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Ala-insertion scanning mutagenesis of the glycophorin A transmembrane helix: A rapid way to map helix–helix interactions in integral membrane proteins

ISMAEL MINGARRO,[1] PAUL WHITLEY,[1] MARK A. LEMMON,[2] AND GUNNAR VON HEIJNE[1]

[1] Department of Biochemistry, Stockholm University, S-106 91 Stockholm, Sweden
[2] Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, New York 10016

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Abstract

Alanine insertions into the glycophorin A transmembrane helix are found to disrupt helix–helix dimerization in a way that is fully consistent with earlier saturation mutagenesis data, suggesting that Ala-insertion scanning can be used to rapidly map the approximate location of structurally and/or functionally important segments in transmembrane helices.

Keywords: α-helix; helix packing; membrane protein; mutagenesis

According to the "two-stage" model for membrane protein assembly (Popot & Engelman, 1990), individually stable transmembrane α-helices develop during the initial insertion of a nascent chain into the membrane, and these preformed helices then pack together during the second stage to form the fully folded structure. Formation of the secondary structure (i.e., the transmembrane helices) and the tertiary structure (i.e., the tightly packed helix bundle) are thus effectively decoupled processes, with little feedback from the tertiary to the secondary structure level.

Although the sequence characteristics that control the membrane insertion step are quite well understood (von Heijne, 1994), much less is known about the molecular interactions that drive helix–helix packing in a lipid environment. This is due both to the lack of good assays for helix–helix interactions, and to the difficulty of obtaining high-resolution structural information for membrane proteins.

As an alternative to direct structure determination methods, molecular genetic approaches to the problem of helix–helix packing have been developed over the past few years (Popot & Saraste, 1995). In particular, saturation mutagenesis has been used to map the helix–helix interfaces in the glycophorin A (GpA) homodimer (Lemmon et al., 1992b) and in the phospholamban homopentamer (Arkin et al., 1994). In the case of GpA, the analysis is especially simple because the homodimer is stable at high temperatures even in detergents such as SDS (Lemmon et al., 1992a). Mutationally induced shifts in the monomer-dimer equilibrium are thus easy to assay by SDS-PAGE.

From an analysis of 282 mutations in the GpA transmembrane helix, seven residues aligned on one face of the helix were found to be critical for dimer formation (Lemmon et al., 1992b) (Fig. 1). All other residues in the helix could be replaced simultaneously by leucines with no detrimental effects on dimerization (Lemmon et al., 1994), providing strong support for a 3D model based on a right-handed supercoil (Treutlein et al., 1992).

Although similar analyses can, in principle, be conducted on transmembrane helices in any membrane protein provided a good assay for structural and/or functional integrity is available, the mutagenesis work involved is considerable. We have therefore sought a way to rapidly identify the most critical segment of a transmembrane helix, which could then be further analyzed by a more focused saturation mutagenesis strategy.

One such approach is insertion mutagenesis. The rationale is that insertion of, say, an alanine (i.e., a residue with a high helix propensity) into a transmembrane helix will displace the residues on the N-terminal side of the insertion by 100° relative to those on the C-terminal side of the insertion, thus effectively disrupting a helix–helix packing interface involving residues on both sides of the insertion (Fig. 1B). If the insertion is outside the critical interface residues, on the other hand, no detrimental effects should be observed. Alanine insertions into helices in globular proteins have been found to sometimes cause looping-out of the inserted residue with only minor effects on helix packing (Heinz et al., 1994); however, in a lipid environment, looping-out should be strongly disfavored because a large number of hydrogen bonds would need to be broken. In fact, Ala

Reprint requests to: Gunnar von Heijne, Department of Biochemistry, Stockholm University, S-106 91 Stockholm, Sweden; e-mail: gunnar@biokemi.su.se.

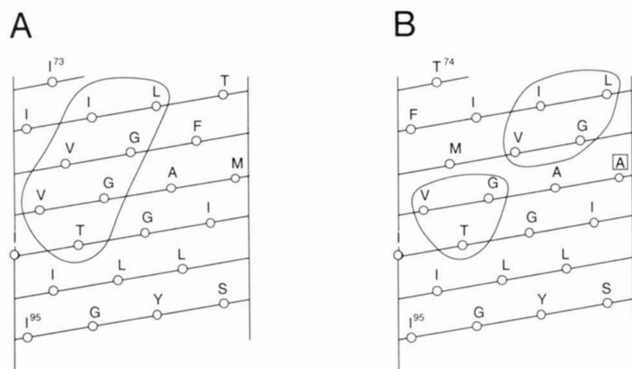

Fig. 1. **A:** The seven critical interface residues in the GpA transmembrane helix mapped onto a helical net diagram. The N-terminus is on top. **B:** Disruption of the interface motif by an Ala insertion (boxed) between positions 81 and 82.

insertions into helix C of bacteriorhodopsin suggest that the only structural consequence is a relative displacement of residues, but no major conformational perturbations (Marti et al., 1992).

