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The Crystal Structure of the Yeast Hsp40 Ydj1 Complexed with Its Peptide Substrate

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Summary

The mechanisms by which Hsp40 functions as a molecular chaperone to recognize and bind nonnative polypeptides is not understood. We have identified a peptide substrate for Ydj1, a member of the type I Hsp40 from yeast. The structure of the Ydj1 peptide binding fragment and its peptide substrate complex was determined to 2.7 Å resolution. The complex structure reveals that Ydj1 peptide binding fragment forms an L-shaped molecule constituted by three domains. The domain I exhibits a similar protein folds as domain III while the domain II contains two Zinc finger motifs. The peptide substrate binds Ydj1 by forming an extra β strand with domain I of Ydj1. The Leucine residue in the middle of the peptide substrate GWLYEIS inserts its side chain into a hydrophobic pocket formed on the molecular surface of Ydj1 domain I. The Zinc finger motifs located in the Ydj1 domain II are not in the vicinity of peptide substrate binding site.

Introduction

Molecular chaperone Hsp40s play critical roles in cell physiology by acting together with molecular chaperone Hsp70 members to promote protein folding, assembly, translocation, and degradation (Hartl, 1996; Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002). Hsp40 proteins can interact with the hydrophobic side chains of nonnative polypeptides through the peptide binding fragment and prevent the polypeptides from aggregating (Langer et al., 1992; Schmid et al., 1994). Hsp40s can then form transient complexes with Hsp70s and present the nonnative polypeptides to Hsp70s for subsequent protein folding (Misselwitz et al., 1998; Laufen et al., 1999; Qian et al., 2002). All Hsp40 proteins contain an N-terminal J-domain that can stimulate the ATPase activities of Hsp70 (Bukau and Horwich, 1998; Wall et al., 1994). Both type I and type II Hsp40s have a peptide binding fragment located at the carboxyl terminus of the proteins. The J-domains are connected to the peptide binding fragments via a G/F rich linker in both type I and type II Hsp40s. However, type I Hsp40 such as *E. coli* DnaJ, yeast Ydj1, and human Hdj2 contain two Zinc finger-like motifs between the J-domain and the C-terminal peptide binding fragment within their primary sequences, while type II Hsp40 proteins such as yeast Sis1 and human Hdj1 do not (Caplan and Douglas, 1991; Banecki et al., 1996). It has been suggested that the Zinc finger motifs are involved in the peptide binding for Hsp40 chaperone activities (Szabo et al., 1996; Lu and Cyr, 1998). The structural differences between the type I and type II Hsp40 proteins may account for the differences in their molecular chaperone activities. It has been reported that the ability to bind nonnative polypeptides for the cytosolic Hsp40 is an essential function in vivo (Johnson and Craig, 2001).

The crystal structure of the peptide binding fragment of Sis1, a type II yeast Hsp40 protein, has been determined in our laboratory (Sha et al., 2000). The crystal structure revealed that the Sis1 functioned as a homodimer with a U-shaped molecule structure. The two Sis1 monomers are associated by a short C-terminal dimerization motif. A large cleft was formed between the two elongated Sis1 monomers. A hydrophobic depression was located on the molecular surface of the domain Is of Sis1 peptide binding fragment monomer. We hypothesized that yeast type II Hsp40 Sis1 dimer may interact with the nonnative polypeptides through the two hydrophobic depressions. Simultaneous binding of a nonnative polypeptide at two sites on Sis1 dimer might serve to hold the substrate in an extended conformation which is preferred by Hsp70 (Flynn et al., 1991; Zhu et al., 1996).

The peptide binding specificity of molecular chaperone Hsp40s and the mechanisms by which Hsp40s interact with nonnative polypeptides are currently unknown. Using yeast type I Hsp40 Ydj1 as the model protein, we have screened a phage peptide display library to obtain the peptide substrate candidates for Hsp40s. Among these candidates, we identified a peptide substrate with the sequence of GWLYEIS that binds Ydj1 with high affinity by utilizing isothermal titration calorimetry (ITC) technique. The 2.7 Å crystal structure of Ydj1 and the peptide substrate complex revealed that the peptide substrate forms an extra β strand with Ydj1 peptide binding fragment. The Leucine residue in the middle of the peptide substrate plays important roles in mediating the binding of the peptide to Hsp40 Ydj1. In the crystal structure, the hydrophobic side chain of this Leucine residue in the peptide substrate fits well into a hydrophobic pocket on the molecular surface of Ydj1. Therefore, this hydrophobic pocket of Ydj1 may define the peptide substrate specificity for the type I Hsp40 Ydj1.

Results and Discussion

Identity of the Peptide Substrate of Type I Hsp40 Ydj1

To obtain the peptide substrates for type I Hsp40 Ydj1, we have screened a 7-mer phage peptide display library (New England Biolabs) using recombinant full-length Ydj1 protein. The phage peptide display library screening has been shown to be a powerful tool to study protein-peptide interactions (Scott and Smith, 1990). After three cycles of bio-panning, 20 colonies of the bound phages by Ydj1 were randomly chosen for subsequent

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Table 1. Peptide Substrate Candidate Sequences Identified by 7-Mer Phage Peptide Library Screening

GWLYEIS	×6
SESDPVA	×4
AWIEVLA	×3
HWTELIE	×2
YTVQLSS	×1
DYRLIIP	×1
SPWNNAN	×1
YTVQLSS	×1
KLFPVTK	×1

The numbers following the sequences indicate the redundancy of the peptide sequence from the screening.

