

The Crystal Structure of *Aspergillus fumigatus* Cyclophilin Reveals 3D Domain Swapping of a Central Element

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Summary

The crystal structure of *Aspergillus fumigatus* cyclophilin (Asp f 11) was solved by the multiwavelength anomalous dispersion method and was refined to a resolution of 1.85 Å with R and R_{free} values of 18.9% and 21.4%, respectively. Many cyclophilin structures have been solved to date, all showing the same monomeric conformation. In contrast, the structure of *A. fumigatus* cyclophilin reveals dimerization by 3D domain swapping and represents one of the first proteins with a swapped central domain. The domain-swapped element consists of two β strands and a subsequent loop carrying a conserved tryptophan. The tryptophan binds into the active site, inactivating *cis-trans* isomerization. This might be a means of biological regulation. The two hinge loops leave the protein prone to misfolding. In this context, alternative forms of 3D domain swapping that can lead to N- or C-terminally swapped dimers, oligomers, and aggregates are discussed.

Introduction

Cyclophilins (CyPs) constitute a family of cytosolic proteins involved in many biological processes. CyP is an enzyme that catalyzes the peptidyl-prolyl *cis-trans* isomerization (PPIase) (Fischer et al., 1989; Takahashi et al., 1989). Belonging to the family of immunophilins, CyP binds the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984). The complex of CyP with CsA binds and inhibits the protein phosphatase calcineurin (Liu et al., 1991), thus suppressing signal transduction in T cells (Jain et al., 1993). Therefore, CsA is one of the most important immunosuppressant drugs used for prevention of graft rejection after transplant surgery (Borel, 1990). CyPs are also assumed to

participate in other biological functions, such as cell surface recognition (Anderson et al., 1993) and heat shock response (Sykes et al., 1993).

CyPs exist abundantly and ubiquitously in a broad range of organisms (Hunter, 1998). They have also been isolated as IgE binding proteins from several fungi (Flückiger et al., 2002; Horner et al., 1995; Lindborg et al., 1999), including CyP from the mold *Aspergillus fumigatus* (Asp f 11) (Crameri, 1999). *A. fumigatus* is the etiologic agent identified in 80% of *Aspergillus*-related human diseases. The ubiquitous mold is considered as an opportunistic pathogen associated with an impressive list of pulmonary and allergic complications in humans and animals (Bardana, 1981). The ability of the active Asp f 11 to act as an allergen in vivo was shown by positive skin reactions of *A. fumigatus*-sensitized individuals (Flückiger et al., 2002). Crossreactivity between Asp f 11 and other CyPs from *Malassezia sympodialis*, *Candida albicans*, *Saccharomyces cerevisiae*, and *Homo sapiens* were shown by Western blot analysis and inhibition ELISA (Flückiger et al., 2002).

Several structures of CyPs have been solved (Table 1). Despite the fact that crystals were grown under different conditions, such as PEG and ammonium sulfate, resulting in different unit cell dimensions and space groups, all of them are monomeric (Table 1). They share the same fold consisting of a β barrel with eight antiparallel β strands and two α helices covering the bottom and top of the barrel. In contrast, the crystal structure of Asp f 11, presented here, reveals a dimer formed by 3D domain swapping of two central β strands and a subsequent loop. 3D domain swapping is the event by which a monomer exchanges part of its structure with identical monomers to form an oligomer, where each subunit has a similar structure to the monomer (Rousseau et al., 2003).

In the present work, inhibition of *cis-trans* isomerization activity as a consequence of domain swapping and as a potential way of functional regulation is discussed. Furthermore, alternative forms of 3D domain swapping leading to misfolded dimers and oligomers are addressed.

Results and Discussion

Structure Determination

Initial crystals of the original protein, which contained a noncleavable His tag and a linker region at the N terminus, diffracted to about 3.0 Å resolution on a home source. CocrySTALLIZATION with the dipeptide Ala-Pro improved the resolution by about 0.5 Å. Removal of the His tag and linker improved the resolution by a further 0.5 Å. The final protein crystals, cocrySTALLIZED with Ala-Pro, diffracted to about 1.8 Å resolution at the Swiss Light Source (Villigen, Switzerland), where all relevant datasets were collected.

Considering the high homology to human CyP (56% sequence identity), the structure was expected to be solvable by molecular replacement. Surprisingly, no solution was found, neither with AMORE (Navaza, 1994)

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Table 1. Structures of Cyclophilins that Have Been Solved to Date

Protein (Ligand)	Resolution (Å)	R Factors, R/R _{free} (%)	Space Group	Quaternary Structure	PDB Code	Reference
Human CyPA	1.63	18.0/—	P2 ₁ 2 ₁ 2 ₁	monomer	2CPL	(Ke, 1992)
Human CyPB (CsA)	1.85	16.0/—	P2 ₁ 2 ₁ 2 ₁	monomer	1CYN	(Mikol et al., 1994)
Human CyPH (U4/U6 SnRNP)	2.00	19.5/21.2	P12 ₁ 1	monomer	1MZW	(Reidt et al., 2003)
Human Nucl CyP20	2.00	17.0/22.4	P2 ₁ 2 ₁ 2 ₁	monomer	1QOI	(Reidt et al., 2000)
Human CyPJ	2.60	17.5/23.6	P3 ₁ 21	monomer	1XYH	(Huang et al., 2005)
<i>E. coli</i> CyP	2.10	16.0/—	C222 ₁	monomer	2NUL	(unpublished data)
<i>E. coli</i> CyPB	1.80	20.1/22.5	P3 ₂ 21	monomer	1J2A	(Konno et al., 2004)
Murine CyPC (CsA)	1.64	19.7/—	P12 ₁ 1	monomer	2RMC	(Ke et al., 1993b)
<i>B. malayi</i> CyP1	1.95	19.9/23.3	P4 ₁ 2 ₁ 2	monomer	1A58	(Taylor et al., 1998)
<i>P. falciparum</i> CyP19 (CsA)	2.10	15.0/19.0	P1	monomer	1QNG	(Peterson et al., 2000)
<i>C. elegans</i> CyP3	1.80	21.5/28.6	P4 ₁ 2 ₁ 2	monomer	1DYW	(Dorman et al., 1999)
<i>C. elegans</i> CyP5	1.75	18.2/25.0	P6 ₁	monomer	1HOP	(Pickens et al., 2002)
Bovine CyP40	1.80	17.8/25.6	C121	monomer	1IHG	(Taylor et al., 2001)
<i>S. cerevisiae</i> CyPA	1.90	19.9/21.6	P1	monomer	1IST	(unpublished data)
<i>M. tuberculosis</i> CyPA	2.60	21.2/22.9	P3 ₁	monomer	1W74	(Henriksson et al., 2004)
<i>A. fumigatus</i> CyP; Asp f 11	1.85	18.9/21.4	P3 ₁ 21	3D d.s. dimer	2C3B	(present work)

One representative of each protein is listed (unligated, if available). *A. fumigatus* CyP (Asp f 11) is the only one forming a 3D domain swapping (3D d.s.) dimer.

nor with MOLREP (Vagin and Teplyakov, 1997). Therefore, selenomethionylated protein was expressed and crystallized in order to perform a multiwavelength anomalous dispersion (MAD) experiment. The X-ray structure was solved by MAD phasing and refined against native data to a resolution of 1.85 Å with R and R_{free} values of 18.9% and 21.4%, respectively. A total of 90.9% of the residues are in the most favored regions of the Ramachandran plot, with those remaining located in the additional allowed regions. Data collection, phasing, and refinement statistics are shown in Table 2.

