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Interaction of the Periplasmic Peptidylprolyl-cis-trans Isomerase SurA with Model Peptides

THE N-TERMINAL REGION OF SurA IS ESSENTIAL AND SUFFICIENT FOR PEPTIDE BINDING

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One of the rate-limiting steps in protein folding has been shown to be the cis-trans isomerization of proline residues, which is catalyzed by a range of peptidylprolyl cis-trans isomerases. To characterize the interaction between model peptides and the periplasmic peptidylprolyl cis-trans isomerase SurA from E. coli, we employed a chemical cross-linking strategy that has been used previously to elucidate the interaction of substrates with other folding catalysts. The interaction between purified SurA and model peptides was significant in that it showed saturation and was abolished by denaturation of SurA; however the interaction was independent of the presence of proline residues in the model peptides. From results obtained by limited proteolysis we conclude that an N-terminal fragment of SurA, comprising 150 amino acids that do not contain the active sites involved in the peptidylprolyl cis-trans isomerization, is essential for the binding of peptides by SurA. This was confirmed by probing the interaction of the model peptide with the recombinant N-terminal fragment, expressed in Escherichia coli. Hence we propose that, similar to protein disulfide isomerase and other folding catalysts, SurA exhibits a modular architecture composed of a substrate binding domain and distinct catalytically active domains.

During the past three decades processes involved in the misfolding, aggregation, and degradation of proteins have attracted increasing interest. However, our understanding of the molecular mechanisms of protein folding and hence protein misfolding is far from being complete. Folding catalysts have been identified that facilitate specific isomerization steps that would otherwise limit the rate of polypeptide folding. Protein disulfide isomerase (PDI) catalyzes the formation, isomerization, and reduction of disulfide bonds (for review see Ref. 1), and peptidylprolyl cis-trans isomerase (PPIases) accelerate the rate of conformational interconversions around proline residues in polypeptides in vitro (2, 3). PPIases are ubiquitous in prokaryotes and eukaryotes (4, 5); however, their biological significance remains elusive. Three classes of PPIases have been identified so far: the cyclophilins, the FK506-binding proteins, and PPIases that have sequence homology with the catalytic domain of parvulin (3).

Some PPIases are thought to have separate substrate binding domains structurally distinct from their catalytic domains (6), and hence it has been postulated that these folding catalysts also can exert chaperone-like activities. Trigger factor, a bacterial cytoplasmic PPIase, was found to have two domains at the N and C terminus that did not influence catalytic activity but are essential for substrate binding (6). FkpA, a periplasmic PPIase, was found to assist the folding of single-chain antibody fragments (scFv) independent of its PPIase activity. It appeared that FkpA was able to interact with early folding intermediates to prevent aggregation and also interact with unfolded or partially aggregated proteins to prevent irreversible aggregation and facilitate their reactivation (7, 8). Interestingly, FkpA had this chaperone effect also on scFv antibody fragments that did not contain any proline residues. In contrast, the coexpression of the periplasmic PPIase SurA produced no increase in the functional scFv fragment level in the periplasm (7, 8).

SurA was originally isolated as a protein product of stationary phase survival genes of Escherichia coli (9–11). Null mutants of SurA resulted in loss of cell viability after 3–5 days of incubation, leading to the conclusion that SurA was essential for stationary phase survival (11). Sequence analysis revealed that SurA contained two domains with strong homology to parvulin, and from this finding it was inferred that SurA has PPIase activity (10, 12, 13). Further functional studies were carried out, and it was concluded that SurA was important in maintaining outer membrane integrity probably through the binding of peptidoglycans (10, 11). It was also found that those null mutants, or mutants with decreased amounts of SurA in the periplasm resulted in a decrease in the folding of the maltose-inducible porin Lam B (11). This led to the conclusion that SurA was important not only in maintaining outer membrane integrity but also in catalyzing the folding of other outer membrane proteins (12).

To address the question of the nature of the interaction between SurA and its substrates we used chemical cross-linkers, powerful tools to study interactions between proteins, which can be applied to proteins available in small amounts even in crude cell extracts (14, 15). Here we report the results of our recent studies using chemical cross-linking of purified SurA and its fragments to model peptides, which mimic substrates.

