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The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequence homologies and of folding of the protein in the membrane

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We report here, for the first time, the primary structure of uncoupling protein as established by amino acid sequencing. Like the ADP/ATP carrier, this protein has a tripartite structure comprising three similar sequences of ~100 residues each. These six 'repeats' exhibit striking conservation of several residues, in particular glycine and proline, at possible structurally strategic positions. Although the two proteins differ strongly in their amino acid composition, their sequences are distantly homologous. Three membrane-spanning α-helices can be deduced from hydropathy plots. A modified plot accounting for amphiphilic helices indicates 5–6 such α-segments. In addition an amphiphilic β-strand of membrane-spanning length can be discerned. The tripartite sequence structure is also distinctly reflected in the hydropathy distribution. Based on the membrane disposition of the segments of the ADP/ATP carrier, a model for the transmembrane folding path of the polypeptide chain of the uncoupling protein is proposed.

Key words: amino acid sequence/hydropathy plots/relation to ADP/ATP carrier/uncoupling protein/secondary structure analysis

Introduction

The structure of biomembrane carriers is a central issue when trying to understand the mechanism of transport through biomembranes. To elucidate the structure-function relationship it seems most promising to concentrate on simple carriers with no superimposed ATP hydrolysis, where the relatively small protein only undertakes transport catalysis. Solute carriers of the inner mitochondrial membrane belong to this category. The ADP/ATP carrier (AAC) was the first biomembrane carrier whose primary structure became known by amino acid sequencing (Aquila *et al.*, 1982a, 1982b). Another functionally quite different carrier, the 'uncoupling protein' (UCP) from brown fat mitochondria, turned out to be structurally quite similar, as is shown below.

Using methods developed originally for the ADP/ATP carrier from beef heart mitochondria (Riccio *et al.*, 1975; Klingenberg *et al.*, 1979), the uncoupling protein from brown adipose tissue of mitochondria was purified a few years ago (Lin and Klingenberg, 1980, 1982). Only recently has the uncoupling protein been successfully reconstituted into artificial liposomes and shown to be a H^+ carrier (unpublished results), substantiating an early suggestion by Nicholls (1976). The protein returns protons into mitochondria ejected by the respiratory chain, thus bypassing the ATP-synthase. As a result, oxidative energy is converted into heat, thus the uncoupling protein is the mechanistic key element in the heat-generating process of brown adipose tissue. An important feature of the uncoupling protein is that it binds purine nucleoside di- and triphosphates, thereby inhibiting the H^+ transport. This binding and its peculiar characteristics are fully retained in the isolated and in the reconstituted protein.

There are a number of structural similarities between the UCP and the AAC. Both proteins form dimers and have a similar mol. wt. With non-ionic detergents both isolated proteins form large mixed micelles, which indicates that both have large hydrophobic surfaces. Furthermore, both proteins bind only one inhibitor molecule per dimer, i.e., ATP in the case of UCP and carboxyatractylate in the case of AAC. The two proteins also share some functional properties, in particular in that they both provide binding sites for purine nucleotides. On the other hand, there are important contrasts: in the case of the UCP, the nucleotide is an inhibitor, whereas in the case of the AAC it is a substrate for transport. The UCP transports protons, the smallest solutes, and AAC translocates ADP and ATP, about the largest solutes known to be transported.

The primary structure of the UCP is presented here for the first time. The experimental details will be presented elsewhere. The comparison of the sequence with that of the ADP/ATP carrier reveals highly significant similarities. The resulting secondary structural similarities between both proteins will be elucidated.

Results and Discussion

Amino acid sequence of the UCP and comparison with that of the AAC

The primary structure of the UCP, as established by amino acid sequencing, is shown in Figure 1. The protein consists of 306 amino acids and has a calculated mol. wt. of 33 215 d. Its length and size are therefore very similar to that of the AAC which has 297 residues and a M_r = 32 906. The N terminus in the UCP is not blocked, starting with a valine, whereas in the AAC it is an acetylated serine. In the UCP a microheterogeneity was detected in position 128, where leucine and phenylalanine are found in about equal amounts. On the basis of this finding one could assume that the UCP from the brown adipose tissue of the golden hamster is either a dimer consisting of two polypeptides identical in all residues but one, or the heterogeneity may be due to differences in the genomes of the individual animals from which the protein was isolated, or it may have its origin in a heterozygocity in one and the same organism.

The amino acid composition of the two proteins is compared in Table I. Both proteins contain ~30% alicyclic hydrophobic amino acids and ~10% aromatic amino acids, i.e., ~40% hydrophobic residues altogether. The proteins have nearly equal amounts of polar residues: AAC 39%, UCP 42%. These values are rather high for integral membrane proteins. Both UCP and AAC contain a surprisingly large number of charged residues. Quantitatively, however, there are important differences between the two proteins, for instance in the number of charged amino acids. The UCP has 28 positive and 19 negative residues, that is an excess of nine positive charges. This compares with a considerably higher content of charged residues in the AAC, namely

