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A C_α−H···O Hydrogen Bond in a Membrane Protein Is Not Stabilizing

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C−H···O H-bonds are quite common in protein structures.[2] Although they are expected to be weaker than traditional H-bonds, quantum mechanical calculations suggest that C−H···O H-bonds involving a backbone C_α−H donor could be roughly one-half the strength of a traditional H-bond (up to 3 kcal/mol in vacuo).[3,4] Thus, they have the potential to be highly significant in stabilizing protein structures and in protein−ligand association. Surprisingly, however, no experimental tests of their energetic contribution to stability have been performed. Here, we probe the strength of a C_α−H···O H-bond in the membrane protein bacteriorhodopsin (bR).

If C_α−H···O H-bonds are energetically significant, they should be particularly important in the membrane environment where there is a low dielectric and minimal competition from water.[5] Indeed, a survey of membrane proteins reveals that they are quite common.[5,6] Chamberlain and Bowie, however, found that many of the observed carbon H-bonds may simply be a consequence of close packing, because incorrectly folded, energy minimized structures contain a number of carbon H-bonds similar to that for correctly folded structures.[7] Thus, the mere presence of these H-bonds does not imply energetic significance.

To evaluate the energetic contribution of an interaction in a protein, it is necessary to delete the interaction and measure the consequences on thermodynamic stability. These measurements have been difficult to perform for membrane proteins because of a lack of suitable model proteins of known structure for which thermodynamic stability can be measured. We have recently developed a method for measuring the thermodynamic stability of the membrane protein bR, enabling us to test the energetic contributions of interactions seen in the crystal structure.[1]

Although bR contains many putative C_α−H···O H-bonds,[5] only one satisfied important selection criteria (details in Supporting Information). The chosen C_α−H···O H-bond, shown in Figure 1A, was also highlighted by Senes et al. and occurs between the γ-O of the Thr24 side chain and the C_α−H of Ala51.[5] It is located near the center of the membrane. Although the C_α−H−O angle of 117° deviates substantially from the ideal angle of 180°, the C_α−O distance is close to ideal at 3.4 Å (ideal is 3.32 Å), and the H−O−C angle of 115° is near the ideal value (ideal is 109°).[3] Moreover, Vargas et al. argue that the angle dependence is not strong.[4] Thr24 also makes an intrahelical H-bond to the backbone carbonyl oxygen of Met20. Because helix A remains intact in the SDS unfolded state, the intrahelical H-bond is not forced to break and should not contribute to our stability measurements.[8]

Prior work strongly suggests that the selected C_α−H···O H-bond between Thr24 and Ala51 must break in the unfolded state, which is essential if we are to observe its contribution to stability. The H-bond occurs between helices A and B. NMR experiments found minimal interhelical interactions in an A/B helix peptide in SDS.[9] Moreover, mutants in the interface between these helices make substantial contributions to stability, suggesting that specific interactions between helices A and B are lost in the unfolded state.[1]

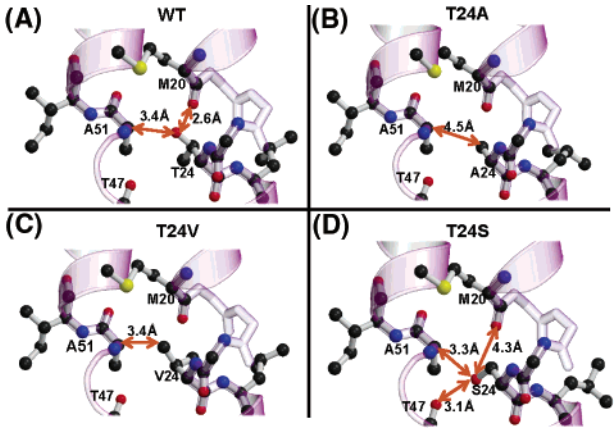

Figure 1. Structures of the wild-type and mutant proteins around position 24. (A) The wild-type structure (1PY6[1]) showing the C_α−H···O between the C_α−H of Ala51 and the O_γ of Thr24 and the traditional hydrogen bond between the backbone carbonyl of Met20 and the O_γ of Thr24. (B) The structure of T24A. (C) The structure of T24V. (D) The structure of T24S showing the new hydrogen bond between the O_β of Ser and the backbone carbonyl of Thr47.

