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The topology of the brown adipose tissue mitochondrial uncoupling protein determined with antibodies against its antigenic sites revealed by a library of fusion proteins

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The uncoupling protein (UCP) of brown adipose tissue mitochondria is a specialized member of the family of evolutionarily related mitochondrial membrane transporters, which also includes the ADP/ATP translocator and the phosphate carrier. We have generated a library of bacterial clones randomly expressing short subsequences of the UCP fused to the MalE periplasmic protein of *Escherichia coli*. Anti-UCP sera were used to select clones expressing antigenic sequences of the UCP. Ten different fusion proteins representing eight non-overlapping subsequences of the UCP were obtained. The ability of fusion proteins to select antibodies directed against a short segment of the UCP was used to study the topological organization of the UCP in the inner mitochondrial membrane. Four different fusion proteins were used to determine the orientation of the N-terminal extremities of the first, second, third and fourth predicted α-helices of the UCP. This topological study together with previous data on the UCP provides an experimental basis for the predicted structure of the UCP and for other homologous carrier proteins.

Key words: Escherichia coli/inner mitochondrial membrane/MalE/membrane transporter/recombinant expression

Introduction

The high thermogenic capacity of brown adipose tissue is due to the activity of the UCP (uncoupling protein), a unique protein located in the inner mitochondrial membrane. The UCP is a 33 kDa protein which allows proton re-entry into the mitochondrial matrix. This proton flux by-passes the ATP synthase and frees brown adipocytes from the restraints of respiratory control, allowing them to develop a very high catabolic activity and to dissipate most of the energy as heat (for reviews see Klingenberg, 1990; Ricquier *et al.*, 1991). The activity of the UCP is regulated. It possesses a binding site for purine nucleotides, and nucleotide binding inhibits the transport activity of the UCP, whereas free fatty acids activate it (Rial *et al.*, 1983). AcylCoA may also affect the UCP (Katiyar and Shrago, 1991). The UCP is also able to transport a variety of anions, including chloride and several synthetic compounds (Jezek and Garlid, 1990). Its sequence is related to those of anion transporters of the inner mitochondrial membrane, such as the ADP/ATP translocator, the phosphate carrier and the oxoglutarate carrier. The sequences of several other proteins deduced from DNA sequences are related to the mitochondrial carriers, although their function is still unknown. Thus, all these proteins form a family of membrane proteins of ~30 kDa, thought to derive by duplication and divergence from a common ancestor (for a general review see Walker, 1992). The relationship of mitochondrial carriers to other proteins has also been noticed, suggesting that their evolutionary history could be more complex than expected (Bouillaud *et al.*, 1992).

The structure of these carriers has attracted great interest since they are relatively small membrane transporters exhibiting substrate specificity and regulation of transport, and also because they catalyse exchanges of importance for the metabolism of eukaryotic cells (Krämer and Palmieri, 1992). Prediction of their structure took place after publication of the first sequences (Saraste and Walker, 1982; Aquila *et al.*, 1985; Runswick *et al.*, 1987). These proteins present a repetition of internal motifs, suggesting that they have arisen by the triplication of an ancestral domain of ~100 amino acids. This triple organization is also apparent in the structure of the genes (Cozens *et al.*, 1989; Ricquier *et al.*, 1991; Iacobazzi *et al.*, 1992). A model for the organization of these carriers in the inner membrane has been proposed taking into account this internal repetition. A general model has thus emerged according to which the mitochondrial carrier is made up of three topologically equivalent domains composed of a hydrophilic segment of ~40 amino acids between two α-helices spanning the membrane (Walker, 1992). Each domain is linked to the next one by a short sequence; thus six transmembrane α-helices have been predicted for each monomer of 30 kDa. These carriers are thought to act as dimers, thus at least 12 α-helices participate as the functional unit. Non-permeating probes, such as proteases and antibodies, reacting with a definite subsequence of such a membrane protein are valuable tools for studying their topology. The N-terminal and C-terminal extremities of the phosphate carrier have been studied (Ferreira *et al.*, 1990; Capobianco *et al.*, 1990) and shown to be exposed on the cytosolic side of the inner membrane as is the N-terminal extremity of the ADP/ATP carrier (Brandolin *et al.*, 1989). For UCP, topological data concerning the orientation of the last α-helix are available: it has been shown that the C-terminal end of UCP protrudes into the inter-membrane space (Eckerskorn and Klingenberg, 1987) and that the N-terminal part of this helix is orientated towards the matrix (Miroux *et al.*, 1992). Other proposals about the actual number of membrane-spanning segments in the ADP/ATP carrier (Marty *et al.*, 1992), and the UCP (Aquila *et al.*, 1985; Klingenberg, 1990) have been made. These proposals have questioned the validity of the general model and point to the need for further experimental data. The use of fusion proteins to sort sequence-specific antibodies in a serum raised against the whole UCP has proved to be an efficient way of performing topological studies, and a

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generalization of this method has been proposed (Miroux *et al.*, 1992). The results of its application to the UCP are presented here.

