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The Periplasmic Chaperone SurA Exploits Two Features Characteristic of Integral Outer Membrane Proteins for Selective Substrate Recognition*

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The *Escherichia coli* periplasmic chaperone and peptidyl-prolyl isomerase (PPIase) SurA facilitates the maturation of outer membrane porins. Although the PPIase activity exhibited by one of its two parvulin-like domains is dispensable for this function, the chaperone activity residing in the non-PPIase regions of SurA, a sizable N-terminal domain and a short C-terminal tail, is essential. Unlike most cytoplasmic chaperones SurA is selective for particular substrates and recognizes outer membrane porins synthesized *in vitro* much more efficiently than other proteins. Thus, SurA may be specialized for the maturation of outer membrane proteins. We have characterized the substrate specificity of SurA based on its natural, biologically relevant substrates by screening cellulose-bound peptide libraries representing outer membrane proteins. We show that two features are critical for peptide binding by SurA: specific patterns of aromatic residues and the orientation of their side chains, which are found more frequently in integral outer membrane proteins than in other proteins. For the first time this sufficiently explains the capability of SurA to discriminate between outer membrane protein and non-outer membrane protein folding intermediates. Furthermore, peptide binding by SurA requires neither an active PPIase domain nor the presence of proline, indicating that the observed substrate specificity relates to the chaperone function of SurA. Finally, we show that SurA is capable of associating with the outer membrane. Together, our data support a model in which SurA is specialized to interact with nonnative periplasmic outer membrane protein folding intermediates and to assist in their maturation from early to late outer membrane-associated steps.

Protein folding in all living cells is monitored and assisted by a network of accessory proteins, the molecular chaperones (1, 2). According to their mechanisms of action the major chaperones residing in the cytoplasm of prokaryotic and eukaryotic cells are divided into two classes: (i) chaperones that bind to nascent and newly synthesized peptide chains as well as to unfolded and damaged proteins protecting them from unspecific interaction and aggregation (*e.g.* Hsp70 chaperones, such as *Escherichia coli* DnaK), and (ii) chaperones that provide an internal cavity in which complete proteins or protein domains can fold shielded from the environment (the Hsp60 chaperonins, such as *E. coli* GroEL/ES). Both classes of chaperones exhibit rather broad substrate specificity and recognize hydrophobic residues and/or unstructured backbone regions exposed by their non-native protein substrates.

Proteins with chaperone-like function have also been identified in the periplasm, the second compartment of Gram-negative bacteria that is located between the inner and the outer membranes of the cells. Their molecular mechanisms and their molecular principles of substrate recognition, however, are poorly understood. These chaperones must function in the absence of known cellular energy sources, the periplasm lacks ATP (3), and thus must be mechanistically distinct from the major cytoplasmic chaperones, most of which use ATP to drive their catalytic cycles of substrate binding and release (1, 2).

The periplasmic chaperone SurA facilitates the correct folding and maturation of outer membrane porins, the pore-forming proteins, which provide for the integrity and exchange function of the outer membrane (4). The porins are synthesized in the cytoplasm and secreted into the periplasm in a mostly non-native state, where they must fold, assemble, and insert into the outer membrane. The timing and order of these processes are still largely unknown, and the only clearly documented role of SurA in this folding pathway thus far relates to an early step, the conversion of unfolded to folded monomer (5).

The function of SurA in porin maturation was first attributed to the peptidyl-prolyl isomerase (PPIase)[1] activity exhibited by one of its two parvulin-like PPIase domains (5, 6). It was shown only recently that the main biological function of SurA is that of a chaperone and that this function resides in its non-PPIase regions, a sizable N-terminal domain and a short C-terminal tail (7). These regions of SurA mediate both chaperone activity and substrate binding and are required and sufficient for its *in vivo* function. Additionally, the N-terminal amino acids 21–133 of SurA were shown to exhibit proline-independent peptide binding activity (8). The structural arrangement of SurA is consistent with these biochemical properties (9). The N- and C-terminal regions and the inactive PPIase domain I of SurA together constitute a compact core module traversed by a broad, deep crevice suggestive of a polypeptide-binding site. The active PPIase domain II is tethered to this core by two extended polypeptide segments. Finally, in contrast to most

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[1] The abbreviations used are: PPIase, peptidyl-prolyl isomerase; Ar, aromatic amino acids; OMPs, outer membrane proteins; SPR, surface plasmon resonance.

cytoplasmic chaperones, SurA is selective for particular substrates, recognizing *in vitro* synthesized outer membrane porins much more efficiently than other similarly sized proteins (7), which suggests that SurA is specialized for the maturation of outer membrane proteins (OMPs).

To characterize the substrate specificity of SurA, we have screened cellulose-bound peptide libraries representing three biologically relevant SurA substrates. The analysis of the SurA-binding peptides derived from these substrates suggests that both specific patterns of aromatic amino acids and the proper orientation of their side chains along two faces of the peptides are important for binding. Our data lead us to propose that the capability of SurA to recognize OMPs selectively is based on the frequent coincidence of aromatic patterns and of β-strand secondary structure in these proteins, because this allows the observed preferred orientation of aromatic side chains. Furthermore, we show that a fraction of the SurA molecules present in the cell associates with the outer membrane. Thus, in addition to its reported role in an early periplasmic step in porin maturation (5), SurA may act in late outer membrane-associated steps in this folding pathway.

MATERIALS AND METHODS

Protein Purification—The C-terminally His_6-tagged SurA and SurAN+I-Ct proteins were produced (from pASKSurA and pASKSurAN+I-Ct, respectively) and purified as described previously (7), except that proteins to be used in SPR studies were purified in TBST buffer (31 mM Tris-HCl, pH 7.6, 170 mM NaCl, 6.4 mM KCl, 0.05% Tween 20) instead of buffer A. pASKSurAN+I-Ct was generated by cloning a PstI/BglII fragment derived from pSurAN+I-Ct (7) into PstI/BglII-cleaved pASKSurA. If required, proteins were further purified by gel filtration (Hiload Superdex 75 prep grade, Amersham Biosciences). Protein concentrations were determined by UV absorbance at 280 nm using absorption coefficients of 29,450 M^{-1} cm^{-1} for SurA and 23,470 M^{-1} cm^{-1} for SurAN+I-Ct as calculated according to Pace *et al*. (10).

Synthesis and Screening of Cellulose-bound Peptides—Peptide libraries (13-mer peptides overlapping by 10 amino acids) were prepared by automated spot synthesis (11). Peptides are attached to cellulose-aminohydroxylpropylether-cellulose via a $(\beta\text{-Ala})_2$ spacer at a density of 90–100 nmol/cm^2 membrane. The peptides were derived from the sequences of the OmpF and LamB porins and the outer membrane protein OmpA. Screening was performed essentially as described previously (12). The libraries were incubated with 170 nM SurA or SurAN+I-Ct protein diluted in MP buffer (31 mM Tris-HCl, pH 7.6, 170 mM NaCl, 6.4 mM KCl, 0.05% Tween 20, 5% sucrose), and the bound proteins were electrotransferred to polyvinylidene difluoride membranes. SurA was immunodetected by using SurA-specific polyclonal rabbit antibody and anti-rabbit alkaline phosphatase secondary antibody (Sigma) at 1:10,000 dilutions. The blots were developed with a chemifluorescence substrate (ECF-Kit, Amersham Biosciences), and the signals were visualized using a PhosphorImager system (Storm 860, Amersham Biosciences). To determine relative binding affinities, for each peptide library the spot intensities of three representative membranes were averaged and normalized to the spot of highest intensity (100%).

