BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 142–145 (1998)
ARTICLE NO. RC988745

Identification of the Catalytic Triad of the Protein D2 Protease in *Pseudomonas aeruginosa*

Eisaku Yoshihara, Hiroshi Yoneyama, Toshihisa Ono, and Taiji Nakae
Department of Molecular Life Science, Tokai University School of Medicine, Isehara 259-11, Japan

Received April 29, 1998

We reported recently that protein D2 (OprD) porin of *Pseudomonas aeruginosa* bears protease activity (*FEBS Letters* 394, 179–182, 1996). To identify the catalytic residues of OprD, we introduced the site-directed mutations replacing the putative catalytic triad His156, Asp208, and Ser296 with glutamine, asparagine, and alanine, respectively. The OprD proteins purified from the chromosomal *oprD*-deficient mutants harboring the plasmids encoding the site-directed mutations showed protease activity less than 0.1% of that of the wild-type OprD. These site-directed mutageneses caused undetectable changes in the pore-forming activity of OprD as measured by single-channel conductance by the planar lipid bilayer. The minimum inhibitory concentration of imipenem in mutants having the replaced catalytic triads was identical with that in the wild-type strain. On the other hand, introduction of the mutation at His367 replacing with glutamine, the site that is supposed to be unrelated to the catalytic sites, showed the unchanged protease activity. These results unequivocally demonstrate that OprD is the protease bearing porin and catalyzes the reaction at His156, Asp208, and Ser296 residues. © 1998 Academic Press

Porins of Gram-negative bacteria form the water-filled diffusion channel for hydrophilic solutes across the outer membrane (1, 2, 3). *P. aeruginosa,* an opportunistic pathogen to the immunocompromised and cystic fibrosis patients, produces three species of porins, namely protein C, D2 (OprD) and E1 in the outer membrane. All these porins form channels with narrower diameter than the *Escherichia coli* OmpF porin probably preventing access of a broad range of structurally unrelated antibiotics (4). Among these porins, the OprD porin has been shown to have the following unique properties. (i) OprD is a porin that allows specific diffusion of imipenem (IPM, a β-lactam antibiotic) (5, 6). (ii) OprD exhibited opening and closing of the channel and this channel activity was constrained to quiescent in the presence of IPM or basic amino acids (7, 8). (iii) OprD showed the weak protease activity (9). The conclusion that OprD porin is protease, is based on the following findings (9). (i) Highly purified OprD protein hydrolyzed several synthetic peptides. (ii) A serine protease-specific inhibitor, diisopropyl fluorophosphate, inactivated the enzyme activity and bound covalently to OprD. (iii) Monoclonal antibody raised against OprD lowered the rate of the substrate hydrolysis. In addition, comparison of the amino acid sequence of OprD with those of chymotrypsin and trypsin revealed that His156, Asp208 and Ser296 may have a similarity with the sequence of the catalytic triad residues of His57, Asp102 and Ser195 of chymotrypsin and His55, Asp99 and Ser192 of trypsin.

To identify the amino acid residues involved in the catalytic reaction and ascertain protease nature of OprD, we constructed the mutant OprD proteins substituting the predicted catalytic residues to other amino acids by means of site-directed mutagenesis. We report here that the substitution of His156, Asp208 and Ser296 with glutamine, asparagine and alanine, respectively, knocked out the protease activity of OprD.

MATERIALS AND METHODS

Chemicals. tert-butoxycarbony (Boc)-Gln-Ala-Arg-methylcoumarin (MCA) was purchased from Peptide Institute Inc. Osaka. N-lauroyl sarcosinate and n-octyl-polyoxyethylene (octyl-POE) were from Nacalai Tesque Inc. Kyoto and Alexis Corp., Switzerland, respectively. Imipenem and DX8739 were from Banyu Pharmaceuticals, Tokyo and Daiichi Pharmaceuticals, Tokyo, respectively. Mutan-K kit was from Takara Shuzo Co. Osaka. All other chemicals were of the highest purity commercially available.

Bacterial strains and plasmids. Bacterial strains, phage, and plasmids used are listed in Table 1.

