Structural and thermodynamic basis of proline-induced transmembrane complex stabilization

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In membrane proteins, proline-mediated helix kinks are indispensable for the tight packing of transmembrane (TM) helices. However, kinks invariably affect numerous interhelical interactions, questioning the acceptance of proline substitutions and evolutionary origin of kinks. Here, we present the structural and thermodynamic basis of proline-induced integrin αIβ3 TM complex stabilization to understand the introduction of proline kinks in membrane proteins. In phospholipid bicelles, the A711P substitution in the center of the β3 TM helix changes the direction of adjacent helix segments to form a $35 \pm 2^\circ$ angle and predominantly repacks the segment in the inner membrane leaflet due to a swivel movement. This swivel repacks hydrophobic and electrostatic interhelical contacts within intracellular lipids, resulting in an overall TM complex stabilization of $-0.82 \pm 0.01$ kcal/mol. Thus, proline substitutions can directly stabilize membrane proteins and such substitutions are proposed to follow the structural template of integrin αIβ3(A711P).

In the evolution of globular proteins, structural complexity and functionality can be increased by combining independently folding protein domains. In contrast, in membrane proteins, individual intramembraneous domains are not apparent beyond transmembrane (TM) helices and an increase in complexity necessitates an increase in the overall number of TM helices. In the human genome, multi-pass (polytropic) membrane proteins are predicted to exhibit an average number of 6.6 TM helices and to contain up to 37 TM helices. To maximize the available structural repertoire, TM helices must cross each other at non-zero angles. However, with increasing distance from helix-helix crossing points, sidechains will lose interhelical contacts. Apparently, this downside is compensated by introducing helix kinks and by wedging either non-helical residues or additional helices into a helix-helix interface (Fig. 1). In contrast to wedges, helix kinks may be created by a single point mutation that introduces proline. The fusion of the proline sidechain to the backbone nitrogen atom and the loss of helical hydrogen bonding introduces a helix kink of varying severity. Mutations to proline consequently may have played a central role in the evolution of membrane proteins.

Indirect support for this hypothesis is abundant. Inspection of membrane protein structures reveals that helix kinks are frequently centered around proline residues (Fig. 1a,b). Even for non-proline kinks, it is likely that a proline first initiated this conformation but became redundant when tertiary contacts solidified the kink conformation. The important function of prolines further extends to preventing membrane protein misfolding. Despite the benefit of prolines, their evolutionary origin is unclear as proline substitutions are difficult to establish. TM sequences from the Human Gene Mutation Database have one of the highest phenotypic incidences for proline substitutions. Moreover, in the seven-helix bundle protein bacteriorhodopsin, 15 proline substitutions were examined and all were found to destabilize the protein. Similarly, in the glycoporphin A homodimer, proline scanning of the TM helix only destabilized the protein. While protein stability may be recoverable by subsequent mutations, the extensive structural perturbations created by the sidechain geometry of proline invariably make such a pathway challenging. In comparison, an initially stabilizing kink followed by destabilizing, adaptive

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bicelles by isothermal titration calorimetry. We found a stabilization of itself. Thus we determined the thermodynamic stability of the wild-type TM complex stability, termed interactions.

Specifically, the inactive ectodomains and associated TM complex stabilize each 

In the family of integrin adhesion receptors, the TM complex between and subunits constrains the receptor in its inactive conformation. Specifically, the inactive ectodomains and associated TM complex stabilize each 

To understand the basis of , we determined the structure of the TM complex in isotropic phospholipid bicelles by multidimensional heteronuclear NMR spectroscopy. In the structure
Specifically, thermodynamic stabilities of four point mutations between the wild-type αIIbβ3 (ΔΔG°,αIIbβ3) and increased towards the C-terminus (Fig. 3c).

On the other hand, in the IMC changes in interhelical distance and swivel orientation were encountered. These changes altered significantly about the helix axis relative to wild type (swivel movement) took place (Fig. 3c). Changes in inter-helical segment was largely invariant, making it suitable to superimpose αIIbβ3 and αIIbβ3(mutant) coordinates to illustrate long-range structural differences. Within the dimer, the A711P substitution caused a 35° kink in the 3 helix. The impact of this kink was minimized by maintaining αIIb interhelical packing against ΔΔG°,αIIbβ3(Asp723) interactions (Fig. 3a).

