM $H^+ \cdot min^{-1} \cdot mg\ protein^{-1} \cdot mV^{-1}$

* Guanylyl imidodiphosphate

4.2. *The proton conductance of proteoliposomes*

In numerous experiments the proton conductance of reconstituted proteoliposomes was calculated to be 20 nM $H^+ \cdot mg\ protein^{-1} \cdot mV^{-1}$. This value can be compared to results in [1] where the proton conductance of isolated whole mitochondria from hamster brown fat was tound to be 350 nM $H^+ \cdot min^{-1} \cdot mg$ uncoupling $protein^{-1} \cdot mV^{-1}$ (considering that the proportion of the uncoupling protein is about 10% of the total mitochondrial proteins [7]). Such calculations suggest that 6% of the uncoupling protein was reconstituted in an active configuration, which is in agreement with two series of experiments involving the reconstitution of an other mitochondrial component [12,13].

4.3. *The regulatory function of nucleotides*

The data of the exp. a in table 1 are in excellent

agreement with data from experiments on whole mitochondria which indicated that the proton conductance was inhibited by GDP or by guanylyl imidodiphosphate but not by CDP or AMP [14]. Nevertheless the expected inhibition of the proton conductance of reconstituted proteoliposomes was rarely observed. Such a result could be explained by a poor interaction between added GDP and the reconstituted protein. Moreover, recent elegant experiments have demonstrated that very small amounts of free fatty acids could be very potent activators of the proton conductance of brown fat mitochondria [2,3]. Thus, it may be postulated that in our conditions free fatty acids were bound to the protein (during purification or liposome preparation) and that they activated the protein and prevented the effect of GDP.

Our data do not fully demonstrate that this uncoupling protein is a proton translocator but they do indicate that this hypothesis is plausible. Such an hypothesis has been recently proposed by authors in [15] who clearly demonstrated that the uncoupling protein binds firmly dicyclohexylcarbodiimide which is a well-known ligand of proton pumping activity of several membrane enzymes.

ACKNOWLEDGEMENTS

This research was supported by grants from the C.N.R.S. The authors wish to thank M.J.-C. Dedieu for expert technical assistance in freeze-fracture studies.

REFERENCES

[1] Nicholls, D.G. (1979) Biochim. Biophys. Acta 549, 1-29.
[2] Locke, R.M. and Nicholls, D.G. (1981) FEBS Lett. 113, 299-303.
[3] Locke, R.M. Rial, E., Scott, I.D. and Nicholls, D.G. (1982) Eur. J. Biochem. 129, 373-380.
[4] Heaton, G.M., Wagenvoord, R.J., Kemp, A. jr. and Nicholls, D.G. (1978) Eur. J. Biochem. 82, 515-521.
[5] Ricquier, D. and Kader, J.C. (1976) Biochem. Biophys. Res. Commun. 73, 577-583.
[6] Ricquier, D., Mory, G. and Hemon, P. (1979) Can. J. Biochem. 57, 1262-1266.
[7] Lin, C.S. and Klingenberg, M. (1980) FEBS Lett. 135, 249-252.
[8] Ricquier, D., Lin, C.S. and Klingenberg, M. (1982) Biochem. Biophys. Res. Commun. 106, 582-589.
[9] Ricquier, D., Barlet, J.P., Garel, J.M., Combes-George, M. and Dubois, M.P. (1983) Biochem. J. 210, 859-866.
[10] Holloway, P.W. (1973) Anal. Biochem. 53, 304-308.
[11] Nichols, J.W. and Deamer, D.W. (1980) Proc. Natl. Acad. Sci. USA 77, 2038-2042.
[12] Brandolin, G., Doussiere, J., Gulik, A., Gulik-Krzywicki, T., Lauquin, G.J.M. and Vignais, P.V. (1980) Biochim. Biophys. Acta 592, 592-614.
[13] Kramer, R. and Klingenberg, M. (1979) Biochemistry 18, 4209-4215.
[14] Heaton, G.M. and Nicholls, D.G. (1977) Biochem. Soc. Trans. 5, 210-212.
[15] Kolarov, J., Houstek, J., Kopecky, J. and Kuzela, S. (1982) FEBS Lett. 144, 6-10.