Porin OmpP2 of *Haemophilus influenzae* Shows Specificity for Nicotinamide-derived Nucleotide Substrates*

Received for publication, December 23, 2002, and in revised form, April 8, 2003
Published, JBC Papers in Press, April 14, 2003, DOI 10.1074/jbc.M213087200

Christian Andersen‡§, Elke Maier‡, Gabrielle Kemmer‡, Julia Blass‡, Anna-Karina Hilpert‡, Roland Benz‡‡ and Joachim Reidel¶

From the ‡Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany and the §Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany

*This work was supported in part by Deutsche Forschungsgemeinschaft Grants Be 865/10 and Re 1561/1 and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of an Emmy-Noether fellowship.

§ Recipient of an Emmy-Noether fellowship.

The only known natural habitat of *Haemophilus influenzae* is the human nasopharynx. *H. influenzae* is Gram-negative and is able to grow under facultative anaerobic conditions. It is also a human pathogen and is responsible for significant morbidity and mortality in young children (1, 2). To cultivate *H. influenzae*, a complex medium is required; and if not blood-based, it must contain two growth factors: NAD and hemin (3). In the nasopharynx, *H. influenzae* finds a relatively constant environment, and the organism has retained a certain degree of flexibility in its ability to utilize nutrients (4) by possessing some limiting metabolic pathway redundancy (5).

Early biochemical investigations have established that NMN

---

Porin OmpP2 of *Haemophilus influenzae* shows specificity for NAD (factor V) because it lacks all biosynthetic enzymes necessary for de novo synthesis of that cofactor. Therefore, growth in vitro requires the presence of NAD itself, NMN, or nicotinamide riboside (NR). To address uptake abilities of these compounds, we investigated outer membrane proteins. By analyzing OmpP2 knockout mutants, we found that NAD and NMN uptake was prevented, whereas NR uptake was not. Through investigation of the properties of purified OmpP2 in artificial lipid membrane systems, the substrate specificity of OmpP2 for NAD and NMN was determined, with *K₅₅* values of ~8 and 4 mM, respectively, in 0.1 M KCl, whereas no interaction was detected for the nucleoside NR and other purine or pyrimidine nucleotide or nucleoside species. Based on our analysis, we assume that an intrinsic binding site within OmpP2 exists that facilitates diffusion of these compounds across the outer membrane, recognizing carbonyl and exposed phosphate groups. Because OmpP2 was formerly described as a general diffusion porin, an additional property of acting as a facilitator for nicotinamide-based nucleotide transport may have evolved to support and optimize utilization of the essential cofactor sources NAD and NMN in *H. influenzae*.

---

Haemophilus influenzae has an absolute requirement for NAD (factor V) because it lacks all biosynthetic enzymes necessary for de novo synthesis of that cofactor. Therefore, growth in vitro requires the presence of NAD itself, NMN, or nicotinamide riboside (NR). To address uptake abilities of these compounds, we investigated outer membrane proteins. By analyzing *ompP2* knockout mutants, we found that NAD and NMN uptake was prevented, whereas NR uptake was not. Through investigation of the properties of purified OmpP2 in artificial lipid membrane systems, the substrate specificity of OmpP2 for NAD and NMN was determined, with *K₅₅* values of ~8 and 4 mM, respectively, in 0.1 M KCl, whereas no interaction was detected for the nucleoside NR and other purine or pyrimidine nucleotide or nucleoside species. Based on our analysis, we assume that an intrinsic binding site within OmpP2 exists that facilitates diffusion of these compounds across the outer membrane, recognizing carbonyl and exposed phosphate groups. Because OmpP2 was formerly described as a general diffusion porin, an additional property of acting as a facilitator for nicotinamide-based nucleotide transport may have evolved to support and optimize utilization of the essential cofactor sources NAD and NMN in *H. influenzae*.

---

The only known natural habitat of *Haemophilus influenzae* is the human nasopharynx. *H. influenzae* is Gram-negative and is able to grow under facultative anaerobic conditions. It is also a human pathogen and is responsible for significant morbidity and mortality in young children (1, 2). To cultivate *H. influenzae*, a complex medium is required; and if not blood-based, it must contain two growth factors: NAD and hemin (3). In the nasopharynx, *H. influenzae* finds a relatively constant environment, and the organism has retained a certain degree of flexibility in its ability to utilize nutrients (4) by possessing some limiting metabolic pathway redundancy (5).

Early biochemical investigations have established that NMN and nicotinamide riboside (NR) can substitute for NAD, whereas nicotinamide, niacin, or other nicotine-based interme-

---

Porin OmpP2 of *Haemophilus influenzae* shows specificity for nicotinamide-derived nucleotide transport may have evolved to support and optimize utilization of the essential cofactor sources NAD and NMN in *H. influenzae*. NMN, or nicotinamide riboside (NR). To address limiting metabolic pathway redundancy (5).

Recently, we presented data showing that two gene products appear to be involved in the NAD utilization pathway of *H. influenzae* (12–14). The gene products were identified as the hel-encoded outer membrane lipoprotein e(P4) and a periplasmic protein termed NadN. Knockout mutations of both genes resulted in growth-deficient phenotypes depending on the NAD concentrations provided in the growth medium. The enzymatic activities of both proteins were characterized. NadN has the ability to act as an NAD pyrophosphatase as well as an NMN 5'-nucleotidase. Furthermore, for e(P4), we showed that it acts as an NMN 5'-nucleotidase and that deletion or point mutants in hel affect the growth of *H. influenzae* and the uptake of NAD and NMN. Finally, an *nadN hel* double mutant was constructed, which was unable to utilize NAD or NMN and could grow only in the presence of NR (13).