In the wild-type αIIbβ3 TM complex, we used selectively methyl-labeled protein and deuterated lipids to obtain interhelical NOE distance restraints. Upon inspecting this structure, we predicted that it is possible to detect a similar number of distance restraints by measuring NOEs between backbone 1H and sidechain 1H nuclei across the helix-helix interface. We thus combined one perdeuterated and one protonated subunit in protonated lipids. Additionally, as described previously, we cross-linked the complex outside of the TM region by a disulfide bond to maximize the concentration of dimer, to suppress residual monomer signals and to improve dimer lineshapes. This approach permitted the detection of interhelical NOEs up to 1H-1H' pairs (Fig. 2), albeit only in the vicinity of glycines packed in the dimerization interface. The reduced range of 1H-1H' as opposed to 1H-CH3-H2O distances is mitigated by the high rigidity of backbone 1H nuclei compared to sidechain 1H nuclei. Moreover, it was further compensated by observing intersubunit NOEs to the indole 1H nuclei of αIIb(W968) and β3(W715), which are located at the N- and C-helix termini, and by detecting NOEs to the aromatic ring of αIIb(F993) in fractionally deuterated samples (Fig. 2). Membrane proteins show an abundance of aromatic residues in the membrane-water interface, which makes the presented approach effective for the structural determination of membrane proteins with packed glycines in the presence of protonated lipids or detergents. Further structural restraints included H-N residual dipolar couplings collected for the perdeuterated complex. An ensemble of 20 structures was calculated by simulated annealing with a coordinate precision of 0.33 Å for backbone heavy atoms (Supplementary Figure 1 and Supplementary Table 1).

In the wild-type αIIbβ3 TM complex, two association motifs were differentiated. The outer membrane clasp (OMC) is characterized by sidechain packing into the helix grooves created by αIIb(G972), αIIb(G976) and β3(G708). The inner membrane clasp (IMC) is characterized by the wedging of αIIb(Phe992-Phe993) to connect the separating TM helices and to maximize electrostatic αIIb(Arg995)-β3(Asp723) interactions (Fig. 3a). In the αIIbβ3(A711P) TM complex, these interactions were maintained albeit with changes. The 15N chemical shift differences between αIIb when complexed with either β3 or β3(A711P) illustrated that structural changes predominantly took place for IMC residues and residues that pack near the mutation site (Fig. 3b). The αIIb(W967-L979) helical segment was largely invariant, making it suitable to superimpose αIIbβ3 and αIIbβ3(A711P) coordinates to illustrate long-range structural differences. Within the dimer, the A711P substitution caused a 35 ± 2° kink in the 3 helix. The impact of this kink was minimized by maintaining αIIb interhelical packing against ΔΔG°,αIIbβ3(Asp723) while distributing the changes in 3 helix directions to both the OMC and IMC (Fig. 3a). In the OMC, no significant rotation about the helix axis relative to wild type (swivel movement) took place (Fig. 3c). Changes in inter-helical sidechain distances were apparently compensated by modifications of sidechain conformations (Fig. 3c). On the other hand, in the IMC changes in interhelical distance and swivel orientation were encountered. These changes altered αIIb contacts with β3 residues L712, W715, K716, I719 and D723 in the dimerization interface and increased towards the C-terminus (Fig. 3c).

To achieve a quantitative context for discussing changes in sidechain contacts, we determined changes in thermodynamic stabilities of four point mutations between the αIIbβ3(A711P) and αIIbβ3 TM complexes. Specifically, ΔΔG°,αIIbβ3(mutant) = (ΔG°,αIIbβ3(mutant) - ΔG°,αIIbβ3) and (ΔG°,αIIbβ3(A711P) - ΔG°,αIIbβ3(A711P)) was quantified to compare the disturbance created by a mutation relative to its respective αIIbβ3 and αIIbβ3(A711P) reference structure. In accordance with largely invariant OMC interactions, ΔΔG° was small for αIIb(G972A) with...
0.16 ± 0.03 kcal/mol (Fig. 3d and Table 1). In the IMC, the swivel for β3(L712) centered its sidechain more directly in the dimerization interface (Fig. 3c,d) and a ΔG°′ of −0.34 ± 0.01 kcal/mol revealed improved sidechain packing. Interestingly, β3(W715) moved in such a way that its pyrrole ring position in αIIbβ3 was replaced by its benzene ring in αIIbβ3(A711P) (Fig. 3c). This swap heightened hydrophobic interactions with αIIb(Phe993) and, with ΔG°′ = −0.51 ± 0.04 kcal/mol for β3(W715Y), contributed to TM complex stabilization. The swivel of the IMC helix segment of β3 rotated Lys716 towards the dimerization interface (Fig. 3c), which allows more favorable hydrogen bonding with αIIb(Phe992/CO) relative to the wild-type structure. Unfortunately, the strongly destabilizing nature of β3(K716) substitutions\(^{18}\) did not allow the direct quantification of ΔG°′ at this site.