The orientations of the first, second, third and fourth predicted α-helices relative to the inner membrane have been determined. These data, combined with the results of other previous studies (Eckerskorn and Klingenberg, 1987; Miroux *et al.*, 1992), demonstrate that the general model is in agreement with the experimental data obtained on the UCP.

Results

Construction and screening of the library

The first aim of this study was to generate a library of bacterial clones randomly expressing subsequences of the UCP fused to the C-terminal end of the MalE protein. A procedure was developed deriving from the strategy used to produce clones for DNA sequencing (Figure 1). It was necessary to plate a relatively high number of clones (a few thousand in several rounds), as statistically only one-sixth of the clones were expected to have the UCP sequence inserted in-frame with MalE and to produce a MalE-UCP fusion protein. Moreover, an unknown fraction of these fusion proteins was expected to contain an antigenic determinant of the UCP. The size of 10–30 amino acids selected for the UCP moiety in fusion proteins was a compromise between the minimum required to permit antigen–antibody recognition and the small size allowing topological conclusions to be made.

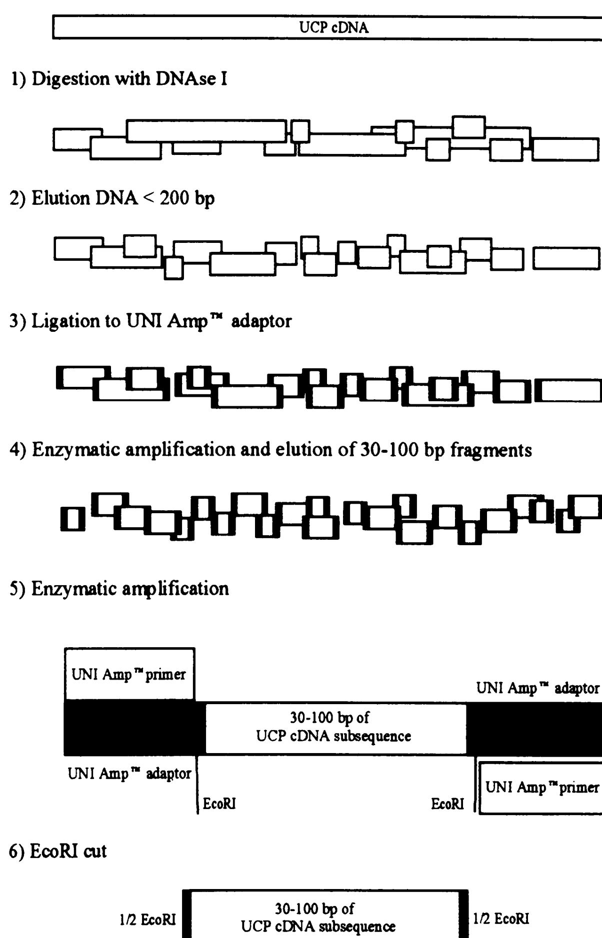

Fig. 1. Construction of the library. Outline of the strategy for generation of fragments of UCP cDNA to be cloned in the pMEC vector. The details of the structure of a DNA fragment that was amplified are indicated, as well as of the final product after *Eco*RI digestion.

Sequences of the UCP recognized by antibodies in the library of fusion proteins

The immunological screening of the library was made according to classical procedures. The nomenclature of bacterial clones was as follows: the first number corresponds to the serum used, and this is followed by a letter or number to discriminate between different clones recognized by the same serum. The first anti-UCP serum used (sheep number 375, see Table I) was chosen because it was highly reactive against the UCP. This serum recognized 13 different clones in a first screening, but only 12 of these contained an insert; one of these 12 clones contained a very large insertion. After sequencing the 11 remaining clones, only four of them (375-B, 375-F, 375-G and 375-I) were retained. The others contained chimeric sequences in which several adaptors or different parts of the UCP cDNA were linked together artefactually. The same serum was used in a second round of screening, leading to recognition of three further antigenic sites (375-4, 375-9, 375-22). Different clones expressing the same fusion protein as in clone 375-22 were found many times in this second round of screening, revealing that the cloning procedure could lead to over-representation of particular sequences, possibly because of variable DNase sensitivity, or differential amplification of sequences. As we progressed, the newly selected clones proved to produce fusion proteins overlapping these seven antigenic sites. When it became clear that serum 375 was not able to reveal further antigenic sites, other sera (sheep numbers 495, 505 and 579) were used, although their titre was much lower when tested against the UCP. Three other clones (495, 505 and 579) were isolated. The loss of efficiency of screening procedures in revealing new antigenic determinants and the availability of sequences of interest for topological studies prompted us to proceed to topological studies. The different fusion proteins are presented in Table I. The UNI-Amp *Eco*RI adaptor is expected to introduce always the same amino acids, Asp-Phe, at the N-terminus, and a more variable sequence at the C-terminus. However, the amino acids found at the boundaries of the UCP subsequences varied, and other unwanted amino acids were frequently found, as a consequence of cloning artefacts generated during ligation and amplification steps. Occasionally, mutations could be found in the UCP sequences, a phenomenon linked to the high number of PCR cycles employed. The size of the UCP subsequences, 11–26 amino acids, was in good agreement with the size of the DNA selected in the elution step.