It becomes more and more apparent that specialized outer membrane proteins (OMPs) play a key role in bacterial adaptation in host niches (15). In *H. influenzae*, a number of OMPs have been characterized, but none of them have been assigned as facilitators for specific substrates. Of the major OMPs, OmpP2, with a molecular mass of ~40 kDa, was described previously (16–18). It was found that *ompP2* knockout mutants are viable under laboratory conditions, but are deleterious in an animal model, thus indicating an important role for bacterial physiology and infection under *in vivo* conditions. Through investigation of permeability channels of outer membrane (OM) samples of *H. influenzae* type b, a 40-kDa OMP was described previously as a diffusion porin with an exclusion size of ~1400 Da (19), which was then found to correspond to OmpP2 (16). Variations in OmpP2 porins of different non-typeable *H. influenzae* isolates were found to cluster in a surface-exposed protein domain (20). OmpP2 porins of different non-typeable *H. influenzae* isolates from antibiotic-treated cystic fibrosis patients have been collected and tested for antibiotic permeability and channel properties (21). The OmpP2 variants have different properties due to variation and accumulation of point mutations in distinct protein domains (21). In addition, it was shown that OmpP2 is involved in binding between bacterial cells and human nasopharyngeal mucin (22).

In this study, we show that OmpP2 has an additional propa-
Nucleotide Specificity of OmpP2 Porin of H. influenzae

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—The *H. influenzae* reference strain Rd (26) and the *H. influenzae* knock out mutant, PCR-conducted DNA fragments containing 500 bp of the immediate upstream sequence and 400 bp of the immediate downstream sequence flanking the *ompP2* gene were generated (see Fig. 1A). A synthesis of these PCR fragments was carried out according to Mullis and Faloona (26) using the following synthetic oligonucleotides (synthesized by MWG Biotech, Ebersberg, Germany): 2P2-3 (5'-CGCAAAGTACAGGGTTTTAC-3') and 3P2SbAI (5'-AAATCTGAAAGCAGGGCGATTGCAAAGA-3'); 5P2-5 (5'-CAGGTTTAATAGTATAGGC-3') and 5P2PstI (5'-AATGCAGCGCTCAATGATGTTCGTCGCC-3'), respectively. In addition, an 1-kbp cat gene, encoding chloramphenicol acetyltransferase, was obtained by fragment isolation via gel separation using QIAGEN-Quick (QIAGEN, Frankfurt, Germany) and was derived from plasmid pKat (27) digested with SsAI and PstI. A ligation reaction was performed with the two PCR DNA fragments (treated with restriction enzymes SsAI and PstI) and the cat gene fragment according to Maniatis et al. (28). The ligation mixture was used as template in PCRs utilizing oligonucleotides 2P2-3 and 5P2-5. As a result, a 1.9-kbp DNA fragment was amplified. Upon restriction analysis, this fragment was identified as a DNA fragment containing the *cat* gene flanked by 500 bp of *ompP2* upstream sequence and 400 bp of *ompP2* downstream sequence (data not shown). Next, this DNA fragment was used and transformed into *H. influenzae* strain Rd, which was grown to A900 = 2 (corresponding to 1.5 × 108 colony-forming units/ml) and used for the uptake of [14C]NAD, [14C]NMN, and [14C]NR, as described previously (13). To determine the Vmax and Km values for NAD and NMN uptake, substrate concentrations ranging from 0.1 to 8 mM were used, and wild-type and mutant *ompP2* strains were tested. For the wild-type strain, a Lineweaver-Burk fit by least-squares linear regression analysis could be applied for NAD and NMN uptake. The specific activity of [carbonyl-14C]NAD was 81,000 cpm/nmol. NNN and NMN uptake was monitored at time points ranging from 0.5 to 2.5 min and from 2 to 18 min, respectively.

**Uptake Studies—*H. influenzae* strain Rd and mutant strain Rd *ompP2*cat from overnight cultures were inoculated in BHI agar (3.8%) supplemented with NAD (10 µM) and hemin chloride (20 µg/ml) and grown to A900 = 2. Cells were then resuspended to A900 = 2 (corresponding to 1.5 × 108 colony-forming units/ml) and used for the uptake of [14C]NAD, [14C]NMN, and [14C]NR as described previously (13). To determine the Vmax and Km values for NAD and NMN uptake, substrate concentrations ranging from 0.1 to 8 mM were used, and wild-type and mutant *ompP2* strains were tested. For the wild-type strain, a Lineweaver-Burk fit by least-squares linear regression analysis could be applied for NAD and NMN uptake. The specific activity of [carbonyl-14C]NAD was 81,000 cpm/nmol. NNN and NMN uptake was monitored at time points ranging from 0.5 to 2.5 min and from 2 to 18 min, respectively.

