Identification of Elements of the Peptide Binding Site of DnaK by Peptide Cross-linking*

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We used photocross-linking of peptides to DnaK to identify elements of the peptide binding site of DnaK. We attached a photoactivatable group (N-hydroxysuccinimidy1-4-azido-salicylic acid (NHS-ASA) or N-iodoacetamidobutyl-4-azido-salicylic acid (I-ABASA)) to different positions on peptide C of the vesicular stomatitis virus glycoprotein, 125I-radiolabeled the cross-linker, and that the N-terminal 45-kDa fragment of DnaK was not cross-linked by these modified peptides. The attachment points of the three peptide C derivatives carrying photoactivatable cross-linkers at different locations on the peptide, PepC-ASA, PepC-SC-ABASA, and PepC-SBC-ABASA, have been identified as Arg-356, Arg-527, and His-541 of DnaK, respectively. Thus all three peptides cross-linked to amino acids located close together in a sequence that includes one end of the long α-helix in the NMR-based secondary structure model of the peptide binding domain of Hsp70 family (Morshauser, R., Wang, H., Flynn, G., and Zuiderweg, E. (1995) Biochemistry 34, 6261-6266).

The DnaK protein of Escherichia coli (1) is a well characterized member of the family of highly conserved Hsp70 proteins (2) which play a variety of physiological roles by functioning as molecular chaperones (3). The Hsp70 family contains members whose expression is induced by a heat shock (5). Furthermore DnaK plays a role in the interaction of DnaK with its protein substrates has been much less well characterized. One strategy for analyzing the interaction of DnaK with its protein substrates has been to employ small peptides such as Peptide C of vesicular stomatitis virus (26–28). Certain peptides have been shown to bind to BiP, Hsc70, and DnaK and to stimulate their ATPase activities (29–33). Such peptides bind to a C-terminal substrate binding domain. The crystal structure of the 44-kDa N-terminal ATPase domain of bovine Hsc70 has been solved and shown to be structurally homologous to the ATPase domains of hexokinase and actin (15, 16). The structure of the ATPase domain of DnaK is likely similar to that of Hsc70 due to their high amino acid sequence similarity (17).

A variety of missense mutations have been characterized that affect the ATPase activity of DnaK and other members of the Hsp70 family of proteins (18–24). DnaK also has a weak autophosphorylation activity that results in the autophosphorylation of threonine 199 (23). Nonconservative substitutions of this residue knock out the ATPase and autophosphorylation activities of DnaK (23) and result in proteins that fail to properly function in the cell (25).

In contrast, the C-terminal domains of Hsp70 proteins have been much less well characterized. One strategy for analyzing the interaction of DnaK with its protein substrates has been to use small peptides such as Peptide C of vesicular stomatitis virus (26–28). Certain peptides have been shown to bind to BiP, Hsc70, and DnaK and to stimulate their ATPase activities (29–33). Such peptides bind to a C-terminal substrate binding domain. The crystal structure of the 44-kDa N-terminal ATPase domain of bovine Hsc70 has been solved and shown to be structurally homologous to the ATPase domains of hexokinase and actin (15, 16). The structure of the ATPase domain of DnaK is likely similar to that of Hsc70 due to their high amino acid sequence similarity (17).

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an ATPase activity, revealing that the 16-kDa polypeptide directly adjacent to the ATPase domain is responsible for protein binding (17). The work by Wang et al. (36) demonstrated that the 18-kDa internal fragment of Hsc70 that immediately follows the N-terminal domain is sufficient for peptide binding. Similarly, the C-terminal domain of DnaK that starts at residue 383 has been shown to have the same peptide binding affinity as wild type DnaK, indicating this C-terminal domain is solely responsible for substrate binding (32).

