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Folding patterns of porin and bacteriorhodopsin

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Porin spans the outer membrane of *Escherichia coli* with most of the protein embedded within the membrane. It lacks pronounced hydrophobic domains and consists predominantly of β-pleated sheet. These observations require the acommodation of polar and ionizable residues in an environment that has a low dielectric constant. Owing to a currently limited understanding of the constraints governing membrane protein structure, a minimal approach to structure prediction is proposed that identifies segments causing polypeptides to reverse their direction (turn identification). The application of this procedure avoids hydrophobicity parameters and yields a model of porin which is in good agreement with all experimental data available. The presence of polar and ionizable residues within membrane boundaries implies a dense (saturating) network of hydrogen bond donor and acceptor groups. Application to a paradigm of hydrophobic membrane proteins, bacteriorhodopsin, reveals a pattern consistent with its α-helical folding. The postulated structure includes significantly more polar residues in the membrane domain than have been assumed previously, suggesting that there are also hydrogen bonding networks in bacteriorhodopsin. Extensive networks permeating protein interior and surfaces would explain the extraordinary stability and the tight interactions between functional units in the formation of crystalline arrays of both proteins.

Key words: hydrophobicity/polarity/secondary structure prediction/transmembrane proteins/turn identification

Introduction

Bacteriorhodopsin, which spans the purple membrane of *Halobacterium halobium*, is a light-activated proton pump (Stoeckenius and Bogomolni, 1982). It is considered to be a paradigm of transmembrane proteins. Its structure consists of seven membrane-spanning α-helical rods, none of which protrudes much beyond membrane boundaries (Henderson and Unwin, 1975). An initial structural model including evidence from limited proteolysis and chemical modifications (Engelman *et al.*, 1980) proposed that the membrane-embedded domain contains mostly hydrophobic residues. Methods relying on hydrophobicity parameters to predict structure have yielded similar models (Kyte and Doolittle, 1982; Engelman *et al.*, 1982).

Porin, which spans outer membranes of *Escherichia coli*, forms voltage-dependent transmembrane channels (Schindler and Rosenbusch, 1978). It resembles bacteriorhodopsin in that it spans the membrane in compact form, with very small domains bulging into the aqueous phase. It differs, however, in that it is very polar (Rosenbusch, 1974), does not contain sizeable hydrophobic domains (Chen *et al.*, 1979) and consists predominantly of β-structures (Rosenbusch, 1974; Schindler and Rosenbusch, 1984). About two thirds of its polypeptide backbone is arranged in antiparallel β-pleated sheet configuration in an orientation approximately normal to the membrane plane, with an average strand length of 10–12 residues (Kleffel *et al.*, accompanying paper). Although this is sufficient for an extended polypeptide to span the hydrophobic membrane core, the consistently polar character of any segment of this length in the sequence of this protein challenges current notions of membrane protein structure and raises the question of how such a molecule could reside in a hydrophobic environment. Of course, this problem only exists if porin does indeed span the membrane. The evidence regarding this contention is as follows.

Firstly, voltage-gated ion-conducting channels are formed in lipid bilayers by this protein (Schindler and Rosenbusch, 1978, 1981). The channels are unlikely to be due to contaminants since various independent extraction and solubilization procedures (Rosenbusch *et al.*, 1982) yielded indistinguishable results. Even after extreme dilution of highly purified protein solutions (1 mol porin trimer/10^9 mol phospholipids), channel recoveries were high (50–80% of the expected conductance). These results are consistent with three channels/trimer. Low mol. wt. contaminants have not been observed under either native or denaturing conditions. Channel formation cannot be attributed to the peculiar environment in which porin resides in the native outer membrane (Nikaido and Nakae, 1979), since conductance measurements with either unextracted outer membranes or reconstituted homogeneous trimers incorporated into typical phospholipid bilayers (Schindler and Rosenbusch, 1981) yielded indistinguishable results. Secondly, 3-dimensional image reconstruction from electron micrographs of porin reconstituted into crystalline sheets revealed three stain-filled structures which pass through each porin trimer (Dorset *et al.*, 1983, 1984). Among the 2-dimensional crystalline lattices studied were two hexagonal crystal forms that differed in lattice constants (77 and 93 Å) but did not exhibit detectable differences in morphology of their triplet channels. The distinctly larger areas between channels in the 93 Å unit cells reflect a 5-fold higher phospholipid content of this large lattice form and suggest that protein and lipids form a single layer. This is supported by phospholipase treatment, which results in the conversion of the large crystal form to the small crystal form (Regenass *et al.*, 1985). Estimates of membrane thickness (45–55 Å) from electron microscopy (Steven *et al.*, 1977) and X-ray crystallography (Garavito *et al.*, 1983) provide evidence that porin, like bacteriorhodopsin, protrudes little on either side of a single membrane layer. Thirdly, studies of detergent binding to solubilized porin by conventional quantitative binding assays and by small-angle neutron scattering experiments showed that trimers bind detergents in amounts and with a geometry that are consistent with a hydrophobic transmembrane zone covered by a single detergent layer (Grabo, 1982; Zulauf and Rosenbusch, 1982). Finally, porin has a receptor site for bacteriophages

