Contributions of the LPPVK Motif of the Iron-Sulfur Template Protein IscU to Interactions with the Hsc66-Hsc20 Chaperone System*

Received for publication, May 20, 2003, and in revised form, July 7, 2003
Published, JBC Papers in Press, July 17, 2003, DOI 10.1074/jbc.M305292200

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Hsc66 (HscA) and Hsc20 (HscB) from Escherichia coli comprise a specialized chaperone system that selectively bind the iron-sulfur cluster template protein IscU. Hsc66 interacts with peptides corresponding to a discrete region of IscU including residues 99–103 (LPPVK), and a peptide containing residues 98–106 stimulates Hsc66 ATPase activity in a manner similar to IscU. To determine the relative contributions of individual residues in the LPPVK motif to Hsc66 binding and regulation, we have carried out an alanine mutagenesis scan of this motif in the Glu98–Cys106 peptide and the IscU protein. Alanine substitutions in the Glu98–Cys106 peptide resulted in decreased ATPase stimulation (2–10-fold) because of reduced binding affinity, with peptide(P101A) eliciting <10% of the parent peptide stimulation. Alanine substitutions in the IscU protein also revealed lower activities resulting from decreased apparent binding affinity, with the greatest changes in K_m observed for the Pro101 (77-fold), Val102 (4-fold), and Lys103 (15-fold) mutants. Calorimetric studies of the binding of IscU mutants to the Hsc66-ADP complex showed that the P101A and K103A mutants also exhibit decreased binding affinity for the ADP-bound state. When ATPase stimulatory activity was assayed in the presence of the co-chaperone Hsc20, each of the mutants displayed enhanced binding affinity, but the P101A and V102A mutants exhibited decreased ability to maximally simulate Hsc66 ATPase. A charge mutant containing the motif sequence of NifU, IscU(V102E), did not bind the ATP or ADP states of Hsc66 but did bind Hsc20 and weakly stimulated Hsc66 ATPase in the presence of the co-chaperone. These results indicate that residues in the LPPVK motif are important for IscU interactions with Hsc66 but not for the ability of Hsc20 to target IscU to Hsc66. The results are discussed in the context of a structural model based on the crystallographic structure of the DnaK peptide-binding domain.

Hsc66 is an Hsp70 class molecular chaperone that is constitutively expressed in Escherichia coli (1–3). The gene encoding Hsc66, hscA, is found in a gene cluster, iscSUA-hscBA-fdx (1, 4), that encodes proteins important for the proper function of iron-sulfur cluster proteins (5). Genetic studies in eukaryotes (6, 7) and bacteria (8, 9) indicate that Hsc66 and its eukaryotic homolog assist in the biogenesis of iron-sulfur proteins. Although the exact role of Hsc66 in this process is unknown, the importance of Hsc66 in iron-sulfur cluster assembly may arise from its interactions with the IscU protein (10). IscU is a small, dimeric protein that is capable of forming labile iron-sulfur clusters in vitro and is proposed to serve as a template for iron-sulfur cluster formation in vivo (10–12). Recent studies have provided evidence that IscU behaves as a substrate for Hsc66 (13), and Hsc66 binding of IscU may serve to regulate the type or stability of iron-sulfur clusters formed on IscU.

Recently, we found that Hsc66 interacts with cellulose-bound peptides containing a conserved motif of IscU (residues 99–103, LPPVK), suggesting that Hsc66 binds a single discrete region of the protein (14). In addition, a synthetic peptide corresponding to IscU residues 98–106 (ELPPVKIHC) was capable of eliciting IscU-like effects on Hsc66. The Glu98–Cys106 peptide stimulates Hsc66 ATPase activity with affinity similar to that of full-length IscU (14), whereas denatured proteins that bind Hsc66 but lack the LPPVK motif fail to stimulate Hsc66 ATPase activity (3). These results indicate that residues in the LPPVK motif play a key role in regulation of Hsc66 by IscU. Studies with a synthetic peptide selected using Hsc66 in affinity panning experiments, SLWPPVSGG, also suggest that the LPPVK motif is important for binding to and regulation of Hsc66 (14). This peptide contains three residues, Pro-Pro-Val, found in the LPPVK motif but exhibits reduced affinity for stimulation of Hsc66 ATPase activity and a decreased ability to affect Hsc66 chaperone activity (14). These results suggest that the LPPVK motif is an important determinant for Hsc66-IscU interactions, but the relative contributions of individual residues in the LPPVK motif to binding Hsc66 and regulation of Hsc66 ATPase activity are unknown.

