Sequence Specificity in the Dimerization of Transmembrane α-Helices†

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ABSTRACT: While several reports have suggested a role for helix–helix interactions in membrane protein oligomerization, there are few direct biochemical data bearing on this subject. Here, using mutational analysis, we show that dimerization of the transmembrane α-helix of glycophorin A in a detergent environment is spontaneous and highly specific. Very subtle changes in the side-chain structure at certain sensitive positions disrupt the helix–helix association. These sensitive positions occur at approximately every 3.9 residues along the helix, consistent with their comprising the interface of a closely fit transmembranous supercoil of α-helices. By contrast with other reported cases of interactions between transmembrane helices, the set of interfacial residues in this case contains no highly polar groups. Amino acids with aliphatic side chains define much of the interface, indicating that precise packing interactions between the helices may provide much of the energy for association. These data highlight the potential general importance of specific interactions between the hydrophobic anchors of integral membrane proteins.

The importance of noncovalent interactions between transmembrane α-helices in the assembly and oligomerization of integral membrane proteins is becoming increasingly apparent (Lemmon & Engelman, 1992). For example, in the assembly of the T-cell receptor complex (TCR),[1] interaction between TCRα and CD3δ is mediated by the single transmembrane domains of these two subunits, partly through the proposed formation of interhelical salt bridges (Manolios et al., 1990; Cosson et al., 1991). Furthermore, in the assembly of the Fcγ receptor of human natural killer cells (FcγRIIIA or CD16), noncovalent interactions are suggested to occur between the transmembrane domain of the α subunit of this receptor and the equivalent domain of the ζ subunit of TCR/CD3 (Kurosaki & Ravetch, 1989; Lanier et al., 1989). Each of these transmembrane domains contains a single aspartic acid residue, which is important for assembly (Lanier et al., 1991; Romeo & Seed, 1991). However, in addition, mutation of a leucine to an isoleucine in the transmembrane domain of the ζ subunit has been shown to reduce its interaction with FcγRIIIA-α by 65% (Kurosaki et al., 1991). The ζ subunit itself exists as a disulfide-linked homodimer, formation of which is mediated by the transmembrane domain. It has recently been shown (Rutledge et al., 1992) that, in addition to a charged residue and a cysteine, other residues in the transmembrane domain are necessary for this dimerization. It has also been suggested (Sternberg & Gullick, 1990; Cao et al., 1992) that a subdomain within the transmembrane domain of $p185^{neu*}$, including the position at which the activating V to E mutation occurs, is at least partly responsible for the dimerization of this EGFR-like molecule and may in turn be important in transmembrane signaling. Glycophorin A (GpA) from human erythrocytes has been shown to dimerize in vitro through specific interactions mediated by its single transmembrane α-helix (Furthmayr & Marchesi, 1976; Bormann et al., 1989). By contrast with the above cases, this association involves no strongly polar groups.

Interactions between transmembrane α-helices are also important in the folding of many integral membrane proteins which cross the bilayer more than once. In the case of bacteriorhodopsin (bR), which has seven transmembrane α-helices, functional protein has been regenerated by separately reconstituting three bR peptides, corresponding to the first and second helices, respectively, and a chymotryptic fragment containing the remaining five helices in lipid bilayer vesicles and then allowing the fragments to associate (Kahn & Engelman, 1992; Popot et al., 1987). The two small peptides are independently stable as transmembrane helices (Hunt et al., 1991) and can associate with the larger five-helix chymotryptic fragment in the bilayer to regenerate the tertiary structure of the protein.

The only structural information concerning the chemical nature of such interhelical interactions comes from the X-ray crystal structures of photosynthetic reaction centers (Deisenhofer et al., 1985; Yeates et al., 1987), as well as electron crystallography (Henderson et al., 1990) and neutron diffraction (Popot et al., 1989; Engleman & Zaccaï, 1980) analysis of bacteriorhodopsin. Analysis of these data has led to the suggestion that the relative polarity of the surface and interior residues is reversed between membrane proteins and water-soluble proteins (Rees et al., 1989a). In the case of the photosynthetic reaction centers, interactions inside the proteins are similar in average polarity and packing density to those seen in the interiors of water-soluble globular proteins. The lipid-facing residues of integral membrane proteins tend, on average, to be more hydrophobic than the interior residues, this resulting in the reversed polarity. Lipid-facing residues are less well conserved between the reaction centers of different

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[1] Abbreviations: TCR, T-cell antigen receptor; EGFR, epidermal growth factor receptor; GpA, glycophorin A; GpATM, transmembrane domain of glycophorin A; bR, bacteriorhodopsin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; LB, Luria broth; HPLC, high-pressure liquid chromatography.

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species than are the protein-buried residues (Williams et al., 1986; Rees et al., 1989a,b).

