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Review Letter

Solute carriers involved in energy transfer of mitochondria form a homologous protein family

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The sequences of three mitochondrial carriers involved in energy transfer, the ADP/ATP carrier, phosphate carrier and uncoupling carrier, are analyzed. Similarly to what has been previously reported for the ADP/ATP carrier and the uncoupling protein, now also the phosphate carrier is found to have a tripartite structure comprising three similar repeats of approx. 100 residues each. The three sequences show a fair overall homology with each other. More significant homologies are found by comparing the repeats within and between the carriers in a scheme where the sequences are spliced into repeats, which are arranged for maximum homology by allowing possible insertions or deletions. A striking conservation of critical residues, glycine, proline, of charged and of aromatic residues is found throughout all nine repeats. This is indicative of a similar structural principle in the repeats. Hydropathy profiles of the three proteins and a search for amphipathic α-spans reveal six membrane-spanning segments for each carrier, providing further support for the basic structural identity of the repeats. The proposed folding pattern of the carriers in the membrane is exemplified with the phosphate carrier. A possible tertiary arrangement of the repeats and the membrane-spanning helices is shown. The emergence of a mitochondrial carrier family by triplication and by divergent evolution from a common gene of about 100 residues is discussed.

Mitochondrial carrier family; ADP/ATP carrier; Uncoupling protein; Phosphate carrier; Amino acid sequence; Protein homology; Hydropathy plot; Secondary structure analysis

1. INTRODUCTION

A particularly active and diversified group of substrate carriers is found in the inner mitochondrial membrane. These carriers catalyze the intense traffic of solutes which link the inner mitochondrial functions with those of the cytosol [1–3].

Because of their intracellular localization, these carriers do not require an elaborate machinery, such as binding proteins, phosphate transfer systems or even ATPases for accumulating substrates from a low-concentration environment. Stripped down to only the translocator function, they have a simple structure and consist of only one type of medium-sized protein chain.

One can differentiate between two groups of solute carriers in mitochondria, those involved in hydrogen and carbon metabolism and those engaged in energy transfer. The second group comprises three carriers, for the transport of ADP and ATP, of phosphate, and of H^+. The function of the ADP/ATP carrier (AAC) and of the phosphate carrier (P_iC) in the energy traffic of the eukaryotic cell is obvious (fig.1). They provide the cytosol with ATP generated by the intramito-

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Abbreviations: AAC, ADP/ATP carrier; P_iC, phosphate carrier; UCP, uncoupling protein

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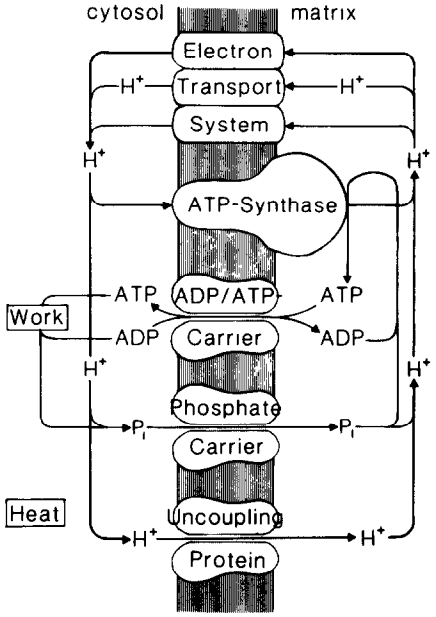

Fig.1. The role of the three mitochondrial carriers (ADP/ATP carrier, phosphate carrier and uncoupling protein) in mitochondrial energy-transfer reactions. The electrochemical membrane potential generated by the electron transport system is either used for ATP synthesis, electrophoretic ATP export, ΔpH-driven phosphate import or – only in brown fat mitochondria – dissipated by the uncoupling protein.

chondrial ATP synthase and play a central role in the symbiosis of the mitochondria with the surrounding eukaryotic cytosol. The transport activity performed by these carriers is probably the most active one in a eukaryotic organism, since about 6 ATP molecules are generated for the consumption of 1 oxygen molecule. The uncoupling protein (UCP) has a function which is somewhat contrary to that of the other two carriers, as it dissipates oxidative energy into heat. It functions as an H^+ carrier and short-circuits the H^+ transport generated by electron transport, thus bypassing the ATP generation [3]. A peculiarity of the UCP is the inhibition of its H^+ current activity by binding ATP or ADP. Thus, although UCP and AAC have in common a nucleotide-binding site, nucleotides have opposite effects on the two proteins; whereas ADP or ATP activate the transport by the AAC, they inhibit H^+ translocation by UCP.

