Antibodies Selected from Whole Antiserum by Fusion Proteins as Tools for the Study of the Topology of Mitochondrial Membrane Proteins

EVIDENCE THAT THE N-TERMINAL EXTREMITY OF THE SIXTH α-HELIX OF THE UNCOUPLING PROTEIN IS FACING THE MATRIX

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Bruno Miroux, Louis Casteilla, Susanne Klauss, Serge Rainbault, Sandrine Grandin, Jean Marie Clément, Daniel Ricquier, and Frédéric Bouillaud

From the †Centre de Recherches sur l’Endocrinologie Moleculaire et le Développement, Centre National de la Recherche Scientifique UPR 1511, 9 rue Jules Hetzel, 92190 Meudon and the ‡Département des Biotechnologies, Unité de Programmation Moléculaire et Toxique, Centre National de la Recherche Scientifique URA 144, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France

The reactivity to freeze-thawed mitochondria or submitochondrial particles of a whole antiserum raised against the uncoupling protein has been investigated. Incubation with freeze-thawed brown adipose tissue mitochondria trapped antibodies reactive toward accessible parts of the uncoupling protein. One-third to one-half of antibodies against uncoupling protein which were present in the serum remained free. These antibodies were highly reactive with the vesicles obtained by sonication of mitochondria, in which the matrixial side of the inner membrane was made accessible. To define epitopes recognized by the antiserum, different fusion proteins made up of MalE protein and uncoupling protein fragments were used. Immunoaffinity chromatography, using an immobilized purified fusion protein containing amino acids 253 to 290 of uncoupling protein, selected antibodies specifically directed against this part of the protein. A more precise localization of the main epitope recognized by these antibodies is proposed. These purified antibodies reacted with the protein only in submitochondrial particles, indicating a matrixial orientation of this epitope. This result, associated with other data concerning uncoupling protein or related mitochondrial carriers such as the ADP/ATP translocator and the phosphate carrier, allowed us to determine the orientation of the sixth α-helix of the uncoupling protein.

The high thermogenic capacity of brown adipose tissue is due to the activity of a unique protein located in the inner mitochondrial membrane: UCP. UCP allows proton re-entry into the mitochondrial matrix, which bypasses the Fo-F1 ATP synthase and frees brown adipocytes from the constraints of respiratory control, allowing them to develop a very high catabolic activity and to dissipate most of the energy as heat. UCP is also able to transport a variety of anions including chloride and several synthetic compounds (Jezequell and Garid, 1990). UCP is now a well known protein (for reviews, see Klingenberg, 1990; Klauss et al., 1991; Ricquier et al., 1991). It belongs to a family of anion transporters of the mitochondrial inner membrane, such as the ADP/ATP translocator (Aquilla et al., 1982; Arends and Sebold, 1984), the phosphate carrier (Runswick et al., 1987; Ferreira et al., 1989; Phelps et al., 1991), the oxoglutarate carrier (Runswick et al., 1990), and proteins of unknown functions such as MRS3, MRS4 (Wiesenberger et al., 1991), and HML7 (Zarilli et al., 1989). All these proteins share extensive sequence homologies and seem to result from triplication and divergence of an ancestral sequence (Saraite and Walker, 1982; Aquilla et al., 1987). These carriers are considered as simple models of membranous transport proteins. The activity of UCP is regulated. It possesses a binding site for purine nucleotides; nucleotide binding inhibits the transport activity of UCP, whereas free fatty acids are its activators (Rial et al., 1983). Several investigators also pointed out a possible effect of acyl-CoA on UCP (Katiyar and Shrago, 1991). Compared to other mitochondrial carriers, the strict tissue specificity of UCP simplifies the design of recombinant expression systems for this protein, since there is no interference with the activity of the endogenous gene (Klauss et al., 1990; Casteilla et al., 1990; Mudra-Ingis et al., 1991).

