

## COMMUNICATION

# Modulation of Substrate Specificity of the DnaK Chaperone by Alteration of a Hydrophobic Arch

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Hsp70 chaperones assist protein folding by reversible interaction with extended hydrophobic segments of substrate polypeptides. We investigated the contribution of three structural elements of the substrate-binding cavity of the *Escherichia coli* homologue, DnaK, to substrate specificity by investigating mutant DnaK proteins for binding to cellulose-bound peptides. Deletion of the C-terminal subdomain ( $\Delta$ 539-638) and blockage of the access to the hydrophobic pocket in the substrate-binding cavity (V436F) did not change the specificity, although the latter exchange reduced the affinity to all peptides investigated. Mutations (A429W, M404A/A429W) that affect the formation of a hydrophobic arch spanning over the bound substrate disfavored DnaK binding, especially to peptides with short stretches of consecutive hydrophobic residues flanked by acidic residues, while binding to most other peptides remained unchanged. The arch thus contributes to the substrate specificity of DnaK. This finding is of particular interest, since of all the residues of the substrate-binding cavity that contact bound substrate, only the arch-forming residues show significant variation within the Hsp70 family.

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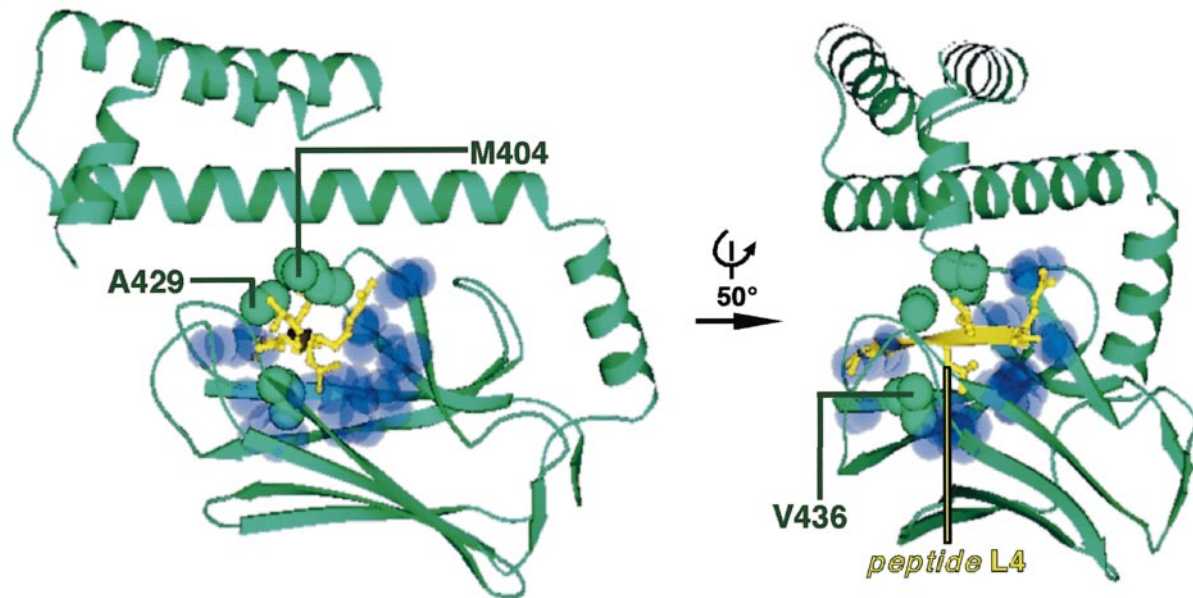
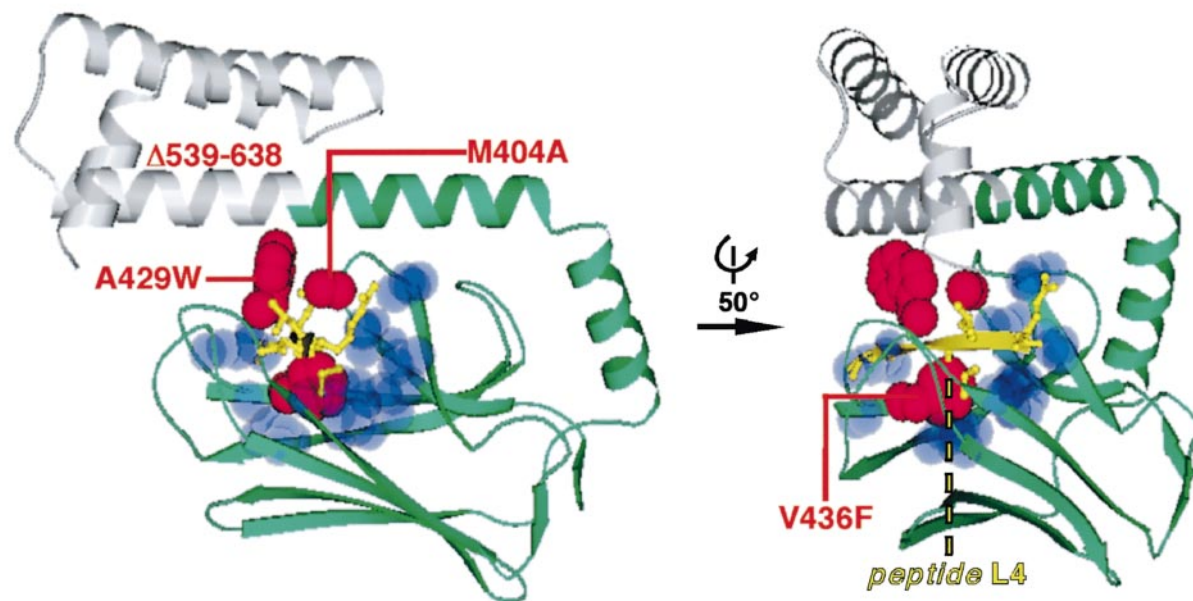
Hsp70 chaperones assist a broad spectrum of protein-folding processes in the cell, ranging from folding and translocation of newly synthesized proteins, to disaggregation of aggregated proteins.<sup>1–3</sup> This functional diversity is achieved by the amplification and specialization of Hsp70 chaperones and the activity of an arsenal of co-chaperones which controls the interactions of Hsp70 proteins with substrates. The diversity may also result from differences among Hsp70 family members in substrate specificity,<sup>4–6</sup> although this possibility has not been investigated in much detail. This study investigates the DnaK homologue of *Escherichia coli* with respect to the structural parameters determining its substrate specificity, thereby exploring the potential for differences in substrate specificity within the Hsp70 family.