**OmpP2 Extraction—**OmpP2 was isolated and purified starting from 1 liter of culture of the *H. influenzae* RD2 strain grown to A900 = 1 under the conditions described above. Cells were harvested by centrifugation at 8000 × g for 10 min in a Sorvall RC5 B centrifuge and washed once with buffer containing 100 mM NaCl. Subsequently, the cells were resuspended in 25 ml of 10 mM Hepes (pH 7.4) supplemented with protease inhibitor (Boehringer Mannheim, Germany) and were passed three times through a French pressure cell. Unbroken cells were removed by centrifugation at 8000 × g for 15 min. The supernatant containing the broken cells was portioned in 2-ml aliquots, and the cell membrane was pelleted by centrifugation in an Eppendorf centrifuge at 13,000 rpm for 30 min at 4 °C. The membrane preparation was carried out according to Carlone et al. (30). In brief, the cell membrane pellets were resuspended in 1% sodium N-lauryl sarcosinate and 10 mM Hepes (pH 7.4) and incubated for 30 min at room temperature to remove most of the soluble inner membrane components. The pellets of the subsequent centrifugation were washed once with 10 mM Hepes (pH 7.4) and resuspended in 50 µl of the same buffer supplemented with protease inhibitor (Bio-Rad). The probes contained 1 mg/ml protein from outer membrane components, including the channel-forming activity.

The probes were pooled, and the OM was pelleted by a subsequent centrifugation at 48,000 rpm for 30 min at 4 °C (Beckman Optima Ti-70.1 rotor). The pellet was resuspended in 2 ml of 0.05% Zwittergent 3-12 (Calbiochem) and 10 mM Tris-HCl (pH 8.0) and pelleted by centrifugation. The pellet was resuspended in 2 ml of the same buffer, and the mixture was centrifuged again. To solubilize OmpP2, the pellet was resuspended in 2 ml of 0.4% Zwittergent, 10 mM Tris-HCl (pH 8.0), and 10 mM EDTA and incubated for 30 min on ice. The OM was pelleted by centrifugation at 48,000 rpm for 30 min at 4 °C (Beckman Optima, Ti-70.1 rotor). The pellet was subjected to the same procedure again.

The supernatants of both extractions were pooled and used for preparative SDS-PAGE or subjected to anion-exchange chromatography.

**SDS-PAGE—**SDS-PAGE was performed according to Laemmli (31) as described previously (13). The gels were stained with Coomasie Brilliant Blue or silver. Preparative SDS-PAGE was used for identification and purification of the channel-forming activity from the extracts of OM fractions obtained by sodium N-lauryl sarcosinate treatment of the cell envelope. 15 µl of the 0.4% Zwittergent supernatant (protein concentration of ~0.2 mg/ml) were mixed with 7 µl of colorless sample buffer and added to one pocket of the preparative SDS-polyacrylamide gel. One lane of the gel was stained with Coomasie Blue to localize the different protein bands. Unstained lanes with different molecular mass ranges were cut into slices and eluted overnight with 10 mM Tris-HCl (pH 7.4) and 1% Glycerol X-100.

**Anion-exchange Chromatography—**The supernatant from the extraction with 0.4% Zwittergent was loaded onto a Q-Sepharose anion-exchange column (1-ml Hitrap Q, Amersham Biosciences, Freiburg, Germany). Bound OmpP2 protein was eluted by a linear NaCl gradient in 0.05% Zwittergent and 10 mM Tris-HCl (pH 8.0). The fractions were collected and checked for purity by SDS-PAGE. Fractions collected at 550 mM NaCl contained pure OmpP2 protein and were used for reconstitution in black lipid bilayer membranes.

**Determination of Conductivity and Titration Experiments with Nicotinamide Compounds—**The methods used for the lipid bilayer experiments have been described in detail previously (22). Black lipid bilayer membranes were obtained from the method described by Ong et al. (29). The transformed cells were plated onto BHI agar containing chloramphenicol. After 2 days, chloramphenicol-resistant *H. influenzae* colonies were observed and purified. Chromosomal DNA was prepared; and utilizing the chromosomal DNA as template and oligonucleotides 2P2-3 and 5P2-5, we verified the cat insertion on the chromosome by PCR analysis (data not shown). The data indicated that the cat gene had been acquired by double crossover and therefore had replaced *ompP2* on the chromosome (see Fig. 1).
was not satisfactory in all cases. This could be explained by the assumption either that the OmpP2 channels did not close completely when they were occupied by the different compounds or that only a fraction of the OmpP2 channels closed completely. In general, a much better fit was possible with Equation 2, which took this problem into account,

\[
\frac{G}{G_{\text{max}}} = \frac{1 - \frac{G_c}{G_{\text{max}}}}{K_c + 1} \quad (\text{Eq. 2})
\]

where \(G_c\) is the conductance at very high substrate concentration, i.e. the fraction of the conductance that did not respond to the nicotinamide compounds.

**RESULTS**

**Phenotype of H. influenzae ompP2::cat Mutants and Utilization of NAD, NMN, and NR**—An ompP2 deletion mutant was constructed by replacing ompP2 with the cat gene (see “Experimental Procedures”) (Fig. 1A). Expression of the 40-kDa OmpP2 protein was analyzed by SDS-PAGE of OMP preparations. As a result, we found that the OmpP2 band was missing in the constructed ompP2 knockout strain (Fig. 1B). Upon comparison of the growth of the ompP2::cat mutant strain on BHI agar and liquid medium supplemented with NAD, NMN, or NR, a general growth-deficient phenotype was observed, similar to that described previously (16). However, this attenuated growth rate could not be compensated by adding increasing concentrations of NAD or NMN and also did not differ in the presence of NR (data not shown). Therefore, a decreased diffusion of substrates such as NAD and NMN in an ompP2 mutant alone is not responsible for a reduced growth rate; thus, other relevant substrates are taken up across the OM via OmpP2.

