Deletion of Amino Acids 261−269 in the Brown Fat Uncoupling Protein Converts the Carrier into a Pore†

M. Mar González-Barroso,‡ Christophe Fleury,§ Corinne Levi-Meyrueis,§ Pilar Zaragoza,‡ Frédéric Bouillaud,§ and Eduardo Rial*,‡

Centro de Investigaciones Biológicas, CSIC, Madrid, Spain, and CEREMOD, CNRS, Meudon, France

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ABSTRACT: The uncoupling protein (UCP) from brown adipose tissue mitochondria is a carrier that catalyzes proton re-entry into the matrix and thus dissipates the proton electrochemical potential gradient as heat. UCP activity is regulated: purine nucleotides inhibit while fatty acids activate transport. We have previously reported that sequence 261−269 of the UCP has a closely related counterpart in the adenine nucleotide translocator, as well as in the DNA binding domain of the estrogen receptor. Site-directed mutagenesis of the UCP showed that deletion of amino acids 267−269 in the UCP abolished nucleotide inhibition [Bouillaud, F., *et al.* (1994) *EMBO J. 13*, 1990−1997]. Complete deletion of the homologous domain (UCPΔ9) produced a highly deleterious mutant that collapsed the mitochondrial membrane potential and halted yeast growth. Since under our growth conditions revertants appeared rapidly, it was not possible to characterize this mutant. In this article, we have designed conditions to isolate mitochondria containing significant amounts of the UCPΔ9 mutant protein. These mitochondria show no respiratory control and are insensitive to nucleotides. Investigation of the permeability properties revealed that UCPΔ9 mitochondria swell rapidly in potassium salts in the absence of valinomycin, thus indicating a loss of specificity. The size exclusion properties of this mutant were determined with polyethylene glycols of various molecular masses (400−20000 Da), and it was found that UCPΔ9 can catalyze permeation of molecules of up to 1000 Da. We conclude that the deletion of amino acids 261−269 converts the UCP into an unspecific pore.

The uncoupling protein (UCP)[1] is a transporter exclusively found in mitochondria from brown fat, a thermogenic organ present in mammals, and as its name suggests, it uncouples respiration from ATP synthesis (*1*). Recently a new protein which has 59% amino acid identity with UCP, and also uncouples mitochondrial respiration, has been identified in other mammalian tissues and thus has been termed UCP2 (*2*). These two proteins are members of the protein superfamily that includes the metabolite transporters of the mitochondrial inner membrane (*3*−*5*). All these carriers present both functional and structural similarities. Their molecular masses are around 30 kDa, and they consist of 3 repeated sequences of 100 amino acids, each repeat including 2 transmembrane domains. Except for two cases, they are anion transporters with an antiport activity and function according to a sequential mechanism (*5*).

UCP function is to allow proton re-entry into the matrix, thus dissipating the proton electrochemical potential gradient as heat (*1*). Its transport activity is still a matter of debate since it can transport a variety of anions while its physiological function is to dissipate the proton gradient. Fatty acids are the cytosolic second messengers that activate UCP (*6*), and two hypotheses have been put forward to explain their role. One proposal is that UCP transports fatty acid anions as a part of a cycling mechanism that would be completed with the translocation of the protonated form across the lipid bilayer, with the net uptake of a proton (*7, 8*). The other hypothesis states that the physiological function of UCP is to translocate protons and that fatty acids act as a prosthetic group that delivers protons to a site from where they are translocated to the matrix (*9*).

The mode of action of purine nucleotides has been less controversial than that of fatty acids. Nucleotides bind to UCP from the cytosolic side of the mitochondrial inner membrane and inhibit its transport activity (*10*). UCP, like the rest of the members of the mitochondrial transporter superfamily, is a dimer formed by two identical subunits, but only one molecule of nucleotide binds per dimer (*11*). The nucleotide binding site must be deep in the protein since photoaffinity labeling experiments (*12, 13*) have revealed the participation of the loop that links the fifth and sixth transmembrane domains and this region is located in the matrix side of the membrane (*14*). A recent report has demonstrated the presence of two UCP conformations with nucleotide bound to the protein, a loose and a tight complex. Only in the tight conformation does binding result in inhibition of proton transport (*15*).