The structure of the Hsp70 peptide binding domain has been the object of considerable speculation. Two studies (37, 38) have hypothesized that Hsp70 proteins have a similar peptide binding structure as the class I major histocompatibility complex (MHC) protein and the class II MHC protein. The peptide binding domains of HLA-A2 (class I MHC) (39, 40) and HLA-DR1 (class II MHC) (41) are composed of eight strands of antiparallel β-sheet that form a floor and two antiparallel α-helixes on the top of the β sheets. The bound peptide, in an extended conformation, lies between the two antiparallel α-helixes. However, the recently published secondary topology of the 18-kDa peptide binding domain of Hsc70 as determined by multidimensional NMR (42) is very different from that predicted from the MHC model. In the NMR-based structure there are eight β-strands in the peptide binding domain similar to that predicted by the MHC-based model, but these eight β-strands are divided into two independent sheets of four strands each, whereas in the MHC model the sheet is contiguous. Another difference is, instead of having two α-helixes as predicted, the peptide binding domain has only one long α-helix at its C-terminal end.

In this report we describe experiments designed to identify elements of the DnaK peptide binding site. Our approach involved the use of peptides carrying photoactivatable cross-linkers attached to particular sites on the peptides. These peptides were shown to interact with DnaK in a manner similar to the unmodified peptides. Our cross-linking studies suggest that a region near the C-terminal end of the peptide-binding domain is important for peptide binding, and we discuss our results in terms of the recently published model for the secondary structure of the Hsc70 peptide binding domain.

**EXPERIMENTAL PROCEDURES**

Proteins, Peptides, and Cross-linking Agents—DnaK protein was purified as described previously (23). The heterobifunctional photoactivatable cross-linkers N-hydroxysuccinimidyl-4-azido-salicylic acid (NHS-ASA) and N-iodoacetamidobutyl-4-azido-salicylic acid (I-ASA) (Fig. 1) were purchased from Pierce. 1-Iodoacetamido-2-phenylethyl chloro methyl ketone-treated trypsin was obtained from Sigma. Peptide C, PepC-STC, and Pep-SBC were synthesized on an automatic peptide synthesizer (Applied Systems, 430A).

ATPase Assay—The ATPase activity of DnaK was assayed as described elsewhere (23), except that pH 7.4 HEPES buffer (100 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 5 mM mercaptoethanol) was used. When assaying the stimulation of the ATPase activity of DnaK by a peptide, a 100 μM relevant peptide was included in the buffer.

Modification of Peptides with Photocross-linkers—1.9 mg (6.9 μmol) of NHS-ASA in 0.5 ml of acetonitrile was added to 10 mg (6.9 μmol) of Peptide C in 0.5 ml of 50 mM, pH 8.0, Tris buffer. The resulting solution was incubated at 37°C for 1 h. The modified peptide (PepC-ASA) tended to precipitate in basic buffer but could be redissolved by adjusting the buffer pH to 6.5. PepC-ASA was purified by HPLC with a Waters Delta-pak C18 cartridge column. The column was run at 30 ml/min started with 100% buffer A (0.1% trifluoroacetic acid) to 100% buffer B (80% acetonitrile, 0.085% trifluoroacetic acid) within 60 min.

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The modification of PepC-STC and PepC-SBC with I-ASA was carried out following the same procedure and purified with the same column. The modified peptides were referred to as PepC-STC-ASA and PepC-SBC-ASA, respectively. The structure of the modified peptides were confirmed by electron spray mass spectrometry: PepC-ASA, calculated, 1618.90; found, 1618.86; and PepC-SBC-ASA, calculated, 1762.96; found, 1762.86.

Radiolabeling of Cross-linker-modified Peptides by 125I-Iodination—Lodination was performed following a previously described procedure (43) with some modifications. 10 μl of NaI (29) in 0.1 N NaOH (Amersham Corp., 1.0 mCi) were added to 50 μl of a 5 mg/ml PepC-ASA water solution. To this solution 20 μl of 5 mg/ml chloramine-T were added, and the reaction was allowed to occur for 5 min, followed by quenching with 30 μl of 5% sodium metabisulfite. The labeled peptide was separated from the free iodide on HPLC with a Waters Delta-pak C18 cartridge column. After sample injection, the column was first washed with 100% buffer A (0.1% trifluoroacetic acid) for 10 min, then to 100% buffer B (80% acetonitrile, 0.085% trifluoroacetic acid) within 30 min. The radiolabeled peptide was pooled and lyophilized to give a final concentration of 55 μM at a specific activity of 630 Ci/mmol. PepC-STC-ASA and PepC-SBC-ASA were radiolabeled by the same procedure, and the radiolabeled peptides were obtained with a similar concentration and specific activity.