Determination of the tridimensional structure of these carriers is a challenging goal. The extreme difficulties encountered in the crystallization of membrane proteins have stimulated the development of alternative strategies. Use of algorithms to predict transmembrane segments is one of these (Fasman and Gilbert, 1990; Jähning, 1990). Its application to known mitochondrial carriers led to the prediction of six membrane-spanning α-helices (Aquilla et al., 1985; Runswick et al., 1987) and is summarized in Fig. 2. Other strategies include chemical modification, proteolysis, and immunological studies. Obviously, nonpermeant probes like proteolytic enzymes and antibodies are highly valuable tools to determine the topology of a membranous protein. Their application to ADP/ATP carrier (Brandollin et al., 1989) and to phosphate carrier (Capobianco et al., 1991; Ferreira et al., 1990) already has given topological information. UCP has been studied extensively by chemical modification and proteolysis (Rial et al., 1989; Jezequell and Drahota, 1989; Katiyar and Shrago, 1989; Fernandez et al., 1987; Klingenberg and Appel, 1989), but up
to now there are inadequate data about its topology. These data are mainly concentrated on the extreme C-terminal end, which, as shown by tryptic cleavage, protrudes on the cytosolic side of the inner membrane (Eckerskorn and Klingenberg, 1987).

In this paper we demonstrate that a serum raised against whole UCP can be considered as a valuable tool to probe UCP topology. Starting from this serum, we were able to purify antibodies directed against a restricted part of UCP by immunofinity chromatography with immobilized fusion proteins. The first batch of antibodies obtained in this way was able to give clear-cut results on the orientation of a region of UCP encompassing a sequence highly homologous to the ADP/ATP carrier (Bouillaud et al., 1987).

The sources of other chemicals were as follows: anti-sheep horseradish peroxidase- or alkaline phosphatase-linked IgG antibodies, 3,3′,5,5′-tetrathionobenzidine, 3,3′-diaminobenzidine, chicken ovalbumin from Sigma; alkaline phosphatase substrate kit (BCIP, NBT) from BioRad; nitrocellulose membrane from Sartorius (Gottingen, Germany).

Restriction and modification enzymes were obtained from Appligene (Illkirch, France) or Bio-Lab Corp. (St. Paul, MN). Expression vectors pPDI40 and pMEC and the ED9 strain were kindly provided by the Laboratory of M. Hofnung (Institut Pasteur). Cross-linked amyllose resin was prepared according to Ferenci and Klotz (1978), or was purchased from Bio-Rad. Anti sera against UCP were those described by Ricquier et al. (1983).

The cDNA fragment and its neighboring vector sequences, according to constructs used in this study have been checked by appropriate restriction vectors for fusion proteins were constructed by addition of plasmid, called pSau131, is coding for a 506-amino-acid fusion protein (370 amino acids of MalE, 131 amino acids of UCP, and 3 amino acids C-terminal before amber codon).

The expression and purification of fusion protein—The growth, induction of expression, and preparation of periplasmic or whole-cell extracts and the purification of MalE hybrids were made exactly as described by Bedouelle and Duplay (1988), except that, for the amyllose column, Tris buffer was replaced by 10 mM phosphate, pH 7. Pvu38 fusion protein was purified from periplasmic extracts, while Sau103 was purified from whole-cell extracts. We were not able to purify Sau131 protein and its mutants, as these proteins seemed to be retained in the membranous fraction of E. coli (not shown).

Construction of Expression Vectors for Fusion Proteins—Expression vectors for fusion proteins were constructed by addition of various fragments of UCP cDNA to plasmids pPDI40 (Duplay et al., 1984) and pMEC (Szmelcman et al., 1990). These vectors are coding for MalE under control of its own promoter. In both of them cloning sites are localized before the last codon of MalE, a site only downstream of UCP coding sequence. The recombinant plasmids from minipreps were screened by Sau3AI digestion and T-ending sites were localized by restriction endonuclease digestion. Sau3AI fragments from mutated cDNA were cloned into pUC18 plasmid, obtained by the use of the "Saub3AI from "XerTro Mutagenesis System" Kit from Promega Corp. (Madison, WI).

