

Structural elucidation of the PDI-related chaperone
Wind with the help of mutants

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The structures of the PDI-related protein Wind (with a C-terminal His₆ tag) and the mutants Y53S, Y53F and Y55K have been determined and compared with the wild-type structure with the His₆ tag at the N-terminus. All five structures show the same mode of dimerization, showing that this was not an artefact introduced by the nearby N-terminal His₆ tag and suggesting that this dimer may also be the biologically active form. Although the mutants Y53S and Y55K completely abrogate transport of the protein Pipe (which appears to be the primary function of Wind in the cell), only subtle differences can be seen in the putative Pipe-binding region. The Pipe binding in the active forms appears to involve hydrophobic interactions between aromatic systems, whereas the inactive mutants may be able to bind more strongly with the help of hydrogen bonds, which could disturb the delicate equilibrium required for effective Pipe transport.

1. Introduction

Wind, a product of the *Windbeutel* gene, is required for dorso-ventral patterning in the developing embryo of *Drosophila melanogaster* along with two other gene products, Nudel (*nudel*) and Pipe (*pipe*), that constitute three of the 11 genes localized in the somatic follicle cells (Konsolaki & Schüpbach, 1997). Wind is an endoplasmic reticulum resident protein and belongs to the protein disulfide isomerase (PDI) related protein subfamily (PDI-D). PDIs are known for their varying redox and chaperone activities (Ferrari & Söling, 1999). The PDI-D group of proteins is characterized by the presence of one or two N-terminal domains of about 100–120 residues in length called the thioredoxin domain (b-domain) followed by an all- α -helical C-terminal domain about 110 residues in length termed the D-domain (Ferrari & Söling, 1999). Pipe is a Golgi-resident type-II transmembrane protein related to the vertebrate glycosaminoglycan-modifying enzyme heparan sulfate 2-O-sulfotransferase. It has been shown recently that Wind is required for correct targeting of Pipe to Golgi (Sen *et al.*, 2000), but the mechanism by which this process takes place is not yet understood.

The crystal structure of wild-type Wind has been described by Ma *et al.* (2003); the asymmetric unit consists of a dimer, with the dimerization-contact surface along the N-terminal b-domain. Based on this structure, a series of mutational studies have been carried out to map substrate-binding sites on the surface of Wind and a putative peptide-binding site in the Wind b-domain has been characterized with the help of *in vitro* binding assays (Barnewitz *et al.*, 2004). Within the Wind dimer, a surface tyrosine cluster formed by Tyr53, Tyr55 and

Received 12 December 2005
Accepted 22 March 2006

PDB References: Wind-His, 2c0e, r2c0esf; Y53F mutant, 2c0f, r2c0fsf; Y53S mutant, 2c0g, r2c0gsf; Y55K mutant, 2c1y, r2c1ysf.

Tyr86 is important for substrate binding. Mutations at these sites (Y53S, Y55K, Y55S, Y86Q and Y86L) completely abrogate Pipe transport in *in vivo* assays. Both Y53S and Y55S are functionally inactive, whereas Y86S retains processing activity. These mutations enhance substrate binding, but reduce Pipe-processing efficiency. The Y55F mutant did not show any negative effect on Pipe processing, which further suggests that the aromatic/hydrophobic behaviour of the inner Tyr53/Tyr55 pair

plays a major role in Wind–Pipe interaction. In order to assess whether structural differences are responsible for these observations, we have determined the crystal structures of the Wind Y53S, Y53F and Y55K mutants and performed some hydrophobic molecular interaction field (MIF) calculations to look at the surface hydrophobic behaviour in these mutants.

Independent mutational studies of the residues at the dimerization interface in ERp29 (a mammalian ortholog of Wind) and a preliminary low-resolution crystal structure of human ERp28 also provide a clue that the dimerization interfaces in Wind and ERp29 are similar (Lippert *et al.*, 2006). The different dimerization interface proposed on the basis of the NMR structure of the rat ERp29 monomer (Liepinsh *et al.*, 2001) may prove to have been rather speculative, as the authors themselves acknowledged.