EXPERIMENTAL PROCEDURES

“Scrambled” ribonuclease A, the homobifunctional cross-linking reagent diisuccinimidyl glutarate (DSG), Juglon, and all other chemicals...
were obtained from Sigma. Cyclosporin A and FK506 were from Calbiochem. 125I-Bolton-Hunter labeling reagent, enhanced chemiluminescence reagent, and x-ray films were purchased from Amersham Pharmacia Biotech. N-Hydroxysuccinimidy biotin and streptavidin-horseradish peroxidase conjugates were from Pierce. The somatostatin derivative without cysteine residues (Δ-somatostatin, Ala-Gly-Ser-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Thr-Ser-Ser) and peptide P (Phe-Thr-Glu-Glu-Thr-Ala-Pro-Lys-Ile-Phe-Gly-Glu-Ile-Lys) were synthesized as described previously for other peptides (16).

Methods—125I-Bolton-Hunter labeling of Δ-somatostatin was performed as recommended by the manufacturer. Scrambled ribonuclease A was biotinylated with N-hydroxysuccinimidy biotin as described by the manufacturer. Bovine liver PDI was purified as described previously (17).

Cloning of SurA and Fragments and Purification of Full-length SurA—The gene encoding SurA was amplified from the E. coli genome by polymerase chain reaction using the following primers. Forward primer, 5'TTAAAAATATGCCCCAGGTATGCTGAAATGTCTG-3', reverse primer, 5'TTTTTTTCTCTCGAGCTATTAGTTGCTCAGGATTTTAACTAGTGGC-3'.

For the amplification of the N-terminal fragment (Ala51-Arg123) the following primers were used. Forward primer, 5'TATAATACATATGGCCCCAGGTATGCTGAAATGTCTG-3'; reverse primer, 5'TATAATAATGCTGATTAGCTGACTTATTAGTTGCTCAGGATTTTAACTAGTGGC-3'.

For the amplification of the fragment without the N terminus (Asp169—An499) the following primers were used. Forward primer, 5'TTAATTATGAGCGCCGACCATGAACTGAGGTGTCTGTCGC-3'; reverse primer, 5'TTAATTATGATGACTATTAGTGGTCAGGATACTGTTAACTAGTC-GCGCCGCTGGC-3'. These primers allowed the insertion of a NdeI site at the N terminus and a SalI site at the C terminus. The primers complementary to the 3'terminal including a stop codon. The inserts were cloned between the NdeI and SalI sites of pLWRP51, a modified pET23d vector, which contained an insert coding for an initiating methionine residue followed by a hexa-histidine tag.2

Gene Expression—Protein production was carried out in E. coli strain BL21(DE3) carrying the pLysS plasmid to control leak-through expression and to allow subsequent cell lysis by freeze thawing. For the production of full-length SurA and its fragments, E. coli BL21(DE3)-pLysS was transformed with the corresponding plasmid-DNA. The cells were incubated at 37°C in LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Three hours after induction with 1 mg isopropyl-thio-β-galactopyranoside the cells were harvested by centrifugation.

For the purification of full-length SurA, the cell pellet was suspended in buffer A (20 mM sodium phosphate, pH 7.3), and DNase to a final concentration of 10 μg/ml was added. After freezing and thawing the suspension twice, the lysate was centrifuged at 12000 × g for 30 min. The filter-sterilized supernatant was loaded onto a nickel-nitrilotriacetate acid column (12-ml volume, Qiagen), which, after activation with NiSO4, was equilibrated with buffer A. Unspecifically bound proteins were removed by washing the column with buffer A containing 500 mM NaCl and 50 mM imidazole, followed by buffer A. Recombinant SurA was eluted with buffer A containing 10 mM EDTA. The elution fraction was loaded onto a Resource Q column (6 ml, Amersham Pharmacia Biotech) equilibrated with buffer A and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer A. Fractions containing homogenous SurA were pooled and dialyzed against 10 mM phosphate buffer, pH 7.2.

Fragmentation of SurA—Purified SurA was incubated with chymotrypsin (30 μg/ml) for 30 min at 0°C. The reaction was stopped by the addition of 10 mM PMSF and subsequent incubation for 5 min at 0°C.

Binding of Peptides and Biotinylated Scrambled RNase—After precipitation with trichloroacetic acid, the radiolabeled Δ-somatostatin was dissolved in distilled water. Labeled Δ-somatostatin (approximately 30 μM) or biotinylated scrambled RNase (approximately 50 μM) was added to buffer B (100 mM NaCl, 25 mM KCl, 25 mM sodium phosphate buffer, pH 7.5) containing purified SurA or PDI (approximately 2 mg/ml) or E. coli lysates containing full-length SurA or fragments of it. The samples (10 μl) were incubated for 10 min on ice before cross-linking (14).