at the external face of the membrane (Chen *et al.*, 1979), whereas at the interior face it appears to interact very tightly with the peptidoglycan (Rosenbusch, 1974).

Thus, the question as to how polar amino acids could be accommodated within a hydrophobic membrane domain does appear to be relevant. As expected, application of structure predictions relying on hydrophobicity (Argos *et al.*, 1982; Kyte and Doolittle, 1982, and references therein) to porin or related outer membrane proteins have led to ambiguous results (Overbeeke *et al.*, 1983; Charbit *et al.*, 1984). An independent approach, consisting of the prediction of secondary structure (Chou and Fasman, 1978, 1979; Garnier *et al.*, 1978) did not provide convincing results either (Inokuchi *et al.*, 1982; Charbit *et al.*, 1984) since the location of polar residues within a bilayer was neglected. Yet, this approach remains the only practicable one to probe the folding of polar membrane proteins and we have, therefore, reassessed the assumptions underlying it. Reducing them to a minimum, we propose a folding pattern for porin which seems consistent with the results of structural studies. The application of this conceptual approach to bacteriorhodopsin should be a critical test of its validity. The folding pattern obtained is compatible with what is known about the structure of this protein.

Results and Discussion

Polarity, hydrophobicity and hydrogen bond formation

In porin many of the ionizable and polar residues within membrane boundaries may be located in pores and would thus be accessible to the aqueous phase. Indeed, over two thirds of the 19 amino groups per monomer, and approximately one half (22 residues) of the carboxyl groups react with small, polar, pore-permeant probes, but not with large hydrophobic reagents (Tokunaga *et al.*, 1981; Schindler and Rosenbusch, 1982, and unpublished results). In the present context, the most pertinent aspect of these results is the complementary conclusion that substantial numbers (5–6 amino groups, >20 carboxyl residues and several guanidinium groups) do not react unless the protein is unfolded. Together with highly abnormal apparent pK values of conformational transitions (Schindler and Rosenbusch, 1984), this suggests that a sizeable number of ionizable residues may be buried in the membrane interior. Substantial shifts of pK values may be expected, since such anomalies have been shown previously to exist in hydrophobic pockets of soluble proteins (Parson and Raftery, 1972).

Considering the evidence on electrostatic and hydrogen bonding interactions in soluble, globular proteins (Thornton, 1982; Rashin and Honig, 1984), in membrane proteins (Engelman, 1982; Kyte and Doolittle, 1982) and in model compounds (Kristof and Zundel, 1980), it appears plausible that polar residues can exist in environments that have low dielectric constants if extensive networks of hydrogen bonds are formed (Baker and Hubbard, 1984). Such bonds, which would be particularly strong in a medium with a low dielectric constant, may exist between potentially ionizable residues (present as neutral species because of their local environment) and residues such as tyrosine, asparagine, and other hydrogen bond accepting and donating groups. Alternatively, ion pairs (Honig and Hubbell, 1984) may be present, particularly if they are stabilized by supplementary hydrogen bonds to other residues (Rashin and Honig, 1984). All these configurations would cause substantially decreased polarity (Finney, 1978), although they would be accompanied by considerable dipole moments. These are likely to be of the same order of magnitude as those of hydrogen bondings of peptide bridges in membrane-traversing backbone segments (Tanford, 1973; Henderson, 1975; Kennedy, 1978), or as those described between polar side-chain residues (Tanford, 1973; Chothia, 1974). In a dense network, such dipole moments may compensate each other to some extent and thus minimize dielectric inhomogeneities (Paul, 1982). Although a critical test of this hypothesis will obviously require high resolution structural data, questioning the general validity of prediction methods which scan primary sequences linearly in search of hydrophobic stretches, while ignoring the local environments of polar and ionizable residues in the tertiary structure of a protein, appears timely.