The evolutionary position of these carriers is still a matter of debate [4]. The AAC appears to be unique for mitochondria since it is not present in prokaryotes, in contrast to other components of the energy-transfer system such as ATP synthase and the respiratory chain. ATP transport in storage vesicles has not yet been identified as being related to the mitochondrial AAC [5,6]. The P_iC should be present in all mitochondria, but phosphate transport also occurs in prokaryotes [7]. The UCP is highly specialized and found only in brown adipose tissue which is potentially present in all mammalian organisms [8]. Thus UCP can be regarded as a carrier developed at a later stage in evolution.

In line with their unique mitochondrial localization, all three carriers are synthesized within the cytosol [9] and are coded for by nuclear genes [10–13]. The primary translation product is identical with the complete carrier and does not contain a processed signal or leader sequence for the insertion into the inner mitochondrial membrane [10–15].

The function of these carriers can be described in translocation cycles similar to the catalytic cycles of enzymes (fig.2). In this scheme the net charge and H^+ transfer during these translocation cycles are specified. In the AAC the exchange of ADP^{3-} against ATP^{4-} results in a transfer of a negative charge [16]. In contrast, P_i^- is translocated together with the H^+ by the P_iC in an electroneutral P_i^--H^+ cotransport [17]. In UCP only an H^+ is transported which results in the export of a negative charge [18]. Transport by the AAC and UCP is driven in both cases by the membrane potential whereas the phosphate transport is dependent on the ΔpH.

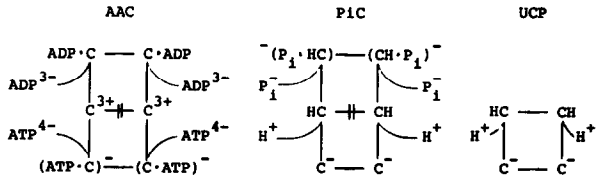

Fig.2. Translocation cycles of the three mitochondrial carriers. The AAC catalyses the counter-exchange of ADP and ATP, while both the P_iC and the UCP catalyse unidirectional transport.

2. BULK STRUCTURE OF THE THREE CARRIERS

The methods used for solubilizing and purifying these carriers are quite similar. They are based on the use of nonionic detergents, in particular Triton X-100. The striking common feature is the separation of the carriers from the majority of the solubilized protein by the passage through hydroxyapatite to which these carriers do not adsorb under appropriate conditions [19–21]. The isolation of the P_iC requires additional measures in order to separate this protein from the large excess of AAC [21–23]. These similarities in isolation procedure reflect a common bulk structure.

Hydrodynamic studies, in particular of the AAC, show that these proteins have a large Stokes' radius of 65 Å due to an extending detergent annulus which covers a major part of the protein surface [24,25]. After subtracting detergent and phospholipids, the molecular mass for both AAC and UCP corresponds to a dimer. This agrees with a functional minimum molecular mass determined by the binding of ligands to the AAC [19] and UCP [20] which shows that there are only 0.5 ligands (carboxyatractylate or ATP) per molecule of carrier polypeptide. One binding center requires two polypeptides and therefore both proteins have a 'half-the-site reactivity' with respect to these ligands.

These results are best explained by assuming that the detergent micelles of both AAC and UCP form an oblate ellipsoid with the 2-fold symmetry axis of the protein, representing the shortest axis of the ellipsoid. Corresponding data for the phosphate carrier are not yet available. It is assumed that they are similar and that all these carriers have a common overall structure.

