An Analysis of the Conserved Residues between Halobacterial Retinal Proteins and G-Protein Coupled Receptors: Implications for GPCR Modeling

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Received January 10, 1996⊗

An alignment of the transmembrane domains of halobacterial retinal proteins (including bacteriorhodopsin) and G-protein coupled receptors (GPCRs) is presented based on the commonality of conserved residues between families. Due to the limited sequence homology displayed by these proteins, an alternative strategy is proposed for sequence alignment that correlates residues within secondary structure elements. The nonsequential alignment developed identifies three proline and two aspartates residues that share common positions and, in the former case, similar functions in the transmembrane domain. The alignment is further applied to model the packing of transmembrane helices 5 and 6 of the β-adrenergic receptor based on the backbone coordinates of bacteriorhodopsin helices 3 and 2, respectively. Unlike models derived from standard sequential alignments, the approach developed here allows the key structural features conferred by the proline residues to be captured during model building. The structure described is also compared with available site directed mutagenesis results as well as existing GPCR models. In addition to the implications to model building, the commonality observed suggests a potential relationship among the GPCRs and retinal proteins.

INTRODUCTION

G-protein coupled receptors play a critical role in a diverse array of physiological processes. Although these receptors have been the primary target of a large number of drug design studies over the years, most, if not all, have been greatly hindered by the lack of structural information available for this class of proteins. For the most part, molecular modeling studies of GPCRs have relied heavily on the structure of bacteriorhodopsin (BR) for model development.[1] This approach is supported in part by sequence analyses of GPCR primary structures and in part by the low resolution projection structure of rhodopsin.[2] Both suggest that GPCR structures, like BR, contain seven transmembrane (TM) helices arranged in a fairly close packed bundle. While many computational studies perform direct alignments of GPCR and BR sequences during model building,[3,4] the distant relationship displayed by these protein families is often problematic. For example, it is not possible to match the highly conserved proline residues in TM helices 4, 5, 6, and 7 of the GPCR family with those found in BR.[5] The BR structural template is simply inconsistent with the GPCR sequence information, producing anomalous structural features in the models that are not easily reconciled. This concern has been raised in previous studies.[5,6] Pardo et al. have suggested that the sequence homology in the helical regions would be greater if the sequential ordering of the helices is ignored. This led to a cross matching of helices in which BR7 matched with GPCR 3, BR3 with GPCR 5, and BR 1 with GPCR 7. The procedure was justified based on evolutionary arguments that cited exon shuffling as the primary cause of helix reorganization in the TM domain.[5]

Since this initial report, two studies have appeared which further address the potential relationship between BR and GPCRs.[7,8] The first suggests that gene duplication occurred within BR in which helices 5−7 were derived from helices 1−3. This hypothesis was supported by the very high sequence homology between BR helices 1 and 5. It was argued that this gene duplication explained the structural homology between BR 7 and GPCR 3 originally observed by Pardo et al. (since BR 7 originated as a duplicate of BR 3). This alternative interpretation, when combined with the observation that the retinal-linked lysine in helix 7 is conserved in both bacterial and mammalian rhodopsins, was also used to support conventional, direct sequence alignments (used in the majority of GPCR homology modeling experiments).[7] The second, more recent report asserts that neither exon shuffling nor gene duplication has occurred in the evolution of the GPCR and BR sequences. Gene duplication for BR was found to be unlikely based on an analysis of BR and related halobacterial retinal protein sequences which showed that homology between BR helices 1 and 5 was unique. Such homology was not shared by other members of the family. Furthermore, a sequence analysis of the retinal family of proteins and the GPCRs was performed which provided no strong evidence to justify systematic similarities between the two families regardless of the ordering of the helices. No indication for an evolutionary linkage between the two families was found. It was therefore concluded, contrary to previous studies, that GPCR model building should not be based on homology with BR.[8]

Herein, we report the combined analysis of sequences of both bacterial retinal proteins and GPCRs in an effort to identify commonalities among conserved residues in the transmembrane domains of these protein families. It is our intent to re-evaluate the potential connection between BR and GPCR sequences, especially as it relates to model building receptor structures. The results of the analysis emphasize the importance of conserved proline residues and the need to incorporate their structural effects in GPCR models. In addition, the commonality of function of various residues conserved in both families is examined. Potential

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

S0095-2338(95)00360-X CCC: $12.00

similarities in helix-helix packing between BR and GPCRs are then used to build a model of helices 5 and 6 of the β-adrenergic receptor. The resulting model is evaluated in light of site directed mutagenesis results of the β-adrenergic receptor as well as other existing models.

