Identification of the High-affinity Substrate-binding Site of the Multidrug and Toxic Compound Extrusion (MATE) Family Transporter from *Pseudomonas stutzeri*[^5]

Laiyin Nie[^1], Ernst Grell[^2], Viveka Nand Malviya[^3], Hao Xie[^3], Jingkang Wang[^4], and Hartmut Michel[^5]

From the ^4^Tianjin University, School of Chemical Engineering and Technology, State Key Laboratory for Chemical Engineering, Collaborative Innovation Center of Chemical Science and Chemical Engineering, 300072 Tianjin, China and the ^6^Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, Max-von-Laue-Strasse 3, 60438 Frankfurt am Main, Germany

Multidrug and toxic compound extrusion (MATE) transporters exist in all three domains of life. They confer multidrug resistance by utilizing H^+^ or Na^+^ electrochemical gradients to extrude various drugs across the cell membranes. The substrate binding and the transport mechanism of MATE transporters is a fundamental process but so far not fully understood. Here we report a detailed substrate binding study of NorM_Ps, a representative MATE transporter from *Pseudomonas stutzeri*. Our results indicate that NorM_Ps is a proton-dependent multidrug efflux transporter. Detailed binding studies between NorM_Ps and 4,6-diamidino-2-phenylindole (DAPI) were performed by isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and spectrofluorometry. Two exothermic binding events were observed from ITC data, and the high-affinity event was directly correlated with the extrusion of DAPI. The affinities are about 1 μM and 0.1 mM for the high and low affinity binding, respectively. Based on our homology model of NorM_Ps, variants with mutations of amino acids that are potentially involved in substrate binding, were constructed. By carrying out the functional characterization of these variants, the critical amino acid residues (Glu-257 and Asp-373) for high-affinity DAPI binding were determined. Taken together, our results suggest a new substrate-binding site for MATE transporters.

The ability of all living organisms to protect themselves against toxic substances is essential for their survival. Organisms acquire this ability through evolutionary force (1). Due to the development of scientific knowledge, more and more medical drugs are being developed and applied. As a consequence, general defense mechanisms to resist antibiotics, drugs, and other toxic compounds have been evolved in bacteria (2). Being one of those defense mechanisms, the active extrusion of the toxic compounds from the cells plays an important role (3). In bacteria, the extrusion is often carried out by multidrug transporters, which are integral membrane proteins. They are classified into five distinct families: ATP-binding cassette, multidrug and toxic compound extrusion (MATE),[^4] major facilitator superfamily, resistance nodulation division, and small multidrug resistance transporters (4).

In 1999, the MATE family proteins were first recognized as a new group of the multidrug transporters, existing in all three domains of life (5). Most members of this family consist of a single chain of 450–550 amino acid residues and exhibit 12 putative transmembrane helices (TMHs). Up to now, about 900 proteins have been annotated as MATE transporters on the basis of amino acid sequence similarities. Moreover, a subclassification of the MATE family has been suggested, NorM-, DinF- (DNA damage-inducible protein F), and eukaryotic subfamily (4, 6, 7). Various compounds can be recognized by NorM proteins, including dyes, fluoroquinolones, and aminoglycosides (7). As secondary active transporters, MATE proteins utilize transmembrane Na^+^ or H^+^ electrochemical gradients to extrude toxic compounds.

So far atomic structures of four MATE transporters have been reported in their putative outward-facing states, in both drug-bound and/or drug-free states. NorM_VC from *Vibrio cholerae* and NorM_NG from *Neisseria gonorrhoeae*, are both Na^+^ driven, whereas PfMATE from *Pyrococcus furiosus* and DinF_BH from *Bacillus halodurans* are published to be H^+^ dependent antiporters (8–11). All these four proteins contain 12 TMHs with intracellular N and C termini, and possess a hydrophobic internal cavity, formed by two symmetric bundles with 6 TMHs each. The two bundles are linked by a cytoplasmic loop between the 6th and 7th TMH. All MATE proteins share about 40% sequence similarity, and they employ the same rocker-switch mechanism for substrate extrusion (12). In addition, the crystal structures have also revealed different locations of cation- and substrate-binding sites, suggesting a mechanistic diversity among MATE transporters. In the structure of drug-bound NorM_NG, the binding site is located close to the membrane-periplasm interface, whereas in the structure of PfMATE, the

[^1]: Supported by the China Scholarship Council and the Max Planck Society.
[^2]: To whom correspondence may be addressed. Tel.: 86-2227405754; Fax: 86-2227374971; E-mail: wangjkch@tju.edu.cn.
[^3]: To whom correspondence may be addressed. Tel: 49-6963031001; Fax: 49-6963031002; E-mail: hartmut.michel@biophys.mpg.de.
[^4]: The abbreviations used are: MATE, multidrug and toxic compound extrusion; TMH, transmembrane helix; TM, transmembrane; β-DMM, β-dodecyl β-D-maltoside; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; CV, column volume(s); SEC-MALS, size-exclusion chromatography coupled to multi angle light scattering; Bistris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; MIC, minimal inhibitory concentration.
Substrate-binding Site of a MATE Transporter

FIGURE 1. Expression and purification of NorM_PS and its variants. a, Western blot of small scale expression screening of NorM_PS and its variants in a E. coli KAM32 cell. The empty plasmid was used as negative control; induction of the WT as positive control. The samples were made out of 150 μl of cell culture at A600 = 2. b, 10 μg of purified NorM_PS was loaded onto a 4–12% SDS-polyacrylamide gel, stained by Coomassie. c, analytical gel filtration profile of purified NorM_PS using a Superdex 200 column in the NorM_PS sample buffer.

The Oligomeric State of NorM_PS—Despite the difficulties in analyzing the oligomeric state of membrane proteins in detergent solution, it is essential to know this parameter for NorM_PS to determine the quantitative thermodynamic parameters. In this study, we used size-exclusion chromatography coupled to multangle laser light scattering (SEC-MALS) to study the oligomeric state of NorM_PS, because this method has been successfully applied on many membrane proteins (14–19). Using a Superdex 200 column, the protein was eluted at 13.35 ml and showed a homogenous single peak detected by all three detectors (Fig. 2). The weight-averaged molecular mass was calculated based on the measured ΔLS (light scattering) and C (the concentration of the protein) values (20). The molecular mass of NorM_PS in detergent solution was determined to be 54.7 kDa, which corresponds well to the theoretical monomeric mass of 53.0 kDa. Surprisingly, the amount of detergent associated with NorM_PS was quite high, the mass ratio between β-DDM and NorM_PS (6) was 2.82. This could be attributed to the high flexibility of NorM_PS, so that more detergent is needed to stabilize the protein in solution.