In 1992, Bouillaud *et al*. (*16*) described the similarities between region 261−269 of the UCP and the DNA binding

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* Correspondence should be addressed to this author at the Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain. Telephone: 34-1-5611800, ext. 4236. Fax: 34-1-5627518. Email: rial@fresno.csic.es.

‡ CSIC.

§ CNRS.

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[1] Abbreviations: UCP, uncoupling protein; AAC, ADP/ATP carrier; OGC, oxoglutarate carrier; PEG, polyethylene glycol; RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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domain of the estrogen receptor. Since this UCP region had already been implicated in the interaction with nucleotides (*12, 13*), the similarities suggested that the two proteins could share a homologous domain that could be related to the nucleotide binding activity or DNA recognition, respectively. This hypothesis was later supported by results obtained with two mutants of this putative 'nucleotide recognition element' in the UCP: UCPΔ3 (deletion of Phe267, Lys268, and Gly269) and UCPΔ9 (complete deletion of the homologous domain, i.e., amino acids 261−269) (*17*). UCPΔ3 was an active UCP and sensitive to fatty acids but insensitive to nucleotides. UCPΔ9 resulted in an extremely deleterious protein that collapsed mitochondrial membrane potential when expressed in *S. cerevisiae* and thus arrested yeast growth. Mitochondrial studies with this mutant have been hampered since their isolation yielded mitochondria almost devoid of protein due to the appearance of revertants (*17*). In the present report, we describe conditions to obtain yeast mitochondria with high levels of functional UCPΔ9, and we demonstrate that the deleterious action is due to its behavior as a pore that allows permeation of solutes of up to 1000 Da.

METHODS

Chemicals. Components for culture media were from Difco Laboratories. Polyethylene glycols (PEGs) were obtained from Sigma. Cytohelicase was from BioSepra (France). All other chemicals were of the highest purity available.

UCP Expression and Isolation of Mitochondria. The diploid yeast (*Saccharomyces cerevisiae*) strain W303 was transformed with the vector pYeDP-1/8−10 where the coding sequences for wild-type UCP and the mutant UCPΔ9 have been introduced (*17*). Control yeast (UCP^-) contained the same vector but with the UCP cDNA in the inverse orientation. For the isolation of mitochondria, yeast were grown aerobically at 28 °C in liquid minimal medium with 2% lactate plus 0.1% glucose as carbon source (*18*). Ten to eleven hours before harvesting, yeast were diluted in the same medium but with 0.05% glucose. Cell density was adjusted to 0.1 OD_{600} for UCPΔ9 and to 0.4 OD_{600} for UCP^+ and UCP^-. Expression was induced for 4.5 h with 1% galactose. Mitochondria were isolated from protoplasts as previously described (*18*).

Determination of Bioenergetic Properties of Mitochondria. The activity of UCP^+ and UCPΔ9 was evaluated from respiration parameters (*18*) and swelling properties. The osmotic behaviour was assayed in potassium salts of acetate, sulfate, and malonate by monitoring the light scattering changes (*18, 19*). Mitochondria were suspended at 17 °C in medium containing either 0.2 M potassium acetate or 0.133 M K_2SO_4 or 0.133 M potassium malonate, plus 5 mM K_2HPO_4, 1 mg/mL fatty-acid-free albumin, 2 mM $MgCl_2$, 10 μM atractylate, 0.5 μg/mL antimycin A, and 10 mM Tes, pH 6.8. Mitochondrial protein concentration was 0.7 mg/mL.

Measurement of the size exclusion properties of the carrier was performed by a method based on that described in ref *20*. Thus, swelling properties were assayed in PEGs of various molecular masses (400−20000 Da). Since the different PEGs do not behave as ideal solutes, the desired osmolality was calculated from the tables of osmotic pressure presented at the Website: "http://aqueous.labs.brocku.ca/osfile.html". Thus, to obtain 0.4 osmolal PEGs, the following solutions were prepared: PEG 400, 11.4% (w/w); PEG 600, 15.3%; PEG 1000, 18.2%; PEG 3350, 22.6%; PEG 6000, 24.3%; PEG 20000, 25.4%. The concentrations of some of the buffer components were decreased to a value that did not affect the swelling behavior of UCP^- mitochondria but at the same time minimized possible interactions with the PEG molecules that could result in a modified osmolality. The swelling buffer contained 0.4 osmolal solutions of the different PEGs, plus 1 mM K_2HPO_4, 0.5 mg/mL albumin, 0.5 mM $MgCl_2$, and 5 mM Tes.