METHODS

Sequence Analysis. Sequences of retinal proteins have been taken from the SWISSPROT database.[9] In all, eight nonidentical sequences are used for the alignment of retinal proteins. Aligned GPCR sequences were taken from the EMBL database.[10] All available sequences for GPCRs which bind cationic neurotransmitters have been selected. Alignments for each of the seven transmembrane regions were extracted from the file amine.ALIGN from the EMBL database.[10] Sequences of opioid and somatostatin receptors are also included in the alignment yielding a total of 201 GPCR sequences. In attempting to match conserved residues between retinal proteins and GPCRs, two criteria were imposed. First, the identity of the residue must be the same. Second, the relative depth of the residue within the lipid bilayer must be in reasonable agreement. For example, a tyrosine residue conserved among the retinal proteins can be matched with any tyrosine conserved among GPCRs to fulfill the first criteria. However, if this tyrosine lies in the middle of the membrane in the retinal proteins and is found toward the intracellular side in GPCRs, then no match exists since the second criteria is not fulfilled. Although the beginning and end points of each helix are not absolutely established for GPCRs, the relative depth in the membrane can be estimated using a generic numbering scheme for aligned GPCRs that has been suggested.[11,12] By comparing the position of the residue in a particular BR helix with its corresponding position in the GPCR numbering scheme, the quality of the match can be assessed.

Model Building. The coordinates of the electron microscopy structure of bacteriorhodopsin[1] were obtained from the Protein Data Bank.[13] The starting backbone and β-carbon atom coordinates for the model of helices 5 and 6 of the β-adrenergic receptor were taken directly from those of helices 2 and 3 of bacteriorhodopsin. The sequence of helix 6 of the adrenergic receptor was laid onto helix 2 of BR with matching of proline residues as shown in the alignment in Figure 1. Likewise, helix 5 of the adrenergic receptor was placed onto helix 3 of BR. Coordinates for side chains of residues (with the exception of β carbons) that did not match in the alignment were generated with the EDIT module of AMBER.[14] Next, side chain torsion angles were rotated to appropriate values in accordance with the backbone dependent rotamer library of Dunbrack and Karplus.[15] The resulting model was subjected to energy minimization using the MIN module of AMBER and the Weiner et al. all-atom force field.[16] 1,4 van der Waals and electrostatic energies were scaled by a factor of 2.0, and no cutoff was applied for nonbonded interactions. A constant dielectric of 2.0 was used to mimic the low dielectric environment of the surrounding lipid. The minimized structure was compared to the starting structure by RMS difference in Cartesian space.

RESULTS AND DISCUSSION

Sequence Analysis. An analysis of the 162 residues comprising the TM domain of the retinal proteins indicates