These 10 fusion proteins represented eight non-overlapping sequences of the UCP (Figure 2). It is noteworthy that only one (375-9) did not contain a predicted helical segment, another (375-F) corresponded to the fourth α-helix, whereas eight of them contained the joint between a predicted α-helix and the preceding or following hydrophilic segment. No antigenic determinant was found at either end of the fifth α-helix. Other than the sequence previously studied (Miroux *et al.*, 1992), no antigenic determinant was found in the last third of the UCP, nor was an antigenic determinant at the C-terminal end of UCP (Ridley *et al.*, 1986) identified by our sera.

Table I. Sequences of fusion proteins recognized by the sera

Name of protein fusion	Number of amino acids	5′ sequence	UCP sequence	3′ sequence	Mutations	Stop codon
375-G	11	C***EF***VSLRAK	MVSSTTSEVQPT 1 11	IT***N***SN	–	AMB
579	18	C***EF***V	TSEVQPTMGVKIFSAGVS 5 22	LPT***N***SN	–	OCH
495	32	C***EF***V	DIITFPLDTAKVRLQIQGEGQASSTIRYKGVL 27 58	VVIADVGTLGYLSW	–	AMB
375-I	19	C***EF***VA	ITTLAKTEGLPKLYSGLPA 61 79	M***N***SK	–	AMB
375-4	18	ACALKVPESI	GLYDTVQEYFSSGRETPA 93 110	H***EF***SEGSRSR	–	AMB+NS-NZLR
375-B	14	C***EF***V	GRETPASLGSKISA 105 118	GWLH***EF***	–	OCH
375-9	20	C***EF***V	EVVKSECTQSHLHGIKPRYT 134 **** 154	PR***IL***TS	aa 138–142 deleted; * unknown aa	OPA
505	21	C***EF***VVR	ATTESLSTLWKGTTPNLMRNV 164 184	TT***N***SN	–	AMB
375-F	26	C***EF***V	NLMRNVIINCTELVTCDLMKGALVNH 179 * 204	T***N***SN	*Y194–C	AMB
375-22	19	C***EF***V	MTMYTKEGPAAFFKGFAPS 255 273	H***EF***	–	OCH

Bold italic letters indicate the amino acids corresponding to the *Eco*RI site. Other, unwanted non-UCP and non-MalE amino acids are also indicated, as well as mutations if they occurred in the UCP sequence. Most of the time these unexpected modifications were due to sequences corresponding to the UNI-Amp adaptors being incorrectly ligated.

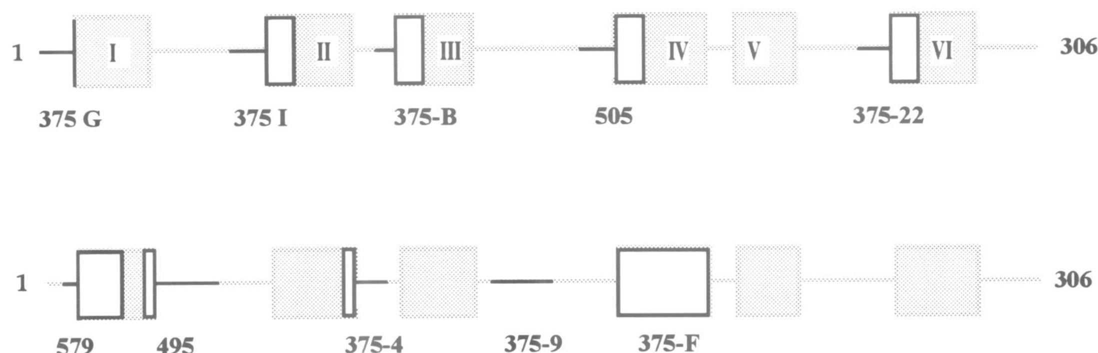

Fig. 2. Location of immunoreactive UCP sequences fused to the MalE protein. Regions of UCP expressed in the immunoreactive fusion proteins presented in Table I are shown. On the shaded background representing the UCP sequence, boxes numbered with roman letters indicate the situation of the six predicted transmembrane α-helices.

