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Mitochondrial targeting sequences

Why 'non-amphiphilic' peptides may still be amphiphilic

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The notion that mitochondrial targeting peptides form amphiphilic α-helices with one apolar and one polar, positively charged face is controversial, since some experimental results seem to imply that non-amphiphilic targeting peptides can also function as import signals. However, the standard methods used to assess the amphiphilicity of a peptide may be misleading, since they do not take the flexibility of the amino acid side chains into account. To demonstrate this, we have developed a new method for calculating the amphiphilicity of helical peptides.

Mitochondria; Targeting; Topogenic sequence; Amphiphilic helix

1. INTRODUCTION

Most mitochondrial proteins are encoded in the nucleus and synthesized as larger precursors in the cytoplasm. The precursors contain amino-terminal extensions that direct import into mitochondria. These presequences are cleaved off once the proteins have reached their final destination [1].

Although no significant sequence homology has been found in mitochondrial targeting signals, these peptides do share some common features. They are rich in hydrophobic, hydroxylated and basic amino acids, especially arginines, but rarely contain acidic residues. Furthermore, most of them have the potential to form an amphiphilic α-helix with one positively charged and one apolar face. Thus, besides positive charge, amphiphilicity may be a requirement for mitochondrial import [2,3].

However, some recent experimental results seem to contradict this hypothesis. For example, mutational analysis of the ornithine transcarbamylase (OTC) leader peptide indicates that the functional structure of the region responsible for import is unlikely to be an amphiphilic α-helix. The wild-type sequence does contain a putative amphiphilic helix [3], but when point mutations were made in order to introduce positive charges on the hydrophobic face of this helix, import was more or less unimpaired. Furthermore, replacement of one or two helix-forming residues with the helix breaker glycine did not disrupt import. In contrast, artificial leader peptides rich in arginine and predicted to assume an α-helical conformation failed to direct import [4].

Similar results have also been reported for other artificial presequences composed of only a few kinds of amino acids (Phe, Leu, Ser, Gln) [5,6]. Presequences with a composition resembling that of natural ones were found to be able to direct import, whereas more hydrophilic sequences were not. The import efficiency did not always correlate with the predicted amphiphilicity of the sequences, but the physical properties of synthetic model peptides nevertheless indicated that the functional presequences behaved as amphiphilic peptides.

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In the interpretation of these experiments, it was assumed that the distribution of residues around the helix axis could be represented by helical wheel diagrams of the type shown in fig.1A. However, we will show that movements of the side chains of an α-helix may produce structures that are much more amphiphilic than that indicated by the idealized wheel projection (fig.1B). Thus, side-chain flexibility might explain some of the seemingly contradictory experimental results.

2. METHODS

In the analysis of α-helical amphiphilicity, it is usually assumed that the side chains are immobile and regularly spaced. This assumption is embodied in the helical wheel projection, where the side chains are represented as sticks protruding perpendicularly to the helix axis at 100° intervals (fig.1A). The so-called hydrophobic moment [7] is a convenient measure of the amphiphilicity of a peptide; it is defined as:

$$\bar{\mu} = \Sigma h_i \bar{s}_i \quad (1)$$

where h_i is the hydrophobicity of residue i and $\bar{s}_i$ is a unit vector pointing from the helical axis towards the geometric center of side chain i. The sum is taken over a given window (typically 11 or 18 residues long) that is moved successively along the sequence.

In most applications only the absolute value of μ is of interest; this is easily computed as:

$$\mu = \{[\Sigma h_i \sin(i\delta)]^2 + [\Sigma h_i \cos(i\delta)]^2\}^{1/2} \quad (2)$$

where δ, the angle between successive residues of the α-helix, is 100°.

The hydrophobic moment of an idealized helical wheel projection will be referred to as the classical hydrophobic moment. When calculating this property, we use eqn 2 and the consensus hydrophobicity scale of Eisenberg et al. [7] (table 1).

In a real α-helix, the side chains will be free to assume various folded conformations, corresponding to a distorted helical wheel (figs 1B,2). Such movements can result in structures with more hydrophobic sidedness than is indicated by the classical helical wheel diagram. We have tried to develop a model that takes this possibility into consideration.