Our original crystal structure of wild-type Wind (PDB code 1ovn) had been determined with a His₆ tag at the N-terminus (His-Wind). To rule out the possibility that the His₆ tag at the N-terminus, which is close to the dimerization region, was responsible for artefacts in the dimerization mode, we also report here the crystal structure of wild-type Wind with the His₆ tag at the C-terminus (Wind-His).

2. Materials and methods

2.1. Expression, purification and crystallization

Wild-type Wind-His protein (with the His₆ tag at the C-terminus) and the His-Wind mutants Y53S, Y53F and Y55K (with His₆ tags at the N-termini) were expressed and purified as described in Ma *et al.* (2003). Crystals of Wind-His and the

Table 1

Crystallization and cryoprotectant conditions of Wind-His and its mutants.

| | Wind-His | Y53S | Y53F | Y55K |
|---------------------------------|---|---|---|--|
| Reservoir | 0.1 M MES pH 5.8–6.6, 50 mM NaCl, 16–20% PEG 400 | 0.1 M MES pH 5.8–6.6, 50 mM NaCl, 16–20% PEG 400, 5% glycerol | 0.1 M MES pH 5.8–6.6, 50 mM LiCl, 16–20% PEG 400 | 0.1 M Tris–HCl pH 8.2–8.8, 25 mM MgCl ₂ , 10% PEG 4000, 5% glycerol |
| Protein† (mg ml ^{−1}) | 15 | 10 | 18 | 5 |
| Cryoprotectant | Reservoir + 25% PEG 400, 10% glycerol | Reservoir + 25% PEG 400, 10% glycerol | Reservoir + 25% PEG 400, 10% glycerol | Reservoir + 20% glycerol |

† Protein in all cases was dissolved in 5 mM HEPES pH 7.5, 25 mM NaCl, 0.0025% (v/v) β-mercaptoethanol.

mutants were obtained using the hanging-drop vapour-diffusion method (McPherson, 1992) at 293 K. The crystallization and cryoprotectant conditions are summarized in Table 1 and the crystals are shown in Fig. 1. Large crystals of Wind-His, Y53S and Y53F grew within about 4 d, whereas crystals of Y55K appeared in about a week.

2.2. Data collection and processing

The crystals of each of the mutants were soaked in a suitable cryoprotectant (Table 1) and mounted in a loop in a cold nitrogen-gas stream. The data sets were collected in two passes: a high-resolution and a low-resolution pass. The data-collection statistics are summarized in Table 2. All data sets were processed with *HKL* (Otwinowski & Minor, 1997) and the space group and data statistics were determined using *XPREF* (Bruker AXS, Madison, WI, USA).

2.3. Structure solution, refinement and validation

The Wind-His, Y53S and Y53F structures were solved by molecular replacement using *EPMR* (Kissinger *et al.*, 1999) with search models taken from the His-Wind structure (PDB code 1ovn). Monomers consisting of one b-domain and one D-domain were used, except for the solution of Y55K, for which individual b-domains and D-domains were employed. Solutions were found with correlation coefficients of 49.6, 52.5 and 51.5% for Wind-His, Y53S and Y53F, respectively. The Y55K mutant proved to be more problematic and so *PHASER* (McCoy *et al.*, 2005) was employed. First the two b-domains were located, followed by a search for the D-domains. It was only possible to locate the second D-