The interaction of IscU with Hsc66 is enhanced by the J domain co-chaperone Hsc20, which directly binds to both IscU and Hsc66 (2, 10). Together Hsc20 and IscU synergistically stimulate the ATPase activity of Hsc66 greater than 400-fold (10). In contrast to the IscU protein, however, the Glu98–Cys106 peptide does not function synergistically with Hsc20, and Hsc20 does not interact with IscU peptides arrayed on a cellulose blot (14). These findings suggest that Hsc20 may bind a region of IscU not present in small, unstructured peptides and that this interaction is necessary for synergistic stimulation of Hsc66 ATPase activity. The contributions of individual residues of the LPPVK motif to direct interactions between Hsc20 and IscU or the ability of Hsc20 and IscU to synergistically stimulate Hsc66 ATPase activity remain unclear.

To better understand IscU interactions with Hsc66, we have investigated the effect of alanine substitutions in the LPPVK motif of the IscU protein and of the Glu98–Cys106 peptide on interactions with Hsc66. In addition, the ability of IscU alanine...
EXPERIMENTAL PROCEDURES

Materials—E. coli DH5αF’IQ cells were from Invitrogen, and BL-
21(DE3)pLysS cells were from Novagen. Materials for peptide synthesis
were from Novabiochem. Enzymes for DNA manipulations were from
Roche Applied Science or U. S. Biochemical Corp. Synthetic nucleotides
were obtained from Sigma-Genosys. Bacterial growth media compo-
nents were from Difco, and other reagents were from Sigma.

Site-directed Mutagenesis—Vectors for expressing mutants of IscU
(pTrcIscU) (10) were constructed using the QuikChange technique
(Stratagene) and confirmed by DNA sequencing (Laragen, Inc.). For
Hsc66 a series of 7-

Experiments were carried out at 25°C as pre-
viously described (10, 13). For Hsc66 a series of 7-

FIG. 1. Stimulation of Hsc66 ATPase activity by peptide mu-
ments. Peptides corresponding to residues 98–106 (ELPPVKIHIC) of
IscU with and without alanine substitutions of the individual residues of
the LPPVK motif were assayed for their effects on Hsc66 ATPase at
peptide concentrations of 10, 40, and 200 μM. The results are reported
as the increase in basal ATPase rates at 23°C. The error bars reflect the
values obtained in two independent experiments.

Isothermal Titration Calorimetry—A Microcal Omega microcalorim-
eter (Anherst, MA) was used to investigate the binding of IscU proteins
to Hsc66 and Hsc20. Measurements were carried out at 25°C as pre-
viously described (10, 13). For Hsc66 a series of 7-

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mutants to bind Hsc20 and to be targeted to Hsc66 by Hsc20
was examined. Our findings indicate that three residues in the
LPPVK motif, Pro101, Val102, and Lys103, play key roles in
interactions with Hsc66. Targeting of IscU to Hsc66 by Hsc20
can override deficiencies in binding affinity caused by these
changes, but substitution of Pro101 or Val102 decreases the
maximal stimulation elicited by IscU in the presence of Hsc20.

The peptide corresponding to this region, Glu 98

Antibodies—Steady-state ATPase rates were determined at 23°C in HKM buffer containing 5 mM DTT and 0.5 mM ATP by measuring phosphate release
using the EnzCheck coupled enzyme phosphate assay kit (20) (Molecular Probes) as previously reported (2, 3, 10, 13, 14, 21). Under
these conditions Hsc66 has a basal turnover number of ~0.10 min
The error bars for all of the figures represent individual values observed
in two separate experiments and are shown when they fall outside the
symbols used.