Surface Plasmon Resonance Measurements—The interaction of SurAN+I-Ct and peptide substrate was measured quantitatively by surface plasmon resonance (SPR) spectroscopy using a BIAcore 2000 system (BIAcore AB, Uppsala, Sweden). Two peptides were synthesized, a good binder (pepH = DVHMIDFYYWDIS) and a non-binder (pepN = TLELGVDYGRANL), and immobilized on a C1 sensor chip (BIAcore AB, Uppsala, Sweden) by thiol-coupling via an additional C-terminal Cys residue to final densities of 20 pg/mm^2 (10.3 fmol of pepH) and 26 pg/mm^2 (15.8 fmol of pepN), respectively. Interaction of SurAN+I-Ct with these peptides was detected by monitoring the mass concentration-dependent changes of the refractive index on the sensor surface, expressed as resonance units. TBST buffer was used as the running buffer. The immobilized peptides were regenerated in 1 M NaCl. All solutions used for SPR were filtered (0.22 μM) and degassed.

Control experiments using different SurAN+I-Ct concentrations and flow rates between 5 and 75 μl/min revealed no notable mass transfer effects. Kinetic measurements were performed at a flow rate of 25 μl/min. Each series of measurements comprised three cycles in which six different SurAN+I-Ct concentrations were injected randomly into the flow cells. For each concentration, the net binding of SurAN+I-Ct to pepH was determined by subtracting the sensorgrams obtained for its binding to pepH from those obtained for its binding to pepN. The equilibrium dissociation constant K_D was determined from the net binding data obtained in two independent series of measurements by using BIAevaluation 3.2 software, assuming a one-to-one binding model without mass transfer.

Flotation Gradient Centrifugation—E. coli wild-type strain CAG16037 (13) was grown in 50 ml of dYT medium at 37 °C to mid-exponential phase ($A_{600} \sim 0.6$). Cells were harvested, washed three times in MP buffer, and disrupted by French press and sonication. Remaining intact cells were removed by low speed centrifugation (4000 rpm, 10 min). Membranes were pelleted from the cell-free crude extract by centrifugation at 100,000 $\times$ *g*, 10 °C for 2 h and resuspended in 1 ml of 60% sucrose. A sucrose step gradient was then formed by overlaying the sample with 1 ml of sucrose solutions of decreasing concentrations in 5% increments (55 to 15% (w/v)) and centrifuged at 100,000 $\times$ *g*, at 10 °C for 16 h. Fractions of 0.5 ml were collected from the top of the gradients, precipitated with trichloroacetic acid, and analyzed by immunoblotting. Primary polyclonal antisera against SurA, OmpA, MalF, and Hsc66 were used at 1:10,000, 1:500, 1:20,000, and 1:100,000 concentrations, respectively. Anti-rabbit and anti-mouse alkaline phosphatase secondary antibodies (Sigma) were used at 1:10,000 concentrations. The blots were developed by incubation in reaction buffer (100 mM Tris-HCl, pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$, 37.5 μg/ml nitro blue tetrazolium, 150 μg/ml 5-bromo-4-chloro-3-indolyl phosphate).

RESULTS

Screening of Peptide Scans for Binding of SurA—SurA has recently been shown to interact selectively with *in vitro* newly synthesized, non-native outer membrane porins (7) and to exhibit a peptide binding activity (8). Therefore, to characterize its substrate-binding specificity and to identify the binding sites within its biological substrates, we screened cellulose-bound peptide libraries that scan the complete primary sequences of three OMPs: the trimeric porins and known SurA substrates LamB and OmpF, and the monomeric OMP and candidate SurA substrate OmpA. The peptide libraries were composed of 13-mer peptides each overlapping with the adjacent peptides by 10 residues and thus presenting all potential linear binding sites in these proteins. For screening, the libraries were incubated to equilibrium either with purified wild-type SurA or with purified SurAN+I-Ct, a SurA variant lacking the active PPIase domain II. Peptide-bound protein was then electrotransferred to polyvinylidene difluoride membranes and immunodetected.

SurA-binding peptides were identified in all peptide scans (Fig. 1). In each of them SurA bound only to a subset of peptides, demonstrating that SurA discriminates between different sequences. Frequently, binding to overlapping peptides was observed, suggesting that neighboring peptides share a SurA-binding site. Unlike the cytoplasmic chaperone DnaK, which binds to hydrophobic patches, including the signal peptides of secretory proteins (12), SurA did not interact with the LamB, OmpF, or OmpA signal sequences. The SurAN+I-Ct protein bound to the same subset of peptides in all three peptide scans (data not shown), demonstrating that the active PPIase domain II of SurA is not required for peptide recognition and binding.

SurA Selects for Peptides Rich in Aromatic Amino Acids—All screened peptides were classified as good, weak, or non-binders of SurA by determining their relative spot intensities. According to this classification, among the 376 library peptides, 41 peptides bound to SurA with high relative affinity (good binders; relative intensity $\geq$ 60%), 37 peptides bound to SurA with low relative affinity (weak binders; relative intensity $<$ 60% $\geq$ 40%), and 298 peptides did not bind to SurA (non-binders; relative intensity $<$ 40%). It should be noted, however, that in the group of peptides classified as non-binders a subset might still bind to SurA with low affinity.

A comparison of the amino acid distribution of the three peptide classes is shown in Fig. 2*A*. Good and weak SurA-

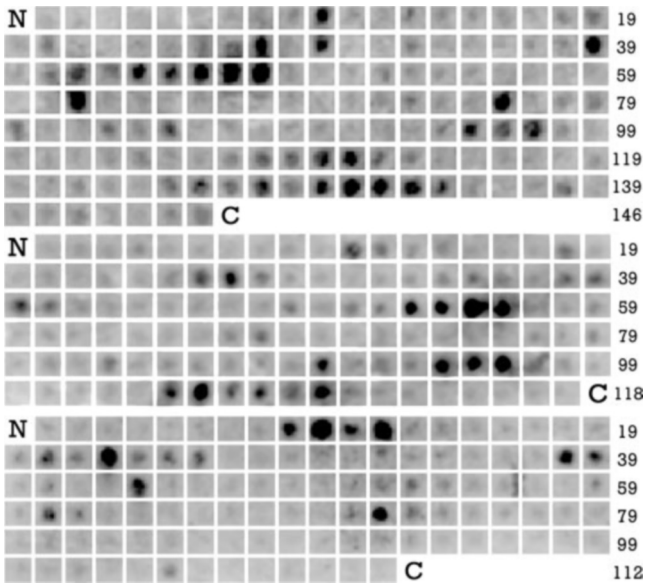

FIG. 1. **Binding of SurA to cellulose-bound peptide libraries (13-mers) derived from the sequences of the LamB (*top*) and OmpF (*center*) porins and the outer membrane protein OmpA (*bottom*).** *Numbers* at the *right* indicate the last peptide spot in the row.

