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Detection of Likely Transmembrane β-Strand Regions in Sequences of Mitochondrial Pore Proteins Using the Gibbs Sampler

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The mitochondrial channel VDAC is presumed to fold as a β-barrel although the number and identity of transmembrane β-strands in the protein are controversial. Previously, a novel multiple alignment algorithm called the Gibbs sampler was used to detect a residue-frequency motif in sequences of bacterial outer-membrane proteins that corresponds to transmembrane β-strands in bacterial porins of known structure (Neuwald *et al.*, 1995, *Protein Science*, **4**, 1618. In the present study, this bacterial motif has been used to screen sets of mitochondrial membrane protein sequences, with matches occurring in only two classes of proteins: VDACs and the outer-membrane protein import pore (ISP42, MOM38). These results suggest a structural (and perhaps evolutionary) relatedness between the bacterial and mitochondrial pore proteins, with the mitochondrial subsequences that match the bacterial motif corresponding to transmembrane β-strands as in the porins.

INTRODUCTION

VDAC (voltage-dependent, anion-selective channel) is a 30-KDa protein in the mitochondrial outer membrane that forms a large-conductance, voltage-gated channel *in vitro* (Colombini, 1979; Mannella *et al.*, 1992). This channel is generally considered to represent the main pathway for passive diffusion of ions and metabolites across the mitochondrial outer membrane. VDAC is often referred to as a mitochondrial "porin," because several of its physical and permeability properties are similar to those of this class of bacterial outer-membrane pores. The structures of several bacterial porins have been determined by x-ray crystallography to be 16- or 18-strand antiparallel β-barrels (Weiss and Schulz, 1992; Cowan *et al.*, 1992; Schirmer *et al.*, 1995). Recent studies from this laboratory (Shao *et al.*, 1994; Shao, 1994) indicate that the circular dichroism spectra of fungal VDAC in detergent micelles and liposomes are similar to those of bacterial porins, suggesting that the mitochondrial channel may also be a β-barrel. Molecular envelopes provided by electron crystallography of membrane crystals of fungal VDAC (Mannella *et al.*, 1989; Guo *et al.*, 1995) are consistent with a β-barrel having a diameter of 3.8 nm at the Cα backbone, somewhat wider than that of bacterial porins. The number of transmembrane β-strands needed to form a lumen of this size may vary from 13 to 23, depending on the tilt of the individual strands (Mannella *et al.*, 1992).

In order to understand properties like selectivity and voltage-gating, it is important to know which regions in the VDAC polypeptide correspond to transmembrane segments and which to external loops. Considerable structural information about VDAC is being provided by immunotopological studies (De Pinto *et al.*, 1991; Stanley *et al.*, 1995) and by electrophysio-

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logical studies of genetically engineered mutant channels (Blachly-Dyson *et al.*, 1990; Peng *et al.*, 1992). Most modelling efforts have employed amphipathicity analysis as the primary method to deduce the locations of transmembrane β-strands (Blachly-Dyson *et al.*, 1990; De Pinto *et al.*, 1991; Rauch and Moran, 1994). This approach entails searching for regions within VDAC sequences having patterns of alternating polar–nonpolar residues, which are presumed to correspond to β-strands with residues pointing into the aqueous lumen and into the lipid phase, respectively. This kind of analysis has drawbacks, in particular, the absence of quantitative criteria for deciding on "hits" vs. "misses," causing different groups to draw disparate conclusions about the number and location of β-strands in VDAC (Fig. 1 below). Also, amphipathicity analysis does not take advantage of information that might be provided by the class of pores with known β-barrel structure, namely, the bacterial porins.

Previous attempts to model VDAC by sequence comparison with bacterial porins have been deterred by the low sequence homology between the two classes of pore proteins (e.g., Forte *et al.*, 1987). Recently, a new algorithm called the Gibbs sampler has been developed which can detect regions of subtle local similarity in sets of weakly related sequences (Lawrence *et al.*, 1993). In this report, we describe results obtained when the Gibbs sampler is used to screen mitochondrial outer-membrane protein sequences with a residue motif that corresponds to transmembrane β-strands in bacterial porins.