UV Cross-linking of 125-I-Labeled Modified Peptide to DnaK—In all the cross-linking experiments, a 1:1 molar ratio of DnaK and peptide was used. A typical cross-linking experiment was carried out as follows. 4 μl of 4.6 mg/ml (65 μM) DnaK and 5 μl of 125-I-labeled PepC-ASA (55 μM) were added to 10 μl of 50 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 5 mM mercaptoethanol buffer. The resulting solution was incubated at 37°C for 1 h, transferred to a microtiter plate, and UV-irradiated at 366 nm for 2 min. The UV lamp (Mineralightlamp, model UVGL-25; UVP, Inc., San Gabriel, CA) was placed 2 cm above the microtiter plate. The UV-irradiated solution was loaded onto a SDS-PAGE gel after boiling with loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) for 3 min. The gel was run at 30 mA until the dye reached the bottom, dried, and subjected to autoradiography. The radioactivity of the DnaK band was also quantitated by a PhosphorImager (Molecular Dynamics) if necessary.

Trypsin Partial Digestion of DnaK and Separation of the Radioactive Fragment for Sequencing—Four μl of 4.6 mg/ml (65 μM) DnaK and 5 μl of 125-I-PepC-ASA were added to 10 μl of 50 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 5 mM mercaptoethanol buffer. The resulting solution was incubated at 37°C for 1 h, transferred to a microtiter plate, and UV-irradiated at 366 nm for 2 min. A UV-irradiated DnaK-peptide solution prepared as described above was transferred to a microcentrifuge tube. Then 2 μl of 1 mg/ml trypsin (trypsin:DnaK, 1:20) were added to the solution of DnaK-125-I-PepC-ASA complex, and the solution was incubated at 37°C for 1, 2, 5, 10, and 30 min, respectively. After incubation, 10 μl of loading buffer were added immediately to the reaction and boiled for 3 min. The digestion solution was separated on a 10% SDS-PAGE gel. After electrophoresis, the gel was soaked in...
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RESULTS

Preparation of Photoreactive Peptide C and Peptide C Derivatives—Peptide C is a basic peptide of 13 amino acids (Lys-Leu-Leu-Gly-Val-Leu-Ser-Ser-Leu-Phe-Arg-Pro-Lys) derived from the vesicular stomatitis virus glycoprotein and was shown to bind to BiP and stimulate its ATPase activity (30). Peptide C was subsequently shown to bind to DnaK as well and to stimulate its ATPase activity (26, 27). To analyze the nature of its binding to DnaK, we first modified peptide C and certain monocyteine derivatives of peptide C with photoactivatable cross-linkers, I-ABASA, and studied the photoreactive cross-linking of the radioactive cross-linker-modified peptides to DnaK. One strategy we employed was to modify peptide C with NHS-ASA, a heterobifunctional photoactivatable cross-linker that specifically reacts with primary amines. There are two lysine residues in peptide C that could be potentially modified by NHS-ASA. The NHS-ASA-modified peptide C (PepC-ASA) is likely a mixture of peptides with modifications at either or both of the lysine residues. The peptide with one modification and the peptide with two modifications were separated by reverse phase HPLC column, and only the peptide with modification at one lysine residue was used in the subsequent cross-linking studies. PepC-ASA was found to stimulate the ATPase activity of DnaK to a degree comparable to the stimulation of the ATPase activity by peptide C (Table I). The ability of PepC-ASA to stimulate the ATPase activity of DnaK to the same extent as peptide C indicated that PepC-ASA interacts with DnaK similarly to peptide C. Monocysteine derivatives of peptide C, PepC-S7C, and PepC-S8C, were synthesized, and a cysteine-specific photocross-linker I-ABASA was attached to the cysteine residue on both of the peptides. The photocross-linker-modified PepC-S7C and PepC-S8C are called PepC-S7C-ABASA and PepC-S8C-ABASA, respectively. The ability of PepC-S7C, PepC-S8C, PepC-S7C-ABASA, and PepC-S8C-ABASA to stimulate the ATPase activity of DnaK was assayed. As shown in Table I, the degree of ATPase stimulation by PepC-S7C, PepC-S8C, PepC-S7C-ABASA, and PepC-S8C-ABASA was similar to that by peptide C, indicating the nature of interactions of the monocysteine derivatives and the cross-linker-modified derivatives of peptide C with DnaK are similar to peptide C itself. PepC-S7C, PepC-S7C-ABASA, and PepC-S8C-ABASA were all radiolabeled through iodination of the aromatic ring of the azido-salicylic cross-linker as described previously (43) to yield the radioactive modified peptides: 125I-PepC-ASA, 125I-PepC-S7C-ABASA, and 125I-PepC-S8C-ABASA, all of which carry a photoactivatable azido group.