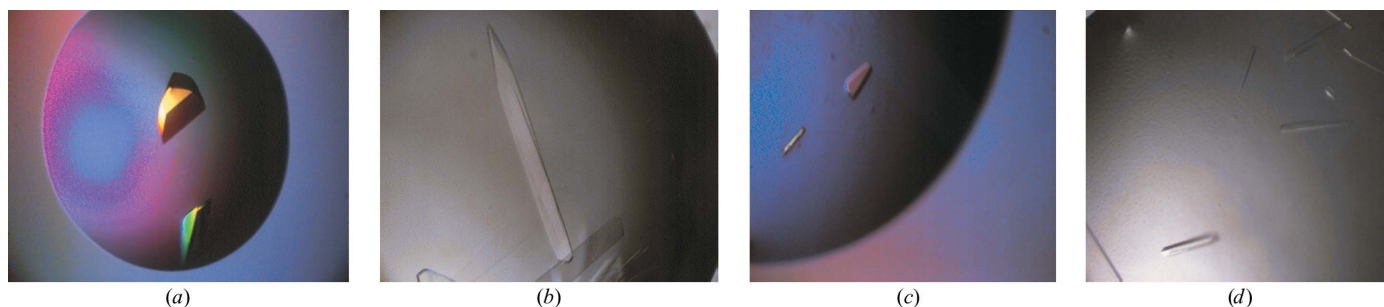


Figure 1

Crystals of (a) Wind-His (0.3 × 0.3 × 0.2 mm), (b) Y53S (0.4 × 0.1 × 0.05 mm), (c) Y53F (0.1 × 0.1 × 0.05 mm) and (d) Y55K (0.2 × 0.1 × 0.05 mm).

Table 2

Data-collection and refinement statistics.

Values in parentheses are for the outer resolution shell.

| | Wind-His | Y53S | Y53F | Y55K |
|---------------------------------------|------------------|------------------|------------------|-----------------------------|
| Data-collection statistics | | | | |
| Wavelength (Å) | 0.8976 | 0.8976 | 0.8976 | 1.0 |
| X-ray source | BESSY BL2 | BESSY BL2 | BESSY BL2 | DESY X13 |
| Detector | MAR IP 345 | MAR IP 345 | MAR IP 345 | MAR CCD |
| Space group | C2 | C2 | C2 | P ₂ ₁ |
| Unit-cell parameters | | | | |
| <i>a</i> (Å) | 107.78 | 108.47 | 109.44 | 35.42 |
| <i>b</i> (Å) | 50.36 | 50.55 | 51.71 | 119.09 |
| <i>c</i> (Å) | 98.67 | 98.9 | 100.65 | 64.03 |
| β (°) | 112.19 | 112.06 | 112.70 | 112.20 |
| Mosaicity (°) | 1.8 | 1.2 | 1.5 | 1.9 |
| Resolution (Å) | 2.35 (2.45–2.35) | 1.75 (1.85–1.75) | 2.28 (2.37–2.28) | 2.25 (2.34–2.25) |
| Reflections (unique) | 19292 | 47523 | 22333 | 22938 |
| Redundancy | 4.11 (3.00) | 3.86 (2.96) | 3.43 (3.0) | 4.67 (2.62) |
| Completeness (%) | 98.28 (99.2) | 99.1 (95.9) | 98.3 (89.9) | 97.8 (91.2) |
| Mean <i>I</i> / σ (<i>I</i>) | 20.05 (9.46) | 19.32 (3.96) | 15.19 (4.55) | 16.83 (3.51) |
| <i>R</i> _{int} † (%) | 4.05 (10.87) | 4.36 (26.2) | 4.99 (24.32) | 6.5 (26.6) |
| Refinement statistics | | | | |
| Final <i>R</i> factor (%) | | | | |
| Working set | 22.60 | 21.92 | 21.92 | 22.27 |
| Working + test set‡ | 22.95 | 22.15 | 22.24 | 22.63 |
| Final free <i>R</i> factor (%) | 28.34 | 26.75 | 28.06 | 29.25 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.025 | 0.021 | 0.031 | 0.033 |
| Bond angles (°) | 2.057 | 1.784 | 2.377 | 2.745 |
| Mean <i>B</i> value (Å ²) | | | | |
| Monomer 1 | | | | |
| Main-chain atoms | 15.707 | 25.125 | 30.770 | 49.889 |
| Side-chain atoms | 15.313 | 28.175 | 29.646 | 49.065 |
| Monomer 2 | | | | |
| Main-chain atoms | 25.458 | 41.263 | 41.375 | 47.438 |
| Side-chain atoms | 22.696 | 37.593 | 38.636 | 46.207 |
| Solvent | 15.338 | 30.284 | 32.510 | 40.634 |
| No. of protein atoms | 3287 | 3306 | 3255 | 2699 |
| No. of solvent atoms | 108 | 167 | 98 | 55 |
| PDB code | 2c0e | 2c0g | 2c0f | 2c1y |

† *R*_{int} = $\sum |F_o^2 - F_c^2(\text{mean})| / \sum |F_o^2|$. ‡ 5% of reflections are selected in thin shells as the test data set.**Table 3**

Dimer-interface interactions in all mutants.