The same strategy was used to generate pSau131-564 and pSau103-564 plasmids coding for mutant proteins (Fig. 6), with the Sau3AI fragments from mutated cDNA for UCP, obtained by the use of the "Saub3AI from "XerTro Mutagenesis System" Kit from Promega Corp. (Madison, WI). The plasmids from minipreps were screened by Sau3AI digestion and T-tracking. pSau103 was selected and is coding for a 474-amino-acid fusion protein (103 amino acids of UCP from Ile-149 to Pro-251 and 1 amino acid before opal codon).

Expression and Purification of Fusion Protein—The growth, induction of expression, and preparation of periplasmic or whole-cell extracts and the purification of MalE hybrids were made exactly as described by Bedouelle and Duplay (1988), except that, for the amyllose column, Tris buffer was replaced by 10 mM phosphate, pH 7. Pvu38 fusion protein was purified from periplasmic extracts, while Sau103 was purified from whole-cell extracts. We were not able to purify Sau131 protein and its mutants, as these proteins seemed to be retained in the membranous fraction of E. coli (not shown).

Purification of Specific Antibodies—Immunoglobulins directed against UCP were partially purified by ammonium sulfate precipitation according to Harlow and Lane (1988). The purified fusion protein for all immunological experiments, is called serum in this paper.

One mg of Pvu38 fusion protein was purified on amyllose column, dialyzed, lyophilized, and solubilized in 4.5 ml of 10 mM phosphate buffer, pH 7. Affi-Gel 15 activated beads (1 ml of suspension) were washed quickly with 2 volumes of bidistilled water and then an and purified Pvu38. The suspension was gently stirred at 4°C overnight. Afterward, beads were transferred into a 10-ml Bio-Rad column and equilibrated with PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4).

Five ml of the serum diluted 5-fold in PBS was added to the affinity matrix, and the suspension was shaken at room temperature. After a 90-min incubation the column was washed with 5 volumes of PBS, then with 1 volume of 10 mM phosphate buffer, pH 6.8, and eluted with 100 mM glycine, pH 2.8. Three fractions of 1 ml were collected and immediately neutralized by 1/20 volume of 1 M phosphate buffer, pH 8. The second fraction contained most of the specific antibodies as assessed by ELISA.
In this case, PBS was replaced by buffer A until peroxidase-conjugate IgG incubation.

Immunodetection of Fusion Protein—Fusion proteins or purified UCP were electrophoresed in a 12% polyacrylamide gel using Hoeffer minigel system. Proteins were transferred to a nitrocellulose sheet. After an overnight incubation with PBS-T, serum diluted 2000-fold or purified antibodies diluted 100-fold were added. After extensive washings with PBS-T, horseradish peroxidase- or alkaline phosphatase-linked anti-sheep IgG were used as second antibodies.

RESULTS

Reactivity of UCP Antiserum with Mitochondria or Submitochondrial Particles—The reactivity of a previously characterized anti-UCP serum (Ricquier et al., 1983) toward freeze-thawed brown adipose tissue mitochondria was examined using back-titration procedure (Fig. 1). Freeze-thawed mitochondria were able to bind some antibodies reacting with UCP, but not all of them, and increasing the amount of mitochondria from 2 to 8 μg/ml had little effect. Nearly 50% of anti-UCP antibodies remained in the 100,000 × g supernatant. The same amount of submitochondrial particles trapped anti-UCP antibodies more efficiently, because less than 20% of UCP reactivity was left over. After incubation of the serum with an excess of mitochondria, antibodies highly reactive against submitochondrial particles were left in the supernatant (Fig. 1B). Other experiments showed that the presence of 10 ng of purified UCP was able to compete with mitochondria or submitochondrial particles and decreased the reactivity of the 100,000 × g supernatant in the subsequent ELISA test (data not shown). Repeated experiments gave identical ratio between mitochondrial and submitochondrial abilities to bind antibodies.