Surface Plasmon Resonance Analysis—Surface plasmon resonance
studies were carried out at 25°C using a Biacore 2000 instrument as
previously described (10, 13, 14). Hsc66 in the presence of 1 mM ATP and 10 mM magnesium chloride was randomly cross-linked to the surface of the sensor chip by amine coupling. The experiments were con-
ducted in HKM buffer containing 5 mM DTT, and maximal signals were measured during 1–2 min injections. Binding of IscU appeared to be specific because no interaction was observed using sensor chips pre-
pared without Hsc66. Binding of IscU proteins was measured sequen-
tially at each concentration to ensure that degradation of the sensor
surface during the experiment did not contribute to differences ob-
served. The curves shown represent a least squares fit of the data to a
hyperbolic saturation function. The amount of immobilized Hsc66 ca-
peptide: 10 μM 40 μM 200 μM

Fig. 1 shows the stimulation of Hsc66 ATPase activity observed using 10, 40, and 200 μM concentrations of each peptide. All of the alanine-substituted peptides were able to stimulate Hsc66 ATPase activity, but stimulatory activity for the alanine

RESULTS

Stimulation of Hsc66 ATPase by Alanine-substituted Peptides—In a previous study, we demonstrated that Hsc66 selec-

1 The abbreviations used are: DTT, dithiothreitol; e., entropy units.

2 Atomic coordinate files for the Hsc66 structural models are avail-
able from the authors.
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Alanine Scanning Mutagenesis of the LPPVK Region of IscU—To ascertain the effects of substitutions in the LPPVK motif in the context of the folded protein IscU mutants L99A, P100A, P101A, V102A, and K103A were constructed using site-directed mutagenesis. All five mutants displayed chromatographic properties, solution molecular masses (>26 kDa, corresponding to the IscU dimer), and circular dichroism spectra similar to wild-type IscU (data not shown). In addition, each of the mutants was able to form iron-sulfur clusters similar to wild-type IscU (10, 24), suggesting that the overall structure of IscU was not affected by the substitutions and that none of these residues are essential for cluster formation (data not shown).

IscU Mutant Interaction with Hsc66-ATP—To investigate which residues of the LPPVK motif are important for IscU binding to the ATP-bound T state of Hsc66, we first investigated binding directly using surface plasmon resonance. Fig. 2 shows the results of titrations in which Hsc66 was immobilized on a sensor chip and exposed to different concentrations of the IscU mutants. Wild-type IscU bound with an apparent affinity of 23 μM with the maximal signal observed corresponding to 16% of the immobilized Hsc66. Each of the mutants displayed reduced binding affinity, and a decrease in the maximal signal was observed relative to wild-type IscU. The largest changes in apparent binding affinity were for IscU(K103A) (95 μM) and IscU(P101A) (102 μM). Because of the differences in maximal binding, however, it is difficult to interpret the results quantitatively. IscU behaves as a dimer in solution, and multidentate binding as well as surface immobilization effects may affect the kinetics of the IscU-Hsc66 interaction (10).

As a separate measure of binding to the ATP state of Hsc66, we examined the effects of IscU alanine mutants on the stimulation of Hsc66 ATPase activity. As shown in Fig. 3 all five mutants were found to stimulate Hsc66 ATPase activity in a concentration-dependent manner and elicit a maximal stimulation of Hsc66 ATPase activity equal or greater than that caused by wild-type IscU (7-fold). These findings suggest that substitutions in the LPPVK motif do not affect the ATPase domain of Hsc66. However, each of these mutants displayed reduced apparent affinity compared with wild-type IscU. Alanine substitution at positions 99, 100, and 102 resulted in small (2-4-fold) increases in the concentration necessary for half-maximal stimulation of Hsc66 ATPase, but substitution at positions 101 and 103 increased the concentration necessary by 70- and 15-fold, respectively. The ability of the IscU(P101A) mutant to stimulate Hsc66 at high concentrations suggests that the low level of stimulation observed using peptide(P101A) may reflect a decreased affinity for Hsc66.