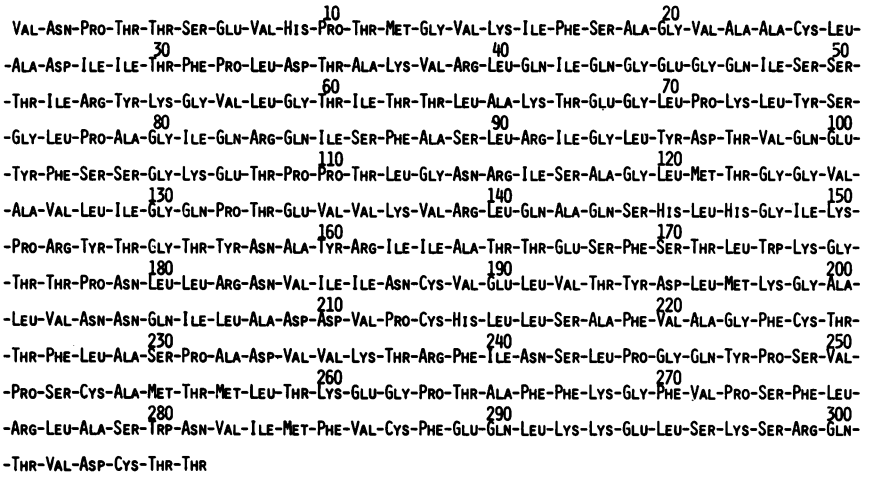

Fig. 1. Primary structure of the UCP. The protein was isolated as described (Lin and Klingenberg, 1982) and the cysteine residues were carboxymethylated. Cyanogen bromide peptides render the basic sequence information, the large peptides CB2 and CB3 were subfragmented with the Lys-specific protease. For enzymatic digestions the protein was solubilized by citraconylation and cleaved with trypsin, thermolysin or *Staphyloccocus aureus* protease. Generally the fragments were prefractionated by gel filtration in 80% formic acid on Sephacryl S-200. The larger peptides were further purified by h.p.l.c. on RP8 columns in 10% formic acid applying propanol gradients, whereas for the smaller peptides RP18 columns in 50 mM ammonium acetate, pH 4.0, and acetonitrile gradients (0–60%) were used. The Edman degradations were performed either with a liquid phase sequenator of our own construction or manually using the dimethylaminoazobenzene isothiocyanate procedure. The phenylthiohydantoin derivatives were identified by h.p.l.c. (Zimmerman and Pisano, 1977) and the dimethylaminoazobenzene thiohydantoin derivatives by t.l.c. (Chang, 1983).

40 positive and 21 negative residues, resulting here in a net positive charge of 18, taking into account the blocked N-terminal amino group. Great differences exist between the two proteins as regards their amino acid content. The number of the hydroxy-amino acids serine and threonine varies widely: the UCP has a total of 54 of each, whereas the AAC contains only 26. Also the UCP has a relatively high proline content, of 17 residues, compared with eight prolines in the AAC. Only two tryptophan residues occur in the UCP, while AAC has five. The UCP contains seven cysteine residues and the AAC four, out of which only one seems to be conserved (position 253 in UCP). In contrast to the AAC where one lysine residue is trimethylated (position 51), the UCP reveals no modified amino acid residue.

Inspection of the UCP sequence reveals a remarkably frequent occurrence of Pro-Thr (five) and Pro-Ser (three) bonds. There is even a clustering of these pairs in the unique sequence Pro-Thr-Thr-Pro. In contrast, the AAC has only one Pro-Thr bond. In the sequence of the AAC, a clustering of charged amino acids had been noted. Besides the unique triplet Arg-Arg-Arg there are about nine ion pairs of positive/negative charged amino acids; often these pairs are separated by only a few residues. The same clustering of charged residues, although less frequent, is observed in the UCP.

Internal sequence homology of the UCP and homology with the AAC

In the course of sequencing a certain sequence repetitions within the UCP became apparent. Therefore, we generated a comparison matrix (MacLachlan, 1971) of the sequence of the UCP with itself (Figure 2). High scores of similarity occur in lines parallel to the diagonal at a distance of ~100 residues. This implies that the sequence is divided up into three similar stretches, each ~100 amino acids in length – the 'repeats'. The strongest similarity appears between repeats 1 and 2, whereas it is only very weakly expressed between repeats 1 and 3.

We shall show that this internal homology is especially interesting in comparison with the AAC. For the AAC an internal homology had been also noted during sequencing and was discussed in Aquila *et al.* (1982b), and then described in a 'Diagon' plot by Saraste and Walker (1982).

Table I. Amino acid composition of UCP and AAC. Data from the amino acid sequences (Figure 1 and Aquila *et al.*, 1982a)

Amino acid	Residues (mol/mol subunit)	
	UCP	AAC
Aspartic acid	8	15
Asparagine	10	8
Threonine	32	11
Serine	22	15
Glutamic acid	11	6
Glutamine	13	14
Proline	17	8
Glycine	24	30
Alanine	22	33
Valine	25	24
Cysteine	7	4
Methionine	6	7
Isoleucine	20	19
Leucine	31/30[a]	22
Tyrosine	9	12
Phenylalanine	15/16[a]	21
Histidine	4	3
Tryptophan	2	5
Lysine	16	23
Arginine	12	17
Total	306	297

[a]Microheterogeneity, see text.