The light scattering changes were monitored from the absorbance at 550 nm. Data were converted to the parameter β, as defined in ref *19*, that correlates quantitatively to solute entry. The parameter "a" was determined as in ref *21* for mitochondria obtained from UCP^- and UCP^+-expressing yeasts. Since there were no significant differences, the value used (0.358) was the average for the two types of mitochondria.

Immunodetection and Quantification of UCP. SDS−PAGE of proteins and Western blotting methods have been described previously (*22*). Immunodetection was achieved with Amersham's ECL detection system and following the manufacturer's protocol. Subsequent quantification of UCPΔ9 incorporation to yeast mitochondria was performed by densitometric analysis with a Molecular Dynamics Densitometer.

RESULTS

Isolation of Mitochondria from UCPΔ9-Expressing Yeasts. We have previously reported that expression of UCPΔ9 is deleterious for yeast growing aerobically on a nonfermentable substrate and that it arrests growth. Flow cytometry showed that mitochondria are uncoupled, thus explaining its deleterious action (*17*). In that report, it was found that, when conditions normally employed to isolate mitochondria from UCP-expressing yeasts are applied to strains expressing UCPΔ9, mitochondria behaved like UCP^-. The explanation for this result was that the selective pressure led to a marked decrease in the number of copies of the expression vector, and therefore mitochondria were almost devoid of the mutant UCP as it was also revealed by immunodetection (*17*).

In order to investigate the transport properties of UCPΔ9, it was necessary to design conditions to isolate mitochondria in the uncoupled state detected by flow cytometry. Two factors appeared essential: first, harvesting should be made at the point when the lowest yeast growth rate had just been attained, and this occurs 4−5 h after galactose induction (*23*). Second, induction should be made at low cell densities (OD_{600} below 0.7, i.e., less than 10 million cells/mL). Induction at higher cell densities suppresses expression of UCPΔ9 while it has no effect on the level of expression of UCP^+ or other UCP mutants tested in our laboratory (data not shown). Consequently, biomass at the time of harvesting is low (OD_{600} below 1.3), and therefore large volumes are needed to get enough mitochondria to perform transport studies on UCPΔ9. Routinely, from 1.5 L of culture at 1.0−1.2 OD_{600}, we obtained around 3 g of cells (wet weight) and from them 12−15 mg of mitochondrial protein. In the case of UCP^-, with 800 mL at 5 OD_{600}, the yield was 7 g of cells and 20−25 mg of mitochondrial protein. Breaking of

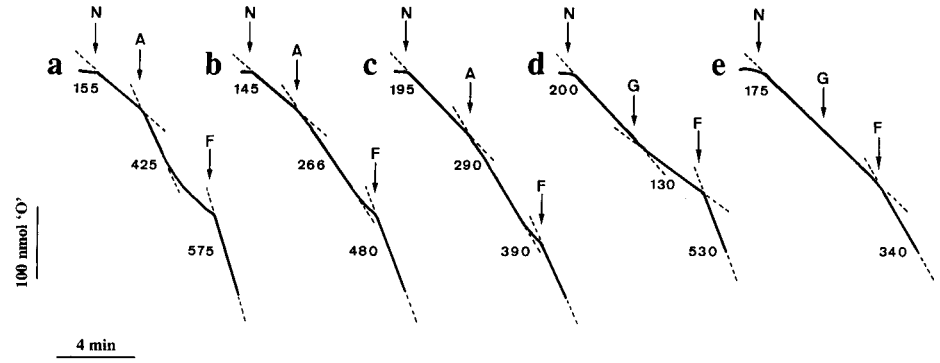


FIGURE 1: Control of respiratory activity in UCP$^-$ (trace a), UCP$^+$ (traces b and d), and UCPΔ9 (traces c and e) yeasts. Traces are from representative experiments; for average values, see text. Rates of respiration are given in nmol of 'O'·min^{-1}·mg^{-1}. Additions: N, 3 mM NADH; A, 0.1 mM ADP; G, 1 mM GDP; F, 10 μM FCCP.

