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Replacement of the Sole Histidinyl Residue in OmpF Porin from *E. coli* by Threonine (H21T) Does Not Affect Channel Structure and Function

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The sole histidine residue in OmpF porin was replaced by threonine using site-directed mutagenesis. This exchange affected neither channel properties nor channel structure, as determined by X-ray analysis to 3.2 Å. Conductance and critical voltage (V_c) were observed in the pH range 4.3–9.4, with results indistinguishable from those observed in the wild-type protein. The validity of these observations is supported by the independence of the methods used, and by the fact that mutants in residues located in the channel constriction yielded significantly different values from wild-type protein. The binding of a glycolipid molecule might be affected.

Porin (OmpF) is a homotrimeric protein that forms water-filled, voltage-gated channels across outer membranes of *Escherichia coli.* Conductance studies have previously revealed effective incorporation into planar lipid bilayers which appeared to be due to the presence of bacterial glycolipids (lipopolysaccharides, see ref. (1)). Three channels in a trimer open in a highly cooperative manner (2). Two distinct conductance states have been observed the larger of which (0.8 nS) is characteristic of the OmpF-porin, while the smaller one (0.5 nS) is observed in the native OmpC-porin, with the favoured state depending on the size of porin aggregates in the planar bilayer, as well as on the lipid used (1, 3). Todt and coworkers also reported two channel sizes the relative occurrence of which appeared to be pH-dependent: acidic conditions favoured the smaller pore-size, while at alkaline pH, the larger conductance state was more frequent. The pH-induced switch was reported to be at $\sim$ pH 7 (4), and the only histidinyl residue of OmpF-porin (H21) was therefore implicated (5).

The structure determination of *E. coli* porin to high resolution revealed that His-21 is located at the outside of the β-barrel which harbours the channel in each subunit (6, 7). Moreover, model calculation of its electrostatic properties (8) suggested that of the three arginyl resides (R42, R82, R132) which occur in close juxtaposition at the channel constriction, the *in situ* pK-values of the middle one is distinctly lower than the values applicable in solution. The calculated pK-value of R82 has been given as $\sim$7, and that of H21 as 4.4 (8). The titration mid-point of the critical voltage (V_c), the threshold potential above which the equilibrium between open and closed states of the channels is shifted in favor of the closed state, was found to be $\sim$7.7 in native porin, and the removal of each of the three guanidinium groups abolished the titration behaviour (9). These results suggested that the charge state of the ariginyl residues rather than that of the histidine might affect channel closing. We have now replaced the histidinyl residue by threonine (H21T) using site-directed mutagenesis, and determined the functional properties of the mutant over a pH-range from 4.3 to 9.4, and its structure to a resolution of 3.2 Å. The results clearly show that the histidinyl group is not responsible for the switch of the conductance states, but that a change in the position

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of an amphiphile, used to crystallize the protein, may reflect an altered interaction of a lipopolysaccharide molecule with the porin trimer.

MATERIALS AND METHODS

Site-specific mutagenesis, protein purification, and planar bilayer measurements were performed as reported (9). The critical voltage (V_c) was determined according to two different methods. Either a transmembrane potential was applied, and after increments of 10 mV, the conductance was allowed to reach equilibrium. Alternatively, V_c was estimated from current-voltage curves obtained by applying ramps of the potentials across the bilayer from 0 to ± 250 mV over a period of 100 s. X-ray analysis was performed as described (10). The space group of the crystal was *P*321, with cell constants $a = b = 118.7$Å, $c = 53.0$ Å; $\alpha = \beta = 90°$, $\gamma = 120°$. The agreement between symmetry related reflections was $R_{sym} = 11.5\%$. The completeness of the data set was 98.6%. Using the wild-type OmpF model (PDB code 2omf) including ordered detergent fragments as the starting model, the crystallographic R-factors was 19.9% initially, and decreased to 15.9% after remodeling of the the site of the mutation and subsequent conventional positional refinement.

RESULTS AND DISCUSSION

Table I shows the conductance values of the wild-type protein and the H21T mutant at three pH-values: 4.3, 7.4, and 9.4. Based on the large number of events, and the small standard deviations observed, the statistics can be regarded as excellent. Significant differences relative to the wild-type protein are not observed in H21T. For a comparison, the replacement of one of the three cationic residues by cysteine, e.g. R42C, or a replacement of an anionic group (D113G) on L3, did not lower the conductance significantly either. Only a deletion mutation (Δ109–114) in the constriction, in which six residues are removed and the following seven disordered (10) showed a decrease in conductance of about one third at all pH-values (Table I).