In this paper we report the results of an extensive mutational analysis that defines the interface of the dimer formed by the single transmembrane α-helix of human glycophorin A (GpATM). We have previously described the use of a chimeric protein to investigate the dimerization of this hydrophobic helix, known to be helical in detergent micelles as well as in lipid bilayers (Lemmon et al., 1992). The transmembrane domain was fused to the C-terminus of staphylococcal nuclease and the resulting chimera expressed in *Escherichia coli* by utilizing a T7 expression vector (Studier et al., 1990). The chimera is readily purified and, like GpA, forms an SDS-stable dimer. This dimer is disrupted upon addition of a synthetic peptide corresponding in sequence to the transmembrane domain of GpA, with concomitant formation of a peptide–protein heterodimer (Bormann et al., 1989; Lemmon et al., 1992), and is not disrupted by addition of heterologous helical transmembrane peptides.

EXPERIMENTAL PROCEDURES

Plasmid Constructs. Molecular biological manipulations were all performed according to published protocols (Sambrook et al., 1989). Construction of the plasmids encoding the chimeric protein (SN/GpA) is described in Lemmon et al. (1992). The construct subjected to in vitro mutagenesis encoded a chimera in which amino acids 60–99 of GpA were fused to the C-terminus of staphylococcal nuclease [SN/GpA99 in Lemmon et al. (1992)]. The hydrophobic transmembrane α-helix (residues 73–95, followed by the sequence RRLI) is joined to nuclease via a 13 amino acid linker. Previous studies have shown that the linker serves only to tether the transmembrane domain to nuclease. It plays no role in the dimerization of SN/GpA (Lemmon et al., 1992).

In Vitro Mutagenesis. Mutations were generated as described (Lemmon et al., 1992) or, where convenient restriction sites existed, through incorporation of the desired mutation using the polymerase chain reaction (Mullis et al., 1986), with subsequent appropriate ligation of the PCR product into the parent vector. The mutagenic oligonucleotide contained either random sequence (NNN) or a restricted set of possible codons at the position under study. In addition, in some cases a variation on the double primer method of oligonucleotide-directed mutagenesis (Sambrook et al., 1989) was employed. Single-stranded template containing SNGpA-(–) was generated from a phagemid generated by the subcloning of SN/GpA into pBS SK$^+$ (Stratagene). The 5′ end of the general primer used for mutagenesis contained unique sequence, noncomplementary to vector sequences, while the 3′ half was complementary to pBS sequence. After extension of the second (mutant) strand, PCR was utilized to amplify this strand specifically, with one primer equivalent to the unique 5′ sequence of the general primer and the second (antisense) primer complementary to the region encoding the C-terminus of the protein. The PCR product was then digested and ligated into the parent vector. After screening, all mutated constructs were sequenced from double-stranded plasmid, using the Sequenase system (U.S. Biochemicals) (Sanger et al., 1977).

Screening of Mutants. For initial screening of mutants, the DNA products of in vitro mutagenesis, in which SN/GpA was under the control of the *phoA* promoter (pSN/GpA), were transformed into *E. coli* MM294, and overnight cultures in Luria broth (LB) were grown. Glucose–MOPS medium (Neidhardt et al., 1974) containing 0.1 mM phosphate was inoculated from these overnight cultures and grown for 8 h, leading to induction of expression of the mutated forms of SN/GpA by phosphate starvation. As previously described (Lemmon et al., 1992), SDS–PAGE of whole cells was performed, followed by Western blotting of the resulting gels with an antibody to staphylococcal nuclease. The apparent ratio of SN/GpA dimer to monomer was estimated from these blots, and mutations were classified as disrupting dimerization completely, having no effect, or having an intermediate effect. Mutations of interest, as well as all of those involving conservative substitutions, were subcloned into pET11a (Studier et al., 1990) for high levels of expression [pT7SN/GpA in Lemmon et al. (1992)]. From these constructs, proteins were expressed in *E. coli* MGT7, extracted and purified as described below. The relative effects of amino acid substitutions in these cases were then determined from Coomassie-stained SDS gels of purified protein, including 50 mM DTT in the gel loading buffer in all cases.

Purification of Mutated Chimerae. Mutated protein was produced and extracted in 1 mM NaCl, 2% Thesit, 25 mM Tris-HCl, and 1 mM EDTA, pH 7.9, as described (Lemmon et al., 1992). Thesit, available in purified form from Boehringer Mannheim Inc., replaced the similar detergent Lubrol PX in this procedure since the latter is no longer available. The extract was diluted to 100 mM NaCl with 50 mM MOPS, pH 6.8, and 2% Thesit, passed through a 0.45-μm syringe filter, and loaded on a Waters Sep-Pak CM cartridge preequilibrated with 100 mM NaCl, and 50 mM MOPS, pH 6.8. After the cartridge was washed with 10 volumes of 300 mM NaCl, 50 mM MOPS, pH 6.8, and 1.5% octyl β-glucoside, protein was eluted with the same buffer containing 700 mM NaCl. The salt concentration was then reduced by buffer exchange in a Centricon 30 concentrator (Amicon). Alternatively, the extract was loaded onto a Vydac semipreparative C4 reversed-phase HPLC column, and protein was eluted with a gradient of 0–80% acetonitrile in water, with a constant 20% 2-propanol.