β3(Ile719) engages in hydrophobic packing below the wedged aromatic rings and its increased distance from αIIb in the αIIbβ3(A711P) TM complex is expected to be destabilizing (Fig. 3d). Likewise, the increased distance of β3(D723) from αIIb requires an adjustment of the αIIb backbone conformation to make electrostatic contacts with αIIb(R995) (Fig. 3d). ΔG°′ of 0.8 ± 0.2 kcal/mol for αIIb(R995A) confirmed the destabilizing nature of this adjustment.

As is the case with β3(A711P), prolines in membrane protein structures are frequently encountered near the center of TM helices\(^{4,22,23}\). Based on the αIIbβ3(A711P) TM complex structure, we propose a general scheme for incorporating proline kinks in membrane proteins: maintain interhelical packing close to the proline kink and predominantly repack either the helix segment preceding or succeeding the kink. In case of αIIbβ3, the OMC with glycine packing interactions was largely maintained (Fig. 3a,b), which is likely of general validity due to the
high structural specificity of this interaction. With respect to β3(G708), A711P created a GXXP motif. Proline generally kinks away from the H-bond that is lost (Fig. 3c), which makes the GXXP spacing well suited for heterodimeric helix-helix packing. In the repacked helix segment, the increasing separation of interhelical interactions tends to diminish interhelical contacts. To achieve a net stabilization of helix-helix interactions, contacts that remain within sidechain packing distances must be optimized and, evidently, the gain in stability must supersedes the destabilization from compromised sidechain contacts. In case of αIIbβ3(3711P), interactions within two helix turns C-terminal to the proline substitution were optimized (Fig. 3d and Table 1). Additionally, based on the β3(A711P)-induced chemical shift changes of αIIb (Fig. 3b), favorable contributions from any repacking of β3(G708) with αIIb(L980) cannot be excluded.

The alternative to maintaining interhelical contacts near the proline kink would be to preserve interactions at the TM helix termini. When inspecting this possibility for αIIbβ3(3711P), it is apparent that mostly αIIb(3795)-β3(D723) benefits whereas packing on β3(G708) and αIIb(G796) would be less intimate (Fig. 3e). This mode of interaction appears generally inferior as it creates a packing void at the β3 helix centre that is difficult to fill even when more TM helices were to be added. Despite the relatively complex and extensive packing of the integrin αIIbβ3 TM complex (Fig. 3), β3(A711P) revealed that it is not as well packed as possible. This is perhaps not surprising for two reasons. First, to accomplish the allosteric regulation of the receptor, ΔG°TM must be balanced with the affinity of intra- and extracellular receptor agonists and with the stability of the inactive versus the active ectodomains. Secondly, the increase in ΔG°TM came at the expense of αIIb (R995)-β3(D723) destabilization. This interaction is disrupted during talin-mediated integrin activation25. With its reduced importance for TM complex stability in αIIbβ3(3711P), talin is now unable to activate the receptor in its presence26. In sum, we have revealed the structural and thermodynamic requirements for incorporating proline into TM helix-helix interactions and gained insight into constraints that underlie the evolution of such kinks.

**Methods**

**NMR spectroscopy.** The disulfide-linked αIIb(A963C)–β3(G690C/A711P) dimer was prepared applying published protocols27 and incorporated human integrin sequences αIIb(A958-P998) and β3(P685-F727) with αIIb(C687S). Peptide-derivatized peptides were produced using 99% d7-glucose, 99% 15ND4Cl and 99% D2O. A fractionally deuterated [1H]31C13N-αIIb(A963C)–β3(G690C/A711P) sample was prepared by growing E. coli cells in 60% D2O using protonated precursors. Freeze-dried peptide was reconstituted in 320 μL of 350 mM 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), 105 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 6% D2O, 0.02% w/v NaN3 buffered by either 25 mM NaH2PO4/Na2HPO4, pH 7.4 or 25 mM HEPES NaOH, pH 7.4 for a final concentration of 0.8 mM and bicelle q-factor of 0.3.