Analogous results emerge from the dependence of the critical voltage V_c from the pH-values (Table II). The V_c of wild-type and H21T mutant were determined by applying positive or negative voltage ramps. They showed comparable values at three pH-values, that is, higher potentials were required to close the channels at alkaline pH. The titration mid-point (9) is ∼pH 8.6 for the H21T mutant. In those mutants that are located in the constriction site, the pH-dependence was essentially abolished. To assess the validity of the results, we examined the V_c-values using positive or negative ramps at all pH-values. The impact of the direction of the ramp was not significant at any of the three pH-values used. At one, pH 7.4, we also explored the effect of allowing the conduc-

TABLE 1
Conductance Values, Given in nano-Siemens (nS), of Wild-Type and Mutant Porins in a pH Range of 4.3 to 9.4

Mutation	pH 4.3		pH 7.4		pH 9.4	
	Positive potential	Negative potential	Positive potential	Negative potential	Positive potential	Negative potential
Wild type	**0.81 ± 0.09** (n = 205)	**0.84 ± 0.08** (n = 129)	**0.82 ± 0.05** (n = 73)	**0.86 ± 0.06** (n = 83)	**0.80 ± 0.07** (n = 129)	**0.83 ± 0.05** (n = 87)
H21T	**0.82 ± 0.08** (n = 97)	**0.85 ± 0.05** (n = 8)	**0.85 ± 0.06** (n = 23)	**0.81 ± 0.04** (n = 33)	**0.78 ± 0.05** (n = 39)	**0.77 ± 0.08** (n = 45)
D113G	0.71 ± 0.03 (n = 41)	0.91 ± 0.04 (n = 40)	0.78 ± 0.06 (n = 144)	0.82 ± 0.04 (n = 139)	0.73 ± 0.03 (n = 34)	0.84 ± 0.03 (n = 31)
Δ109–114	0.53 ± 0.05 (n = 62)	0.58 ± 0.05 (n = 49)	0.50 ± 0.06 (n = 68)	0.50 ± 0.04 (n = 79)	0.41 ± 0.06 (n = 42)	0.62 ± 0.08 (n = 47)
R42C	0.78 ± 0.06 (n = 29)	0.80 ± 0.08 (n = 25)	0.80 ± 0.07 (n = 60)	0.75 ± 0.06 (n = 48)	0.80 ± 0.09 (n = 39)	0.79 ± 0.07 (n = 37)

Note. The H21T mutation and wild-type porin (**bold**) show no significant differences. The values of the two point-mutants in the constriction site (R42, D113G) are slightly lower, while the conductance of the mutant with a deletion in the constricting loop (L3) is decreased by ∼40% over the entire pH range. Differences between measurements performed at positive or negative polarities of the potentials applied can be noticed (e.g., at pH 9.4) in the deletion mutant Δ109–114, but the significance of this result is not clear. The table exhibits a total of >2000 observed events.

TABLE 2
The Critical Threshold Voltage (V_c, Indicated in millivolts, mV) of Wild-type and Mutant Proteins

Mutation	pH 4.3		pH 7.4		pH 9.4		pH 7.4
	Positive ramp	Negative ramp	Positive ramp	Negative ramp	Positive ramp	Negative ramp	With equilibration
Wild type	**132 ± 10** (n = 8)	**125 ± 12** (n = 8)	**155 ± 10** (n = 10)	**172 ± 14** (n = 10)	**228 ± 18** (n = 10)	**220 ± 18** (n = 10)	**116 ± 5** (n = 4)
H21T	**141 ± 14** (n = 9)	**111 ± 7** (n = 10)	**140 ± 11** (n = 10)	**190 ± 12** (n = 10)	**209 ± 16** (n = 7)	**214 ± 13** (n = 8)	**108 ± 5** (n = 6)
D113G	165 ± 13 (n = 11)	145 ± 12 (n = 11)	168 ± 11 (n = 10)	160 ± 10 (n = 10)	165 ± 4 (n = 10)	161 ± 10 (n = 10)	123 ± 5 (n = 5)
Δ109–114	72 ± 5 (n = 10)	86 ± 4 (n = 10)	111 ± 12 (n = 10)	101 ± 6 (n = 10)	111 ± 13 (n = 10)	115 ± 8 (n = 10)	86 ± 7 (n = 8)
R42C	70 ± 4 (n = 10)	57 ± 5 (n = 10)	116 ± 9 (n = 10)	110 ± 10 (n = 10)	100 ± 10 (n = 10)	147 ± 14 (n = 10)	88 ± 7 (n = 5)

Note. The substitution mutant H21T shows a titration behaviour comparable to that of wild-type porin (**bold**). Threshold voltages for the other three mutations shown reveal changed values, but a much weaker dependence on pH. Measurements shown were obtained by applying a potential ramp (250 mV over a period of 100 s) in either a positive or a negative direction. A comparison with values at pH 7.4, obtained by allowing equilibration after each 10-mV increment, yields values that are comparable to those measured previously (1). The values in the last column are lower than, but consistent with, those exhibited for ramp measurements at the same pH of 7.4 (exhibited in the table in columns 3 and 4), with values that are higher than those obtained with the equilibration method. This is expected since during the equilibration time, the voltage continues to rise to a higher value. Both methods seem applicable, but the results cannot be cross-compared. Measurement using ramps are much more rapid (the >2000 values shown were obtained in a net time of 4 hr), while the observations with the equilibration method took 20 hr net for the 28 values obtained.

tance to reach equilibrium after voltage-increments of 10 mV each. The absolute values obtained were lower, a result to be expected as in the ramp experiments, the potentials keep rising during channel opening and closing. These results, together with the small standard deviations and the independence of the method applied (see legend to Table II), shows that the confidence-level of the results shown is high. An earlier result from experiments in which porin was carbethoxylated (4) remains unexplained. Reversible modification with this reagent appeared to affect the channel size. Although preferential reaction with histidine residues has been reported, its specificity is nonetheless not unequivocal (11).