RESULTS AND DISCUSSION

For each amino acid in the 23-residue-long hydrophobic stretch of the GpA transmembrane domain, the codon in the chimeric construct was subjected to oligonucleotide-directed saturation mutagenesis. Altered forms of the chimera were analyzed for their ability to dimerize in SDS–PAGE, with the assumption that this reflected an equilibrium between monomers and dimers of transmicellar α-helices (as opposed to surface-lying). The resultant changes in dimerization were interpreted in terms of structure. A total of 282 different amino acid sequences have thus been generated, and analysis of their respective propensity for dimerization illustrates some interesting points. The mutagenesis results are summarized in Figures 1 and 2. It is of note that, with the exception of G83 (where all mutations observed disrupted the dimer completely), it is possible to mutate any position in the transmembrane domain of GpA and retain at least some propensity for dimerization. Mutations were separated into three groups: those to residues with relatively hydrophobic side chains (A, C, V, L, I, M, F, W) (Figure 1); those to residues with somewhat polar side chains (Y, G, S, T) (Figure 2); and those to residues with strongly polar side chains (R,K,E,D,H,N,Q), to proline, or to stop codons (not shown). The 117 mutations which fall into the last of these groups abolish dimerization completely or almost completely. In many cases, the mobility of the monomeric protein in SDS–PAGE is also affected. This indicates that the association of

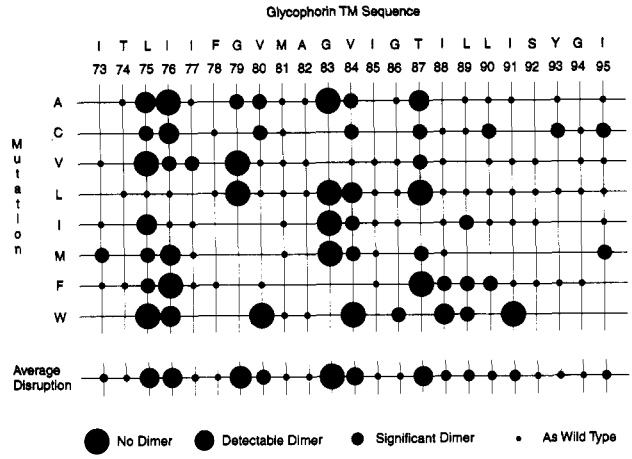

FIGURE 1: Summary of the effects of substitution with nonpolar residues (A, C, V, L, I, M, F, W) upon helix–helix association at each position along GpATM. The relative degree of disruption by each substitution is indicated by the area of the black circle at each position. Those mutations which show no detectable effect are represented by a small circle. Mutations not found have no circle at their respective grid points. The bottom row of the table presents a circle for each position, the area of which indicates the average effect of amino acid substitutions at this position upon helix–helix interaction.

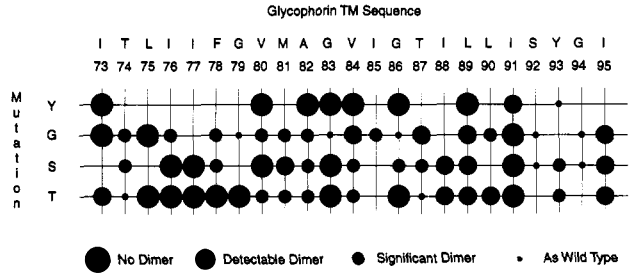

FIGURE 2: Summary of the effects of substitution with relatively polar residues (Y, G, S, T) upon helix–helix association at each position along GpATM. Details are as for Figure 1.

these mutated proteins with the detergent micelle is altered. The introduced highly polar group is likely to interact with the micelle surface, resulting in the stabilization of a monomeric state which does not resemble a transmicellar α-helix. Substitution with proline is likely to have a similar effect as a result of helix distortion that could create an unsatisfied main-chain hydrogen bond acceptor, which may interact with the micelle surface. Consequently, we are not able to make conclusions from mutations of this type with confidence.

The first two groups include 165 substitutions with residues naturally represented in the transmembrane domain of GpA (GpATM), with the addition of W and C. In none of these cases was mobility of the protein in SDS–PAGE affected, indicating that there are no significant differences in the interactions of the mutated proteins with the detergent micelle. The data presented in Figure 2 are not amenable to any straightforward interpretation. Substitution with tyrosine, the most hydrophilic of these residues from the scale of Engelman et al. (1986), results in almost complete disruption in all cases. In general, relatively polar residues in the transmembrane domain (excepto G79, G83, and T87) can be replaced with G, S, or T with relatively little disruption of the dimer. G79, G83, and T87 are excluded since they are also very sensitive to substitutions with hydrophobic residues (see Figure 1), and G86T is an exception for which we have no clear explanation. Substitution of the hydrophobic residues of GpATM with G, S, or T, however, is generally rather disruptive, possibly as a result of introducing a polar group (S and T) or exposing the peptide backbone (G). The effect seems to be more pronounced for hydrophobic residues in the regions I73–I77 and I88–I95, i.e., toward the ends of the helix, and may possibly indicate an effect at the surface of the detergent micelle. Given these difficulties with the data of Figure 2, we restrict subsequent analysis largely to the data presented in Figure 1.

