Structural and Functional Characterization of OmpF Porin Mutants Selected for Larger Pore Size

I. CRYSTALLOGRAPHIC ANALYSIS*

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Kuo-Long Lou, Nathalie Saint, Alexej Prilipov, Gabriele Rummel, Spencer A. Benson, Jurg P. Rosenbusch, and Tilman Schirmer‡

From the Departments of Structural Biology and Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and the Department of Microbiology, University of Maryland, College Park, Maryland 20740

OmpF porin is a nonspecific pore protein from the outer membrane of Escherichia coli. Previously, a set of mutants was selected that allow the passage of long maltodextrins that do not translocate through the wild-type pore. Here, we describe the crystal structures of four point mutants and one deletion mutant from this set; their functional characterization is reported in the accompanying paper (Saint, N., Lou, K.-L., Widmer, C., Luckey, M., Schirmer, T., Rosenbusch, J. P. (1996) J. Biol. Chem. 271, 20676–20680). All mutations have a local effect on the structure of the pore constriction and result in a larger pore cross-section. Substitution of each of the three closely packed arginine residues at the pore constriction (Arg-42, Arg-82, and Arg-132) by shorter uncharged residues causes rearrangement of the adjacent basic residues. This demonstrates mutual stabilization of these residues in the wild-type porin. Deletion of six residues from the internal loop (ΔI09–114) results in disorder of seven adjacent residues but does not alter the structure of the β-barrel framework. Thus, the large hollow β-barrel motif can be regarded as an autonomous structure.

OmpF porin is one of the major outer membrane proteins of Escherichia coli (1). The protein forms three large water-filled channels per trimer, allowing the diffusion of small hydrophilic molecules such as nutrients and waste products across the outer bacterial membrane (2–4). Several antibiotics, including β-lactams, use the porin pathway to cross the outer membrane and to find their targets (5).

The three-dimensional structure of the OmpF porin has been solved at 2.4 Å resolution (6). Each monomer consists of a 16-stranded β-barrel that contains the channel. Six loops are exposed at the surface of the cell, and one is involved in subunit contact within the trimer. The longest loop, L3, is bent into the channel at a height corresponding to the center of the membrane, constricting the pore considerably. In this narrow region, a positively charged cluster, formed by basic residues that protrude from the barrel wall near the 3-fold molecular axis, faces two negatively charged acidic side chains located on L3 (see Fig. 1). This establishes a strong electrostatic field parallel to the membrane plane (7). The size and charge distribution of the pore constriction apparently allows the fine-tuning of the channel characteristics. A similar pore architecture and segregation of charge has been found for porins from photosynthetic bacteria (8, 9) that lack sequence homology with OmpF porin. The x-ray structure of maltoporin, a porin specific for translocation of maltodigosaccharides, also exhibits a pore constriction, but there is no charge segregation (10).

The pore properties of porins can be modified drastically by mutations at the pore constriction. By application of pertinent selection pressure, a colicin-resistant mutant with a single-residue replacement in loop L3 has been obtained (11) that resulted in a considerably reduced pore cross-section (12). Benson and co-workers (13) selected OmpF porin mutants that were able to translocate long maltodextrins that do not pass through the wild-type protein. Growth tests, antibiotic sensitivities and rates of [14C]maltose uptake suggested enlargement of the pore size. Later, the x-ray structure of OmpF porin (6) showed that all of the latter mutations had occurred at the pore constriction where, in each case, a specific side chain protruding into the channel was pruned, or part of the internal loop was deleted. Here, we report the crystal structures of five mutant proteins (R42C, R82C, R132P, D113G, and ΔI09–114). In the accompanying paper, the functional changes of the mutant proteins are analyzed (14).

MATERIALS AND METHODS

Sequencing of OmpF Mutants—The following E. coli strains were used: Top10 (Invitrogen); OC 1509, OC 5101, OC 5104, IB 910, and IB 915 (13). The oligonucleotides used were: ompf-0, 5'-CTGAGACA-LATAAAGACACCAACTCTCTCA-3'; ompf-and, 5'-GTGCATTAGAA-CTGGTAAACGATACCCAC-3'; S620, 5'-ACAGTCATGTCGCAATG-3'; and S840, 5'-CGGTGTGGTTATATGC-3'.