The similarity of the two proteins in the bulk structure may suggest similarities in the sequence also. Therefore, the sequences of these two proteins were compared in a Diagon plot (Figure 3). High scores of similarity can be detected along the diagonal and along parallel lines to the diagonal, again at a distance of ~100 residues. The density of the points indicates a distant, but concurrent homology between the full sequences as well as between the individual repeats. Parallel shifts within the repeats, indicating insertions or deletions, are rare and do not exceed five residues.

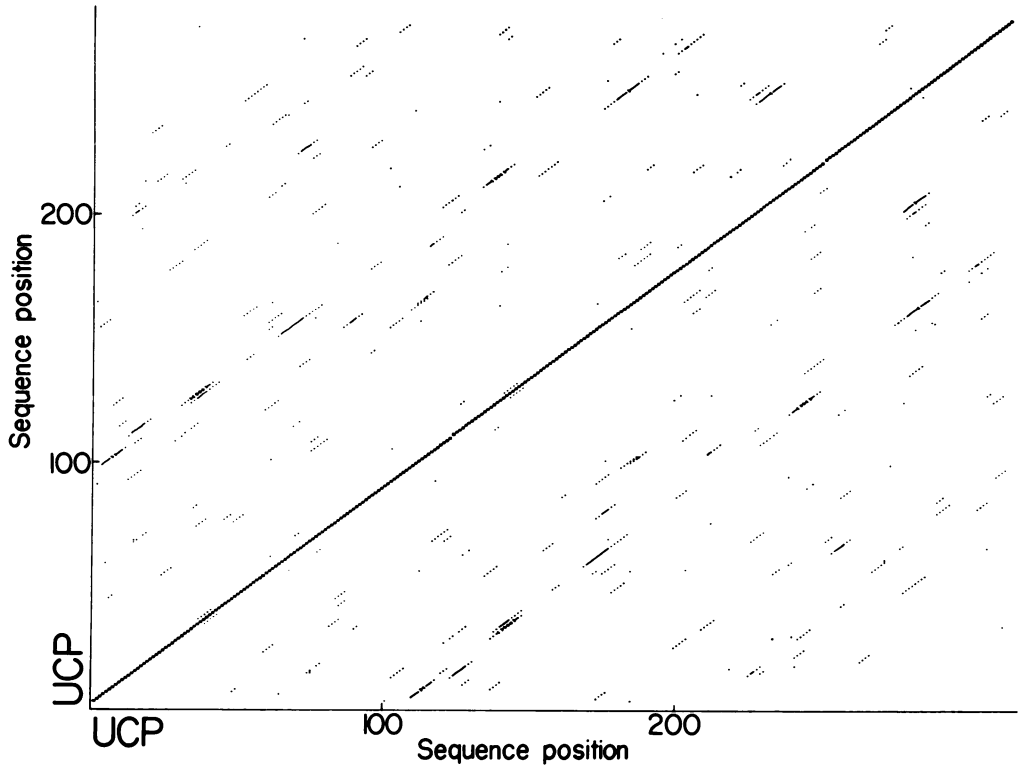

Fig. 2. Diagon-plot comparing the sequence of the UCP with itself according to McLachlan (1971), using the substitution frequencies given to one digit and a span setting of 11.

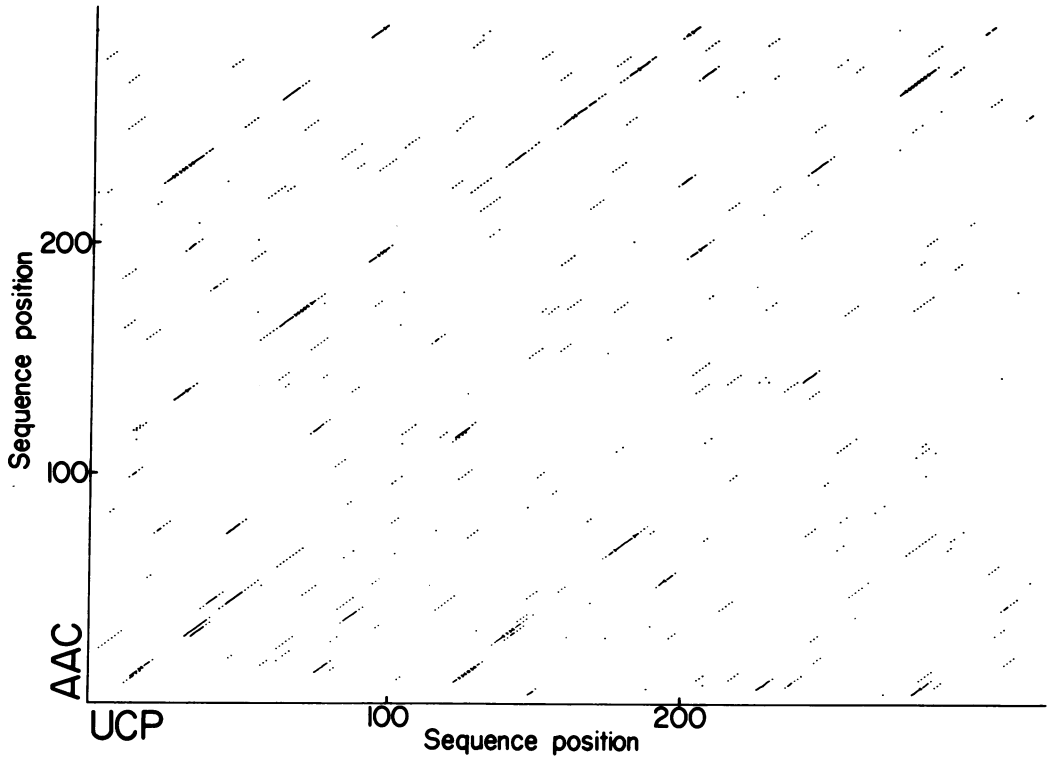

Fig. 3. Diagon-plot of the AAC from beef heart mitochondria versus UCP.