binders are highly enriched in aromatic residues (Phe, Trp, and Tyr) and histidine. This preference for aromatic amino acids is unlikely to simply reflect nonspecific hydrophobic interactions because Ile, Leu, and Val are not enriched and alanine is even disfavored in SurA-binding peptides. Moreover, a closer analysis revealed that neither Phe, nor Trp or Tyr was favored for binding to SurA, simply because it was enriched in peptides that contained one of the other two aromatic amino acids. All three aromatic amino acids were statistically distributed with respect to each other among the library peptides (p values $>$ 0.1; data not shown). Likewise, no linkage was observed between His and Trp or His and Tyr. His was, however, enriched in Phe-containing peptides with a statistical significance of $<$1% ($p = 0.004$), suggesting that its occurrence in a peptide is linked to the occurrence of Phe in the peptide. Nevertheless, we included His in the group of aromatic amino acids for our further analyses of the library peptides, because, despite its linkage to Phe, it might still contribute to peptide binding by SurA, and because it represented only a small fraction of the aromatic amino acids contained in both the total library and in SurA-binding peptides (8.4 and 8.5%, respectively). In addition, glutamine and glutamate are disfavored in good and weak binders, and, from 16 cysteines present in the libraries, none was recovered in a SurA-binding peptide. Again, a statistical analysis revealed no linkage between these amino acids in the library peptides (p values $>$ 0.1; data not shown), suggesting that they are disfavored for binding to SurA independent of each other. Finally, evaluation of the data using the method of Kluck *et al.* (14) resulted in essentially the same relative occurrence of each amino acid in a theoretical peptide with the characteristics of the library that has the highest affinity to SurA as obtained for our good and weak SurA-binders (data not shown), indicating that the arbitrary thresholds were set at reasonable levels for these two classes.

Classification of the peptides according to their content of aromatic residues revealed that with increasing numbers of aromatic residues in the peptides the fraction of non-binders decreases continuously in favor of good and weak SurA-binders (Fig. 2*B*). All peptides without aromatic residue and all except one of 111 peptides containing a single aromatic residue were non-binders. In contrast, all peptides with more than four aromatic residues bound to SurA with high affinity. The average content of aromatic residues increases from 1.7 for non-binders to 2.9 and 3.3 for weak and good binders, respectively (Fig. 2*C*). These data indicate a correlation between the number of aromatic amino acids in a peptide and its affinity for SurA. However, the quite broad overlap of the binding and non-binding peptide classes in the range of two, three, and even four aromatic residues per peptide (Fig. 2, *B* and *C*) suggests that additional determinants may contribute to binding.

Because the occurrence of glutamate was reduced in good and weak SurA-binders (Fig. 2*A*), we asked whether charge could possibly affect binding. However, only plotting the average net charge of binding and of non-binding peptides as a function of the number of aromatic residues in the peptides revealed a noticeable difference (Fig. 2*D*). Although a linear relationship ($R^2 = 0.98$) between both parameters became apparent for the non-binding peptides, the values for the binding peptides scattered strongly due to the small sample size. Interestingly, however, the corresponding trend line ($R^2 = 0.71$) runs parallel to but shifted by about +0.6 charge units from that of the non-binding peptides. Although this difference may not be statistically significant, it is remarkable that in each group of peptides with identical aromatic residue count the average net charge of SurA-binding peptides is more positive than that of non-binders.

Finally, in accord with Webb *et al.* (8), who showed binding of SurA to a model peptide devoid of proline residues, we found prolines not to contribute to substrate binding (Fig. 2*A*). Among all binding peptides only 22% contain prolines, demonstrating that this amino acid is not required for the interaction of peptides with SurA.

SurA-binding Peptides Contain Specific Patterns of Aromatic Residues—The limited size of the data set did not allow for a reliable prediction of a consensus binding motif for SurA. However, we observed that 73% of the good and 62% of the weak binders contained the sequences Ar-Ar or Ar-*X*-Ar (with Ar = F, W, Y, or H and *X* = any other residue). Both sequence motifs occurred with about identical frequency in the class of good SurA-binders, whereas the Ar-Ar motif was less frequent in weak binders (Table I). Because the Ar-*X*-Ar tripeptide motif has recently been shown to be diagnostic of membrane proteins (15), we asked whether the Ar-Ar motif might also be characteristic of OMPs. The analysis of known integral OMPs and of periplasmic proteins however revealed that the Ar-Ar motif is not prevalent in integral OMPs, occurring at even lower frequency than expected (Table II). In contrast, and consistent with Ref. 15, the Ar-*X*-Ar motif is slightly enriched in integral OMPs especially when compared with periplasmic proteins. Thus, the Ar-Ar motif would not be characteristic of OMPs but yet occurs frequently in SurA-binding peptides (Table I), suggesting that Ar-Ar is only part of a more complex recognition pattern for SurA-binding.

Indeed, we found that in the majority of the SurA-binding peptides both the Ar-*X*-Ar and the Ar-Ar motifs formed more complex patterns of the following types (Fig. 3): 1) Ar_n with n = 3–4 (Fig. 3*c*); 2) Ar-*X*-Ar-*X*-Ar (Fig. 3*d*); 3) Ar-Ar-X_n-Ar or Ar-X_n-Ar-Ar, with n = 2–7 (Fig. 3*e*); and 4) Ar-*X*-Ar-*X*$_n$-Ar or Ar-X_n-Ar-*X*-Ar, with n = 3, 5, and 7 (Fig. 3*f*).