However, by analyzing the uptake of \([^{14}\text{C}]\text{NAD}\), \([^{14}\text{C}]\text{NMN}\), and \([^{14}\text{C}]\text{NR}\), we found a significant uptake deficiency for NAD and NMN (Fig. 2, A and B). In contrast, if NR was used as substrate, the mutant ompP2 strain showed at least the same level of accumulation compared with the wild-type strain (Fig. 2C). In addition, we determined the \(V_{\text{max}}\) and \(K_m\) values for substrates NMN and NAD (Table I). For the wild-type strain, it was possible to obtain the absolute values according to Michaelis-Menten kinetics. In contrast, for the ompP2 mutant, no absolute values for \(V_{\text{max}}\) and \(K_m\) could be determined. This was because up to the highest possible amount of labeled substrate (8 \(\mu\)M), the uptake rates still increased linearly, and no saturation could be observed. This indicated that the concentration range used in the experiments was below the \(K_m\); and therefore, the Michaelis-Menten equation can be reduced to

\[
\frac{V}{V_{\text{max}}} = \frac{V_{\text{max}}}{K_m + [S]}
\]

indicating that the slope of the linear dependence between the uptake rate and the substrate concentration is \(V_{\text{max}}/K_m\). This procedure allowed quantitative comparison with the wild-type strain. We found that the \(V_{\text{max}}/K_m\) ratios were...
Conductivity of OM:\(\text{p}\)k of the Wild-type and Mutant ompP2 Strains—We studied the OM fractions of wild-type \(H.\) influenzae (strain Rd KW20) and the \(\text{p}\)mpP2 deletion mutant for channel formation in lipid bilayer membranes. The OMs were dissolved in buffer containing 10 mM Tris-HCl (pH 8) and 1% Genapol X-80. Lipid bilayer experiments demonstrated that the OM of the wild-type strain contained a channel-forming protein with a single-channel conductance of \(-1.6\) nS in 1 M KCl. Only small amounts of the dissolved membrane fraction had to be added for high reconstitution rates. In contrast, the probability for channel formation observed with the dissolved OM fraction of the \(\text{p}\)mpP2 deletion strain was very low. Here, the channel-forming protein with a conductance of 1.6 nS was absent. The majority of channels observed with the \(\text{p}\)mpP2 deletion strain had a single-channel conductance of \(-2.5\) nS in 1 M KCl. We conclude that OmpP2 protein is the origin of the 1.6-nS conductance steps.

Purification of OmpP2—Many porins expressed by enteric bacteria are firmly attached to the peptidoglycan layer (34), indicating that they can be isolated starting from the peptidoglycan-protein complex. OmpP2 represents an exception because it is only loosely associated with the peptidoglycan (19), and different solubilization and isolation procedures have been used starting from whole cells or the cell envelope (19, 35). To purify OmpP2, we started from the OM fraction obtained by \(N\)-lauryl sarcosinate treatment of the cell envelope. The OM pellet was resuspended twice in buffer containing 0.05% Zwittergent, followed by centrifugation. The supernatants from the pellet were subjected to the same procedure. The supernatants of both extractions contained high conductance steps in the lipid bilayer assay.

SDS-PAGE demonstrated that the 0.4% Zwittergent extracts contained OmpP2 as the major band (Fig. 3, lane 2). Final purification of OmpP2 was achieved by preparative SDS-PAGE or by anion-exchange chromatography. For purification by preparative SDS-PAGE, the supernatant was loaded onto a 10% SDS-polyacrylamide gel. Different molecular mass bands were excised and eluted overnight with 10 mM Tris-HCl (pH 8) and 1% Genapol X-80 at 4 °C. The fraction with the highest channel-forming activity contained pure OmpP2 protein essentially free of contaminants as proven by SDS-PAGE (data not shown). Alternatively, OmpP2 was purified by anion-exchange chromatography. The supernatant was loaded onto a HiTrap Q column, and a linear salt gradient was used to elute bound protein. Fractions collected at 550 mM NaCl contained pure protein, which was identified as OmpP2 by SDS-PAGE (Fig. 3, lane 3). OmpP2 protein purified by ion-exchange chromatography formed the same channels as Genapol X-80 extracts of the OM and the protein derived from preparative SDS-PAGE. This shows that purification by preparative SDS-PAGE does not affect the channel-forming ability of OmpP2.

Characterization of OmpP2—OmpP2 has been characterized previously in experiments using lipid bilayer membranes (36). To verify that the channel properties of purified OmpP2 used in this study are similar to those described in the previous work, we performed single-channel and multichannel measurements using lipid bilayer membranes from diphytanoylphosphatidylcholine/\(n\)-decane. The addition of pure OmpP2 at a very low concentration (100 ng/ml) to a black lipid membrane caused a substantial conductance increase over several orders of magnitude similar to that caused by other bacterial porins (37). Under conditions of appropriate amplification and low protein concentration, the recording of single events of pore integration into the membrane could be resolved as conductance steps (see Fig. 4 for a single-channel recording in 1 M KCl). Fig. 5 shows a histogram of all current fluctuations observed with OmpP2 in 1 M KCl. The data suggest that the current fluctuations are fairly homogeneous.
Ion Selectivity of OmpP2—The presumed low selectivity of OmpP2 was checked by multichannel experiments under zero-current conditions. For a 5-fold salt gradient, the potential on the more dilute side of the membrane was positive for KCl and potassium acetate, suggesting preferential movement of cations through the OmpP2 channel for these salts. However, the zero-current membrane potential was slightly negative using LiCl as electrolyte, suggesting preferential movement of chloride over lithium ions. The calculation of the permeability ratios of cations and anions through OmpP2 using the Goldman-Hodgkin-Katz equation (39) is given in Table III together with the zero-current membrane potential. The results suggest that the selectivity of the OmpP2 channel is indeed very low, in agreement with the study of Vachon et al. (36). The channel showed a small preference for cations over anions for KCl and potassium acetate ($P_0/P_+$ = 1.5 and 2.5, respectively), whereas it was slightly anion-selective for LiCl ($P_0/P_+ = 0.8$).