Following the Diagon plot, an alignment was made in the following way. In the sequence of the UCP, one residue was inserted between positions 43 and 44, four residues between positions 131 and 132 and five residues between positions 246 and 247. In the sequence of the AAC, two insertions of one amino acid each had to be assumed between residues 202/203 and 205/206.

Quantification of the sequence similarities of UCP and AAC

With this alignment the similarity between the sequences was evaluated according to Wieland *et al.* (1984). The statistical

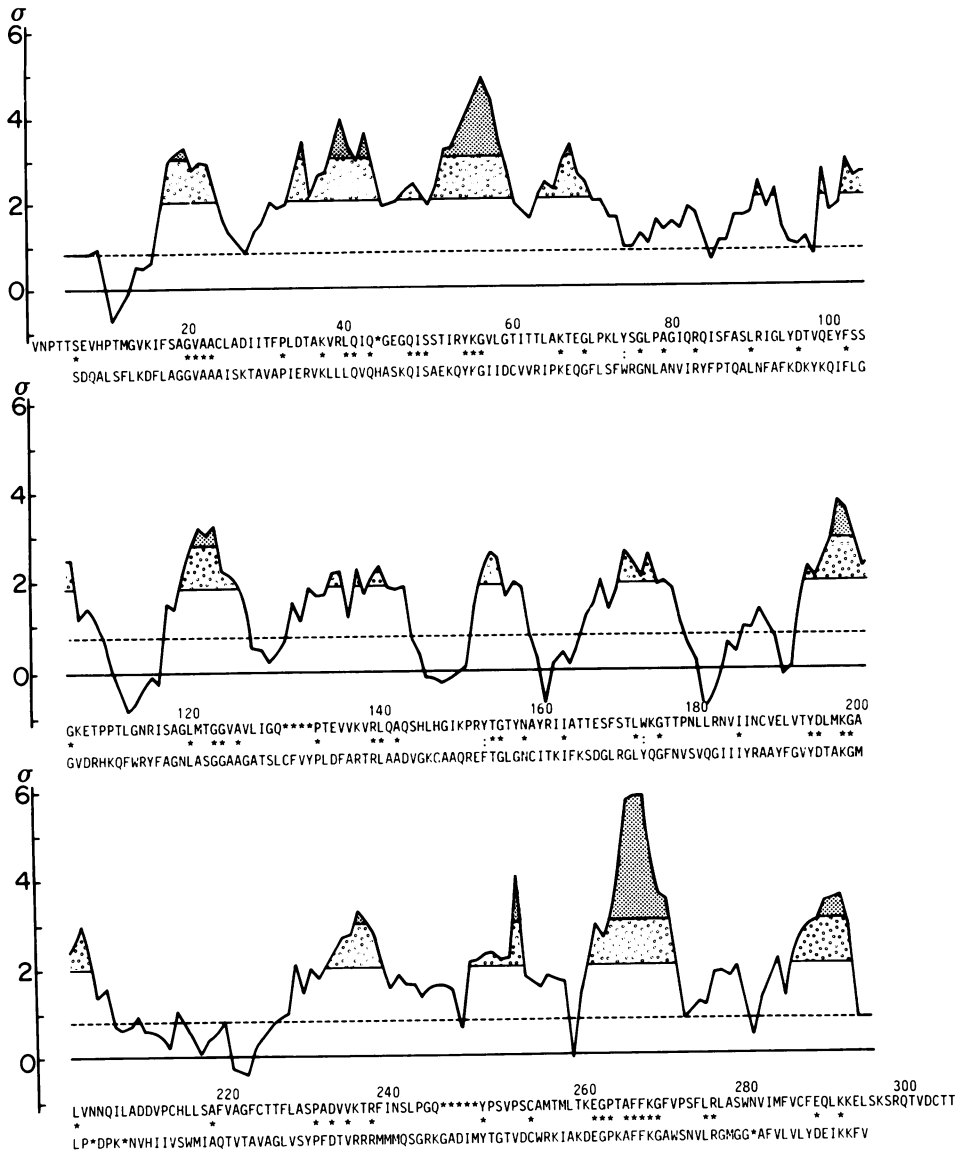

Fig. 4. Plot of local similarities between the AAC and the UCP according to Wieland *et al.* (1984). The alignment of the sequences was performed by inserting necessary residues by hand. A span setting of 7 was used. The natural substitution frequencies MDM_{78} were taken from Dayhoff (1978). The ordinate scale is given in standard deviation units.

analysis was performed by overlaying all possible segments of seven residues of the UCP and the AAC. The distribution of the 90 830 overlays is approximately Gaussian. Thus the standard deviation of the scores from their mean value could be calculated and each score expressed by its distance from the mean as measured in standard deviation units.