Results and discussion

To systematically test the structural consequences of insertions in a well-defined system, nine initial alanine insertions were made throughout the GpA transmembrane helix (Fig. 2A). Mutant GpA C-terminal domains including the transmembrane helix (residues 60–131) and a terminal His_6-tag were fused to staphylococcal nuclease and cloned into plasmid pT7SN/GpA (Lemmon et al., 1992a). All proteins were expressed in *Escherichia coli* BL21(DE3) (containing plasmid pLYS-S) from the T7 promoter, and purified in one step using a Ni-NTA agarose resin.

As shown in Figure 2B, the results correlated well with the positions of the critical residues identified previously. Thus, Ala insertions in positions 82 and 86, inside the central GVxxGVxxT motif, nearly abolished dimerization, whereas insertions outside this motif gave rise to a more graded response. To pin-point the ends of the dimerization motif, further Ala insertions were made in positions 76, 78, 87, and 89 (Fig. 2C). As expected, the Ala 87 mutation again abolished dimerization, whereas the Ala 89 mutation had almost no effect. Thr^{87} can thus be precisely identified as the C-terminal residue in the dimerization motif directly from the Ala-insertion results.

The N-terminal end of the motif is not so cleanly identified, because mutants Ala 75–Ala 78 only reduce dimerization by about 50% compared to the wild-type protein. This suggests that Leu^{75} and Ile^{76} are less critical than the other interface residues and can be replaced by other hydrophobic residues. Thus, in the A77 mutant, the equivalent positions of residues Leu^{75} and Ile^{76} are occupied by Ile-Ala. By itself, the $Leu^{75} \rightarrow Ile$ mutation is somewhat disruptive (41% dimer; data not shown) and the $Ile^{76} \rightarrow Ala$ mutation is highly disruptive (Lemmon et al., 1992b), yet the A77 insertion still allows 40% dimer formation. However, mutants $Leu^{75} \rightarrow Ile$ and $Ile^{76} \rightarrow Ala$ can form a stable heterodimer (our unpublished data), as can mutants $Leu^{75} \rightarrow Val$ (another disruptive mutation) and $Ile^{76} \rightarrow Ala$ (Lemmon et al., 1992b), suggesting that certain mutations in positions 75 and 76 may be compensatory. To test this further, the double

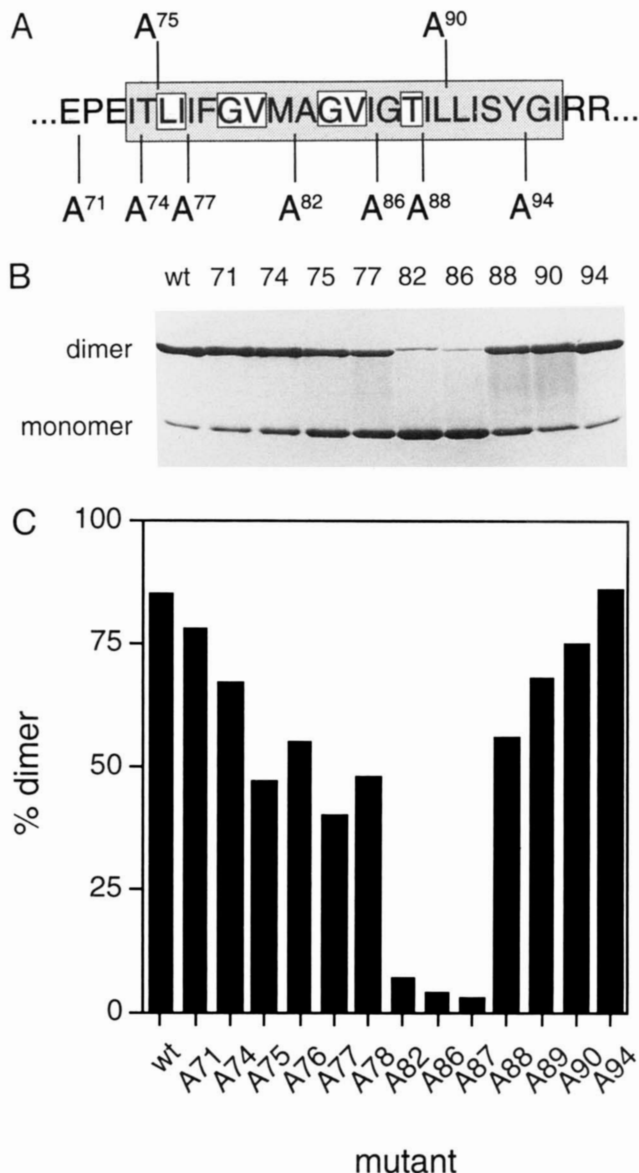

Fig. 2. Mapping of the critical segment in the GpA transmembrane helix by Ala-insertion scanning. **A:** Positions of the insertion mutations in the GpA transmembrane helix. The seven critical interface residues defined by saturation mutagenesis (Lemmon et al., 1992b) are boxed. **B:** SDS-PAGE analysis of the wild-type (wt) GpA transmembrane helix and Ala-insertion mutants. The position of the insertion is shown above each lane. **C:** Percent dimer for the wild-type GpA helix and Ala-insertion mutants.