3. PRIMARY STRUCTURE AND HOMOLOGIES

Because of the relative ease of isolation and high abundance of these carriers in the mitochondria, the primary structure of the three proteins could be established by amino acid sequencing. In the case of the AAC it was the first amino acid sequence of a biomembrane carrier to be determined [26,27]. The primary structure of the UCP was established some time later [28] and the amino acid sequence of the P_iC was also determined recently in our laboratory. An N-terminal fragment of the P_iC-containing residues 1–47 had previously been reported by Kolbe et al. [29]. Because of the lack of favorable cleavage sites in the P_iC, a stretch of about 28 residues in the last third of the protein has resisted our efforts so far. This gap of about 8% of the sequence does not impair the striking evidence of the homology features and structural similarities of the P_i carrier within the energy-transfer carrier group.

Some of the characteristics of the amino acid composition of the three carriers are compared in table 1. All three carriers have an about equal size of around 300–310 residues. For membrane proteins there is a relatively high percentage of polar amino acids, with the basic residues strongly outnumbering the acidic ones. The result is a large positive net charge which is about twice as high in the AAC as in the P_iC and UCP. In most membrane carriers intact cysteine groups have been found to be essential for their function. This is also true for these three carriers. The best known example is the high sensitivity of the P_iC to SH reagents [29]. In the AAC the reactivity of the SH group has been used to define conformational states [30–34]. In reconstituted UCP the transport is inhibited by SH reagents (unpublished). The amount of

Table 1

Amino acid characteristics of the carriers

Amino acids	AAC (BHM)	P_iC (BHM)	UCP (Hamster)
Total	297	≈310	306
Acidic	21	>24	19
Basic	40	>35	28
Net charge	18+	≈11+	9+
Histidine	3	1	4
Cysteine	4	≈6	7
Tryptophan	5	≈5	2
Aromatic	38	>42	26
Hydrophobic	126	>111	119
Polarity (%)	39.7	≈38	37.6
Molecular mass (kDa)	32.9	≈35	33.2

Data are from amino acid sequences [27,28]

hydrophobic amino acid residues, in particular of aromatic amino acid residues, is not very high compared to lactose permease [35]. As a result, the polarity as calculated by Capaldi and Vanderkoi [36] is between 38 and 40%. In summary, already the overall amino acid patterns of the proteins clearly indicate a certain similarity.

Before discussing the sequence homologies of the three carriers we would like to explain briefly some structural features of the AAC. The sequences of AAC from three other sources, *Neurospora crassa* [10], *Saccharomyces cerevisiae* [12] and *Zea mays* [11], have now been established by cDNA sequencing. The carriers are remarkably strongly conserved (identity >50%) over the long evolutionary distances. Highly conserved regions alternate with more variable ones. The conserved regions are concentrated in the hydrophobic segments and the variable ones more in the polar segments. One interesting feature is the non-conservation of histidine. Not a single histidine is found in maize and *Neurospora*. This fact is remarkable from the viewpoint that histidine is generally involved in P_i transferases and hydrolases, and strikingly demonstrates that histidine is not necessary in proteins which do not chemically modify the nucleotides.

A tripartite structure of the AAC was deduced from the amino acid sequence [27,37] and later confirmed by a computer analysis of homologies [38]. Also the UCP is composed of three similar segments of about 100 residues each and is strikingly homologous to AAC [28]. A corresponding analysis of the third carrier reveals now that the P_iC is also constructed according to the same principle. Its sequence is homologous to both AAC and UCP and is made up of three repeats. All nine repeats of AAC, of UCP and of P_iC can be aligned as shown in fig.3. A number of highly conserved amino acid residues (proline, glycine, several acidic and aromatic residues) are detectable. The type of conserved amino acid residues (proline and glycine in the folding of the protein, charged residues in the formation of ion pairs within the protein or in the binding of phospholipids) indicates that a common structural principle is retained despite the development of different carrier functions. There is no room to discuss the possible implications here in more detail.