As shown in Figure 4, regions of highly significant sequence similarity (exceeding three standard deviations) are found in the N-terminal and the C-terminal repeats. In the latter there is a short region exceeding five standard deviations. For the second repeat only two short regions exceeding three standard deviations are found. There are two long regions from position 70 to 97 and from 208 to 228 with a standard deviation between 1 and 2 or even less. The second of these contains the most hydrophobic stretch of the AAC which is assumed to be a membrane-spanning helical segment (Aquila *et al.*, 1982a). Sixty-four amino acid residues, i.e., ~20%, are conserved, including 12 of the 24 glycine residues of the UCP and four of the eight proline residues of the AAC. We conclude that the compared chains are homologous. Regions of strong conservation alternate with regions of great divergence. It is expected that this pattern can be interpreted in terms of functional similarities and functional differences between the two transport proteins.

Statistical analysis of the triplicate structure

The conservation of amino acid residues is even more striking when the three repeats of UCP and AAC are compared with each other (Figure 5). The most obvious features are: the conservation of one proline and one glycine residue throughout all six repeats at the positions corresponding to residue numbers 32 and 76 of the UCP. In all repeats three charged residues (once positive at number 37 and twice negative at numbers 34 and 96) and two aromatic residues (at numbers 54 and 74) are conserved. Identity of residues in all repeats but one is noted in eight positions. Hydrophobic residues are found frequently either in five or all six repeats. We conclude that the repeats have diverged from a common ancestor. Thus a common sequence can be constructed that shows greater similarity with each of the repeats than the

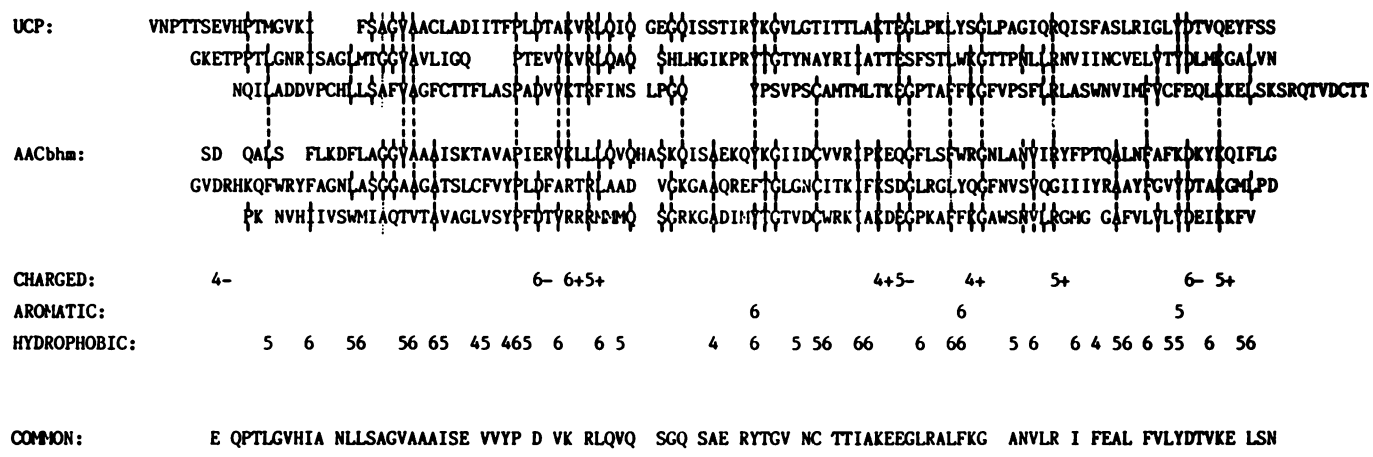

Fig. 5. Sequence homology of the six repeats of UCP and AAC. Three and more identical residues in the same position in all six repeats are marked by a stroke above and below the residue. Positions with at least four charged, or four aromatic or four hydrophobic residues are also indicated. The common sequence was derived by choosing the amino acid with the highest sum of similarity scores compared with all the residues at this position throughout the six repeats. The one-digit substitution frequencies of McLachlan (1971) were used. If the sum of the scores was <27, no residue was printed.

repeats with each other (Figure 5).

Statistical analysis of the six repeats in comparison with each other and with the common sequence reveals that the first repeat of the UCP and the third repeat of the AAC show greater similarity with each other and in relation to the common sequence than any of the other repeats (Table II). The direction of decreasing similarity with the common sequence is: first-second-third repeat of the UCP and third-first-second repeat of the AAC. There are two possible explanations for this observation: either this pattern of similarities represents the evolutionary distance between the individual repeats; this would indicate that this evolutionary separation between uncoupling protein and the nucleotide carrier has occurred before the triplication; or the second explanation would be that the pattern of similarities represents the functional requirements of the two membrane proteins, accounting for their identical as well as their different properties; the two proteins may thus have evolved from a common triplicated ancestor.