The range of flexibility for different side chains was estimated by the following method: model α-helices with the sequence $(Ala)_4$-X-$(Ala)_4$, where X designates one of the 20 standard amino acids, were studied using the computer program HYDRA [8] running on an IRIS 3130 graphics workstation (Silicon Graphics, Mountain View, CA). For each amino acid X, the total energy was calculated for a conformation where all the torsion angles of the side chain were set to 180° (fig.2). By varying the torsion angles, we looked for conformations where the side chain was maximally removed from the radial direction and with the total energy not exceeding that of the initial conformation. The 'maximal flexibility' (MF) angles (defined in fig.2) found in this way were taken as measures of the range of flexibility for each side chain X. The values of the MF angles

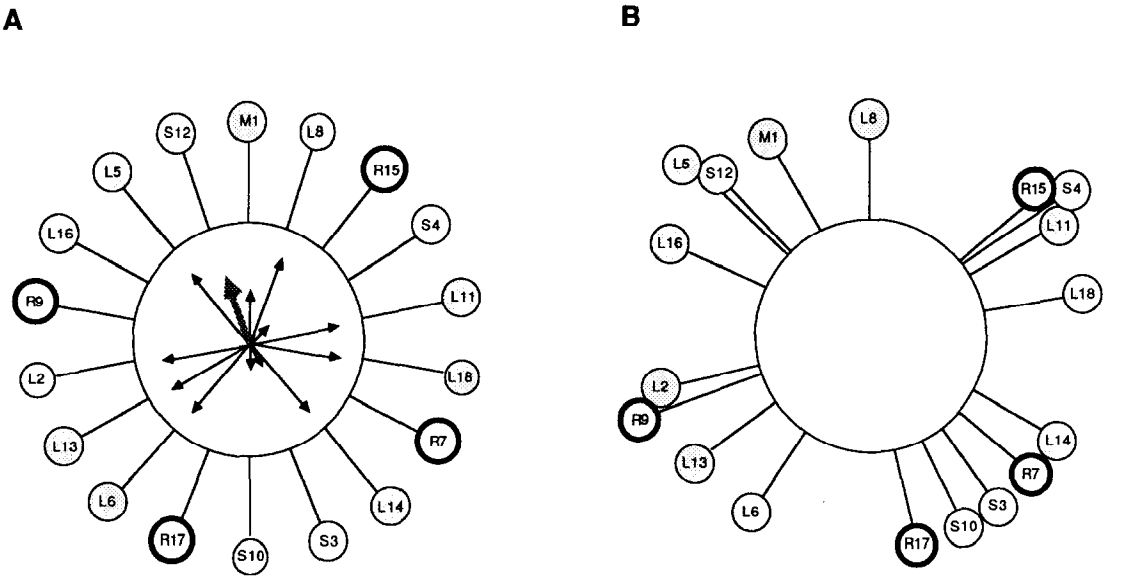

Fig.1. (A) Helical wheel projection of the SynC peptide [5]. The numbers indicate the positions in the sequence. The hydrophobic moment (grey arrow) of the helix is the vector sum of the individual hydrophobicity vectors (the length of the Arg vectors has been put equal to zero). Apolar residues are shaded; arginines are outlined. (B) Modified helical wheel projection of the SynC peptide. The hydrophobic moment of this conformation is $\mu_{mod} = 6.0$, whereas that of the conformation shown in A is $\mu_{class} = 2.6$. Note that all polar residues have moved towards Ser_3 and all apolar residues towards the opposite side of the helix.

Table 1

Hydrophobicity [7] and maximal flexibility angles (ϕ_- and ϕ_+, see text) of the 20 standard amino acids

Amino acid	H_i	Reference atom (defines position of head group)	ϕ_- (°)	ϕ_+ (°)
A, Ala	0.62	C_β	0	0
C, Cys	0.29	S_γ	18	23
D, Asp	−0.9	C_γ	19	20
E, Glu	−0.74	C_δ	25	16
F, Phe	1.19	halfway between C_γ and C_z	30	34
G, Gly	0.48	H	0	0
H, His	−0.4	halfway between $N_{\delta 1}$ and $N_{\epsilon 2}$	33	30
I, Ile	1.38	$C_{\gamma 1}$	22	20
K, Lys	−1.5	N_z	54	30
L, Leu	1.06	C_γ	18	9
M, Met	0.64	S_δ	47	20
N, Asn	−0.78	C_γ	19	19
P, Pro	0.12	–	0	0
Q, Gln	−0.85	C_δ	43	15
R, Arg	−2.53	C_z	56	29
S, Ser	−0.18	O_γ	18	18
T, Thr	−0.05	C_β	0	0
V, Val	1.08	C_β	0	0
W, Trp	0.81	C_ϵ	39	47
Y, Tyr	0.26	C_z	34	32

The ϕ angles are measued from the center of the helical wheel to the reference atom in the side chain as in fig.2