There are two monomers per asymmetric unit. The final model reveals an intertwined dimer consisting of chain A and B. Both chains run from the start methionine

to the last residue (Leu171) of the protein. The hinge region (aa 126–129) between the two subunits can be traced unambiguously within a good quality electron density map resulting from solvent flattening done without NCS averaging. Correlation coefficient values higher than 0.7 resulting from the real space correlation analysis indicate the correctness of the tracing. Two loops are missing in the final model: Phe70 to Phe91 and Asn105 to Ser113 in chain A and Asp69 to His95 and Asn105 to Gly112 in chain B. These loops are highly flexible resulting in a poor electron density. In chain A, the last residue of the thrombin cleavage site (Ser0) is also visible; 123 water molecules and 2 sulfate ions have been added. They are all well defined in the electron density.

Table 2. Data Collection, Phasing, and Refinement Statistics

	Native	Se (Inflection)	Se (Peak)	Se (Remote)
Data collection				
Wavelength (Å)	0.99990	0.97988	0.97894	0.97128
Unit cell axes a = b, c (Å)	64.83, 156.29	64.85, 157.01	64.86, 156.83	64.99, 157.37
Resolution (Å)	45.6–1.85 (1.92–1.85)	38.3–2.05 (2.12–2.05)	38.3–2.05 (2.12–2.05)	38.4–2.27 (2.35–2.27)
Unique reflections	33,079	46,229	46,153	34,063
Redundancy	5.9	5.6	5.6	5.0
Completeness (%)	99.1 (99.9)	99.7 (99.6)	99.5 (99.7)	99.0 (93.2)
R _{sym} (%)	8.7 (48.8)	4.5 (23.5)	4.7 (24.2)	6.1 (28.8)
Average I/σ	19.2 (2.6)	20.2 (4.8)	20.5 (5.0)	17.4 (3.3)
MAD Phasing				
Phasing power (iso/ano)		—/0.66	0.96/0.82	0.14/0.48
R _{cullis} (iso/ano)		—/0.94	0.75/0.78	1.00/0.90
FOM (SHARP/RESOLVE)		0.32/0.60		
Refinement statistics				
Resolution (Å)	45.6–1.85			
Number of reflections	33,035			
Number of atoms	2308			
R _{crist} (%)	18.9			
R _{free} (%)	21.4			
Mean B factor (Å ²): All atoms	43.7			
Main chain atoms (chain A/B)	40.8/40.9			
Side chain atoms (chain A/B)	46.1/46.2			
Solvent atoms	46.0			
Rmsd bond lengths (Å)	0.014			
Rmsd bond angles (°)	1.95			

In spite of the improvement in resolution, the ligand Ala-Pro is not visible in the electron density, neither the active site, nor on the surface. Due to dimerization, the active site is not accessible. Therefore, Ala-Pro cannot bind, despite its affinity to CyP. The K_d of Ala-Pro for Asp f 11 is not known, but it is most probably comparable to the K_d of other CyPs; Asp f 11 shows comparable activities to other CyPs, and all the active site residues are conserved. The enzymatic activity of Asp f 11 was demonstrated by its ability to catalyze the *cis-trans* isomerization of N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Flückiger et al., 2002). The human CyPA-Ala-Pro complex was obtained by soaking native crystals in 100 mM Ala-Pro (Ke et al., 1993a). A K_d of 23.3 mM was measured for Ala-Pro with *Caenorhabditis elegans* CyP3 (Wu Sy et al., 2001). Therefore, at a concentration of 80 mM Ala-Pro, one would expect binding of the dipeptide to Asp f 11. This is probably true for the monomeric form.

Overall Structure

The intertwined chains A and B form two similar subunits. Subunit A consists of the N-terminal amino acids 1–69 and the C-terminal amino acids 128–171 of chain A plus the domain-swapped residues 96–127 of chain B. Subunit B consists of the N-terminal amino acids 1–68 and the C-terminal amino acids 128–171 of chain B plus the domain-swapped residues 92–127 of chain A (Figures 1A and 1B). As in all other cyclophilins, the fold of each subunit consists of an eight-stranded antiparallel β barrel, and two α helices covering the top and the bottom of the barrel. In subunit A, there is an additional small 3_{10} helix formed by Thr122, Ser123, and Trp124 of chain B. In subunit B, Arg93 to Lys97 of chain A adopt an α -helical conformation, whereas in subunit A and in all other structurally known cyclophilins, this stretch is coiled.

Superposing human CyPA on either subunit of Asp f 11 reveals some structural deviation (Figure 1C). The rmsd between the C α positions of subunit A and CyPA is 1.18 Å, using a cut-off distance of 3.5 Å (120 out of 128 alignable pairs). For subunit B the corresponding rmsd is 1.26 Å (120 out of 130 alignable pairs). There is also some deviation between subunit A and B—mainly in the domain-swapped part—as shown by the rmsd of 1.15 Å (no cut-off). Using a 3.5 Å cut-off, the rmsd between 130 out of 135 alignable pairs drops to 0.65 Å.

Compared to human CyPA, there are some structural differences due to residue inserts (see sequence alignment, Figure 2). One difference in sequence and structure is the prolongation of the first loop after the first β strand by 5 residues. Another insert of two amino acids (Glu138/Lys139) leads to the elongation of helix 2, and a third insert (Asn160/Thr161) prolongs the loop after helix 2 (Figure 1C).

A further structural deviation is found in the loop after the first α helix (R45-PAG-E49), a divergent loop, where some CyPs have an insert (e.g., *C. elegans* CyP3 [Dorman et al., 1999]). Compared to human CyPA and CyPB, which feature the sequence GEKGF in the corresponding region, and form a type I β turn, the turn of Asp f 11 swings into the opposite direction, forming a type II β turn (Figure 1C). The same conformation has been observed in the structure of *C. elegans* CyP5 (Picken et al., 2002), featuring a similar sequence (KPKGE) as Asp f 11.

The protein forms a disulfide bond between Cys43 of the N terminus and Cys168 near the C terminus. The cysteines oxidize very slowly over time. This results in a protein, which is half oxidized and half reduced after purification and storage at 4°C for several days. Treatment with oxidized glutathione has no effect, probably due to the inaccessibility of the cysteines. The electron density reflects the situation very well. About 50% of the cysteines are visible in their reduced and 50% in their oxidized form. The sulfur atoms of the reduced cysteines are separated by 4.58 Å and 4.49 Å in subunit A and B, respectively. Simple rotation about the C α -C β cysteine side chain bonds toward each other allows disulfide bond formation with a bond length of 2.04 Å and chi torsion angles around 180° for all oxidized cysteines in both subunits. In the electron density, no disorder or conformational change is observed in the proximity of the disulfide bonds. Potential roles of this disulfide bond could be the stabilization of the protein in an oxidizing environment or a signaling mechanism in response to oxidative stress, as hypothesized for *C. elegans* CyP3 (Dorman et al., 1999). Both cysteines are conserved in many cyclophilins (Figure 2).