It is clear from Figure 1 that even a very subtle alteration in the side-chain structure at certain positions has a profound effect upon the propensity of the helices to dimerize. For example, removal of a single methylene group from the side chain of L75, by substituting a valine at that position, prevents the helices from associating. Furthermore, mutation of I76 to either A or F, for example, disrupts the dimer almost completely, as does mutation of T87 to F or L. By contrast, similar mutations at I91 and T74 have no observed effect upon dimerization, and M81 and I85 can be replaced with a number of different residues with no detectable effect upon helix–helix association. A comparison of circular dichroism (CD) spectra (not shown) of a number of transmembrane peptides derived from the mutated chimeric protein by trypsin digestion (Lemmon et al., 1992) shows that conservative substitutions which disrupt dimerization have no detectable effect upon the α-helical secondary structure in SDS or in DMPC bilayers. We make the assumption, supported by the mutant complementation data reported below, that an amino acid side chain is likely to be at the dimer interface if its replacement with a series of side chains of similar hydrophobicity but different structure results in significant disruption of the dimer in our assay. This assay provides information only on the relative stabilities of the dimeric and monomeric states, and we cannot formally determine whether mutations which disrupt dimerization do so by destabilizing the dimer or by stabilizing the monomeric state. In the case of soluble proteins, mutations can alter ΔG for folding through effects upon the free energy of the denatured state (Shortle et al., 1990). Dimerization of transmicellar α-helices involves the loss of helix–detergent interactions and the gain of interhelix and detergent–detergent interactions. A mutation may reduce the association constant through a loss of favorable helix–helix interactions in the dimeric state, by increasing favorable helix–detergent interactions in the monomeric state, or both. We consider that disruption of helix–helix interactions by mutation of interfacial residues is the most likely explanation for our results, but we cannot formally exclude the alternative possibilities.

Alteration of both Polar and Nonpolar Residues Disrupts Dimerization. For a given native side-chain identity, the effect of mutations upon dimerization is very dependent upon its position in the sequence. Consider first the relatively polar amino acids: there are four glycine (79, 83, 86, and 94) as well as two threonine (74 and 87) residues in the transmembrane domain. Replacement of G79, G83, or T87 with any nonpolar amino acid significantly disrupts dimerization, whereas an identical set of substitutions at G86, G94, or T74 has no effect at all (see Figure 1). Interpretation of the role of the glycines in dimerization is not straightforward. It is possible that G79 and G83 play specific roles in defining the backbone structure of the interacting helices, alteration of which completely abrogates the interaction. Additionally, there may simply be a requirement for a small side chain at these positions, in order to allow close approach of the interacting helices in this region. Indeed, all substitutions studied at G83 disrupt the dimer completely, as does

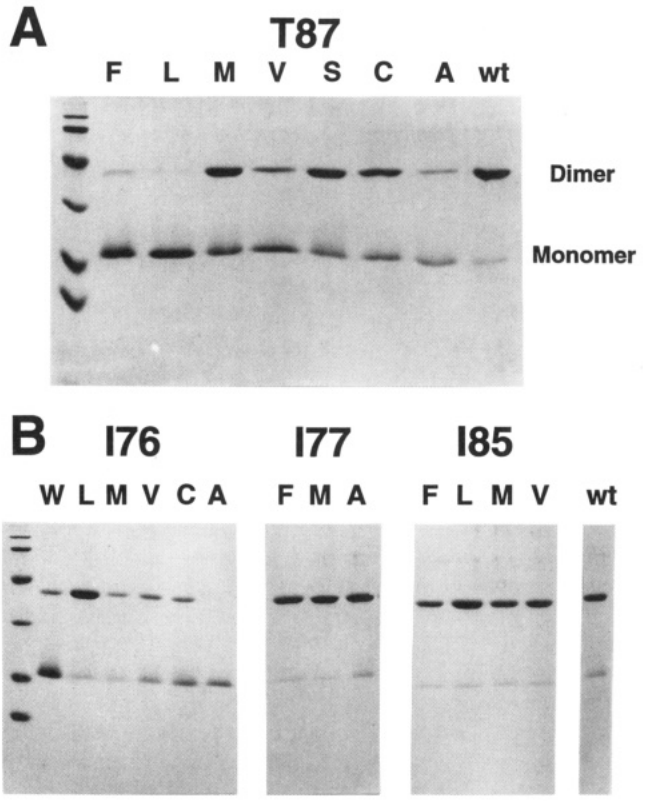

FIGURE 3: (A) 12.5% SDS–polyacrylamide gel depicting the relative dimerization of a number of mutants of T87. Protein was loaded, in sample buffer containing 50 mM DTT, at a concentration of 0.22 mg/mL, as assessed by OD_{280} of protein prepared as described under Experimental Procedures. Lanes: 2, T87F; 3, T87L; 4, T87M; 5, T87V; 6, T87S; 7, T87C; 8, T87A; 9, wild-type. Lane 1 shows low molecular mass markers (Bio-Rad) of 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa. (B) SDS–PAGE of mutants of I76, I77, and I85, demonstrating the different sensitivity of these three positions. Procedures are as in (A). Lanes: 1, molecular mass markers; 2, I76W; 3, I76L; 4, I76M; 5, I76V; 6, I76C; 7, I76A; 8, I77F; 9, I77M; 10, I77A; 11, I85F; 12, I85L; 13, I85M; 14, I85V; 15, wild-type.