Results

Production and Purification of NorM_PS and Its Variants—Expression of NorM_PS could be detected in the cell lysates by Western blot analysis with the anti-polyhistidine antibody. The protein band of NorM_PS was found in the gel between 30 and 40 kDa (Fig. 1a). Of all the tested variants (D38N, E257A, E257Q, E257D, and D373N), E257A showed an undetectable expression level under our standard conditions (Fig. 1a), and all other variants could be produced in KAM32 cells with similar amounts as the wild-type (WT) protein.

NorM_PS was purified using a combination of Ni2+-affinity and size-exclusion chromatography in the presence of n-dodecyl β-D-maltoside (β-DDM). A single peak in the size exclusion chromatography indicated that NorM_PS could be purified to homogeneity (Fig. 1c); the corresponding Coomassie-stained SDS-PAGE gel demonstrated a relatively high purity (Fig. 1b). A typical purification yielded up to 2.5 mg of NorM_PS from 12 liters of cell culture. Among the tested variants, D38N, D373N, and E257D could be purified to a homogeneous state as the WT, whereas E257Q could not be isolated using our standard procedure, suggesting this residue is likely to play a critical role in maintaining the stability of NorM_PS.

Drug Resistance of NorM_PS—NorM_PS is annotated to be a multidrug transporter based on general sequence alignment. MIC tests were carried out to identify its potential substrates. The obtained MIC results are listed in Table 1. Elevated MIC values were observed for DAPI, doxorubicin, daunorubicin, tetracycline, kanamycin, and gentamycin, implying that Escherichia coli KAM32 cells harboring NorM_PS exhibited a higher resistance to those toxic compounds. Among all tested compounds, the highest

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relative resistance was observed for DAPI, so DAPI was chosen as a suitable and interesting substrate for further studies.

In this work, three residues (Asp-38, Glu-257, and Asp-383) were changed by mutagenesis, because they are acidic, located in the TM region (D38N in the N-bundle, E257D and D373N in the C-bundle), and are highly conserved within the NorM subfamily. As an initial screen, the MIC test was carried out for all 5 variants in the presence of DAPI. The extrusion of DAPI was totally abolished for these variants, because the MIC values were the same as the negative control, suggesting the critical role of these three residues.

**Cation Specificity of DAPI Antiport**—To obtain information on the driving force for the active extrusion, MIC tests were carried out using LB medium in the absence of NaCl and in the presence of DAPI. For control cells (KAM32/pBADA2 vector and pBADA2-NorM_PS), the MIC value of DAPI in LB medium was 0.25 μg/ml. For NorM_PS-containing membrane vesicles elicited by DAPI, which clearly suggested the DAPI/H^+ antiport (open arrow). The excitation and emission wavelength are 495 and 530 nm, respectively.

To confirm the ion specificity for H^+, a DAPI/H^+ antiport assay employing everted membrane vesicles was implemented. Acridine orange was chosen as a probe to monitor H^+. Because this fluorescent dye is membrane permeable in its deprotonated form and, if a pH gradient is established, will accumulate in their protonated form and get quenched on the side of the membranes where the pH is lower (21). In a typical measurement, upon addition of lactate (solid arrow) in Fig. 3, the respiration was initiated and thus a pH gradient (acidic inside) was generated as monitored by the quenching of the fluorescence. The addition of DAPI (open arrow) in Fig. 3 caused an increase in fluorescence intensity (red dots). Using normal LB medium containing NaCl, both control and test cells could withstand DAPI concentrations up to 0.5 and 2 μg/ml (Table 2). The reduced absolute MIC values in minimal medium could be the result of insufficient nutrients. However, the relative resistance between control and test cells in both media showed no dependence on the presence/absence of Na^+; excluding the possibility of considering Na^+ as the counter ion of toxic compound extrusion.
formed at pH 7.4 and at constant ionic strength in the temperature range between 10 and 85 °C. The protein samples exhibited an irreversible endothermic transition at around 53 °C. In the absence of ligand (solid line in Fig. 4), a very broad, almost symmetric transition of NorM_PS with a maximum at 53.5 °C was recorded. In the presence of 1 mM DAPI, the transition temperature was increased about 3 °C on average (Table 3). A very characteristic change, however, was the marked decrease of the width of the protein transition in the presence of DAPI (Fig. 4, Table 3), observed for all protein samples. The small change in thermal stability as indicated by the increase of transition temperature and the marked decrease of the transition width provides strong evidence for DAPI binding to NorM_PS.

Spectrofluorometric Titration—DAPI is twice positively charged at physiological pH and has been widely applied for DNA detection in biology due to its fluorescent properties (22). Besides, the interaction between DAPI and negatively charged proteins and lipid vesicles has been investigated (23, 24). In general, DAPI binding was associated with a blue shift of the emission spectrum and a marked increase of the emission intensity. There are two different rotamers of DAPI in solution and only one undergoes an intramolecular proton transfer, from the 6-amidinium group to the indole aromatic ring, in the excited singlet state of DAPI alone (23). However, upon binding to BSA and DNA or when dissolved in DMSO, this transfer does not occur and consequently causes a large increase of fluorescence (23).

In this study, we observed that the fluorescence intensity of DAPI increased upon the addition of NorM_PS (Fig. 5a), which we attribute to binding. Meanwhile, the excitation maximum of DAPI at 345 nm exhibited a 14-nm red shift in the presence of NorM_PS, whereas the main emission maximum at 465 nm was blue shifted.