3D Domain Swapping

A striking feature is the dimerization of the protein by 3D domain swapping. A central segment—the end of the first hinge loop (aa 92–99 in chain A, aa 96–99 in B), the fifth and sixth β strand (aa 100–118) and the subsequent loop (aa 119–127)—changes into the neighboring subunit, thus displacing its corresponding element (Figures 1A and 1B).

3D domain swapping is quite rare. To date, about 60 structures of domain-swapped proteins have been reported, most of them exchanging their N or C terminus (Liu and Eisenberg, 2002). Only few proteins are known to swap a central element. For example, blood coagulant factor IX/X binding protein, an anticoagulant isolated from the venom of the habu snake, consists of two homologous subunits linked by an intermolecular disulfide bond. The two subunits form a heterodimer by exchanging a loop in the central part of the molecule (Mizuno et al., 1997). The NC1 Domain of type IV collagen forms a heterotrimer consisting of two α 1 chains and one α 2 chain, involving domain swapping of two central β strands (Sundaramoorthy et al., 2002). The structure of Asp f 11 reported here also exchanges a central element, but forms a homodimer. Because monomeric nonswapping structures of homologous CyPs are known, and because the swapped domain in Asp f 11 adopts exactly the same conformation as the corresponding nonswapped domain in homologous structures, this kind of domain swapping is termed quasi-domain swapping (Liu and Eisenberg, 2002). To our knowledge, Asp f 11 is the only quasi-domain-swapped homodimer exchanging a central element.

This kind of 3D domain swapping requires two hinge loops that adopt a different conformation in the domain-swapped dimer and in the monomer. The second hinge loop is very short in Asp f 11. It runs from amino acid 126 to 129 (DGKH) and adopts a different conformation in each chain. All backbone atoms of this hinge loop and the side chains of the aspartate and the histidine are clearly defined in the experimental electron

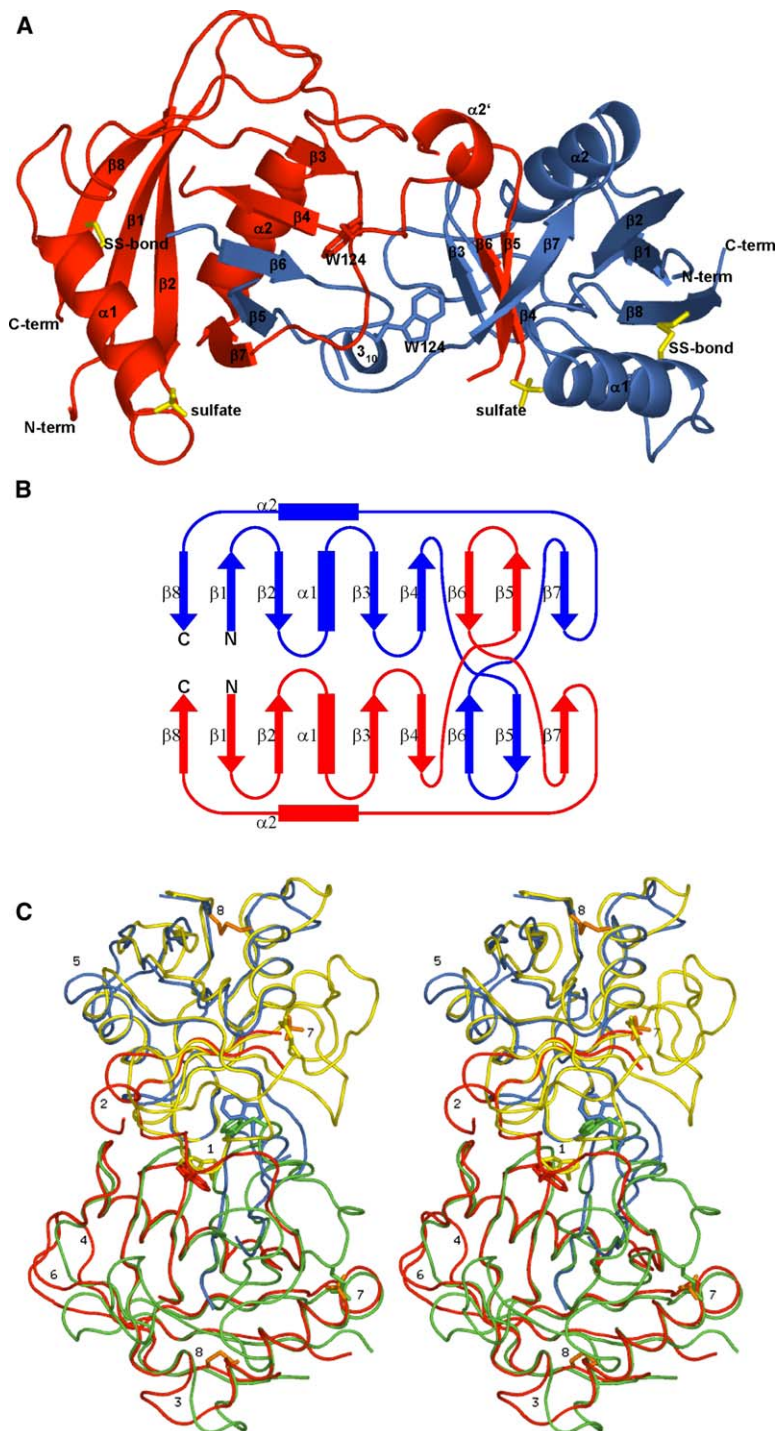


Figure 1. Crystal Structure of Asp f 11

(A) Overall structure of the 3D domain-swapped Asp f 11 dimer. Chain A and B are in red and blue, respectively. The conserved Trp124 is shown as capped stick model.

(B) Topology plot of secondary structure elements of the dimer schematically showing the 3D domain swapping.

(C) Superposition of two independent monomers of human CyPA (2CPL)—one in green, one in yellow—on subunit A (core in red, swapped domain in blue) and on subunit B (core in blue, swapped domain in red) of Asp f 11, respectively. The view is the same as in (A), but rotated by 90° perpendicular to the plane. The moieties of the protein diverging from human CyPA have been numbered from 1 to 8. The conformation of Trp124 of Asp f 11 is different from Trp121 of human CyPA (1). The end of the first hinge loop is α -helical in chain A of Asp f 11, whereas it is coiled in human CyPA (2). The divergent loop of Asp f 11 forms a type II β turn, whereas in CyPA it forms a type I β turn (3). The first loop is prolonged in Asp f 11 by 2 residues (4). Helix 2 is prolonged in Asp f 11 by 2 residues (5). The loop after helix 2 is elongated by another 2 residues (6). The side chain carboxyl of Glu86 of human CyPA (homologous to Glu89 of Asp f 11) superposes on the sulfate ion in the Asp f 11 crystal structure (7). Asp f 11 forms a disulfide bond, human CyPA doesn't (8).

density, whereas the side chain of the lysine is not visible (Figure 3A). All distances, angles, and torsions in the hinge loop are chemically sound with torsion angles lying in the most favored or additional allowed regions of the Ramachandran plot. There are three H bonds between the hinge loops of chain A and B (A:His129;N bonded with B:Leu125;O, A:Leu125;O with B:His129;NE2, and A:Gly127;N with B:Trp124;O). An additional H bond is formed within the hinge loop of chain A (Trp124;O with His129;NE2) (Figure 3A).