Hsp70 proteins share the ability to associate with extended linear peptide segments of polypeptides in an ATP-dependent manner.<sup>7</sup> The binding motif

has been disclosed for the *E. coli* DnaK homologue by screening of cellulose-bound peptides.<sup>8</sup> It is characterized by a core of four or five consecutive amino acid residues enriched in hydrophobic residues, especially Leu, and flanking regions enriched in basic residues. Negatively charged residues are disfavored. The eukaryotic homologue of the endoplasmic reticulum, BiP, recognizes similar side-chains,<sup>9</sup> indicating conservation of the general binding pattern. However, some differences in substrate recognition were suggested by comparative analysis of peptide binding to DnaK, Hsc70, and BiP<sup>4,5</sup> (S.R., J.S.-M. & B.B., unpublished results).

Most information on the structural determinants of substrate specificity exists for DnaK. The crystal structure of the substrate-binding domain of DnaK in complex with a heptameric peptide substrate (NRLLLTG; Figure 1(a))<sup>10</sup> revealed that the substrate is bound in a cavity formed by the strands and connecting loops of a  $\beta$ -sandwich. Although structural information exists for all parts of DnaK,<sup>10–12</sup> no other substrate-binding site could be identified. This is further supported by biochemical

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**A DnaK wild type****B Alterations in DnaK mutant proteins**

**Figure 1.** Structure of the DnaK substrate binding domain. (a) The structure of the DnaK fragment 389-607 (green), which comprises most of the DnaK substrate binding domain (residues 384-638) in cocystal with the peptide NRLLLTG (yellow),<sup>10</sup> is shown in the standard view and rotated counterclockwise by 50°. The peptide side-chains are represented as yellow sticks. The side-chains of the residues of DnaK, which were altered in the present study, are shown as green space fillings. All other residues that make hydrophobic contacts to the cocystalized peptide are shown as blue transparent space fillings. (b) The mutations investigated in this study are indicated: the point mutations M404A, A429W and V436F are shown as red space fillings. The new Phe436 side-chain overlies the central Leu4 of the peptide. The deletion in DnaK-G2-Q538 is indicated by grey helices and coils. The Figure was produced using MOLSCRIPT.<sup>26</sup>

evidence demonstrating that, in the absence of cofactors, DnaK binds with a higher level of affinity to peptide compared to protein substrate,<sup>13</sup> and differs with respect to this for example from chaperonines.<sup>14</sup>