Photoaffinity Labeling of DnaK with Peptide C and Peptide C Derivatives—The radiolabeled peptides carrying a photoactivatable azido group were incubated with DnaK at a molar ratio of 1:1 at 37 °C for 1 h before UV irradiation. Although the maximum absorption wavelength of the azido-salicylic acid group is 305 nm, irradiation with the long wavelength (366 nm) light of a UV lamp gave efficient cross-linking (44). With a Mineralight® 366-nm UV lamp at 2 cm above the sample, the photocross-linking of 125I-PepC-ASA to DnaK was complete in less than 1 min (Fig. 2A). Longer irradiation time did not increase the efficiency of the reaction, rather it led to the degradation of protein. The efficiency of the photocross-linking was also dependent upon the concentration of the 125I-labeled peptides (Fig. 2A). The higher the peptide concentration, the greater the amount of DnaK that was cross-linked to the radioactive peptide. The cross-linking efficiency of the radiolabeled PepC-ASA to DnaK decreased by 80% in the presence of ATP (Fig. 2C), indicating ATP is able to release 125I-PepC-ASA efficiently from DnaK as expected on the basis of previous studies (26–28). ADP and ATP-5 did not have much effect on the efficiency of peptide cross-linking to DnaK again as expected from previous studies (26–28). Taken together with the data for stimulation of the DnaK ATPase activity, these observations provide additional evidence that we are observing results from binding of the modified peptides in the normal peptide-binding site. The photocross-linking of 125I-PepC-S7C-ABASA to DnaK was inhibited by the unmodified PepC-S7C (Fig. 2B). At 1.1 molar ratio of PepC-S7C to 125I-PepC-S7C-ABASA, 20% of the cross-linking of 125I-PepC-S7C-ABASA to DnaK was inhibited. At 2.1 PepC-S7C to 125I-PepC-S7C-ABASA, there was 50% inhibition. Similar inhibitions of 125I-PepC-ASA to DnaK by peptide C and 125I-PepC-S7C-ABASA to DnaK by PepC-S7C were also observed. The inhibition of the cross-linking of the cross-linker-modified peptides to DnaK by the unmodified peptides provides additional evidence that the modified radiolabeled peptides are binding specifically to the DnaK protein.
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FIG. 2. A, dependence of cross-linking efficiency upon radiation time and peptide concentration. 5 μl of 5 mg/ml (70 μM) DnaK and 10 μl of 125I-PepC-S8C-ABASA (55 μM) was mixed and incubated at 37 °C for 1 h. Lane 1, DnaK; lane 2, DnaK and 125I-PepC-S8C-ABASA, but with no UV irradiation; lanes 3–6, UV-irradiated at 366 nm for 1, 2, 5, and 10 min, respectively; lanes 7–10, 5 μl of 5 mg/ml (70 μM) DnaK with 1.0, 2.0, 5.0, and 10 μl of 125I-PepC-S8C-ABASA (55 μM) incubated at 37 °C for 1 h and UV-irradiated at 366 nm for 10 min. DnaK protein was separated from the free peptide on a 10% SDS-polyacrylamide gel. B, competition of 125I-PepC-S8C-ABASA binding to DnaK by PepC-S8C. Lanes 1–4, 0, 2.5, 5, and 10 μl of 66 μM PepC-S8C were added to 5 μl of 5 mg/ml (70 μM) DnaK and 2.5 μl of 125I-PepC-S8C-ABASA solution. Samples were then incubated at 37 °C for 1 h and UV-irradiated at 366 nm for 2 min. C, the effects of nucleotides on the cross-linking of 125I-PepC-S8C-ABASA to DnaK. 5 μl of 5 mg/ml (70 μM) DnaK and 10 μl of 125I-PepC-S8C-ABASA (55 μM) were incubated at 37 °C for 1 h, and then 2 μl of water (lane 1), 2 μl of 10 mM ATP (lane 2), 2 μl of 10 mM ADP (lane 3), and 2 μl of 10 mM AMP (lane 4) were added. The resulting samples were incubated at 37 °C for 5 min and UV-irradiated at 366 nm for 2 min.