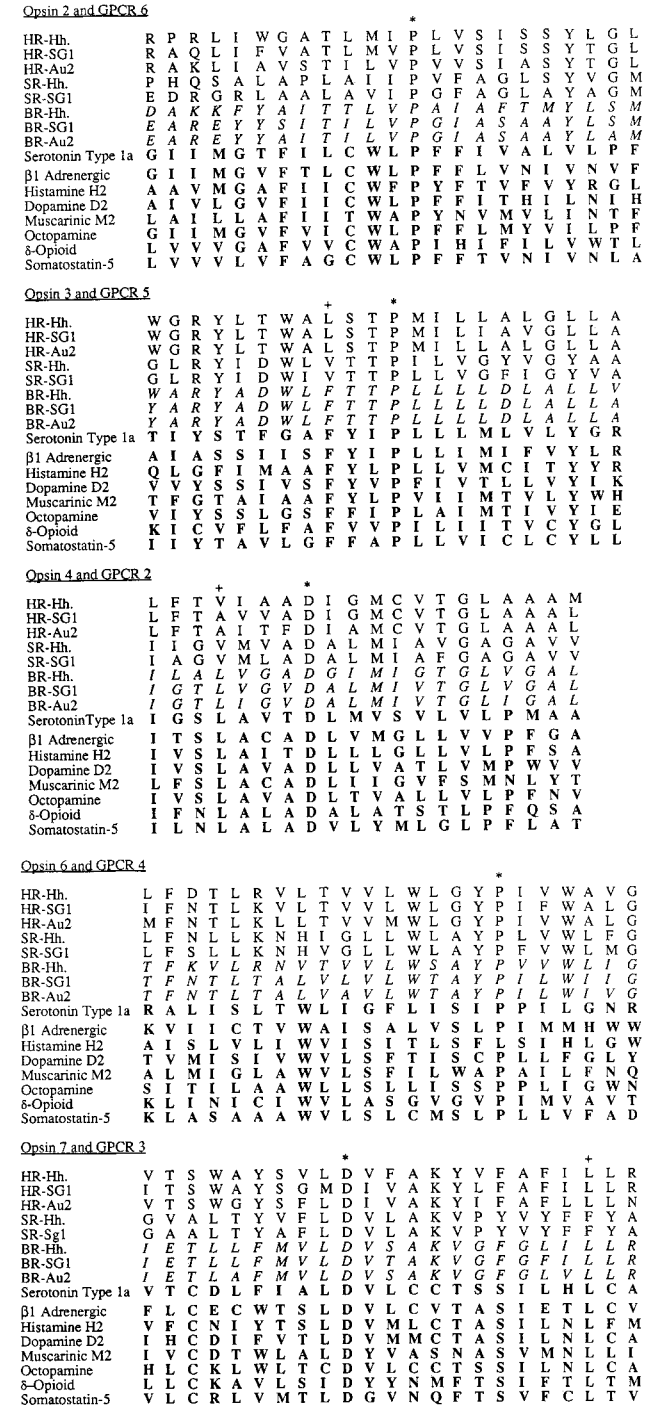

Figure 1. Overlay of halobacterial retinal and G-protein coupled receptors sequences. An asterisk (*) notes the conserved positions common to both families. A plus sign (+) indicates residues found in BR that align with residues highly conserved among GPCRs. Sequences for the halobacterial retinal proteins were taken from the Swissprot database.[9] Representative GPCR sequences were taken from the EMBL database.[10] Bacteriorhodopsin sequences are in italics. GPCR sequences are in bold. BR = bacteriorhodopsin, HR = halorhodopsin, SR = sensory rhodopsin I, Hh = *H. Halobium*, SG1 = *Halobacterium* sp. strain SG1, Au2 = *Halobacterium* sp. strain Au2.

that 22 positions are absolutely conserved. Among the aligned GPCR sequences, 21 such positions can be identified. The conserved residues of the bacterial retinal proteins were then compared with residues conserved among GPCRs to see if any were common to both families. If the TM domains of retinal proteins and GPCRs are aligned directly (i.e., TM 1 onto TM 1, .., etc.), there is a match of only one conserved

Table 1. Identity and Percent Conservation of All Conserved Residues (>95%) Among 201 Aligned GPCR Sequences

helix 2		helix 3		helix 5		helix 6	
Leu	96	Asp	100	Phe	100	Phe	100
Asp	99	Ser	100	Pro	100	Trp	100
		Leu	96	Tyr	100	Pro	100

residue, a tryptophan in helix 6. In the BR structure this tryptophan is the 17th residue in helix 6. The tryptophan conserved in TM6 of GPCRs is in position 13 suggesting that the relative depth is reasonably similar. This residue does not appear to play a vital role in proton transport in BR,[17] and its role in GPCRs is not yet clear. Alternatively, if the sequential ordering of the helical domains is ignored, it is possible to match five residues between the two families. In particular, the prolines conserved in retinal protein helices 2, 3, and 6 correspond to prolines in GPCR helices 6, 5, and 4, while aspartates in retinal protein helices 4 and 7 correspond to GPCR helices 2 and 3, respectively. For each of these matches, the position of the residue with respect to the depth in the membrane is very consistent. The greatest difference between the position in BR and the generic GPCR numbering system for all cross matches is 3. The cross alignment of transmembrane domains between halobacterial retinal proteins and GPCRs is shown in Figure 1. For simplicity, only a representative set of GPCR sequences is given. However, the key residues indicated are at least 95% conserved among all 201 GPCR sequences.

Studies of proline residues in both BR and GPCRs suggest that these conserved residues serve a similar function in each family. Mutation of proline residues in helix 5 as well as helix 6 in the m3 muscarinic receptor yielded many less functional receptors than would be expected based on mRNA levels, suggesting these prolines play a key role in protein folding and helix−helix packing.[18] Mutation of the proline in helix 4 in the same system yielded drastically reduced binding affinities for both agonists and antagonists.[18] The prolines in BR appear to play parallel roles to their proposed GPCR counterparts. Mutations of prolines in BR helices 2 and 3 yielded proteins which regenerated the chromophore at markedly slower rates than the wild type which suggests these residues play a key role in helix packing.[19] Studies of the proline in BR 6 indicate possible interactions with the retinal chromophore.[20] Thus, the proline which is located closer to the extracellular side (BR6-GPCR4) appears to be in a position to interact with the ligand, while the other prolines (BR6-GPCR2 and BR5-GPCR3), which are both located near the center of the transmembrane bundle, evidently serve a structural role.