In the patterns 1 and 2, the Ar-Ar and Ar-*X*-Ar motifs are arranged in clusters of three to four aromatic residues in a row (Ar-Ar-Ar-Ar, Fig. 3*c*) or in every second position (Ar-*X*-Ar-*X*-Ar, Fig. 3*d*; no histidine was observed in this pattern). In patterns of the types 3 and 4, the motifs are flanked by at least one additional aromatic residue (or by a second motif) with a spacing of two to seven residues (Fig. 3, *e* and *f*). Thus, a

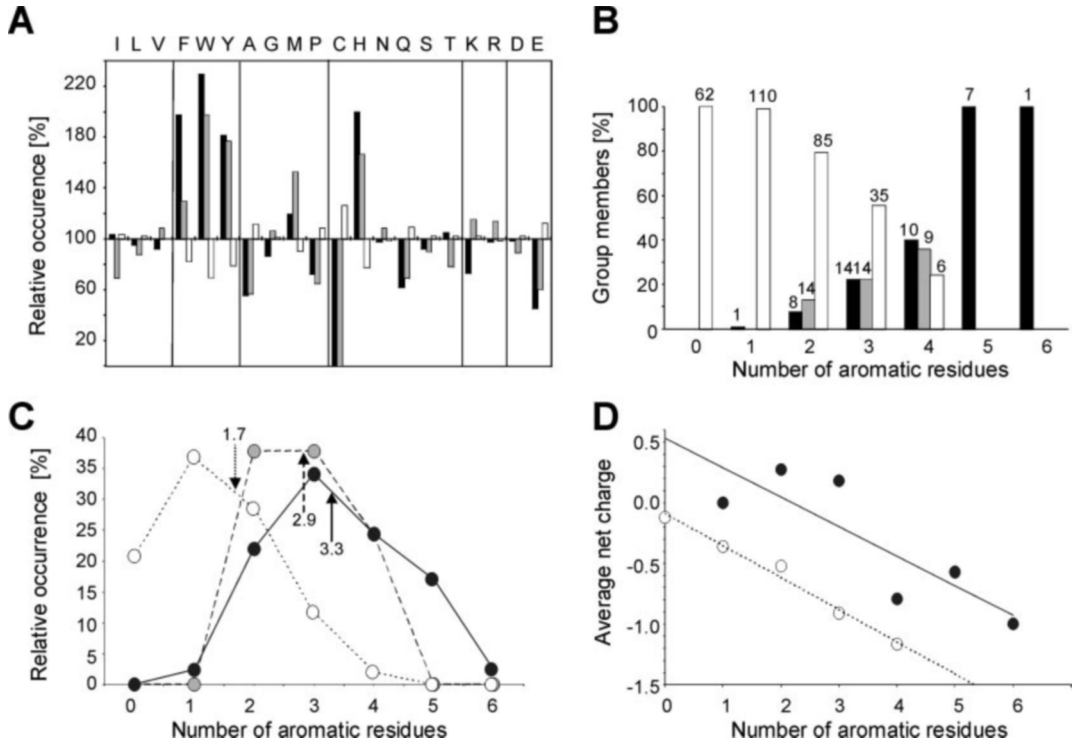

FIG. 2. **Substrate specificity of SurA.** *A*, relative amino acid occurrence for the 376 peptides representing the LamB, OmpF, and OmpA protein sequences. The relative occurrence of each amino acid in peptides with high (*black bars*), low (*gray bars*), and no affinity (*white bars*) for SurA was normalized to its occurrence in the whole peptide libraries (= 100%). Differences between the binding and non-binding peptide populations are not statistically significant for Ile, Leu, Val, Met, Gly, Pro, Asn, Ser, Thr, Lys, Arg, and Asp ($p > 0.05$). Differences between both peptide classes are significant on the 5% level for Cys ($p = 0.041$) and on the 1% level for Phe, Trp, Tyr, His, Ala, Gln, and Glu ($p \leq 0.01$). *B*, classification of good, weak, and non-binders according to their content of aromatic residues (Phe, Trp, and Tyr, including His). Color-coding as in *A*. The actual number of peptides is shown *above each column*. *C*, distribution of good, weak, and non-binding peptides with respect to the number of aromatic residues in the peptides. *White circles*, non-binders; *gray circles*, weak binders; *black circles*, good binders. *Arrows* indicate the average value for each peptide class. *D*, average net charges of binding (*filled circles*, trend line $R^2 = 0.71$) and non-binding (*open circles*; trend line $R^2 = 0.98$) peptides as a function of the number of aromatic residues in the peptides.

TABLE I
Frequency of occurrence of Ar-X-Ar and Ar-Ar motifs in library peptides

	Number of Ar-*X*-Ar[a]	Ar-*X*-Ar/peptide	Number of Ar-Ar	Ar-Ar/peptide
Good binders	29	0.71	31 (35)	0.76 (0.85)
Weak binders	19 (21)	0.51 (0.57)	8 (13)	0.22 (0.32)
Non-binders	27 (28)	0.09 (0.091)	18 (23)	0.06 (0.08)
Total library peptides	75 (78)	0.199 (0.21)	57 (71)	0.15 (0.19)

[a] Ar represents F, W, or Y (values in parenthesis also include H), and *X* represents any amino acid.

TABLE II
Frequency of occurrence of aromatic amino acids and of aromatic motifs in E. coli cell surface proteins

Proteins (35 integral OMPs and 118 periplasmic proteins) were taken from annotations available at the *E. coli* Cell Envelope Protein Data Collection (www.cf.ac.uk/biosi/staff/ehrmann/tools/ecce/ecce.htm) excluding hypothetical proteins.

Protein(s)	Occurrence of Ar[a]	Ar-*X*-Ar/100 amino acids				Ar-Ar/100 amino acids			
		Observed	Expected[b]	Variance	*p* value	Observed	Expected[c]	Variance	*p* value
	%								
Integral OMPs	12.12	1.70	1.44	0.908	0.105	1.11	1.45	0.365	0.0006
Periplasmic proteins	7.89	0.54	0.61	0.252	0.13	0.72	0.62	0.301	0.048
MalE	10.1	1.77	1.00			1.01	1.01		

[a] Ar represents F, W, or Y, and *X* represents any amino acid.
[b] Calculated from the frequency of occurrence of aromatic amino acids as $98 \times (\text{freq. (Ar)})^2$ (15).
[c] Calculated from the frequency of occurrence of aromatic amino acids as $99 \times (\text{freq. (Ar)})^2$.

complete binding pattern may comprise up to eleven residues. Although even- and odd-numbered spacing was observed between an Ar-Ar motif and its flanking aromatic residue (Ar-Ar-X_n-Ar, with $n = 2$–7), the spacing between Ar-*X*-Ar motifs and the flanking aromatic residues in most cases was odd-numbered (Ar-*X*-Ar-X_n-Ar, with $n = 3$, 5, and 7), suggesting that the orientation of the aromatic side chains may be important for binding.