IscU Mutant Binding to Hsc66-ADP—ATP hydrolysis by Hsc66 results in a conformational change that converts Hsc66 from the low affinity T state to the high affinity R state (3, 13, 21). To investigate whether substitution of Pro101, Val102, and Lys103 affects binding to the high affinity R state of Hsc66, we measured binding affinities of IscU and IscU alanine mutants directly using isothermal titration calorimetry (Fig. 4). Under
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**Fig. 4. Binding of IscU alanine mutants to the ADP complex of Hsc66.** Isothermal titration calorimetry measurements were carried out using a series of injections of IscU or IscU mutant into a cell containing Hsc66 in HKM buffer with 3 mM DTT and 1 mM ADP. Integrated heat caused by binding ($Q_{mix}$) is plotted versus the molar ratio of IscU to Hsc66 in the titration cell. The solid lines represent the best fit curve to the data: IscU:Hsc66, 0.90 binding sites, $K_d = 3.1 \mu M$, $\Delta H = 8.3 \text{ kcal/mol}$, $\Delta S = 53.0 \text{ e.u.}$; IscU(P101A):Hsc66, 0.88 binding sites, $K_d = 22.0 \mu M$, $\Delta H = 6.5 \text{ kcal/mol}$, $\Delta S = 43.0 \text{ e.u.}$; IscU(V102A):Hsc66, 1.0 binding sites, $K_d = 2.1 \mu M$, $\Delta H = 10.2 \text{ kcal/mol}$, $\Delta S = 60.3 \text{ e.u.}$; IscU(K103A):Hsc66, 0.83 binding sites, $K_d = 8.5 \mu M$, $\Delta H = 11.2 \text{ kcal/mol}$, $\Delta S = 60.8 \text{ e.u.}$

The conditions of the experiments, Hsc66-ADP binds wild-type IscU with an affinity of $\approx 3 \mu M$. This value is 7-fold higher than the apparent affinity of IscU for Hsc66-ADP ($K_m$, ATPase stimulation $= 22 \mu M$). The IscU(V102A) mutant bound Hsc66-ADP with similar affinity ($K_d = 2 \mu M$) to wild-type IscU. Replacement of Pro$^{101}$ ($K_d = 22 \mu M$) or Lys$^{103}$ ($K_d = 8.5 \mu M$) with alanine decreased the affinity of IscU for Hsc66-ADP $\approx$ 3-fold, respectively, compared with wild-type IscU. These findings indicate that residues Pro$^{101}$ and Lys$^{103}$ found to be important for IscU interactions with the ATP complex of Hsc66, are also important for binding to the ADP complex.

**Fig. 5. Effect of Hsc20 on stimulation of Hsc66 ATPase by IscU mutants.** ATPase assays were carried out in the presence of 50 $\mu M$ Hsc20, and data are reported as stimulation above the basal hydrolysis rate. The curves represent best fits to the data using the following values for maximal stimulation and concentration giving half-maximal stimulation: wild-type IscU (○), 455-fold, 4.98 $\mu M$; IscU(K103A) (■), 262-fold, 8.13 $\mu M$; IscU(P101A) (●), 110-fold, 4.05 $\mu M$. The error bars are shown when the fall outside the symbol used and represent the range of values for two experiments.

Hsc20 Effects on IscU Mutant Stimulation of Hsc66 ATPase—J domain co-chaperones behave as specificity factors that guide substrates to their cognate chaperone (25–27), and the interaction of Hsc20 with IscU is consistent with this behavior (10). Hsc20 enhances both the binding of IscU to Hsc66 (10) and the degree of stimulation of Hsc66 ATPase activity (3). To determine whether changes in the LPPVK sequence affect the ability of Hsc20 to target IscU to Hsc66, we investigated Hsc66 ATPase activity in the presence of Hsc20 over a range of IscU concentrations. A concentration of Hsc20 (50 $\mu M$) was used that had previously been shown to elicit maximal synergistic stimulation (10). Fig. 5 shows that IscU and Hsc20 synergistically stimulate Hsc66 ATPase activity $\sim$ 450-fold with half-maximal stimulation occurring at $\approx 5 \mu M$ IscU. Hsc20 also enhanced the binding of each of the alanine mutants to Hsc66 with the concentration of each mutant necessary for half-maximal stimulation ranging from 4 to 8 $\mu M$. These results indicate that substitutions in the LPPVK motif do not significantly affect the ability of Hsc20 to enhance the affinity of IscU for Hsc66. The striking enhancement in binding affinity for the P101A and K103A mutants, which exhibit very low affinity in the absence of Hsc20 ($K_m \approx 300 \mu M$), establishes that Hsc20 binding to IscU can override the effects of changes in the LPPVK motif.