The OmpP2 Channel Binds NAD and NMN—The in vivo uptake experiments revealed that OmpP2 is necessary for the transport of certain nicotinamide compounds such as NAD and NMN, but not NR, across the OM. This result suggests that OmpP2 could be a channel specific for nicotinamide compounds containing a binding site for NAD and NMN in a similar way as LamB and ScrY are carbohydrate-specific channels with a binding site for malto-oligosaccharides (25, 40, 41). To test this hypothesis, we performed similar titration experiments as described previously for specific porins of Escherichia coli (24, 33). OmpP2 was reconstituted into lipid bilayer membranes. After the insertion process had slowed down considerably, concentrated solutions of different nicotinamide compounds were added to the aqueous solutions at both sides of the membrane while stirring to allow equilibration. Fig. 6 shows the experiment using NMN as a prospective substrate of OmpP2. Interestingly, the addition of NMN led to a dose-dependent block of OmpP2-mediated membrane conductance, which decreased by ~40% at 7.4 mM NMN.

The titration experiments with OmpP2 were analyzed in a way similar to that previously used with the carbohydrate-binding channels of Gram-negative bacteria (25, 33), which means that the stability constants could be calculated by a direct fit of the experimental data using Equation 1 or 2. Fig. 7 shows the fit of the experimental data of Fig. 6 using Equation 1 (dashed line), which resulted in a stability constant ($K$) of 108 $M^{-1}$ ($K_0 = 9.2$ mM). Unfortunately, the fit of the titration data of Fig. 6 using Equation 1 was not satisfactory, as the dashed line in Fig. 7 clearly indicates. This could be explained by the assumption either that OmpP2 did not close completely when it was occupied by NMN or that the OmpP2 probe contained another highly active channel-forming protein that could not be detected by SDS-PAGE and that did not respond to NMN.

![Fig. 4. Single-channel recording of a diphytanoylphosphatidylcholine/n-decane membrane in the presence of ~50 ng/ml OmpP2 from H. influenzae. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; $T = 20\, ^\circ\text{C}$.](image)

![Fig. 5. Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed from diphytanoylphosphatidylcholine/n-decane in the presence of 50 ng/ml OmpP2 from H. influenzae in different salt solutions.](image)

| Salt             | Concentration | $G$ (nS) |
|------------------|---------------|----------|
| KCl              | 0.1           | 0.10     |
| KCl              | 0.05          | 0.05     |
| KCl              | 0.05          | 0.10     |
| KCl              | 0.1           | 0.20     |
| KCl              | 0.3           | 0.60     |
| KCl              | 1.0           | 1.60     |
| KCl              | 3.0           | 4.00     |
| KCH$_3$COO (pH 7)| 0.1           | 0.10     |

| Salt             | $V_m$ (mV) | $P_0/P_+$ |
|------------------|------------|-----------|
| KCl              | 6.6        | 1.5       |
| LiCl             | −3.8       | 0.8       |
| KCH$_3$COO (pH 7)| 15         | 2.5       |

The membranes were formed from diphytanoylphosphatidylcholine dissolved in n-decane. The aqueous solutions were unbuffered and had a pH of ~6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20°$\text{C}$. The average single-channel conductance ($G$) was calculated from at least 80 single events.

**Table II**

Average single-channel conductance ($G$) of OmpP2 from *H. influenzae* in different salt solutions

| Salt             | Concentration | $G$ (nS) |
|------------------|---------------|----------|
| KCl              | 0.1           | 0.10     |
| KCl              | 0.05          | 0.05     |
| KCl              | 0.05          | 0.10     |
| KCl              | 0.1           | 0.20     |
| KCl              | 0.3           | 0.60     |
| KCl              | 1.0           | 1.60     |
| KCl              | 3.0           | 4.00     |
| KCH$_3$COO (pH 7)| 0.1           | 0.10     |

**Table III**

Zero-current membrane potentials ($V_m$) of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of OmpP2 from *H. influenzae* measured for a 5-fold gradient of different salts $V_m$ is defined as the difference between the potential at the dilute side (100 m$m$) and the potential at the concentrated side (500 m$m$). The aqueous salt solutions were buffered with 10 m$m$ Tris-HCl (pH 8); $T = 20\, ^\circ\text{C}$. The permeability ratio $P_0/P_+$ was calculated using the Goldman-Hodgkin-Katz equation (39) from at least three individual experiments.