The conserved interactions are shown in bold.

| Mutant | Residue 1 | Residue2 | Distance (Å) | Charge interaction | Hydrogen bonding |
|----------|------------------|------------------|--------------|--------------------------------|----------------------|
| His-Wind | Gly26 N | Asp31 OD2 | 2.47 | N()...O(−) | Hydrogen bond |
| | Asp75 OD1 | Arg41 NH2 | 2.68 | O(−)...N(+) salt bridge | Hydrogen bond |
| | Val28 CG1 | Val28 CG1 | 3.30 | C()...C() | |
| | Arg41 NH2 | Lys74 O | 2.80 | N(+)...O() | Hydrogen bond |
| Wind-His | Gly26 N | Asp31 OD2 | 2.59 | N()...O(−) | Hydrogen bond |
| | Asp75 OD1 | Arg41 NH2 | 2.79 | O(−)...N(+) salt bridge | Hydrogen bond |
| | Val28 CG1 | Val28 CG1 | 3.64 | C()...C() | |
| | Lys37 NZ | Thr73 O | 2.71 | N(+)...O() | Hydrogen bond |
| Y53S | Gly26 N | Asp31 OD2 | 2.66 | N()...O(−) | Hydrogen bond |
| | Asp75 OD1 | Arg41 NH2 | 2.51 | O[−]...N(+) salt bridge | Hydrogen bond |
| | Val28 CG1 | Val28 CG1 | 3.28 | C()...C() | |
| | Arg41 NH2 | Lys74 O | 2.85 | N(+)...O() | Hydrogen bond |
| Y53F | Gly26 N | Asp31 OD2 | 2.74 | N()...O(−) | Hydrogen bond |
| | Asp75 OD1 | Arg41 NH2 | 2.38 | O(−)...N(+) salt bridge | Hydrogen bond |
| | Val28 CG1 | Val28 CG1 | 3.28 | C()...C() | |
| | Arg41 NH2 | Lys74 O | 2.99 | N(+)...O() | Hydrogen bond |
| Y55K | Gly26 N | Asp31 OD2 | 2.89 | N()...O(−) | Hydrogen bond |
| | Asp75 OD1 | Arg41 NH2 | 2.81 | O(−)...N(+) salt bridge | Hydrogen bond |
| | Val28 CG1 | Val28 CG1 | 3.76 | C()...C() | |
| | Arg41 NH2 | Lys74 O | 2.99 | N(+)...O() | Hydrogen bond |

domain with a much lower log-likelihood gain and a very low *Z* score. Though there was a solution for both the D-domains, the second D-domain had to be discarded because the *B* values were very high and there was no traceable electron density in this region during refinement. Prime-and-switch phasing in *RESOLVE* (Terwilliger, 2004) was used to minimize model bias. The model was checked and rebuilt manually from the bias-minimized electron-density map from *RESOLVE*. All structures were refined against *F* using TLS refinement in *REFMAC* (Murshudov *et al.*, 1997), alternating with model building in real space using $2mF_o - DF_c$ and $mF_o - DF_c$ maps in *COOT* (Emsley & Cowtan, 2004). The refinement statistics are summarized in Table 2. The structures were validated with *PROCHECK* (Laskowski *et al.*, 1993). All residues lie within the allowed regions of the Ramachandran plot. Figures were drawn with *CHIMERA* (Pettersen *et al.*, 2004), *MOLSCRIPT* (Kraulis, 1991) and *RASTER3D* (Merritt & Bacon, 1997).

