Method To Assess Packing Quality of Transmembrane α-Helices in Proteins. 2. Validation by "Correct vs Misleading" Test

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We describe a set of tests designed to check the ability of the new "membrane score" method (see the first paper of this series) to assess the packing quality of transmembrane (TM) α-helical domains in proteins. The following issues were addressed: (1) Whether there is a relation between the score (S^{mem}) of a model and its closeness to the "nativelike" conformation? (2) Is it possible to recognize a correct model among misfolded and erroneous ones? (3) To what extent the score of a homology-built model is sensitive to errors in sequence alignment? To answer the first question, two test cases were considered: (i) Several models of bovine aquaporin-1 (target protein) were built on the structural templates provided by its homologs with known X-ray structure. (ii) Side chains in the spatial models of visual rhodopsin and cytochrome *c* oxidase were rebuilt based on the backbone scaffolds taken from their crystal structures, and the resulting models were iteratively fitted into the full-atom X-ray conformations. It was shown that the higher the S^{mem} value of a model is, the lower its root-mean-square deviation is from the "correct" (crystal) structure of a target. Furthermore, the "membrane score" method successfully identifies the rhodopsin crystal structure in an ensemble of "rotamer-type" decoys, thus providing the way to optimize mutual orientations of α-helices in models of TM domains. Finally, being applied to a set of homology models of rhodopsin built on its crystal structure with systematically shifted alignment, the approach demonstrates a prominent ability to detect alignment errors. We therefore assume that the "membrane score" method will be helpful in optimization of in silico models of TM domains in proteins, especially those in GPCRs.

1. INTRODUCTION

There has been a certain progress in the field of protein structure prediction (both ab initio and template-based approaches) during several last years (see the first paper of this series for a brief review). One of the most serious bottlenecks limiting wide applications of these methods is the assessment of quality of the resulting models. Usually, closeness of a protein model to the "native" state (if the latter is known) is expressed in terms of root-mean square deviation (rmsd) of the atomic coordinates from the experimental structure. In a case of ab initio predictions, a threshold of 6.5 Å C_α-rmsd is used to mark a "correct" model,[1,2] while practical modeling approaches that might be useful for structure-based drug design (SBDD) tasks are accepted to be correct, if rmsd is much lower (e.g., 1−2 Å), and precise side-chain orientations are given by a modeling procedure.

There are few community-wide tests to assess the power of existing structure prediction methods; among them are CASP[3,4]—the competition to evaluate human predictors, and CAFASP[5,6]—a similar procedure for fully automated, Web-based prediction platforms. Modeled targets, being the same for both experiments, are newly discovered protein structures, preferentially with novel structural scaffolds, which are held unpublished for a certain period of time to make these experiments "truly blind". Another two tests—Live Bench[7,8] and EVA[9]—supplement the aforementioned annual initiatives in a more continuous manner, launching the prediction tests as soon as a novel experimental structure becomes available. Another common way to demonstrate the possibilities of a modeling approach is to predict the structure of a protein as close to the experimental one as possible, without relying on structures of closely related templates (the so-called "half-blind test"[10−12]).

The protein models' "quality-checkers" represent another important independent group of methods that along with structure prediction techniques are used to obtain realistic theoretical models of proteins. These methods are designed in such a way that some computational "scoring" function would correlate with the model's closeness to the "nativelike" state (e.g., according to rmsd criterion). The scoring function might be developed using either "first-principle" (e.g., based on empirical conformational energy) or statistical approaches. The latter ones incorporate some structural features that are observed in proteins with already determined spatial structures and check whether the theoretical models are compat-

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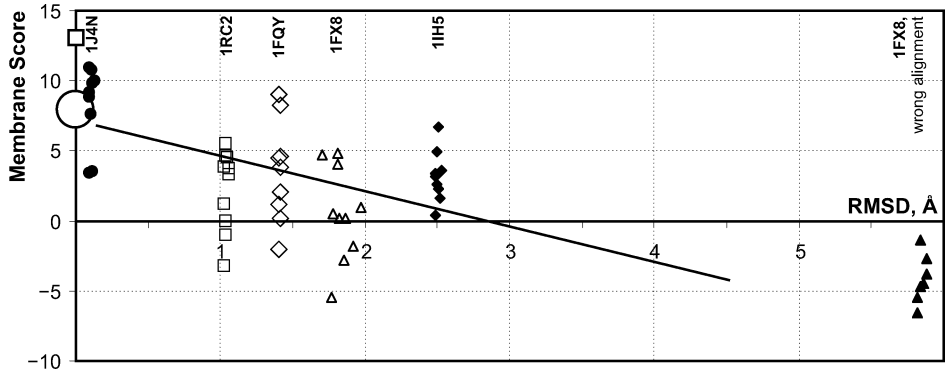