A helical subdomain that spans over the cavity and contacts the cavity-forming loops has been proposed to constitute a lid-like structure.<sup>10</sup> The peptide is bound to DnaK through H bonds to its main-chain and van-der-Waals contacts to its five

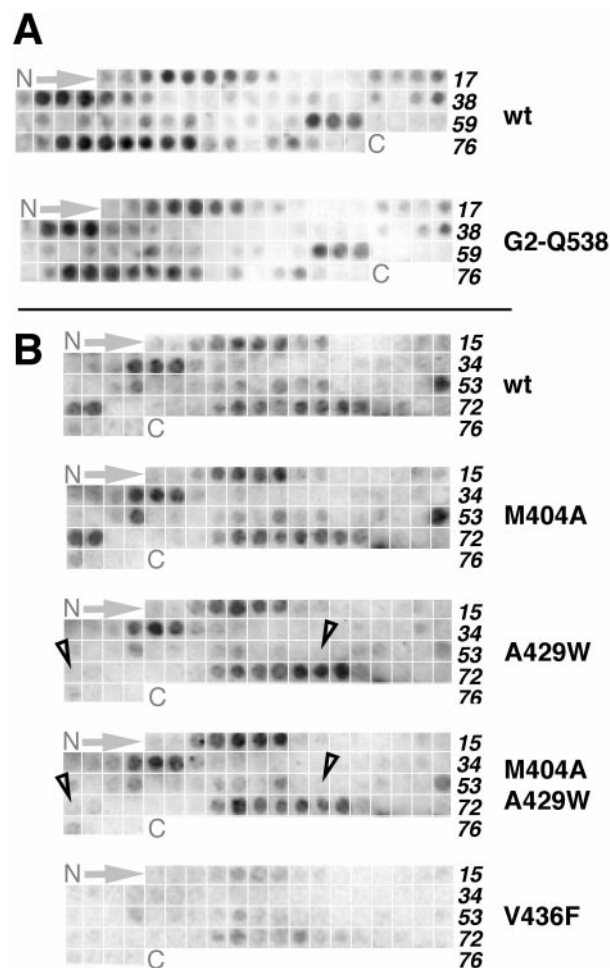
central side-chains, which are largely hydrophobic in nature. Most of the residues of DnaK that provide these hydrophobic contacts are conserved within Hsp70s. Key elements of the substrate binding cavity are: (i) a deep pocket for a single hydrophobic residue (Leu4 of the substrate peptide; position 0); (ii) a hydrophobic arch formed by M404 and A429 of DnaK which wraps over the peptide backbone and contacts the residues adjacent to the residue bound to the hydrophobic pocket (Leu3 and Leu5 of the peptide; positions  $-1$  and  $+1$ ); and (iii) the lid-forming  $\alpha$ -helical subdomain, which does not contact the substrate but must open to allow substrate exchange. ATP binding to the adjacent ATPase domain induces most likely an opening of the lid, perhaps at a proposed hinge point at position Arg536 to Gln538, and further conformational changes in the  $\beta$ -subdomain.<sup>10,13,15</sup>