Site-directed mutagenesis of the cloned oprD gene. Site-specific oligonucleotide-directed mutagenesis of *oprD* was carried out according to the procedure described by Kunkel (10). We subcloned the *Bam*HI through *Kpn*I fragment from pTN003 into the vector, pBluescript II SK(−) (STRATAGENE) to yield pOPRD001. Oligonucleotide primers employed in mutagenesis of the putative catalytic amino acids were H156Q (AGGCAGGCCAGTTCACCGAG), D208N (GAACTCGAAAACATCTATCG), and S296A (GTGGCGACGCGA-

0006-291X/98 $25.00

TABLE I

Bacterial Strains, Phage, and Plasmids

	Relevant properties	Reference
Strains		
E. coli CJ236	*dut*1, *ung*1, *thi*-1, *relA*1/pCJ105(F′*cam*^{r})	Takara
E. coli BMH71-18 *mutS*	Δ(*lac-proAB*), *supE, thi, mut*S215::TN*10* (*tet*^{r})/F′(*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)	Takara
E. coli MV1184	*ara*, Δ(*lac-proAB*), *rpsL, thi*(ϕ80*lac*ZΔM15), Δ(*srl-recA*)306::Tn*10*(*tet*^{r})/ F′(*tra*D36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)	Takara
P. aeruginosa TNP031	*P. aeruginosa* PAO2003 derivative with OprD deficiency	FEBS Lett. (1991) **283,** 177–179
P. aeruginosa TNP100	TNP031 (oprD$^-$) derivative carrying pTN006	This study
P. aeruginosa TNP101	TNP031 (oprD$^-$) derivative carrying pH156Q	This study
P. aeruginosa TNP102	TNP-31 (oprD$^-$) derivative carrying pD208N	This study
P. aeruginosa TNP103	TNP031 (oprD$^-$) derivative carrying pS296A	This study
Plasmid		
pTN006	pKT240 derivative which has *oprD*	AAC (1993) **37,** 2385–2390
pBluescript II SK(−)	Multipurpose vector, ABPC^r	Toyobo
pKT240-1	pKT240 derivative deleting *Pst*I fragment, KM^r	This study
pOPRD001	pBluescript II SK(−) derivative carrying *oprD* fragment	This study
pH156Q	Fusion plasmid between pKT240-1 and pOPRD001 derivative carrying mutagenized oprD with change from His (156) to Gln	This study
pD208N	Fusion plasmid between pKT240-1 and pOPRD001 derivative carrying mutagenized oprD with change from Asp (208) to Asn	This study
pS296A	Fusion plasmid between pKT240-1 and pOPRD001 derivative carrying mutagenized oprD with change from Ser (296) to Ala	This study

TTTTCCT). To examine the effect of the site-directed mutagenesis at unrelated site to the putative catalytic site, H367 was replaced with glutamine using the primer, ATGGCAAGCAGCACGAAACC. These primers were annealed with single-stranded DNA prepared from *E. coli* CJ236 (TAKARA) harboring pOPRD001 and treated with T4 DNA polymerase and with DNA ligase. *E. coli* BMH71-18 *mutS* (TAKARA) cells were transformed with the extended DNA, followed by recovery of single-stranded DNA from the transformed cells with a helper phage, VMC-13 (STRATAGENE). Next, *E. coli* MV1184 cells were infected with the phage and colonies were selected on L-agar (1.5%) plate containing 150 μg/ml ampicillin. The nucleotide sequence of the mutagenized DNA fragments was determined by the dideoxy chain-termination method (11). To express these *oprD* genes in *P. aeruginosa,* the plasmid, pTN006, pH156Q, pD208N and pS296A, were transferred by the conjugation into *P. aeruginosa* TNP031, a OprD deficient strain.

Determination of protease activity. OprD protein was purified as reported earlier (4) and the protease activity was measured according to the method described by Kawabata et al. (12).

Single channel conductance measurement. The channel activity was measured by the planar lipid bilayer technique as described before (7).

Minimum inhibitory concentration (MIC) measurement. Antibiotic susceptibility was determined by the 2-fold agar dilution method using the Mueller Hinton Medium (Difco). The MIC was defined as the lowest drug concentration that inhibited the growth of the test strain after 20 h of incubation at 37°C.