Expression in E. coli and Purification of Fusion Proteins—Fusion proteins were used to overcome the difficulty of E. coli producing UCP as insoluble inclusion bodies of incorrectly folded protein. Based on restriction sites present in rat UCP cDNA, a first generation of fusion proteins is presented here (Figs. 2, 3, and 6); MalE coding sequence-derived vectors (Duplay et al., 1987; Szmelczman et al., 1990) were used. Induction of maltose operon led to the production of fusion proteins. Several procedures were used to purify them (see “Experimental Procedures”). Usually, the fusion protein was accompanied by various amounts of a protein having a size close to MalE protein and being poorly reactive with UCP antibodies. This product was probably due to a cleavage of the fusion protein near the junction between MalE and UCP sequences, because we were using MalE-minus strains. The results obtained with Pvu38 fusion protein are presented here (Fig. 3). Purification of pure Pvu38 protein is feasible because the first fractions eluted with maltose were enriched in the expected 44-kDa species (Fig. 3, lanes 2 and 3).

Selection and Characterization of Anti-Pvu38 Antibodies—Antibodies selected for their affinity to Pvu38 corresponded to a nearly undetectable amount of proteins and were assayed against UCP or different fusion proteins using ELISA (Fig. 4). These antibodies were strongly reactive to UCP, or to Pvu38, but were unable to recognize Sau103 fusion protein which has no sequence in common with Pvu38. Anti-Pvu38 antibodies were a minor component of the whole serum,

Fig. 2. Location of UCP sequences fused to MalE protein.

The sequence of UCP is drawn according to the structural prediction of Aquila et al. (1985). Numbers indicate the boundaries of transmembrane a-helices in the protein sequence. a-Helices are presented as open boxes. The UCP moiety present in Pvu38 or Sau131 and Sau103 is indicated. Underlined numbers indicate the first and last amino acids of UCP present in each fusion protein. The restriction sites of rat UCP cDNA (Bouillaud et al., 1986) used to make the constructs are indicated.

Fig. 3. Purification of Pvu38. Periplasmic extracts were loaded on an amylase column. After a wash of 5 column volumes, the fusion protein was eluted with 10 mM maltose. Ten fractions of 1.2 ml were collected, and 10 μl of each fraction were analyzed by SDS-PAGE. Lanes 1–10 correspond to the fraction numbers.

Fig. 4. ELISA test for reactivity of anti-Pvu38 antibodies. Antibodies were selected against UCP, or different fusion proteins, using ELISA (Fig. 4). These antibodies were strongly reactive to UCP, or to Pvu38, but were unable to recognize Sau103 fusion protein which has no sequence in common with Pvu38. Anti-Pvu38 antibodies were a minor component of the whole serum.

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values of absorbance at 450 nm.

were subjected to electrophoresis in 12% SDS-PAGE, electroblotted experiments.

genic indicator antibody, with hydrogen peroxide as substrate and TMB as chromogenic indicator (cf. "Experimental Procedures"). Ordinates represent values of absorbance at 450 nm.

because purified Pvu38 protein was hardly recognized by this serum (data not shown), whereas Sau103 was recognized in ELISA or Western blots by the whole serum. This led to the conclusion that this immunoaffinity procedure allowed the preparation of these specific anti-Pvu38 antibodies with little or no contamination with antibodies against other parts of UCP. This preparation will be called anti-Pvu38 antibodies. The same blot was then incubated with anti-UCP serum, and bound antibodies were revealed by alkaline phosphatase-conjugated peroxidase-conjugated antibody (A). The same blot was then incubated with anti-UCP serum, and bound antibodies were revealed by alkaline phosphatase-conjugated antibody (B). Lane 1: UCP (0.5 µg); lane 2: Sau131 (5 µg); lane 3: Sau131-564 (7 µg); lane 4: Sau131-565 (5 µg).