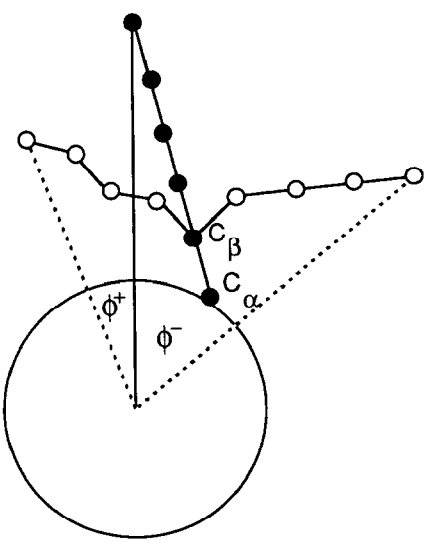

Fig.2. Initial (black) and folded (white) conformations of a model side chain. ϕ_+, positive maximal flexibility angle; ϕ_-, negative maximal flexibility angle. For most residues, $\phi_- > \phi_+$. Note that the movements of long side chains (like Arg and Lys) may encompass rather wide angles.

are listed in table 1. In the actual calculations described below, we only allowed side chains to move out to half of their MF angles, since steric hindrance from neighboring side chains will often be greater in real helices than in our model poly(Ala) helix. This restriction does not lead to any substantial differences in the qualitative results.

An algorithm was developed where the angles determined by the above method were used to estimate how much the amphiphilicity of a helical peptide can be increased by side-chain flexibility. In this algorithm, amino acid sequences were analyzed using an 18-residue moving window as follows. Each of the 18 residues in the window was considered in turn as the 'reference' residue. All other residues in the window were moved an angle equal to one-half of their ϕ_- or ϕ_+ values (table 1) so as to maximize the hydrophobic moment in the direction defined by the reference residue (fig.1B). By repeating this procedure for each residue in the window, we obtained 18 modified conformations. The hydrophobic moment of the most amphiphilic of these conformations will be referred to as the modified hydrophobic moment.

For each amino acid sequence, the segment which yielded the maximal modified hydrophobic moment was identified. The maximal hydrophobicity of the hydrophobic face, H_{max}, was calculated for this segment. This was done by finding those seven neighboring residues that had the highest summed hydrophobicity. The minimum hydrophobicity of the polar face, H_{min}, was calculated in a similar manner.

3. RESULTS AND DISCUSSION

We applied the algorithm described above to 51 mitochondrial targeting sequences collected from the literature. In 45 cases, the maximal modified hydrophobic moment ($\mu_{mod,max}$) and the maximal classical one ($\mu_{class,max}$) were found in windows shifted by less than three residues relative to each other. The mean value of the moments for the whole sample increased from $\mu_{class,max} = 8.5$ to $\mu_{mod,max} = 10.5$ (not shown).

For each of the targeting peptides, the sequence in the window of $\mu_{mod,max}$ was randomly scrambled 10 times. 81% of the scrambled copies had lower modified hydrophobic moments than the original sequence. For the sequences in the $\mu_{class,max}$ windows, 82% of the copies had lower classical hydrophobic moments.

To ascertain whether side-chain flexibility could explain the experimental results referred to in section 1, we performed detailed calculations for the mutant and artificial presequences described in [4–6] (table 2). The results imply that modified amphiphilicity analysis is a more sensitive tool for identifying import-competent sequences than standard helical wheel diagrams or hydrophobic moment calculations. The modified H_{max} value seems to be the best discriminator between active and inactive sequences. The inactive leader peptides in table 2 all have very low $H_{mod,max}$ values, indicating that they are unable to produce a significant hydrophobic face. In contrast, judging from the $H_{mod,max}$ values, all the functional peptides have fairly good hydrophobic faces. Some do not meet the requirements for surface activity proposed in [3] ($\mu_{class} \geq 7.3$ and $H_{class,max} \geq 4.5$) but, with the exception of SynC, they still have reasonably high classical hydrophobic moments. The modified hydrophobic moment analysis of SynC suggests that even this sequence may be amphiphilic in an α-helical conformation (fig.1B), as is observed experimentally [6].