Cross-linking—Cross-linking was performed using the homobifunctional cross-linking reagent DSG (18). The samples were supplied with one-fifth volume of cross-linking solution (2.5 mM DSG in buffer B). The reaction was carried out for 60 min at 0°C. Cross-linking was stopped by the addition of SDS-polyacrylamide gel electrophoresis sample buffer (18).

The samples were subjected to electrophoresis in 10, 12.5, or 17.5% SDS-polyacrylamide gels with subsequent autoradiography. For protein sequencing the proteins were electrotransferred onto polyvinylidine difluoride membranes and subjected to N-terminal Edman degradation (492 Protein Sequencer, Applied Biosystems). Biotinylated products were detected using streptavidin-horseradish peroxidase conjugates. The detection was carried out with enhanced chemiluminescence reagent.

RESULTS

Radiolabeled Peptides Can Be Cross-linked to Purified SurA—Chemical cross-linking of radiolabeled peptides has

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2 L. W. Ruddock, unpublished results.
Fig. 2. The binding of Δ-somatostatin to SurA shows saturation and is inhibited in the presence of detergent A, various concentrations of radiolabeled Δ-somatostatin (Δ-SRIF, 9–150 μM) were incubated with purified SurA (0.6 μM) for 10 min at 0 °C prior to cross-linking with DSG. After cross-linking the samples were analyzed on 10% polyacrylamide gels with subsequent autoradiography. B, radiolabeled Δ-somatostatin (9 μM) was incubated with 0.6 μM purified SurA or PDI in the presence of 0.1% (v/v) Triton X-100 with subsequent cross-linking with DSG. After cross-linking the samples were analyzed on 10% polyacrylamide gels with subsequent autoradiography.

been successfully employed to study the interactions between various folding catalysts, specifically members of the protein disulfide isomerase family, and small peptides. Here we extended our studies and asked whether this technique can also be employed to study the interactions between the periplasmic PPlase SurA and peptides. To this aim 125I-Bolton-Hunter-labeled peptide P was cross-linked to purified SurA (Fig. 1A), using the chemical cross-linker DSG. Purified bovine liver PDI served as a positive control whereas a sample without SurA or PDI, supplemented only with buffer, served as a negative control (lanes 5 and 6). In the absence of DSG, no cross-linking products could be detected (lanes 1–4). In the presence of purified SurA a single cross-linking product with an apparent molecular mass of 48 kDa could be observed (lane 10). This indicates that a radiolabeled model peptide containing a proline residue can be cross-linked to purified SurA.

To address the question whether or not the interaction between SurA and peptides depends on the presence of proline residues in the peptide, we used another radiolabeled model peptide, Δ-somatostatin (Δ-SRIF). As with the proline-containing peptide, a cross-linking product could be detected (lane 9), indicating that the interaction of SurA with peptides does not depend on the presence of proline residues in the peptide. From these data we conclude that peptides without proline residues can be cross-linked specifically to purified SurA.

After heat inactivation of purified SurA (5 min at 95 °C) prior to chemical cross-linking, the interaction between Δ-somatostatin and SurA was strongly inhibited (Fig. 1B). Thus, the interaction of radiolabeled Δ-somatostatin with purified SurA depended on its native conformation.

The Interaction of Δ-Somatostatin with SurA Shows Saturation and Requires Hydrophobic Interactions—Chemical cross-linking after increasing the concentration of radiolabeled Δ-somatostatin while keeping the concentration of purified SurA constant (2 μM) revealed that there was saturation of binding at concentrations above 45 μM (Fig. 2A).

To reveal the nature of the interaction between SurA and non-proline-containing peptides, radiolabeled Δ-somatostatin was chemically cross-linked to purified SurA in the absence or presence of Triton X-100. Purified PDI, the interaction of which with peptides is inhibited in the presence of Triton X-100, served as a positive control. As with PDI, the binding of radiolabeled Δ-somatostatin to purified SurA was completely abolished in the presence of Triton X-100 (Fig. 2B). This experiment clearly demonstrates that the interaction of SurA, like PDI, with peptides is detergent-sensitive and presumably relies on hydrophobic interactions.