4. SECONDARY STRUCTURE ANALYSIS AND FOLDING

For determining the secondary structure and the transmembrane folding of membrane proteins, analysis of the hydrophobicity distribution has gained wide acceptance [47]. Application of the average hydrophobicity distribution analysis [39] to the AAC and UCP led to the prediction of only a few transmembrane α-helices (fig.4). In the AAC only one stretch comprising 20 residues is sufficiently hydrophobic to make it a clear candidate

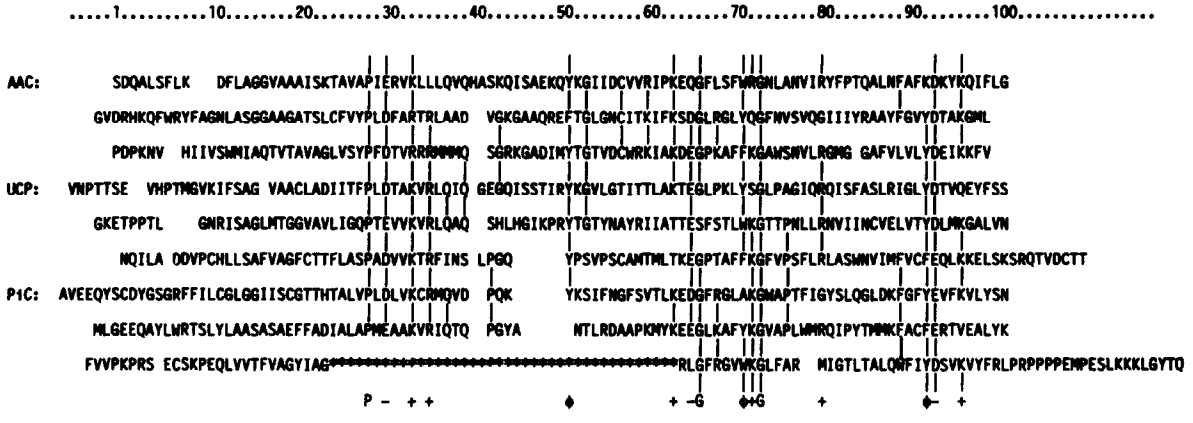

Fig.3. Alignment of the amino acid sequences of the three mitochondrial carriers into three repeats each. The numbering corresponds to the first repeat of the AAC. Several functionally conserved residues (proline, glycine, charged and aromatic residues) are marked by vertical lines and indicated in the bottom line.