Whereas all of the mutants displayed binding affinity similar to wild-type IscU, IscU(P101A) and IscU(V102A) were impaired in their ability to elicit maximal stimulation of Hsc66 ATPase activity. The maximal stimulation with IscU(P101A) in the presence of Hsc20 was only 24% of that of wild-type IscU, and the maximal stimulation with IscU(V102A) was 58%. These findings indicate that the nature of the amino acid side
Chains at these positions affects the allosteric communication between the substrate-binding domain and the ATPase domain of Hsc66.

**IscU Mutant Interactions with Hsc20**—The enhanced binding affinity of Hsc66 for IscU mutants in the presence of Hsc20 could arise from an increase in the affinity of these mutants for Hsc20. To investigate this possibility, the binding affinities of Hsc66 for the two IscU mutants that exhibited the greatest enhancement in affinity for Hsc66 in the presence of Hsc20, IscU(P101A) and IscU(K103A), were examined using isothermal titration calorimetry (Fig. 6). Neither mutant displayed enhanced affinity compared with wild-type IscU (Kd = 28.0 μM, ΔH = −5.1 kcal/mol, ΔS = 3.82 e.u.; IscU(P101A):Hsc66, 1.28 binding sites, Kd = 33.6 μM, ΔH = −4.96 kcal/mol, ΔS = 3.84 e.u.; IscU(K103A):Hsc66, 0.97 binding sites, Kd = 75.9 μM, ΔH = −3.18 kcal/mol, ΔS = 8.16 e.u.).

**Fig. 6. Binding of IscU mutants to Hsc20.** Isothermal titration calorimetry was carried out using a series of injections IscU or IscU mutant into a cell containing Hsc20 in HRK buffer plus 5 mM DTT. Integrated heat caused by binding (Qinj) is plotted versus the molar ratio of IscU or IscU mutant to Hsc20 in the titration cell. The solid line represents the best fit to the data using the following values: IscU:Hsc20, 1.1 binding sites, Kd = 28.0 μM, ΔH = −5.1 kcal/mol, ΔS = 3.82 e.u.; IscU(P101A):Hsc66, 1.28 binding sites, Kd = 33.6 μM, ΔH = −4.96 kcal/mol, ΔS = 3.84 e.u.; IscU(K103A):Hsc66, 0.97 binding sites, Kd = 75.9 μM, ΔH = −3.18 kcal/mol, ΔS = 8.16 e.u.

**Effect of the V102E Substitution on IscU Interactions with Hsc66 and Hsc20**—In addition to IscU, diazotrophic organisms contain a second iron-sulfur template protein, NifU, that functions in the assembly of the nitrogenase protein (28, 29). The N-terminal regions of NifU proteins display sequence homology to IscU proteins, but there are no known chaperones associated with iron-sulfur cluster assembly specific to the nitrogen fixation machinery (5, 12). Comparison of IscU proteins with NifU proteins reveals that the valine residue in the LPPVK motif is replaced by glutamic acid (LPPEK) in NifU sequences. To investigate the effect of this substitution on interactions with Hsc66, we prepared the IscU(V102E) mutant. The V102E protein exhibited similar general properties to the wild-type protein (chromatographic behavior, dimerization, and iron-sulfur cluster formation; data not shown), indicating that this substitution does not have major effects on IscU structure.

In contrast to the IscU(V102A) mutant, IscU(V102E) failed to stimulate the ATPase activity of Hsc66 even at concentrations as high as 800 μM (Fig. 7). This suggests that introduction of a negative charge at this position significantly impairs the ability of IscU to interact with the T state of Hsc66. To determine whether this change affects the ability of IscU to bind to ADP complex of Hsc66, we carried out isothermal titration calorimetry experiments. As shown in the inset to Fig. 7, no significant enthalpic changes were observed upon titration into Hsc66:ADP, suggesting that the mutant protein may not bind the R state of Hsc66. We also investigated IscU(V102E)-Hsc66 interactions using surface plasmon resonance, and these experiments also failed to reveal binding to either the ATP state or the ADP state (data not shown). Together these results indicate that replacement of Val102 with glutamic acid greatly weakens the ability of IscU to interact with both conformational states of Hsc66. This finding is consistent with earlier affinity panning experiments indicating that acidic residues are disfavored in Hsc66 substrates (14).