As discussed below, one of these mutants involves a traditional H-bond, three residues away from Thr24, indicating that interactions are broken in this particular area of the A/B interface. Thus, it is unlikely that the C_α−H···O H-bond made by Thr24 will be maintained in the unfolded state.

To probe the energetic importance of this H-bond, we changed the Thr24 side chain to Ala, Ser, and Val, and we measured the thermodynamic stability of the bR mutants using an SDS unfolding assay.[1,10] For both T24A and T24S, the midpoint of the unfolding curves occurs at higher SDS concentrations than that of the wild-type protein (Figure 2), while T24V unfolds at a slightly lower SDS concentration. At an SDS concentration of 0.6 mole fraction, the corresponding changes in the free energy, $\Delta\Delta G_u$, are 0.6 ± 0.2 kcal/mol for T24A, 0.3 ± 0.2 kcal/mol for T24S, and −0.2 ± 0.5 kcal/mol for T24V. Thus, none of the mutations are significantly destabilizing. Indeed, removal of both the Thr hydroxyl and the methyl groups in the T24A mutant is stabilizing. It is therefore possible that the Thr side chain is actually destabilizing because of modest steric conflicts, which outweigh any contribution of the H-bond. These results suggest that the C_α−H···O bond between T24 and A51 is not making a significant contribution to the stability of the protein.

It is possible that structural adjustments occur in the mutants that compensate for the loss of the C_α−H···O H-bond. We therefore solved the crystal structures of each of the mutants at residue 24. T24S, T24A, and T24V were solved at 2.0, 2.2, and 2.3 Å resolution and were refined to R_{free} values of 27.2, 28.7, and 26.6, respectively. All of the structures are globally similar to the wild-type protein (C_α RMSDs to the wild-type are 0.17, 0.17, and 0.13 for T24A, T24V, and T24S, respectively), but exhibit interesting changes in the local region of the mutations.

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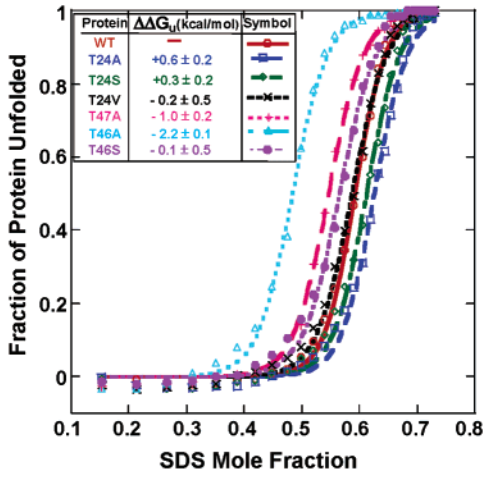

Figure 2. Stability of bR variants. The curves are SDS unfolding isotherms for the different bR proteins. The inset lists the symbols used for each protein and presents the calculated change in unfolding free energies, $\Delta\Delta G_u$, for each of the proteins relative to wild-type at a SDS mole fraction of 0.6. The curves were fit to a two-state unfolding model as described by Faham et al.[1]

The structure of the T24A mutant is essentially identical to the wild-type protein in all but the deleted atoms (Figure 1B). We observed no apparent packing adjustments to fill the gap left by the mutation. Thus, the increased stability of T24A is most likely explained by the loss of some steric repulsion caused by the threonine side chain, rather than improved packing in the mutant protein, implying that the extra atoms of the Thr side chain are actually destabilizing due to steric repulsion.

In the crystal structure of T24V (Figure 1C), Val adopts a different rotamer ($\chi_1 \approx -60°$) than Thr ($\chi_1 \approx 180°$). The Val side chain replaces the Thr hydroxyl with a larger methyl group that cannot make the $C_\alpha-H\cdots O$ bond with Ala51. The reason for the rotamer change is not clear, but probably has to do with the loss of H-bonding potential, freeing the Val to flip. The C_γ atom is within 3.4 Å of the C_α atom of Ala51, possibly producing a mild steric clash that could explain the slightly reduced stability of the T24V mutant.