DNA extraction and sequencing. Table 1 lists the sequences of the peptide substrate candidates of Ydj1 revealed by peptide library screenings.

The affinities between these Ydj1 peptide substrate candidates and Ydj1 protein were then measured by utilizing isothermal titration calorimetry (ITC) technique. These peptide substrate candidates for Hsp40 Ydj1 were synthesized and purified to more than 95% homogeneity (Invitrogen). The ITC experiments were carried out by injecting the peptide solutions into the buffers containing purified Ydj1. The measured amount of the heat releases from the injections was used to calculate the dissociation constants and steochiometry between Ydj1 and its peptide substrate candidates. The sensitivity of ITC instrument allows us to detect the binding affinity with the K_d up to ~150 μM. Out of the 10 peptide substrate candidates obtained from screening peptide display library, 1 peptide with the sequences of GWLYEIS showed significant binding affinity to Ydj1 with the dissociation constant K_d of 12 μM (Figure 1A) and the enthalpy ΔH −6067 ± 2270 cal/mol. The ITC studies also indicated that one Ydj1 dimer can bind two peptide substrate molecules. Presumably one Ydj1 monomer may bind one peptide substrate molecule. The ITC studies did not show that other peptides had measurable affinities to Ydj1.

Crystallization of Ydj1 F335D Monomer and Peptide Substrate Complex

Crystallization trials of the full-length yeast Hsp40 Ydj1 complexed with the peptide substrates GWLYEIS failed to produce diffraction quality crystals. We reasoned that the G/F rich linker between the N-terminal J-domain and the C-terminal peptide binding fragment in Hsp40s generate flexibility within the molecules that may interfere with the protein crystallization. Moreover, the crystal structure of the yeast type II Hsp40 Sis1 peptide binding fragment indicated that the Hsp40 monomers dimerized through a short C-terminal dimerization motif (Sha et al., 2000). It is likely that the short dimerization motif may not hold the two Ydj1 monomers rigidly enough for crystallization.

To eliminate the possible intramolecular flexibility, we intended to construct a monomeric form of Ydj1 peptide binding fragment without the N-terminal J-domain. Limited proteolysis showed that Ydj1 peptide binding fragment dimer covered amino acid residues 102–384 (Lu and Cyr, 1998). The crystal structure of the type II Hsp40 Sis1 peptide binding fragment dimer indicated that Y336 played an important role for its dimerization through hydrophobic interactions. The sequence alignment between type I Hsp40 Ydj1 and type II Hsp40 Sis1 predicts that F335 of Ydj1 may function as the counterpart of Y336 of Sis1 to contribute to Ydj1 dimerization. To generate a monomeric form of Ydj1, we constructed a mutant Ydj1 F335D (102–384) that covers the amino acid residues 102–384 of the full-length Ydj1 molecule with the missense mutation Phe to Asp at the position 335 to change its hydrophobic nature. The recombinant Ydj1 F335D (102–384) from *E. coli* was quickly degraded to Ydj1 F335D (102–350) during purification. Ydj1 F335D (102–350) forms a monomer in solution indicated by size exclusion chromatography as we expected. Ydj1 F335D (102–350) still retains the wild-type chaperone function to bind the peptide substrates GWLYEIS with the dissociation constant of ~10 μM from ITC studies (data not shown). The crystallization trials of Ydj1 F335D (102–350) and the peptide substrate GWLYEIS complex produced diffraction quality crystals. The attempts to crystallize Ydj1 as dimer or monomer without peptide substrate never succeeded.

The Ydj1 Peptide Substrate GWLYEIS Compete with the Denatured Luciferase in Hsp40/Hsp70 Molecular Chaperone System

To confirm that the identified Ydj1 peptide substrate GWLYEIS interacts with Ydj1 through the same binding sites as the nonnative polypeptides do, we have tested whether the peptide GWLYEIS could compete with the denatured Luciferase in the Hsp40/Hsp70 molecular chaperone refolding machineries (Figure 1B). Purified recombinant yeast Hsp40 Ydj1 was mixed with purified yeast Hsp70 Ssa1 to constitute the in vitro Hsp40/Hsp70 system. The data from this competition assays clearly revealed that the identified Ydj1 peptide substrate GWLYEIS can inhibit the refolding ability of Ydj1/Ssa1 in a dose-dependent manner (Figure 1B). The peptide substrate showed significant inhibition (~30%) at the concentration of 80 μM, which is about five times of the K_d between Ydj1 and the peptide substrate. The control peptide GLYEIS with undetectable binding affinity to Ydj1 by ITC studies showed much less inhibition for Luciferase refolding. It is highly likely that the peptide GWLYEIS inhibits the refolding ability of Ydj1/Ssa1 by competing with the denatured Luciferase to bind Ydj1 through the same sites. By utilizing the combination of peptide display library screening, ITC studies and Luciferase competition assays, we have successfully identified a peptide substrate GWLYEIS for yeast type I Hsp40 Ydj1.