Non-binding Peptides Containing Ar-X-Ar or Ar-Ar Motifs—Forty-two library peptides are classified as non-binders even though they contain Ar-*X*-Ar or Ar-Ar motifs (14.1% of non-binders). With relative spot intensities of 35–39% five of these peptides fell just below the arbitrary threshold set for low affinity binders (40%). For most other peptides one or several of the following features applied: (i) the aromatic motifs or the flanking aromatic residues reside at the C terminus of the

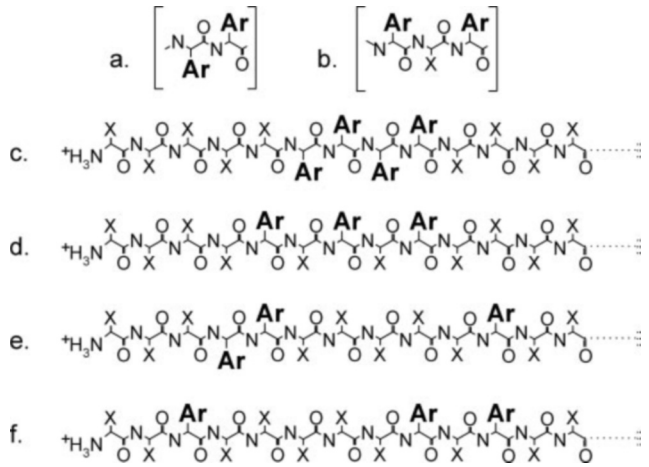

FIG. 3. **Patterns of aromatic amino acids in SurA-binding peptides.** Simplified illustration of linear peptide 13-mers C-terminally coupled via a linker to a cellulose-membrane (*dashed lines*). Two sequences Ar-Ar (*a*) and Ar-*X*-Ar (*b*) recur as basic sequence elements in preferred SurA-binding patterns (with Ar = *F*, *W*, *Y*, or *H*, and *X* = any other residue). They form clusters of aromatic residues arranged in a row (*c*) or in every second position (*d*; no His was observed in these patterns) or are combined with additional flanking aromatic residue(s) located two to seven residues from the Ar-Ar or Ar-*X*-Ar motifs (*e* and *f*). *Odd-numbered spacing* was preferred between Ar-*X*-Ar motifs and the flanking aromatic residue (*f*).

peptides and thus may be located too close to the cellulose-membrane surface to be accessible to SurA (12 peptides, including three peptides presenting the C-terminal Ar-Ar and Ar-*X*-Ar motifs of LamB and OmpF, respectively; see Fig. 4). At least for four peptides this possibility is very likely to apply, because their overlapping peptides bound to SurA. (ii) The peptides contain an Ar-*X*-Ar or Ar-Ar motif but no additional, flanking aromatic residue and thus no complete aromatic pattern (16 peptides). However, six library peptides bound to SurA despite carrying merely a basic aromatic motif, interestingly in five cases an Ar-*X*-Ar motif. Thus, the Ar-*X*-Ar motif, which is also part of the consensus binding pattern recently proposed for SurA (15), may closely match with the required binding criteria but is not generally sufficient for binding. (iii) The peptides contain complete aromatic patterns, but the ratio between negatively charged and aromatic amino acids in the peptides is $\geq$0.67 (15 peptides), again suggesting that negative charge may adversely affect binding (see above and Fig. 2*D*).

Aromatic Side Chains in SurA-binding Peptides Have Preferred Orientations—Our analysis of the frequency of occurrence of Ar-Ar and Ar-*X*-Ar motifs in known integral OMPs and in periplasmic proteins (Table II) revealed that the periplasmic maltose-binding protein MalE, which is no substrate of SurA (7), contains just as many Ar-*X*-Ar motifs per 100 amino acids as integral OMPs and at higher than random frequency. This finding suggests that OMP-characteristic features in addition to patterns of aromatic residues contribute to the capability of SurA to recognize OMPs selectively.

One feature that distinguishes integral OMPs from most periplasmic proteins is their β-barrel structure and thus their markedly high content of β-strand secondary structure. An extended β-strand would place the side chains of at least two aromatic pattern residues (Fig. 3) on one face and an additional aromatic side chain on either the same or the opposite face of the strand. To test whether this also applies to the SurA-binding peptides, we predicted the secondary structures of all library peptides using the PSIpred V2.3 program (16). The analysis revealed that peptides with segments of predicted β-strand secondary structure are slightly enriched among the good and weak SurA-binders, whereas peptides with α-helical

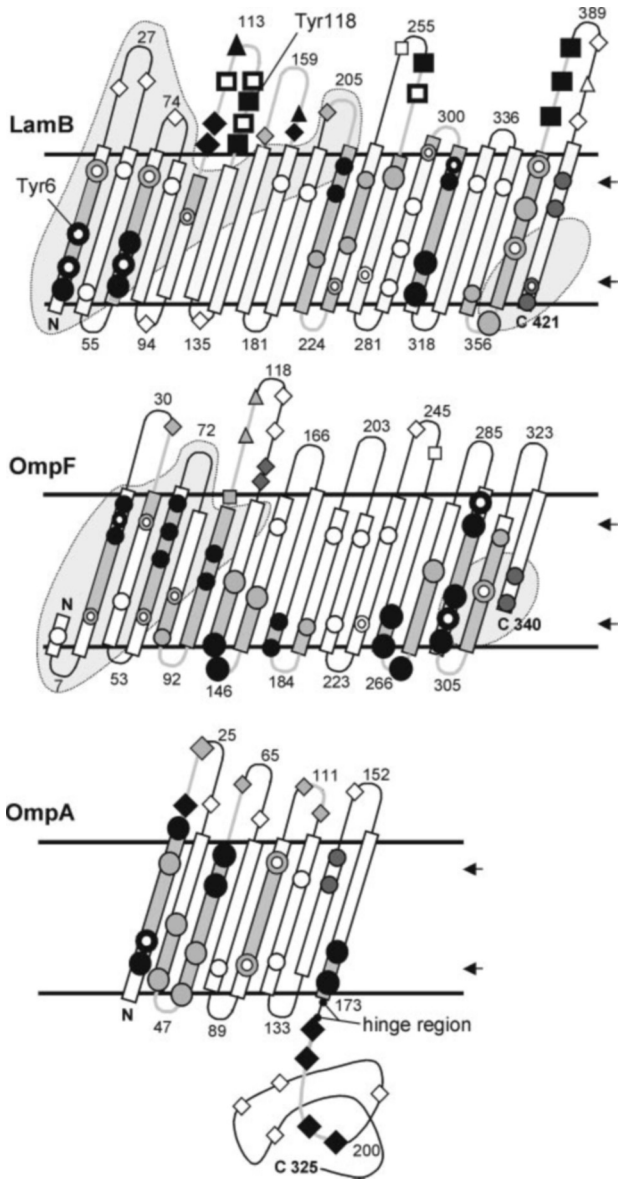

FIG. 4. **Localization of aromatic residues occurring in SurA-binding peptides within the structures of LamB, OmpF, and OmpA.** The topology of the proteins is shown viewed from the outside of the unrolled, antiparallel β-barrels. Amino acid positions are indicated. Aromatic residues are shown as *circles* if their side chains stick out of, or as *doughnuts* if their side chains face into the β-barrel. Aromatic residues in loops and turns are shown as *diamonds*. They are shown as *triangles* if they locate to a loop α-helix, or as *squares* and *frames* if they reside in loop β-strands facing one or the other side of the strand, respectively. Aromatic residues occurring in SurA-binding peptides are colored *light gray* or are highlighted in *black*, if they form aromatic motifs (Ar-*X*-Ar and Ar-Ar). They are connected with a *light gray line* if they form a putative binding site (in most cases a complete aromatic pattern as shown in Fig. 3) identified in one or in overlapping SurA-binding peptides. Aromatic motifs that were not found in SurA-binding peptides are shown as *small dark gray circles*. *Large symbols* emphasize aromatic residues occurring in good SurA-binding peptides. Trimer subunit interface regions are *shaded*, and *arrows* indicate the approximate position of the "aromatic belts."

elements are disfavored at least in the class of good binders (Table III). Strikingly, according to HelicalWheel predictions, in all but one of the binders with predicted α-helical elements at least two aromatic side chains face the same or opposite sides of the helix (data not shown).