substitution of G79 with any residue other that A, which is tolerated to some extent. In the modeling study presented in Treutlein et al. (1992), the two helices approach each other most closely in the region around G79 and G83, with some electrostatic attraction between the backbones of the two helices in this region. This provides one possible explanation for the requirement for a small side chain at both of these positions in the dimer. By contrast, G86 and G94 seem to play no such role. Since they can be replaced with a variety of different amino acids without effects upon dimerization, we suggest that they are less important in the interhelical interactions. In the model of Treutlein et al. (1992), the distance between the helix axes in the dimer is at least 1 Å greater in the regions of these glycine residues than at G79 or G83.

Similarly, whereas T74 appears to play no direct role in the association, accommodating all substitutions studied, T87 seems to be intimately involved (Figure 3A). Substitution of this residue with M, V, S, or C is slightly disruptive, substitution with A or G is rather more disruptive, and substitution with either F or L is almost completely disruptive. Thus, substitutions with residues that are most closely isosteric with T are the least disruptive. Significant dimerization is still observed (albeit reduced) if the hydroxyl group of T87 is replaced with a methyl group (T87V) or if the methyl group of T87 is replaced by hydrogen (T87S). Furthermore, significant dimer remains if the methyl group of T87 is removed and the hydroxyl group is replaced by a thiol group (T87C) or by $-CH_2-S-CH_3$ (T87M). However, removal of the hydroxyl group and replacement of the methyl group with a branched hydrocarbon chain (T87L and T87F) or removal of all substituents beyond the β-carbon (T87A and T87G) results in much more significant disruption. The model of the dimer (Treutlein et al., 1992) predicts the possibility of interhelical hydrogen bonding and/or strong electrostatic interactions involving the side-chain hydroxyl group of T87. The effects of mutations at this position suggest that this may indeed play a role in stabilizing the dimer. However, other factors are also likely to be important given the results presented above. A comparison of wild-type, T87S, and T87V, for example, suggests that van der Waals interactions mediated by the methyl group of T87 may also be involved in the association of the helices.

Substitution of certain nonpolar residues of GpATM can similarly disrupt the dimer. At both V80 and V84, the residues immediately following the glycine residues discussed above, some conservative substitutions result in disruption of the dimer. Substitution of W for V80 is disruptive, possibly through steric hindrance of the close approach of the two helices in this region, as discussed above. It should be noted, however, that this mutation also introduces a polar group, which may be partly responsible for this disruption, as discussed for the data in Figure 2. Mutation of V80 to A or C is slightly disruptive, which may indicate the loss of some favorable packing interactions. V84 is more sensitive. As with V80, V84W does not dimerize, and V84A and V84C are slightly disrupted. However, unlike V80L, V84L is significantly disrupted. Thus, insertion of a single methylene group in this side chain has an adverse effect upon dimerization. It may do so through hindrance of the close approach of the two helices in this region or by altering the specific packing of side chains. The fact that V84I and V84M are less disruptive would argue for the latter.

Several of the isoleucines show some sensitivity to substitution with residues with significantly larger side-chain volume. For example, substitution of I88 with F or W disrupts the dimer to some extent. I77 and I85 accommodate all conservative substitutions observed with relatively little effect upon dimerization (Figure 3B, lanes 8–14). By contrast, at I76, all conservative substitutions, with the exception of L, disrupt significantly (Figure 3B, lanes 2–7). That GpATM dimerizes to a greater extent with I or L at position 76 than with any other residues argues for specific interhelical packing interactions involving the side chain at this position. Significant alteration of the size of this side chain seems to perturb these interactions. Substitution such that the branch occurs at the γ-carbon rather than the β-carbon in the side chain appears to have no effect (I76L). Removal of a single methylene group (I76V) permits significant dimer to form. However, substitution with unbranched residues (I76A, I76C, I76M) or those which extend beyond the δ-carbon (I76F, I76W) results in significantly reduced formation of dimer.

A similar situation is seen for the three leucine residues in the helix. Most conservative substitutions at L89 and L90 have relatively little effect upon dimerization. However, at L75 similar substitutions have a significant effect. For example, removal of a single methylene group (L75V) abrogates dimerization completely, and substitution such that the branch position is at the β-carbon rather than the γ-carbon is significantly disruptive (L75I). In this regard it is of note that L75F, a substitution with a γ-branched side chain, dimerizes as well as L89F and L90F, whereas L75I forms

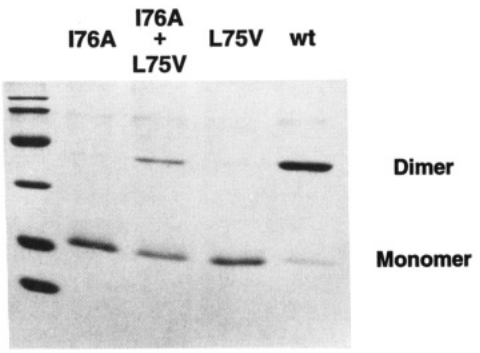

FIGURE 4: SDS–PAGE, as in Figure 3A, demonstrating the heterodimer formation between L75V and I76A. Lanes: 1, molecular mass markers; 2, I76A; 3, I76A/L75V; 4, L75V; 5, wild-type. The total concentration of chimeric protein loaded in each lane was 0.22 mg/mL.

significantly less dimer than L89I or L90I. We conclude that the side chain of L75 is intimately involved in van der Waals interaction with the opposing helix in the dimer.