normal peptide-binding site of DnaK.

Partial Trypsin Digestion of the DnaK-Peptide Complex—After demonstrating that the modified radiolabeled peptides were specifically binding to the same site on DnaK as the unmodified peptides, we determined the fragment of DnaK that was cross-linked by the photolabile peptide. The cross-linked DnaK-125I-PepC-ASA complex was digested by trypsin for limited time, and the digested fragments were separated on a 13% SDS-PAGE gel. The separated fragments were electrotransferred to a polyvinylidene difluoride membrane which was first stained with Coomassie Blue and then subjected to autoradiography to identify the radioactive bands. After one minute of trypsin digestion, two bands with molecular masses of 28 and 25 kDa were found to be radioactive. The 28-kDa fragment disappeared with increased digestion time, and the 25-kDa fragment was the only remaining radioactive fragment after a 10-min digestion (Fig. 3). Edman sequencing of the first seven residues of this 25-kDa fragment gave a sequence of DVLLELDV, indicating that the fragment had been generated by a trypsin cut at lysine 387. We also observed that a 45-kDa fragment generated by trypsin digestion was not radioactive. Amino acid sequencing of this 45-kDa fragment gave a sequence of GKIIGID which is the predicted N-terminal sequence of DnaK without the first methionine (1, 45). These sequencing data revealed that 125I-PepC-ASA binds specifically to a C-terminal region of DnaK starting at residue 388 and that it does not interact with the N-terminal 45-kDa fragment. The complexes of DnaK-125I-PepC-S7C-ABASA and DnaK-125I-PepC-S8C-ABASA gave the same trypsin digestion pattern and the same pattern of radioactive labeling as DnaK-125I-PepC-ASA, suggesting the three peptides with different photoreactivatable cross-linkers at different positions on the peptide all specifically interact with the same C-terminal domain of DnaK.

Determination of the Points of Cross-linking of the Peptides to DnaK—We were interested in determining which amino acids in the C-terminal of DnaK had been cross-linked by the various peptide C derivatives carrying a photoreactivatable group. In order to do this, we carried out a procedure consisting of complete trypsin digestion of the DnaK-peptide complexes, HPLC separation to purify the radioactive cross-linked peptides, and subsequent complete amino acid sequencing of the radioactive peptides to determine the cross-linked residues. The UV-irradiated DnaK and 125I-PepC-ASA solution was extensively dia lyzed to get rid of the free 125I-PepC-ASA before being subjected to trypsin digestion. Complete trypsin digestion of the radiolabeled PepC-ASA cross-linked DnaK sample and subsequent HPLC separation of the radioactive fragment gave two radioactive peaks (Fig. 4A). One of these radioactive peaks was in the void volume and is likely due to the free iodide because the radioactivity can be precipitated by silver nitrate. Complete Edman sequencing of the other radioactive peak gave a peptide starting from residue 518 with Arg-536 missing (Table II), implying Arg-536 is the cross-linking site of DnaK by 125I-PepC-ASA. The sequence of this radioactive peptide gave a sequence starting from residue 518 with Arg-536 missing (Table II), implying Arg-536 is the cross-linking site of 125I-PepC-ASA. The sequence of this radioactive peptide gave a sequence starting from residue 518 with Arg-536 missing (Table II), implying Arg-536 is the cross-linking site of 125I-PepC-ASA. We cannot tell whether Arg-536 of DnaK became cross-linked by an ASA moiety attached to the N terminus or the C terminus of peptide C. However, the N-terminal half of peptide C is known to bind relatively strongly to DnaK (35). Therefore, it is possible that the relevant ASA is at the N-terminus of peptide C. The complete trypsin digestion of DnaK-125I-PepC-S7C-ABASA complex and HPLC separation of the digested fragments also gave one major radioactive peak (Fig. 4B). Complete sequencing of this radioactive peptide gave a sequence starting from residue 518 with Arg-527 missing (Table III). Thus Arg-527 is likely the cross-linking site of DnaK-125I-PepC-S7C-ABASA. Similar experiments led to the identification of His-541 as the cross-linking site of DnaK-125I-PepC-S8C-ABASA (Table IV). These three peptides with different photocross-linkers at different positions on the peptide cross-linked to the same region of DnaK, suggesting this fragment of DnaK is likely involved in peptide binding or close to the peptide binding site of DnaK.