Figure 1. Dependence of the membrane score for homology-built models of bovine aquaporin-1 on C_α-rmsd from the X-ray structure (white circle). The models were built on the following templates: bovine aquaporin-1 (PDB Id: 1J4N, filled circles), aquaporin Z (PDB Id: 1RC2, empty rectangles), human aquaporin-1 (PDB IDs: 1FQY, empty diamonds and 1IH5, filled diamonds), and aquagliceroporin (PDB Id: 1FX8, empty and filled triangles correspond to correct and incorrect sequence alignments, respectively; see text for more details). For each template, a series of ten models was constructed. The solid line is the least-squares fit to the data. The white square denotes a "theoretical" S^{mem} value estimated based on the length of the TM domain for aquaporin-1 (148 residues). This value was derived using the regression equation given in the first article of this series.

ible with these features. One of the most famous methods to estimate the packing "quality" of a protein is the 3D environment profiles technique developed using a set of 16 globular proteins structures.[13] As discussed in the first paper of this series, assessment of the packing quality of membrane proteins (MP) requires that peculiarities of known experimental structures of MPs are taken into account.

In this series of two papers we present a novel method for assessment of packing quality of transmembrane (TM) α-helical domains of MPs. In the first paper we developed the "membrane score" method, using a nonredundant set of high-resolution X-ray structures of MPs to derive residues' preferences to the predefined classes of membrane-protein environment. Definitions of the classes were given, and their borders were optimized. The resulting scoring function was designed to either whole-structure or residue-level assessment of "nativity" of the packing characteristics, including the degree of membrane exposure and the polarity of the proximal protein parts. For proteins from both training and test sets it was shown that there is a good correlation between the sequence length of TM domain and the corresponding score value (S^{mem}). This gives a possibility to check whether a certain model of MP has a packing quality comparable to that observed for experimental structures with the same length. Also, based on the found dependence of the membrane score on rmsd from the "native" structure, we demonstrated the possibility of delimiting a well-packed (e.g., X-ray) structure of rhodopsin and the set of its (mostly erroneous) theoretical models.

In this paper we strengthen the findings that were made in the first article and further validate the "membrane score" method's ability to delimit the "nativelike" and "far-from-native" structures of MPs. Thereto we explore the connection between the membrane score values and the closeness to the X-ray structure for the following theoretical models, including their artificial "decoy" variants: (1) a set of homology models of bovine aquaporin-1 built on various structural templates; (2) a set of "rotameric" decoy conformations of bovine rhodopsin with TM α-helices systematically rotated around their axes; and (3) a set of rhodopsin and cytochrome *c* oxidase structures with the backbone identical to the crystal structures but with completely altered orientations of side chains.

The first test addresses the problem of the choice of an optimal structural template, if several are available. The second one gives an idea on model optimization by slight whole-domain rearrangements (e.g., rigid-body rotation of TM α-helices) if the only template is available. The third test considers the problem of "high-resolution refinement" of theoretical models that still represents a great challenge,[14] especially given the fast progress in ab initio techniques for structure prediction.[15] It is a widespread opinion in molecular modeling that if the backbone conformation of a model is close to the "native" one, the task of the structure prediction is successfully solved. But the spatial arrangement of side chains on a conservative backbone may be very important for a variety of structure-based tasks (e.g., drug design). The problem of adequate treatment of side-chain mobility is yet to be solved by means of sophisticated modeling techniques.

Moreover, we also tested the method's ability to detect alignment errors in an ensemble of rhodopsin models built using its own crystal structure as a template but with systematically shifted alignments. The problem of correct alignment, especially in a case of low target-template homology, is one of the most serious in molecular modeling, along with problems mentioned in the previous paragraph.

2. RESULTS AND DISCUSSION

2.1. rmsd vs Membrane Score Dependencies in Homology Models. Because very few structures of MPs are available for homology modeling, it is very important to choose an appropriate spatial template, if several are available. Here we decided to build a number of homology models of bovine aquaporin-1 (whose structure is known; PDB Id: 1J4N) based on the templates provided by the X-ray structures of several of its homologs: human aquaporin-1 (PDB Ids: 1FQY, 1IH5), aquaporin Z (PDB Id: 1RC2), and aquagliceroporin (PDB Id: 1FX8) (see Methods). The aim of this test was to further validate the connection between the membrane score and the models' "nativity" as well as to check whether the template corresponding to the most homologous protein as is obligatory as the best choice for modeling. In Figure 1 the score values are plotted against