While there is no clear functional connection between aspartate residues in the two families, each aspartate is known to play a very important role in GPCR systems. The aspartate in helix 3 has been shown, via site directed mutagenesis experiments, to be vital for ligand binding in a wide variety of GPCR systems.[21] Similarly, the aspartate in helix 2 has been shown to be either directly or indirectly involved in both ligand binding and/or signal transduction in various GPCR systems.[21]

Within the alignment of GPCR sequences, there are a total of eight residues that are conserved at a level of 95% or better in helices 2, 3, and 5. These residues are listed in Table 1. Of these eight residues, three have conserved correlates in the retinal protein family (i.e., the two aspartates and one of the prolines mentioned above). An additional three of these residues conserved in GPCRs are identical with those in the corresponding positions in the bacteriorhodopsins. Thus, in all, six of the eight conserved residues in the GPCR set correspond with BR in the nonsequential alignment.

Model Building. This proposed relationship between BR and GPCRs has important consequences for GPCR model building. While the majority of GPCR models are based on the direct sequence alignment of BR onto the GPCR, these models cannot represent the kinking effects induced by prolines. This effect is the most distinctive feature in terms of secondary structure for transmembrane α-helices. The cross matching of helices proposed here between retinal proteins and GPCRs retains several elements originally proposed by Pardo et al. (BR3-GPCR5 and BR7-GPCR 3).[5] This initial match was made on the basis of sequence homology. The alignment of sequences shown in Figure 1 in several cases yields as many as five identical matches between BR and the GPCR sequences. We suggest that by using the nonsequential alignment the helices taken from the BR structure may be used as secondary structural templates for GPCR model building. The direct correspondence of proline residues alone makes this method useful by taking advantage of the few proline-containing transmembrane helices whose structure has been determined.

With regard to the bundle formed by the transmembrane helices, there is no basis for the assumption (implicit in BR based models) that the helix packing in GPCRs is like that of BR. Although we present a nonsequential alignment between GPCR and BR helices, we do not propose an alternative arrangement of the helices in the bundle as had previously been suggested by Pardo. The basis of the alternative arrangement suggested by Pardo was that specific interhelical contacts found in BR would be preserved in GPCRs.[5] Any alternative overall packing scheme based our cross matching scheme with BR would involve crossing of interhelical loops across the bundle which we believe to be very unlikely. Indeed, detailed sequence analysis of the GPCR superfamily strongly suggests that each helix packs next to its nearest neighbors in the primary sequence.[22] However, it is possible that the packing of BR helices 2 and 3 corresponds to that of GPCR helices 5 and 6 since both helices possess helix-kinking prolines and are adjacent to each other in the primary sequence.

To test the possibility of common packing between proline containing helices in BR and GPCRs we have overlaid the sequence of the β adrenergic receptor for helices 6 and 5 onto the backbone coordinates of BR helices 2 and 3 with the matching of proline residues as shown in Figure 1. This model was then subjected to energy minimization to relieve any close steric contacts in the side chains. Although an unconstrained minimization is performed, the RMS deviation of the backbone atoms is only 0.53 Å, and visual inspection of the minimized structure indicates essentially no changes in secondary structure relative to the initial model. The only significant deviations in backbone atoms occur at the ends of the helices. The overall RMS value of 0.80 Å indicates that there is somewhat more movement among side chains as would be expected.