The procedures followed standard methods throughout (15). The polymerase chain reaction (PCR)1 was carried out using Expand Long Template PCR System from Boehringer Mannheim according to recommendations of the manufacturer. The conditions used were: 92 °C for 2 min for the first denaturation; subsequently 24 circles at 92 °C for 10 s, at 65 °C for 30 s, and finally at 68 °C for 4 min. DNA sequencing was performed using the T7 Sequencing Kit (Pharmacia Biotech Inc.) according to the instructions of the manufacturer. E. coli cells were grown overnight in 10 ml of 2YT medium to which either 10 μg/ml tetracycline (in the case of OC strains) or 50 μg/ml kanamycin (in the case of IB strains) were added. Cells were pelleted, resuspended in TE medium, and treated twice with phenol/chloroform mixture. The DNA was then precipitated with ethanol and redissolved in water in case of OC strains carrying mutations of the ompF gene in the chromosome. In the case of IB strains, low copy number plasmids were purified with Qiagen 5 columns. For PCR, either 10 ng of chromosomal or 1 ng of plasmid DNA were used. For PCR, ompf-0 and ompf-and oligonucleotides were used to

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1 The abbreviation used is: PCR, polymerase chain reaction.

1GFO, 1GFP, and 1GFQ) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

¶ To whom correspondence should be addressed. Fax: 41-61-267-2109; E-mail: schirmer@abau.unibas.ch.

‡ From the Departments of Structural Biology and Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and the Department of Microbiology, University of Maryland, College Park, Maryland 20740.
amplify the complete ompF gene. After digestion of the PCR-generated fragments (1208 base pairs) with restriction enzymes HindII and BglII, the resulting fragments (900 base pairs) were ligated with pGEM-7zf(-) (Promega) cleaved with BamHI and Smal. After transformation into E. coli Top10, all colonies from each plate were removed, and the plasmid DNA was purified. The fraction of blue colonies was about 30% for all plates. This mixture of plasmids either containing or lacking the insertion were sequenced with ompF-specific oligonucleotides S620 and S640 to avoid possible errors caused by Taq-polymerase. The resulting sequence changes are summarized in Table I. Insertion were sequenced with a French press, and envelopes were recovered by centrifugation. Purification was performed by ion-exchange chromatography (DEAE-cellulose, Merck), followed by chromatofocusing (PBE94, Pharmacia) and finally by gel filtration on Sephadex G-150 (Pharmacia). The proteins. Further purification was performed by ion-exchange chromatography (DEAE-cellulose, Merck), followed by chromatofocusing (PBE94, Pharmacia) and finally by gel filtration on Sephadex G-150 (Pharmacia). The protein crystals were obtained by pre-extracting five times with a buffer containing 0.5% octyl-polyoxyethylene (Bachem), which eliminated the majority of contaminants. Five extraction steps with a buffer containing 3% octyl-polyoxyethylene allowed solubilization of the integral membrane proteins.

Further purification was performed by ion-exchange chromatography (DEAE-cellulose, Merck), followed by chromatofocusing (PBE94, Pharmacia) and finally by gel filtration on Sephadex G-150 (Pharmacia). Purity was checked by SDS-polyacrylamide gel electrophoresis.

X-ray Crystallography—Crystallization of the porin mutants was performed as described for the wild type (17). The crystals diffracted to varying resolutions in the range of 2.7–3.5 Å and were of space group P321 with similar cell constants as the wild-type protein (Table II).

Data were collected on a FAST area detector or an image plate detector (for mutant R82C) and processed by the programs MADNES (18) and MOSFLM (19). Data reduction and map calculations were carried out by programs from the CCP4 package (20). Initial maps were phased with the wild-type porin model (Protein Data Bank code 2omf, (6)). Model rebuilding was carried out with the help of program O (21). Crystallographic refinement was performed with X-PLOR (22).

RESULTS

Trigonal crystals (space group P321) of the OmpF porin mutants were readily obtained using the established protocol for the wild-type protein (17). The structures were solved by the difference Fourier method at the resolutions listed in Table II.