Table 1. Structures Used To Build Homology Models of Bovine Aquaporin-1

no.	PDB Id	name	resolution, Å and method	length	identity to 1J4N,[b] %	rmsd,[c] Å	$S^{mem\ b}$
1	1J4N	aquaporin-1, *Bos taurus*	2.2, X-ray	271	100	0.00	7.98
2	1FQY	aquaporin-1, *H. sapiens*	3.8, EC[a]	269	80	1.45	13.31
3	1IH5	aquaporin-1, *H. sapiens*	3.7, ED[a]	269	80	2.54	3.83
5	1RC2	aquaporin Z, *E. coli*	2.5, X-ray	231	35	1.00	19.79
4	1FX8	aquagliceroporin, *E. coli*	2.2, X-ray	281	25	1.33	19.95

[a] EC — electron crystallography; ED — electron diffraction. [b] Over the TM region, homologous of that of 1J4N (148 residues in eight helices). [c] C_α-rmsd from the 1J4N structure over the TM region.

RMSDs from the "native" structure for several groups of bovine aquaporin-1 models built on different templates, including its own structure. As seen from the figure, there is some anticorrelation between S^{mem} and rmsd from the crystal structure. However, this dependency is clear only if we account for all of the models, including those built using the crystal structure of aquaporin-1 as a template, and another group based on erroneous alignment. If we do not consider these groups of models, the dependency appears to be statistically insignificant and not steady. In this case the membrane score method seems to be not very sensitive in the rmsd range 1−3 Å. However, we still can use the "theoretical" S^{mem} value, estimated based on the length of the TM domain of aquaporin-1 (square in Figure 1) to figure out that all constructed models still require optimization and that the models with negative scores contain gross errors.

This example along with the data in Table 1 pictorially illustrates that the most homologous template is not obligatory as the best choice for modeling. Here, the models built on the high-resolution structure of the remote homolog aquaporin Z (1RC2) appear to be closer to the "native" structure than those built on low-resolution scaffolds of highly homologous human aquaporin-1 (1FQY, 1IH5). This might be due to poor quality of the refinement procedure of the low-resolution electron crystallography models, which place protein atoms relatively far from the "native" structure, as compared to the X-ray models. In this case, we cannot unambiguously select the best template for model building, relying solely on the membrane score values. However, in another situation, if, e.g., one of the possible templates has remote spatial similarity (rmsd from structure of the modeling protein (which is unknown) higher than, say, 4 Å), we could eliminate an unsuitable template because of its negative score.

Since models for this test were built using a fully automated alignment and modeling procedures, there might be some gross errors that can be easily corrected by human intervention. Indeed, this happened for the models built on the aquagliceroporin template. All these models (marked with filled triangles in Figure 1) reveal negative scores, and on closer examination we found that one of the TM α-helices in the target was aligned with a loop region in the template. This yielded an unrealistic environment for residues in this segment and, as a consequence, poor quality of the model. Comparison of the models with the X-ray structure (which was not used in homology building) shows that the resulting rmsd values are much higher than those between the crystal structures of bovine aquaporin-1 and aquagliceroporin. The latter result is surprising because generally RMSDs from the "native" state are very similar for the template and the homology-built models. Correction of the alignment resulted in bringing the models closer to the rmsd region typical for the template, and the scores became mainly positive.

It is well-known that taking into account predicted information on protein topology and/or secondary structure is able to improve alignment quality or at least to avoid serious errors. The "membrane score" method helps in recognition of such mistakes. In order to evaluate this ability in a systematic manner, we built an ensemble of rhodopsin models based on its own structure but with artificially shifted alignment.

2.2. Detection of Errors in Sequence Alignment. Traditional comparative modeling strongly relies on the alignment between sequences of the modeled protein and the template. A single amino acid shift may lead to dramatic structural changes in the model. For instance, in case of α-helix, this corresponds to 100° rotation of the misaligned segment. In order to check if the "membrane score" method can detect such alignment errors, a special test was designed. It implies an ensemble of "decoy" homology models of bovine rhodopsin, built from its own structure, but with systematically shifted alignment. The target sequence was cycled against the template sequence like the bicycle chain (see Methods). In total, 348 series of models (50 models for each alignment) were generated. Only one of them had an unshifted alignment. Average values of the membrane score calculated for the resulting series of models are given in Figure 2. It is seen, that only "unshifted" models have positive scores, close to the crystal structure, while all other "chains" models reveal extremely low quality. There are not any "intermediate" states: even models, based on the alignments shifted by just one residue, are absolutely as "bad" as any other models with larger shifts. Very few models have positive scores (see inset on Figure 2), although much smaller than the "unshifted" models. This gives strong evidence that the alignment errors in comparative modeling of MPs can be recognized when applying the "membrane score" method. The obvious explanation is the following. If someone is building a model of a protein, homologous to a template with high packing quality (as indicated by the membrane score), he (or she) should get a structure of comparable quality (depending on the homology degree). But if there is an alignment error (most likely, only in a part of the model), the proportion of residues that fall into an unrealistic environment will be rather high, and even the total score may indicate this. The closer by-residue examination should reveal a continuous part of the model with negative scores.