Turn identification

Conventional methods of secondary structure predictions have yielded ambiguous results for both hydrophobic and polar membrane proteins (Khorana *et al.*, 1979; Inokuchi *et al.*, 1982; Charbit *et al.*, 1984). Re-examination of the basic assumptions raised two questions. The first concerns the validity of using conformational parameters derived from soluble proteins and is widely recognized. It has been partially circumvented by focussing on polypeptide chain reversals (Khorana *et al.*, 1979; Argos *et al.*, 1982), since turns are the regions most likely to be exposed to aqueous environments (Kuntz, 1972; Rose *et al.*, 1983) in both types of proteins. The second problem concerns the length of a segment required to reverse the direction of a polypeptide. In soluble proteins it appears that tetrapeptides are encountered most frequently (Crawford *et al.*, 1973). Since a systematic test of the effect of segment lengths on computational prediction neither favored nor disfavored tetrapeptides, they were selected for automated computer searches (Chou and Fasman, 1979). However, shorter turns (1–3 turns, or γ-turns) have been predicted (Nemethy and Printz, 1972) and observed in synthetic oligopeptides (Madison *et al.*, 1974; Pease and Watson, 1978; Thompson and Gierasch, 1984) and in soluble proteins (Mathews, 1972). Although their frequency in the latter is low, the constraints existing in membrane proteins may be significantly different. Further, backbone angles of just two peptide bonds appear to be sufficient to define reverse turn conformation (Robson and Pain, 1974; Engelman and Steitz, 1981). We therefore consider segments of three residues to be sufficient for chain reversal, without excluding more open turns or turn clusters.

Which residues are involved in turn formation? We adopted the concept that residues failing to stabilize hydrogen bonding between peptide bridges (Kossiakoff, 1982, and unpublished results) would contribute to turn formation. Such residues would include glycine (which fails to protect such bonds from solvent interaction), proline (which does not contribute a hydrogen) and asparagine, aspartic acid and serine (whose side-chains can readily form hydrogen bonds to the backbone themselves). These five residues coincide with those observed most frequently in turn configurations in soluble proteins, where they surpass all other residues with respect to high and most unequivocal turn propensities (Levitt, 1978; Chou and Fasman, 1978). Therefore, they may also be regarded as turn promoting in membrane proteins. The remaining amino acids have been assigned tentatively to one of two classes. The first comprises residues occurring at low frequency in turns (Chou and Fasman, 1978). These residues are defined as turn blocking. The second class comprises amino acids that appear intermediate on all counts and are therefore considered to be indifferent towards turns. Turns may now be identified as segments ≥ 3 residues, of which at least one is turn promoting and none turn blocking. Turn clusters are defined as groups of turns linked by segments <6 residues long (see below). Segments between turns (or turn clusters) are considered to be arranged

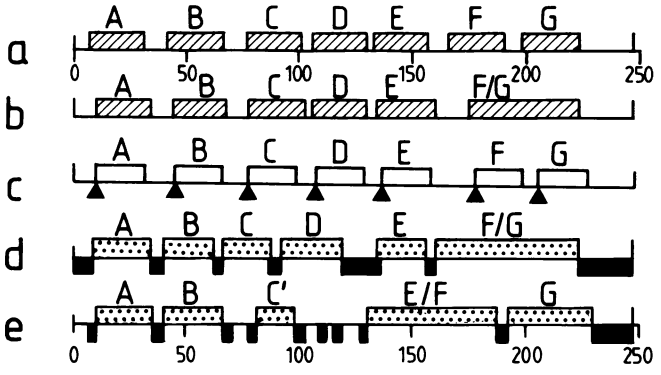

Fig. 1. Folding of bacteriorhodopsin. (**a**) Adaptation of a model based on various criteria (Engelman *et al.*, 1980). The seven helical segments (Henderson and Unwin, 1975) are indicated by hatched bars. Similarly derived models exhibit quantitative differences, but maintain the same pattern. (**b**) Prediction adapted from the hydropathy profile (Kyte and Doolittle, 1982). Hydrophobic segments are indicated as above. (**c**) Adaptation of a prediction based on computing transfer free energies of constituent amino acyl residues (Engelman *et al.*, 1982). Arrows indicate helix starts. Empty bars represent the standard size segment of 21 residues assumed in that analysis. (**d**) Turn identification proposed in this report. Turns and turn clusters are indicated as solid bars below the line, while the intervening segments are shown as dotted bars above it. (**e**) Adapted from the computer prediction (Chou and Fasman, 1979) of bacteriorhodopsin according to Khorana *et al.* (1979). Turns and segments are shown as in **d**. The numbers on the abscissae show residues 1 – 248 according to the sequence of Khorana *et al.* (1979).