Reactivity of Anti-Pvu38 with Mitochondria or Submitochondrial Particles—Anti-Pvu38 antibodies were assayed with mitochondrial preparations to determine whether they were able to recognize freeze-thawed mitochondria or only submitochondrial particles (Fig. 7). Back-titration experiments showed that they were unable to recognize UCP in freeze-thawed mitochondria, but were reactive toward sonicated mitochondrial preparations. We verified also that the specificity of the antibodies retained by submitochondrial particles was the same as described before: reactive against Sau131 and Sau131-565, but not against Sau131-564.

**DISCUSSION**

Brown Adipose Tissue Mitochondria Are Suitable for Topological Experiments—Three properties are essential for the use of brown adipose tissue mitochondria in topological stud-
ies on UCP. First, the removal or damaging of the outer membrane should give access to the cytosolic side of inner membrane. Second, the matricial side of inner membrane should not be exposed in these mitochondria. Third, the disruption of these mitochondria should give access to the matricial side of inner membrane. Freeze-thawing of beef heart mitochondria is known to damage outer membrane, and it allows antibodies or proteases to act on the cytosolic side of the inner membrane. On the contrary, the matrix side is kept inaccessible to these macromolecules (Brandolin et al., 1989; Ferreira et al., 1990; Capobianco et al., 1991). Sonication of mitochondria produces small vesicles called submitochondrial particles in which the matricial side of the inner membrane is exposed. These procedures were applied to brown adipose tissue mitochondria. The cytosolic side of the inner membrane was accessible in our mitochondrial preparations, because tryptic cleavage of the C-terminal end of UCP (data not shown) occurred as described in former studies (Eckerskorn and Klingenberg, 1987) (Fig. 8). A serum directed against UCP is expected to contain antibodies directed against epitopes scattered on UCP sequence; some of them must be oriented toward the matrix of mitochondria. As a consequence titration of this serum against mitochondria fulfilling the conditions described above should reveal anti-UCP antibodies unable to reach their epitopes, whereas these antibodies must be trapped by submitochondrial particles. In order to perform a topological study, the back-titration of antibodies first allowed to react with mitochondrial suspension is the best procedure avoiding artifacts due to damage of the membranes during the adsorption to microtiter plates (Brandolin et al., 1989). Titration of the anti-UCP serum toward freeze-thawed mitochondria showed that only a little more than half the anti-UCP antibodies present in the serum were bound to these mitochondria. On the contrary, most antibodies reacted with UCP in submitochondrial particles. This increase of reactivity with the serum indicates unmasking of epitopes of UCP, hidden in freeze-thawed mitochondria and accessible in the membranous vesicles obtained by sonication. Therefore, these new epitopes must be considered as sequences of UCP facing the matrix of mitochondria. Submitochondrial particles were a mixture of inside-out and inside-in structures since they also titrated antibodies reacting with freeze-thawed mitochondria and allowed apparition of the tryptic cleavage of the C-terminal end of UCP (Eckerskorn and Klingenberg, 1987; this study, data not shown). Most of the freeze-thawed mitochondria were impermeable to macromolecules, because even a large excess of them was unable to titrate antibodies which were otherwise trapped by a much lower amount of submitochondrial particles. Partial enrichment of vesicles in UCP cannot be involved to explain their better reactivity, because sonicated mitochondria gave the same result (for example, see Fig. 7). All these data confirmed that brown adipose tissue freeze-thawed mitochondria and the vesicles obtained by their sonication can be used reliably to examine UCP topology.