A Heterodimer Will Form between Two Different Nondimerizing Mutants. One piece of data suggests that the side chain of L75 interacts, at least in part, with that of I76 on the adjacent helix. In Figure 4, an SDS–polyacrylamide gel is presented, in which lane 2 shows the mutant I76A and lane 4 the mutant L75V, both loaded at 0.22 mg/mL. In both cases, virtually no dimer is seen. However, after these two mutants are mixed and the mixture is loaded at the same total protein concentration, approximately 40% of the protein runs at a position corresponding to dimer (Figure 4, lane 3). Thus, the heterodimer formed between I76A and L75V is significantly more stable than the homodimer of either mutant. Addition to this mixture of a 10-fold molar excess of a synthetic peptide corresponding to native GpATM disrupts the I76A/L75V heterodimer, with the resultant formation of peptide–protein heterodimer (not shown). We assume that the inability of L75V and I76A to form homodimers results from a loss of favorable interhelical packing interactions. Since both mutations result in the reduction of side-chain volume, yet heterodimer will form, we consider it unlikely that these residues interact in the dimer directly with their respective counterparts on the adjacent helix. Rather, we suggest that L75 of one helix interacts, at least in part, with I76 of the opposing helix. To illustrate the argument, a dimer of native GpATM helices (helix 1 and helix 2) can be considered. If the simplifying assumption is made that two sites of interaction are represented by L75(1)–I76(2) and I76(1)–L75(2), then it may be argued that mutation of L75 will disrupt both of these sites and that this is sufficient to abrogate homodimer formation. Mutation of I76 will have a similar effect upon this mutant homodimer. However, if I76 of helix 1 and L75 of helix 2 are mutated, then only one of these sites of interaction will be perturbed in a heterodimer of helices 1 and 2 [the L75(1)–I76(2) interaction], and the other will remain as it is in the wild-type dimer. That this loss results in a relatively small degree of disruption is suggested by the fact that significant heterodimer is formed between I76A and L75V (Figure 4, lane 3). Similarly, in considering the formation of heterodimer with the wild-type peptide, again only one of the two sites in our simplification will be perturbed, and some heterodimer will form.

Mixtures of a large number of nondimerizing mutants have been analyzed for their ability to form heterodimers. Apart from L75V/I76A, the only example that was found was the mixture of L75V/I76W, the heterodimer for which is less

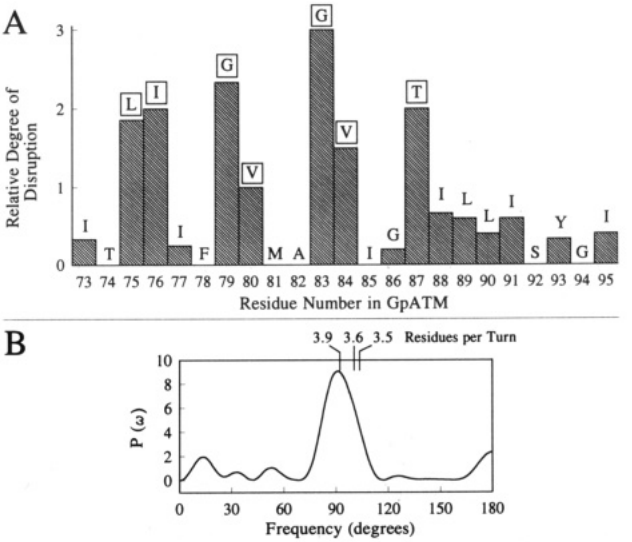

FIGURE 5: (A) Histogram showing the relative degree of disruption of the dimer of GpATM caused by substitution of each residue with the set of nonpolar amino acids. Each mutation observed was scored for its effect upon dimerization as in Figure 1, and values were assigned: for appearance of no dimer, a value of 3.0 was assigned for the mutation; where a small but detectable amount of dimer remained, a value of 2.0 was assigned; where there was significant dimer, but clear disruption, a value of 1.0 was assigned; and for no effect, a value of 0.0 was assigned. The histogram plots the average value for mutations at each position in the helix, using this scale. Residues defined as sensitive (i.e., having a relative degree of disruption greater than 1.0) are boxed. (B) Fourier transform power spectrum generated as described (Cornette et al., 1987) from the profile shown in (A). The frequency axis corresponds to the angle about the helix axis subtended between residues. The only significant peak seen is centered at 92°, corresponding to 3.9 residues per helix turn. Expected peak positions corresponding to 3.9, 3.6, and 3.5 residues per turn are marked along the upper axis.