**TABLE 3**

DSC data (mean values) of NorM_PS in presence and absence of DAPI

| C<sub>DAPI</sub> (mM) | T<sub>m</sub> (°C) | ΔH (kcal mol<sup>−1</sup>) | WHMH<sup>a</sup> (°C) | n<sup>b</sup> |
|---------------------|--------------------|-------------------|------------------|--------|
| 0                   | 52.5 ± 2.0         | 39.5 ± 4          | 125 ± 1.0        | 6      |
| 0.075               | 55.0 ± 0.5         | 43.0 ± 4.5        | 9.5 ± 0.8        | 3      |
| 1.1 ± 0.1           | 55.0 ± 0.5         | 45.0 ± 4.5        | 8.5 ± 0.5        | 5      |

<sup>a</sup> WHMH, width of transition at half-maximum height.

<sup>b</sup> n, number of experiments performed.

**FIGURE 4.** DSC profile of NorM_PS (25 μM) in the absence (solid line) and presence of 75 μM (dot line) as well as 1.0 mM (dash line) DAPI in 20 mM HEPES-NaOH, pH 7.5, adjusted with sodium lactate to total ionic strength of 20 mM. Transition temperatures, enthalpy changes, and widths at half-maximum height are: 53.5 °C, 40.0 kcal mol<sup>−1</sup>, 13.5 °C; 54.5 °C, 41.5 kcal mol<sup>−1</sup>, 10.0 °C; and 56.0 °C, 42.0 kcal mol<sup>−1</sup>, 8.5 °C, respectively.

**FIGURE 5.** Spectrofluorometric titration of 0.5 μM DAPI in the presence of NorM_PS and its variants. a, the concentrated protein sample (up to 250 μM) was titrated into 20 mM HEPES-NaOH, 0.025% (w/v) β-DDM, 0.5 μM DAPI, 11 mM sodium lactate, pH 7.4. The titration experiments were repeated three times. b, excitation (E<sub>e</sub> = 450 nm) and c, emission (E<sub>e</sub> = 360 nm) spectra of DAPI in the absence (black) and presence of 50 μM different proteins.
by 25 nm (Fig. 5, b and c). The fluorescence changing and shifting also clearly indicate binding of DAPI to NorM_PS.

To determine the binding affinity, spectrofluorometric titrations were carried out (Fig. 5a). This determination was done under conditions where the total absorbance at the excitation wavelength (360 nm) did not exceed 0.09 per 0.5-cm light path, which allowed neglecting of the 1st order filter effect. At concentrations of NorM_PS above 10 μM, the titration exhibited a tendency of saturation. An evaluation of four titrations based on a 1:1 stoichiometry provided a mean dissociation constant (K_d) of 3.5 ± 1.0 μM (Fig. 5a), which corresponded well to the high-affinity binding observed by the ITC measurements (see the ITC results below for details).

The DAPI binding study was carried out with variants D38N, E257D, and D373N. Being negatively charged in their deprotonated form and located halfway in the TMHs, these residues could also be involved in DAPI binding. Within the same concentration range, E257D and D373N behaved differently from the WT protein. The blue shift in the emission spectra decreased from 25 to 13 nm. Apart of this smaller spectral shift, the emission was smaller than for the WT protein (Fig. 5c). The determination of a K_d value was not possible due to the absence of an apparent saturation (up to 60 μM). This observation likely indicates that both variants, E257D and D373N, are still somehow able to bind to DAPI but not with high affinity.

In contrast to E257D and D373N, the excitation and emission maxima of DAPI in the presence of D38N were similar to those of the WT protein and the titration also exhibited saturation behavior. The calculated K_d value of D38N was 4.9 ± 1.0 μM (3 titrations). On the other hand, the emissions of titrations with D38N were significantly lower than in the case of the WT protein (Fig. 5a). This observation could be due to a change within the local surrounding of the fluorophore when bound to D38N.

Isothermal Titration Calorimetry—To achieve more insights into DAPI binding to NorM_PS and the related energetic aspects, a detailed ITC study was carried out at 25 °C in 20 mM HEPES-NaOH, 0.025% (w/v) β-DDM, pH 7.4, I = 20 mM (sodium lactate). The general titration behavior is shown in Fig. 6a. Here, the dominating and resolved exothermic titration process was characterized by a K_d value not possible due to the absence of an apparent saturation (up to 60 μM). This observation likely indicates that both variants, E257D and D373N, are still somehow able to bind to DAPI but not with high affinity.

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of about 0.1 mM. However, the 1st full DAPI injection, following “m,” exhibited a surprisingly large heat change (Fig. 6a), which may be assigned to an unresolved, additional exothermic binding process of higher affinity.

Another unexpected observation was made during the starting phase of reference titrations “R” (Fig. 6b, full additions 1 to 14). Reference titrations are usually expected to show only comparatively narrow signals of similar magnitude due to the quickly equilibrating heat changes. The observed exothermic, broad signal could be attributed to a chemical process such as a de-aggregation of DAPI upon dilution. This process was independent of the presence of detergent in the buffer, its ionic strength, and the stirring rate (data not shown). If this process is assigned, for example, to a dissociation of DAPI dimers, the related dimer/monomer dissociation constant is around 25 μM and the enthalpy change around −2 kcal mol⁻¹, a result of a separate investigation. Ligand dilution together with the de-aggregation process contributed also to the titrations in the presence of NorM_PS (Fig. 6, b and c), which are assumed to be additive for the purpose of evaluation. For this study, the aggregated form of DAPI is considered as reference state for the evaluations. Nevertheless, we assume that only DAPI monomers are bound to NorM_PS and its variants. The thermodynamic parameters resulting from our evaluations are apparent ones, characteristic for our experimental conditions.