The Ydj1 Peptide Binding Fragment Structure

The crystal structure of Ydj1 F335D (102–350) complexed with its peptide substrate GWLYEIS was determined to 2.7 Å resolution by the MAD method using the intrinsic Zn atoms as the anomalous scattering centers (Table 2) (Hendrickson, 1991). The resultant electron density map from the MAD phasing was readily traceable (Figure 2A) and the main chain was uninterrupted from residue 110–337. The 8 residues in the N terminus

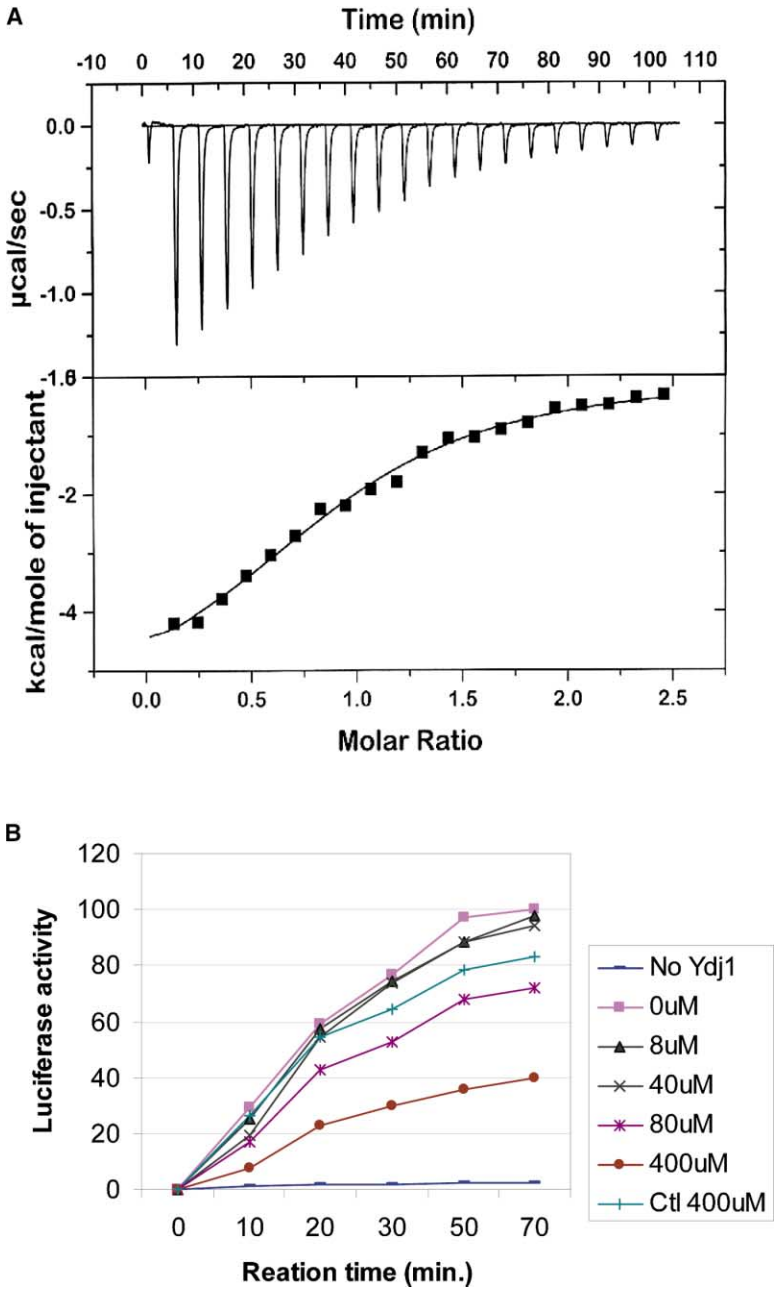

Figure 1. Identification of the Ydj1 Peptide Substrate

(A) Isothermal titration calorimetry (ITC) data of Ydj1 with the peptide GWLYEIS. The top panel shows the heat release data for injecting the buffer containing the peptide GWLYEIS in the buffer containing Ydj1. Twenty injections were performed. The lower panel shows the data fitting for the released heat from the reactions with the standard model curve.

(B) The Ydj1 peptide substrate GWLYEIS compete with the denatured Luciferase in Hsp40/Hsp70 molecular chaperone system. Purified recombinant (1.6 μM) Ydj1 was mixed with 0.8 μM purified yeast Hsp70 Ssa1 in 25 mM HEPES buffer (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, and 1.6 mM ATP to constitute the renaturing buffer. Various concentrations (0 μM, 8 μM, 40 μM, 80 μM, and 400 μM) of the Ydj1 peptide substrate was added into the buffer. Denatured Luciferase (50 nM) was diluted into the renaturing buffer and the Luciferase activities were measured at different time points. The reaction volumes are 125 μL. The horizontal axis indicates the reaction time in minutes. The refolded luciferase activity with wild-type Ydj1 and Hsp70 Ssa1 after 70 min reaction time is defined as 100% in this figure. As the negative control, no Ydj1 was added into the reaction buffer to generate the data curve labeled as "No Ydj1." A control peptide with the sequence of GLYEIS showed little inhibition for Luciferase refolding at the concentration of 400 μM (labeled as "Ctl 400 μM"). The control peptide GLYEIS did not show detectable binding affinity to Ydj1 by ITC studies. All data are the averaged values of three independent experiments.

and the 13 residues in the C terminus appeared to be flexible and were not visible in the electron density map. All the residues of the bound peptide substrate GWLYEIS showed up nicely in the electron density map (Figure 2A).

The structure of the Ydj1 F335D (102–350) monomer consists of 13 β strands (B1–B13) and two short α helixes (A1 and A2). Three distinct domains (I, II, and III) are readily to be identified from the crystal structure (Figure 2B). Domain I covers residues 110–142 and residues 209–256. The domain II protrudes out from the domain I and contains residues 143–208. The domain III comprises of residues 257–337. The crystal structure reveals that the Ydj1 F335D (102–350) monomer forms an L-shaped molecule. Domain III is connected to domain I in a head-to-tail fashion to generate the long arm (about 100 Å) for the L-shaped molecule while the domain II forms the short arm (about 40 Å). The two arms form an angle of about 90°.