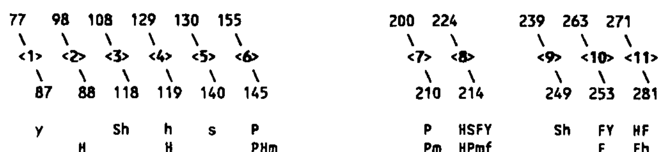
METHODS

The problem in local multiple sequence alignment is to find and align in a collection of sequences those subsequences which are related, i.e., which share a common pattern. The Gibbs sampling algorithm for multiple sequence alignment is a Bayesian procedure which iterates between two objects: the alignment and the residue frequency model which describes the common pattern. The reader is referred to recent papers by Lawrence, Liu, Neuwald and co-workers (Lawrence *et al.*, 1993; Neuwald *et al.*, 1995; Liu *et al.*, 1995) for detailed mathematical descriptions of the method. In essence, the algorithm works as follows.

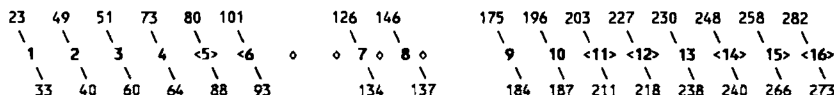
Given a set of N protein sequences, select at random a set of M subsequences of some specified residue length, L. M is an initial guess of the number of such elements to be found in the sequence set. From the M randomly selected subsequences, one calculates the probability of each of the 20 amino acids occurring at each of the L residue positions. This is the initial probability model for the sequence motif, the "site model." (When $p > 1$ motifs are expected to occur in the sequences, then p motif models are initiated in this manner.) A "non-site model" is also calculated which is the overall probability of each of the 20 amino acids occurring at positions in the sequences that do not contain the motif. Next, a subsequence of length L is arbitrarily selected and the residue counts of this segment are removed from the model from which it was previously a member. The probability that this subsequence belongs to each model (site and non-site) is calculated, and the ratio of the probability of membership in the site model to that of the non-site model is calculated. This ratio is normalized by the ratio of sites to non-sites currently in the sequences. Next an "electronic coin" for which the probability of heads to tails is equal to the normalized model membership ratio is flipped. If the coin turns up heads, the subsequence is added to the site model, or if tails to the non-site model, and the models are updated. Additional subsequence are randomly selected as this process is iterated. Once a few "correct" subsequences are selected by chance, the model becomes biased (albeit slightly) toward the correct solution and convergence takes off exponentially (see Fig. 1 in Lawrence *et al.*, 1993). A nonparametric statistical test is employed to determine if the final convergent model is significant (Neuwald *et al.*, 1995; Liu *et al.*, 1995).

A motif model derived from one set of sequences using the Gibbs sampler can be used to screen a different set using a procedure described by Neuwald *et al.* (1995). Individual subsequences are scored in terms of the probability that their observed degree of agreement with the motif might occur in the particular sequence by chance alone. (In Table II, only subsequences for which this probability is less than or equal to 0.5 are shown.) Similarly, each sequence is scored in terms of the probability that its observed subsequence matches might occur by chance in the sequence set (where the observed number of motifs is optimally determined). In the present study, a residue frequency model derived from application of the Gibbs sampler to bacterial protein sequences was used to screen sequences of mitochondrial outer-membrane proteins. Sequences were obtained from SWISS-PROT and GenBank/Genpept databases, and the program PURGE (Neuwald *et al.*, 1995) was used to remove closely related sequences from the analyses.