Altogether these data imply that not only patterns of aromatic residues but also the orientation of their aromatic side chains is important for peptide binding by SurA. From the

TABLE III
Predicted secondary structure of library peptides
The secondary structure of the library peptides was predicted using the PsiPred version 2.3 program (16).

	CE[a]	CH	C
		%	
Good binders	56.1	4.9	39.0
Weak binders	56.7	13.5	29.7
Non-binders	31.5	15.8	52.7
Total library peptides	36.7	14.4	48.9

[a] C, random coil; E, β-strand; and H, α-helical.

observed aromatic patterns (Fig. 3) and the predicted secondary structures we conclude that SurA prefers peptides in which at least two aromatic side chains are placed on the same face of the peptide at maximally seven amino acids distance (this applies for 92% of all good SurA-binding peptides) and a third aromatic side chain is placed on either the same or the opposite face of the peptide. This requirement would also explain the capability of SurA to selectively recognize OMPs: in integral OMPs most aromatic patterns coincide with β-strands or β-turns (Fig. 4), allowing the above preferred orientation of their side chains, whereas four of five aromatic patterns in MalE coincide with α-helices in the final structure (data not shown).

Localization of SurA-bound Aromatic Patterns within the Native OMP Structures—In integral OMPs aromatic amino acids form two girdles, the "aromatic belts," around the β-barrels close to the bilayer surfaces at about 10 Å from the membrane center (17). In the maltose-specific LamB porin they furthermore constitute the "greasy slide," a contiguous row of aromatic amino acids that runs down the inner channel wall and mediates the transport of maltose (18). To examine whether these aromatic residues contribute to SurA-binding, we have determined the localization of all aromatic residues and aromatic patterns found in SurA-binding peptides within the known LamB, OmpF, and OmpA structures (Fig. 4).

Of all Ar-Ar and Ar-*X*-Ar motifs present in the three proteins only five were not found in SurA-binding peptides. These comprise the two C-terminal motifs in LamB, the C-terminal motif in OmpF, an Ar-*X*-Ar motif at the end of strand 7 in OmpA, and an Ar-Ar motif at the end of loop 3 in OmpF. With the exception of the latter motif, all these motifs or their flanking aromatic residues reside at the C termini of the respective library peptides and thus, as discussed above, may not have been accessible to SurA in the peptide scan. However, the C-terminal peptide of OmpF contains no complete aromatic pattern but only the C-terminal Ar-*X*-Ar motif, suggesting that even in solution it may not or only weakly bind to SurA. Indeed, a peptide with an OmpF-like C-terminal sequence (DNRDGN-VYQF; underlined amino acids are identical to the C-terminal OmpF sequence) has been shown by isothermal titration calorimetry to interact only very weakly with SurA (15). Finally, the Ar-Ar motif residing at the end of loop 3 in OmpF is directly preceded by two negatively charged amino acids, and the respective library peptides all have a negatively charged/aromatic amino acid ratio ≥ 0.67 (see above).

In the OMP structures the aromatic patterns of SurA-binding peptides match with transmembrane β-strands, and internal turns as well as external loops, but without obvious regularity. In LamB, half of the SurA-bound aromatic patterns reside in external loops (L3, L4, L6, and L9), the other half in transmembrane strands (S1, S3, S10, S11, S14, and S17), whereas in OmpF all SurA-bound aromatic patterns reside in transmembrane strands and internal turns. Furthermore, greasy slide or aromatic belt residues are not *a priori* targets for SurA-binding but are bound only as part of aromatic motifs or patterns. Residues of both aromatic belts however, constitute the binding patterns in strands S1, S3, S10, and S14 of LamB, in strands S1, S3, S5, and S14 of OmpF, and in strands S1 and S3 of OmpA, suggesting that these strands may be bound completely by SurA. Interestingly, strands S1 and S3 are part of the subunit interface in the LamB and OmpF trimers (Fig. 4). In addition, all three proteins contain regions without or with only few putative SurA-binding sites. In the LamB and OmpF trimers these regions constitute a substantial portion of the membrane-exposed surface (OmpF, strands S7 to S11) or are only partially buried in the subunit interface (LamB, strands S4 to S9). In OmpA this region comprises the C-terminal half of the barrel and the periplasmic domain. Only the Ar-*X*-Ar motif terminating the β-barrel, and two aromatic motifs that form a binding pattern in the periplasmic domain immediately downstream the proline-rich hinge region, were found in SurA-binding peptides (peptides 61–62 and 71–73; Fig. 1).

Strikingly, loop L3 in LamB, which in all trimeric porins folds back into the barrel to form a constriction of the pore about halfway through the membrane instead of being exposed at the cell surface, is completely covered with two overlapping patterns of aromatic residues both of which were found in peptides with highest affinity to SurA (peptides 44–48, Fig. 1). In this context, it should be mentioned that the Ar-Ar-Ar-Ar pattern in this loop, as well as the SurA-bound aromatic motifs in the LamB loops L6 and L9, match with loop β-strands. Thus, the above postulated preferred orientation of the aromatic side chains also holds for these loop-localized putative SurA-binding sites.

Analysis of SurA-peptide Interactions by Surface Plasmon Resonance—To verify the observed relative binding affinities and to determine the K_D of peptide binding by SurA we studied the interaction of SurA with a good and with a non-binding peptide by surface plasmon resonance (SPR). To allow direct comparison, both peptides were chosen from the LamB library (Fig. 1): the good binder #46 (DVHMIDFYYWDIS = pepH) and the non-binder #71 (TLELGVDYGRANL = pepN), respectively. Both peptides were synthesized and coupled to the surface of separate cells of a C1 BiaCore chip (10.3 fmol of pepH; 15.8 fmol of pepN), and the cells were injected with various concentrations of the SurAN+I-Ct protein. Because wild-type SurA behaved less well in these experiments, the main analysis was performed with SurAN+I-Ct.

The net binding of SurAN+I-Ct with pepH was calculated from the gross binding data as described under "Materials and Methods" (Fig. 5). With increasing concentrations of SurAN+I-Ct, the protein associates faster with pepH but dissociates slower from the peptide, suggesting that SurAN+I-Ct binds to pepH with significantly higher affinity than to pepN. Essentially the same results were obtained in independent experiments and with different batches of SurAN+I-Ct protein. To calculate the equilibrium dissociation constant K_D, the data have been fit with a theoretical curve assuming a one-to-one binding model, because this model fitted the data best. From two independent series of measurements, a K_D of 1.6–2.7 μM was determined, which is in good accordance with the K_D of 1–14 μM recently determined for the interaction of SurA with a 7-mer peptide by isothermal titration calorimetry (15).