Interestingly, the resulting model (Figure 2) exhibits features that are consistent with site directed mutagenesis

Figure 2. Stereoview of helices 5 (right) and 6 of the β2 adrenergic receptor model built on the backbone coordinates of BR helices 3 and 2. Residues involved in ligand binding are shown in green, conserved aromatic residues in magenta, and conserved prolines in red.

results as well as existing models of the β adrenergic receptor. For example, residues that are believed to be involved in ligand binding are on the same side of the helical pair. The two serine residues in helix 5 (positions 204 and 207 in the β receptor) and the phenylalanines (289 and 290) in helix 6 are in close proximity.[23] These residues are conserved and believed to make up a key part of a binding site among dopaminic and serotonergic as well as adrenergic receptors.[3] In general, the interior face of the two helices aligns well with a recent model of the β adrenergic receptor in which the facial profile of each helix was determined by Fourier-transform periodicity sequence analysis.[24] Curiously, the orientation of the prolines is in essentially the opposite position with respect to the lipid than their respective BR counterparts. However, this result is not contrary to the idea that their primary role is in helix packing. Another interesting feature is the proximity of conserved aromatic residues between helices 5 and 6. This involves the conserved Phe/Tyr in helix 5 along with Trp residue in helix 6. These residues along with the Phe 290 in helix 6 discussed above appear to be close enough to form a tight knit aromatic cluster. The side chains of these aromatic residues may undergo conformational changes as part of an overall ligand induced conformational change that enables G-protein coupling.[3] These conserved aromatic residues as well as the ligand binding residues are highlighted in Figure 2.

Another issue raised by this two helix model regards the "chirality" of the GPCR models. As mentioned above, GPCR helical bundles should pack with each helix adjacent to its neighbor in the primary sequence. Within this constraint, two possibilities still exist. GPCR models based on a direct alignment onto the BR structure necessarily assume counterclockwise packing (when viewed from the extracellular side). By contrast, other GPCR modeling efforts have suggested that a clockwise arrangement is more plausible.[25,26] Given the arrangement of helices 5 and 6 for the β adrenergic receptor model shown in Figure 2, only a counterclockwise arrangement is possible. Although our evidence is indirect, the structural details presented may provide new insight to the development of critical experiments to resolve this structural dilemma. In our preliminary model of the β adrenergic receptor for instance, helix 6 contains two phenylalanine residues which both reside on the interior face. It is possible that one or both of these residues forms specific interhelical contacts with residues on adjacent helices (Figure 2). For example, Phe 290 appears to interact with Phe 208 of helix 5. Carefully designed site-directed mutagenesis experiments or peptide NMR studies[27] may allow these specific interactions to be further resolved, addressing a major piece of the GPCR modeling puzzle.

CONCLUSION

In this report, we have presented a nonsequential alignment of GPCR helices with BR based on common conserved residues between the GPCR superfamily and the halobacterial retinal proteins. While the connection established here is limited in sequence homology, the similarity of the relative depth in the membrane of the common conserved residues as well as the conservation of the directionality for each helix in the alignment cannot be overlooked. In addition, there is evidence of a functional correspondence between proline residues conserved among the two families. As we have shown above, the resulting nonsequential alignments have important implications for GPCR model building. GPCR models based on a direct sequence alignment with BR cannot produce appropriate secondary structures for GPCR helices primarily due to the lack of correspondence of proline residues. Furthermore, such models assume that the helix packing in GPCRs is the same as that in BR. Projection structures of bovine rhodopsin provide strong evidence that this is not the case. This alignment allows for an alternative use of BR as a template for GPCR modeling which eliminates the shortcomings of those based on direct alignments. Using the BR X-ray coordinates, direct sequence overlays can be performed that capture the conformational features conferred by the highly conserved Pro residues in GPCR secondary structures. With respect to the helix−helix packing in GPCRs, the preliminary structure of helices 5 and 6 for the β adrenergic receptor nicely demonstrates the utility of this approach to GPCR modeling. Although further work is required to pack the remaining helices in the TM bundle, the clues provided here and those derived from sequence analyses and experimental studies[27,28] should prove advantageous in the development of complete GPCR structures.

In addition to providing an alternative use of BR as a structural template, our analysis may reopen the debate regarding potential evolutionary relationships among retinal proteins and GPCRs. The link between these families has been the subject of some controversy. Although a recent analysis has concluded that no relationship exists among these sequences, the commonality reported here is provocative. While our results by no means prove an evolutionary relationship between BR and GPCRs, the possibility of exon shuffling raised previously cannot be ruled out. A recent

report involving sequence analysis of a new class of GTP binding proteins suggests that exon shuffling may be occurring, providing some evidence for the viability of this mechanism.[29] Alternatively, the results may reflect a more general relationship stemming from structural homology. The sequence dependence of secondary structure motifs is certainly well-known and widely applied in knowledge-based protein modeling. It is certainly reasonable to assume that these concepts are extendable to TM domain sequences across families with similar global structures and functions.

ACKNOWLEDGMENT

Support for M. G. Paterlini has been provided by NIOA training Grant T32DA07234.

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CI950360J