Hydrophobicity distribution analysis

The similarity of the two proteins also becomes evident when their hydropathy plots (see next paragraph) are compared (Figure 6). The UCP contains two short polar extensions at the N and C termini; apart from this, the agreement between UCP and AAC in the pattern of hydrophobic regions alternating with hydrophilic regions is striking. Also the existence of three similar repeats of ~100 residues each can be seen in both proteins. On average, the repeat starts with about five polar residues, followed by a stretch of ~25 hydrophobic residues. The sequence continues with ~18 polar residues followed by ~10 hydrophobic residues. A stretch of ~16 indifferent residues ensues, then a slight hydrophobic stretch of ~12 residues follows. A stretch of about eight polar residues terminates the repeat.

Search for membrane-spanning segments

As a result of their deep embedding in the membrane, the UCP and the AAC should dispose of a large hydrophobic surface which faces the acyl chains of the phospholipid matrix. For both proteins this interface is reflected in the voluminous detergent micelle surrounding the isolated proteins. Therefore, the distribution of hydrophobic/hydrophilic stretches might denote those segments of the chain which delineate this surface. Experience with synthetic peptides (Rosenblatt *et al.*, 1980), compilations of von Heijne (1981) and the model bacteriorhodopsin (Engelman *et al.*, 1980) suggest that these segments are made up of hydrophobic α-helices. A stretch of ~20–23 amino acids would correspond to an α-helix of 35 Å which transverses the hydrophobic phase. As pointed out previously, in the AAC only two sections (segments C and E in Figure 6) are not interrupted by polar amino acids and one other (segment D) has only one arginine inserted. These three segments were put forward as candidates for membrane spanning α-helices (Aquila *et al.*, 1982a, 1982b). However, circular dichroism studies show that the AAC and the UCP have a nearly equal content of ~40–42% α-structure, whereas three hydrophobic segments of ~20 residues each account for only 16% α-structure.

Hydropathy plots according to Kyte and Doolittle (1982) were applied to detect other possible α-structures. These are shown in Figure 6 for both UCP and AAC, using the hydropathy index assignment to amino acids of these authors. Mean hydropathy values >1.6 for a span of 19 residues have been postulated to be required for membrane-spanning α-helices (Kyte and Doolittle, 1982). However, in the AAC only one stretch, segment E, and in UCP only three stretches, segments A, C and E, are found where the mean hydropathy of a span of seven residues

Table II. Segment-averaged similarities were attained by averaging the local similarities between different repeats of the AAC or the UCP (cf. Figure 4) over the whole range. Numbers are given in standard deviation units