Another task which often arises in homology-based modeling of MPs is related to the recognition of crystal structure among models with altered packing characteristics in TM domain. In the next section we demonstrate that the

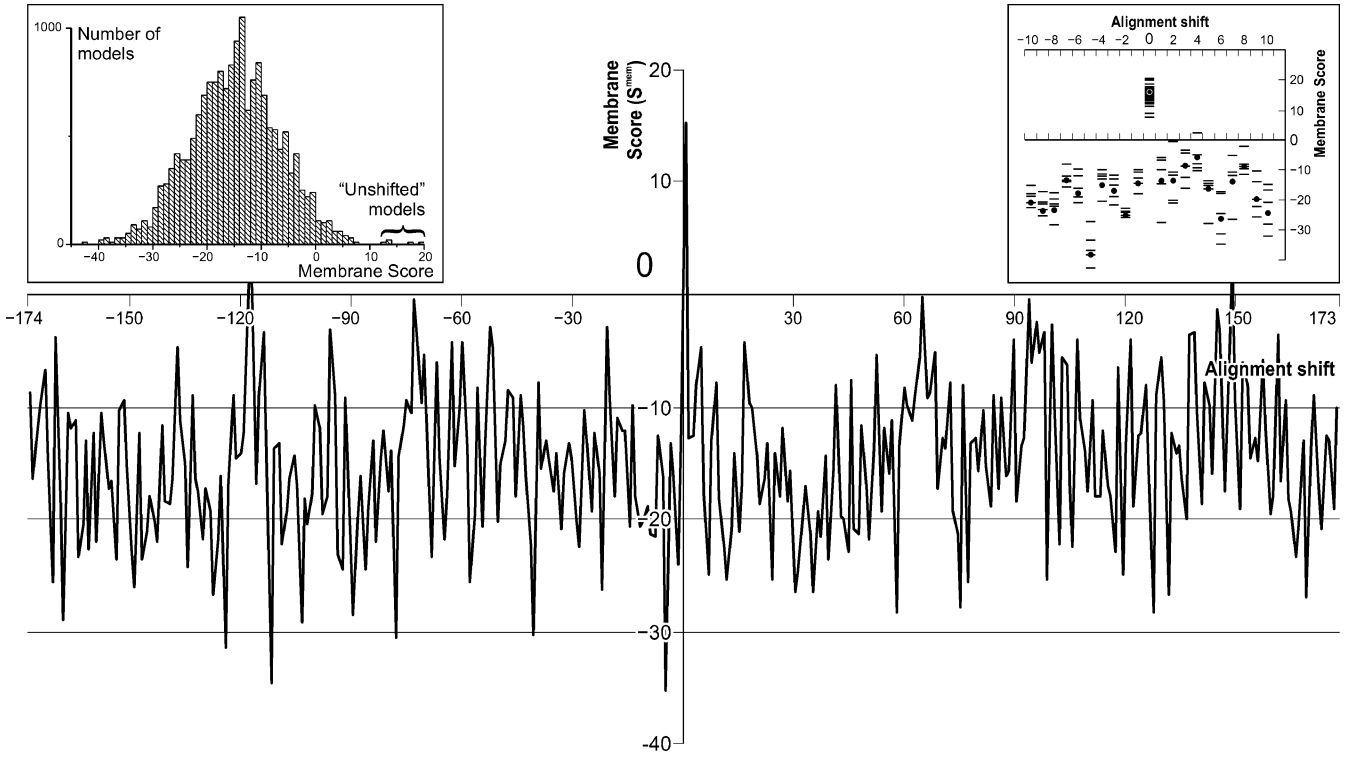

Figure 2. Evaluation of the ability of the "membrane score" method to detect alignment errors. Values of the membrane score (S^{mem}) for 348 sets of homology models of bovine rhodopsin (based on its X-ray structure) with systematically shifted alignment (see Methods). The "zero" position corresponds to "unshifted" models. Each value S^{mem} is an average score for a set of 50 models (standard deviations are omitted for clarity since they do not change the qualitative picture). *Left inset:* Distribution of S^{mem} values for all models. Scores of the "unshifted" models are shown with a brace. *Right inset:* A close-up view of S^{mem} values for the alignment shift ± 10 residues. For all positions except the "unshifted" ones, only the scores for the randomly chosen 5 of the 50 models are presented for clarity; the data for all fifty models are given for the "unshifted" position. Circles denote the average S^{mem} value for all models built with the current alignment.