The domain I of Ydj1 peptide binding fragment has the similar folding topology as the domain III. Each domain has a core formed by a major β sheet and a minor β sheet that are connected by a short helix. The major β sheet of domain I is formed by B1, B6, and B7. In domain III, B8, B9, B12, and B13 comprise the major β sheet. The minor antiparallel β sheets are composed of B2 and B5 in domain I and B10 and B11 in domain III, respectively. Domain III differs from domain I in the fact that it has a larger major β sheet that contains an additional antiparallel strand B8 at the flank of B9; otherwise,

Table 2. Statistics for MAD Data Collection and Structure Determination

Data Collection				
	Peak	Edge	Remote I	Remote II
Resolution (Å)	2.7	2.7	2.7	2.7
R_{sym}	0.057 (0.129)	0.056 (0.133)	0.052 (0.129)	0.054 (0.145)
Completeness (%)	97.5% (86.0%)	97.6% (86.8%)	98.1% (89.6%)	95.0% (73.1%)
$<I/\sigma>$	28.1 (10.4)	28.2 (10.9)	28.2 (11.0)	27.3 (8.7)
Redundancy	5.36	5.38	5.43	5.22
Numbers in parentheses are for the outer resolution shell 2.87–2.7 Å				
Anomalous Diffraction Ratios				
	λ1	λ2	λ3	λ4
λ1	0.049 (0.029)	0.032	0.038	0.041
λ2		0.047 (0.030)	0.043	0.046
λ3			0.044 (0.027)	0.038
λ4				0.041 (0.030)
MAD Phasing Statistics				
FOM = 0.45				
Refinement				
Resolution range (Å)		3.0–2.7		
Number of used		7827 (786 used for R_{free} calculation)		
R_{factor} (%)		26.8 (32.5 for outer resolution shell)		
R_{free} (%)		29.6 (32.9 for outer resolution shell)		
Number of model atoms		1819 (147 water molecules)		
Average value of B factors		47.8		
Rms Deviations from Ideality				
Bond lengths (Å)		0.008		
Bond angles(°)		1.91		
Impropers (°)		1.10		
Dihedrals (°)		26.0		

the structures of these two domains are very similar. The structures of these two domains can be superimposed remarkably well except that an insertion domain II protrudes out from the junction region between B2 and B5 of domain I. If the β strand B8 in domain III is ignored, the rms deviation of the coordinates for the main chain atoms between the domain I and domain III is 3.51 Å once the two domains are superimposed. The crystal structure of yeast type II Hsp40 Sis1 has revealed that Sis1 peptide binding fragment also contains two domains (I and II) with similar folds (Sha et al., 2000). Therefore, it may be a common feature for Hsp40 peptide binding fragments to maintain two structurally similar domains for their molecular chaperone functions.

The domain II of Ydj1 peptide binding fragment forms the shorter arm of the L-shaped Ydj1 F335D (102–350) monomer. The domain II covers two Zinc finger motifs and a β-hairpin containing B3 and B4 (Figure 2B). The Zinc finger motifs have little contacts with other domains of Ydj1 structure. Protein sequence alignments indicated that type I Hsp40 proteins contain four 100% conserved CXXCXG patterns within their primary sequences (Martinez-Yamout et al., 2000). The crystal structure of Ydj1 peptide binding fragment reveals that the residues C143 and C146 in the first CXXCXG motif (Z1) coordinate one Zn atom with the residues C201 and C204 in the fourth CXXCXG motif (Z4) to construct the first Zinc finger motif. C159 and C162 from the second CXXCXG motif (Z2) form the second Zinc finger motif with C185 and C188 from the third CXXCXG motif (Z3). The two Zinc finger motifs constitute the 90° turn between the long arm and the short arm for the L-shaped Ydj1 peptide binding fragment. The solution structure of the Zinc finger motifs of *E. coli* type I Hsp40 DnaJ determined by NMR revealed that the DnaJ Zinc finger motifs formed a V-shaped molecule (Martinez-Yamout et al., 2000). This is consistent with our crystal structure of Ydj1.

The Ydj1 and the Peptide Substrate Interactions

The bound peptide substrate GWLYEIS forms an antiparallel β strand with B2 of the minor β sheet of the domain I in Ydj1 peptide binding fragment (Figure 2). The minor β sheet of the domain I contains three β strains after binding to the peptide substrate. Out of the 7-mer peptide substrate GWLYEIS of Ydj1, the main chain atoms of the six residues GWLYEI form the typical β sheet hydrogen bond networks with B2 of Ydj1. The last residue Ser in the peptide substrate does not make significant interactions with the protein Ydj1.

Hsp40 Ydj1 interacts with the side chains of the peptide substrate GWLYEIS. The Leucine residue in the middle of the peptide substrate GWLYEIS makes the most contacts with Ydj1 among the seven amino acid residues. The side chain of this Leucine is fully buried in a hydrophobic pocket formed on the surface of domain I of Ydj1 (Figure 2C). The hydrophobic pocket is located between the two β strains B1 and B2 from the major β sheet and minor β sheet of domain I. This pocket is constructed by a number of hydrophobic residues that

includes I116 from B1, L135 and L137 from B2, L216 from B5 and V247, and F249 from B7. The hydrophobicity of these residues are nicely conserved among the family members of type I Hsp40s, indicating that this hydrophobic pocket may be a common feature for this molecular chaperon family. In addition to the interactions between the Leucine residue from the peptide substrate and the hydrophobic pocket located on Ydj1 domain I, the side chain of residue Ile from the peptide substrate GWLYEIS makes contact with a small hydrophobic region formed by residues A136 and I215 from Ydj1. The side chains of the residues Trp and Tyr of the peptide substrate GWLYEIS of Ydj1 are not in contacts with Ydj1 in the crystal structure.