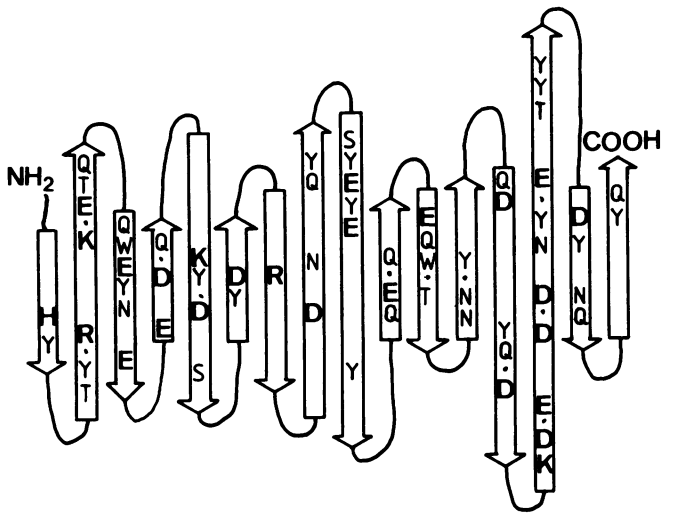

Fig. 2. The array of β-strands in porin located on the basis of the turn identification proposed here. Presumed periodically arranged segments (11 ± 5 residues long) are shown as arrows corresponding to β-pleated sheet. The structure is less regular than that of bacteriorhodopsin. The linker regions containing turns or turn clusters are drawn purely schematically. They contain segments of 3 – 20 residues. In the β-strands shown, ionizable residues and other hydrogen bonding acceptor or donor groups are indicated in one-letter code. Dots represent single non-hydrogen bonding residues between polar ones. Empty spaces indicate hydrophobic stretches. Due to the long loop segments, assignments of parallel or anti-parallel strands are not possible.

in periodic arrays: if their length exceeds 18 residues, they could traverse the membrane core in α-helical configuration whereas strands ≥ 6 residues are potentially in β-configuration. This very low figure accounts for membrane-spanning segments within the protein domain, and for potential variation of membrane thickness (Mouritsen and Bloom, 1984). In porin in particular, shorter chains may be contiguous to its tilted channels (Dorset *et al.*, 1984). For the manual application of turn identification see Materials and methods.

Turn identification applied to bacteriorhodopsin and porin

At present, combined knowledge of sequence and structure in membrane proteins is restricted to bacteriorhodopsin. It therefore constitutes an obligatory first test for any procedure aimed at an understanding of the folding of membrane proteins. A comparison of the turn identification procedures with various other methods is shown in Figure 1. The prediction procedures that have been applied to this protein rely on hydropathy profile and transfer free energies (panels b and c). They yield models which are similar to that (panel a) of Engelman *et al.* (1980). Evaluating the results obtained with the standard automated computer prediction (panel e) also reveals several segments long enough to span membranes as α-helices, but turns appear both over- and underestimated. Overall, the turn identification procedure (panel d) is in agreement with the first three models. It recognizes six helical domains (Rosenbusch, unpublished) of which the last long segment is likely to contain the helices F and G of Engelman *et al.* (1980). A similar conclusion was drawn from the hydropathy profile (panel b). The sites of onset of helices A, B, and E are consistently predicted by all methods, while those of the onset of helices C, D, F and G appear more ambiguous (Argos *et al.*, 1982). The most consistent differences between the present turn identification and other methods are located in the regions of helices C and D of the model of Engelman *et al.* (1980). This is due to two apparently chain-reversing tripeptides, located in the very center of these helices by the turn identification procedure, but not by the automated computer search. Reshuffling helical segments leads to the structure (panel d) which is compatible with previous experimental data. Approximately 75% of the secondary structure is predicted to be α-helical in the model we propose which is similar to the experimental value of Long *et al.* (1977).