### Alterations in three key elements of DnaK's substrate binding domain

We investigated by mutational analysis the role of the three key elements of the substrate-binding cavity of DnaK in providing substrate specificity (Figure 1). The central hydrophobic pocket was blocked by replacement of Val436 with Phe (DnaK-V436F). The arch was altered: (i) by a Met404-to-Ala exchange (DnaK-M404A) to reduce the potential of hydrophobic contacts in the positions  $-1$  and  $+1$  of the substrate-binding cavity and to prevent formation of the arch with Ala429; (ii) by an Ala429-to-Trp exchange (DnaK-A429W) to increase on the one hand the potential of hydrophobic contacts in the positions  $-1$  and  $+1$ , and on the other hand to increase the steric hindrance in the arch, which may prevent access of certain substrates; and (iii) both changes were combined in the DnaK-M404A/A429W mutant. These changes represent the most drastic alterations in the arch that can be achieved with the 20 proteinogenic amino acid residues. The lid was eliminated by truncation of the DnaK polypeptide at the proposed hinge point at residue 538 (DnaK-G2-Q538). The biochemical properties and chaperone activities of these mutant proteins are described in detail elsewhere.<sup>13,16-18</sup> The mutational alterations affected the association or dissociation rates of complexes of DnaK with model peptide and protein substrates resulting in reduced affinity of DnaK for substrates.<sup>13</sup> However, the ATP-dependent control of the substrate release and the interaction with the DnaJ co-chaperone was not affected.<sup>13</sup> Furthermore, it should be emphasised that the degree of the stimulation of the ATPase of these altered proteins depends strictly on their affinity for substrate even in the presence of DnaJ.<sup>13</sup> Therefore, it is important also with respect to the physiological situation where Hsp70s are accompanied by DnaJ cochaperones, to investigate the principles governing Hsp70 specificity.

For determination of the substrate specificity of these mutant proteins we chose 76 13mer peptides

scanning the sequence of the  $\lambda$  CI protein (236 residues) by an overlapping window of ten residues (Figure 2). Since DnaK-binding sites occur frequently in protein sequences including  $\lambda$  CI,<sup>8</sup> scans of this size constitute a representative sample of the entire range of binding and non-binding



**Figure 2.** Binding of DnaK wild-type and mutant proteins to cellulose-bound peptide scans. Peptide scans<sup>27,28</sup> derived from the sequence of  $\lambda$ CI were screened for binding to wild-type DnaK and mutants as indicated. The arrowheads indicate spots reduced in affinity for the DnaK mutants A429W and M404A/A429W. Peptide libraries were prepared by automated spot synthesis.<sup>28-30</sup> Peptides are C-terminally attached to cellulose *via* ( $\beta$ -Ala)<sub>2</sub> spacer. Each peptide is shifted in the sequence of  $\lambda$ CI by three residues compared to the peptide before. The peptides were investigated for DnaK binding by the electrotransfer technique as described.<sup>8</sup> All DnaK proteins were incubated at a concentration of 150 nM for 60 minutes at 25 °C. The cloning, purification and characterization of the proteins are described elsewhere.<sup>13</sup> The detection was performed by an immunological assay utilising (a) a chemiluminescence kit (Boehringer Mannheim) or (b) by chemifluorescence (ECF kit, Amersham-Pharmacia) and a fluorimaging systems (FLA2000, Fuji). The different detection procedures in (a) and (b) are responsible for differences in the contrast of the spots.

peptides. Furthermore, a scan of this size allows the simultaneous investigation of several mutant proteins under identical conditions.

### The hydrophobic pocket and the lid do not regulate DnaK's substrate specificity

Deletion of the lid (DnaK-G2-Q538) did not change the binding pattern as compared to wild-type DnaK protein (Figure 2(a)), indicating that the determinants of the substrate specificity of DnaK localize to the  $\beta$ -subdomain.

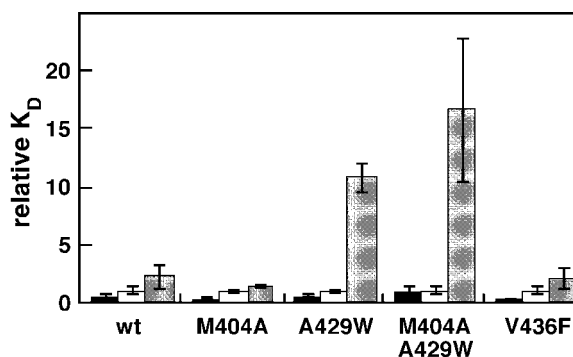
Blockage of the hydrophobic pocket in the DnaK-V436F mutant protein strongly reduced the affinity for all peptides (Figure 2(b)). Weak signals were obtained only for some peptides that had a strong affinity for wild-type DnaK (e.g. Figure 2, spots 5-7). At a fivefold increased concentration of DnaK-V436F compared to wild-type DnaK, the signals were intensified but found to show an overall similar binding pattern compared to wild-type DnaK (not shown). In particular, we did not identify peptides that bind with the same or greater affinity to DnaK-V436F as compared to wild-type DnaK, indicating that this alteration has a global negative effect on substrate binding. We noted that even peptides with a short hydrophobic core of exclusively bulky side-chains and flanking negatively charged residues (e. g. TKKASDSAFWLEV, spot 45 in Figure 2), which should be precisely positioned in the cavity of wild-type DnaK because of the incompatibility of the negatively charged flanking residues for binding to the cavity,<sup>10</sup> show weak but detectable affinity for the V436F mutant protein and are not particularly disfavored. Thus, even bulky side-chains can be accommodated to some extent at the blocked position 0. The remaining weak affinity for peptides can be explained by assuming that the hydrophobic pocket is not completely blocked by the Val-to-Phe exchange. This possibility is indicated by the NMR structures of the substrate binding domains of DnaK and Hsc70, which show a central hydrophobic pocket at position 0 that is larger than the pocket identified in the X-ray structure.<sup>19,20</sup> Reorganisation of the pocket before substrate binding is congruent with the finding that only the association rate constants ( $k_{on}$ ) of DnaK-V436F is 20 to 40-fold lower for protein and peptide substrates as compared to wild-type DnaK, while this particular exchange does not affect the substrates dissociation rates.<sup>10,13,15</sup>