To determine whether Hsc20 could overcome the effects of the V102E substitution, we carried out Hsc66 ATPase assays in the presence of saturating levels of Hsc20 (Fig. 6). Under these conditions IscU(V102E) was capable of stimulating Hsc66 ATPase activity, and the concentration necessary for half-maximal stimulation (Km = 2 μM) is similar to that seen for wild-type IscU in the presence of Hsc20 (Fig. 5). However, the maximum stimulation observed (20-fold) is much lower than that elicited by wild-type IscU (455-fold) or IscU(V102A) (262-fold). To determine whether the decreased stimulation might reflect an altered interaction with the co-chaperone, we examined the binding of IscU(V102E) to Hsc20 using isothermal titration calorimetry. As shown in the inset to Fig. 7, the stoichiometry and binding affinity observed are similar to those found for wild-type IscU (Fig. 6), indicating that the reduced activity observed likely results from altered interactions with Hsc66. Thus, although Hsc20 targeting can overcome the effect of the V102E substitution on the affinity of Hsc66 for IscU, introduction of a negative charge in the LPPVK motif has dramatic effects on communication between the substrate-binding domain and the ATPase domain.

**Fig. 7. Interaction of IscU(V102E) with Hsc66 and Hsc20.** Stimulation of Hsc66 ATPase activity in the absence (○) and presence (○) of 50 μM Hsc20. The curve for stimulation in the presence of Hsc20 represents a best fit to the data assuming 20-fold maximal stimulation and half-maximal stimulation at 2.0 μM. The error bars for the deviation of two experiments are shown when they fall outside the symbols used. Inset, isothermal titration calorimetry analysis of IscU(V102E) binding to Hsc66 (○) and to Hsc20 (○). The solid line represents the best fit to the data for IscU(V102E) binding Hsc20 assuming 1.05 binding sites, Kd = 29.8 μM, ΔH = −2.6 kcal/mol, and ΔS = 12.1 e.u.
The results described herein provide new evidence that residues in the conserved LPPVK motif corresponding to residues 99–103 of IscU are important for interactions with Hsc66. Studies with synthetic peptides revealed that substitution of alanine for any of the residues in this motif decreased the ability to stimulate the ATPase activity of Hsc66 at low concentrations. The largest effect on peptide-induced stimulation of Hsc66 ATPase activity was observed when the central proline of the LPPVK motif (corresponding to Pro\textsuperscript{101} of IscU) was replaced with alanine, suggesting that this residue is a key determinant for peptide stimulation of Hsc66. Studies of alanine mutants of IscU provided additional information about the roles of individual residues in the context of the natural protein substrate. Substitution of each of the residues of the recognition motif with alanine reduced the affinity of IscU for Hsc66-ATP as evidenced by surface plasmon resonance binding studies and the increased concentrations necessary for half-maximal stimulation of Hsc66 ATPase. The largest changes were observed with IscU(P101A), IscU(V102A), and IscU(K103A). The P101A and K103A mutants also exhibited decreased affinity for the R state of Hsc66. The decrease in binding affinity for Hsc66-ADP displayed by these mutants was not as pronounced as for the ATP-bound states but establishes that Pro\textsuperscript{101} and Lys\textsuperscript{103} are important for IscU binding to both of the conformational states of Hsc66. Because initial binding of IscU to Hsc66 in vivo is most likely to occur with the ATP-bound state, the larger effects observed in the ATPase assay are more relevant to the physiological interaction between the proteins.

Although substitutions in the LPPVK motif decrease binding affinity of IscU for Hsc66, the addition of Hsc20 can override this deficiency. In the presence of Hsc20, all of the mutants displayed concentrations for half-maximal stimulation (4–8 \( \mu \text{M} \)) of Hsc66 ATPase similar to wild-type IscU. Hsc20 targeting of the IscU(V102E) mutant also occurred with affinity similar to wild-type IscU, even though no binding of this mutant could be detected in the absence of Hsc20. These findings indicate that the exact sequence of the LPPVK motif does not affect the affinity enhancement caused by Hsc20 and suggest that Hsc20 binding to IscU will be a key determinant of Hsc66 interactions with IscU. Hsc20 may function as a scaffold to aid in positioning IscU on the substrate-binding domain of Hsc66. A role for Hsc20 in specific targeting of IscU to Hsc66 (10, 13) is consistent with the notion that the functional specificity of Hsp70-type chaperones is determined to a large degree by interactions of their ATPase domain with other components of the cellular machinery (30).