| Salt             | $V_m$ (mV) | $P_0/P_+$ |
|------------------|------------|-----------|
| KCl              | 6.6        | 1.5       |
| LiCl             | −3.8       | 0.8       |
| KCH$_3$COO (pH 7)| 15         | 2.5       |
much better fit was possible with Equation 2, which took this problem into account. Fig. 7 shows also the fit of the data of Fig. 6 using Equation 2 (solid line). It was much better and yielded a stability constant \( K \) of 330 \( M^{-1} \) \( (K_S = 3.0 \text{ mM}) \). The average of six titration experiments of the same type yielded, on average, a stability constant of 260 \( M^{-1} \) \( (K_S = 3.8 \text{ mM}) \), and we found that 45% of the OmpP2-mediated conductance was not blocked by NMN (Table IV). As pointed out above, this could mean that ~45% of the initial conductance was ascribed to channels that did not react to NMN or, as a second possibility, that OmpP2 channels occupied by NMN could still conduct KCl at ~45% of the conductance in the open state (without NMN).

To investigate the two possibilities, we performed single-channel conductance experiments with OmpP2 in the presence of different concentrations of NMN. The results (summarized in Table V) indicate that OmpP2 does not contain a channel-forming impurity because the conductance of the 1.6-nS channel at 1 \( \mu \)M KCl and that of the 0.2-nS channel at 0.1 \( \mu \)M KCl were found to be dependent on the presence of NMN. Analysis of the single-channel conductance as a function of NMN concentration using Equation 2 resulted in a similar half-saturation constant as derived above from the titration experiments \( (K_S = 3.8 \text{ mM}) \). Interestingly, the ionic strength of the aqueous phase had a substantial effect on the binding of NMN to OmpP2. An increase in potassium chloride concentration from 0.1 to 1 \( \mu \)M resulted in a 2.6-fold increase in the half-saturation constant for NMN binding from 3.8 to 10.0 mM (Table IV). Again, the channels were not completely blocked by NMN and conducted by ~70% at very high concentrations of NMN, similar to the situation at 0.1 \( \mu \)M KCl. The dependence of NMN binding on the electrolyte concentration indicates a possible ion-ion interaction between OmpP2 and the substrate NMN.

We also performed current inhibition measurements with other nicotinamide compounds to investigate whether the interaction between NMN and OmpP2 is specific and if special structural requirements of the molecules are necessary for binding. As demonstrated above, the stability constant \( K \) for binding of these molecules to the binding site inside the OmpP2 channel was derived from a direct fit of the experimental data using Equation 2. The results for \( K \) and the half-saturation constant \( K_S (1/K) \) are listed in Table IV. They suggest that, besides NMN, also NAD binds to the binding site inside OmpP2. On the other hand, we did not observe binding of ATP, ADP, AMP, GTP, GMP, and NR to OmpP2 within the concentration range that could be used here. This indicates that the half-saturation constant for binding of these molecules, if it exists, is at least above 100 mM, which is above the physiologically relevant concentration.

The influence of the different nicotinamide compounds was also tested on the single-channel conductance of OmpP2 in 0.1 and 1 \( \mu \)M KCl. As described above, the conductance of the channel was decreased by the addition of NAD. The molecules, which had no effect in titration experiments, showed also no

---

**Table IV**

| \( K \) | \( K_S \) | Maximum inhibition of channel conductance |
|--------|--------|------------------------------------------|
| In 1 \( \mu \)M KCl | | |
| NMN | 100 | 10.0 | 40 |
| In 0.1 \( \mu \)M KCl | | |
| NMN | 260 | 3.8 | 55 |
| NAD | 125 | 8.0 | 70 |
| NADH | <10 | >100 | |
| NR | <10 | >100 | |
| ATP | <10 | >100 | |
| ADP | <10 | >100 | |
| AMP | <10 | >100 | |
| GTP | <10 | >100 | |
| GMP | <10 | >100 | |

**Table V**

| | \( G \) |
|----------------|-------|
| In 1 \( \mu \)K Cl | | |
| NAD, 9 mM | 1200 |
| NMN, 7.4 mM | 900 |
| In 0.1 \( \mu \)K Cl | | |
| NAD, 9 mM | 90 |
| NMN, 5 mM | 90 |
| NR, 9 mM | 200 |

**Fig. 6.** Titration of membrane conductance induced by OmpP2 of \( H. \) influenzae with NMN. The membrane was formed from diphytanoylphosphatidylcholine/\( n \)-decane. The aqueous phase contained ~100 ng/ml OmpP2 protein, 0.1 \( \mu \)M KCl, and NMN at the concentrations shown. The temperature was 20 °C, and the applied voltage was 20 mV. For further explanations, see “Results.”

**Fig. 7.** Fit of the results of the titration experiment shown in Fig. 6 with Equation 1 (dashed line) or with Equation 2 (solid line). The fit of the data using Equation 1 yielded \( K_S = 9.2 \text{ mM} \); that using Equation 2 yielded \( K_S = 3.0 \text{ mM} \) and \( G_S = 44\% \) of \( G_{\text{max}} \). For further explanations, see “Results.”
The growth of *H. influenzae* depends strictly on two growth factors: hemin and NAD (3). Regarding NAD, it is known that *H. influenzae* lacks genes encoding enzymes necessary for *de novo* biosynthesis of NAD (9); and therefore, this compound has to be imported by the cells. The mode by which NAD enters the cell, especially how it crosses the OM, was the focus of our study. In addition to NAD, also the uptake of NMN and NR was investigated because these substances can substitute for NAD.