The initial low R-values (Table II), obtained with the wild-type OmpF model, indicated that all mutant crystals were virtually isomorphous to the wild-type crystals, except the deletion mutant Δ109–114. Remodeling was straightforward and crystallographic refinement resulted in models with good R-factors and stereochemistry (Table II). Restricted B-factor refinement was carried out only for those OmpF mutants for which a sufficient number of observations was available (R42C and R82C).

Structures of OmpF Mutants with Residue Replacements at the Pore Constriction—Comparison of the models of all OmpF point mutants with that of the wild-type porin showed that the backbones of the mutant structures are virtually unchanged. After superposition, the respective root mean square differences between all Cα-positions are below the expected coordinate errors (mutant R42C, 0.10 Å; R82C, 0.09 Å; R132P, 0.13 Å; D113G, 0.06 Å). Comparison of the atomic B-factors of the models of mutants R42C and R82C with those of the wild-type protein showed no indication for increased flexibility of the main chains.

### Table I

| Name of strain | Sequencing of OmpF Mutants |
|----------------|---------------------------|
| OC 1509        | Δ108–114                  |
| IB 910         | Δ109–114                  |
| OC 5101        | R42S                      |
| OC 5104        | R82C                      |
| IB 915         | R132P                     |

### Table II

| OmpF mutant | Cell constantsa | Resolution | Rmerge b | Number of reflections | Completeness | R-factor | Root mean square deviation of bond |
|-------------|-----------------|------------|----------|-----------------------|--------------|----------|-----------------------------------|
|             | A | % | Å | % | Å | % | Å |
| R42C        | 118.5 | 52.7 | 2.70 | 7.4 | 9917 | 81.0 | 17.7 | 16.4 | 16.4 | 0.014 | 1.6 |
| R82C        | 118.6 | 52.9 | 2.80 | 10.0 | 10271 | 99.4 | 20.0 | 18.6 | 16.1 | 0.012 | 1.7 |
| R132P       | 118.2 | 52.8 | 3.30 | 12.8 | 5100 | 78.8 | 22.7 | 20.3 | 18.7 | 0.011 | 1.8 |
| D113G       | 118.3 | 52.8 | 3.50 | 15.0 | 4533 | 80.1 | 20.4 | 20.3 | 18.8 | 0.010 | 1.7 |
| Δ109–114    | 117.7 | 52.7 | 3.10 | 9.0 | 7211 | 97.8 | 30.1 | 26.0 | 18.8 | 0.010 | 1.7 |

*Wild-type OmpF porin crystals: a = b = 118.5 Å; c = 52.7 Å.

### Footnotes

1. Initial refinement with the wild-type OmpF porin.
2. R-factor = Σ||Fobs| - |Fcalc||/Σ|Fobs|, where |Fobs| is the intensity measurement of a particular symmetry-related reflection.
3. R-factor = Σ|Fobs| - |Fcalc|/Σ|Fobs|, where |Fobs| is the structure factor.
4. Fcalc from wild-type model.
5. After positional and B-factor (for mutants R42C, R82C, and Δ109–114) refinement.
In the wild-type porin, the side chains of arginines 42, 82, and 132 are stacked upon each other, forming the most tightly packed part of the pore constriction (Fig. 1). Substitution of each of these arginyl residues by shorter uncharged groups (R42C, R82C, and R132P) results in well defined, short range structural changes that break up the front line at the wall.
structure. In all cases, neighboring side chains bend toward the empty space left by the truncation of the long side chain (Figs. 2, a–c, and 3). The increase of the cross-sectional area at the height of the pore constriction is rather modest (5–7%; see e.g. Fig. 4).

In mutant R42C, the adjacent side chain of Arg-82 has changed its conformation and partly fills the space vacated by the mutation (Figs. 2a and 3). The mean side chain B-factor of Arg-82 has increased by 20 Å² compared with the wild-type porin; all other B-factor changes are \(<10 Å²\). The guanidinium group of Arg-82 sandwiched in the wild type between Arg-42 and Arg-132 (Fig. 1) is now within H-bonding distance (2.5 Å) to Cys-42.