"membrane score" method may be efficient in solving this problem.

2.3. "Rotameric Test": Reassembly of an α-Helical Bundle. The main idea of this approach was to generate a voluminous ensemble of artificial "misfolded" structures via alterations of the existing X-ray model. The next step was to discriminate the crystal ("correct") model based on the analysis of the membrane score data. It was therefore assumed that even small intervention into the delicate architecture of a given MP will result in a model inconsistent with the general principles of MPs' structural organization. The X-ray structure of the TM part of rhodopsin was taken as a reference object. The set of altered conformations was generated by rotation of TM α-helices around their axes as rigid bodies (see Methods). The quality of helix packing in these "rotamers" was further evaluated using the "membrane score" method. As in the first article of this series, performance of the classic 3D-1D profiles method[13] was also tested. Figure 3a shows the distribution of the rotamers over their 3D-1D score. It is seen that about 40% of the generated conformers demonstrate higher scores than the crystal structure does. An attempt to overcome the "membrane exposition artifact" and to account for only the scores of buried residues in the total sum moderately improves the result, but 22% of the rotamers still score better than the crystal structure (Figure S1). Like in the example from our previous article, this proves that the method developed for globular proteins loses its sensitivity if applied to MPs.

The "membrane score" method allows overcoming this difficulty (Figure 3b). It ranks the crystal structure practically at the top of the list containing more than 1.6×10^4 rotamers (see Methods). Moreover, the score attributed to the X-ray structure falls outside the 3σ interval from the mean value of the distribution (σ is the standard deviation), thus indicating a very high ($p < 0.003$) statistical significance of the result. The crystal structure, however, is not the only high-scoring model. As seen in Figure 3b, five rotamers demonstrate even larger values of S^{mem} as compared to the crystal structure, though the differences are quite small. It is interesting that in all such top-scoring models only the helices TM1 and/or TM2 are rotated with respect to the X-ray structure. (In one case the helix TM4 is also rotated). To inspect in more detail the sensitivity of the membrane score to the conformational rearrangements of individual helices, the latter were rotated independently with an increment of 5°, and the resulting angular distributions of the values S^{mem} were analyzed. Only the rotation of TM2 leads to a sharp maximum at 280° (clockwise rotation, if seen from the extracellular side), whereas rotation of other helices is not accompanied with an increase of the total score (data not shown). It is unclear whether the top-scoring models of rhodopsin deviating from the crystal structure have any biological significance or this is just a computational artifact. If, for example, similar results were obtained for helices TM3 and TM6, whose rotation is known to be coupled with receptor activation,[16] one might speculate about the existence of stable (well-packed) intermediate states alternative to the crystal structure. At the same time, recent structural work on the activated state of rhodopsin[17] demonstrates that this type of mobility of the helices TM3 and TM6 may be