Two other comments on this control experiment can be made. First, we cannot rule out the possibility that antigenic determinants were still hidden in submitochondrial particles, since we are unable to decide whether the weak immunoreactivity still detected in the 100,000 x g supernatant of submitochondrial particles was only a nonsignificant background, or indicated the presence of some antibodies unable to reach their target owing to the membranous localization of UCP. Second, this experiment suggests that the repartition of epitopes on UCP is more or less equal on both sides of the membrane. However, we must take into account the fact that an antiserum obtained in one animal cannot describe all antigenic determinants of a protein (Geysen et al., 1987).

**Pvu38 Fusion Protein Allows Purification and Characterization of Sequence Specific Antibodies**—The present work (Figs. 2 and 3) gives evidence that it is possible to obtain and purify Pvu38 fusion protein containing 38 amino acids of UCP. This is the first report on the purification of a fusion protein derived from a mitochondrial carrier. We cannot yet present results on the purification of longer proteins such as Sau31 or Sau103. We used purified Pvu38 to select, by an immunoaffinity technique, antibodies directed against this part of UCP. We showed that this procedure was efficient to purify antibodies strictly specific for the sequence present in the fusion protein (Fig. 4). We made several attempts to define the part of Pvu38 responsible for this antigenic recognition (Fig. 5), and we observed that (i) anti-Pvu38 antibodies were nearly as reactive with Sau131 as they were with Pvu38, and (ii) anti-Pvu38 antibodies were not able to recognize the mutated Sau131-564 fusion protein.

These results suggest that the main epitopes are localized in the sequence shared by Pvu38 and Sau131 (residues 253–279 of UCP), and that amino acids essential for this immunological recognition have been removed by the deletion of amino acids 267–269. Moreover differences between human and rat UCP sequences could explain the important decrease in reactivity of anti-Pvu38 antibodies. Comparison of the sequences of rat, human, and mutated proteins between amino acids 253–279 is shown in Fig. 6. Prediction of UCP secondary structure (Aquila et al., 1985) suggested that this sequence (Figs. 2 and 6) contains a short segment of a hydrophilic loop of UCP (residues 253–262) followed by the N-terminal half of the sixth α-helix. This prediction also indicates that all amino acids of this sequence should be on the same side of the inner membrane. Thus anti-Pvu38 antibodies could be used as accurate tools to study the localization of their epitopes relative to the membrane.

**The Sequence Recognized by Anti-Pvu38 Antibodies Is Accessible Only on the Matrix Side of the Inner Membrane**—Anti-Pvu38 antibodies did not react against freeze-thawed mitochondria, but they were trapped efficiently by submitochondrial particles. This unambiguous reactivity showed that the epitope(s) located between residues 253 and 279 of UCP were accessible only from the matrix side of the inner membrane. This result is in agreement with previously published

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![FIG. 8. Proposed arrangement of the C-terminal part of UCP according to the prediction of Aquila et al. (1985), to experimental data (Eckerskorn and Klingenberg, 1987), and to this study. Amino acids thought to be involved in the reaction with anti-Pvu38 antibodies are boxed.](image-url)
predictions and experimental data concerning the structure of mitochondrial carriers. It has been demonstrated that the C-terminal extremity is accessible in freeze-thawed mitochondria, either after tryptic cleavage of UCP (Eckerskorn and Klingenberg 1987), or by the use of antibodies directed against the C-terminal peptide of the phosphate carrier (Capobianco et al., 1991). Thus we provide an experimental confirmation that the sixth α-helix of UCP crosses the membrane, with its N-terminal part facing the matrix.