Even in the presence of Hsc20, however, mutations at two positions, Pro\textsuperscript{101} and Val\textsuperscript{102}, caused a decrease in the maximal stimulation of Hsc66 ATPase elicited by IscU. Replacement of Pro\textsuperscript{101} with alanine resulted in a 76% decrease in maximal stimulation, and alanine substitution at Val\textsuperscript{102} led to a 42% decrease. Introduction of a negatively charged residue had an even greater effect. The IscU(V102E) mutant gave 86%–90% of the maximal stimulation of wild-type IscU. Substitution of each of the residues of the recognition motif with alanine reduced the affinity of IscU for Hsc66 in the presence of co-chaperone, they are important for communication between the ATPase domain and the substrate-binding domain of Hsc66. Specific interactions between the side chains of Pro\textsuperscript{101} and Val\textsuperscript{102} of IscU and the substrate-binding domain of Hsc66 appear to be necessary for the allosteric regulation of Hsc66 ATPase activity.

**A Model for the Interaction of IscU with Hsc66**—The specificity of Hsc66 for the LPPVK motif and the effects of mutations in this region raise questions regarding how the substrate-binding domain of Hsc66 recognizes this amino acid sequence. Crystallographic studies of *E. coli* DnaK (31) and NMR studies of DnaK (32–34) and rat Hsc70 (35) have revealed that bound peptide exists in an extended conformation and lies in a groove in the \( \beta \)-sandwich subdomain of the substrate-binding domain. Interactions occur between both backbone and side chain atoms of the bound peptide and the chaperone with the most extensive contacts occurring with five central residues at peptide positions –2, –1, 0, 1, and 2. The side chain of the central residue 0 projects into a hydrophobic pocket, and the peptide backbone at this position is covered by an arch or bridge formed by residues from \( \beta \)-strand loops of the chaperone. Structural features that contribute to peptide binding specificity are not well understood, but comparisons of sequences among different Hsp70 species (36) and mutational studies on DnaK (37, 38) suggest that the identity of residues forming the hydrophobic pocket and the arch contribute to selectivity.

The substrate-binding domain of Hsc66 exhibits 43% sequence identity and 60% similarity with that of DnaK, suggesting that Hsc66 is likely to interact with peptides in a manner similar to DnaK (1). To identify structural differences between Hsc66 and DnaK that might contribute to their different peptide recognition specificities, we used the Swiss model program (22) to construct a model of Hsc66 based on the crystal structure of DnaK (31). Fig. 8 shows the structure of the substrate-binding domain of DnaK complexed with the synthetic heptapeptide NRLLLTGT. This view is as in Fig. 2 of Ref. 31 and shows the peptide oriented with its N terminus facing out of the plane on the “front side” of the \( \beta \)-sandwich subdomain and the C terminus projecting into the plane on the “back side” (hereafter designated “N \( \rightarrow \) C orientation”). The central leucine residue of the peptide projects downward into a hydrophobic pocket at position 0. Residues Met\textsuperscript{404} (from \( \beta \)-loop L\textsubscript{1,2} ) and Ala\textsuperscript{429} (from \( \beta \)-loop L\textsubscript{0,1} ) form an arch over the peptide backbone and make contact with the side chains of leucine residues at positions –1 and 1. A model for the substrate-binding domain of Hsc66 generated in the absence of a peptide substrate is shown in the lower panel of Fig. 8. The general structure of the Hsc66 model is similar to that of the DnaK template, but there are a number of dissimilarities that may contribute to differences in the peptide binding specificity of the two chaperones. A phenylalanine residue (Phe\textsuperscript{426}) occurs in the arch of Hsc66 in place of Ala\textsuperscript{429} in DnaK, and the phenyl side chain projects into the substrate binding cleft. Hsc66 also has a methionine (Met\textsuperscript{435}) at the base of the pocket forming position 0, whereas the corresponding residue in DnaK is a valine (Val\textsuperscript{436}). The bulky side chains of Phe\textsuperscript{426} and Met\textsuperscript{435} partially block the peptide binding site in the Hsc66 model, reducing its size compared with that observed in the DnaK-peptide complex. There are also differences in surface charge distribution between Hsc66 and DnaK. The front side of Hsc66 contains acidic residues (Asp\textsuperscript{422} and Glu\textsuperscript{406}), whereas this side of DnaK contains mostly nonpolar side chains near the region the peptide exits the binding groove (Fig. 8). The back side of Hsc66 has basic residues (Arg\textsuperscript{453} and Arg\textsuperscript{457}), whereas this side has neutral or acidic residues (Gln\textsuperscript{456} and Asp\textsuperscript{460}) in DnaK (31). Differences in surface charge distribution combined with differences in residues comprising the peptide pocket may contribute to the differences in substrate specificity observed for the two chaperones (3, 39) and to the failure of Hsc66 to complement the phenotype of dnaK\textsuperscript{–} cells (39, 40).