DISCUSSION

In order to explore the nature of the interactions of peptides with DnaK, we employed a strategy of attaching a radiolabeled photoactivatable group to different positions on a peptide, allowing the modified peptides to bind to DnaK, cross-linking the peptides to DnaK by exposure to UV irradiation, and then determining the point of attachment to DnaK. The peptides used in this study were all derivatives of peptide C, a fragment of the vesicular stomatitis virus glycoprotein that has been well characterized for its ability to bind to DnaK. The cross-linker modified peptide C derivatives interacted with DnaK in a fashion that was very similar to that of unmodified peptide C on the basis of several observations. (i) The modified peptides stimulated the ATPase activity of DnaK in a fashion similar to peptide C; (ii) the modified peptides bind less well to DnaK in the presence of ATP as in the case of peptide C; and (iii) peptide C competes with the modified peptide C derivatives for binding to DnaK.

Strikingly, all three of the peptide C derivatives cross-linked
to amino acids (Arg-527, Arg-536, and His-541) located close together in the linear sequence of the C terminus of DnaK. Phenyl-azide photocross-linkers have been widely used in the identification of the substrate binding sites of proteins (46, 47). After UV irradiation the light-generated short-lived phenylnitrene-related highly reactive intermediate covalently incorporate to the residues that are in direct contact with or close to the photocross-linker. Due to the high reactivity and short lifetime of the reactive intermediate, only the residues that are close to the photocross-linker have the possibility of reacting with the photocross-linker. Less than a few microseconds after its generation, the reactive intermediate generated from the photolysis of phenyl-azide either forms covalent bonds with certain residues or is converted to other nonreactive molecules.

**Fig. 4.** HPLC separation of the tryptic fragments of peptide cross-linked DnaK. The HPLC separations were run at 2 ml/min with a gradient from buffer A (0.1% trifluoroacetic acid) to buffer B (0.085% trifluoroacetic acid and 80% acetonitrile). 50 μl of each 1-ml collection were applied to liquid scintillation to determine the radioactivity. A, tryptic digestion of DnaK-^125I^-PepC-ASA complex. It was run with a gradient of 0–25% B over 10 min, 25–75% B over 30 min, and 75–100% B over 10 min. The tryptic digestion of DnaK-^125I^-PepC-S7C-ABASA complex (B) and DnaK-^125I^-PepC-S8C-ABASA complex (C) were separated with a gradient of 0–25% buffer B over 10 min, 25–75% buffer B over 60 min, and 75–100% buffer B over 10 min.
it is bound to DnaK.

residues that became cross-linked are closest to the peptide when (46, 47). It therefore seems likely that the DnaK amino acid bimolecular reaction (49). Didehydroazepine has a lifetime of expansion product, the didehydroazepine, that undergoes the not permit its bimolecular reaction. Rather it is the ring expansion product, the didehydroazepine, that undergoes the same chemical reaction when subject to photolysis.