The most important conclusion to be drawn from table 2 is that point mutations of the kind studied in the experiments described above cannot easily perturb the amphiphilic character of a sequence sufficiently to make it non-amphiphilic. Due to the flexibility of the amino acid side chains, α-helices should often be able to assume conformations with much higher amphiphilicity than that indicated by a helical wheel diagram. In particular, for basic residues (Arg, Lys), which have long, flexible side chains, the precise position in the sequence is not very crucial to the amphiphilicity of

Table 2

Hydrophobic moment calculations for mutant and artificial targeting peptides (the segment with maximal μ_{mod} is given)

Sequence	Ref.	Import	Segment	μ		H_{max}		H_{min}	
				Class	Mod.	Class	Mod.	Class	Mod.
CoxIV, wt	[5]	+	3–20	11.8	13.6	5.1	5.1	−10.0	−10.0
SynA2	[5]	+	4–21	8.8	12.2	4.1	4.1	−8.2	−11.8
SynB2	[5]	−	5–22	8.2	11.3	−0.9	−0.9	−11.0	−11.0
SynC	[5]	+	5–22	3.6	6.9	1.8	3.0	−5.4	−8.2
SynD	[5]	−	5–22	6.2	7.8	−0.9	−0.9	−8.0	−8.0
Δ11,12	[6]	+	3–20	5.9	9.0	2.8	4.0	−7.1	−8.4
OTC, WT	[4]	+	14–31	10.0	12.4	4.3	4.3	−8.7	−8.7
Met 21 → Arg	[4]	+	14–31	7.2	10.0	1.9	3.9	−8.7	−8.7
Asn 24 → Arg	[4]	+	14–31	8.7	11.9	3.3	6.2	−8.7	−8.7
Met 21, Asn 24 → Arg	[4]	+	14–31	5.7	9.2	0.2	3.1	−8.7	−8.7
A1	[4]	−	2–19	6.3	11.8	0.1	0.1	−9.1	−11.4
A2	[4]	−	1–18	3.6	10.5	−6.2	−7.0	−13.0	−13.0

CoxIV, presequence of yeast cytochrome oxidase, subunit IV; WT, wild-type sequence; SynA2 and SynC, artificial presequences composed only of leucine, arginine, and serine; SynB2 and SynD, artificial presequences similar to SynA2 and SynC but containing glutamine instead of leucine; Δ11,12, deletion mutant where residues 11 and 12 of the wild-type CoxIV presequence have been removed; OTC, presequence of human ornithine transcarbamylase; Met 21 → Arg, OTC mutant where Met 21 has been replaced with arginine; Asn 24 → Arg, OTC mutant where Asn 24 has been replaced with arginine; Met 21, Asn 24 → Arg, OTC mutant where Met 21 and Asn 24 have been replaced with arginine; A1, artificial presequence composed predominantly of alanine and arginine; A2, artificial presequence composed predominantly of serine and arginine

the peptide. This could explain why the artificial presequence SynC behaves like an amphiphilic α-helix, in spite of the fact that it was designed to have four arginines evenly distributed around the helix axis (fig.1A). Evidently, the effects of point mutations that introduce arginines or lysines on the hydrophobic face of a putative amphiphilic α-helix [4] must be interpreted with caution.

In summary, our analysis suggests that amphiphilicity depends primarily on the overall amino acid composition rather than on the precise sequence. Conceivably, almost any sequence with a minimum number of hydrophobic residues, a couple of arginines, and lacking acidic residues could promote measurable levels of mitochondrial import, provided that it is exposed on the surface of the precursor protein. Indeed, recent studies [9,10] indicate that sequences capable of promoting a measurable degree of import when present at the amino-terminus of a mitochondrial protein lacking its own targeting peptide are found with a high frequency even in essentially random DNA.

Although our analysis implies that peptides of suitable overall amino acid composition may be able to form amphiphilic helices irrespective of the precise amino acid sequence, leader peptides with high classical hydrophobic moments should on average be more efficient as mitochondrial targeting signals. For a peptide with a low $\mu_{class,max}$ the number of amphiphilic conformations will be only a small fraction of all possible conformations and recognition events based on amphiphilicity will take longer and be less efficient. Over evolutionary times, targeting peptides would thus evolve towards structures with a larger number of amphiphilic conformations, i.e. sequences with a high $\mu_{class,max}$.

It has been proposed that non-helical amphiphilic presequences may promote mitochondrial import in some cases [6]. This assumption is supported by the properties of SynA2, a functional artificial leader peptide that by CD measurements contains a great deal of β-sheet structure even in detergent micelles. However, this sequence also has the potential to form a reasonably amphiphilic α-helix (table 2). It may be that a small percentage of the SynA2 leader peptides assume helical, import-competent conformations in the presence of mitochondrial membranes. Import of this population would continually shift the α-β equilibrium and eventually lead to import of a significant fraction of the precursor molecules.

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