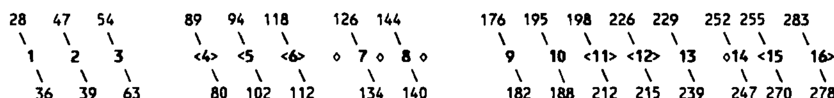
GIBBS SAMPLER



MODEL A



MODEL B



MODEL C

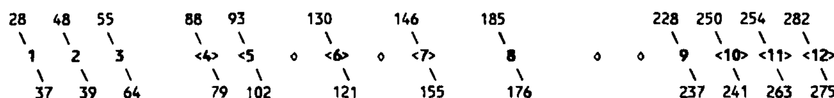


Fig. 1. Comparison of Gibbs sampler results with existing folding models for VDAC; Model A (De Pinto *et al.*, 1991), mammalian VDAC; Models B (Rauch and Moran, 1994), and C (Blachly-Dyson *et al.*, 1990), yeast VDAC. Putative transmembrane β-strands are indicated by diagonal lines connecting two residue positions (nonbold numbers) and labeled by bold numbers, which are inside diamond brackets in the Gibbs diagram. [Residues are numbered according to the yeast sequence using the alignment schemes of Troll *et al.* (1992) and Heins *et al.* (1994).] Strands in Models A–C that correspond closely to those found by the Gibbs sampler are also enclosed in diamond brackets, with partial matches indicated by a single bracket. Empty diamonds in Models A–C indicate positions of β-strands predicted by the Gibbs sampler and not present in the models. The two rows of letters below the strands in the Gibbs sampler diagram indicate the sequences in which a match was found for that segment with a particular bacterial residue frequency model for two different motif scans. The top row corresponds to the results of Table II, using the motif in Table I. The bottom row corresponds to matches found with a closely related bacterial protein motif, not shown. The eleven β-strands correspond to ten found in the first scan plus a unique strand, ⟨2⟩, found in the second scan. Abbreviations: H, human; Y, yeast; F, fungal; S, slime mold; M, maize; P, garden pea. Upper case letters refer to matches with E value less than 0.25, lower case letters indicate E valves between 0.50 and 0.25 (see footnote *c*, Table II).

RESULTS AND DISCUSSION

Application of the Gibbs Sampler to Bacterial Protein Sequences

In a recent study, the Gibbs sampler was applied to sets of sequences of bacterial membrane proteins (Neuwald *et al.*, 1995). By specifying a motif length L of 11 (the number of residues needed for β-strands in porins to span the lipid bilayer), a motif was found that occurs repeatedly in numerous bacterial outer-membrane proteins. For porins of known structure included in the sequence set, the polypeptide segments that match the motif correspond to transmembrane strands in the β-barrels that form the lumen of these pores. In particular, the matches occur in the part of the lumen in contact with lipid and not that at the protein–protein interface within the porin trimer.

Table I is the probability model derived from an analysis of a set of 32 sequences of bacterial outer-membrane transport proteins. This 11-residue motif is subtle but has several recognizable features. There is a general pattern of alternating polar residues (even-numbered positions) and nonpolar residues (odd-numbered positions) from positions 2 to 11. This amphipathicity is expected for β-strands at a water–lipid

Table I. Residue Frequency Motif[a]

POS	C	G	A	S	T	N	D	E	Q	K	R	H	W	Y	F	V	I	L	M	P
1	.	.	.	.	.	15	21	.	.	14	11	.	6	.	.	.	.	.	.	.
2	.	.	13	.	10	15	.	.	9	.	8	4	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	5	16	15	11	14	.	.	.	6
4	.	14	.	.	13	.	.	.	.	.	.	5	.	13	.	.	.	.	.	.
5	.	.	13	.	.	.	.	.	.	.	.	.	.	.	15	22	.	24	.	.
6	.	28	.	21	.	.	.	.	.	.	15	.	.	.	.	.	.	.	.	.
7	.	.	17	.	10	.	.	.	.	.	.	.	.	.	.	21	.	30	.	6
8	.	37	.	.	.	15	10	.	.	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	.	.	.	.	.	.	.	.	27	8	22	.	11	5	.
10	.	.	.	.	.	.	13	.	.	10	20	.	.	.	.	.	.	.	.	.
11	.	.	.	.	.	.	.	.	.	.	.	.	.	46	27	.	.	.	.	.