All are hydrophilic molecules, which makes it unlikely that they cross the OM by diffusion through the lipid layer. Therefore, a hydrophilic pathway in the form of a pore-forming protein must exist, making possible the passage of these substances into the periplasmic space. The only pore-forming protein of *H. influenzae* described so far is the outer membrane protein OmpP2 (19). The three molecules NAD, NMN, and NR have molecular masses below the exclusion limit of OmpP2 of $\sim 1400$ Da and should be able to pass through the channel (19). To test the role of OmpP2 in the uptake of NAD, NMN, and NR, we constructed an *ompP2* deletion strain. Simple accumulation experiments showed that the uptake of NAD and NMN was significantly reduced in the deletion strain. For quantitative comparison of the uptake kinetics, we calculated $V_{\text{max}}$ and $K_m$. Although this was possible for the wild-type strain, the uptake of the *ompP2* deletion strain was so low that only values for the $V_{\text{max}}/K_m$ ratio could be determined. We found that this ratio decreased $\sim$10-fold in the *ompP2* mutant compared with the wild-type strain for NAD and NMN. This indicates that a loss of OmpP2 leads to a significant decrease in $V_{\text{max}}$ and/or increase in $K_m$. We cannot resolve which parameter is affected by the mutation.

So far, we have excluded the possibility that NAD nucleotidase or NMN phosphatase activities are associated with OmpP2 because the purified OmpP2 samples (data not shown) and whole OMP preparations of *hel* knockout mutants do not show either of these activities (15). Because the accumulation of NR in the *ompP2* deletion strain was comparable to that in the wild-type strain, we suggest that OmpP2 provides the hydrophilic pathway for NAD and NMN and that NR is able to cross the OM through a different or additional pathway, most likely through a yet not identified pore-forming protein. It should be mentioned that the knockout of *ompP2* strongly affects the growth rate of *H. influenzae* (16), even in the presence of NR in the growth medium (data not shown).

In wild-type bacteria, OmpP2 protein was expressed in high copy number, as it was responsible for one of the major bands on an SDS gel of OMP preparations. The knockout of OmpP2 indicates a strong reduction of the OM permeability concomitant with limited nutrient uptake. A reduced growth rate can be observed also in other Gram-negative bacteria with a knockout of major porins such as OmpF and OmpC in *E. coli* (42, 43). Lack of the major porins stimulates the cells to promote the increased expression of other pore-forming proteins (34). In the case of *H. influenzae*, this could be a pore-forming protein with the ability to transport NR across the OM, explaining the observed *ompP2*-independent uptake rate for NR. We observed pore activity of the OM extract from the *ompP2* knockout strain. Further characterization of the responsible proteins will be part of future studies.

Upon revealing that OmpP2 provides the hydrophilic pathway for NAD and NMN across the OM, we tested whether OmpP2 is a specific channel for these nicotinamide compounds. Therefore, we purified OmpP2 from *H. influenzae* Rd KW20 and reconstituted the porin in black lipid bilayers. Detergent extracts of the OM fractions showed a high reconstitution rate of pores with a single-channel conductance of 1.6 nS in 1 M KCl, which was not observed in OM extracts of the OmpP2-deficient strain. OmpP2 was further purified by preparative SDS-PAGE or ion-exchange chromatography. Preparative SDS-PAGE has been previously used for purification of other pore-forming proteins (44–46). One should mention that OmpP2 forms trimers, which are not stable in SDS. Therefore, only the monomeric form could be cut out of the gel. Nevertheless, this preparation was active, and pores showed no difference compared with those seen before SDS-PAGE. One can assume that OmpP2 trimers assemble properly *in vitro*, as shown previously and as also known for *E. coli* porin (47, 48). Alternatively, OmpP2 was also purified by ion-exchange chromatography to exclude that SDS treatment changes channel characteristics. Either purification protocol resulted in channels with the same characteristics, which are similar to those determined in a previous study (36). The single-channel conductance of OmpP2 measured in our study was a little higher than in the previous study, which could be explained by the use of different *H. influenzae* strains. The primary sequence of the different OmpP2 proteins varies within the region of loop 4, which is known to influence single-channel conductance (49). The reconstituted pores were voltage-independent up to a range of $\pm 100$ mV, and single-channel conductance showed a linear dependence on the electrolyte concentration, revealing that there is no ion-binding site inside the channel. Concerning ion selectivity, the OmpP2 pores showed a small preference for cations over anions for KCl and potassium acetate ($P_{\text{Ka}} / P_{\text{Pa}} = 1.5$ and 2.5, respectively) and were slightly anion-selective for LiCl ($P_{\text{Ka}} / P_{\text{Pa}} = 0.8$). Compared with the partially highly cation-selective general diffusion pores of enteric bacteria such as *E. coli* and *Salmonella typhimurium* with $P_{\text{Ka}} / P_{\text{Pa}}$ ratios between $-4$ and $-20$ for KCl, the selectivity of OmpP2 porin is not very pronounced (40, 50). Although, in enteric bacteria, the OM acts as a filter against negatively charged harmful compounds such as bile salts, OmpP2 porin might not select passing compounds according to their charge. This is underscored by the observation that the OM of *H. influenzae* shows a higher permeability for anionic $\beta$-lactam antibiotics than the OM of *E. coli* (51). The two substrates NAD and NMN, which enter the periplasmic space via OmpP2, carry no net charge, but are zwitterionic with a positively charged nicotinamide and a negatively charged phosphate group. The addition of these substances to a membrane with reconstituted OmpP2 channels resulted in a dose-dependent decrease in the membrane conductance. This indicates that NAD and NMN block the flow of the electrolyte by binding reversibly to a binding site inside the channel. Thus, it is possible to perform titration experiments similar to those performed to study the malto-oligosaccharide binding of carbohydrate-specific channels and to calculate the stability constant of the binding process. The binding constant of NMN was double that of NAD (260 and 125 $\text{M}^{-1}$ in 0.1 M KCl, respectively), corresponding to half-saturation concentrations of 3.8 and 8.0 mM, respectively. Compared with the binding of malto-oligosaccharides to sugar-specific channels (e.g. the binding constant ($K$) for binding of maltopentaose to maltoporin is $\sim 14,000$ $\text{M}^{-1}$) (24), these values appear to be low. One has to consider that the number and strength of interactions between the protein and the substrates determine the stability constant. In the case of maltoporin, the stability constant for maltose binding ($K_{\text{maltose}} = 110 \text{ M}^{-1}$) is 2 orders of magnitude lower compared with that for maltopentaose binding, and that for glucose binding is even lower ($K_{\text{glucose}} = 9.5 \text{ M}^{-1}$) (24). An important question that results from these studies is what parts of the substrates are involved in binding to the binding site of OmpP2? Comparing the structures of NAD and NMN, one has to assume that the adenosine monophosphate group of
the NAD molecule does not contribute to the binding. Otherwise, one would expect a higher stability constant for NAD compared with NMN. This was confirmed using AMP as substrate for titration experiments. No binding of AMP was observed, as the conductance remained unchanged up to 100 mM AMP in the aqueous solution. Also, ADP and ATP had no effect, showing that a nicotinamide ribosyl group is necessary for binding. However, titration experiments with NR failed, revealing that this group is not able to interact with the binding site. This shows that a combination of the nicotinamide ribosyl group and the phosphate group may be necessary to become a target for the specific binding site of OmpP2. Both groups are charged, and one can assume that ionic interactions play a role in the binding of the substrates to the binding site.