RESULTS

To ascertain the expression and correct localization of the modified *oprD* gene product, the outer membrane fractions were prepared by the method described earlier (4) and subjected to SDS-PAGE. The result showed that all the mutants produced the OprD proteins in the amounts comparable with the wild-type strain and proteins were localized in the outer membrane. Electrophoretic migration of the mutant OprD proteins was indistinguishable from that of the wild-type protein (Fig. 1).

The protease activity of the mutant OprD proteins. To compare the enzymatic activities of the mutant OprD proteins with that of the wild-type OprD, rate of Boc-Gln-Ala-Arg-MCA hydrolysis was determined in the solution containing octyl-POE and phosphate buffer, pH 8.2. Under these conditions, the rate of catalysis of the wile-type OprD protein appeared to be 78.17 nM/min/μM protein and those of the mutant proteins with H156Q, D208N and S296A were 0.023, 0.031 and 0.067 nM/min/μM protein, respectively. These values in the H156Q, D208N and S296A were 0.03, 0.04, and 0.09%, respectively, of the activity of the wild-type OprD (Table II). On the one hand, we modified a histidine residue probably unrelated to the catalytic site replacing with glutamine. The activity in the mutant protein with H367Q was 93% of that in the wild-type OprD (Table II). These results clearly indicate that the site-specific replacement of the putative catalytic amino acid residues at His156, Asp208 and Ser296 caused total inactivation of the protease activity and suggested that His156, Asp208 and Ser296 are most likely the catalytic triad residues of the OprD protease.

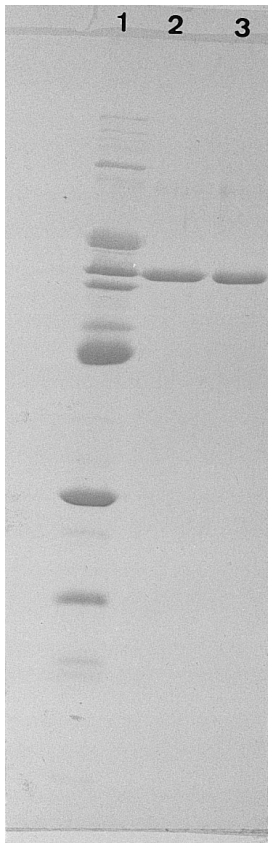

FIG. 1. SDS-polyacrylamide gel electrophoretogram of the purified OprD proteins. Lane 1, whole outer membrane proteins of *P. aeruginosa* PAO1; lane 2, wild-type OprD; lane 3, OprD (H156Q).

Channel activity of the mutant OprD proteins. To test whether the amino acid substitution in the catalytic sites affects porin activity, we measured the single channel conductance of the mutant OprD and compared the results with that of the wild-type protein using the planar lipid bilayer technique (7). All mutant proteins showed the single channel conductance of about 100 pSiemens in 0.1 M NaCl, which is indistinguishable from the conductance of the wild-type OprD, suggesting that the substitution may not exert gross structural alternation of the channel interior (Fig. 2). To test whether the specific interaction of OprD with carbapenem antibiotics such as DX8739 was altered by the amino acid substitution, the channel conductance of the mutant protein was measured in the presence of 3 mM DX8739. Under this condition the mutant OprD showed the closure of the channel, which is unique to OprD channel, indicating that the amino acid replacement does not affect the carbapenem binding activity.

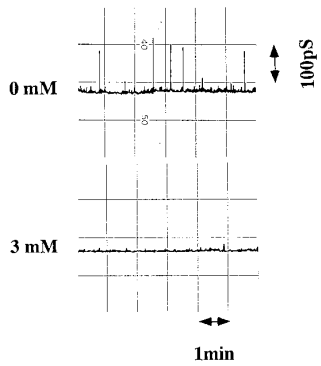

FIG. 2. Effect of DX8739 on the channel conductance of the mutant OprD protein. The conductance of the mutant OprD (D208) was measured in 0.1 M NaCl solution with or without 3 mM DX8739.

The effect of the site-directed mutagenesis on the IPM susceptibility. OprD protein has been reported to be the porin, which facilitates the diffusion of the basic amino acids and their structural analogue, IPM (5, 6). To test whether the amino acid substitution affects the IPM permeability, we examined the minimum growth inhibitory concentration of IPM in the mutants and found that MICs of IPM in TNP101, TNP102 and TNO103 were all 0.78 μg/ml that is indistinguishable from the MIC of IPM in TNP100 harboring the plasmid carrying the wild-type *oprD* (Table III). These results suggest that the catalytic residues for the protease and IPM recognition site are separate.