Given that the domain III of yeast Hsp40 Ydj1 has structural and sequence homology with the domain I, the domain III contains a similar hydrophobic pocket on the molecular surface constituted by L266 and Y268 from B9 and L285 and V288 from B10. However, the hydrophobic pocket is occupied by F259 from the β strain B8, which is unique for the domain III from domain I. The Ydj1 domain III is not involved in peptide substrate GWLYEIS binding.

The domain I of yeast type II Hsp40 Sis1 is structurally similar with that of Ydj1 (Figure 2D). When we superimpose the domain I of Sis1 to that of Ydj1, the rms derivation for the coordinates of the main chain atoms in domain I is only 1.49 Å (Figure 2D). The crystal structure of Sis1 peptide binding fragment showed that a hydrophobic depression existed at domain I that may be responsible for nonnative polypeptide substrate binding (Sha et al., 2000; Lee et al., 2002). The Sis1 hydrophobic depression is also located between the two β strains B1 and B2 from the major β sheet and minor β sheet of domain I. In the structure superposition of Ydj1 and Sis1, the peptide binding site of Ydj1 is very similar to the putative peptide binding site of Sis1 (Figure 2D). Therefore, it is likely that both type I and type II Hsp40 proteins may utilize these hydrophobic regions located on the domain I to interact the hydrophobic side chains of the nonnative polypeptides.

The crystal structure of Hsp40 Ydj1 complexed with the peptide substrate uncovers a novel binding model for molecular chaperones to interact with their nonnative polypeptide substrates for subsequent protein folding. The crystal structures of the members of the two major molecular chaperone families of Hsp70 and Hsp60 have been determined in complex with the peptide substrates (Zhu et al., 1996; Chen and Sigler, 1999). The crystal structure of *E. coli* Hsp70 DnaK peptide binding domain complexed with the peptide substrate indicated that Hsp70 binds the peptide substrate in the extended conformation through a peptide binding groove (Zhu et al., 1996). *E. coli* Hsp60 GroEL interacts with the peptide substrates surrounding the opening of the central cavity (Chen and Sigler, 1999). It has been reported that the bacteria periplasmic chaperone PapD can prime the pilus assembly by donating a β strand to its protein substrate (Sauer et al., 2002). The crystal structure of Hsp40 Ydj1 complexed with its peptide substrate shows for the first time that the nonnative peptide substrate may form an extra secondary structure with a major molecular chaperone such as Hsp40. It is possible that by forming the β strand with its peptide substrate, Hsp40 may facilitate to stretch the nonnative polypeptide into the extended conformation for subsequent Hsp70 recognition.

Hsp40 Peptide Substrate Specificity

The crystal structure of Hsp40 Ydj1 F335D (102–350) monomer and the peptide substrate complex revealed a hydrophobic pocket on the surface of Ydj1 domain I. This hydrophobic pocket is accommodated by the side chain of the Leucine residue in the middle of the peptide substrate GWLYEIS. This binding represents the major interaction between Hsp40 Ydj1 and the peptide substrate side chains. The hydrophobic pocket has the dimensions of 5 × 7 × 7 Å (Figure 2C). The size of the pocket may easily allow the midsized hydrophobic side chains of the residues such as Leu, Ile, Met, Ala to fit in. It may also be able to accommodate the bulky sides of the residues of Phe, Tyr, and Trp by some subtle structural adjustments. This hydrophobic pocket of Ydj1 may define the peptide substrate specificity for the molecular chaperone family of Hsp40. This is consistent with the previous report that Hsp40s prefer hydrophobic residues as their substrates (Rudiger et al., 2001).

To confirm the proposition that the Hsp40 may prefer to bind a hydrophobic residue in the middle of its peptide substrate through the hydrophobic pocket, we synthesized several peptides in which the leucine residue has been replaced by others in the peptide substrate sequence GWLYEIS. The binding affinities between the synthesized peptides and Hsp40 Ydj1 were measured by ITC technique (Table 3). The results indicated that the leucine residue in the middle of GWLYEIS may be substituted by the hydrophobic residues such as Trp, Phe, or Ala without affecting much the binding affinity. However, replacing the leucine residue by a polar residue of Gln may abolish the peptide binding affinity for Ydj1 though the side chain from Gln has the similar size as that from Leu. A Gly residue with no side chain at this position also greatly reduces the binding between Hsp40 Ydj1 and the peptide (Table 3). This data strongly support our structural observations that the hydrophobic pocket on the surface of Hsp40 Ydj1 structure may be responsible for the peptide substrate specificity of Hsp40 proteins.