SurA Is Capable of Targeting to the Outer Membrane—The only clearly documented role of SurA in porin biogenesis thus far relates to an early step, the conversion of unfolded to folded monomer (5). To address the possibility that SurA has additional functions in earlier or later steps of the maturation pathway, we re-examined the cellular location of SurA, particularly asking whether SurA targets to the inner or outer mem-

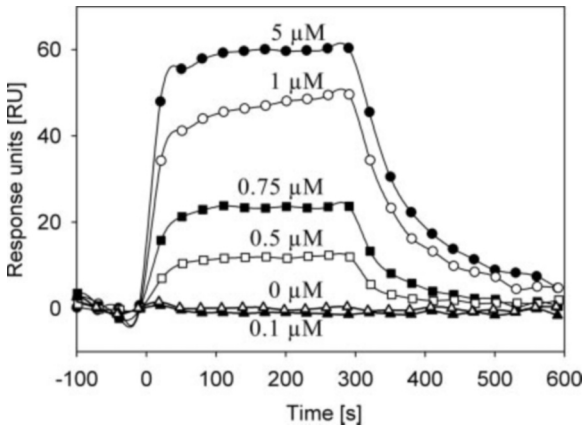

FIG. 5. **Interaction of SurAN+I-Ct with a good binding peptide determined by SPR.** The interaction of SurAN+I-Ct with the good binder pepH (LamB#46, DVHMIDFYYWDIS) and the non-binder pepN (LamB#71, TLELGVDYGRANL) was analyzed at the protein concentrations indicated and the net binding of SurAN+I-Ct to pepH determined as described under "Materials and Methods." The protein was injected at time point zero.

branes. To this end, crude membranes were prepared from wild-type cells and subjected to flotation gradient centrifugation. The inner and outer membranes and the SurA protein were localized in the gradient fractions by immunoblotting using polyclonal antisera directed against the inner membrane protein MalF, the outer membrane protein OmpA, and SurA, respectively (Fig. 6). SurA was present in the main outer membrane fractions (fractions 12 and 13) but not in the main inner membrane fractions (fractions 9 and 10). In contrast, the cytoplasmic protein Hsc66 was not detectable in any of the 18 gradient fractions (data not shown), making a contamination of the membrane fractions with soluble extract unlikely. Moreover, when purified SurA protein was subjected to flotation gradient centrifugation, the protein was absent from membrane fractions but exclusively fractionated in the two bottom fractions (17 and 18), corresponding to soluble and insoluble proteins (19) (data not shown). These data demonstrate that SurA is capable of associating with the outer membrane.

DISCUSSION

Recent studies suggest that the periplasmic chaperone and PPIase SurA may be specialized for the biogenesis of OMPs (7). Its only demonstrated role in this folding pathway thus far, however, is promoting the formation of a native-like folded porin monomer (5). We have investigated the molecular basis for substrate recognition by SurA and have asked whether SurA possibly works at additional steps in porin maturation.

Substrate Binding Specificity of SurA—To analyze the substrate specificity of SurA, we have screened cellulose-bound peptide libraries representing three of its biologically relevant substrates for SurA-binding. We verified by SPR that a peptide that was identified as a good binder in the peptide scans binds to SurA with significantly higher affinity than a peptide that was identified as a non-binder. Our studies show that SurA has strong preference for peptides enriched in aromatic residues, specifically for peptides in which the aromatic residues are arranged in specific patterns with two sequences, Ar-*X*-Ar and Ar-Ar, as the basic sequence elements (Fig. 3). Furthermore, binding of SurA to the library peptides was independent of the presence of prolyl residues, and the SurAN+I-Ct protein, which lacks the active PPIase domain II, bound to the same set of library peptides. Taken together, these findings demonstrate that the observed binding specificity of SurA mainly, if not completely, relates to its chaperone function, which is provided by the N- and C-terminal regions of SurA (7).

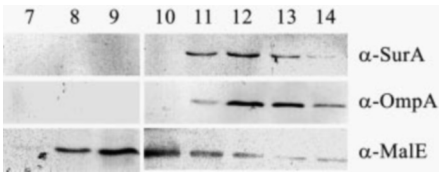

FIG. 6. **Localization of SurA in membrane fractions.** Membranes were prepared from wild-type cells and subjected to sucrose density centrifugation. From two gradients, fractions were collected from the *top*, precipitated with trichloroacetic acid, and analyzed for OmpA, MalF, or SurA, respectively, by Western blotting. Fraction numbers are indicated. The displacement of the main SurA (*11* and *12*) and OmpA fractions (*12* and *13*) can be ascribed to slight differences in the fractionation of the two gradients.

Except for the Ar-*X*-Ar motif, the aromatic SurA-binding patterns identified in our screen do not fit with the recently proposed aromatic-polar-aromatic-nonpolar-proline consensus binding pattern, which was derived from SurA-binding peptides selected from a phage display library of random heptameric peptides (15). Peptides containing this consensus motif were suggested to be mimics of natural substrates, because they bound SurA competitively and with affinities comparable to unfolded OMPs (20). Yet, sequences completely fitting this consensus pattern are rare in integral OMPs and do not even exist in the natural SurA substrates used in our screen. Even the less restrictive sequence Ar-polar-Ar seems not generally sufficient for binding, because only some but not all of our library peptides containing this tripeptide motif, but no complete aromatic pattern, bound to SurA. However, in 88% of all SurA-binding peptides with Ar-*X*-Ar motifs the second residue was polar, confirming a preference for polar residues in this position. Finally, our data clearly demonstrate that proline is not required for peptide binding by SurA and only two overlapping peptides carried both an Ar-*X*-Ar motif and a proline at the proposed consensus position. These differences most likely can be ascribed to the different peptide lengths and the different amino acid compositions of the libraries used in the two alternative approaches. In the phage display experiment, binders were selected from short peptides in which proline occurs about twice as frequently as aromatic amino acids (13.1 and 7.9%, respectively; library Ph.D.-7, New England Biolabs), suggesting that finding proline in the binding peptides could be due simply to a statistical overrepresentation in the library. In contrast, in the LamB, OmpF, and OmpA protein peptide libraries, aromatic amino acids are more frequent than proline (12.3 and 2.86% respectively). However, one possibility that needs to be considered is that SurA might interact with multiple peptides at multiple binding sites. This would also explain why so far we were unable to identify a defined substrate-binding site in SurA, using various approaches.[2] Multiple binding sites with different, perhaps overlapping, substrate specificities would allow SurA to bind various subsets of peptides with different affinities, some of which may not have been detected in our screen.