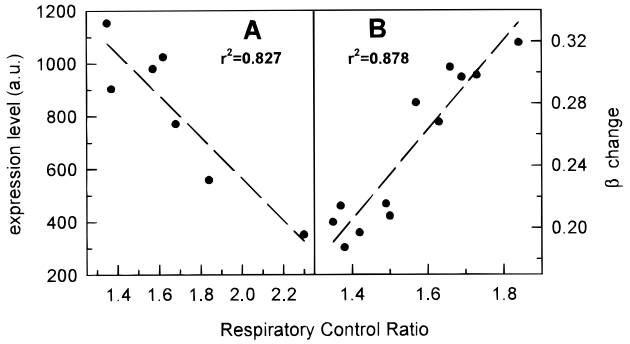


FIGURE 2: Variation in respiratory control ratios (RCR) when mitochondria are isolated from batch cultures of UCPΔ9 after 4.5 h of induction with 1% galactose. Respiratory control ratios were determined from the stimulation of NADH oxidation upon addition of 0.1 mM ADP. Panel A shows the correlation between the RCR and the level of protein incorporated into the mitochondria as revealed by inmunodetection after SDS−PAGE. Panel B shows the correlation between the RCR and the amplitude of the matrix volume change (expressed as β) after nigericin addition to mitochondria suspended in potassium acetate.

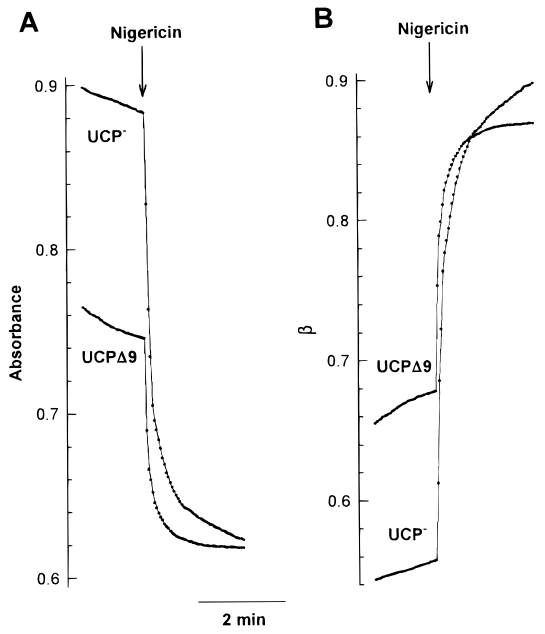


FIGURE 3: Osmotic swelling of UCP$^-$ and UCPΔ9 mitochondria suspended in potassium acetate. Panel A shows the variation in the absorbance at 550 nm of the mitochondrial suspensions. Panel B shows these variations after conversion to the parameter β, calculated as in (*21*).

the cell wall with cytohelicase is more efficient in low grown-cultures.

We have previously described (*23*) that when mitochondria are isolated from yeasts expressing wild-type UCP (UCP$^+$ strain) they present lower coupling than those from UCP$^-$ yeasts. Thus, respiratory control is 2.67 ± 0.07 (n = 12) for UCP$^-$ and 1.75 ± 0.03 (n = 28) for UCP$^+$ (Figure 1). Mitochondria isolated from UCPΔ9 yeasts show variable respiratory control ratios (RCR), but they correlate inversely with the level of protein incorporated to the mitochondrial inner membrane, as revealed by immunodetection (Figure 2A). Under our standard conditions, the lowest RCR obtained has been 1.35. Since we have never observed by flow cytometry a single population at low potential (*17*), the mitochondrial preparation will probably be a mixture (UCPΔ9 and UCP$^-$-like revertants), and thus the RCR observed would be the weighted average of two mitochondrial subtypes. It is possible to estimate the percentage of UCPΔ9 mitochondria if we take into consideration the flow cytometry data that reveal that these mitochondria are totally uncoupled, and thus it would be reasonable to assume that they would respire at the FCCP rate. Since the stimulation by FCCP in UCP$^-$ is 5.1 ± 0.46 (n = 9), for a preparation of UCPΔ9 showing an RCR of 1.35, the percentage of mitochondria containing the mutant would be around 42%. Respiration experiments also reveal that mitochondria isolated from UCPΔ9-expressing yeasts do not respond to 1 mM GDP (Figure 1, trace e), thus in agreement with the postulated loss of the nucleotide binding site (*17*). For UCP$^+$ mitochondria, GDP addition lowers the state 4 rate by 33% ± 1.7 (n = 17) (Figure 1, trace d).