The first hinge loop is very long, running from amino acid 68 to 99. In homologous structures, this part is very well defined. It forms a long loop, which runs like a handle from one side of the molecule to the other side, connecting β strands 4 and 5. In contrast, the larger part of this loop is disordered in Asp f 11 and thus not visible in the electron density. Therefore, its role as hinge loop is ambiguous. Nevertheless, the electron density implies that this loop must be arranged in a different way than in homologous structures. First, a well-defined

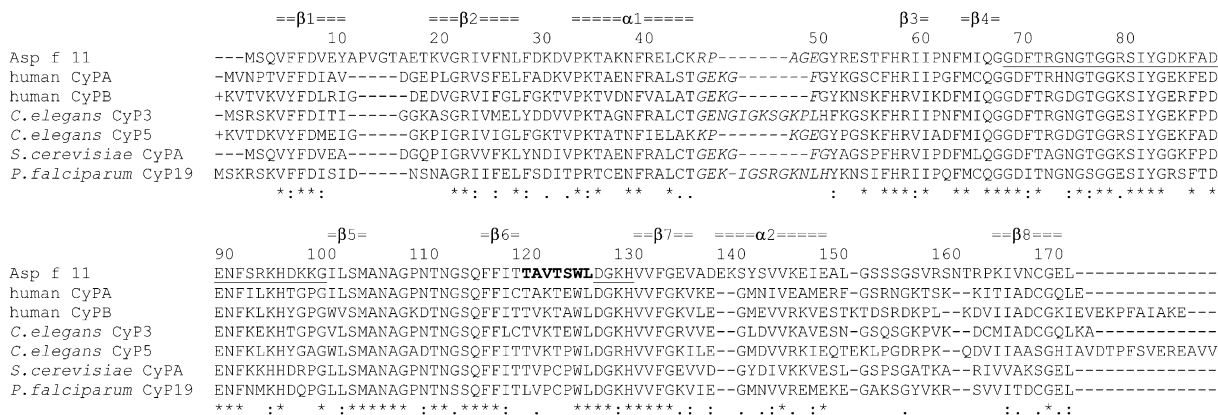


Figure 2. Structure-Based Sequence Alignment of Asp F 11 with Homologous CyPs

The top line shows the secondary structure of Asp f 11 assigned by DSSP (Kabsch and Sander, 1983). The 3₁₀-helix formed by chain B (Thr122–Trp124) and the additional α helix 2' formed by chain A (Arg93–Lys97) are not indicated. The second line shows the sequence numbering of Asp f 11. A "plus" sign at the N terminus indicates truncation of the signal sequence. The first and second hinge loop of Asp f 11 are underlined, and the W loop is in bold. The divergent loop (aa 45–49) is in italics. The aligned CyPs are human CyPA (PDB code 2CPL), human CyPB (1CYN), *C. elegans* CyP3 (1DYW), *C. elegans* CyP5 (1HOP), *S. cerevisiae* CyPA (1IST), and *P. falciparum* CyP19 (1QNG) (Table 1).

sulfate ion is positioned on top of α helix 1 beside the pyrrolidine ring of Pro33, where—in homologous structures—the side chain carboxyl of Glu89 is situated (Figure 1C). This implies that Glu89 of the long loop of Asp f 11 is differently positioned. Both residues, Pro33 and Glu89, are highly conserved in all CyPs (Figure 2).

Second, the visible end of the long loop of Asp f 11 adopts a different conformation from the corresponding loops of homologous structures (Figure 1C). Going backward from β strand 5, this part of the loop folds back in homologous CyPs, running toward β strand 4 within the same subunit. In Asp f 11, this part of the

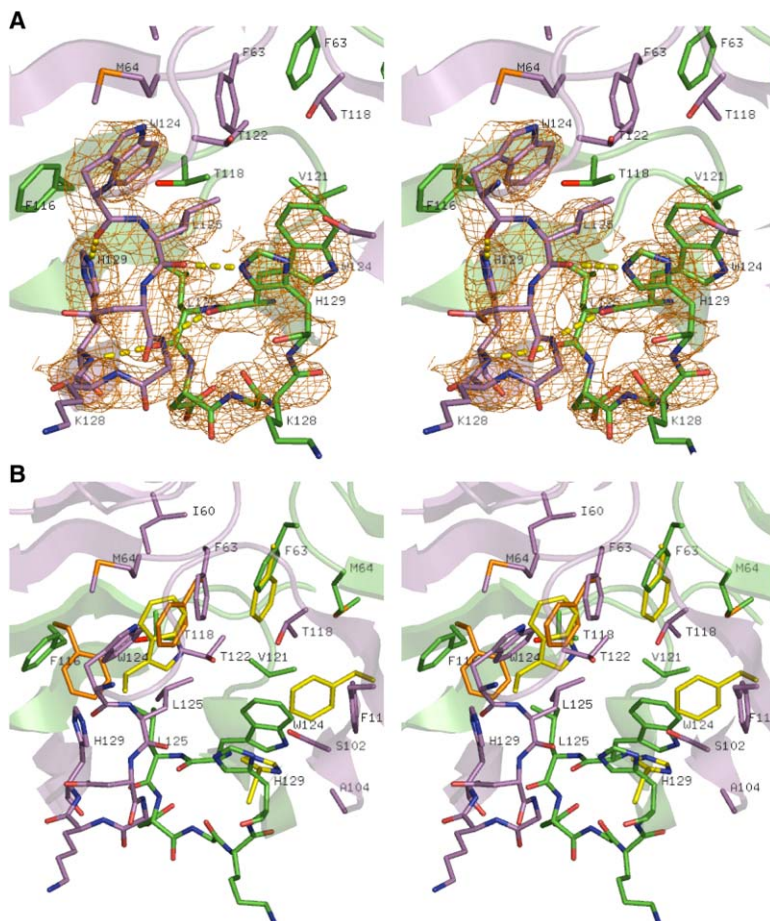


Figure 3. W Loop Bound to Active Site and Second Hinge Loop

(A) Electron density of the second hinge loop and parts of the adjacent W loop (aa 124–129). The experimental map is contoured at 1σ using solvent-flattened experimental phases calculated without NCS averaging. H bonds within and between the hinges of both chains are indicated by yellow, dashed lines. Residues of chain A and B are colored in violet and green, respectively. (B) Hydrophobic binding of the W loop into its own active site. Trp124 and Leu125 of chain A sit into the hydrophobic pocket of subunit A, making hydrophobic interactions with surrounding residues of both chains. In subunit B, Val121 and Trp124 of chain B sit into its active site. Position and conformation of some distinctive residues of human CyPA (2CPL) are shown for comparison. Two independent monomers of CyPA were superimposed on either subunit of Asp f 11 (see Figure 1C). Side chains of CyPA superposed on subunit A and B are colored in orange and yellow, respectively.