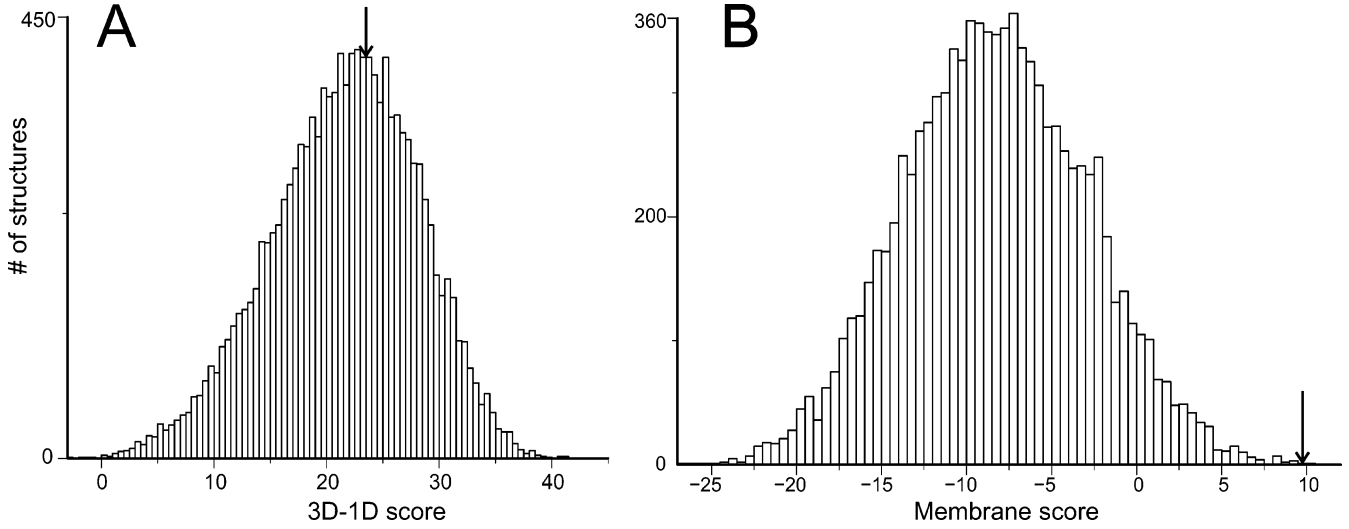

Figure 3. Distributions of 3D-1D[13] (**A**) and membrane (**B**) scores for the ensemble of "rotameric" conformations of visual rhodopsin. The position of the crystal structure is shown with an arrow. In case "**B**", only five "rotamers" are superior to the crystal structure in terms of the membrane score: (270; 270; 0; 0; 0; 0; 0): 11.72, (180; 90; 0; 0; 0; 0; 0): 11.41, (180; 270; 0; 0; 0; 0; 0): 11.23, (0; 180; 0; 0; 0; 0; 0): 10.63, (0; 270; 0; 180; 0; 0; 0): 10.54. Here, the seven numbers in brackets indicate the rotation angles of helices 1−7, as seen from the extracellular side of the membrane, and the numbers after the colon are the related membrane score values. The (0; 0; 0; 0; 0; 0; 0): 10.46 rotamer corresponds to the crystal structure.

overestimated and suggests that TM1 and TM2 may play an important role in physiologically relevant dimer formation. This question, obviously, requires additional studies.

The reported result means, in fact, that one could effectively reconstruct the crystal structure of rhodopsin starting from any arbitrarily built 7th helix scaffold with predefined distances and tilt angles between helix axes. Then the objective lies in optimization of their orientation in order to maximize the membrane score. In practical applications some uncertainty introduced by five conformations scoring better than the crystal structure may be eliminated by engaging some experimental constraints on helices' positions and orientation. Furthermore, the "membrane score" method could be applied to modeling MPs' structures based on low-resolution templates, similar to variability[18] or packing[19] moments methods. Not only rotational degrees of freedom might be explored in this case but also helical shifts and tilts. However, this approach is much more computationally demanding and therefore is a subject of a separate study. We should outline, however, that in the "rotameric test" conformations of the side chains were almost identical to those in the crystal model. To what extent is the membrane score sensitive to the placement of side chains in TM helices?

2.4. The Way to "High-Resolution Refinement" of the Models. To answer this question, we studied dependence of the membrane score of a model on RMSDs from the "native" structure in the high-resolution range (here, it means alternative packing of side chains with the backbone taken from the corresponding crystal structure). We built models of two proteins (namely, rhodopsin and cytochrome *c* oxidase) with completely altered side-chain conformations (large side-chain RMSDs from the X-ray structures) and a set of "intermediate" models with smaller rmsd values. All the models were then assessed via calculation of the membrane score. The results are shown in Figure 4. It is seen that the rebuilding of side chains leads to a considerable drop of the membrane score (especially in case of Rh), indicating that such a procedure seriously disrupts intimate residue/residue interhelical contacts inherent in the "native" structure. Similar results were reached when the side chains were rebuilt based solely on coordinates of backbone or C_α atoms of the corresponding crystal structures. Such "reconstructed" models have much lower values of S^{mem} as compared to the situation when an all-atom template is taken, while the increase of rmsd is rather small (data not shown). Furthermore, as seen in Figure 4, simple energy minimization of the "reconstructed" models slightly raises the S^{mem} value, while minimization of the crystal structures leads to a decrease in the membrane score (data not shown). This gives evidence that the native organization of MPs is very delicate, and even such a "simple" procedure can impair it (this has also been demonstrated for globular proteins). On the other hand, application of energy minimization to a molecule with somehow "randomized" orientations of side chains may drive it closer to the native state.

A general conclusion can be made that the "movement" of a model toward the "native" structure is accompanied by steady increase of the membrane score. In this case the values S^{mem} and rmsd are highly anticorrelated (with a coefficient of $-0.8 \div -0.9$), and the membrane score value can help to pick one of the closest to "native" conformations. Additional studies that will permit more reliable detection of "close-to-native" structures are in progress now in our group.