### Variations in DnaK's arch modulate the affinity for a specific subset of substrates

For most peptides, the binding pattern of the arch mutants DnaK-M404A, DnaK-A429W and DnaK-M404A/A429W did not differ from that of wild-type DnaK (Figure 2(b)). However, we identified two peptide binding regions around spots 47 and 54 (arrowheads in Figure 2(b)) which had significantly less affinity for both mutant proteins carrying the A429W exchange. This effect is less

pronounced in the case of the DnaK-M404A/A429W double mutant. To ensure that these results were not biased by the C-terminal coupling of the peptides to the cellulose matrix, we determined the binding affinity of the peptide corresponding to spot 53 ( $\lambda$  CI-F160-Q172) in solution (Figure 3). The affinity of this peptide was determined by measuring its ability to compete with a fluorescent labeled peptide ( $\sigma^{32}$ -Q132-Q144-C-IAANS) for DnaK binding. We included in this assay two other peptides ( $\sigma^{32}$ -Q132-Q144,  $\sigma^{32}$ -M195-N207) which were previously characterized as high-affinity DnaK binders ( $K_D = 0.1 \mu\text{M}$ ).<sup>21</sup> We found that the relative affinity of DnaK for  $\lambda$  CI-F160-Q172 (compared to  $\sigma^{32}$ -M195-N207 as standard) was strongly reduced for DnaK-A429W and DnaK-M404A/A429W, but not for the DnaK-V436 and DnaK-M404A mutant proteins, consistent with the results of the peptide-scan analysis (Figure 2(b)). In contrast, the relative affinity for  $\sigma^{32}$ -Q132-Q144 was not decreased. We conclude that the A429W exchange leads to a selective decrease in affinity of DnaK for a subset of peptides.

We investigated the molecular basis for this counterselection. Both peptides, Figure 2(b) spot 47 and Figure 2(b) spot 53, which were disfavored for binding to DnaK-A429W and DnaK-M404A/A429W on the peptide scan contain a consecutive patch of large hydrophobic or aromatic residues flanked by acidic residues (ASDSAFWLEVEGN and FPDGM LILVDPEQ). The negatively charged residues which are strongly disfavored in the substrate-binding cavity are likely to impose a positioning of the peptide in the binding cavity of wild-type DnaK. Negatively charged residues in the regions flanking the hydrophobic core are disfavoured by DnaK wild-type, too, but this effect is severely increased for DnaK-A429W and DnaK-M404A/A429W. This positioning may be prevented by the A429W exchange that may sterically



**Figure 3.** Relative affinities of wild-type and mutant DnaK proteins to peptides. The  $K_D$  values of the interactions of DnaK proteins with the peptides  $\sigma^{32}$ -Q132-Q144-C-IAANS,  $\sigma^{32}$ -M195-N207 and  $\lambda$ CI-F160-Q173-C were normalized to the  $K_D$  of each mutant protein to  $\sigma^{32}$ -M195-N207, and thus are represented as relative  $K_D$  values. The experiment was performed according to a published protocol.<sup>21</sup>

hinder the binding of bulky side-chains in the positions +1 and -1 through collision with the large Trp side-chain. To investigate this hypothesis we modulated the sequence of the above mentioned and similar peptides by selective exchange of crucial residues.