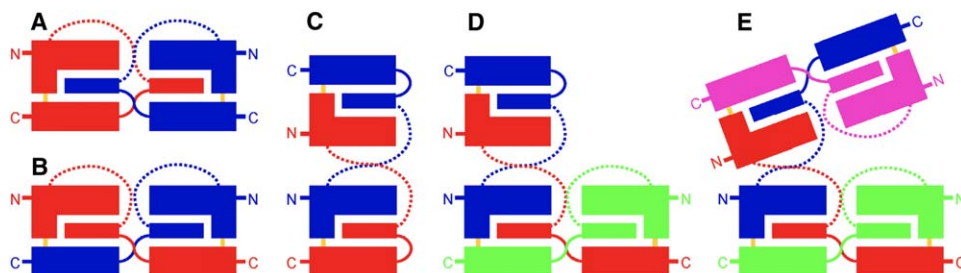


Figure 4. Illustrations of Putative Forms of 3D Domain Swapping

The first hinge loop, which is not visible in the crystal structure, is in dashes. The second hinge loop is fully drawn.

(A–E) (A) 3D domain swapping of the central element, as proposed in the crystal structure, leads to intramolecular disulfide bonds (shown in yellow). (B) 3D domain swapping of the C terminus would lead to intermolecular disulfide bonds; this case can be ruled out for the crystal structure by a nonreducing SDS-PAGE (Figure 5). (B–E) C-terminal (B) and N-terminal domain swapping (C) can lead to misfolded dimers, which are covalently linked by intermolecular disulfide bonds. A combination of central, C-terminal and N-terminal domain swapping can lead to covalently linked trimers (D) and tetramers (E).

loop goes straight on, pointing toward β strand 4 of the neighboring subunit. It forms an α helix in chain A (aa 93–97), whereas, in chain B, it adopts a rather extended conformation (aa 96–99).

Nevertheless, the missing electron density leads to two putative scenarios in respect to domain swapping. First, this long loop represents a hinge loop, thus swapping—together with the second hinge loop—the central element, as suggested in this work (Figure 4A). Second, this loop doesn't represent a hinge loop. Thus, the remaining second hinge loop would domain-swap the whole C terminus (aa 128–171) (Figure 4B) instead of the central element. Taking distances and crystal packing into account, both cases would be possible: β strand 4 connected to β strand 5 of the neighboring subunit or to β strand 5 of the same subunit. In the first case, we end up with an intramolecular disulfide bond (Cys43–Cys168) between the N and the C terminus, as shown in Figure 4A. The second case would lead to an intermolecular disulfide bond (Figure 4B). This second case can be ruled out easily by a denaturing, non-reducing SDS-PAGE. Figure 5 shows that the crystallized protein runs as two very close bands, both at the height of the monomer and both with about the same intensity. The lower band represents reduced protein, the upper band oxidized protein containing an intramolecular disulfide bond. This finding reflects the situation in the electron density, where 50% of the cysteines are reduced and 50% oxidized. The disulfide bonding is intramolecular, connecting the N and C terminus within the same chain. Therefore, the long loop must represent a hinge loop, swapping—together with the second hinge loop—the central element. Interestingly, both regions depicted here as hinge regions are conserved in length and sequence between the homologous proteins (Figure 2), suggesting that domain swapping could also occur in other CyPs. The structural analysis clearly shows that 3D domain swapping is the only solution, allowing fitting of the electron density map and structural refinement. Thus, a similar dimerization arrangement would be expected for homologous proteins undergoing the same event, while for the structures showing the monomeric state (Table 1), different crystal packing arrangements have been observed.

There is one other small loop not visible in the electron density, connecting β strands 5 and 6 (aa 105–112). But

here—considering distances—the connectivity is unambiguous.

Dimer Interface and Binding of W Loop into Active Site

Asp f 11 dimerizes by 3D domain swapping, which leads to a highly intertwined molecule. The calculated surface area of the interface between chain A and B is 2310 $\text{\AA}^2$ per monomer, which makes up about one fourth of the whole surface area of chain A (8880 $\text{\AA}^2$). The interface formed by purely nonswapped parts—excluding hinges

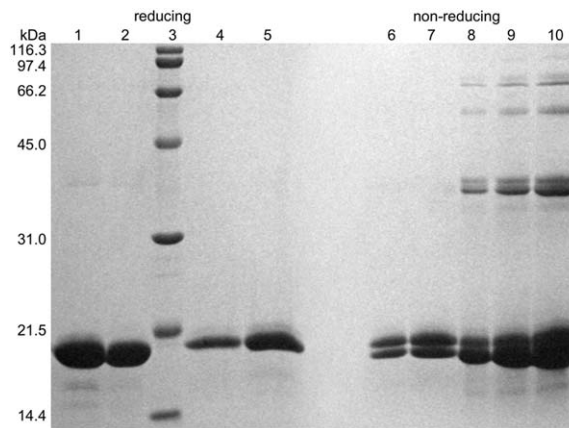


Figure 5. Reducing and Nonreducing SDS-PAGE of Crystallized and Misfolded Asp f 11 Protein

Molecular weights of the marker proteins (lane 3) are indicated on the left side. The shown lanes correspond to: crystallized protein under reducing (lane 5) and nonreducing (lane 7) condition. As a control, protein before crystallization under reducing (lane 4) and non-reducing (lane 6) condition, each 3 μg . Misfolded protein under non-reducing conditions at 4 μg (lane 8), 8 μg (lane 9), and 16 μg (lane 10). As a control, misfolded protein under reducing conditions, 8 μg (lane 1). Reduced protein is shown as a reference (lane 2). Under nonreducing conditions, the upper band around the 21.5 kDa marker represents the oxidized form of the protein, whereas the lower band indicates the reduced one. The small difference in running behavior between reducing and nonreducing condition is due to the presence or absence of DTT/ β -mercaptoethanol as shown by the analysis of partially reduced batches of protein (data not shown).

and swapped domains—is very small, at 180 Å² (less than 8% of the whole interface). Thus, most interactions in the dimer interface are contributed by either of the swapped domains.

The swapped β strands 5 and 6 replace the corresponding elements in the neighboring subunit, adopting exactly the same conformation (Figure 1C). Together with β strands 3 and 4, they form the hydrophobic pocket where the active site for the *cis-trans* isomerization function is situated. The subsequent domain-swapped loop (aa 119–125) contains a highly conserved tryptophan (W124), and is thus called the W loop. It also replaces the corresponding W loop in the neighboring subunit, but, in this way, comes to lie in its own active site. This additional intramolecular interaction is not observed in other CyPs. The conformation of the two corresponding W loops compared to each other and to the homologous W loop in human CyPA is not identical, but similar (Figure 1C).

The binding mode of the W loop within the active site is different in each subunit of Asp f 11. In subunit A, the side chains of Trp124 and Leu125 of chain A make hydrophobic interactions with the side chains of Ile60, Phe63, Met64, and His129 of chain A and with the domain-swapped side chains of Phe116, Thr118, Val121, Trp124, and Leu125 of chain B (Figure 3B). In subunit B, the side chains of Trp124 and Val121 of chain B make hydrophobic interactions with the side chains of residues Phe63, Met64, and His129 of chain B and Phe63, Ser102, Ala104, Phe116, Thr118, Thr122, and Leu125 of chain A (Figure 3B). Interestingly, the position and orientation of the side chain of Trp124 are different in the two subunits. In subunit B, the benzene ring of Trp124 makes π - π interactions with the imidazole ring of His129, while in subunit A, it only makes van der Waals interactions.

The side chains of the W loop, which interact with the active site (Trp and Leu in chain A, Trp and Val in chain B) are completely buried in Asp f 11. In homologous structures, these residues lie on the rim of the active site, and the side chains of the tryptophan and the valine are solvent accessible.