For the WT protein titration, an exothermic binding of DAPI to NorM_PS with an affinity higher than 0.1 mM (Fig. 6a) was resolved by carrying out titrations with lower DAPI concentration and by adding different ligand volumes (Fig. 6b). The titration in Fig. 6b represents a combination of two successive titrations using the same titrant concentration, combined using the program CONCAT. To subtract the reference titration data, two different modes were applied: (i) by subtracting the non-uniform reference titration in the integrated form (Fig. 6c) and (ii) by subtracting a nearly constant value characterizing merely the mean heat of dilution. The resulting heat changes (Δq) in dependence of the DAPI/protein concentration ratio, obtained by employing both subtraction modes i and ii, were applied for final evaluations (Fig. 6d). Both resulting point sequences clearly revealed two distinct binding phases, a very steep phase characterizing high-affinity and a gradually growing one characteristic of low-affinity binding. The evaluation method to obtain thermodynamic parameters was based on the “two binding sites” model, in which two types of independent sites for high- and low-affinity binding were taken into consideration. Even if N1 (stoichiometric coefficient for high-affinity binding) and N2 (stoichiometric coefficient for low-affinity binding) were set equal 1, still at least four parameters (the dissociation constants K₁ and K₂, and the enthalpy changes ΔH₁ and ΔH₂), had to be determined. No direct fits could be achieved under such circumstance using the standard evaluation program. Therefore, a partial analysis of the high- and low-affinity parts of the titration in Fig. 6d was carried out separately, with N1 and N2 fixed to 1. Subsequently, the obtained parameters were used to do simulations related now to the complete titration. The resulting parameters finally could be used as suitable starting values to achieve possible solutions.

For the titration in Fig. 6d, high-affinity binding (1:1 complex) is characterized by a Kₐ value around 0.95 μM and a ΔH₁ value of about −7.2 kcal mol⁻¹, and a very low value for ΔS₁. Cricit entropy is not considered in this study. For low-affinity binding (N2 = 1), K₂ and ΔH₂ values of around 0.13 mM and −78 kcal mol⁻¹, respectively, were obtained. This ΔH₂ value is extremely high for the binding of a single ligand. We assume therefore, for low-affinity binding, that at least two DAPI molecules were bound per NorM_PS (N₂ = 2), leading to a ΔH₂ value of −39 kcal mol⁻¹. The complete set of parameters including errors obtained for the titration shown in Fig. 6d is given in Table 5. All subsequent low-affinity data for the WT protein and its variants were given for N₂ = 2. The mean values of the thermodynamic parameters, based on subtraction mode i, resulting from several independent experiments are listed in Table 4. The reference subtraction mode i was considered to be more realistic due to the chemical process of DAPI de-aggregation, so it was used for all the data analysis. Nevertheless, adopting mode ii, similar results were obtained (Table 5).

It is important to determine experimentally the value of the stoichiometric coefficient N₁ for high-affinity DAPI binding. Therefore, a detailed high-affinity titration with 12 μM NorM_PS up to a DAPI/protein concentration ratio of about 5.5 was carried out (Fig. 6e). The evaluation could only be successfully performed when ΔH₁ was fixed, and a value of 0.91 was resulted for N₁. The number of −9 kcal/mol for ΔH₁ was used based on a simulation performed on this dataset. This supports our earlier assumption that for high-affinity binding a 1:1 DAPI-protein complex is formed under saturating conditions. The initially assumed N₁ value of 1 will from now on be used for all subsequent calculations.

If the high-affinity DAPI binding is dominated by electrostatic interactions, an increase of the ionic strength should lead
to an increased shielding of the involved charges. Therefore, high-affinity binding would be expected to be weaker at high ionic strength (>20 mM). This expectation was indeed confirmed qualitatively by the result of our titration at I = 100 mM (sodium lactate), given in Table 4 (titration not shown).

The calorimetric titration was performed with the three variants, D38N, D373N, and E257D. The titration pattern of D38N is very similar to that of the WT protein, the two characteristic phases of exothermic high- and low-affinity binding were clearly observed (Fig. 7a). The resulting thermodynamic parameters (Table 4) are close to those of the WT protein, only the $K_a$ value is slightly higher. The masking of the negative side chain charge of Asp-38 did not markedly influence the binding behavior, indicating that Asp-38 is not directly involved in high-affinity DAPI binding.

Unlike D38N, in the case of the variant D373N, the characteristic initial, strong exothermic high-affinity binding almost disappeared, whereas the low-affinity binding still remained (Fig. 7b). For data evaluation, proper fit could be achieved by using either fixed $K_a \Delta H_1$ or $\Delta H_1$ value (solid line in Fig. 7b). The $K_a \Delta H_1$ value is higher than that in the case of the WT and D38N, and the binding became entropy driven due to the positive $\Delta H_1$ and $\Delta S_1$ values; for low-affinity DAPI binding, the mean parameters essentially correspond to those mentioned before (Table 4). However, the contribution of the low-affinity binding below the DAPI/protein concentration ratio around 12 was missing because the first 10 equal DAPI additions led to heat changes of similar magnitude (inset in Fig. 7b). This unexpected point sequence can be attributed to a superposition of an endothermic binding process and the initial part of the exothermic low-affinity binding process. As a consequence, Asp-373 can be assumed to be involved in DAPI binding.

For E257D, the combination of two titrations showed a behavior close to that of D373N (Fig. 7c), and the calculated

| Protein (Figure) | $K_a$ | $\Delta H_1$ | $\Delta S_1$ | $K_a$ | $\Delta H_2$ | $\Delta S_2$
|------------------|-------|-------------|-------------|-------|-------------|-------------|
| WT (Fig. 6d)     | 1.1 ± 0.4 | -7.2 ± 0.8 | 3.5         | 7.9 ± 1.4 | -39 ± 4.1   | -114        |
| D38N (Fig. 7a)   | 0.4 ± 0.2 | -3.0 ± 0.7 | 16          | 9.5 ± 1.5 | -40 ± 3.2   | -117        |
| D373N (Fig. 7b)  | 0.05 ± 0.08 | 2           | 28          | 4.4 ± 2.2 | -41 ± 2.2   | -122        |
| E257D (Fig. 7c)  | 0.06 ± 0.007 | 6           | 44          | 4.6 ± 1.0 | -90 ± 10    | -280        |

Data were evaluated using reference subtraction mode i, all the other experiments in this table were evaluated using this mode.