TABLE II

The Protease Activity of the Mutant and Wild-Type OprD Proteins

Protein	Velocity (nM/min/μM protein)	Relative activity (%)
Wild-type	78.17 ± 5.0	100.0
H156Q	0.023 ± 0.005	0.03
D208N	0.031 ± 0.004	0.04
S296A	0.067 ± 0.004	0.09
H367Q	72.80 ± 11.0	93.1

Note. The OprD protein was mixed with 10 μM of Boc-Gln-Ala-Arg-MCA in 150 μl fo 20 mM sodium phosphate buffer, pH 8.2 containing 0.5% of octyl-POE and incubated at 37°C for various periods of time. The hydrolysis of the peptide was measured as described in Materials and Methods. The relative activity (%) is the rate compared with that of the wild-type protein.

TABLE III

Antibiotic Susceptibility of *P. aeruginosa* Strains Harboring the Mutant OprD Gene

Strains	MIC (μg/ml)	
	Imipenem	Ceftazidime
TNP100 (wild-type)	0.78	1.56
TNP101 (H156Q)	0.78	1.56
TNP102 (D208N)	0.78	1.56
TNP103 (S296A)	0.78	1.56

DISCUSSION

We reported recently that the OprD porin of *P. aeruginosa* bears the protease activity (9). However, the weakness of this conclusion was that the amino acid residues involved in the catalytic reaction were not identified and it remained a possibility that the OprD preparation was contaminated by other protease(s). To ascertain these ambiguities, we constructed the mutant OprDs having amino acid substitution at the predicted catalytic residues, His156, Asp208 and Ser296.

We demonstrated unequivocally in this paper that OprD porin is a member of the serine protease family and His156, Asp208 and Ser296 residues constitute the catalytic triads. One may argue that the mutagenesis may induce gross conformational change of OprD resulting in the inactivation of enzyme activity. This possibility cannot be ruled out but less likely, since the planar lipid bilayer experiments and MIC measurements showed the intactness of the channel activity and specific interaction with IPM.

Although His156, Asp208 and Ser296 residues of OprD protease were identified to be the catalytic residues of the OprD protease, the enzyme activity seems to be lower than that of trypsin. Therefore, the physiological role of the OprD protease could be to bind ligands and to facilitate the diffusion through the channel. It is possible that the ligand recognition site was phylogenically derived from protease and this site was preserved in OprD, but the catalytic residues became secondary to the function of OprD. On the other hand, it still remains possible that OprD protease binds the substrate peptides and degrades them so as to ease passing through the OprD channel. Another possibility could be that this protease degrades the proteins located at the host cell membrane, to which the bacteria colonize. To this function, high catalytic activity may not be essential.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture, Tokai University School of Medicine Research Aid, "study of dug-resistant bacteria" funded by the Ministry of Health and Welfare of Japan, and Japan Association of Promoting Science and Tokai University School of Medicine Research Project.

REFERENCES

1. Nakae, T. (1986) *CRC Crit. Rev. Microbiol.* **13,** 1–62.
2. Nikaido, H., and Vaara, M. (1985) *Microbiol. Rev.* **49,** 1–32.
3. Hancock, R. E. W. (1987) *J. Bacteriol.* **169,** 929–933.
4. Yoshihara, E., and Nakae, T. (1989) *J. Biol. Chem.* **264,** 6297–6301.
5. Trias, J., and Nikaido, H. (1990) *Antimicrob. Agents Chemother.* **34,** 52–57.
6. Trias, J., and Nikaido, H. (1990) *J. Biol. Chem.* **265,** 15680–15684.
7. Ishii, J., and Nakae, T. (1993) *FEBS Lett.* **320,** 251–255.
8. Ishii, J., and Nakae, T. (1996) *FEMS Microbiol. Lett.* **136,** 85–90.
9. Yoshihara, E., Gotoh, N., Nishino, T., and Nakae, T. (1996) *FEBS Lett.* **394,** 179–182.
10. Kunkel. T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
11. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T., and Sakakibara, S. (1988) *Eur. J. Biochem.* **172,** 17–25.