The binding of Trp and Leu/Val within the active site of Asp f 11 leads to a change in the side chain conformation and/or in the position of the C α atom of all the residues involved in the hydrophobic pocket when compared to superposed human CyPA. The most distinctive displacements include the C α positions of A:Phe63 (2.0 Å), B:Phe63 (2.2 Å), B:Val121 (2.6 Å), A:Trp124 (3.0 Å), and the side chain positions of A:Phe116;CZ (5.2 Å), B:Phe116;CZ (5.2 Å), and B:His129;CH2 (5.4 Å), to name but a few (Figure 3B). These findings explain the relatively high rmsd between the human CyPA and the chain A and B of Asp f 11 (see above).

Biological Relevance

Is the domain-swapped Asp f 11 dimer physiologically relevant, or is it just a crystallographic artifact? For domain-swapped proteins in general, both cases have been reported (Liu and Eisenberg, 2002). Domain swapping was originally proposed to be a mechanism for the emergence of oligomeric proteins and as a means of functional regulation (Bennett et al., 1995). It can also be a potentially harmful process leading to misfolding,

aggregation, and amyloid formation of some proteins (e.g., cystatin C [Janowski et al., 2001; Staniforth et al., 2001] and human prion protein [Knaus et al., 2001]). Several artifacts of domain-swapped proteins have been reported, where the biological function for the dimers are unknown (Liu and Eisenberg, 2002). They were obtained under nonphysiological low pH, and, often, the domain swapping only occurred as a consequence of the truncation of the whole protein.

The Asp f 11 crystals were grown under physiological pH, but under nonphysiological high salt and high protein concentrations. Despite these facts, dimerization cannot be excluded *in vivo* due to effects like local concentration, other binding partners, or macromolecular crowding in cells (Minton, 2001). Binding of other molecules to Asp f 11 or an environmental change could induce domain swapping and dimer formation. The effect of other macromolecules on a specific macromolecule in cells has been studied and termed macromolecular crowding (Minton, 2001). Macromolecular crowding increases local protein concentration and facilitates protein oligomerization (Lindner and Ralston, 1995; Rivas et al., 1999).

In vitro, the Asp f 11 protein is clearly monomeric under physiological conditions, even at high protein concentration, as shown by gel filtration (Figure 6A). The protein elutes at 12.55 ml, forming one single peak. Calibration with marker proteins yields a molecular weight of 14.9 kDa, which is in agreement with the calculated monomer weight of 18.9 kDa. Under these conditions, the protein also shows *cis-trans* isomerization activity (Flückiger et al., 2002).

However, dimerization can be induced in solution with a modified crystallization buffer at an ammonium sulfate concentration of 20% (Figure 6B). Besides the monomer peak, a second peak, corresponding to a dimer, is obtained. The height of the second peak is dependent on the concentration of the protein. The higher the protein concentration, the bigger the fraction of the dimeric form. Gel filtration of protein at 7.5 mg/ml results in a dimer fraction of about 10%. Both peaks exhibit shoulders, which might represent reequilibrating protein or intermediate states, such as partly unfolded protein. To assess the peptidylprolyl *cis-trans* isomerase activity of the monomeric and dimeric fraction of the gel filtration experiment performed with modified crystallization buffer, enzymatic activity was measured with the assay described by Kofron (Kofron et al., 1991). The enzymatic assay clearly shows that the dimer fraction is inactive while the monomer fraction is active (Figure 6D). This indicates that the dimer observed in the gel filtration experiment is arranged in a similar way as the dimer seen in the crystal structure. Furthermore, it implies a putative regulation mechanism.

In general, CyPs are active in their monomeric form. No dimerization, and thus no potential role for this hypothetical state, have been reported to date. For Asp f 11, both the monomer and the dimer form can be demonstrated (Figures 6A and 6B). Dimerization, as shown by the crystal structure, obviously inactivates the *cis-trans* isomerization function, since both active sites are occupied by their own W loops. Therefore, dimerization of Asp f 11 *in vivo* could be a means of regulating its own biological function.

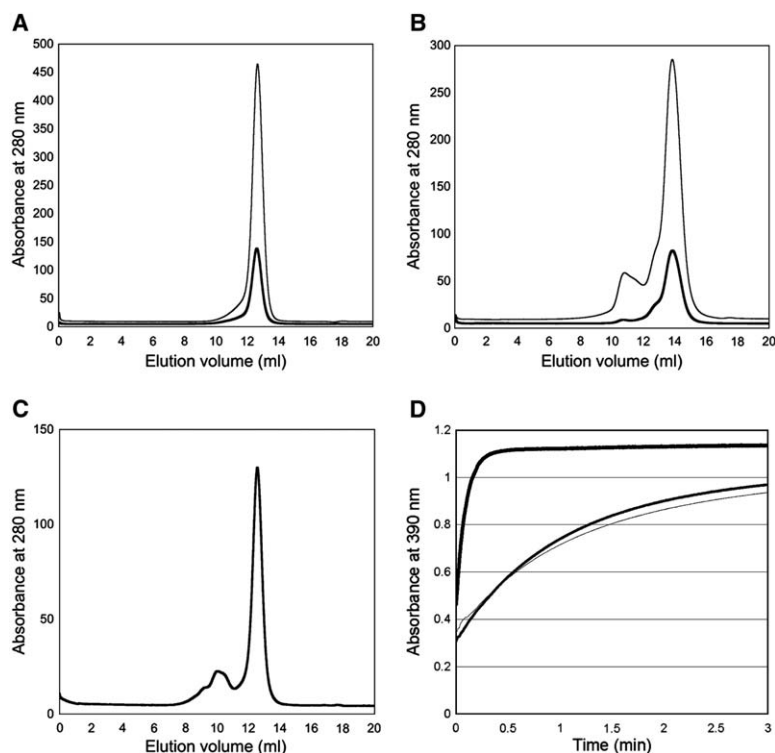


Figure 6. Gel Filtration of Normal and Misfolded Asp F 11 Protein and Peptidylprolyl *cis-trans* Isomerase Activity of the Monomeric and Dimeric Fraction

(A) Protein at 2 mg/ml (bold line) and 7.5 mg/ml (thin line) under physiological conditions (100 mM NaCl, 50 mM Tris, pH 7.5); the chromatogram shows only a monomer peak, which elutes at 12.55 ml. Calibration with four marker proteins yields a molecular weight of 14.9 kDa ($R^2 = 0.98$).

(B) Protein at 2 mg/ml (bold line) and 7.5 mg/ml (thin line) in a modified crystallization buffer (20% ammonium sulfate, 0.1 M MES, pH 6); the chromatogram shows an additional, smaller dimer peak, which is concentration dependent. The main fraction elutes at 13.77 ml, whereas the second peak elutes at 10.68 ml. The high salt conditions used cause the observed shift in the elution times. Despite a low correlation factor ($R^2 = 0.94$), calibration with the four marker proteins indicates a monomeric state for the major peak and a dimeric state for the minor peak.

(C) Misfolded protein at 2 mg/ml under physiological condition (100 mM NaCl, 50 mM Tris, pH 7.5). The major peak elutes at 12.5 ml corresponding to the monomeric state. The minor peak, the shoulder to the right, and the shoulder to the left elute at 10.05, 10.30, and 9.31 ml, which results in calibrated weights of 34.2, 31.5, and 43.7 kDa, respectively, indicating at least the formation of dimers and possibly a trimer.