The mutation R82C does not change the conformation of the flanking arginines to a large degree (Figs. 2b and 4). However, Lys-80, which in the wild-type protein points upwards, has swung into the cleft and is within H-bonding distance (2.6 Å) to Cys-82, replacing the H-bond between Arg-82 and Glu-62 of the wild-type structure (Fig. 1). There is some indication for an alternative and less populated conformation of the side chain of Lys-80 (Fig. 4b) in which the side chain amino group is no longer in H-bonding distance to Cys-82 but exhibits a wild type-like conformation.

Resequencing the mutant porin R132P confirmed the presence of a proline at position 132. The electron density rather suggests an asparagine, but because the resolution of the map is limited (3.3 Å), the assignment from the sequence is used. The side chains of three residues, Glu-71 from loop L2 of an adjacent subunit, Tyr-102, and especially Arg-82, are shifted. This also affects the mode of H-bonding interactions. The polar and ionic interactions, which are observed between the guanidinium group of Arg-132 and the side chains of Glu-71 and Tyr-102 in the wild-type porin, are replaced by an H-bond between Tyr-102 and Arg-82 in the mutant protein (Fig. 2c).

The resolution for the structure of the mutant D113G is the worst among all mutants (Table II). Apart from the truncation of the side chain, no structural changes are apparent.

Structure of the OmpF Mutant with a Deletion in the Internal

**Fig. 4. Stereo diagrams of porin mutant R82C.** a, 2F₀ − F c electron density (contoured at 1σ) with model superimposed. b, model of R82C with partial wild-type model superimposed. Only those side chains of the wild-type model that differ significantly from the mutant model (Arg-42, Lys-80, Arg-82, and Arg-132) are shown (in brown). Both conformations of Lys-80 are shown (see text). Magenta, major conformation with side chain amino group forming a salt-bridge with Cys-82 (see also a); yellow, minor conformation.
Loop—In the wild-type porin residues 109–114 constitute part of the internal loop L3 (Fig. 1) and form a multitude of interactions with the barrel wall. This segment that is absent in the deletion mutant is not positioned at the tip of L3 in the wild type, and major rearrangements of the flanking segments were, therefore, anticipated in this mutant porin. The difference Fourier density (Fig. 5a) shows large positive and negative values at the positions of the deleted part and the adjacent stretch at its C-terminal side. In all other regions, and notably at the β-barrel wall, the density is low. This provides strong evidence that the large structural perturbation of the internal loop does not affect the structure of the framework. Indeed, after refinement the root mean square difference between the Cα positions of the wild-type and the mutant porin carrying the deletion was only 0.24 Å. In particular, the residues N-terminal to the deleted region, i.e. all residues up to number 108, show wild-type conformation. Interestingly, the first two residues C-terminal to the deletion, Leu-115 and Pro-116, adopt approximately the positions of wild-type residues Leu-109 and Gly-110 at the C terminus of the small α-helix. The conformation of segment 117–123 is not defined by observed electron density and is probably highly disordered. From Tyr-124 to the C terminus, the structure shows again wild-type conformation (Figs. 2d, 5, and 6). The deletion of six residues and the resulting disorder of residues 117–123 increases the pore section by about 50% as defined by electron density (Fig. 5b), i.e. by ordered structure.

**DISCUSSION**

The mutant selection procedure employed previously (13) has allowed the successful identification of residues and polypeptide segments that are critical in affecting the pore size in OmpF porin. Subsequently, the x-ray structure (6) showed that all the mutations map to the constriction of the pore. The results of the mutation study thus demonstrate the prime role of this part of the molecule as a pore determinant. In the present study, we have determined the structures of five of these OmpF mutants by x-ray crystallography.