phenyl-azides we used in the cross-linking study should undergo the same chemical reaction when subject to photolysis. The adducts of didehydroazepine with glutamic acid or aspartic acid (carboxyazepines) are extremely sensitive to hydrolysis and it is unlikely these adducts will withstand the rigors of protein chemistry (51). Therefore, the amino acid residues that are likely reactive to phenyl-azide under UV irradiation and the adducts could be detected by protein chemistry techniques are limited to lysine, arginine, histidine, serine, threonine, cysteine, glutamic acid, and aspartic acid. The adducts of didehydroazepine with glutamic acid or aspartic acid (carboxyazepines) are extremely sensitive to hydrolysis and it is unlikely these adducts will withstand the rigors of protein chemistry (51). Therefore, the amino acid residues that are likely reactive to phenyl-azide under UV irradiation and the adducts could be detected by protein chemistry techniques are limited to lysine, arginine, histidine, serine, threonine, and cysteine. The relative activity of these residues to didehydroazepine is unknown. Due to the high photochemical reaction specificity of phenyl-azide to amino acid residues with nucleophiles, caution must be exercised in making inferences about the precise chemical environment around the photoactivatable group on a modified peptide. Because of these characteristics of phenyl-azide photochemistry, the amino acid residues of DnaK that become cross-linked may be in actual contact with the photocross-linker or may be just close enough to form an adduct but not directly involved in making specific contacts that are critical for the binding of the peptides. Thus, the fact that we identified Arg-527, Arg-536, and His-541 as the cross-linking points of peptide C and its derivatives was clearly influenced by the fact that arginine and histidine residues are nucleophiles. Although it may not mean Arg-527, Arg-536, and His-541 are the residues that are directly involved in peptide binding of DnaK, nevertheless the fact that all three peptides with photocross-linker at different positions on the peptides all cross-linked to the same fragment of DnaK appears to indicate the importance of this region in the peptide interaction with DnaK. The results reported in this study were obtained prior to the publication of a study describing the determination of the secondary structure of the Hsc70 peptide binding domain (42) and were not particularly easy to interpret on the basis of the MHC-based publication of a study describing the determination of the secondary structure of the Hsc70 peptide binding domain (42) and were not particularly easy to interpret on the basis of the MHC-based model for the Hsp70 peptide binding domain. However, it is interesting to consider the DnaK residues that become cross-linked in our experiments in light of the secondary structure model. Although the C-terminal domain of Hsp70s is less conserved than the N-terminal ATPase domain, there are significant homologies between the peptide binding domain of Hsc70 and the corresponding region of other members of the Hsp70 family, including DnaK. Furthermore, a fragment of DnaK extending from residue 383 is similar to that of Hsc70.

indicated that this domain has a secondary structure topology similar to that of Hsc70. The cross-linking points of125I-PepC-ASA (b), 125I-PepC-S7C-ABASA (a), and 125I-PepC-S8C-ABASA (c) are shown.

No amino acid could be determined at this position.

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* No amino acid could be determined at this position.

(46, 47). It therefore seems likely that the DnaK amino acid residues that become cross-linked are close to the peptide when it is bound to DnaK.