Data were evaluated using reference subtraction mode ii.

a

b

c

D38N

D373N

E257D

FIGURE 7. DAPI binding to variant D38N and D373N by ITC at 25 °C in the medium of Fig. 6. a, combined titration of 4 μM D38N with 0.5 mM DAPI and corresponding reference titration $R$ (offset shifted) as shown in the inset; heat change after subtraction of reference titration plotted versus concentration ratio and evaluated on the basis of model NSe (parameter list in Table 5 and mean values of 2 measurements are listed in Table 4). Injection was 3 μl (m) + 12 × 6 μl + 11 × 18 μl, 3 μl (m) + 15 × 18 μl. b, combined titration of 6.0 μM D373N with 0.8 mM DAPI and corresponding reference titration $R$ (offset shifted) as shown in the inset; heat change after subtraction of reference titration plotted versus concentration ratio and evaluated on the basis of model NSe (parameter list in Table 5 and mean values of 2 measurements are listed in Table 4). Injection was 3 μl (m) + 9 × 3 μl + 16 × 15 μl, 3 μl (m) + 17 × 15 μl. c, combined titration of 4 μM E257D with 0.5 mM DAPI and corresponding reference titration $R$ (offset shifted) as shown in the inset; heat change after subtraction of reference titration plotted versus concentration ratio (parameter list in Table 5 and mean values of 2 measurements are listed in Table 4). Injections were 3 μl (m) + 12 × 6 μl + 11 × 18 μl and 3 μl (m) + 15 × 18 μl.
thermodynamic parameters were also similar (Table 4), only that the enthalpy and entropy changes for the low-affinity binding were larger than those for D373N. This observation may be due to the fact that a single binding equilibrium related to only one dissociation constant may not be suitable to describe low-affinity ligand binding to E257D quantitatively. However, similar titration behavior as D373N regarding the high-affinity binding might indicate a direct participation of Glu-257 in high-affinity DAPI binding.

**Discussion**

**Physicochemical and Biochemical Aspects**

*NorM_PS Is a Multidrug/H+ Antiporter*—In this study, we characterized a multidrug transporter from *P. putida*. The drug-hypersusceptible *E. coli* strain KAM32 overexpressing NorM_PS displays increased resistance to 6 toxic compounds, which demonstrates that the protein functions as a toxin extruder in *vivo*. NorM_PS exhibits a moderate level of cellular resistance against substrates (2–4-fold), which is in accordance with former studies on MATE transporters (10, 25). The low level of cellular resistance might be due to low expression levels of transporters (10), however, so far this suggestion could not be substantiated by experimental results. Moreover, based on sequence similarities, the MATE family belongs to the multidrug/oligosaccharidyl-lipid/polsaccharide (MOP) export superfamily (TCDB 2.A.66), sharing a certain similarity as the prokaryotic polysaccharide transporter family. The prokaryotic polysaccharide transporter family is known to be involved in the lipopolysaccharide O-antigen repeat unit as well as exopolysaccharide or capsular polysaccharide export in bacteria (26). Instead of extruding drugs or toxic compounds like DAPI, the MATE transporters from bacteria might play an important role in the extrusion of lipid precursors or metabolic waste products. It therefore might be comprehensible that MATE transporters from bacteria confer only modest multidrug resistance.

With regard to ion specificity of NorM_PS, our results support that NorM_PS utilizes the H+ potential across the membrane as its energy source, and no signs of simultaneous Na+ coupling have been observed. The result of the MIC tests in Na+-deficient medium showed no difference to that in normal LB, proving that Na+ did not affect the function of NorM_PS in *vivo*. In the in *vitro* ITC experiment, Na+ was titrated into purified NorM_PS up to a molar ratio of 1600, but no sign of binding could be observed (data not shown). Among all functionally characterized NorM subfamily proteins, most confer a Na+ dependence, only NorM_PS from *P. putida*, PmpM (homologue of NorM_PS) from *Pseudomonas aeruginosa*, and AbeM from *Acinetobacter baumannii* show an ion specificity for H+. Particularly, NorM_VC, previously assumed to be Na+ dependent, was reported of being able to simultaneously bind Na+ and H+ (27). This diversity of the counter cation for substrate extrusion shows a high adaptability of MATE transporters.

**Qualitative Ligand Binding**—After incubating with DAPI, the peak width at half-maximum intensity of the thermal NorM_PS transition decreases dramatically in DSC, which is usually a sign of a more compacted structure. Thus, there is likely to be a conformational change induced by DAPI binding. As for the local surroundings of bound DAPI, some insights could be obtained by comparing its fluorescence spectra with those of the fluorophore in different media.

For this purpose, the influence of solvent polarity on the fluorescence properties of free DAPI, its excitation and emission spectra were measured in water, methanol, and isopropyl alcohol. Decreasing polarity of the solvent leads to an increase of the red shift of the excitation and a blue shift of the emission maximum together with an increase of emission intensity (data not shown). The same changes were observed when DAPI was bound to the WT protein and the variant D38N (Fig. 5, a–c). This observation is considered as an indication that the aromatic residues of bound DAPI are preferentially located in a rather nonpolar environment of the protein such as between amino acid side chains of the hydrophobic TM region.

Another unexpected observation in our substrate binding study is the additional low-affinity binding. In general we consider the low-affinity binding event to be qualitatively characterized, the calculated binding affinity has more significance in terms of the range rather than the exact number itself. Although the transfer of DAPI across the TM region of the protein may require more than one binding site, it appears unlikely to us, to assign more than one postulated site to the TM region of NorM_PS. The observed large ΔH2 value is characteristic of a strong interaction process, counter compensated by the large negative ΔS2 value. In the purified NorM_PS preparation, phospholipid molecules were detected (data not shown), which could be negatively charged. Thus, the weak binding of DAPI to NorM_PS could be due to the interaction between DAPI and the bound lipids.

**Quantitative Ligand Binding**—So far, only limited information regarding substrate binding is available for the MATE transporters. The binding between DinF-BH and rhodamine 6G was studied using fluorescence polarization, and the *Kd* value was determined to be 3.1 μM (10). Using the same technique, another quantitative binding study was performed on NorM_VC, and the *Kd* value for rhodamine 6G and doxorubicin binding to NorM_VC was 2.1 and 1.0 μM, respectively (8). For many other multidrug transporters, dissociation constants in the micromolar range were reported for substrate binding (28–32). One of the reports regarding MdfA is of particular interest, because MdfA is a multidrug transporter of *E. coli*, belonging to the major facilitator superfamily, which shares some structural and functional similarities with NorM_PS. MdfA also has 12 TMHs and functions in the monomeric form. The *Kd* value of MdfA and chloramphenicol was determined to be 75 μM using ITC (32). The binding affinity is lower than the observed affinity between NorM_PS and DAPI in our study. This difference could be due to the dominating binding interaction. In the case of MdfA, the carboxyl side chain of Asp-34 forms two H-bonds with the O4 and O5 hydroxyl groups of chloramphenicol (32), whereas in this study, the high-affinity binding between NorM_PS and DAPI is supposed to be caused by electrostatic interactions.