Transport Properties in UCPΔ9. The aim of the present study is to determine the transport properties of UCPΔ9. The transport specificity of UCP has often been investigated with the use of the technique based on the swelling in isosmotic salts (*24*, *25*). The conversion of the absorbance changes to a parameter linearly related to A^{-1} (β) has been shown to be quantitatively related to solute uptake (*19*, *21*). The light scattering technique has already been used to demonstrate the conservation of the transport properties of UCP when incorporated to yeast mitochondria (*18*). Nucleotide-sensitive swelling in potassium acetate plus valinomycin has been the usual procedure to determine the UCP-induced proton permeability (*26*). When UCPΔ9 mitochondria were suspended in potassium acetate, the initial absorbance was always lower than that obtained with suspensions of either UCP$^+$ (data not shown) or UCP$^-$ mitochondria of the same protein content (Figure 3). It should be mentioned that the protein content indeed reflected the same amount of mito-

chondrial protein since the corresponding respiratory activity in the swelling assay (calculated from the rate of respiration in the presence of uncoupler in a parallel experiment) was the same, 177 ± 23 nmol of 'O'/min assay for UCP$^-$ and 189 ± 16 for UCPΔ9 (4 determinations in each case). When the extent of swelling in potassium acetate was determined with the addition of nigericin (Figure 3), it was demonstrated that the low initial absorbance (prior to the ionophore addition) resulted in a decrease in swelling amplitude or, in other words, that the final value of β was the same regardless of the source of mitochondria (UCPΔ9, UCP$^-$, or UCP$^+$). This low initial absorbance, in the absence of ionophore, for UCPΔ9 was due to fast swelling of a fraction of the mitochondrial population that was almost completed within the mixing time (approximately 15 s), and it must correspond to the subpopulation containing the mutant protein because it correlates with the level of protein incorporated (Figure 2). Since this rapid swelling occurred in the absence of ionophore to catalyze the entry of K^+, we must assume that UCPΔ9 also allows K^+ permeation.

It should be stressed at this point that data presented below correspond to mitochondrial preparations where the RCR were less than 1.55 and thus that swelling amplitudes (variation in β) in potassium acetate after the addition of nigericin were less than 0.23. It should also be noted that when error bars are shown, they reflect not only the normal experimental error but also the fact that preparations are intrinsically different, although within the limits that we have just mentioned.

The apparent loss of transport specificity was further investigated by studying the swelling behavior in K_2SO_4 and potassium malonate. The two salts were chosen because neither of the anions are transported by UCP (25). In the absence of ionophores, UCP$^-$ mitochondria did not swell in malonate and swelled slowly in K_2SO_4. Addition of valinomycin only increased the swelling rate in sulfate (data not shown). UCPΔ9 mitochondria suspended in these salts behaved in the same way as in potassium acetate: the initial light scattering readings were low due to fast swelling, even in the absence of ionophores. Thus, while the average initial β for UCP$^-$ was 0.62 in malonate, 0.61 in sulfate, and 0.58 in acetate, for UCPΔ9 they were 0.77, 0.76, and 0.69, respectively. Therefore, the loss of transport specificity in the mutant protein is again revealed.

The possibility that this deletion was inducing the formation of a porelike structure in UCP was tested with polyethylene glycols of different molecular mass. The approach used was similar to that developed by Pfeiffer *et al.* (*20*) to determine the size exclusion properties of the permeability transition pore. Mitochondria were suspended in 0.4 osmolal solutions of PEG ranging from 400 to 20 000 Da (see Methods for details on the preparation of PEG solutions). Figure 4 shows the swelling properties of UCP$^-$ and UCPΔ9 mitochondria, and it is observed that for PEG 3350, PEG 6000 and PEG 20 000 both types of mitochondria gave similar initial β values. However, UCPΔ9 mitochondria suspended in PEG 400, PEG 600, and PEG 1000 gave β values that were significantly higher than those obtained with UCP$^-$ mitochondria. We must conclude that the difference reflects the activity of the mutant protein and thus that it can catalyze the movement of PEG molecules of up to 1000 Da.