This result is surprising with respect to predictions about the localization of the nucleotide binding site in UCP. UCP is homologous to the ADP/ATP translocase. These two proteins are both able to bind free ATP or ADP, and it is thus highly probable that their nucleotide binding sites share common characteristics. Several years ago, the localization of the strongest homology recorded between UCP and the ADP/ATP translocase led us to propose that this part of the two proteins was somehow implicated in nucleotide binding (Bouillaud et al., 1986; Castella, et al., 1989). This speculation gained some experimental support, because photoaffinity labeling of these two carriers with nucleotide derivatives showed that this sequence was located close to a labeled region (Mayinger and Klingenberg, 1988; Dalbon et al., 1989). The highest homology domain comprises residues 261–269 of UCP. In UCP the nucleotide binding site is accessible to cytosolic nucleotides. This is somehow contradictory to our results concerning the matricial orientation of a segment of UCP extending from residues 253 to 279. Either this proposal about the nucleotide binding site was wrong, or one must admit that nucleotides coming from the cytosol are able to reach a binding site deeply embedded in the protein.

Comparison between the Use of Recombinant Fusion Proteins and of Synthetic Peptides—Fusion proteins already have been used as a means to define the localization of epitopes (Tunwell et al., 1991). However, up to now the topology of mitochondrial carriers has been studied through the use of antipeptide antibodies combined with the use of proteases. All previous reports using antipeptide antibodies concluded that N- or C-terminal extremities of carrier proteins are located on the cytosolic side of the inner membrane. The present work, based on the use of a fusion protein, led to the purification of antibodies directed against an internal sequence of UCP on the matrix side of the inner membrane. Several other characteristics of fusion proteins as a tool for such topological experiments deserve discussion. (i) It is possible to use a serum raised against the whole protein and to select antibodies directed against a protein fragment expressed in a fusion protein. As studies about carriers often led to the purification of the protein, and to the obtaining of antisera, it may be concluded that these antisera are potential tools to study the topology of several carriers. (ii) Immunization with these fusion proteins is possible; expression vectors used here have been designed for the production of vaccines and have already been used to raise antibodies in animals. Immunization can be obtained even with crude preparation of bacteria (O’Callaghan et al., 1990; Leclerc et al., 1990). In these respects fusion proteins can provide a valuable and inexpensive alternative to synthetic peptides (Martineau et al., 1991). (iii) Fusion proteins offer the opportunity to randomize fragmentation of UCP in a library of clones expressing UCP fragments. Such a library could allow an easier determination of antigenic determinants of UCP which could be used efficiently in topological studies.

Perspectives—The experiment described in Fig. 1 was essential to demonstrate that the lack of reactivity of anti-Pvu38 antibodies was not due to the inability of antibodies to reach UCP in freeze-thawed mitochondria. We also demonstrated in this experiment that antibodies not retained by freeze-thawed mitochondria were still able to react with a small amount of submitochondrial particles (Fig. 1B). This opens the way to a systematic exploration of UCP topology. Moreover, procedures to define epitopes recognized by antisera in a protein have been developed by Geysen et al. (1987). In these experiments the antisera were assayed against a collection of peptides covering the complete sequence of the myohemerythrin. Antisera against UCP could be assayed with such a collection of peptides before or after preincubation with freeze-thawed mitochondrial or submitochondrial particles. This procedure will reveal epitopes along UCP, as well as their orientation relative to the membrane. Such an approach could be facilitated by the abundance of UCP in brown adipose tissue mitochondria, but it is difficult to anticipate if this attractive approach will be feasible with other less abundant proteins. Alternatively a library of clones expressing randomly UCP fragments could be used to determine matrixial or cytosolic antigenic determinants on UCP.

The use of fusion proteins may not be restricted to immunological aspects. There is now experience in the crystallization of MalE-derived fusion proteins (Rodseth et al., 1990). It must be noted that Pvu38 is fully soluble without detergent; so is Sau105, which contains one third of UCP’s sequence. The use of MalE derivatives for structural studies implies that the fusion protein allows the UCP sequence to fold in the same way as in the inner membrane. We have no such evidence for Pvu38. However, there is usually a good correlation between the functionality of MalE, the efficient export of the fusion protein across the inner membrane of E. coli, and its correct folding. Pvu38 was exported efficiently to the periplasmic space and was purified with a procedure based on the functional properties of MalE.

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