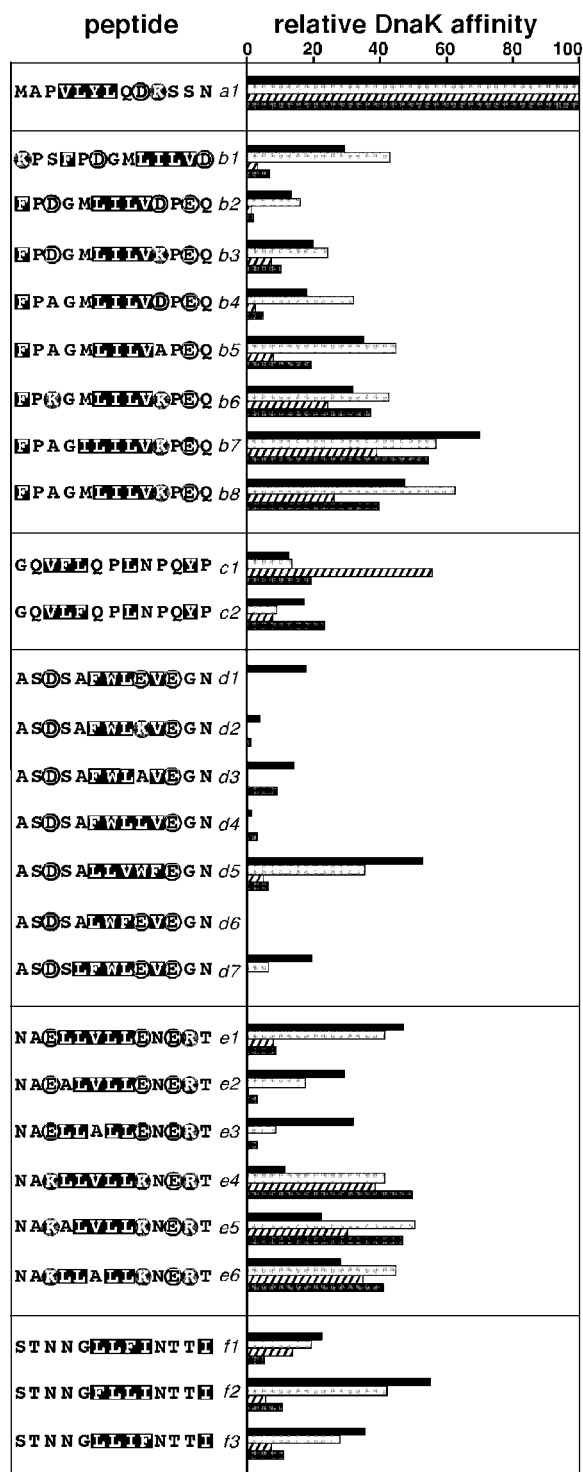
### Binding of wild-type and arch mutant proteins to site-directed altered peptides

The selected peptides were investigated for binding to wild-type and arch mutant proteins by quantitative analysis of cellulose-bound peptide scans (Figure 4). The affinity pattern of the DnaK-M404A/A429W mutant protein was similar to the pattern of DnaK-A429W, although the binding intensity of the double-mutant protein was in general slightly stronger. This indicates the dominant contribution of the A429W exchange to the observed binding phenotype of the double mutant. Consistent with this finding is that the peptide binding pattern of the DnaK-M404A mutant protein was in most cases similar to wild-type.

Exchange of the flanking acidic residues by basic residues eliminated the disadvantage of the DnaK-A429W and DnaK-M404A/A429W mutant proteins for the association to peptides with four or five consecutive large hydrophobic or aromatic residues (Figure 4, peptide *b2* versus *b6* and peptides *e1-e3* versus *e4-e6*). Replacement of acidic residues by Ala had an intermediate effect (Figure 4, peptide *b2* versus *b5*). This emphasizes the importance of positive contributions of basic residues outside the hydrophobic core to the affinity for DnaK, while negatively charged residues have the opposite effect. These results support the hypothesis that the flanking acidic residues position the peptide in the DnaK-binding cavity, and that this positioning is problematic for the A429W arch mutants when the peptide has a bulky hydrophobic peptide core.