To identify the minimum length that is needed for the peptide substrate to bind Hsp40 Ydj1, we have synthesized peptides with the N-terminal or C-terminal residues removed from the current Ydj1 peptide substrate GWLYEIS. The binding affinities between the synthesized peptides and the Hsp40 Ydj1 were measured by ITC technique (Table 3). The data suggested that the C-terminal serine residue was dispensable for the peptide substrate binding to Ydj1. This is in excellent agreement with the structural observation that the C-terminal serine residue is not in contact with Hsp40 Ydj1 in the complex structure. Deletion of more residues from the C-terminal of the peptide substrate abolished the binding affinity. Removal of the N-terminal glycine residue from the peptide substrate GWLYEIS also eradicated the binding between the peptide and Hsp40 Ydj1 (Table 3). Thus, yeast type I Hsp40 Ydj1 may interact with the

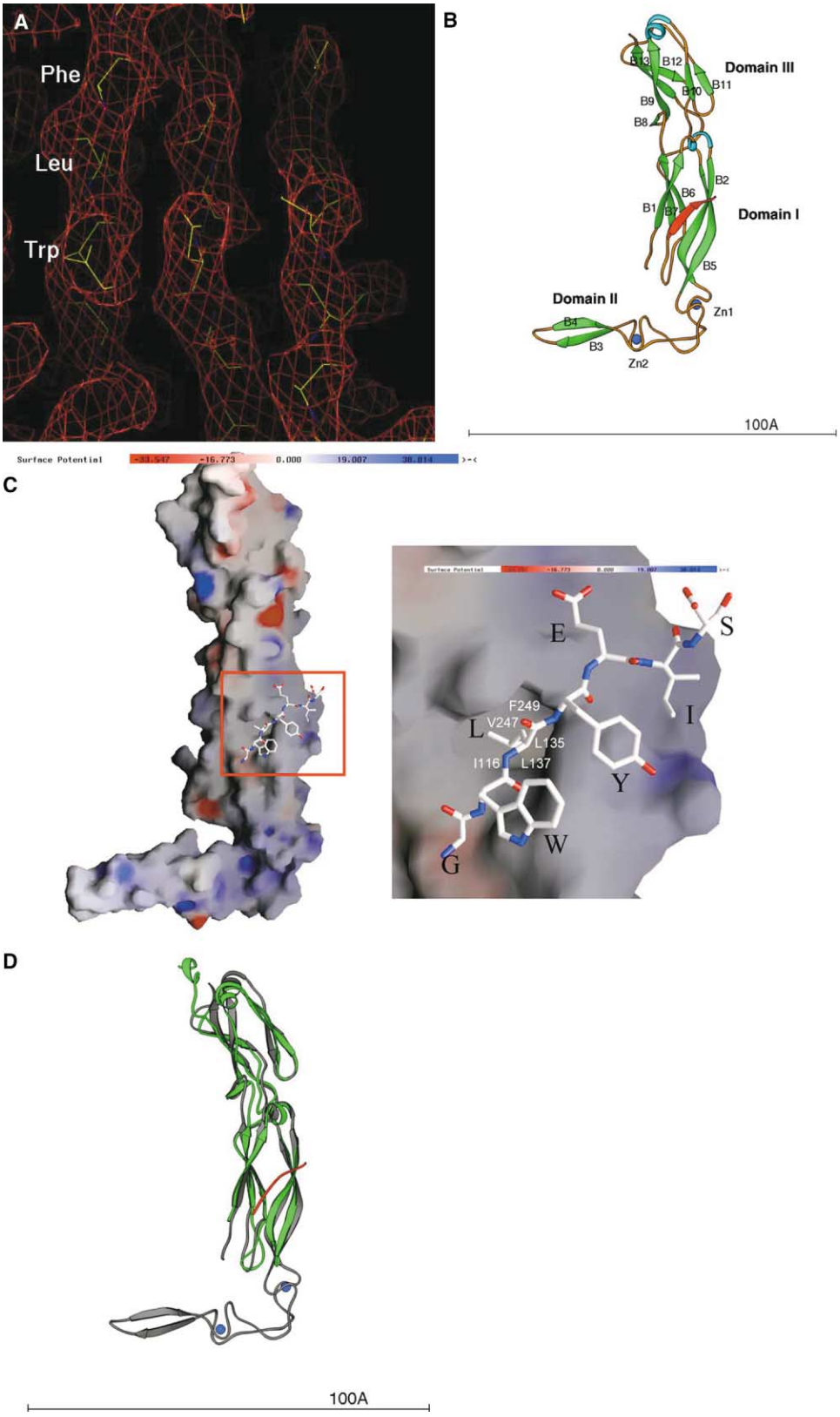

Figure 2. Ydj1 and the Peptide Substrate Complex Structure

(A) The electron density map around the peptide binding site after solvent flattening by using the program RESOLVE. The initial phases were determined by the program SOLVE utilizing the anomalous scattering information from Zn atoms. The residues Trp, Leu, and Phe within the peptide substrate are labeled. The electron density map clearly showed that the peptide substrate formed a β strand with the Ydj1 protein.

Table 3. The Binding Affinities between Ydj1 and the Synthesize Peptides Measured by ITC

Peptides	Dissociation Constants (μM)	Binding Molar Ratios
GWLYEIS	12 ± 5.0	1.0 ± 0.21
GWWYEIS	23 ± 10.5	1.2 ± 0.24
GWAYEIS	13 ± 6.6	1.0 ± 0.28
GWNYEIS	ND	ND
GWGYEIS	ND	ND
WLYEIS	ND	ND
GWLYEI	31 ± 13.7	0.8 ± 0.22
GWLYE	ND	ND

The ITC studies also generated the molar binding ratios between the peptides and the Ydj1 monomer by fitting the experimental data to the standard curve.
ND, not detectable.

peptide substrates with the minimum length of six amino acid residues.