stable than the wild-type dimer but more stable than either mutant homodimer (not shown). Substitution of I76 with W is less disruptive than I76A (Figure 3B, lanes 2 and 7). Therefore, some of the interactions disrupted in the homodimer of I76A remain, or are replaced, in the homodimer of I76W. In the context of the simplification presented above for the I76A/L75V heterodimer, it might again be argued that, in the I76W/L75V heterodimer, one of the two sites of interaction [L75(1)–I76(2) and L75(2)–I76(1)] in the wild-type dimer is perturbed. A slightly larger proportion of the protein runs as heterodimer in the I76W/L75V mixture than in the case of I76A/L75V. The perturbed interaction [W76(2)–V75(1)] may contribute to this slightly increased stability, which is consistent with the fact that perturbation of both interactions in the I76W homodimer is not completely disruptive.

That these arguments are an oversimplification is evident by the fact that not all mixtures of disruptive mutants at I76 with those at L75 (e.g., L75A/I76A, L75V/I76C) showed enhanced heterodimer formation. It is possible that the nature of the interactions in the cases of the mutant heterodimers discussed above is different in detail from that in the wild-type dimer. It is also possible that new interactions are formed in these cases, which have no equivalent in the wild-type dimer. In the absence of specific structural information, we are unable to address this issue.

Sensitive Residues Occur with a Period of 3.9 Residues. Figure 5A shows a histogram representing the relative degree of disruption (defined in the legend to Figure 5A) caused by substitutions with the set of apolar residues at each position in the transmembrane domain of GpA. It is clear that residues likely to be involved in helix–helix interactions occur at

intervals over approximately two-thirds of the length of the helix, with a striking periodicity, consistent with the dimer being a closely fit supercoil. This result contrasts with suggestions for the TCRα–CD3δ (Manolios et al., 1990; Cosson et al., 1991) interaction described in the introduction and the interpretation of association in the case of the *neu* oncogene product (Sternberg & Gullick, 1990; Cao et al., 1992), where a more localized motif is proposed to be important for helix–helix association. It is of note that G79 and G83, both clearly important in the dimerization of GpATM, occur in a sequence that is included by the motif suggested by Sternberg and Gullick (1990) to be responsible for transmembrane helix dimerization in growth factor receptors with tyrosine kinase activity. G79 would correspond to P0 and G83 to P4 in their motif. A82 would correspond to P3, the position equivalent to that at which a V to E substitution in the *neu* oncogene results in constitutive activation of the kinase domain.

Generation of a Fourier transform power spectrum from the disruption data in Figure 5A (Rees et al., 1989a; Cornette et al., 1987) gives a single significant peak, at a frequency of 92° per residue around a helical wheel (Figure 5B). This corresponds to a period between residues important in helix–helix association of approximately 3.9 residues, which is consistent with an interaction between two slightly underwound α-helices or a pair of α-helices in a right-handed supercoil (in the latter case the apparent number of residues per turn is elevated by projection along the helix axis). The same analysis of a variability profile (Rees et al., 1989a) determined from the set of nondisruptive conservative substitutions gives a power spectrum that is almost identical (not shown). This further supports our assumption that disruption by conservative substitutions occurs through the simple loss of helix–helix contacts rather than as a result of some more global conformational change.

Hydrophobicity profiles (Rees et al., 1989a) of both the native GpA transmembrane domain and the set of altered sequences that still dimerize show no significant helical periodicity. Indeed, assuming that the set of mutationally sensitive residues comprise the interface of the helix dimer, it is apparent that this set contains 4 residues with aliphatic side chains (L75, I76, V80, and V84) and 3 relatively polar residues (G79, G83, and T87). The presumed outside of the dimer contains 11 nonpolar and 5 relatively polar residues. The average hydrophobicity of residues in the presumed interface is not significantly different from that for those on the outside of the dimer. This suggests that the dimer of GpATM, at least, does not have reversed relative polarity compared to soluble globular proteins. Figure 6 compares helical wheel plots for GpATM generated by assuming a helical periodicity of 3.9 residues (Figure 6A) and of 3.6 residues per turn (Figure 6B). In the latter case a semicircle depicts the dimer interface predicted by Argos et al. (1982), based upon assumptions that the helices are straight and that the most polar face comprises the dimer interface. It is clear that most of the positions at which conservative substitutions disrupt the dimer are present in this predicted interface. However, they are rather more scattered than in Figure 6B, and several insensitive residues are also included.