Integral OMPs are characterized by a high content in aromatic amino acids and, as recently shown (15), are enriched in Ar-*X*-Ar motifs. Thus, the observed substrate specificity of SurA for peptides with specific patterns of aromatic residues directly reflects an OMP-characteristic feature. However, it does not adequately explain the selective interaction of SurA with OMPs. The maltose-binding protein MalE, which binds to SurA ~50-fold less efficiently than outer membrane porins (7), has OMP-like contents of aromatic residues and Ar-*X*-Ar motifs (Table II). This raises the question of how SurA discriminates

[2] G. Hennecke and S. Behrens, unpublished data.

between aromatic motifs exposed by folding intermediates of periplasmic proteins and of integral OMPs. One possible explanation relates to the final native structure of the proteins. In the native MalE protein all but one of the aromatic patterns localize to helical structures while in the OMPs most aromatic patterns align with β-strands (Fig. 4), which places the aromatic side chains along the two opposite faces of the strands. Our finding that SurA preferentially binds to peptides with predicted β-strand secondary structure indicates that such an orientation of the aromatic side chains may be preferred for binding. Consistent herewith, Glu and Pro, the two amino acids with the strongest potential to break β-regions (21), are disfavored in SurA-binding peptides (which furthermore is reflected in the finding that negative charge adversely affects binding). Moreover, in agreement with our data the somatostatin peptide AGSKN**FFW**KT**F**TSS, which has recently been shown to bind to an N-terminal fragment of SurA (8), not only contains a complete aromatic pattern (bold) without proline but also has a predicted β-strand secondary structure (data not shown). However, 39% of the good and 29% of the weak SurA-binding peptides, respectively, were predicted to have a random coil secondary structure (Table III). These peptides might still adopt the required orientation of their aromatic side chains upon binding to SurA by an induced fit-like mechanism.

We therefore postulate that a prerequisite for selective substrate recognition by SurA is the proper orientation of aromatic side chains along two faces of a peptide: at least two aromatic side chains on one and at best a third on either the same or the opposite face of the peptide. This requirement is best accomplished by integral OMPs, in which preferred aromatic patterns coincide more frequently with β-strand secondary structure than in other proteins.

Localization of Putative SurA-binding Sites in the Native OMP Structures—Consistent with the distribution of aromatic amino acids in OMPs (17), most putative SurA-binding sites localize to the transmembrane regions of the LamB, OmpF, and OmpA structures, including the inwardly folded constriction loops L3 (Fig. 4). Thus, just as in soluble proteins, where chaperone binding sites are mostly buried in the central core of the native protein structures (2), in the native outer membrane-embedded proteins the majority of binding sites would be buried and inaccessible to SurA, at the membrane interface or in the central pore. In accord with this, it has recently been shown that SurA does not interact with native OMPs (20). Interestingly, in all three proteins, strands S1 and S3, which in LamB and OmpF are part of the trimer subunit interface, are putative targets of SurA. In contrast, only very few putative SurA-binding sites are localized in the regions of the β-barrels that constitute the exterior surface of the native trimers. This suggests that regions in the porins that mediate subunit contacts are protected by SurA from incorrect and premature interaction during folding, whereas regions that interface with the lipid bilayer remain mostly unprotected. In case of the monomeric OmpA protein, the Ar-*X*-Ar motif terminating its β-barrel domain and the aromatic pattern located in its periplasmic domain immediately downstream of the proline-rich hinge region were found in high affinity SurA-binding peptides, in addition to strands 1 and 3. Possibly, binding of SurA to these sites allows both domains to fold unhampered by each other.

Most eye-catching about LamB is the even complete coverage of its constriction loop L3 with two overlapping patterns of aromatic residues, both of which were found in peptides with highest relative affinity for SurA. This apparent particularly tight binding might relate to the functional importance of L3 in the native protein. Residue Tyr-118, which is part of one of the two aromatic patterns in L3 (Fig. 4), mainly constricts the LamB channel and directly interacts with the maltodextrins during transport (18). Moreover, in the final structure, Tyr-118 locates in close vicinity to the greasy slide residue Tyr-6 (Fig. 4), which also was found in a good SurA-binder. Finally, binding of SurA to the L3 β-strand, which harbors the second aromatic pattern, may prevent its interaction with the membrane or with the folding barrel β-strands, allowing the loop to fold back into the barrel pore. Further studies will have to address which of the putative SurA-binding sites suggested here are truly the main targets of SurA binding in the full-length porins and which are relevant for correct porin folding.

Role of SurA in Porin Maturation—The only documented role of SurA in porin maturation thus far relates to the conversion of unfolded to native-like folded monomers, an early step in the maturation pathway (5, 7). Another periplasmic chaperone, the small Skp protein, is likely to collaborate with SurA, because the combined deletion of both proteins causes synthetic lethality (7, 22). Both proteins may act in either the same or, as genetic evidence suggests (22), in a parallel folding pathway. Skp has been shown to interact with early OMP folding intermediates at the periplasmic side of the inner membrane and to be required for the release and the maintenance of soluble periplasmic intermediates (23, 24). Whether Skp also acts in late, outer membrane-associated steps of OMP maturation is unknown. Here, we have shown that a fraction of the SurA molecules present in the cell associates with the outer but not with the inner membrane, suggesting that SurA acts in both early periplasmic and late outer membrane-associated steps, but not as early as Skp, in porin maturation. Interestingly, monomeric porin assembly intermediates have also been found associated with the outer membrane (25, 26). Thus, it seems likely that SurA interacts with early porin folding intermediates in the periplasm and that the resulting complex associates with the outer membrane either before or after formation of the native-like folded monomer. The interaction with the outer membrane may be mediated either by the substrate or by SurA itself. Kinetic *in vitro* folding experiments suggest that OMPs fold into the lipid bilayer from membrane-associated folding intermediates but that a closed β-barrel is only formed after insertion (27). In this model the role of SurA at the outer membrane could be to keep porins in a native-like, insertion- and assembly-competent state. However, it is still unclear whether porins trimerize before or after their insertion into the outer membrane. Previous data suggest that trimerization takes place at or in the outer membrane (28, 29), or that trimerization is initiated in the periplasm by formation of a central core involving the subunit interface regions of the final trimer (30). Our data indicate that many putative SurA-binding sites are located in the subunit interface regions of the trimeric porins. Therefore, to allow their trimerization before insertion into the outer membrane, SurA would first have to be released, which raises the question how the energy required for this process is provided unless perhaps from the trimerization process itself. However, the association of SurA with the outer membrane still suggests that these events take place rather at the outer membrane than in the free periplasmic space. Alternatively, the existence of many putative SurA-binding sites in the subunit interface regions, but only few such sites in the membrane interface regions of the porins, would be consistent with a model in which SurA prevents porins from premature trimerization but allows their insertion into the outer membrane in a process that was suggested to be spontaneous and thermodynamically driven (25). In this model, trimerization could take place either at the outer membrane during insertion or in the outer membrane after insertion. Binding of SurA to

the LamB loop L3 (see above) would even allow the barrel to fold into the membrane in the correct orientation, at the same time placing the loop in the central pore. Moreover, thermodynamically driven insertion of the OMPs might concomitantly provide the energy required for the release of SurA, providing one possible explanation of how SurA promotes folding in the absence of known sources of energy.

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