[a] Numbers under amino acid symbols correspond to 100× frequency of that amino acid occurring at the position (POS) indicated in left-hand colujmn. For clarity, values are shown only for those amino acids with elevated frequencies of occurring at each position.

interface. However, this motif also has several more specific features:

(1) Charged residues occur at highest frequency at the ends of the segments, i.e., positions 1 (46%) and 10 (43%).

(2) Bulky aromatics tend to occur at the ends of the segments, most frequently at position 11 (73%), and at lower frequency at two other odd-numbered (nonpolar) positions, 3 (42%) and 9 (35%).

(3) Glycines are common at the middle of the segments at even-numbered (polar) positions, e.g., 37% at position 8 and 28% at position 6.

The first two tendencies have been noted in analyses of the atomic structures of porins solved by x-ray crystallography (Weiss and Schulz, 1992; Cowan *et al.*, 1992). Charged residues at the ends of the strands may interact with phospholipid headgroups, and the bulky, polarizable aromatic rings might be energetically favored to reside at the hydrophopic–hydrophilic interface, where they might serve to anchor the strands in the bilayer. The third tendency has not been previously described and its significance is unclear. It is possible that having glycines at these middle, lumen-facing positions in the transmembrane strands may serve to maximize the internal volume of these passive diffusion pores.

Application of the Bacterial Model to Mitochondrial Protein Sequences

The 11-residue bacterial residue frequency model represented in Table I was used to scan various sets of sequences of mitochondrial membrane proteins. *Significant matches occurred with the bacterial motif in only two classes of mitochondrial proteins:* the outer-membrane voltage-gated channel, VDAC (Colombini, 1979), and the outer-membrane protein import pore, MOM38 or ISP42 (Baker and Schatz, 1991). Examples of segments of these proteins from different species that were found to match the bacterial motif are summarized in Table II. These results provide evidence for a previously undetected relatedness among the sequences of bacterial porins, VDACs, and the mitochondrial protein import pore. Also, since the motif used to screen the mitochondrial sequences corresponds to a particular structural element in porins of known structure, i.e., transmembrane β-strands in the lipid bilayer tilted about 30° with respect to the cylinder axis, matching segments in the mitochondrial proteins are likely to have a similar structure.

In five VDAC sequences included in one analysis, seventeen 11-residue segments were found to match the bacterial motif (Table II). Comparison of the locations of these segments in the different sequences indicates that there are only 10 different or unique subsequences among these 17 matches (Fig. 1). The above analysis was repeated several times on somewhat different sets of mitochondrial protein sequences, using residue frequency models derived from application of the Gibbs sampler to sets containing various combinations of bacterial (and sometimes mitochondrial) outer-membrane proteins. In these analyses, the same 10 VDAC subsequences were consistently detected, along with a few others (generally at low significance levels). For example, in another scan using a slightly different motif and mitochondrial sequence

Table II. Subsequences of Mitochondrial Outer-Membrane Proteins That Match the Bacterial Motif