The reactivity of anti-UCP sera against fusion proteins

The reactivity of the different sera against these different fusion proteins was studied. It was found that antibodies directed towards the 375-B sequence were largely predominant in serum 375, and were probably responsible for its high reactivity. In contrast, in sera of lower reactivity, such as 505, 579 and 495, the titre of antibodies against the different fusion proteins was more homogenous (data not shown). This explains why these sera allowed poorly immunoreactive fusion proteins to be isolated. The apparent molecular weights of the different fusion proteins and the intensities of the immunological signals in Western blots are shown in Figure 3A. The signal was highly variable and the periplasmic extracts from clones 375-F, 375-9, 495, 589 had barely detectable reactivity. The heterogeneity of the immunological reactivity was confirmed by ELISA (Figure 3B) of different fusion proteins, and indicated the necessary dilutions of anti-UCP antibodies in back-titrations. The periplasmic extracts from clones 375-B, 375-G, 375-I gave a signal higher than the background and thus could be used directly for back-titration, without further purification either of the fusion protein or of the anti-UCP serum. With the sera no significant signal could be seen in ELISA using the fusion protein 505. According to the structure prediction of the UCP it was of interest to examine the topology of the region 505 (Figures 2 and 6). Thus, after the purification

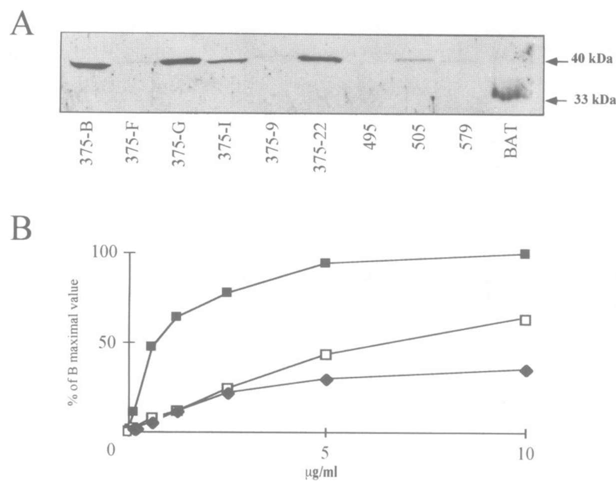

Fig. 3. Immunoreactivity of the fusion proteins. (**A**) Western blot analysis of periplasmic extracts containing the fusion proteins. For each recombinant clone, 5 µg of periplasmic proteins were loaded on to the gel. The last lane contained 1 µg of mitochondrial protein from brown adipose tissue. (**B**) ELISA test. Microtitre plates were coated with increasing concentrations (0.16–10 µg/ml) of 375-B (■), 375-G (□) and 375-I (◆) fusion proteins. Immobilized proteins were incubated with anti-UCP serum diluted 15 000-fold. The subsequent steps are described in the text and by Miroux *et al.* (1992). The background [values obtained with periplasmic extract containing the MalE protein alone (pMEC vector)] has been subtracted. To facilitate comparisons, the maximal value of absorbance obtained with the fusion protein B was considered to be 100%.

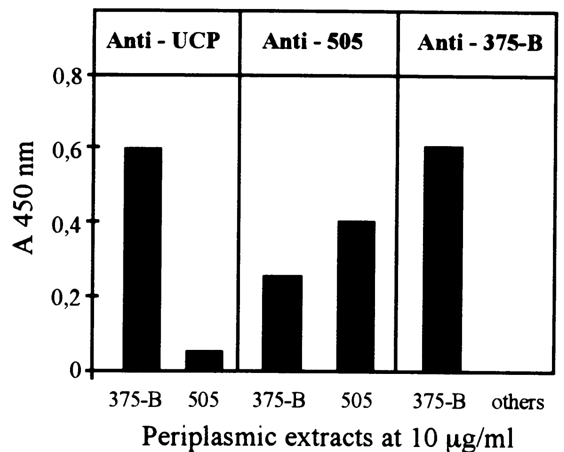

Fig. 4. Reactivity of antibodies assayed by ELISA after purification by affinity chromatography on 375-B or 505 fusion proteins. The purification of the antibodies 505 and 375-B was followed by ELISA. Wells were coated with periplasmic extracts of bacteria producing the fusion proteins. Immobilized proteins were incubated with anti-UCP serum, purified anti-505 antibodies or purified anti-375-B antibodies; the dilutions were respectively 1:16 000, 1:150 and 1:200. Peroxidase activity was monitored by absorbance at 450 nm.

of this fusion protein on amylose, an affinity column was made by crosslinking the purified 505 fusion protein to activated beads. This affinity column was then used to purify anti-505 antibodies present in serum 375. The resulting anti-505 antibodies were usable in back-titration, although their purification was not complete, since other antibodies recognizing 375-B were still present (Figure 4). It should be noted that the titre of anti-505 antibodies was not significantly higher in serum 505 than in serum 375 (data not shown). The same procedure was applied to purify sequence-specific anti-375-B antibodies.