The locally confined changes observed for all point mutations corroborate the earlier conclusion that the residues affected are directly involved in determining the pore size and are particularly responsible for the size limit of the maltooligosaccharides that can be translocated across the outer membrane. The increase of the cross-sectional area of the pore constriction appears, however, rather modest (10%). Two possibilities for the altered permeation properties are 1) change of the flexibility of the structure, 2) change in the local electric field, or 3) both. In the case of the arginine mutations, an increased plasticity of the pore constriction is indeed to be expected, because the packing of the closely stacked guanidinium groups in the wild-type protein (Fig. 1) is perturbed. The close proximity of the arginine residues was expected to cause large shifts in their pK values, and calculations suggested an unusual charging behavior (7). Because the channel gating of OmpF porin has been shown to be pH-dependent (23) with a titration point at neutral pH, the arginine cluster may well be the titrating group (7). The confinement of the arginines into close proximity may, thus, have evolved to promote a shift of their pK values into a physiologically relevant pH range. The electrostatic behavior of the arginine mutants is discussed in the accompanying article (14) on the basis of their functional parameters.

The confinement of the arginine residues is relieved by replacement of any one of them by a smaller side chain. Whereas in the case of mutants R42C or R132P the newly created space is taken by the adjacent arginine, in the mutant R82C the flanking arginines Arg-42 and Arg-132 keep essentially their native conformation. This contrasting behavior can be explained by the mutual repulsion of the positively charged guanidinium groups.

In both of the mutants in which cysteine residues were introduced (R42C and R82C), H-bonds between the cysteine sulfhydryl groups and a neighboring basic side chain were found. It is likely that in both cases the positively charged environment lowers the in situ pK values of the sulfhydryl group and that the cysteine is present in its anionic form and participates in an ion pair interaction (Cys-42-Arg-82 and Cys-82-Lys-80). A salt bridge between a lysine and a cysteine residue has recently been found in human glutathione transferase (24). The cysteine residues at the pore constriction appear to be...
Crystallographic Analysis of OmpF Selection Mutants

Figure 6. Mean differences of atom positions between the models of porin mutant Δ109–114 and wild type after superposition. a, main chain atoms. b, side chain atoms.

well suited for covalent attachment of groups that may block the channel or report atomic motions in spectroscopic studies. The deletion mutant Δ109–114 shows a major disordering of a 7-residue segment (residues 117–123). Hence, a total of 13 residues that are ordered in the wild-type structure are missing in the electron density map and the pore size appears considerably increased (Fig. 5).

In wild-type OmpF porin there are two completely buried and uncompensated acidic groups (Glu-296 and Asp-312) that are in H-bonding distance with respect to each other. Their side chains, originating from the β-barrel, are covered by the internal loop. Electrostatic calculations based on the x-ray model of wild-type porin predicted (7) that both groups are protonated, i.e., uncharged, at neutral pH. In the deletion mutant, these acidic residues are exposed (Fig. 2d) and are, in fact, no longer H-bonded to each other (25). It is likely that the groups assume normal pK values in the mutant and that the charged carboxylates repel each other. It has been proposed that in wild-type porin, the closed state of the channel may be defined by an alternative conformation of the internal loop, which would also lead to the exposure of these acidic groups and concomitant liberation of electrostatic energy. The involvement of the only histidine present in the OmpF sequence (His-21), which is located at the outer surface of the barrel, as a candidate for the pH sensor (26) can be ruled out, because a His-21 → Thr mutant shows wild-type behavior (14). Also, the x-ray structure of this mutant, though revealing distinct changes around the site of mutation, does not show alterations at the channel lining (25).

It is interesting that the OmpF deletion mutant maintains the structure of the hollow β-barrel framework. It appears unlikely that this is caused by trapping of the structure in the wild-type crystal lattice. The structure of the barrel thus appears to be ensured by the main chain H-bonds, the packing of side chains, and the stabilization by the extensive contact with the neighboring barrels in the trimeric aggregate. However, the lack of the internal loop as a brace decreases the thermal stability by 20 degrees.

The notion that transmembrane channels are formed by simple motifs, such as helical bundles or β-barrels, and are supplemented by selectivity and gating loops seems attractive. In OmpF and PhoE porins there is a single constricting loop (L3) oriented parallel to the plane of the membrane (6). In maltoporin, a channel specific for maltoligosaccharides, a similar constriction is further narrowed by the contribution of two additional loops (10). Although no high resolution structures are currently available for ion-specific channels, related arrangements have been suggested (for a review, see Ref. 27). It will be interesting to determine whether similar or different structural principles are involved in channel-forming transmembrane proteins in general.

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