mutant $Leu^{75} \rightarrow Val/Ile^{76} \rightarrow Ala$ was made; this dimerized nearly as efficiently as the A77 insertion mutant (33% versus 40%; data not shown). We also note that Leu^{75} and Ile^{76} were not required for dimerization when the GpA dimerization motif was introduced into the *neu* transmembrane helix (Lemmon et al., 1994). In conclusion, both Ala-insertion scanning and replacement mutagenesis suggest that the most critical interface residues are Gly^{79}, Val^{80}, Gly^{83}, Val^{84}, and Thr^{87}, and that a number of substitutions of Leu^{75} and Ile^{76} still allow significant dimer formation.

Insertion mutagenesis should, in general, be more disruptive to helix–helix interactions than replacement mutagenesis and

thus better suited for an initial screen for functionally or structurally important segments in transmembrane helices. A standard alanine-replacement scan of the GpA transmembrane helix would only have picked up residues Leu^{75}, Ile^{76}, Gly^{83}, and Thr^{87}, and a Cys-scan only residue Ile^{76} (Lemmon et al., 1992b). Had the Ala-insertion analysis been conducted prior to saturation mutagenesis, the latter could have been focused on a stretch of ~10 residues rather than on the whole 23-residue long transmembrane segment. In fact, because the C-terminal residue of the dimerization motif (Thr^{87}) can be identified directly from the Ala-insertion results (as can presumably the C-terminal residue Gly^{79}), other candidate interface residues such as Gly^{83} could have been identified easily on a helical net diagram (cf., Fig. 1A)

Cys-scanning of the LacY protein has so far identified only a handful of functionally critical residues of more than 300 that have been mutated (Kaback et al., 1994); in the Trg chemoreceptor transmembrane segments, only one of 54 Cys mutants was found to be functionally inactive (Lee & Hazelbauer, 1995); and random mutagenesis of the diacylglycerol kinase protein uncovered only a handful of functionally critical residues of more than 120 mutated residues (Wen et al., 1996). In general, integral membrane proteins thus appear to be very resilient to replacement mutagenesis, and we suggest that an Ala-insertion scan followed by saturation mutagenesis may be a more efficient strategy for mapping helix-helix interactions.

Materials and methods

Plasmid constructs

Construction of the plasmids encoding the chimeric protein (SN/GpA) is described in Lemmon et al. (1992a). The *Hin*d III-*Bam*H I fragment from pSN/GpA was cloned into the *Hin*d III-*Bam*H I sites of M13mp18. For purification purposes, a His_6 tag was added by site-directed mutagenesis at the extreme C-terminus of the coding region. All site-directed mutagenesis was performed by the Kunkel method (Kunkel, 1985) as modified by Geisselsoder (Geisselsoder et al., 1987). After mutagenesis, the mutated *Hin*d III-*Bam*H I fragment was cloned between the *Hin*d III and *Bam*H I sites in the high level expression vector pT7SN/GpA (Lemmon et al., 1992a).

Expression, extraction, and purification of SN/GpA

For SN/GpA production, pT7SN/GpA was transformed into *E. coli* BL21(DE3) strain containing the plasmid pLYS-S (Novagen). Colonies were picked and grown to logarithmic phase in LB at 37 °C. Cultures were diluted 1:100 into terrific broth (TB), and were grown to an A_{600} of 2.5. Isopropyl β-D-thiogalactopyranoside was then added to 0.8 mM, and growth was continued for a further 3 h. After harvesting by centrifugation, cells were resuspended 1:20 in 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1 mM PMSF, 0.025% NaN_3. Cells were lyzed by three rounds of freeze-thaw in this suspension, lysis being aided by the constitutive expression of T7 lysozyme directed by the pLYS-S plasmid. $CaCl_2$ was added to 10 mM to activate the nuclease moiety of SN/GpA. The resulting cellular DNA hydrolysis was complete after incubation for 15 min on ice. The lysate was clarified by centrifugation, and protein was extracted from the resulting pellet by sonication for 1 min at 4 °C (at 1:10 dilution) in a solution containing 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, TBS, 1% SDS, 1 mM PMSF. Nonsolubilized products were removed by centrifugation and proteins were purified using Ni-NTA agarose resin (Qiagen). Samples were washed with 10 mM imidazole, 0.5% SDS in TBS, and eluted with 100 mM imidazole in the same solution. The presence of the His_6 tail was found not to affect the dimerization efficiency and the chimeric product had the expected mobility on SDS-PAGE (data not shown). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard.

SDS-PAGE analysis

Two microliters of a 1 mg/mL (38 μM) solution of purified protein was loaded onto 12% SDS polyacrylamide gels (Hoefer). The loading buffer contained 2% SDS, and samples were boiled for 5 min prior to electrophoresis. Gels were stained with Coomassie blue, and the percentages of monomer and dimer were estimated by laser densitometry.

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