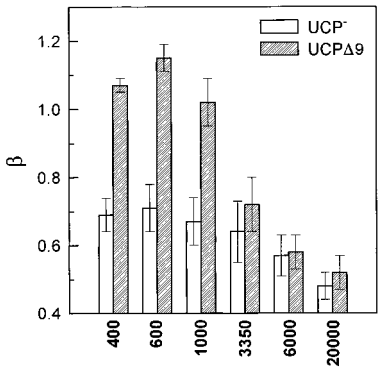
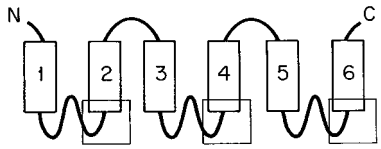


FIGURE 4: Osmotic behavior of UCP$^-$ and UCPΔ9 mitochondria suspended in 0.4 osmolal solutions of polyethylene glycols of average molecular mass ranging from 400 to 20 000 Da. The data represent the light scattering of the mitochondrial suspensions and are the mean values ± SEM of 2−3 determinations in 5 (UCP$^-$) or 3 (UCPΔ9) different mitochondrial preparations.

DISCUSSION

The sequences of mitochondrial carrier proteins are characterized by their triplicated structure where the occurence of three 100 amino acids related domains constitutes the entire protein (*3*, *5*, *27*). This organization is clearly apparent in the folding model proposed for these proteins (*28*, and Figure 5). Two conserved patterns are found at the limits between α helices and the hydrophilic loops orientated toward the matrix. The first one is characterized by a strictly conserved proline residue and is defined in the PROSITE database (PS00215). The second one is characterized by the conservation of glycines residues (*27*, *29*). Positions 261−269 of the UCP fit to the second (EG...+++G, in Figure 5), and according to the folding model proposed for mitochondrial carriers, these amino acids would constitute the N-terminal end of the sixth transmembranous α-helix (*5*). Similar sequences are also found at the end of the second and fourth helices (Figure 5). Experimental studies on the UCP showed that amino acids 267−271 participate to an antigenic site that could be reached from the matrix side, thus in agreement with the model but suggesting that this region is not deeply embedded in the membrane (*14*). Observation of similarities between region 261−269 of UCP and the DNA binding domain of the estrogen receptor suggested that these amino acids in the UCP could form an α-helix, and led to the proposal that the two proteins shared a homologous domain that was involved either in nucleotide or in DNA binding (*17*). Deletion of three amino acids (Phe267, Lys268, and Gly269) of the UCP led to the loss of nucleotide inhibition of the proton conductance, thus validating the starting hypothesis. The deletion of the entire homologous domain resulted in a highly deleterious mutant (UCPΔ9) that halted subsequent studies of the properties of the mutated protein. In this article, we have established conditions to obtain mitochondria containing UCPΔ9, and we have characterized its transport properties. This deletion results not only in the loss of the nucleotide inhibition but also in the gating ability, thus allowing passage of large uncharged molecules.

The loss of transport selectivity has also been described for various members of the mitochondrial transporter family (*30*−*35*). This behavior has been detected after modification of cysteine residues with mercurials, a process that can be reversed by addition of dithioerythritol (*30*, *34*). The

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Folding Pred.   ----------hhhhhhhhhh