Segments	Internal		
	1/2	1/3	2/3
AAC	1.77	1.58	1.32
UCP	1.44	1.98	1.69

AAC/UCP

UCP \ AAC	1	2	3
1	2.05	1.87	1.31
2	1.72	1.39	1.46
3	2.10	1.71	1.91

—/Common

	1	2	3
AAC	2.79	2.59	3.16
UCP	3.28	2.77	2.41

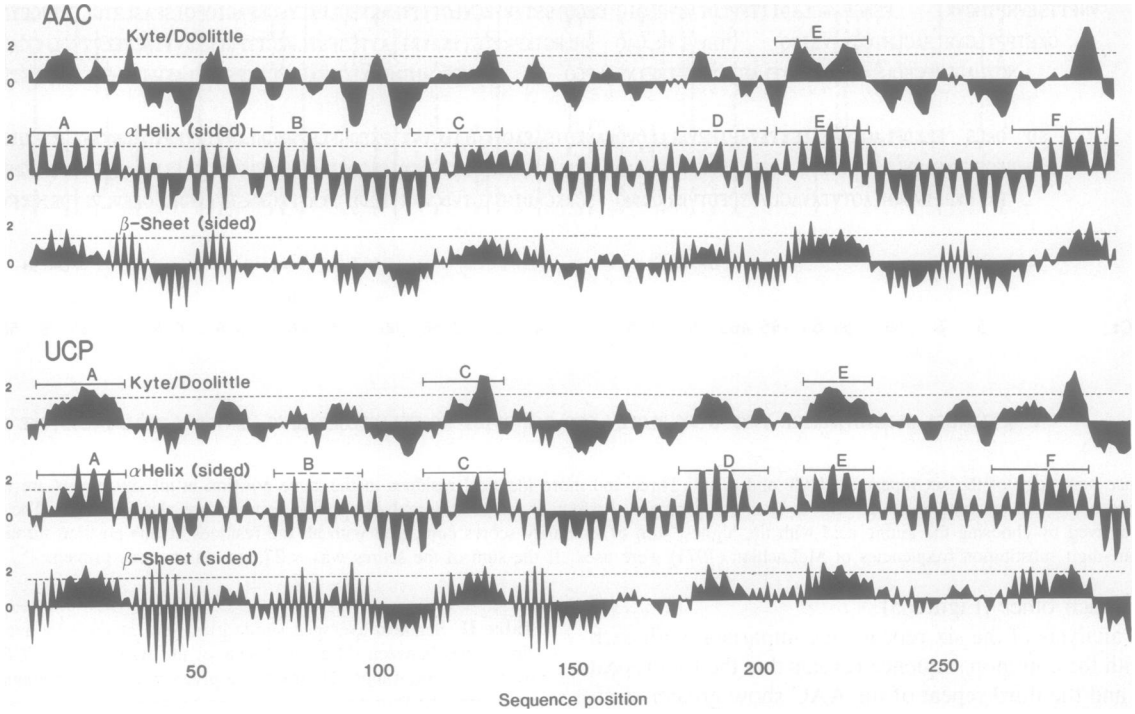

Fig. 6. Hydropathy plots of the AAC and of the UCP according to Kyte and Doolittle (1982) and amphiphilic hydropathy plots of the same proteins. The dotted line indicates a hydropathy value of 1.5. For the weighted average (upper curves) the hydropathy indices of the residues n, n±1 (weight), n±2 and n±3 (weight 1/2) were used. For the sided α-helix (centre curves) the residues n, n±4 (distance 1 turn, 40°) (weight), n±3 (distance 1 turn, 60°), and n±7 (distance 2 turns, 20°) (weight 1/2) were taken into account. For the sided β-sheet (lower curves) the residues n, n±2 (weight), n±4 and n±6 (weight 1/2) were considered.

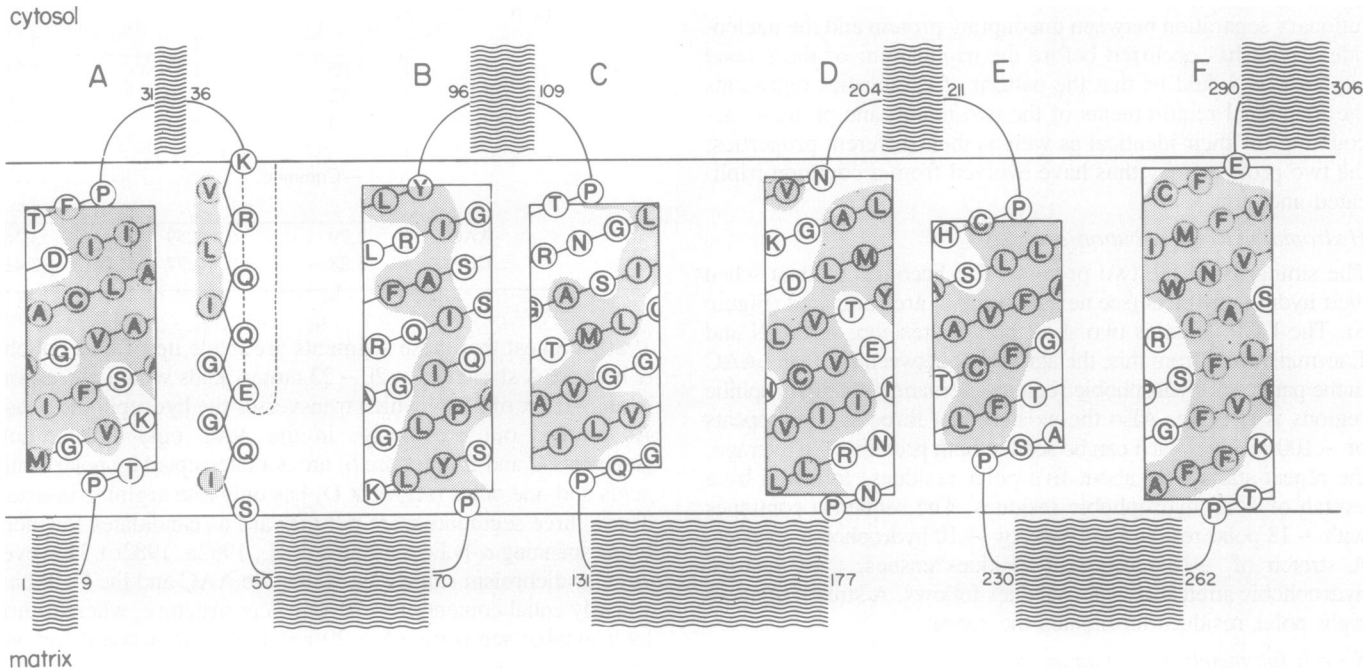

Fig. 7. Suggested arrangement of the polypeptide chain of the UCP across the membrane. The six membrane spanning helices are labelled A–F starting from the amino terminus. For visualization of the geometrical relationship between the residues of an α-helix, the cylindrical plot presentation is used (Schiffer and Edmundson, 1967). Areas with residues more hydrophobic than glycine are grey. A possible β-strand near a pore (dotted line) is shown between helix A and B. Stretches of unknown structure are depicted as shaded boxes of different size. Their sided location is only graphical, as these segments are suspended between the ends of the helices.

approaches 1.5 (dotted line) for ~20 residues. By a more refined use of hydropathy indices additional α-structures may be revealed with significance for the localization in the membrane. Thus some of the α-helices may have widely different degrees of hydrophobicity on the opposite sides of the cylindrical surface. The less hydrophobic side may be in contact with other stretches of the

polypeptide chain or even with a hydrophilic channel. Therefore, following a suggestion of F.Jaenich, hydropathy plots were constructed according to a sided helix pattern by stressing an averaging over the 3rd, 4th and 7th residues, as described in Figure 6. With this evaluation, membrane-spanning α-helices can be detected in segments where for a length of ~20 residues the amphiphilic hydropathy values of at least every third and/or fourth residue are >1.5. For the AAC these are observed to be segments A, E and F and for the UCP segments A, C, D, E and F. Segments C and D in the AAC are less hydrophobic than the corresponding segments in the UCP, but in analogy they can also be expected to form membrane-spanning segments. The B segments are less likely candidates.