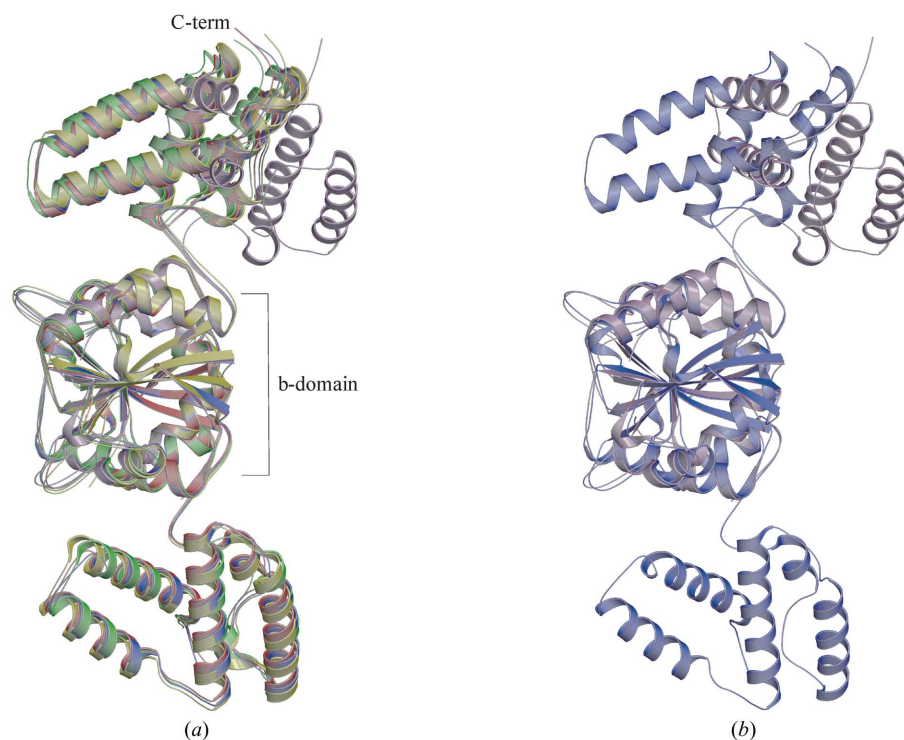
2.4. Hydrophobic molecular-interaction field

The hydrophobic molecular-interaction fields at the surfaces of the different mutants were analysed using a DRY probe in the program package *GRID* (Goodford, 1985). The hydrophobic probe finds favourable locations that interact with other molecule(s) on the surface of a protein in an aqueous environment (Liljefors, 1998). All *GRID* calculations were performed for the whole volume of the protein using a grid spacing of 1.0 Å.

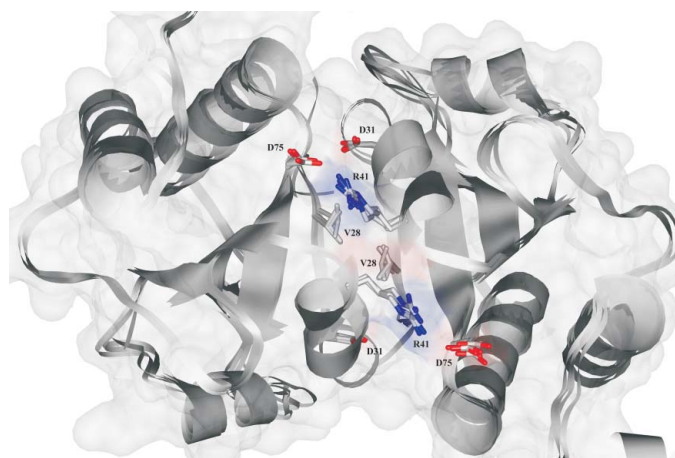
3. Results and discussion

3.1. Structure of Wind-His and its mutants

The overall fold of all three mutants is quite similar to the wild-type structure. The mutants crystallize as homodimers in the asymmetric unit, each monomer consisting of two domains, the N-terminal b-domain (118 residues) and the C-terminal D-domain (107 residues), connected by a flexible linker of

**Figure 2**

Superposition of dimers of (a) Wind-His (blue) with Y53S (green), Y53F (yellow), Y55K (plum) and His-Wind (red). (b) Superposition of Wind-His dimer (blue) with Y55K dimer (plum). The N-terminal b-domain and C-terminal D-domains are labelled.

**Figure 3**

Superposition of the five structures viewed approximately down the non-crystallographic twofold axis of the dimer, with the key residues in the structurally strongly conserved Wind-His dimer interface highlighted.