The structure of the T24S mutant revealed unexpected structural changes. Although the Ser hydroxyl could adopt the same position as the Thr hydroxyl and make the same H-bonds, it chooses to do something else. As shown in Figure 1D, the Ser hydroxyl rotates closer to the threonine methyl group's position. The $C_\alpha\cdots O$ distance (3.3 Å) remains essentially unchanged, but the $C_\alpha-H\cdots O$ H-bond geometry has degraded substantially. The $C_\alpha-H-O$ angle is 94° (ideal is 180°), and the $H-O-C$ angle is 73° (ideal is 109°). Another key change is the loss of the intrahelical H-bond between the hydroxyl of Thr24 with the carbonyl of Met20. Instead, Ser24 reaches across to helix B, making a new interhelical H-bond to the backbone carbonyl oxygen of T47. The new H-bond is facilitated by a small local backbone shift of about 0.5 Å toward transmembrane helix B. Thus, although the Ser side chain has a choice of making a better $C_\alpha-H\cdots O$ H-bond, it opts for a different conformation that apparently improves packing or provides a more favorable traditional H-bond. We speculate that the methyl group of Thr blocks this alternative structure. Clearly, the energetic differences are quite subtle and do not support an important role for the $C_\alpha-H\cdots O$ H-bond.

Can we detect the energetic contribution of a traditional H-bond made by a Thr side chain? In prior work,[1] we made two Ala substitutions at Thr residues involved in interhelical H-bonds, which can serve as controls for this work. Although not seen in our structure (1KME), in the 1.55 Å bR structure (1C3W), T47 is seen to be involved in an indirect, water-mediated H-bond to the backbone carbonyl oxygen of F27.[11,12] The T47A mutation was found to reduce stability by 1.0 ± 0.2 kcal/mol. The T47A mutant is particularly notable because the deleted H-bond is located only three residues away from the $C_\alpha-H\cdots O$ H-bond tested here and also links helices A and B. The fact that T47A is destabilized indicates that interactions between helices A and B in this region of the protein are broken upon unfolding and that we can easily measure the contribution of even a weak, water-mediated traditional H-bond. A second Ala substitution was made at Thr46 of bR, which forms an interhelical H-bond with the O_δ atom of Asp96. This mutation diminishes stability by a substantial 2.2 kcal/mol. To test whether this drop in stability was due to the H-bond and not some other packing effect, we made a T46S mutant. As shown in Figure 2, T46S is about as stable as the wild-type protein with a $\Delta\Delta G_u$ of -0.11 ± 0.5 kcal/mol, suggesting that the H-bond has been recovered. We solved the structure of T46S at 2.0 Å resolution ($R_{free} = 26.6$) and found that the H-bond between D96 and T46S is indeed present, with a 3.0 Å distance between the Oδ atom of the Asp96 side chain and hydroxyl of Ser46 (not shown). Thus, our methods are clearly sensitive to the loss of an energetically significant H-bond and specifically in H-bonds made near Thr24.

Our results provide a first experimental test of the strength of a $C_\alpha-H\cdots O$ H-bond and do not support suggestions that $C_\alpha-H\cdots O$ bonds play an important role in protein stabilization. At best, the $C_\alpha-H\cdots O$ bond studied here, made by a hydroxyl oxygen, is energetically neutral, or its contribution is overwhelmed by other contributions, such as packing or traditional H-bonds. Because it is difficult to find $C_\alpha-H\cdots O$ H-bonds that can be tested without possible confusing influences, we have only been able to test one of these interactions (see Supporting Information). Thus, it remains possible that the rare $C_\alpha-H\cdots O$ H-bonds with ideal geometry or those made by more electronegative carbonyl oxygen atoms would show a stabilizing effect, although perhaps not as strong as suggested by theoretical calculations. We also cannot rule out the possibility of stronger energetic contributions in a natural bilayer. Even if most $C_\alpha-H\cdots O$ H-bonds are not strongly stabilizing, it is likely that they do play an important role, because they allow closer packing than would be allowed if electron orbital overlaps could not occur. For example, in the T24V mutant, the Val side chain was forced into a steric conflict with the $C_\alpha-H$ of Ala51, a close interaction that was better tolerated in the wild-type protein. Thus, $C_\alpha-H\cdots O$ H-bonds may facilitate packing rather than provide a strongly favorable energetic stabilization of the folded structure.

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Supporting Information Available: Methods, H-bond details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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