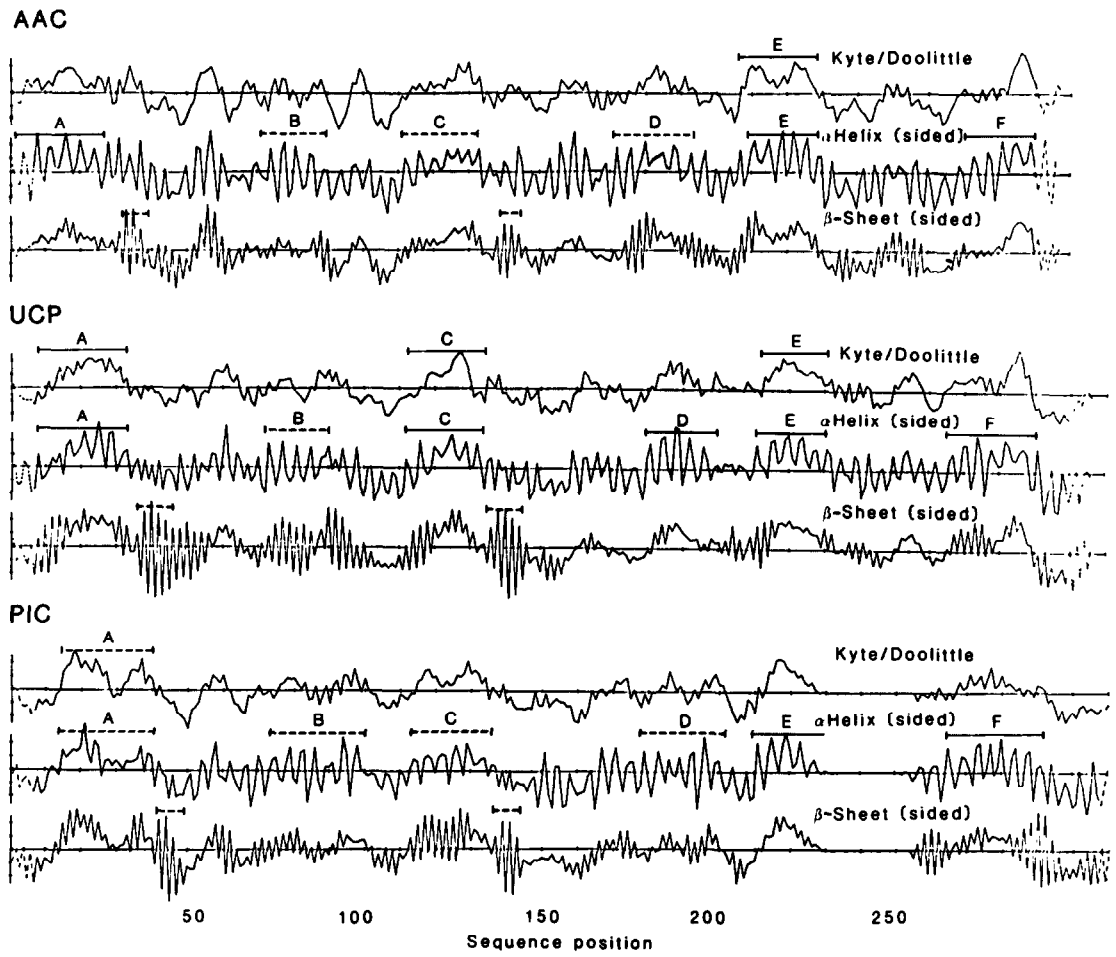

Fig.4. Hydropathy profiles of the three mitochondrial carriers. The hydropathy values of a residue n were obtained by adding the hydropathy indices of the respective residues using the weights given in brackets: weighted average: residues n (1.0), $n \pm 1$ (0.5), $n \pm 2$ (0.5), and $n \pm 3$ (0.5); sided α-helix: residues n (1.2), $n \pm 1$ (0.1), $n \pm 3$ (0.6), $n \pm 4$ (0.6), $n \pm 7$ (0.1); sided β-sheet: n (1.0), $n \pm 2$ (1.0), and $n \pm 4$ (0.5). The hydropathy indices of Kyte and Doolittle [39] were used throughout.

for an α-helix spanning the membrane. A corresponding analysis in UCP yielded three possible transmembrane α-helices [28]. On the other hand, both proteins contain about 41% α-structure according to CD measurements (unpublished). Also, the fact that both proteins are integral membrane proteins, embedded deeply in the membrane, strongly suggests that the number of transmembrane α-helices must be considerably greater.

Further analysis of sided hydropathy profiles allows for the prediction of amphipathic helices in all three carriers (fig.4). In this way the similarity of the hydrophobicity distribution of the three carriers becomes obvious. In particular the tripartite structure of the three proteins can be clearly detected in the sided hydropathy profiles. Each repeat contains one stretch of around 20, more hydrophobic residues and a second more amphipathic α-helical stretch. These predictions are based on a minimum length of 18 residues and a critical hydrophobicity value of 1.5 units [28]. The suggested helical α-structures become more significant by the conservation of the hydropathy distribution throughout the nine repeats of the

three carriers. The general principle of the transmembrane arrangement for each repeat can be visualized as consisting of two membrane-spanning α-helical segments, the first being more hydrophobic and the second being more amphipathic.

The search for amphipathic β-structures also suggests an overall similarity between all the repeats. A significant amphipathic β-pattern is seen near the center of the first repeat and, in UCP, also of the second repeat. It has been suggested that this amphipathic β-structure, corresponding to about 12 residues, may also span the membrane [28].