Back-titration experiments

Anti-UCP antibodies were incubated with a suspension of freeze–thawed brown adipose tissue mitochondria, or with submitochondrial particles obtained by sonication of mitochondria. Antibodies remaining free were assayed by ELISA (Brandolin *et al.*, 1989). In these experiments, purified sequence-specific anti-505 antibodies were trapped by submitochondrial particles only. For the other fusion proteins the direct use of the anti-UCP serum was possible since the sequence-specificity was determined by the fusion protein bound to the ELISA plate. With the anti-UCP serum it was demonstrated that anti-375-I antibodies also reacted only with submitochondrial particles (375-I Figure 5). In contrast, antibodies against 375-B and 375-G were trapped by freeze–thawed mitochondria as well, suggesting that these sequences were accessible on the cytosolic side of the inner membrane; however, the titration was not complete, and the shapes of the titration curves obtained with mitochondria were different from those obtained with submitochondrial particles (data not shown). In order to obtain a complete titration of these antibodies with freeze–thawed mitochondria it was necessary to purify the IgG on protein A–Sepharose (375-G, Figure 5), or to prepare sequence-specific antibodies (375-B, Figure 5). Significant differences could be seen between the amounts of mitochondria or of submitochondrial particles able to titrate each site-specific antibody. This probably reflected different affinities of these antibodies.

Discussion

The power of recombinant expression in *Escherichia coli* has been little used for the study of mitochondrial carriers, except for work on the phosphate carrier (Ferreira and Pedersen, 1992) and the oxoglutarate carrier (G.Fiermonte, J.Walker and F.Palmieri, in preparation). This is partly due to the dependence of the folding on the presence of mitochondria. Thus no active mitochondrial carrier has been expressed in the membrane of bacteria, and the genetic approaches developed in *E.coli* for the study of the topology of membrane proteins (McGovern *et al.*, 1991; Botfield *et al.*, 1992) have not been applied hitherto. Consequently, most of the work on the structure of mitochondrial carriers had been carried out by biochemical techniques, including the use of antibodies raised against synthetic peptides. Antibodies cannot permeate into mitochondria and so they have been used to study the topology of three carriers (Brandolin *et al.*, 1989; Ferreira *et al.*, 1990; Capobianco *et al.*, 1990; Miroux *et al.*, 1992). In these studies, it was assumed that antibodies reacted with sequences of the carrier exposed on the cytoplasmic surface of the inner membrane in freeze–thawed mitochondria. It was also assumed that antibodies directed against sequences exposed on the matrix side did not react with freeze–thawed mitochondria, but did react with submitochondrial particles obtained by sonication of mitochondria, since these submitochondrial particles are a mixed population of inside-in and inside-out inner membrane vesicles. It has previously been demonstrated that titration against either freeze–thawed brown fat mitochondria or submitochondrial particles allowed these two types of antibodies to be distinguished in the anti-UCP serum