By measuring the binding affinities of NMM in different electrolyte concentrations, we showed that binding depends on the ionic strength of the aqueous solution. Binding was significantly reduced in 1 × KCl compared with that in 100 mM KCl (260 and 100 mM, respectively). Charges (which might be involved in the binding process) are shielded more at higher ionic strength. This reduces the strength of interaction between the binding site and substrate and leads to a lower binding constant. Another indication that point charges are involved in binding is the observation that NAD in its reduced form (NADH) lacking the positive charge at the nicotinamide group did not bind to OmpP2.

In contrast to the carbohydrate-specific porins, binding of NAD and NMN to OmpP2 does not completely block the passage of electrolytes. The remaining conductance is the result of partially blocked OmpP2 channels and not of impurities, as we showed by single-channel measurements in the presence of substrate. The maximal percent inhibition depends of the size of the substrate. In the case of NMM (334.2 Da), the conductance is reduced by 55%; for NAD (663.4 Da), this value is 70%. One can imagine that NMM, when bound to the binding site, does not block the channel constriction completely and leaves a gap for ions passing through. The additional adenosine monophosphate group in the NAD molecule leads to a further reduction of the remaining open channel diameter, explaining the higher maximal inhibition of the channel conductance.

The in vitro uptake study revealed that NMM is taken up somehow faster than NAD (Fig. 2, A and B). For NMM, the \( V_{\text{max}} \) is 0.59 pmol min \(^{-1}\) (3.5 × 10\(^{9}\) colony-forming unit/ml), and the \( K_m \) is 6.4 μM, compared with 0.13 μM min \(^{-1}\) and 5.5 μM measured for NAD, respectively. In a previous study, it was calculated and experimentally proven that, at low substrate concentrations, the OM is rate-limiting for uptake (52). The substrate concentration in our uptake experiment was 1 μM; thus, it is possible that passage through OmpP2 is the limiting step for NAD and NMN uptake. The stability constants for NAD and NMN binding to OmpP2 measured by \( \text{in vitro} \) titration experiments reflect the ratios of the on- and off-rates of the binding process. We assume that the off-rates of the NMM- and NAD-binding processes, reflecting the strength and duration of binding, are similar because interaction between the substrates and the binding site should be the same. Therefore, the difference in stability constants must be a result of different on-rates for NAD and NMN. This is in good agreement with the uptake study because, at low concentrations, uptake depends on the on-rate, and the binding constant and the uptake rate of NMN are higher than those of NAD.

In conclusion, we have shown that OmpP2 reveals the hydrophilic pathway for NAD and NMN into the periplasmic space. Uptake of these vital substances through OmpP2 seems to be further improved by the presence of a binding site inside the channel. Our study is the first step in the further characterization of the binding site in the future.
Porin OmpP2 of *Haemophilus influenzae* Shows Specificity for Nicotinamide-derived Nucleotide Substrates

Christian Andersen, Elke Maier, Gabrielle Kemmer, Julia Blass, Anna-Karina Hilpert, Roland Benz and Joachim Reidl

*J. Biol. Chem. 2003, 278:24269-24276.*

doi: 10.1074/jbc.M213087200 originally published online April 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213087200

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 20 of which can be accessed free at http://www.jbc.org/content/278/27/24269.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 24, 2018