Sequence[a]	E^b	Subsequence												E^c
Human VDAC	0.0004													
		270–280:	G	G	H	K	L	G	L	G	L	E	F	0.03
		214–224:	S	N	T	R	F	G	I	A	A	K	Y	0.06
		120–130:	E	H	I	N	L	G	C	D	M	D	F	0.40
		109–119:	K	N	A	K	I	K	T	G	Y	K	R	0.50
		238–248:	N	S	S	L	I	G	L	G	Y	T	Q	0.50
Slime mold VDAC	0.001													
		208–218:	K	A	P	S	F	N	V	G	T	Q	Y	0.02
		232–242:	N	N	R	K	V	N	I	S	Y	I	Y	0.06
		100–110:	S	K	Q	N	F	S	T	E	F	Q	Y	0.13
		123–133:	N	N	K	S	F	N	T	S	L	A	F	0.20
Fungal VDAC	0.005													
		253–263:	E	G	V	T	L	G	V	G	A	S	F	0.01
		215–225:	N	T	V	G	L	E	V	A	T	K	Y	0.05
		271–281:	A	T	H	K	V	G	T	S	F	T	F	0.13
Plant VDAC	0.23													
		138–148:	N	A	L	A	F	G	A	D	I	S	F	0.05
		194–204:	S	N	T	A	V	G	V	D	I	S	H	0.32
Yeast VDAC	0.23													
		253–263:	P	G	V	T	L	G	V	G	S	S	F	0.08
		215–225:	S	N	V	N	I	E	F	A	T	R	Y	0.30
		77–87:	N	T	N	N	L	Q	T	K	L	E	F	0.50
Yeast protein import pore	0.07													
		299–309:	G	S	T	T	I	G	A	K	Y	E	Y	0.01
		349–359:	N	D	T	K	I	G	C	G	L	Q	F	0.06
		133–143:	N	D	L	S	V	S	G	R	L	N	Y	0.32

[a] The first five sequences were included in one scan of mitochondrial outer-membrane proteins using the bacterial motif of Table I. The last sequence was included in a different scan using the same motif but a slightly different subset of mitochondrial proteins. The SwissProt or GenBank identifiers of the proteins in the table are, respectively:

PORI_HUMAN, PORI_DICDI, PORI_NEUCRA, S36454, PORI_YEAST, ISP42_YEAST.

[b] The number of sequences in the scanned set expected to show the observed matches to the motif by chance alone.

[c] The number of subsequence matches this good expected to occur in the particular sequence by chance alone.

set, seven of the eight unique subsequences found were among the 10 picked up in the first scan. Figure 1 shows the 11 unique matching sequences detected in the two scans combined.

In general, significant matches with the bacterial motif fall in two regions of the VDAC sequence, between residues 77 and 155 and between residues 200 and 281 (Fig. 1), suggesting that these two regions may form continuous sections of antiparallel β-sheet in a porin-like β-barrel. The number of residues between neighboring segments is usually small (5 or less), consistent with turns or short loops between adjacent strands in an antiparallel β-sheet. In a few cases, there are no intervening residues between adjacent segments, which appears to violate standard β-sheet geometry since at least two residues are needed for a turn. These discrepancies might be explained by one of the segments in question being one residue shorter than the neighboring segment in the turn region.

The lack of significant matches with the bacterial motif in regions of the VDAC sequence outside residues 77–155 and 200–281 is not necessarily evidence for the absence of β-strand structure from these regions, since the Gibbs sampler typically finds fewer than half of the β-strands in sequences of bacterial porins of known structure (Neuwald *et al.*, 1995). The amino-terminal quarter of bacterial porin sequences generally do not match the motif, which is understandable since this region contains β-strands tilted at about 60° (instead of 30°) with respect to the long axis of the β-barrel and are involved in forming contacts with protein (instead of lipid). The absence of motif matches in the amino-terminal regions of VDAC sequences suggests that the mitochondrial pore may be structur-

ally analogous to bacterial porin in this region. Preliminary attempts to find a second motif that might correspond to another class of β-strands in the bacterial and mitochondrial sequences have failed.

It should be noted that screening of several other membrane protein sequences with the 11-residue bacterial outer-membrane protein motif did not yield matches like those obtained with VDAC. For example, significant matches were not obtained with other mitochondrial proteins (e.g., MOM70, ISP6, MAS6, MIM44), with the mammalian sodium channel, or with the glucose transporter, which has been hypothesized to be a β-barrel (Fischbarg *et al.*, 1993).