11 residues. The b-domain adopts an α/β -fold with the order of secondary-structure elements $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\alpha 2$ - $\beta 3$ - $\alpha 3$ - $\beta 4$ - $\beta 5$ - $\alpha 4$. The strands of the β -sheet form a central core surrounded by the four α -helices. This fold, characteristic of protein-disulfide isomerases, is called the thioredoxin fold. The D-domain has a five-helix fold with all the helices in antiparallel arrangement. Both the N-terminal His₆ tag in the case of Y53S, Y53F and Y55K and the C-terminal His₆ tag of Wind-His were not

visible in the density. The second D-domain was completely absent in Y55K.

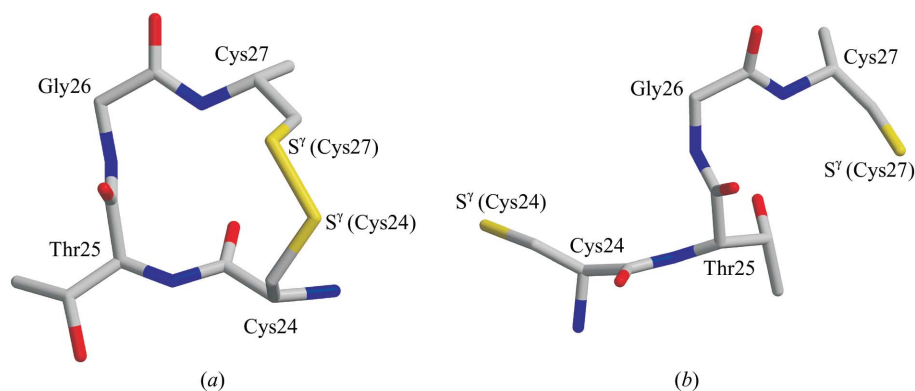
3.2. Superposition of His-Wind and its mutants on Wind-His

Least-squares superposition of the main-chain atoms of dimers of all the mutants (Y53S, Y53F, Y55K) and wild-type His-Wind on Wind-His gave an r.m.s.d. (root-mean-square deviation) of 0.247, 0.272, 0.365 and 0.434 Å for His-Wind, Y53S, Y53F and Y55K, respectively. The program *ESCET* (Schneider, 2002) was used to determine conformationally invariant regions between different pairs of mutants. The CSI (conformational similarity index) relative to His-Wind, calculated using *ESCET*, is in the range of 90% for all the structures except for the Y55K mutant, which has a CSI of around 70%. In all the structures, the D-domains show the least significant variations, which may in part be a consequence of their higher *B* values. There are some minor differences in the loop regions of the b-domains as illustrated in Fig. 2. All

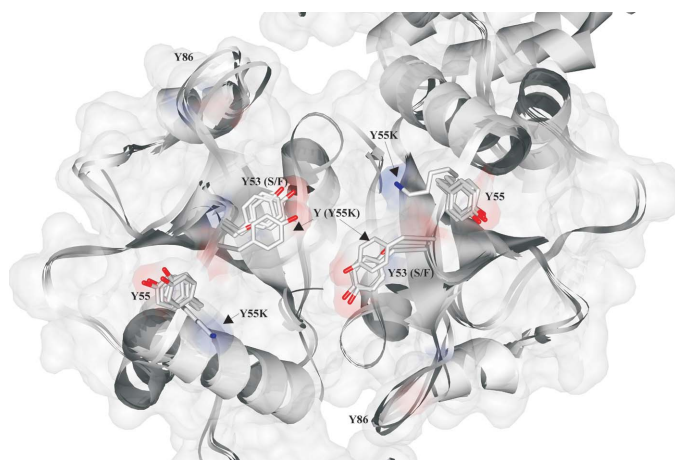
the structures show significant variations in the conformations of some side chains. Compared with the other structures, the D-domain of Y55K exhibits an appreciably different orientation relative to the b-domain, with a rotation of about 45° around Gly145. With such an orientation of the D-domain with respect to the b-domain, residue Cys149 in the linker region is closer to Tyr143, with a C^α–C^α distance between them of about 4.4 Å, compared with about 6.9 Å in the wild-type and other mutant structures. Cys149 is also closer to Tyr194 (3.65 Å) and Asn154 (3.94 Å), thus stabilizing the movement of the D-domain. The relevance, if any, of these observations with respect to a recent suggestion of a key structural role of Cys125 in rat ERp29 (homologous to Cys149 in Wind) in providing stability to the D-domain (Hermann *et al.*, 2005) remains to be established.