(D) Peptidylprolyl *cis-trans* isomerization by monomeric (bold line) and dimeric (normal line) Asp f 11 and, as a negative control, in the absence of protein (thin line). The substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide was isomerized, the *trans*-isomer was cleaved by α -chymotrypsin, and the increase in absorbance measured at 390 nm. The monomer fraction is active, whereas the dimer fraction is inactive.

Misfolding

Two flexible hinge loops could leave the protein prone to misfolding. 3D domain swapping can be a harmful mechanism for misfolding, aggregation, and amyloid formation (Rousseau et al., 2003).

Indeed, misfolding of Asp f 11 can be induced by freeze-thawing the protein in its reduced form. Freeze-thawing of oxidized protein does not lead to unfolding and refolding/misfolding, probably due to the stabilization of the protein by the intramolecular disulfide bond. After freeze-thawing, about 30% of the reduced protein precipitated. The remaining soluble part was changed into a nonreducing buffer, in order to allow oxidation. A subsequent gel filtration under physiological conditions clearly showed absorption peaks in the dimer to tetramer range, in addition to the monomer peak (Figure 6C). Analysis of the gel filtration fractions by non-reducing SDS-PAGE shows monomers as well as covalently linked dimers, trimers and tetramers (data not shown). The ratio between the peak heights was independent of the protein concentration; the oligomer peaks also appear at low concentration (data not shown). The protein used for gel filtration was analyzed by a nonreducing, denaturing SDS-PAGE (Figure 5). Again, the gel showed two close bands at the monomeric weight, which correspond to nonoxidized and to intramolecularly oxidized protein. Moreover, there are bands visible at the dimer, trimer, tetramer, and at higher oligomer masses. These bands must represent oligomers covalently linked by intermolecular disulfide

bonds. They disappear under reducing conditions (Figure 5, lane 1), as expected for oligomers formed by intermolecular disulfide bonds. These results suggest that partial unfolding and refolding occurs during freeze-thawing under reducing conditions.

The observed covalently linked multimers can be explained by alternative forms of 3D domain swapping. The protein can refold/misfold into a dimer, where the whole C terminus after the second hinge loop or the whole N terminus before the first hinge loop is domain swapped (Figures 4B and 4C). These two kinds of domain swapping only involve one hinge loop each. After oxidation, the two chains of the dimer will be covalently linked. A combination of N-terminal, C-terminal, and/or central domain swapping can lead to linear trimers and tetramers (Figures 4D and 4E), and also to cyclic tetramers. Linear multimerization could carry on to higher oligomers, aggregates, and, eventually, precipitate, which indeed can be observed *in vitro* after freeze-thawing. Therefore, incorrect 3D domain swapping could serve as a model for the mechanism of misfolding and precipitation of proteins in general.

Another explanation for the covalently linked multimers could be unspecific disulfide bonding between independent, nonswapping subunits, but this seems very unlikely. There are only two cysteines in Asp f 11 that can form the aforementioned disulfide bond. Both cysteines are involved in secondary structure elements. Cys43 is situated in α helix 1, Cys168 in β sheet 8. This makes the whole region more rigid, despite the fact that

Cys168 lies close to the C terminus. The sulfur atoms are completely inaccessible by solvent as calculated by NACCESS (Hubbard and Thornton, 1996), both in the reduced and the oxidized form. During freeze-thawing under reducing conditions, neither a specific nor a nonspecific disulfide bond can form. Once the protein is refolded and after the buffer is exchanged against a non-reducing buffer, disulfide bonding is more likely to occur within the same subunit, due to solvent inaccessibility of the cysteines.

One wonders, why nature preserves features such as two flexible hinge loops and—as observed in all other CyP structures—a solvent-accessible tryptophan, which leave the protein highly prone to misfolding. The two hinges, as well as the tryptophan and the leucine of the W loop are highly conserved among all CyPs (Figure 2). This leads to the assumption that the domain-swapped protein, as observed in the present crystal structure, could have a biological meaning, but its biological role has yet to be found. Further work could focus on the characterization of the different misfolded multimers and on homologous CyPs in order to investigate whether they show the same multimerization behavior as that of Asp f 11.

Experimental Procedures

Cloning, Protein Expression, and Purification

The original full-length CyP from *A. fumigatus* was cloned by phage surface display (Cramer et al., 1994) as N-terminal, noncleavable His-tagged protein containing a linker of 9 amino acids between the His tag and the start methionine (Cramer, 1999). In order to clone a thrombin-cleavable His-tagged protein, the *Asp f 11* gene was amplified from the original clone by PCR with the primers 5' BamHI-1 5'-CCGCGGATCCATGTCTCAGGTCTTCTTC-3' and 3' HindIII-1 5'-GCCAAGCTTTACAGCTCACCACAGTTGAC-3' by Deep Vent Polymerase (NEB, Beverly, MA). The PCR product was digested with BamHI and HindIII restriction endonucleases (NEB), cleaned with QIAquick PCR purification kit (Qiagen, Hilden, Germany), and ligated into a modified pQE32 vector containing an N-terminal His₆ tag followed by a thrombin cleavage site (HHHHHLLVPRGS), where GS corresponds to the BamHI site. The recombinant vector was transformed into *Escherichia coli* strain XL1-Blue and the sequence of the insert verified by DNA sequencing.

The XL1-Blue cells were grown at 37°C in LB medium to an OD₆₀₀ of 0.6, induced with 1 mM IPTG, harvested after another 15 hr of incubation by centrifugation at 6000 × g for 10 min at 4°C, and stored at −20°C. The cell pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by French press. The insoluble material was removed by centrifugation at 20,000 × g (20 min, 4°C).

The His-tagged recombinant protein was purified by nickel affinity chromatography with a 5 ml HiTrap Chelating HP column (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein was eluted in a linear buffer gradient (10–250 mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). The N-terminal His tag was cleaved off by thrombin (20 U/mg protein) in 300 mM NaCl, 50 mM Tris, 2 mM CaCl₂, pH 7.5, by incubation for 18 hr at 22°C. The cleaved protein was further purified by gel filtration on a Superdex 75 column (FPLC, Pharmacia, Uppsala, Sweden) equilibrated with 100 mM NaCl, 50 mM Tris, pH 7.5. The eluted protein was diluted 1:10 with H₂O and concentrated to 16 mg/ml. Protein identity and purity were assessed using MALDI-TOF mass spectroscopy and SDS-PAGE.

The selenomethionine-substituted protein was expressed under minimal media conditions that inhibit methionine synthesis (Van Duyne et al., 1993). The XL1-Blue cells were incubated overnight in LB medium at 37°C and harvested at 6000 × g for 10 min. The pellet was washed twice in H₂O and then inoculated into 1 liter M9 medium (supplemented with the appropriate antibiotics, 1 mM MgSO₄, 0.4%

glucose, 0.00005% thiamine, 100 mg Lys, Phe, and Thr each, and 50 mg Ile, Leu, Val, and SeMet each) at 37°C until an OD₆₀₀ of 0.45 was reached. After induction with 1 mM IPTG, the cells were grown at 37°C for another 15 hr. The SeMet protein was purified and thrombin-digested by the same procedure as the native protein, except that all buffers were supplied with 10 mM β-mercaptoethanol. The digested SeMet protein was further purified by gel filtration on a Superdex 75 column (FPLC, Pharmacia) equilibrated with 100 mM NaCl, 50 mM Tris, 10 mM DTT, pH 7.5. The eluted protein was diluted 1:10 with H₂O, 10 mM DTT, and concentrated to 9 mg/ml. The incorporation of SeMet was verified by MALDI-TOF mass spectroscopy.