Model Peptides and Scrambled Ribonuclease A Interact with the Same Peptide Binding Site of SurA—Chemical cross-linking of 10 μM radiolabeled Δ-somatostatin to purified SurA was reduced in the presence of an excess (100 μM) of unlabeled Δ-somatostatin (Δ-SRIF) or peptide P (Fig. 3A). This experiment demonstrates that the interaction between SurA and radiolabeled peptides is not due to an unspecific binding of peptides via the 125I-Bolton-Hunter labeling reagent.

To further investigate whether SurA interacts with misfolded proteins as well as with model peptides, we performed chemical cross-linking of radiolabeled Δ-somatostatin (10 μM) to purified SurA in the presence of an excess of unlabeled scrambled ribonuclease A (scRNase) (100 μM).

Fig. 3A clearly shows that unlabeled scrambled ribonuclease A competed with radiolabeled Δ-somatostatin for the binding to SurA, and hence we conclude that a misfolded protein and a small peptide interact with the same principal binding site in SurA. This was confirmed when biotinylated scrambled ribonuclease A was used as a substrate for SurA. As with radiolabeled peptides, a single cross-linking product could be observed, the intensity of which was strongly reduced in the presence of a 10-fold excess of unlabeled scrambled ribonuclease A (scRNase) (100 μM).

The Interaction between SurA and Radiolabeled Δ-Somatostatin Is Reversible—When an excess of unlabeled Δ-somatostatin (S) was added to purified SurA simultaneously (Fig. 4, lane 4) or before 125I-Bolton-Hunter-labeled Δ-somatostatin (S*) was added (lane 3), the same decrease in the intensities of the cross-linking products could be observed as when unlabeled Δ-somatostatin (S) was added after preincubation with radiolabeled Δ-somatostatin (lane 2). Thus we conclude that the interaction between SurA and somatostatin is reversible.

The N Terminus of SurA Is Essential and Sufficient for Peptide Binding—SurA shows a modular architecture consisting of an N-terminal region with no significant similarities to other E. coli proteins and two parvulin-like domains (Fig. 5B). From the observation that the parvulin-specific inhibitor Juglon (19) had no effect on the binding of radiolabeled Δ-somatostatin (data not shown), we hypothesized that the activity of the parvulin-like domains is not required for peptide binding.
tostatin (S) lane 2 for 5 min at 0°C (*).

**Fig. 5** lane 4

Oble band with an approximate molecular mass of 32 kDa (radiolabeled)
tease activity with PMSF, the products were cross-linked to

When these fragments were probed by cross-linking of radiolabeled \( \Delta \)-somatostatin, we did not detect any significant interaction between them and radiolabeled \( \Delta \)-somatostatin (Fig. 5A, lane 8). Full-length SurA that was not proteolytically cleaved, however, still showed binding of radiolabeled \( \Delta \)-somatostatin indicating that the conditions did not affect cross-linking in general. This was confirmed by using control samples in which SurA was incubated with PMSF alone (lane 6) or PMSF-inactivated chymotrypsin (lane 7) prior to cross-linking.

The N-terminal amino acid sequences of both bands were determined and compared with the sequence of SurA (Fig. 5B). Both fragments contained the two parvulin-like domains but were devoid of the N-terminal 108 and 116 amino acids, respectively. These results indicate very clearly that the N-terminal part of SurA is required for the interaction with radiolabeled \( \Delta \)-somatostatin. To confirm this result, we cloned and expressed the N-terminal fragment of SurA (Ala\(^{21-428}\)) and the fragment containing the parvulin domains (Asp\(^{168-428}\)). The expression of both fragments, as well as full-length SurA (Ala\(^{21-428}\)), resulted in soluble products (data not shown) that were probed for their interaction with radiolabeled \( \Delta \)-somatostatin without purification from the *E. coli* lysates. Fig. 6 shows that radiolabeled \( \Delta \)-somatostatin could be cross-linked to full-length SurA and the N-terminal fragment but did not interact with the C-terminal fragment containing the two parvulin domains. These results clearly indicate that the N-terminal fragment of SurA is essential and sufficient for the interaction with model peptides.

**DISCUSSION**

Although the folding of many proteins can occur spontaneously, the folding of other proteins requires the assistance of folding catalysts and molecular chaperones. Molecular chaperones bind to partially folded intermediates in nascent polypeptide chains or newly synthesized proteins in a reversible process to prevent aggregation and misfolding. Folding catalysts enhance the rate of certain rate-limiting steps, e.g., formation and isomerization of disulfide bonds and the cis-trans isomerization of peptidylprolyl residues, to ensure that efficient folding occurs (1, 3). There is increasing interest in elucidating the underlying principles of protein folding and the mechanisms by which molecular chaperones and folding catalysts interact with their substrates; however, those interactions are still poorly understood. To reveal the nature of the interaction between the purified PP1ase SurA from *E. coli* and its substrates, we employed a cross-linking approach, which we have used previously to determine the interaction between peptides and other folding catalysts (14, 15, 18, 20–22).