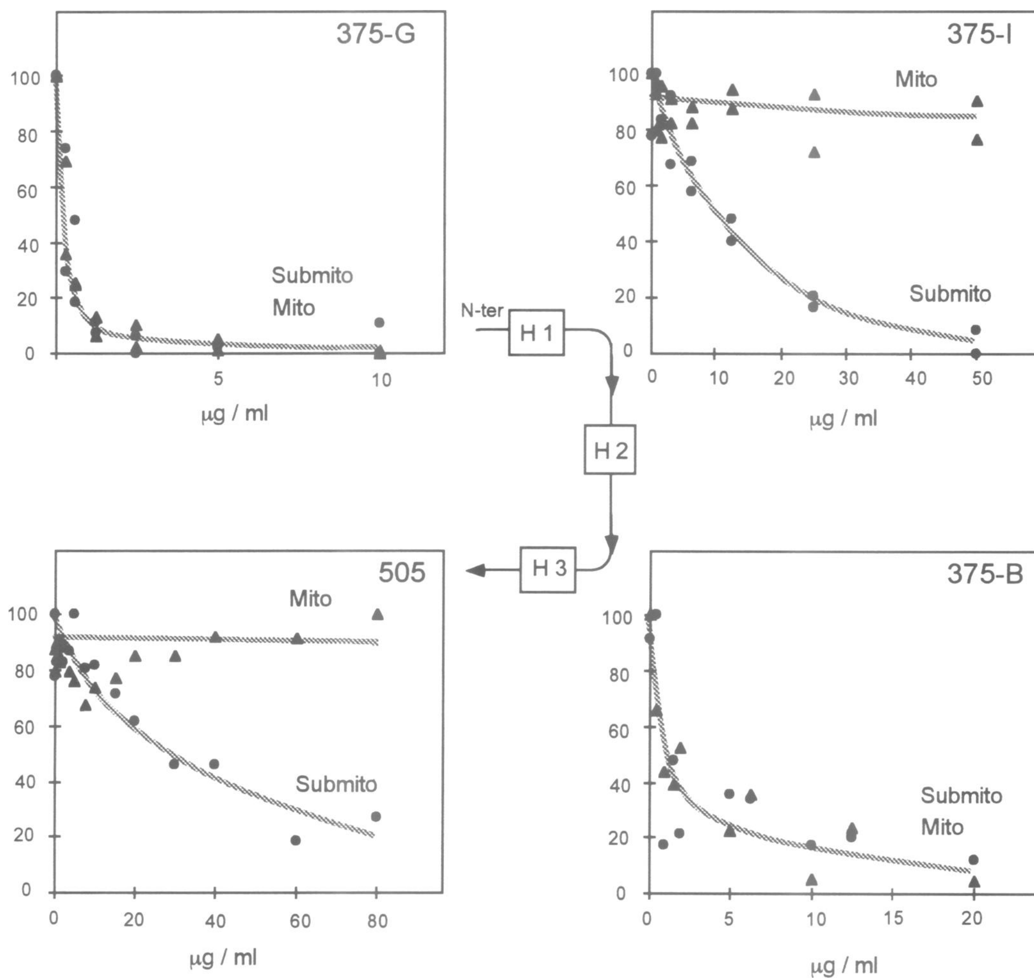

Fig. 5. Back-titration experiments. Back-titration of antibodies after reaction with freeze–thawed rat brown adipose tissue mitochondria (▲) or submitochondrial particles (●). Antibodies were incubated with an increasing concentration of mitochondrial preparations. Details of the back-titration are given in the text. 375-G was incubated with IgG diluted 200 times, 375-I with anti-UCP serum at a dilution of 1/1500. Purified anti-505 or 375-B antibodies diluted 1:100 and 1:200 respectively were used for the two other back-titration experiments. Values were obtained from two independent experiments. To highlight the successive inversions of orientation observed from one fusion protein to the other, the progression along the UCP sequence is schematized in the middle, boxes indicating the proposed transmembrane segments.

(Miroux *et al.*, 1992). The identification of the UCP sequences that were recognized by these two types of antibodies was expected to result in a map of the exposed antigenic sites of the UCP, and to give information about their orientations.

First of all an inventory was constructed of the antigenic determinants recognized by our anti-UCP sera. Previous systematic studies on the immunological response used a large collection of synthetic peptides (Geysen *et al.*, 1987), but we decided to use another strategy and to construct a library of fusion proteins in order to produce a collection of UCP subsequences. Strategies used to make libraries containing randomly generated subsequences were adapted to our purposes. Ten fusion proteins scattered along the UCP sequence were selected; they are likely to represent all the antigenic determinants that our sera could recognize (Table I). Remarkably, five N-terminal extremities of the six predicted α-helices and adjacent amino acids were represented and gave strong immunological signals. This probably reflects common properties of these sequences, making them able to elicit a strong immunological response. It is also striking that the most strongly immunoreactive fusion proteins (375-G and 375-B) (Figure 3) represent two

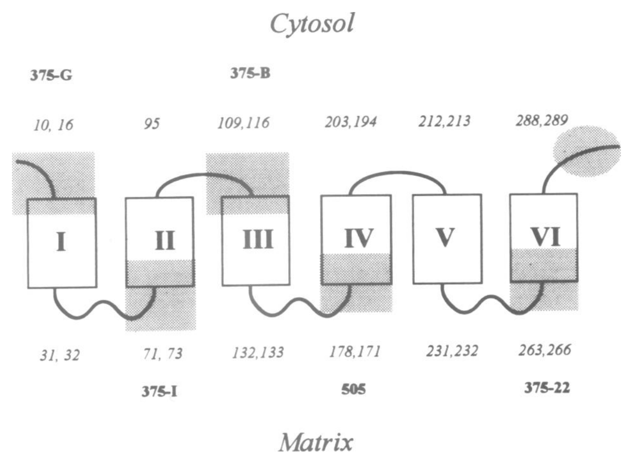

Fig. 6. Experimental evidence and deduced arrangement of the UCP in the membrane. The UCP was drawn as predicted in the general model (Walker, 1992). Numbers in italics separated by a comma indicate the limits of α-helices in two different predictions (Aquila *et al.*, 1985; Klingenberg, 1990). Shaded areas correspond to UCP subsequences of which the location is supported by experimental data (this work and Miroux *et al.*, 1992). The C-terminal end has been studied by Eckerskorn and Klingenberg (1987).