In this study, two independent methods, spectrofluorometric titration and ITC, were applied to quantify substrate binding. Only one binding event was identified using spectrofluorometric titration and the corresponding affinity is about 5 times
lower than the $K_{\text{a}}$ obtained from ITC. This difference is easy to understand because the binding event observed in the spectrofluorometric titration had a contribution from the low-affinity binding site, but due to the limitations of our experimental conditions, it is difficult to resolve the two binding events using this method.

**Structural Aspects**

Asp-38 Is Involved in $H^+$ Translocation—Asp-41$^\text{PIMATE}$ (corresponding to Asp-38$^{\text{NorM,PS}}$) was reported to be involved in $H^+$ translocation by changing its protonation state: the change may trigger the reorganization of the interaction network, thereby inducing the structural transition between the straight and bent conformations of TMH1 (11). Moreover, another orthologue of Asp-38$^{\text{NorM,PS}}$, Asp-40$^{\text{DinF-BH}}$ was proposed to be a competition site for substrate and $H^+$ (10). From our study, Asp-38 of NorM_PS seems to possess a crucial function but we have not obtained any evidence to support a role in substrate binding. The substitution of Asp by Asn led to a deficiency of DAPI extrusion, but the DAPI binding was hardly affected (Figs. 5a and 7a). This result suggests that Asp-38 is not required for DAPI binding. Therefore it is unlikely that there is a direct competition between the substrate and proton for Asp-38. However, D38N did not show any $H^+$ translocation in the antiport assay (Fig. 3), which can be the reason for the loss of its function.

Critical Role of Glu-257—Among the NorM subfamily proteins, the glutamate residue in TMH7 is highly conserved, and Glu-251$^{\text{NorM,VP}}$, Glu-255$^{\text{NorM,VC}}$, and Glu-261$^{\text{NorM,NG}}$ have been proposed to be important. Electron density for a Cs$^+$ ion was observed in the x-ray structure of NorM_VC between Glu-255 and Asp-371; in NorM_NG, Glu-261 and Tyr-294 could coordinate a Na$^+$ ion (8, 9, 33). Unlike both proteins mentioned above, NorM_PS is $H^+$ dependent, and Glu-257 in NorM_PS is likely to stabilize the protein structurally as well as involved in DAPI binding.

Of the three different variants constructed (E257A, E257Q, and E257D), only E257D could be purified successfully using our standard procedure. This result indicates that the negative charge is important for structural stability. Given that Glu-257 is located in the middle of TMH7, it is possible that Glu-257 stabilizes the protein by interacting with the N-bundle or the amino acid residues close by via electrostatic interactions.

No high-affinity binding to DAPI could be observed for E257D, indicating a participation in substrate binding. But the question whether Glu-257 plays a role in $H^+$ translocation remains unanswered.

Proposed High-affinity DAPI Binding Site—In contrast to the WT protein, a resulting endothermic binding in the process of DAPI to D373N as well as to E257D was observed by ITC. This observation might be caused by a protein conformational change required for the formation of the 1:1 DAPI-protein complex. It is thus not surprising that the affinity became lower than in the WT protein. This assumption is supported by the results of the spectrofluorometric titrations. The blue shift in the emission spectra of DAPI was smaller when bound to D373N and E257D compared to the WT protein (Fig. 5c), suggesting a less hydrophobic environment for DAPI.

The C-bundle cavity lined by Asp-373, Glu-257, and many hydrophobic amino acid residues is proposed to be the high-affinity DAPI binding site for three reasons. (i) The distance between Asp-373 and Glu-257 is $\sim 14 \text{ Å}$, and the distance between the two positive charges of DAPI molecule was 13.4 Å (Fig. 8). Although the distance was calculated based on an outward-facing homology model, and for mechanistic reasons, high-affinity binding was to be expected to take place in a different conformation such as in the postulated inward-facing state, no dramatic change of the distance in the inward-facing conformation should be expected. Moreover, the fact that the variant E257D presented a different binding behavior shows the importance of the distance. (ii) The results of fluorescence titration also provide evidence for this assumption. Glu-257 and Asp-373 are surrounded by many hydrophobic amino acid residues (Val-254, Phe-255, Ala-256, Ile-260, Phe-290, Met-291, Val-374, Val-375, Val-377, Leu-294, Leu-434, and Phe-398), which confirms the conclusion that DAPI is exposed to a very hydrophobic environment when bound to the protein. (iii) The observation of an exothermic binding in ITC is likely to imply charge neutralization. If Glu-257 and Asp-373 are part of the binding pocket, electrostatic interactions would dominate the binding process, and the charge neutralization should be the consequence. Taken together, Glu-257 and Asp-373 should be major contributors to the high-affinity DAPI binding site.

Summarizing, we can say that DAPI binds to solubilized NorM_PS with two affinities, and residues Glu-257 and Asp-373 are directly involved in the high-affinity binding event. For the low-affinity binding event, so far no evidence has been obtained in favor of a physiological function. Kinetic binding studies will undoubtedly increase our understanding of these two binding events.

**Experimental Procedures**

Bacterial Strains and Expression Vectors—The E. coli strain DH5$\alpha$ (Invitrogen) was used for cloning and site-directed mutagenesis, and the E. coli strain TOP10 (Invitrogen) was used for heterologous protein production. For functional studies, E. coli strain KAM32 (a kind gift of Dr. Teruo Kuroda from Okayama University) was used, which lacks AcrB and YdhE.
transporters and thus becomes drug-hypersensitive (34). For heterologous protein production in *E. coli*, a modified pBAD vector (Invitrogen), pBAD2A, which contains a tobacco etch virus protease cleavage site and a decahistidine tag (His tag) attached at the C terminus of the target protein, was used (35).