2.5. Limitations of the Approach. Due to the statistical nature of the present method, dependence S^{mem}(rmsd) may have a different character in distinct rmsd ranges. The membrane score value is defined to a large extent by mutual orientations of side chains, so the dependence of this scoring function on the backbone conformation (i.e., ability of "low-resolution" optimization) is strongly mediated by side-chain conformations. Conceivably, the method's capability of packing quality assessment might be different in distinct resolution ranges and not be straightforward in a case when only coordinates of C_α atoms in the template are available.

Furthermore, there is some doubt whether the "membrane score" approach is applicable to assessment of incomplete models, e.g., containing only a part of TM α-helices as compared to the native protein. Environments of the residues

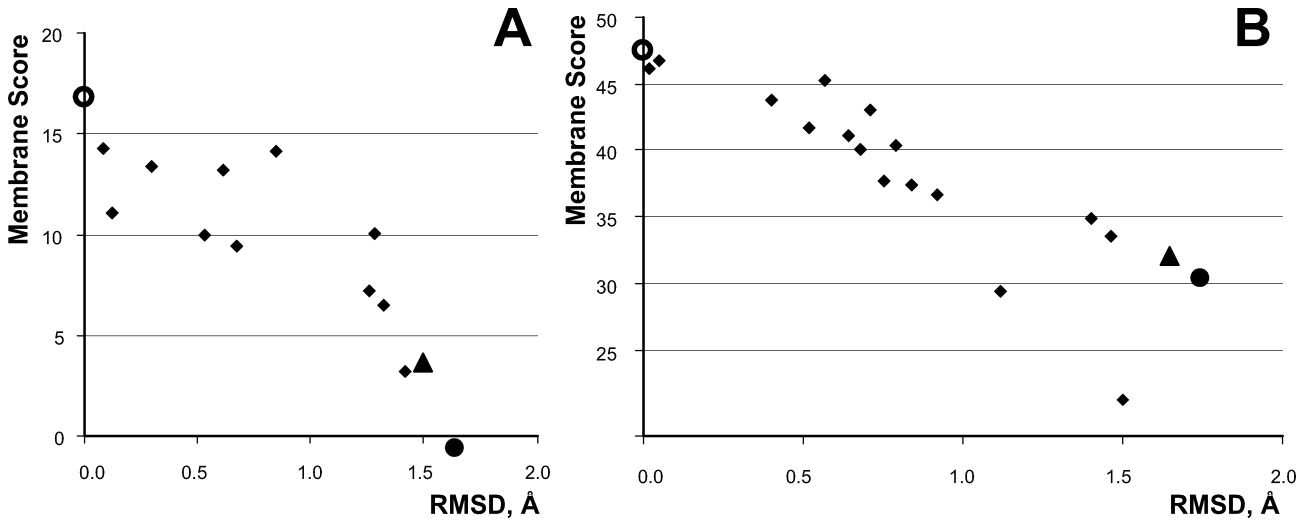

Figure 4. Influence of side-chain positions in protein models on the membrane score (S^{mem}). The values of S^{mem} as a function of heavy-atom rmsd from the X-ray structure for rhodopsin (**A**) and cytochrome *c* oxidase (**B**) models with an alternative packing of side chains. The backbones are those of the crystal structure (marked with circles); black triangles denote energy-minimized conformations "reconstructed" without any additional constraints except the fixed backbone. Black circles point to structures with an artificially reconstructed configuration of side chains (see text). Gray diamonds correspond to models with intermediate closeness to the X-ray conformation.

that lose their partners in such models can drastically change. So, not only the total score values got impaired but also the ability to find "near-from-native" models might be lost.

CONCLUSIONS

Here we presented the method for assessment of packing quality of α-helical TM domains. A variety of computational tests was employed to check the applicability of the approach. Among them are such important issues in molecular modeling like (1) identification in a set of theoretical models those which are the closest to the "native" structure; (2) detection of alignment errors creeping into the models upon their construction; (3) optimization of helix packing in TM bundles; and (4) refinement of side-chain positions in MPs' models. The results obtained demonstrate that the "membrane score" algorithm provides important new data concerning structural and hydrophobic organization of MPs. Often, it allows refinement of the models generated using standard approaches. The developed technique therefore may serve as a powerful tool that complements existing methods destined to produce physically reliable 3D models of MPs. Given the high biological impact and complexity of this problem, only such a combined approach joining the results obtained by independent techniques (including the "membrane score" method) may be efficient for further applications in rational drug design.