**Specificity of the Interaction between Radiolabeled Peptides and SurA**—We found that the 14-amino acid peptide \( \Delta \)-somatostatin, which does not contain proline residues, and peptide P, a proline-containing peptide, after \(^{125}\)I-Bolton-Hunter radio-
labeling can be chemically cross-linked to purified SurA using an homobifunctional cross-linker, specific for the N terminus or the \( \epsilon \)-amino group of lysine residues.

To validate the cross-linking approach and to demonstrate that the interaction between SurA and model peptides was specific we performed various control experiments and compared the results with previous findings obtained for the specific interaction between model peptides and purified PDI (14).

As with purified PDI, the interaction between radiolabeled peptides and SurA was abolished after heat denaturation of purified SurA prior to cross-linking, indicating that the interaction required native SurA.

We found that the interaction between SurA and a model peptide was independent of the presence of a proline residue within the peptide. This result is in line with the findings of Scholz et al. (23), who showed very clearly that the PPIase trigger factor recognizes protein substrates independently of proline residues (23). These observations are not limited to PPIases, and similar results were obtained with purified PDI, which is a potent catalyst of disulfide bond formation and isomerization in the ER. Using chemical cross-linkers we showed previously that PDI interacts with model peptides and that this interaction is independent of the presence of cysteine residues within the model peptide (14). It appears that folding catalysts in general interact with a range of model peptides, even if those peptides do not contain the amino acids the folding catalysts act on.

When radiolabeled \( \Delta \)-somatostatin was cross-linked to a crude cell extract of \( E. \ coli \) overexpressing SurA, cross-linking of the peptides to SurA still could be observed (Fig. 6). This is similar to results obtained when crude cell extracts of \( E. \ coli \) overexpressing PDI were used (15).

The binding of radiolabeled peptides to purified SurA showed
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saturation, and as with purified PDI (14), cross-linking of radiolabeled Δ-somatostatin to purified SurA could be inhibited by the addition of unlabeled Δ-somatostatin. Half-maximal intensity of the cross-linking product was observed at a 1:1 ratio of unlabeled:radiolabeled Δ-somatostatin (data not shown), indicating that the interaction with purified SurA was not due to an unspecific binding of the radiolabeled Δ-somatostatin via the 125I-Bolton-Hunter labeling reagent. The interaction between SurA and Δ-somatostatin prior to cross-linking was found to be reversible, as demonstrated by the addition of unlabeled Δ-somatostatin to SurA, which has been preincubated with radiolabeled Δ-somatostatin. Unlabeled Δ-somatostatin displaced radiolabeled Δ-somatostatin indicating that the interaction between SurA and peptides is highly dynamic, which is very similar to results obtained previously for purified PDI (14).

As with PDI (14) we observed that the interaction between SurA and radiolabeled peptides is sensitive to Triton X-100. Although we cannot completely exclude the possibility that Triton X-100 interferes with the structure of the N-terminal fragment of SurA, this detergent is widely used for the functional recovery of intracellular soluble and membrane-bound proteins, and hence this sensitivity suggests that hydrophobic interactions play an important role in the initial binding of peptides to SurA.

When scrambled ribonuclease A was used in a competition experiment, we found that this inhibited the interaction between radiolabeled Δ-somatostatin and SurA, similar to the inhibition observed with purified PDI (14). We also observed that the interaction between biotinylated scrambled ribonuclease A and SurA could be inhibited by an excess of unlabeled Δ-somatostatin, which indicates an interaction with the same principal binding site in SurA.

Taken together, these data indicate that there is specific binding of Δ-somatostatin and other peptides and that this models the interaction of protein substrates with SurA.

The Peptide Binding Site of SurA Is Different from the Active Site Involved in Peptidylprolyl cis-trans Isomerization—To investigate whether the peptide binding site of SurA resides in the catalytically active site involved in peptidylprolyl cis-trans isomerization, we performed experiments using limited proteolysis. Our results show very clearly that under the chosen conditions the first 108 and 116 amino acids, respectively, of SurA did not show binding activity, indicating that the interaction between SurA and peptides is not essential for peptide binding.

To in—

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