Starting from the [1H, 15N, 13Cα, 13Cβ, and 15C′] assignment of the αIIbβ3 TM complex and the β3(A711P/K716A) backbone assignments of [1H, 15N, 13Cα-1H/15N-αIIb(A963C)–H413C/15N-β3 (G690C/A711P) were acquired employing HNCA, HNCO, HNCA CB and NOEY-TROSY experiments. 11N-edited NOEY-TROSY experiments using [1H, 15N-αIIb(A963C)–313B(G690C/A711P) or αIIb(A963C)–2H/13C/15N-αIIb(A963C)–313B(G690C/A711P), an aromatic 13C-edited NOEY-HSQC experiment (mixing time 150 ms) was recorded. Sidechain assignments started again from the αIIbβ3 TM complex and were similar to the aforementioned NOEY spectra. In a general case, NOEY experiments for [1H, 15N-α–1H/15N-β] and [1H, 15N-α–1H/15N-β] can establish sidechain assignments in combination with standard experiments. Sidechain and NOE assignments were carried out manually using the program CARA. H-N residual dipolar couplings (RDC) were measured twice in compressed polyacrylamide gels (scalar product 0.983) using [1H, 15N, 13Cα, 13Cβ, and 15C′] assignment of the αIIbβ3 TM complex and the β3(A711P/K716A) backbone assignments of [1H, 15N, 13Cα-1H/15N-αIIb(A963C)–H413C/15N-β3 (G690C/A711P) dimer28. All NMR experiments were carried out on a cryoprobe-equipped Bruker Avance 700 spectrometer at 40 °C.

**Structure calculation of the integrin αIIbβ3(3711P) TM complex.** Structure calculations were carried out by simulated annealing, starting at 3000 K using the program XPLOR-NIH27. Backbone torsion angle restraints were extracted from 13N, 13Cα, 13Cβ, and 15C′ chemical shift patterns28. With experimental uncertainties, H-N RDCs measured for the αIIbβ3(3711P) TM dimer fitted the αIIb and β3(A711P/K716A) TM monomer structures29. This congruence permitted the use of H-N, Cα-C′, N-C′ RDCs measured for these monomers to further restrict the individual αIIb and β3(3711P) backbone conformations. An employed torsion angle potential of mean force29 was biased to use the experimental χα angles detected in the monomeric αIIb and β3A(711P/K716A) TM segments, which mostly corresponded to their default values. Moreover, the sidechains of αIIb(Phe992) and β3(Lys176) were adjusted to snorkel. Aside from standard force field terms for covalent geometry (bonds, angles, and improper dihedrals) and nonbonded contacts (Van der Waals repulsion), dihedral angle restraints were implemented using quadratic square-well potentials. In addition, a backbone-backbone hydrogen-bonding potential was employed29. A quadratic harmonic potential was used to minimize the difference between predicted and experimental residual dipolar couplings (RDC, ΔD). The final values for the force constants of the different elements in the simulated annealing target function were as previously described30. Supplementary Table 1 summarizes the structural statistics for all 20 calculated structures. The structures together with the energy-minimized average structure and structural constraints have been deposited in the Protein Data Bank and BMRB with accession numbers 2n9y and 25920, respectively.

**Isothermal titration calorimetry.** ITC measurements of the peptides listed in Table 1 were carried on a Microcal VP-ITC calorimeter. 10 μM of β3 peptide in the 1.425 ml sample cell was titrated with αIIb peptide by injecting 9 μl aliquots over a period of 10 s each. Measurements were carried out in 43 mM 1,2-dihexanoyl-sn-glycerol-3-phosphocholine (DHPC), 17 mM 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), 1 M NaCl buffered by either 25 mM NaH2PO4/Na2HPO4, pH 7.4 or 25 mM HEPES NaOH, pH 7.4 for a final concentration of 0.8 mM and bicelle q-factor of 0.3.
(POPC), 25 mM NaH₂PO₄/Na₂HPO₄ pH 7.4 at 28 °C. Prior to data analysis, the measurements were corrected for the heat of dilutions of the αIIB and β3 peptides. The αIIB/β3 complex stoichiometry was fixed at 1:1 and the reaction enthalpy (ΔH°) and Kₓₓ were calculated from the measured heat changes, ΔH, as described previously. The entropy change, ΔS°, is obtained as (ΔH° − ΔG°)/T.

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Author Contributions
T.S. and T.S.U. collected and analyzed NMR data and performed structure calculations. A.J.S. collected and analyzed ITC data. T.S.U. prepared the manuscript with input from all authors.
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