For the uncoupling protein we would predict the α-helical segments A, C and E to extend between residues 11 to 31, 111 to 131 and 213 to 230, respectively, since these three segments are confined by proline residues which are known to be helix breakers. Further, we would predict α-helical segments from positions 72 to 95 (B), 179 to 202 (D) and 264 to 288 (F); these proposed helices have a proline at their N terminus and residues with high reversed-turn-propensities at their C-terminal ends. Segments B and F, however, would contain one proline residue within the helix. It is interesting to note that the same constellation is found in three out of the seven helices of bacteriorhodopsin (Engelman *et al.*, 1980). As noted by Richardson (1981) proline fits well into the first turn of an α-helix, but further within the helix it produces bends.

Similarly, averaging of hydropathy was performed in a search for hydrophobic amphiphilic β-strands (Figure 6). In the UCP the residues covering positions 35 to 55 and 133 to 147 show strong oscillations with a repetitive length of two residues. One would expect this result if these stretches formed β-strands adjacent to the phospholipid bilayer or hydrophobic areas of the protein on one side and a polar environment on the other side. The location of the strongly conserved prolines 32 and 132 at the N-terminal ends of these segments is remarkable. This interpretation gains support from an analysis of bacterial porin which displays a similar pattern with a repetitive length of two residues in the amphiphilic hydropathy plot. From X-ray diffraction analysis it is known to have β-pleated sheets perpendicular to the membrane (Garavito *et al.*, 1983).

How are the various hydrophobic and hydrophilic segments arranged in the membrane? Chemical modification of the AAC has been performed with [^{3}H]pyridoxal phosphate to determine the membrane disposition of lysine residues. Residues 22, 91, 93, 95, 106, 162, 198, 205, 259, 262 and 267 were shown to be accessible from the cytosol, whereas residues 42, 48 and 146 could be attacked from the matrix space (Bogner *et al.*, 1982, 1983). No similar experiments have so far been performed for the UCP. However, the similarity in hydropathy profile between the two proteins may indicate a similar arrangement in the membrane. On the basis of this assumption and on the grounds of the hydropathy distribution plot, the following model is proposed for the UCP (Figure 7).

The suggested helices are depicted as barrels with grey hydrophobic surfaces. The α-helices are drawn in such a way that the significantly hydrophobic surface extend approximately in the axial direction. These surfaces have rather irregular shapes. On the other hand, there are more or less well defined hydrophilic areas giving these amphiphilic helices a somewhat asymmetric distribution of polarity. The unassigned sections are drawn on both sides of the membrane because of the folding pattern. However, this does not imply that these sections are located outside the membrane. In fact, probably most of the protein mass is located within the bilayer surfaces, as suggested by the ultracentrifugation data of the protein-detergent micelle (Lin *et al.*, 1980).

Conclusions

The most striking observation when comparing the primary structures of the AAC and the UCP is the occurrence of three repeats and the recurrence of the triplicate structure in both proteins. A number of obviously important residues are fully or at least extensively conserved in the six repeats of the two proteins. These residues may be essential for maintaining structural features such as helical breaks, β-turns or ionic anchoring of the phospholipid headgroups. The triplicate character is also borne out by the hydropathy pattern. This may indicate similar secondary structures within each repeat. As a result it can be suggested that each of the two proteins is composed of three structurally similar domains.

If one assumes that the similarity of the repeats is based on a true homology, it can further be speculated that other mitochondrial anion metabolite carriers, for instance for phosphate, dicarboxylate etc., are also derived from the same ancestral gene. Following this hypothesis, a hierarchical family of metabolite carriers can be derived (Klingenberg, 1985), in which the uncoupling protein belongs to the subgroup of H^+-anion co-transporters as we find them in the phosphate carrier and the citrate-malate exchange carrier in the mitochondria. The uncoupling protein would be derived from these carriers by deleting the anion binding site. This implies that the uncoupling protein is not an elementary H^+ carrier from which other H^+ co-transporters are derived, but rather a degenerated H^+-anion co-transporter. This would also agree with its highly specialized and unique occurrence in brown fat mitochondria, whereas all mitochondria contain, e.g., carriers for phosphate and ADP/ATP. This makes it easier to comprehend the function of the uncoupling protein as an H^+ transporter rather than as an anion transporter for OH^- and Cl^-, as originally suggested (Nicholls, 1976).

Functionally, the AAC and the UCP differ drastically in the selection of their transported solutes, as pointed out in the Introduction, but both proteins have binding sites for purine riboside di- and triphosphates, which can be expected to be due to similar nucleotide binding domains. However, the two binding sites differ strongly in their properties. In the AAC the binding site is highly flexible and involved in the translocation, whereas in the UCP it is more rigid and involved in inhibiting the translocation. At least one H^+ translocating binding site should exist in the UCP, whereas in the AAC an H^+ co-transport does not occur.

Under these circumstances, it should be highly rewarding to obtain primary structures of other mitochondrial metabolite carriers not only in order to establish further homologies, but also to see how the suggested divergence from the ancestral carrier produces the observed functional differences.

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