Crystallization

Crystallization was performed with the hanging drop vapor diffusion method at 23°C. The protein was crystallized by mixing 3 μl of protein solution (7 mg/ml with or without 80 mM Ala-Pro) with an equal volume of crystallization solution (13% ammonium sulfate, 0.1 M MES, pH 6), equilibrating it against 500 μl of reservoir solution (50% ammonium sulfate, 0.1 M sodium citrate, pH 5.5). Crystals grew to a size of 300 × 300 × 150 μm within 3–5 days. They belong to space group P3₁21, with cell parameters *a* = *b* = 64.8 Å, *c* = 156.3 Å, and contain two monomers per asymmetric unit.

The selenomethionylated protein crystals were obtained under the same conditions, adding 80 mM Ala-Pro to the protein solution and 5 mM DTT to the reservoir solution. They grew to 200 × 140 × 140 μm, and belong also to space group P3₁21, with cell parameters *a* = *b* = 64.9 Å, *c* = 157.0 Å. Crystals were cryoprotected by soaking them stepwise for 20 s in reservoir solution complemented with 80 mM Ala-Pro, increasing the ethylene glycol concentration from 6% to 12% and then 19%. The crystals were flash-cooled in a stream of gaseous nitrogen and measured at 100 K.

Data Collection, Phasing, and Refinement

Data were obtained on the synchrotron beamline X06SA at Swiss Light Source (Villigen, Switzerland). A dataset of a native crystal was collected to 1.85 Å resolution (Table 2). MAD data were collected with a selenomethionylated crystal to a resolution of 2.05 Å in order to determine the structure by the MAD technique (Table 2).

Data were processed and scaled with DENZO and SCALEPACK of the HKL program package (Otwinowski and Minor, 1997). The selenium atom positions were found with SHELXD (Schneider and Sheldrick, 2002) with MAD *F_A* values calculated with XPREP (Bruker Nonius). Experimental phases were calculated and refined with SHARP (de La Fortelle and Bricogne, 1997). The initial electron density was improved by solvent flattening with 2-fold NCS averaging using RESOLVE (Terwilliger, 1999). A polyaniline model of human CyPA, Protein Data Bank (PDB) code 2CPL (Ke, 1992) was fit into the improved electron density by phased molecular replacement using MOLREP (Vagin and Teplyakov, 1997), followed by manual rebuilding using XtalView (McRee, 1999). The structure was refined against the native dataset with REFMAC (Murshudov et al., 1997) as implemented in the CCP4 program suite (CCP4, 1994). Initially, NCS restraints were used, which were stepwise released and finally omitted. Water molecules were introduced using ARP (Lamzin and Wilson, 1993). Final rounds of refinement were carried out with TLS refinement, defining the domain-swapped central element and the N terminus before, together with the C terminus thereafter, of each chain as separate TLS groups. Statistics from phasing and refinement are provided in Table 2. The stereochemical quality of the final model was assessed with PROCHECK (Laskowski et al., 1993) and WHATCHECK (Hooft et al., 1996). Secondary structure elements were assigned automatically with DSSP (Kabsch and Sander, 1983). Surface areas were calculated with the program NACCESS (Hubbard and Thornton, 1996), an implementation of the Lee and Richards solvent accessibility algorithm (Lee and Richards, 1971), with a probe radius of 1.4 Å and a slice width of 0.01 Å. In order to obtain a good experimental map of the hinge regions (aa 126–129) between the two subunits, solvent flattening was redone without NCS averaging. The resulting electron density map (Figure 3) makes tracing unambiguous. Building of the model in a nonswapped way was not feasible with this experimental map. To assess the correctness of the tracing of the hinge region, a real-space map correlation was calculated by overlapmap

(CCP4, 1994) and plotted along the sequence, showing reasonable correlation coefficients higher than 0.7 in the main chain of the connecting loops as well as the rest of the protein.

Characterization of Quaternary Structure and Activity Assay

The molecular weight of the Asp f 11 protein in solution was determined by gel filtration on a Superdex 75 column (Pharmacia), equilibrated in 100 mM NaCl, 50 mM Tris, pH 7.5. A total of 100 μ l of protein at two different concentrations (2.0 and 7.5 mg/ml) were eluted with the same buffer at a flow rate of 0.8 ml/min at 23°C. Aprotinin, cytochrome C, carbonic anhydrase, and BSA (molecular weight marker kit; Sigma, St. Louis, MO) served as marker proteins.

Dimerization behavior was studied in a modified crystallization buffer (20% ammonium sulfate, 100 mM MES, pH 6) with the same column and the same parameters. The protein (at two different concentrations: 2 and 7.5 mg/ml) was incubated in the modified crystallization buffer for 3 hr at RT before application.

Oligomerization behavior of misfolded protein was studied in 100 mM NaCl, 50 mM Tris, pH 7.5, with the same column and parameters. Misfolding was induced by freeze-thawing. The protein was purified under reducing conditions (as described above) and frozen at -20°C in 100 mM NaCl, 50 mM Tris, 10 mM DTT, pH 7.5. After thawing at RT, the precipitated part (about 30%) was removed by centrifugation at 15,000 $\times$ g, 5 min, 4°C, and the buffer exchanged against a nonreducing buffer (100 mM NaCl, 50 mM Tris, pH 7.5). The protein was applied on the column at 2 mg/ml.

The nature of intra- or intermolecular disulfide bonding of protein, which was used for gel filtration and crystallization, as well as crystallized protein, which was dissolved in H₂O, was analyzed by reducing and nonreducing SDS-PAGE with 15% polyacrylamide gels. For reducing SDS-PAGE, the samples were mixed with 3 $\times$ SDS sample buffer, supplemented with 10% (v/v) β -mercaptoethanol and 0.5 M DTT, and boiled at 95°C for 20 min. For nonreducing SDS-PAGE, samples were mixed with 3 $\times$ SDS sample buffer containing no reducing agents.

Peptidylprolyl *cis-trans* isomerase activity of the monomeric and dimeric fraction of the gel filtration experiment with modified crystallization buffer was measured by the assay described by Kofron (Kofron et al., 1991) with minor modifications. A total of 25 μ l of 0.5 μ M protein solution (final concentration, 25 nM) were diluted with 437.5 μ l 50 mM HEPES, 100 mM NaCl, pH 8 (all solutions cooled on ice). Then, 25 μ l of 10 mg/ml α -chymotrypsin (Sigma) in 1 mM HCl was added and the reaction was started by adding this mixture to 12.5 μ l of 4 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma) in trifluoroethanol, 470 mM lithium chloride, in a semimicrocuvette. The increase in absorbance at 390 nm was measured for 3 min at 0°C in a Cary 50 Conc spectrophotometer. The background, which is due to thermal *cis-trans* isomerization of the substrate, was measured with a blank sample without protein.

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Accession Numbers

Coordinates and structure factors have been deposited in the PDB with accession code [2C3B](#).