Modeling the Ydj1 Peptide Binding Fragment Dimer

It has been reported that both type I and type II Hsp40 proteins function as dimmers (Langer et al., 1992). The crystal structure of the yeast type II Hsp40 Sis1 has been determined as a homodimer (Sha et al., 2000). The wild-type Ydj1 peptide binding fragment forms a dimer in solution as indicated by gel filtration chromatography. In our crystal structure, Ydj1 F335D (102–350) forms a monomer because the residue F335 that is critical for dimerization has been mutated to Asp. It is highly likely that the wild-type yeast type I Hsp40 Ydj1 may dimerize through the C-terminal motif as Sis1 does. We intend to model the Ydj1 dimer structure on basis of the crystal structure of Sis1 peptide binding fragment dimer. The domain III of Ydj1 and the domain II of Sis1 share high structure homology. Therefore, we modeled the Ydj1 peptide binding fragment dimer by aligning the structure of the domain III (residues 258–337) of Ydj1 to that of the domain II (residues 259–336) of Sis1 peptide binding fragment dimer (Figure 3). The rms derivation for the main chain atom coordinates between these two domains is only 3.51 Å when the two domains are superimposed.

The modeled Ydj1 peptide binding fragment dimer indicated that a large cleft was formed between the two Ydj1 monomers. At the bottom of the modeled dimer, the short arm of the L-shaped molecule containing the Zinc finger motifs point inside the cleft. The two short arms are almost anti-parallel to each other. The distance between the two Zinc finger domains is about 30 Å (Figure 3). It has been proposed that the large cleft within

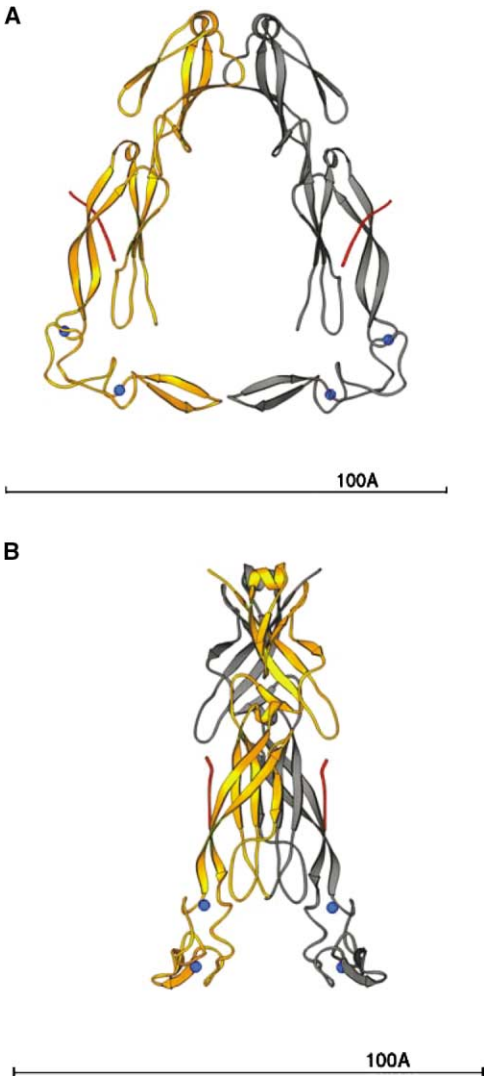

Figure 3. The Modeled Dimer of Type I Hsp40 Ydj1 Peptide Binding Fragment

(A) The Ydj1 protein structures are shown by ribbons drawings and the bound peptide substrates are shown by two red strings. One Ydj1 monomer is in gold and the other is in silver.
(B) The Ydj1 dimer in this figure is rotated along the vertical axis by 90° from its orientation in (A).

(B) The ribbon drawing of the Ydj1 peptide binding fragment complexed with the peptide substrate GWLYEIS (Carson, 1987). For Ydj1 structure, the α helices are shown in blue and the β strands are shown in green. The β strands B1–B13 are labeled. The bound peptide GWLYEIS is shown in red. The three domains (I, II, and III) within the structure are labeled. The two Zinc finger motifs are labeled as Zn1 and Zn2. The two Zinc atoms are shown in blue spheres.
(C) GRASP presentations of the Ydj1 and the peptide substrate complex structure. The left panel shows the surface potential drawing of the Ydj1 F335D (102–350) determined by GRASP (Nicholls et al., 1991) . Blue and red denote positively and negatively charged regions, respectively. The right panel shows the magnified version of the area within the red box in the left panel. The residues within Ydj1 that are responsible for forming the pocket are labeled in white. The residues of the peptide substrate GWLYEIS are labeled in black. The bound peptide GWLYEIS is shown in rod model. In the rod model, carbon atoms are shown in white, oxygen atoms are shown in red, and the nitrogen atoms are shown in blue.
(D) The structure comparison between Ydj1 and Sis1. The domain I of Ydj1 is superimposed with that of the Sis1 structure. Both molecules are in ribbons drawings. The Ydj1 molecule is in gray and the Sis1 molecule is in green. The bound peptide by Ydj1 is shown in red.

yeast type II Hsp40 Sis1 dimer may be the docking site for Hsp70 (Qian et al., 2002; Sha et al., 2000). It is likely that the large cleft in type I Hsp40 Ydj1 plays a similar role in Hsp40/Hsp70 interactions. Hsp40 interacts with the extreme C-terminal 15 amino acid residues of Hsp70; therefore, the protruding Zinc finger regions may not hinder the interactions between Hsp40 and Hsp70.