The transmembrane folding pattern of AAC was investigated by probing the lysine distribution at the membrane surfaces with pyridoxal phosphate [40–42]. In combination with the amphipathic hydrophobicity analysis a folding pattern was developed for the AAC in which definite regions are assigned to the 'c'- or 'm'-surface of the membrane [42]. A similar model was then derived for UCP, including six membrane-spanning α-helices [28]. With the inclusion of a transmembrane β-span the protein chain would fold through the membrane seven times. For the UCP there is evidence that the C-terminal is situated on the cytosolic surface (unpublished) which would agree with an uneven number of spans and the N-terminal on the matrix side.

The demonstrated homologies between the three carriers permit us to assume that the same folding pattern also applies to the phosphate carrier (fig.5). As shown in fig.3, some prolines are strikingly conserved throughout all repeats. However, the highly conserved glycine residues do not occur at the ends of the predicted membrane-spanning helices although glycine often occurs in type II reverse turns or in flexible loops [43]. Therefore, another functional importance of these residues may be assumed, probably in establishing helix-helix contacts as, for example, in hemoglobin.

5. COMMON CARRIER MODEL

A model for the arrangement of the transmembrane helices can be proposed on the basis of the intriguing triplicate structure of these carriers

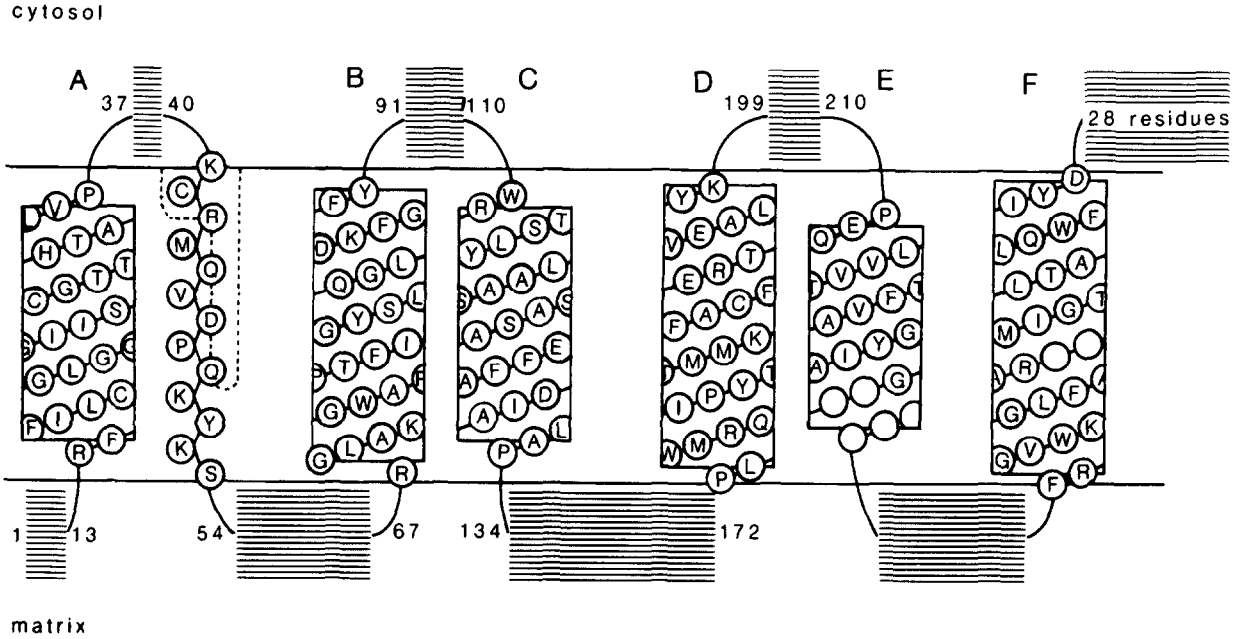

Fig.5. Suggested arrangement of the polypeptide chain of the P_iC in the membrane, as representation for the three mitochondrial carriers. The six membrane-spanning α-helices are shown as boxes and are labeled A–F starting from the amino-terminus. A possible β-strand near a pore (dashed line) is shown between helices A and B. The intervening hydrophilic segments are indicated by hatched boxes as their structure is unclear.