The folding pattern of porin predicted by the turn identification procedure is shown in Figure 2. The displayed segments lie between turns (or turn clusters) and are presumed to be transmembrane sequences. The 16 strands are between 6 and 24 residues long, which is consistent with a β-structure folding pattern. The mean value of 11 ± 5 residues agrees well with the determination of spectroscopic and diffraction methods (Kleffel *et al.*, accompanying paper) which yielded an average strand length of 10 – 12 residues. A spatial segregation of polar *versus* hydrophobic residues is not detected (polar residues are indicated in the figure in one-letter code). Therefore, extensive side-chain hydrogen bonding is likely to exist within or between β-strands for the reasons mentioned above. Dense networks of hydrogen bonds could explain also the unusual stability of porin. The following pairs may serve as examples of the kinds of interactions that can be expected. (i) A strong hydrogen bond (Kyte and Doolittle, 1982; Engelman, 1982) between uncharged Arg and Tyr may be seen in strand 2 (Figure 2). (ii) A bond between protonated carboxyl residues (Suck *et al.*, 1974) may be envisaged within or between strands 13 and 14. (iii) Pairing of ionizable residues (Asp-Arg) may occur in strands 6 and 7. The latter bonds may be stabilized by saturation of hydrogen bonding potentials by neighboring donor and acceptor groups.

Segments containing turns are indicated schematically as loops in Figure 2. They comprise 3 – 20 residues and contain ~60% of the total ionizable and polar residues of porin. Based on their resistance to proteases, to polar and non-polar solvents, to salts and protons (Schindler and Rosenbusch, 1984; Kleffel *et al.*, ac-

companying paper), they are unlikely to be exposed at the protein surface. The following alternatives may be envisaged. (i) Segments may interact at subunit interfaces, (ii) they could line transmembrane channels, (iii) interactions with polar lipid head-groups could occur, or (iv) they may be present as short β-strands (defying identification) and fill in spaces between tilted channels and the membrane surface (see above). Additional β configuration is indeed suggested by the spectroscopic results which indicate 65% of the backbone in β-strands, as compared with 50% derived from the pattern shown in Figure 2. The overall folding pattern appears in any event consistent with the experimental results.

Scope and limitations

The procedure can be tested on three classes of membrane proteins. (i) Compact, membrane-spanning proteins. The solution of the 3-dimensional structure of bacteriorhodopsin (Michel, 1982) and porin (Garavito *et al.*, 1983) should soon provide an ultimate test of this procedure. (ii) Proteins with soluble domains, anchored in the membrane by relatively short polypeptide segments. In this class, cytochrome b_5 is used as an example. The structure of its soluble domain has been solved to high resolution (Matthews *et al.*, 1971). The sequence of the complete protein is known (Fleming *et al.*, 1978). A small C-terminal domain has been shown by chemical modification (Takagaki *et al.*, 1983) to span the membrane. It contains a hydrophobic segment of 28 residues. Turn identification recognizes an uninterrupted run of 21 residues between two turns which is contained within the 28-residue segment. Another example in which high resolution structure allows assignment of a small membrane anchoring-domain is haemagglutinin of influenza virus (Wilson *et al.*, 1981). Its transmembrane segment is also recognized by turn identification (not shown). (iii) Proteins with two soluble parts separated by a transmembrane domain. The best understood protein in this class exhibits a bulky membrane-spanning domain. It is a reaction center of photosynthetic bacteria whose structure has recently been solved to 3 Å (Deisenhofer *et al.*, 1984). Once its sequence is known, it may prove of unusual value. An example with an apparently single membrane-spanning segment is glycophorin. Its hydrophobic segment (Tomita *et al.*, 1978) consists of 37 residues (62 – 98). Turn identification recognizes a segment of 27 residues (65 – 91). Thus, a potentially membrane-spanning segment 20 residues long is recognized by both predictions. An interesting question arises in a third example in this class. It is the epidermal growth factor receptor protein which transduces signals across the membrane. Its growth factor binding site is likely to be located at the cell surface, while the active (kinase) site is thought to be on the cytoplasmic side. The sequence of this protein exhibits a single hydrophobic stretch (23 residues) which is thought to span the membrane (Ullrich *et al.*, 1984). The criteria used in the approach proposed here are less stringent with respect to both polarity and length of membrane-spanning segments. Several additional domains of this protein could therefore occur within membrane boundaries. Thus the question arises as to whether single membrane-spanning α-helices are capable of transducing a signal, or whether larger domains are required. Tolerating significantly more polar domains and shorter segments prevents turn identification from explicitly identifying membrane-spanning segments, in the manner that has been claimed for other methods (Steitz *et al.*, 1982; Kyte and Doolittle, 1982). Overcoming this limitation depends on obtaining complementary experimental evidence from limited proteolysis, chemical modifications, detergent binding studies or spectroscopic methods. Thus, this approach must be considered a conceptual tool rather than a comprehensive prediction method.