Consensus       .+..+.+.EG...+++G...
                        **      *
UCP2(m)    64   TILTMVRTEGPRSLYNGLVA
OGC(b)     67   ALISILRAEGLRGIYTGLSA   2
AACs       56   CVVRIPKEQGVLSFWRGNLA
UCPs       58   TITTLAKTEGLPKLYSGLPA
                      **      *
UCP2(m)   162   AYKTIAREEGIRGLWKGTSP
OGC(b)    167   ALFRIVQEEGVPTLWRGCIP   4
AACs      159   CLVKIFKSDGIRGLYQGFNV
UCPs      159   AYRIIATTESLSGLWKGTTP
                      **      *
UCP2(m)   256   CALTMLRKEGPRAFYKGFMP
OGC(b)    265   VLVKVVRYEGFFSLWKGFTP   6
AACs      256   CWRKIAKDEGGKAFFKGAWS
UCPs      253   CAMTMLTKEGPTAFFKGFVP
                      **  ****
EstRec    205   YHYGVWSCEGCKAFFKRSIQ
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FIGURE 5: Folding model for mitochondrial carriers and alignment of the amino acid sequences at the N-terminal boundaries of the second, fourth, and sixth transmembrane domains. The amino acid sequences of the boxed areas are given below; numbers indicate the position of the first residue listed. The first line represents the folding prediction, with the letter h indicating positions where secondary structure is expected to be α-helix. The second line shows the strictly conserved residues (E, G) and positions where only conservative substitutions occur (+). Asterisks enhance the remarkable conservation of residues between carriers as well as the identities between the UCPs, AACs and the estrogen receptor sequences. UCP2 (m), mouse UCP2 sequence (translated from GenBank U69135); OGC (b), bovine oxoglutarate malate carrier (Prosite: P22292); AACs, consensus sequence for mammalian ADP/ATP translocases after alignment of rat, human, and bovine sequences of the three isoforms (Prosite: Q05962, P12235, P02722, Q09073, P05141, P12236 and P32007); UCPs, consensus sequence of the brown fat uncoupling protein after alignment of mouse, rabit, bovine, human, hamster, and rat sequences (Prosite: P12242, P14271, P10861, P25874, P04575, P04633); EstRec, sequence of the human estrogen receptor. Cysteines underlined in AAC and OGC correspond to residues identified as reacting with maleimides to alter carrier function. In UCP, underlined residues are those deleted in the mutant UCPΔ9.

resulting carrier form loses the substrate specificity at the internal site, but not at the external site, and converts the antiport activity to a uniport that does not display saturability. The low substrate specificity affects not only the size but also the charge; thus in the aspartate/glutamate carrier, the highly specific exchange of aspartate for glutamate is converted into a uniport where the protein catalyzes the permeation of negatively charged solutes like sulfate or succinate, cations such as arginine or lysine, and uncharged substrates such as glucose (*35*). The carrier is not just converted into an unspecific pore since it maintains the activation energies and turnover numbers, thus indicating that a carrier mechanism is still involved; i.e., a conformational change is required and it is the rate-limiting step (*35*, *36*). It should also be mentioned that modification with maleimides of sulfhydryl groups in the UCP also converts the carrier into a highly conducting product that remains nucleotide-sensitive (*37*).

The AAC and the UCP have also been shown to behave as channels under patch-clamp conditions. The first report concerned the AAC, and it showed that Ca^{2+} induced a high-conductance channel of up to 500 pS which displayed low selectivity (*38*). The similarity between the channel properties observed in the AAC and those reported for the mitochondrial permeability transition pore (*39*) may indicate that the channel activity in these latter cases could be due at least partially to the AAC (*38*). The Cl^- channel observed with UCP under patch-clamp conditions has no common features with the rest of the mitochondrial channels (*40*). It has a unit conductance of 75 pS, is strongly anion-selective, and can be inhibited with the same nucleotides that are known to inhibit UCP transport activity. Moreover, the channel behavior of the UCP is not Ca^{2+}-dependent; thus, it appears as an intrinsic property of the protein (*40*).

The current view of the functional organization of the mitochondrial transporters would suggest the presence of two protein domains. One common to all transporters, and perhaps other channel proteins, would be an evolutionary conserved element responsible for channel functions. The other domain would conform the gate and would therefore confer the transporter its specific properties (*34*). The intrinsic channel would only be apparent after modification or removal of the gating domain, and two such ways would be the above-mentioned sulfhydryl modification (*30*−*35*) and the Ca^{2+}-induced pore opening in the AAC (*38*). It is tempting to speculate that in UCP the region comprising residues 261−269 forms part of the gate that allows the selection of the molecules that will permeate and that nucleotide binding to this region results in the inhibition of transport. Its complete removal (UCPΔ9) results in the formation of an unspecific large size pore. Since there are homologous sequences in the other two sequence repeats of the carrier molecule, it is possible that these regions also contribute to the gate. In this context, it should be mentioned that in the oxoglutarate carrier, sulfhydryl reagents bind to Cys184 to inhibit transport (*41*). Similarly, different maleimides can react rather selectively either to Cys56 or to Cys159 of the AAC, and in both cases, transport is inhibited (*42*). All these cysteine residues are located close to the homologous domains in the first and second repeats of the carriers (Figure 5).

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