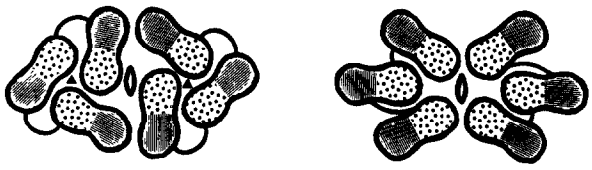

Fig.6. Two possible arrangements of the transmembrane helices of the mitochondrial carrier dimers in a view perpendicular to the membrane. The more hydrophobic helices (A,C,E; hatched) are directed towards the phospholipid environment while the amphipathic helices (B,D,F; stippled) are situated in the centers. In A, each monomer has a central pore accommodating an axis of pseudo 3-fold symmetry and the two monomers have a central axis of 2-fold symmetry (indicated by ◊). In B, the dimer has one central pore accommodating a central axis of pseudo 2-fold symmetry (indicated by ◊).

(fig.6). It is visualized that in each repeat the hydrophobic α-helix is exposed to the phospholipids, while the amphipathic helix is directed towards the inside of the protein. The larger hydrophilic surface would connect to the hydrophilic surface of the next repeat. In this manner the three repeats are arranged around a pseudo 3-fold axis. Two of these triplicate structures form the dimer around the 2-fold central axis of the whole carrier.

It is remarkable that a somewhat similar situation appears to exist in the voltage-dependent Na^+ channel. For this protein a structural hierarchy of more hydrophobic and more polar membrane-spanning α-helices has been reported [44]. Furthermore, the protein is made up of 4 repeats, each containing 3 membrane-spanning α-helices. Why are all these carriers constructed according to the same principle? Is the arrangement as suggested in fig.6A around a pseudo 3-fold axis within the monomers intended to form relatively hydrophilic translocation channels? This important question awaits further clarification; however, the fact that these carriers appear to have only one binding site per dimer seems to suggest that the central 2-fold axis can form a translocation channel.

These problems are closely connected with the localization of the binding center. For the AAC, using an azido derivative of atractylate, a rough localization between positions 156 and 200 was reported [45]. By means of pyridoxal phosphate, Lys 22 was identified as being involved in atractylate binding [42]. These experiments would relate the binding center to helices A and D. Furthermore, pyridoxal phosphate incorporation data suggested that the areas around Lys 48, 106 and 162 participate in the translocation path.

In many proteins nucleotide-binding sites have been found to comprise a glycine-rich sequence pattern, GXXGXGK [46,47]. This pattern is not present either in the AAC or in the UCP. The C-terminal region of AAC from beef heart, starting with Arg 279, resembles the sequence B, RXXXGXXXLhhhD, of some adenine nucleotide-binding proteins [48]. However, Gly 283 is not conserved in the AAC from yeast and Val 288 is replaced by serine in *Neurospora*, maize and yeast. This amino acid pattern is not detected in UCP. At present there are no sound arguments in favor of a participation of this area in nucleotide binding.

In conclusion, no nucleotide-binding site can be detected in the AAC and UCP by mere analogy to the amino acid patterns characteristic for nucleotide-binding sites in a great number of other proteins. This may be rationalized by considering that in the AAC and UCP the nucleotide is not chemically cleaved, nor is Mg^{2+} involved in the binding, in contrast to all the other analyzed nucleotide-binding proteins.

The question arises as to whether there is an amino acid sequence homology to bacterial solute carriers. Recently the sequence of three carriers, for lactose [35], melibiose [49] and citrate [50], has become known. There is no amino acid homology of the three mitochondrial carriers with these bacterial carriers. All these carriers are considerably larger, comprising up to 450 residues, and they are much more hydrophobic. The hydrophobicity distribution also suggests a quite different transmembrane architecture.

The particular triplicate structure in all the three mitochondrial carriers suggests that they evolved first by gene triplication and subsequently by diversification. All these three carriers are encoded by nuclear genes. For these mitochondrial carriers no prokaryotic relatives as possible ancestors are known as yet. This is understandable from the viewpoint both of their intracellular function and of their differences from the known structures of prokaryotic carriers.

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