Comparisons of Results of Gibbs Sampler with Previous Models for VDAC

Several models for folding VDAC into an antiparallel β-barrel have been proposed, three of which are shown in Fig. 1. While specifics vary, the β-strands in these models were generally identified by "amphipathicity" analyses, i.e., by finding patterns of alternating polar–nonpolar residues. Final assignments have been modified based on other data, such as effects of site-directed mutations on ion selectivity (Blachly-Dyson *et al.*, 1990) and accessibility of regions of the protein to antibodies or proteases (De Pinto *et al.*, 1991). As can be seen in the figure, there is considerable disagreement among the three models. Models A and B differ in the assignment of three of their 16 putative β-strands, and both models disagree significantly with Model C, which posits only 12 β-strands. Model C agrees best with Model B in the amino-terminal part of the molecule, having five β-strands in common between residues 28 and 102, while it most closely matches Model A in the location of four β-strands between residue 237 and the carboxy terminus.

Since amphipathicity is a characteristic of the residue frequency model found by the Gibbs sampler, it is not surprising that several of the VDAC subsequences that match the motif also are identified as β-strands in Models A–C. If anything, it is surprising how few of the β-strands predicted by each model agree with the 11 sampler-defined segments: seven strands in each of the Models A, B, and C. Particularly noteworthy are segments which have significant matches in multiple VDAC sequences in the Gibbs sampler analyses and which are not designated as β-strands in one or more of the models (Fig. 1). These include segment 108–118, not designated a β-strand in Models A and C; 145–155, not designated a β-strand in Models A and B; and 200–210, not designated a β-strand in Model C. Perhaps most surprising is that segment 214–224 is not designated as a β-strand in Model C; based on the number and quality of matches found by the Gibbs sampler, it is the segment in VDAC most likely to fold as a porin-like β-strand. Each of these four segments, in fact, has detectable amphipathic character and has been excluded as a β-strand in one or more of the models for other reasons. Given the fact that the segments in bacterial porins of known structure that match the 11-residue pattern correspond, without exception, to transmembrane β-strands, the reasons for not designating these four segments as such in existing VDAC models warrant reexamination. Two such rationale are accessibility of these segments of the protein to proteases and antibodies (unexpected for intramembrane regions), and failure of charge-altering mutations in these segments to detectably change the ion selectivity of the channel (as expected for segments forming part of the lumen).

Accessibility: There is recent evidence that some consensus β-strand regions of the VDAC protein (such as the carboxy-terminal segment, see Fig. 1) can react with antibodies at the mitochondrial outer-membrane surface (Stanley *et al.*, 1995). This may be explained by a mechanism proposed for VDAC closure that involves removal of several β-strands from its lumen wall (Peng *et al.*, 1992). Since the free-energy difference between open and closed states of the channel is small (Colombini, 1989), both channel conformers may be in dynamic equilibrium in the outer membrane. Thus, antibodies (and proteases) may react with segments of the VDAC protein which are accessible in closed states but which are an integral part of the β-barrel in the fully open state. Sorting out this topology will require experimentation on VDAC locked into particular conformations, e.g., by lattice forces (in crystals) or by voltage clamping (in bilayers).

Charge-altering mutations: There are two ways that mutations in a transmembrane segment of the channel might not result in detectable changes in ion selectivity. The first is suggested by the structure of bacterial porins, which contain one or more loops that fold back inside the lumen (Weiss and Schulz, 1992; Cowan *et al.*, 1992; Schirmer *et al.*, 1995). Residues in the β-barrel covered by such loops would have little or no effect on ion selectivity. Also, electron crystallography of fungal VDAC suggests that the lumen has an irregular shape (Guo *et al.*, 1995; Mannella, 1994). Residues in narrower parts of the lumen

would have larger effects on selectivity than those in wider parts, so mutations in the latter might result in small or undetectable changes in channel properties.

CONCLUSIONS

Analysis of sequences of bacterial and mitochondrial membrane proteins with the Gibbs sampler indicates that VDAC and the mitochondrial protein import pore are related structurally (and perhaps evolutionarily) to bacterial porins. An 11-residue pattern that corresponds to transmembrane β-strands in bacterial porins has significant matches at numerous locations in VDAC sequences, suggesting that these segments are also transmembrane β-strands. These results support a β-barrel structure for VDAC, and should help to guide future experiments aimed at establishing the identity of the transmembrane segments in this pore protein.

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