3.3. The dimer interface

The Wind dimer is formed by a head-to-tail arrangement of b-domains with the participation of residues constituting the $\beta 1$ strand and residues within and after the $\alpha 1$ and $\alpha 2$ helices, mediated through a variety of hydrophobic and hydrogen-bonding interactions shown in Table 3. The partially conserved residues Gly26, Val28, Gln31 and Arg41 contribute to the dimer interface (Fig. 3). In wild-type Wind-His and all the mutants, the hydrogen-bonding distances in the region of the dimer interface agree within experimental error with those in His-Wind. The similarities in dimerization modes of Wind-His and His-Wind also indicate that the N-terminal His₆ tag in His-

**Figure 4**

(a) Intact disulfide bond in Wind-His (His-Wind, Y53S and Y55K are similar) and (b) the open disulfide bridge in Y53F, which is accompanied by an approximately 180° rotation about $\psi(\text{Thr25})$.

**Figure 5**

Superposition of the residues at the putative substrate binding site in Wind-His, His-Wind and the Y53F, Y53S and Y55K mutants. The orientations of the residues Tyr53 and Lys55 in the Y55K mutant are slightly different to those in the other four structures. The binding site stretches over both monomers and in the wild type presents a non-polar surface composed of aromatic residues at the opposite end of the non-crystallographic twofold axis of the dimer to that shown in Fig. 3.

Wind does not interfere with dimerization. The χ_1 angle of Val28 is not conserved in the mutant structure Y53F; this could be because of the changes in oxidation state of the Cys pair, which might further affect the dimerization surface and possibly the monomer/dimer ratio. In solution, Wind is present partly as a monomer but mainly as a dimer.

3.4. Opening of the CTGC loop in Y53F

In the crystal structure of Y53F, the CTGC loop in both monomers has lost its disulfide bond, as shown in Fig. 4. As a result of a change of approximately 180° in $\psi(\text{Thr25})$, Cys27 is about 4.2 Å from the carboxyl O atom of Glu32. The distance between Tyr53 and Cys27 is about 11.8 Å, which rules out the possibility of the mutation affecting this opening of the cysteine loop. Whether such an opening has any physiological relevance or is a consequence of radiation damage is

unknown. The diffraction data did not show any other indication of radiation damage.

3.5. Substrate-binding site and hydrophobic molecular-interaction surface

The substrate-binding site of the b-domain containing the Tyr53, Tyr55 and Tyr86 residues was examined for possible structural differences amongst the mutants Y53S, Y53F and Y55K. This region is close to the pseudo-twofold axis of the dimer, so it is possible that the sites in the two monomers combine to make a single Pipe-binding site. The

mutants Y53S and Y53F are conformationally very similar, but in Y55K the lysine side chain points upwards 90° away from the plane of the tyrosine ring (Fig. 5). It is possible that this results in a tighter association of the substrate (Pipe) with the Wind dimer, leading to a stronger complex, accounting for the observation in *in vivo* assays with the Wind-Y55K mutant and Pipe that Pipe translocation from Golgi was abrogated.

To analyse the hypothesis that the Wind–Pipe interaction is predominantly hydrophobic, involving aromatic interactions, the relative hydrophobic potentials on the surface shown in Fig. 6 were estimated using a DRY (hydrophobic) probe in the program *GRID* (Goodford, 1985). The net charge on each of the dimers as calculated using *GRIN* (Goodford, 1985) are –7.38, –9.05, –11.23 and –13.21 for Wind-His, Y53F, Y53S and Y55K, respectively: the two inactive mutants are appreciably more polar. In both wild-type Wind-His and the Y53F mutant, the substrate-binding site has a much larger hydrophobic interaction area at the ‘tyrosine triad’ than in the inactive mutants Y53S and Y55K.