Furthermore, the presented data provoke a number of speculative ideas concerning subsequent lines of research. In particular, the sensitivity of S^{mem} values to side-chain conformations may be exploited to search for the "nativelike" states in a voluminous database of models with similar backbone. Such a task arises when only a low-resolution template with predefined backbone is available—a typical case for ab initio MPs' structure predictions. Such a template can be used to generate a representative ensemble of conformers with varying orientations of side chains—e.g., via exhaustive conformational sampling in Monte Carlo or molecular dynamics simulations of the model. Then the most "nativelike" states can be identified based on their values of the membrane score. Obviously, further work is required to verify the feasibility of this idea.

In conclusion, we believe that one of the most intriguing perspectives of the proposed approach is related to optimization of GPCRs' models built via homology modeling techniques. Substantial progress in this area has been made over the last several years, but the vast majority of the models are still constructed based upon a single structural template, namely the X-ray structure of visual rhodopsin. Large sets of such models are often generated in a fully automatic regime via available Web services. Quality of the resulting models is therefore difficult to assess in a proper way. Production of homology-based models suitable for structure-based drug design tasks, on the other hand, requires delicate and careful optimization encompassing both experimental constraints (mutagenesis data, probable GPCRs activation mechanism, and so on) and theoretical methods based on general physicochemical principles of the structural and hydrophobic organization of MPs. The present "membrane score" approach may serve as one of the latter. Computationally, the technique is highly efficient and can be implemented in up-to-date high-throughput applications. Additionally, the method seems useful for evaluation of the ability of individual α-helices or their bundles to form oligomers in membranes—by comparing the scores before and after association. This work is currently in progress in our group. In the future, similar algorithms might be developed for TM β-sheet domains.

3. METHODS

3.1. Homology Modeling of Bovine Aquaporin-1. A summary on the structural templates used to build models of bovine aquaporin-1 (whose X-ray structure is also available) is given in Table 1.

Sequence alignments were generated automatically by the Clustal software.[21] A series of 10 models for each template was built automatically using the Modeller program.[22] The values of S^{mem} for them are plotted in Figure 1. In the case

of the aquagliceroporin template, two series of models were built; because the automatically produced alignment gave incorrect results in the TM8 region (black triangles in Figure 1), the second series of models was constructed based on the manually corrected alignment (see section 2.1).

3.2. Models of Rh Based on Erroneous Sequence Alignments. A series of 348 homology models of bovine rhodopsin (Rh) was generated based on its own crystal structure,[23] with the target sequence systematically shifted along that of the template. At this stage, a part of the target sequence that became unpaired at the termini was transferred to the opposite end of the sequence (analogous to a bicycle chain). Accordingly, only the "zero-chain" set of the models was unshifted thus revealing 100% identity between both sequences. The other 347 sets of models had a sequence shift ranging from −174 to +173. Fifty models were built based on each of such alignments. This was done using the Modeller software.[22]

3.3. "Rotameric" Test. The artificial models ("rotamers") of Rh were generated by systematic rotation with a certain increment of TM α-helices as rigid bodies around their axes. The resulting structures were subjected to short energy minimization to avoid sterical bumps. If necessary (e.g., when an aromatic ring was pierced by a chemical bond), the bumps were removed by changing the torsion angle χ_1 of the ring-containing side chain. Energy minimization was done using the GROMACS 3.14 software.[24]

Two kinds of the rotameric test were used: (1) A single helix was rotated with 5° increment, yielding 72 structures per one rotating helix. (2) All helices were rotated simultaneously with 90° increment, yielding $(360°/90°)^7 = 16\,384$ structures. The former approach was employed to assess the sensitivity of the "membrane score" method to local structural changes, while the latter technique was used to generate a large number of 3D models, the vast majority of which had an improper fold.

3.4. Decoy Models with Variable Remoteness from the "Native" Structure. A series of models with the backbone identical to that in the crystal structure and altered side-chain conformations was built as follows. First, the side-chain atoms were removed from the X-ray structure (state S_0) and rebuilt using the SwissPDB Viewer software[20] (state S_1). To generate models lying between (in terms of rmsd) the states S_0 and S_1, the *Template_Force* option of the *DISCOVER* program[25] was used. It allows imposition of geometry restraints, thus "driving" the S_1 structure to the S_0 one. The backbone atoms were kept fixed during that procedure, which means that all the differences between the models were due to positions of the side chains. Since the template forcing is a kind of potential energy minimization procedure, none of the resulting models contain crude errors in molecular geometry (such as bumps and clashes).

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Supporting Information Available: Distributions of the "membrane score", Eisenberg's 3D-1D score, and two of its adaptations for membrane proteins for the ensemble of "rotameric" conformations of visual rhodopsin (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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