**Cloning of Genes**—Phusion DNA polymerase (Finnzymes) was used for regular PCR. The primer sequences are listed in **supplemental Table S1**. The gene encoding the NorM_PS (GenBank™ EHY79494.1) was amplified from the genomic DNA of *P. stutzeri* strain ZoBell (ATCC14405). The resulting 1623-bp PCR product was cloned into the pJET1.2 vector using CloneJET PCR cloning kit (Thermo Fisher Scientific). Subsequently, a second PCR was performed to add BamHI and EcoRI restriction enzyme sites to flank the target gene. The resulting PCR product was digested with BamHI–EcoRI endonucleases (Thermo Fisher Scientific) and ligated into the BamHI and EcoRI sites of pBAD2A. Site-directed mutations were constructed using the QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies). All constructs were checked by DNA sequencing (Seqlab).

**Protein Expression and Purification**—The expression vector pBAD2A-NorM_PS was transformed into TOP10 cells for large-scale production or into KM32 cells for MIC tests. After selection on agar plates, a single colony was used to inoculate 50 ml of LB medium containing 50 μg/ml of carbenicillin and incubated at 37 °C overnight. 25 ml of this pre-culture was used to inoculate 2 liters of LB medium. The culture was incubated at 37 °C overnight. 25 ml of this pre-culture was used to inoculate 2 liters of LB medium. The culture was incubated at 37 °C at 180 rpm until the optical density (OD) at 600 nm reached 0.5–0.7. The protein production was induced by addition of 0.05% (w/v) L-arabinose at 37 °C for 2.5 h. To detect the expression, a 1-ml sample was harvested by centrifugation and the pellet was resuspended in 100 μl of 10% SDS. After 30 min of incubation at room temperature, the sample was centrifuged (14,000 × *g*, room temperature, 30 min). 15 μl of the supernatant were subsequently mixed with 5 μl of NuPAGE® SDS Sample Buffer (×4) (Invitrogen), and subsequently analyzed on Novex 4–12% Bistris gels (Invitrogen) by SDS-PAGE and evaluated by Western blot using an alkaline phosphatase-conjugated anti-His antibody (Sigma) according to the manufacturer’s instructions.

Cells were harvested by centrifugation (10,500 × *g*, 4 °C, 20 min) and resuspended in the lysis buffer (20 mM HEPES-NaOH, 100 mM NaCl, pH 7.4, 2 mM phenylmethylsulfonyl fluoride (PMSF; Carl Roth)), at a ratio of 1 g of wet cell/5 ml of buffer, and a small amount of DNase I (Roche Applied Science) was added. The cells were disrupted twice by passing through a M-110LA microfluidizer (Microfluidics) under ice at 8,000 psi. The cell debris was removed by centrifugation (14,000 × *g*, 4 °C, 1 h). The resulting membrane vesicles were collected from the lysate supernatant after ultracentrifugation (145,000 × *g*, 4 °C, 2 h). The pelleted vesicles were resuspended in 20 mM HEPES-NaOH, 100 mM NaCl, pH 7.4. The total protein content in the membrane fraction was determined using the BCA assay (Pierce) according to the manufacturer’s instructions. The final protein concentration of the vesicle suspension was adjusted to 10 mg/ml. The vesicles were frozen in liquid nitrogen and stored at −80 °C.

All solubilization and purification steps were performed at 4 °C. Membranes were solubilized in the presence of 2% (w/v) β-DDM (Glycon Chemicals) for 1 h with slow agitation. The insoluble membrane fraction was removed by ultracentrifugation (170,000 × *g*, 4 °C, 1 h). The supernatant containing the solubilized NorM_PS was mixed with nickel-nitritiotriacetic acid-agarose beads (Qiagen) at a ratio of 200 mg of total membrane protein/ml of beads. The nickel-nitritiotriacetic acid beads were pre-equilibrated with binding buffer (20 mM HEPES-NaOH, 300 mM NaCl, 0.025% (w/v) β-DDM, and 30 mM imidazole, pH 7.4). The mixture was incubated with slow agitation for 1 h. After incubation, beads with bound proteins were collected in a gravity column and washed with 20 column volumes (CV) of binding buffer. Subsequently, the beads were further washed with 10 CV of washing buffer I (binding buffer + 50 mM imidazole), 5 CV of washing buffer II (binding buffer + 80 mM imidazole), and the target protein was eluted with 8 CV of elution buffer (20 mM HEPES-NaOH, 100 mM of sodium lactate, 300 mM imidazole-HCl, and 0.025% (w/v) β-DDM, pH 7.4). The eluted fraction was concentrated 30-fold in an Amicon concentrator with 50-kDa cut-off membrane (Millipore) and loaded onto a Superdex 2000 10/300 column (GE Healthcare) pre-equilibrated with the NorM_PS sample buffer (20 mM HEPES-NaOH, 11 mM sodium lactate, and 0.025% (w/v) β-DDM, pH 7.4). For analytical chromatography, a Superdex 200 PC 3.2/30 column (GE Healthcare) was used to investigate the homogeneity of the purified protein.

**Size Exclusion Chromatography Coupled to Multi-angle Light Scattering**—The oligomeric state of NorM_PS was determined by SEC-MALS and differential refractive-index measurements (Viscotec SEC-MALS 20, Malvern). Prior to the NorM_PS run, the system was calibrated with 3.5 mg/ml of BSA sample in the NorM_PS sample buffer. 100 μl of the purified protein (3.8 mg/ml) were loaded onto a Superdex 200 10/300 column. For data evaluation, the molar extinction coefficient of NorM_PS was at 280 nm (ε<sub>280</sub> (63,745 M<sup>−1</sup> cm<sup>−1</sup>) was used. The specific refractive index increment of the protein was 0.187 g<sup>−1</sup>, the ε<sub>280</sub> and refractive index increment of β-DDM were 0.001 mg<sup>−1</sup> cm<sup>−1</sup> and 0.133 mg<sup>−1</sup>, respectively, all values were taken from the literature (20). The calculation was performed using the “Copolymer method” using the software provided by the manufacturer (Malvern OmniSEC).