3.6. Crystal-packing interactions in Y55K and Wind-His

Y55K crystallized at a different pH and in a different space group to all the other mutants and attempts to crystallize Y55K at the pH of the wild-type crystallization conditions failed, although there is no difference in dimerization mode and in the secondary structure of the b-domains. In Y55K, Tyr53 of one of the b-domains forms a hydrogen bond with Arg218 and the second Tyr53 forms a hydrogen bond with Tyr44 of a symmetry-equivalent molecule. One of the Lys55 residues forms a hydrogen bond with Ser137 of a symmetry-equivalent molecule and the second Lys55 is involved in a hydrophobic interaction with a symmetry-equivalent Phe42. In wild-type Wind-His, except for one tyrosine (Tyr55) that forms a hydrogen bond with symmetry-equivalent Arg237, all other tyrosines are involved in hydrophobic interactions. These interactions may give an indication of the differences in the substrate binding of Y55K, which has been shown to bind much more strongly than wild-type Wind-His to Pipe or to peptides derived from fragments of the Pipe sequence (Barnewitz *et al.*, 2004).

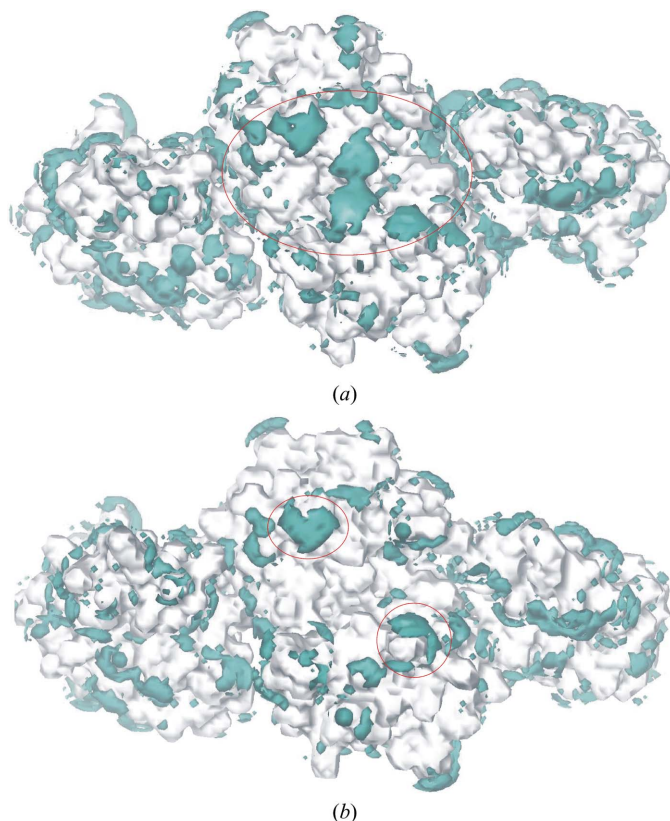


Figure 6

Hydrophobic molecular-interaction field of (a) the Wind-His dimer and (b) the Y53S dimer, contoured at $-0.71 \text{ kJ mol}^{-1}$, highlighting the putative substrate-binding surface (encircled). For Y53F the surface is similar to (a) and for Y55K it is similar to (b).

4. Conclusions

Although the mutants Y53S and Y55K completely abrogate Pipe transport, the structural differences in the binding regions between these two mutants and the functional Y53F mutant and wild-type Wind protein in the Pipe-binding region are subtle, despite a large difference in the orientation of the b-domain and D-domain to one another in the Y55K mutant. The interactions with symmetry-equivalent molecules suggest that Y53S and Y55K may bind more strongly to Pipe, disturbing the delicate equilibrium based on predominantly hydrophobic interactions necessary for Pipe transport. The very similar structures observed for His-Wind and Wind-His indicate that the N-terminal His₆ tag, although close to the

dimer interface, does not interfere with dimerization. The consistent form of dimerization for His-Wind and the four structures reported here lend support to the view that it also dominates *in vivo*, contrary to inferences based on the NMR monomer structure of a related protein, ERp29, from rat.

We are grateful to the Fonds der Chemischen Industrie, the Deutsche Forschungsgemeinschaft (SFB416) and to the European Community (Access to Research Infrastructure Action of Improving Human Potential Programme to the EMBL Hamburg Outstation, contract No. HPRI-1999-CT-000017) for support and to EMBL/DESY, Hamburg and BESSY, Berlin for generous allocations of beam time.

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