Experimental Procedures

Protein Expression and Purification

All the Ydj1 constructs were cloned into the vector pET28b. The plasmids were then transformed into *E. coli* stain BL21(DE3) for protein expression. The expressed proteins were purified using metal chelating and gel filtration chromatography. The yeast Ssa1 was expressed and purified using yeast strain MW141.

Phage Peptide Display Library Screening

The Ph.D. 7-mer phage display library kit was purchased from New England Biolabs. The full-length Ydj1 was coated on the sterile polystyrene petri dish and incubated with 10μl original phage library in 1 ml of TBS buffer (Tris 50 mM [pH 7.5], NaCl 150 mM) with 0.1% Tween-20 for 1 hr at room temperature. The dish was then extensively washed 10 times with TBS buffer with 0.1% Tween-20 to minimize the nonspecific interactions between Ydj1 and the peptides expressed on phage surfaces. The bound phages were eluted by 1 ml TBS buffer with 0.2 mg/ml Ydj1 protein after 1 hr incubation at room temperature. The eluted phages were amplified by infecting *E. coli* host strain ER2738. In the next three rounds of panning, approximately 2×10^{11} pfu was put in the incubation with Ydj1. The Tween-20 concentration in the washing buffer was raised to 0.5%. After the fourth panning, 20 phages were randomly selected and their DNA encoding the peptide substrate was sequenced.

Isothermal Titration Calorimetry Assay

Measurement of binding between Ydj1 and peptide substrates was carried out by use of an isothermal titration calorimeter (MicroCal) at room temperature. Ydj1 (or the mutants) as well as peptides were dialyzed against the same buffer (MES 10 mM [pH 6.0], NaCl 100 mM, 2-mercaptoethanol 1 mM). Ydj1 (or the mutant) was filled in the calorimetric cell and the synthetic peptide was injected into the cell by a 250 μl injection syringe. The released heat was obtained by integrating the calorimetric output curves. Pure buffers were injected into the Ydj1 protein as control experiments. The heat releases from the control experiments were subtracted from the experimental data before the data were utilized for K_d fitting. The K_d values and the binding ratios were calculated by the software supplied with the calorimeter. By using the proteins at the concentration of 0.2 mM and the peptide substrates (all the variants) of 2 mM, the ITC instrument can detect the binding K_d up to ~150 μM.

Luciferase Refolding Assay

Luciferase (15 mg/ml, Promega) was diluted by 42 times into the denaturant solution (HEPES 30 mM [pH 7.5], KCl 50 mM, $MgCl_2$ 10 mM, urea 7M) and incubated at room temperature for 40 min. Then the denatured Luciferase was diluted 125 times into the refolding solution 125 μl (HEPES 30 mM [pH 7.5], KCl 50 mM, $MgCl_2$ 10 mM, ATP 1 mM, Ydj1 or various mutants 1.6 μM, Hsp70 Ssa1 0.8 μM). Luciferase activity was determined by Luciferase assay kit (Promega).

Crystallization and Data Collection

Purified Ydj1 peptide binding fragment Ydj1 F335D (102–350) and peptide substrates were mixed together in an approximately 1:1.2 molar ratio in MES buffer 10 mM (pH 6.0), NaCl 150 mM to produce the Ydj1-peptide complex. The complex was concentrated to 20 mg/ml. Rod-shaped crystals can be obtained by the vapor diffusion method using the mother liquid of Tris 100 mM (pH 7.0), 15% ethylene glycol, and 5% PEG 4K. The crystals ($0.5 \times 0.05 \times 0.05$ mm) were flash frozen at 100 K in a nitrogen gas stream in the cryoprotectant consisting of 100 mM Tris buffer (pH 7.0), 10% PEG 4K, and 20% ethylene glycol. The MAD data were collect at CHESS beamline F2 and APS beamline BMD-14 and processed by use of software Denzo and Scalepack (Otwinowski and Minor, 1997). The crystals diffracted X-ray to 2.7 Å resolution and belong to space group of $P3_221$ with unit cell parameters of a = 55.21 Å, c = 161.87 Å. Crystal analysis shows that the asymmetric unit contains one molecule of Ydj1 peptide binding fragment, which corresponds to a solvent content of 42% (V_M = 2.55 $Å^3Da^{-1}$) (Matthews, 1968).

Structure Determination and Refinement

The MAD method was utilized to determine the structure of Ydj1-peptide complex. Two Zinc atoms were found and phases were calculated by using the program SOLVE. Program RESOLVE was utilized to carry out density modification and solvent flattening. The resultant electron map had continuous electron density and was readily to interpret. Residues 110–337 of Ydj1 and all the residues of the peptide substrate were modeled into the electron density map with program O (Jones et al., 1991). The model was refined by program CNS against the 2.7 Å native data collected at APS (Brunger et al., 1998). Six cycles of positional refinement were then carried out. Restrained individual B-factor refinement was not performed until the last cycle. After each cycle of refinement, the model was manually rebuilt according to the resultant $2F_o - F_c$ and $F_o - F_c$ maps. The refinement gave reasonable rms derivation from the ideal geometry at this resolution (Table 2). A Ramachandran plot of the final model by use of program Procheck revealed that 78.6% of the nonglycine residues in the structure were in the most favorable region.

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Accession Numbers

The coordinates and structure factors of Ydj1 peptide binding fragment complexed with its peptide substrate have been deposited to Protein DataBank with an accession number of 1NLT.