The structural investigation using X-ray analysis revealed that the replacement of H21 by a threonine led only to the expected local change (Fig. 1), with no changes observed at longer range. Residue 21 is located in the crevice between subunits, at the outside of the barrels. This renders an immediate effect of channel conductance unlikely. Since the cooperativity of the opening events in the mutant are unchanged if compared to wild-type, a direct effect on subunit interactions seems unlikely. This is in agreement with studies of the overall conformation by means of Fourier-transformed infrared spectroscopy, which did not reveal significant changes (4). A test concerning the heat stability of the mutant protein yielded a temperature of trimer dissociation (90°C) that is indistinguishable from that of the wild-type protein (unpublished). Changes in heat lability have been found to be very sensitive indicators of conformational changes.

An interesting observation pertains to that amphiphile molecule that is bound to porin at the crevice between subunits. While the position of the octyl-moiety of alkyl-oligooxyethylene is unchanged, the first ethylene-oxide unit of the polar headgroup, which is tightly bound and hence visible in the X-ray analysis, is rotated by about 90° (Fig. 1). Electron microscopic investigations have suggested that lipopolysaccharide binds in this position (12). Since the earlier conductance studies were performed mostly with “LPS-enriched” porin and “LPS-depleted fractions” (4), the possibility that the differences we observe are due to the absence of lipopolysaccharides cannot be

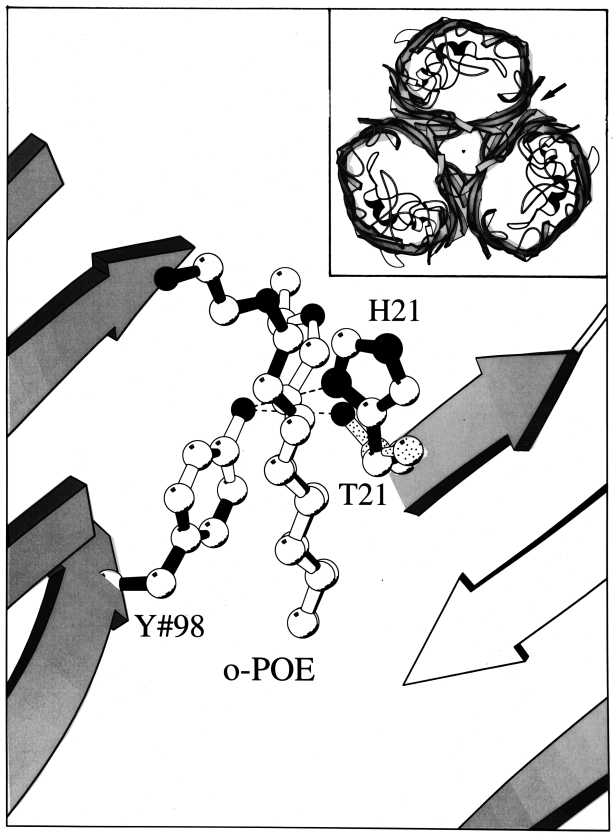

FIG. 1. Effects of the substitution of a histidinyl residue by threonine as determined by X-ray analysis at 3.2 Å. Replacement of His-21 (dark) by threonine (stippled) causes a small, local change. The view is roughly perpendicular to the trimer axis, and is from the outside looking onto the crevice between two barrels (arrow in the inset). The hydrogen bond to a tyrosyl residue in a neighbouring subunit (Y98) is maintained but slightly displaced (hatched lines). Of the one tightly bound detergent molecule, octyl-oligooxyethylene (polydisperse, o-POE), only the first ethylene-oxide group (–O–CH_2–CH_2–O–) and the alkyl chain can be seen. The alkyl chain exhibits only minor changes, but the polar moiety is rotated from a direction towards the viewer (in the wild-type protein; shown with black bonds) to one pointing backwards (mutant, shown with white bonds). The location of the magnified area in the trimer is indicated in the inset (modified from (13)) by an arrow at one of the intersubunit crevices.

excluded, since our experiments were performed with glycolipid-free porin (6). Although a direct role of the histidinyl residue 21 appears unlikely, the possibility of a shift of the F-type channels to the C-type (1), due to a change in glycolipid binding, deserves further investigation.

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