We observed that changes in the hydrophobic core region of substrate peptides result in affinity differences, even if only the positions of hydrophobic residues were switched, without affecting the total amino acid composition of the core (Figure 4, peptides *d1/d6*, *d4/d5* and *f1/f2/f3*). This result indicates that even in the case of DnaK wild-type protein the nature and the order of the hydrophobic side-chains within the hydrophobic core of the peptide substrate are determinants for the substrate specificity. This finding is consistent with the recently identified feature of DnaK to bind substrates in a directional manner (S.R., J.S.-M. & B.B., unpublished results).

The Ala429-to-Trp alteration caused in most cases a reduced affinity of DnaK for peptides (Figure 4). However, we found exceptions in which peptides showed stronger binding to DnaK-A429W compared to wild-type DnaK and, in one case, also to the other mutant proteins (Figure 4, peptide *c1*). The comparison with peptide *c2* suggests that in case of peptide *c1*, the distance of



**Figure 4.** Modulation of peptide binding for alterations in the arch. DnaK wt (black bars) and the arch mutant proteins DnaK-M404A (light grey bars), DnaK-A429W (hatched bars) and DnaK-M404A/A429W (dark grey bars) were analysed for binding to specific cellulose-bound peptides as described in the legend to Figure 1. The intensities of the signals of three independent experiments were quantified and normalized for each protein to the signal of  $\sigma^{32}$ -M195-N207 (peptide *a1*, intensity set to 100) and are represented as bars. For each peptide the sequence and the name to which is referred to in the text is indicated.

the two Leu or the position of the Phe residue might be rather critical for the DnaK-A429W mutant protein, while the affinity of wild-type DnaK to both peptides was similar. In the cases of peptides *e4*, *e5*, and *e6* all arch mutant proteins bound better than wild-type DnaK for unknown reasons. Together, these findings indicate that alterations of the arch-forming residues are also able to increase the affinity of DnaK to specific subsets of peptides.

## Conclusions

This study investigated the role of structural key elements of the substrate-binding cavity of DnaK in affecting the substrate specificity of this chaperone. The C-terminal  $\alpha$ -helical subdomain was found not to contribute to substrate specificity. The relatively low conservation of this subdomain within the Hsp70 family may therefore play a role unrelated to the peptide-binding properties *per se*. An important role of the C-terminal subdomain may be to allow for association of co-chaperones, which then may affect the interaction of Hsp70 proteins with substrates.<sup>22</sup>

The steric crowding in the central hydrophobic pocket at position 0 by the Val436-to-Phe exchange globally reduced the affinity of DnaK for peptides, but did not selectively affect peptide binding. The global effect of DnaK-V436F is consistent with inactivity *in vivo*.<sup>13</sup> The pocket-forming residues are highly conserved between Hsp70 family members, exhibiting 95-100% similarity and 63-100% identity.<sup>6</sup> We conclude that contributions of this hydrophobic pocket are required for all Hsp70-substrate interactions. Given our findings and the high level of conservation of the pocket within the Hsp70 family, it seems unlikely that some homologues allow the association of polar residues at position 0.

In contrast, the arch mutants described here are the first reported mutational alterations in the substrate-binding cavity which modulate the specificity of an Hsp70 chaperone for a subset of substrates. This finding is particularly interesting in view of the low evolutionary conservation of the residues forming the hydrophobic arch, in contrast to the highly conserved residues which mediate additional hydrophobic contacts.<sup>6</sup> While many eukaryotic Hsc70s have an inverted arch compared to DnaK, in which the corresponding residues of Met404/Ala429 are changed to Ala404/Tyr429 (e.g. bovine Hsc70 and yeast Ssa), Hsp70 proteins probably dedicated to specific tasks, such as yeast Ssb<sup>23</sup> or *E. coli* HscA<sup>24,25</sup> have unique combinations (Gln404/Cys429 and Met404/Phe429, respectively) that could be related to their specific functions (all residue numbers given are the corresponding numbers in DnaK). Changes in the arch, therefore, have the potential to change the chaperone activity of Hsp70 proteins *via* the described alterations in substrate specificity, and perhaps further changes in the kinetics of substrate inter-

action.<sup>13</sup> DnaK-A429W mutant proteins do not efficiently complement the temperature-sensitive phenotype of  $\Delta$ *dnaK52* mutant strains, while DnaK-M404A is as active as DnaK wild-type.<sup>13</sup> These findings may reflect that alterations in the arch can be of physiological relevance.

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