of the more variable parts of the UCP, when the sequences of different species are compared. The power of the immunological screening of a library is illustrated by the isolation of the fusion protein 375-22, containing residues 255−273; this permitted a precise localization of this antigenic site to be made after only one cloning step, whereas in our previous study (Miroux *et al.*, 1992) it was necessary to use several fusion proteins to deduce its localization with comparable precision (residues 253−279). Other fusion proteins were poorly recognized in Western blot analysis or in ELISA tests (data not shown) and so were unsuitable for topological experiments. The five fusion proteins were also of considerable interest as they were expected to reveal the position relative to the inner membrane of four new sites in the UCP, and the overall topological organization of the first two repeats of the UCP which had not been examined before.

Taking into account previous data and this study, a fairly complete description of the overall topology of UCP can now be proposed (Figure 6). This agrees with previous work which demonstrated that the C-terminal end of the UCP is oriented towards the cytosol (Eckerskorn and Klingenberg, 1987). It is also demonstrated that the last (sixth) transmembrane α-helix is indeed present in the UCP as shown by the back-titration of antibodies recognizing its N-terminal end (represented here by 375-22) proving that this site is accessible only in submitochondrial particles (Miroux *et al.*, 1992). This study also demonstrates that the N-terminal extremity of the UCP (375-G) is accessible on the cytosolic side of the inner membrane. Thus, both ends of the UCP are cytosolic, as found also in the mitochondrial phosphate carrier (Capobianco *et al.*, 1990). In the case of the ADP/ATP carrier only the cytosolic orientation of the N-terminal extremity has been demonstrated (Brandolin *et al.*, 1989). This indicates that the UCP is made up of an even number of transmembrane segments. Each change in orientation toward the matrix or cytosol observed between two successive antigenic sites on the UCP is proof of a transmembrane segment in the protein (Figures 5 and 6). Consequently there is a transmembrane segment between the antigenic sequences present in 375-G residues 1−11 and 375-I residues 60−78, corresponding to the first predicted α-helix. Two other pairs of fusion proteins (375-I/375-B and 375-B/505) demonstrated the presence of the second and third transmembrane α-helices. There is no change in orientation between 505 and 375-22 regions (Figure 6). As yet there is no experimental confirmation of the fourth and fifth transmembrane α-helices of the UCP, but the presence of one implies the occurrence of the other.

Altogether, the experimental data on the topology of the UCP have given experimental support to the general model (Figure 6). The applicability of the general model to other mitochondrial carriers has been questioned, and it has been proposed for example that a seventh transmembrane segment is present in the UCP and that a transmembrane β-sheet is found between residues 36 and 50 (Aquila *et al.*, 1985). This model is incorrect. In the ADP/ATP carrier the presence of the second transmembrane α-helix predicted between residues 72 and 90 has also been questioned (Marty *et al.*, 1992) but there is now strong evidence that it is present in the UCP. The three larger hydrophilic segments of the UCP are linked to the matrix-facing ends of the transmembrane α-helices. Therefore a substantial and important part of the protein is orientated toward the matrix, although regulators of UCP function, such as nucleotides and fatty acids, originate from the cytosol.

Materials and methods

General procedures

Restriction and modification enzymes were obtained from Appligene (Illkirch, France) or New England Biolabs (Beverly, MA). Anti-sheep-IgG antibodies linked to horseradish peroxidase or alkaline phosphatase were from Sigma (St Louis, MO). Nitrocellulose membrane was purchased from Schleicher & Schuell (Dassel, Germany). Other reagents were from Sigma, Prolabo (Paris, France) or Merck (Darmstadt, Germany). Antisera against the UCP were those described by Ricquier *et al.* (1983).

Mitochondrial preparations, expression and purification of fusion proteins, and the preparation of sequence-specific antibodies by affinity chromatography towards a fusion protein, were done as previously described (Miroux *et al.*, 1992). Purification of IgG was performed on a protein A−Sepharose column (Pharmacia, Uppsala, Sweden).

Back-titrations of antibodies (Brandolin *et al.*, 1989) were used to evaluate the accessibility of antibodies to the UCP. Freeze−thawed mitochondria or submitochondrial particles were diluted in mitochondrial preparation buffer (pH 7.2, 10 mM Tris, 0.25 M sucrose, 1 mM EDTA). Antibodies diluted in the same buffer (for dilution factors see legend to Figure 5) were added. This suspension was incubated for 90 min at room temperature. After ultracentrifugation at 100 000 *g* for 15 min the supernatant was collected and unreacted antibodies present in the supernatant were assayed by ELISA (enzyme-linked immunosorbent assay) against fusion proteins or purified UCP.