Conclusions

Algorithms that have been routinely used to predict the structure of membrane proteins have failed to yield a model for porin which is consistent with its known structural properties. A re-examination of the basic assumptions of such methods led us to devise a simple manual procedure which we have applied to porin and bacteriorhodopsin. The conclusions we draw may be summarized as follows. (i) The result that turn identification yields folding patterns of hydrophobic bacteriorhodopsin and polar porin which are consistent with their respective structures indicates that the information content in turn regions appears sufficient to derive the folding of two very different membrane proteins. In bacteriorhodopsin, the model is rather similar to, though not identical with, the structure obtained from its hydropathy profile. This suggests that these two independent criteria are complementary. Once the high resolution structure is available, it will be interesting to focus on the differences involved. (ii) Short loops (tripeptides) appear to be sufficient for polypeptides to reverse direction. In bacteriorhodopsin, the procedure identifies three such tripeptide turns. This could be the reason why the turn prediction procedure based on tetrapeptides (Chou and Fasman, 1979) has yielded a structure which is least reconcilable with the physical properties of bacteriorhodopsin. (iii) Residues judged likely to promote turn formation from simple physical principles coincided with those empirically found to occur most frequently in turns also in soluble proteins. This encouraged qualitative assessments of turn blocking residues on corresponding criteria. The results obtained with bacteriorhodopsin and porin seem to justify this classification as a first approximation. (iv) A very conservative approach was taken with regard to the length of membrane-spanning segments. In an extended β-configuration of a polypeptide, 10 residues may be expected to traverse the hydrophobic membrane core. Since the surface of membrane proteins may be uneven, for instance by the presence of channels, we have chosen a minimal length of six residues. This results in an estimate of secondary structure of porin which is 15% below that observed experimentally. If we had assumed segments of 10 residues as minimal β-strand lengths, periodic structure would have been underestimated by 30%. (v) The most significant difference compared with previous notions is the admission of polar and ionizable residues within the membrane domain. Porin with its densely interspersed polar residues all but necessitates this as a condition for attaining a meaningful concept of its folding. The model of bacteriorhodopsin suggested here, with many more polar residues included within the membrane boundary than assumed previously, yields a folding pattern which appears compatible with its structure. (vi) An immediate corollary of a relatively high content of polar residues is the postulate that dense hydrogen bonding systems pervade the entire membrane domain. Although apparently unavoidable for porin, it cannot as yet be put to proof. With bacteriorhodopsin, full saturation of all hydrogen bonding donor and acceptor groups appears possible. The extensive hydrogen bonding systems implied here for porin could explain its extraordinary stability. Also, porin trimers associate to form large, stable crystalline lattices. Since this protein does not contain appreciable extramembranous domains, but rather seems penetrated by saturated hydrogen bond systems which may be exposed even on surfaces facing the membrane core, interchanges of intratrimeric to intertrimeric hydrogen

bonds in these locations may explain the tight interactions involved. The existence of such bonds throughout the molecule and even at surfaces exposed to the membrane interior would clearly challenge the notion of inside-out proteins (Engelman and Zaccai, 1980).

Materials and methods

Segments with high turn propensity are identified as follows. Constituent amino acids are divided into three groups. Turn promoters comprise Asn, Asp, Gly, Pro and Ser. Turn blockers comprise Ala, Gln, Glu, Ile, Leu, Met, Phe, Trp and Val whereas turn indifferent residues are Arg, Cys, His, Lys, Thr and Tyr. Membrane protein sequences are searched for turn promoting and neighboring indifferent residues. A segment of three or more of these residues (containing turn promoting but no turn blocking residues) is considered to cause reversal of the polypeptide. If turn blocker containing segments between turns comprise ≤ 5 residues, they are considered to occur within turn clusters. Longer stretches are regarded as potential membrane-spanning segments. This notion is imprecise on purpose (see Results). Since the numerical values guiding the allocation of residues to these three classes are those applying to α-helices, experimental evidence of β-structure would significantly affect assignments of Glu and Tyr (Chou and Fasman, 1978). In that instance, Glu would be turn promoting, while Tyr would block them. An application to porin shows that in this instance, such adjustments yield somewhat more uniform and slightly increased chain lengths, but it does not affect the results qualitatively. To avoid conveying a sense of precision which is neither warranted nor intended, the results shown in the figure are left uncorrected.

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The results of applying the conceptual approach described to bacteriorhodopsin, quoted as unpublished, are now in press (J.P. Rosenbusch, 1985, *Bull. Inst. Pasteur*, **83**).