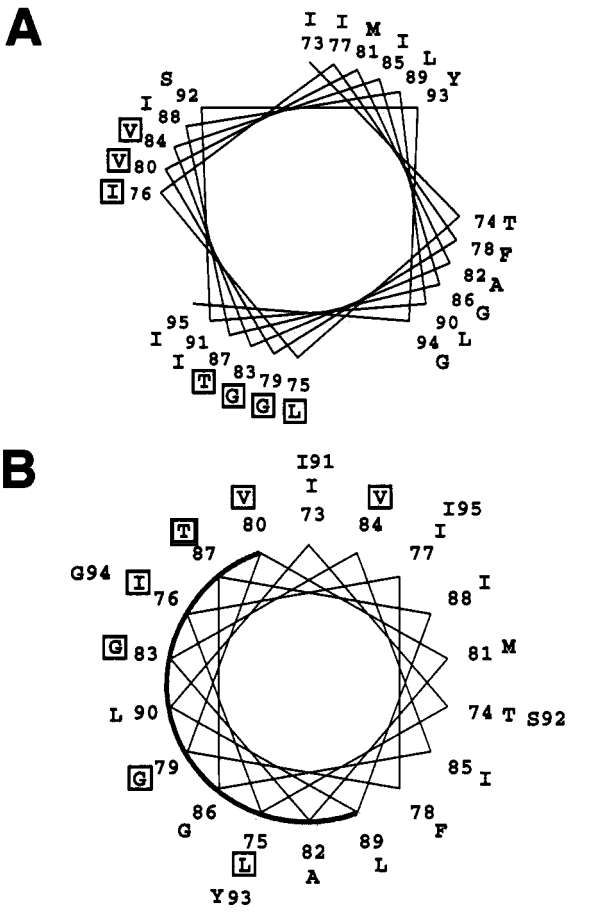

FIGURE 6: (A) Helical wheel projection of C_α positions, assuming a periodicity of 3.9 residues per turn, for the transmembrane helix of GpA. Positions determined to be sensitive in the mutational analysis, and therefore likely to be at the dimer interface, are boxed and are seen to cluster at one face of the helix. The apparent number of residues per turn in this projection may be altered by the transformation of the actual helix axis to a straight line. For a helix involved in a right-handed supercoil, this transformation elevates the apparent number of residues per turn, while in the case of a left-handed coiled coil, the value is reduced. It should be noted also that this method of projection can be misleading, since the positions of the side-chain atoms may deviate from this periodicity. (B) The same as in (A), but with the assumption of canonical α-helix geometry (3.6 residues per turn). Sensitive (interfacial) residues are boxed and are seen to be dispersed about more than half of the circumference of the projection. The dimer interface predicted by Argos et al. (1982) is marked with a bold semicircle and is seen to include several residues that are not sensitive in our mutational analysis (A82, G86, L90, Y93, G94) and not to include V84, which we found to be sensitive.

CONCLUSIONS

We have shown in this paper that there is a high degree of sequence specificity, over an extensive contact region, in the dimerization of the transmembrane α-helix of human GpA. Similar helix–helix packing is clearly important in water-soluble proteins as well as in polytopic integral membrane proteins such as the photosynthetic reaction centers and bR (Rees et al., 1989b). In the growing number of cases for which there are data suggesting that interactions between individual transmembrane α-helices are major determinants in the oligomerization of bitopic integral membrane proteins (Lemmon & Engelman, 1992), highly polar groups are always implicated. GpA, by contrast, has no such groups in its transmembrane α-helix, and this domain forms remarkably stable dimers through specific interactions at a presumed interface consisting of relatively polar as well as hydrophobic amino acids. Our results, combined with the model of the dimer proposed by Treutlein et al. (1992), suggest that GpATM helix association is driven by some or all of the following: (i) electrostatic attraction in a region where close approach of the peptide backbones is possible; (ii) hydrogen bonding and/or strong electrostatic interactions involving the side-chain hydroxyl group of T87; (iii) van der Waals

interactions between aliphatic side chains, most notably involving L75 and I76, which may interact with one another across the dimer interface. In the case of the aliphatic side chains, close packing of complementary faces could result in the maximization of van der Waals interactions compared with the case for their packing with lipids, as proposed by Popot and Engelman (1990). Further, the relatively defined spatial correlation of groups on a helix could promote the coordination of van der Waals contacts so that many small interactions could add, producing a strong interaction between complementary interfaces. Considerable specificity may similarly be afforded in the assembly of other oligomeric membrane proteins. An illustration of this is seen in the case of the FcγRIII-α/ζ-chain interaction described in the introduction (Kurosaki et al., 1991). As a further example, Cosson and Bonifacino (1992) have recently shown that the single transmembrane domains of class II MHC α and β chains form heterodimers and that these interactions are important in the assembly of class II MHC molecules. Several glycine residues, which would be aligned on one face of a putative α-helix in both chains, were shown to be important in this interaction. Furthermore, the specificity of the association could be altered by mutation of residues on this face of the α-chain helix. Thus it appears that the interactions which stabilize this heterodimer are similar in nature to those which drive dimerization of GpATM.

Finally, we note that the periodicity of sensitive residues seen for GpATM (3.9 residues per turn) would place this interaction in the 4-4 class (right-handed) of helix-to-helix packing of Chothia et al. (1981), if a closely packed parallel dimer is considered. Modeling of the dimer of GpATM described in another paper also suggests that GpATM dimerizes as a right-handed supercoil of α-helices (Treutlein et al., 1992). A recent electron crystallographic study of the pea light-harvesting complex shows a dimer of parallel α-helices in the center of the complex with a similar arrangement (Kühlbrandt & Wang, 1991).

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