Construction of the fusion protein library

UCP cDNA was obtained by enzymatic amplification of rat UCP cDNA (Bouillaud *et al.*, 1986) using vent-DNA polymerase (New England Biolabs, Beverly, MA) and the oligonucleotides PCROL1 (CCTTTTGGTCTCTG-CCCTCCGAGCC) and PCROL2C (GCATAGGAGCCCAGCATAGGA-GCCC), which match the 5′ and 3′ non-coding sequences of the UCP cDNA. 2−5 μg of the amplification product was subjected to the action of DNase I in the presence of 1 mM $MnCl_2$ at 37°C. Samples were taken after 15, 20, 30 and 40 min and analysed on a 5% acrylamide gel. Slices of the gel were taken in order to select DNA according to its size, using the DNA coming from the four different times of digestion, in order to diminish the influence of differential DNase sensitivity. The fraction of DNA below 200 bp in size was repaired with Klenow enzyme and ligated to the UNI-Amp *Eco*RI adaptor (Clontech, Palo Alto, CA). This DNA was amplified using the UNI-Amp primer (Clontech, Palo Alto, CA) and *Taq* DNA polymerase (Cetus, Norwalk, CT), according to the manufacturer's instructions. Amplification products were separated on an 8% polyacrylamide gel and DNA fragments from 30 to 100 bp were eluted. Eluted DNA was subjected to a second amplification step (16 cycles) in the same conditions. The final amplification product was digested with *Eco*RI. This DNA was ligated in the *Eco*RI site of the pMEC vector expressing the MalE protein (Smelczman *et al.*, 1990). Chimeric plasmids were introduced into JM103 or JM109 strains of *E.coli* by electroporation.

Screening of the library of bacterial clones expressing fusion proteins

Agar plates containing recombinant clones were duplicated on to plates supplemented with 0.2% maltose by replica plating. Plates were incubated for 4 h at 37°C and colonies were transferred on to nitrocellulose membranes. Membranes were boiled in 0.1% SDS solution for 30 s to lyse bacteria, washed with PBS (phosphate buffered saline), and then incubated in PBS supplemented with 2% dried milk for 1 h at room temperature to block non-specific sites. Membranes were left at 4°C for 16 h in the presence of anti-UCP serum diluted 1:1000 in PBS-T (PBS + 0.05% Tween-20). Membranes were washed extensively with PBS-T, and alkaline phosphatase-linked anti-sheep IgGs were used as the second antibodies. The alkaline phosphatase substrate kit (BCIP, NBT from Bio-Rad) was used to detect phosphatase activity. The fragment of UCP cDNA present in selected clones and its neighbouring vector sequences were amplified with *Taq* DNA polymerase, using oligonucleotides 622 (GTCGATGAAGCCCTGAAAGA) and OmalE (GGAGCTGCATGTGTCAGAGGTTTT), which match pMEC sequences 35 nucleotides before and 100 nucleotides after the *Eco*RI cloning site respectively. The amplification product was sequenced according to the dideoxynucleotide chain termination procedure.

Immunological methods

Fusion proteins or purified UCP were electrophoresed in a 12% polyacrylamide gel using a Hoefer minigel. Proteins were electro-transferred to a nitrocellulose membrane. This membrane was treated for 1 h with PBS supplemented with 2% dried milk, and incubated for 16 h with the anti-UCP serum diluted 1000-fold in PBS. The membrane was washed extensively with PBS-T, and horseradish peroxidase-linked anti sheep IgGs were used as the second antibodies. The ECL detection system (Amersham, UK) was used to reveal peroxidase activity.

ELISA on polystyrene microtitration plates (NUNC ref. no. 4-39454) was used to assay the reactivity of antibodies towards fusion proteins or the UCP: 200 μl of periplasmic extract (10 μg protein/ml) or 200 μl of purified UCP (1 μg/ml) was added to each well. After a 2 h incubation at 4°C for fixation, this solution of antigen was removed; all the following steps were performed at room temperature. Non-specific sites were saturated for 1 h with 250 μl of 2% dried milk solution in PBS. The solution of antibodies to be tested was diluted in PBS and 200 μl of it was added to each well. The reaction was allowed to proceed overnight and the plate was then washed with PBS. Peroxidase-conjugated anti-sheep IgG antibodies diluted 10 000-fold in PBS-T were used as second antibodies. The subsequent steps were carried out as described previously (Miroux *et al.*, 1992). For the back-titration of antibodies present in the 100 000 *g* supernatant, PBS was replaced by the mitochondrial preparation buffer until the incubation with second antibodies.

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