To better understand the interaction of Hsc66 with IscU, we modeled a peptide corresponding to IscU residues 98–104 (ELPPVKI) into the Hsc66 substrate-binding domain. The conformation of this region of the IscU protein is not known, but it
is unlikely to be highly structured based on the finding that the Glu\textsuperscript{98}–Ile\textsuperscript{104} peptide is capable of stimulating Hsc66 ATPase with similar affinity to that of full-length IscU (14). The ELP-PVKI peptide was modeled in an extended conformation similar to that observed for the DnaK-NRLLLTG complex. Because Pro\textsuperscript{101} is the central residue of the peptide and because of its importance for binding, the equivalent peptide residue was placed in central position 0. The peptide was initially aligned in an orientation similar to that of the NR-LLLTTG peptide bound to DnaK (N → C; Fig. 8), and the model was subjected to energy minimization using simulated annealing (23). A, front view of the substrate-binding domain of Hsc66 modeled with the peptide orientation (N → C) similar to that observed for the DnaK-NRLLTG complex. B, top view of the N → C peptide orientation. C, reverse peptide orientation (C → N). The Glu\textsuperscript{98}–Ile\textsuperscript{104} peptide is shown in green in each panel with residue labels boxed. The sulfur atom of Met\textsuperscript{401} is highlighted yellow, and the side chain of Phe\textsuperscript{426} is purple. Nitrogen atoms of side chains of basic residues are blue, and oxygen atoms of side chains of acidic residues are red.

**Fig. 8.** Models of the substrate-binding domains of DnaK and Hsc66. Top panel, crystal structure model of the substrate-binding domain (SBD) of DnaK complexed with the peptide NRLLTG. Arch residues Ala\textsuperscript{429} and Met\textsuperscript{404} are indicated. This is the standard view (from Ref. 31) showing the front side of the substrate-binding domain. The residues closest to the substrate binding region are shown as spheres representing their van der Waals radii, and the backbone structures of other residues are shown as ribbons. Bottom panel, homology model of the Hsc66 substrate-binding domain based on the coordinates of the DnaK structure generated by Swiss model (22). Two differences between Hsc66 and DnaK in the central region of peptide-binding pocket are highlighted (Met\textsuperscript{403}, pink; Phe\textsuperscript{426}, purple).

**Fig. 9.** Models for peptide complexes with the substrate-binding domain of Hsc66. Peptide Glu\textsuperscript{98}–Ile\textsuperscript{104} was modeled into the Hsc66 model structure, and the complex was energy-minimized using simulated annealing (23). A, front view of the substrate-binding domain of Hsc66 modeled with the peptide orientation (N → C) similar to that observed for the DnaK-NRLLTG complex. B, top view of the N → C peptide orientation. C, reverse peptide orientation (C → N). The Glu\textsuperscript{98}–Ile\textsuperscript{104} peptide is shown in green in each panel with residue labels boxed. The sulfur atom of Met\textsuperscript{401} is highlighted yellow, and the side chain of Phe\textsuperscript{426} is purple. Nitrogen atoms of side chains of basic residues are blue, and oxygen atoms of side chains of acidic residues are red.

Effects of LPPVK Mutations on IscU-Hsc66 Interactions
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