**Determination of Protein Concentration**—The protein concentration was roughly measured by the BCA protein assay according to the manufacturer’s instructions. To precisely determine the protein concentration, quantitative amino acid analysis (Functional Genomics Center Zurich) was performed. 1 mg of protein determined by the BCA test corresponded to 1.24 mg. The resulting factor of 1.24 was used to correct all future values obtained from the BCA assay for further ITC, DSC, and fluorescence studies; the molecular mass of NorM_PS was 53,066.7 Da based on its amino acid composition.

**Drug Resistance Assay**—The drug susceptibility test was performed based on established protocols with minor modifications (36). Freshly transformed negative control cells (KAM32 cells + pBAD2A vector) and test cells (KAM32 cells + pBAD2A-NorM_PS vector) were grown in LB at 37 °C until the A<sub>600</sub> reached 0.5 and induced with 0.05% (w/v) L-arabinose.
After incubation for 45 min, the cells were used to inoculate 1 ml of test medium (LB medium containing 0.005% (w/v) l-arabinose, 50 μg/ml of carbenicillin, and different concentrations of antibiotics, drugs, and other toxic compounds). The inoculum corresponded to 10^5–10^6 colony-forming units/ml of media. Cells were incubated at 37 °C for 24 h, and the growth was monitored by measuring A_600.

The drug resistance test was also performed using LB without NaCl (FORMEDIUM) to study the cation dependence of the transport. We defined the MIC as the lowest concentrations of toxic compounds that inhibit the visible growth of cells after overnight incubation under our experimental conditions. DAPI, doxorubicin, daunorubicin, tetracycline, kanamycin, gentamycin, benzamidine, streptomycin, chloramphenicol, ciprofloxacin, norfloxacin, rhodamine 6G, acriflavine, propidium iodide, berberine, ethidium bromide, and tetrphenylphosphonium chloride were tested. All assays were repeated at least three times independently.

**DAPI/H^+ Antiport Assay**—Everted membrane vesicles were prepared for the antiport assay. pBAD2A and pBAD2A-NorM_PS were transformed into E. coli KAM32 strain. After 2.5 h of induction, cells were harvested and the everted vesicles were prepared according to Ref. 37. Total protein concentration was measured by the BCA assay and adjusted to 20 mg/ml. Aliquots of 200 μl were frozen in liquid nitrogen and stored at −80 °C.

The antiport assay was conducted at 25 °C in a reaction mixture (1 ml) consisting of modified TCS buffer (5 mM Tris-HCl, 140 mM choline chloride, 5 mM MgCl₂, pH 7.4) containing 2 μM acridine orange (Sigma) and 400 μg of total protein in everted vesicles. 10 μl of 1 M Na-DL-lactate (Fluka) was added to the mixture to initiate respiration. 2.5 μl of 20 mM DAPI dihydrochloride (Sigma) were added after fluorescence quenching reached a steady state. The sample was continuously stirred during the measurement. All fluorescence measurements were performed with a Hitachi F-4500 fluorescence spectrophotometer in the time-scan configuration, with excitation at 495 nm and emission at 530 nm. Slit width was 5 nm for both excitation and emission, PMT voltage was set to 700 V. DAPI concentration was determined by measuring its absorption, with ε₅₁₀ = 30,000 M⁻¹ cm⁻¹ (in water) (38).

**Differential Scanning Calorimetry**—The DSC measurements were performed using a VP-Capillary DSC system (MicroCal, Malvern, Inc.). Typically, 350 μl of protein in the NorM_PS sample buffer at a concentration of 25 μM was injected into the cell (MicroCal, Malvern, Inc.). Scanning was performed from 10 to 85 °C at a rate of 120 °C/h in the low feedback mode with a 5-min pre-equilibration phase. The system pressure was provided by N₂ and about 60 psi during measurement. Data analysis was processed using Origin 7.0 software (Microcal Origin); thermograms were corrected by subtracting buffer-only blank scans.

**Spectrofluorometry**—The fluorescent binding assay was carried out by spectrofluorometric titrations at 25 °C. 0.5 μM DAPI in the NorM_PS sample buffer was titrated with concentrated protein samples (up to 250 μM). Fluorescence measurements were done with a Hitachi F-4500 fluorescence spectrophotometer in the wavelength scan configuration. Excitation wavelength was 360 nm, and emission wavelength was measured from 380 to 580 nm. Emission at 450 nm was used to record the excitation spectra and for titration analysis. To minimize inner filter effects, a 5 × 5-mm quartz cuvette (101-058-40, Hellma Analytics) was used. Corresponding absorption spectra before and after titrations were conducted in absorption cuvettes (104-5-K-40, Hellma Analytics) with 5-nm light path. Under the chosen condition, the concentration of bound DAPI was always small and thus neglectable compared with that of the total concentration. Volume change corrected intensities at 450 nm together with associated total DAPI concentration were evaluated according to the Henderson-Hasselbach equation based on a 1:1 binding equilibrium. The dissociation constants, K_d, were given as mean values of three independent titrations.

**Isothermal Titration Calorimetry**—ITC measurements were performed at 25 °C with a VP-ITC calorimeter consisting of a 1.45-ml reaction cell and a 278-μl syringe (Malvern, MicroCal, Inc.). The experimental conditions for each measurement are specified in figure legends. To achieve a higher concentration range, two successive titrations with the same ligand solution were performed and combined by the program CONCAT (Malvern, MicroCal, Inc.). The general settings of the instrument were as follows: data interval was 360 s, the stirring speed was 351 rpm, and gain mode was “high.”

Data evaluation was employed using software (MicroCal Origin) based on the “two binding sites” model. All the variants titrations were evaluated in the same way as the WT protein titration. The 1st injection usually had an inaccuracy in volume, so it was not taken into consideration for further analysis and marked with “m” in Figs. 6 and 7.

**Homology Modeling**—The homology model of NorM_PS was constructed using the web-based protein structure modeling server I-TASSER (39–41). The three-dimensional model was generated on the basis of the x-ray structure of the NorM_VC protein (Protein Data Bank code 3MKU), which shares a 43% overall sequence identity with NorM_PS.

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Substrate-binding Site of a MATE Transporter

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