In order to appreciate the implications of our cross-linking data for peptide binding by DnaK, a brief consideration of the specificity of the phenyl-azide photochemistry is necessary. In the solution phase, photolysis of phenyl-azide produces a single phenylisotoprene species, which undergoes ring expansion to give didehydroazepine within 10–100 ps (48). The substituted phenyl-azides we used in the cross-linking study should undergo the same chemical reaction when subject to photolysis. The extremely fast rate of ring expansion of phenylisotoprene does not permit its bimolecular reaction. Rather it is the ring expansion product, the didehydroazepine, that undergoes the bimolecular reaction (49). Didehydroazepine has a lifetime of about 10 μs and reacts only with nucleophiles (50). Nucleophiles, such as amine, hydroxyl, thiol, and carboxy groups, are present in amino acid residues of lysine, arginine, histidine, serine, threonine, cysteine, glutamic acid, and aspartic acid. The adducts of didehydroazepine with glutamic acid or aspartic acid (carboxyazepines) are extremely sensitive to hydrolysis and it is unlikely these adducts will withstand the rigors of protein chemistry (51). Therefore, the amino acid residues that are likely reactive to phenyl-azide under UV irradiation and the adducts could be detected by protein chemistry techniques are limited to lysine, arginine, histidine, serine, threonine, and cysteine. The relative activity of these residues to didehydroazepine is unknown. Due to the high photochemical reaction specificity of phenyl-azide to amino acid residues with nucleophiles, caution must be exercised in making inferences about the precise chemical environment around the photoactivatable group on a modified peptide. Because of these characteristics of phenyl-azide photochemistry, the amino acid residues of DnaK that become cross-linked may be in actual contact with the photocross-linker or may be just close enough to form an adduct but not directly involved in making specific contacts that are critical for the binding of the peptides. Thus, the fact that we identified Arg-527, Arg-536, and His-541 as the cross-linking points of peptide C and its derivatives was clearly influenced by the fact that arginine and histidine residues are nucleophiles. Although it may not mean Arg-527, Arg-536, and His-541 are the residues that are directly involved in peptide binding of DnaK, nevertheless the fact that all three peptides with photocross-linker at different positions on the peptides all cross-linked to the same fragment of DnaK appears to indicate the importance of this region in the peptide interaction with DnaK. The results reported in this study were obtained prior to the publication of a study describing the determination of the secondary structure of the Hsc70 peptide binding domain (42) and were not particularly easy to interpret on the basis of the MHC-based publication of a study describing the determination of the secondary structure of the Hsc70 peptide binding domain (42) and were not particularly easy to interpret on the basis of the MHC-based model for the Hsp70 peptide binding domain. However, it is interesting to consider the DnaK residues that become cross-linked in our experiments in light of the secondary structure model. Although the C-terminal domain of Hsp70s is less conserved than the N-terminal ATPase domain, there are significant homologies between the peptide binding domain of Hsc70 and the corresponding region of other members of the Hsp70 family, including DnaK. Furthermore, a fragment of DnaK extending from residue 383 is capable of binding peptides, and preliminary NMR studies have indicated that this domain has a secondary structure topology similar to that of Hsc70.

The three residues to which our peptide became cross-linked are not located in the region of the DnaK peptide binding domain that consists of eight β-sheets divided into two independent groups of four strands each. Rather these amino acids are located at the extreme C-terminal of the peptide binding domain. As shown in Fig. 5, it seems likely that Arg-527 is

2 E. Zuiderweg and G. Flynn, personal communication.
located in the long α-helix while Arg-536 and His-541 are likely to be located in region which is not included in the long α-helix, at least in the isolated 18 kDa peptide binding domain.

It is not clear how to correlate the relative position of the cross-linked residues on DnaK with the relative position of photocross-linkers on peptide C without knowing some additional aspects of the three-dimensional structure of the peptide binding domain and the bound peptides. It is possible that steric constrains would prevent the modified amino acids in these peptide C derivatives from projecting into the postulated pockets within the peptide binding domain (29). If this were the case, then the modified amino acids carrying the photoactivatable cross-linker might project above the extended peptide as it is bound to DnaK. This line of reasoning suggests the possibility that at least part of the α-helix and the region immediately C-terminal of it might constitute some type of lid that could help to maintain the peptide in the peptide binding region. If such a region were to move in response to ATP binding or ATP hydrolysis by the N-terminal domain, it could play a role in coupling the ATPase cycle of the N-terminal domain to the C-terminal of it might constitute some type of lid that could help to maintain the peptide in the peptide binding region. If such a region were to move in response to ATP binding or ATP hydrolysis by the N-terminal domain, it could play a role in coupling the ATPase cycle of the N-terminal domain to the peptide binding and release cycle of the C-terminal domain.

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Note Added in Proof—While this paper was in press, Zhu et al. (52) reported the crystal structure of a fragment of DnaK (amino acids 389–607) that contains the peptide binding domain. The structure shows that the long helix at the C terminus of the peptide binding domain extends to residue 553 and thus includes Arg-536 and His-541 as well as Arg-527. Furthermore, the structure is consistent with our suggestions that the region of DnaK identified by our peptide cross-linking experiments functions as a lid that helps maintain the peptide in the peptide binding region and that its movement might be driven by DnaK’s ATP hydrolytic cycle.

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