Cell Permeabilization and Uptake of Antisense Peptide-Peptide Nucleic Acid (PNA) into Escherichia coli*

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Magdalena Eriksson†, Peter E. Nielsen‡‡, and Liam Good**

From the †Department of Physical Chemistry, Chalmers University of Technology, Gothenburg SE-41296, Sweden, the ‡Center for Biomolecular Recognition, Institute for Medical Biochemistry and Genetics, Biochemistry Lab. B, Panum Institute, Blegdamsvej 3C, DK-2200 Copenhagen N., Denmark, ‡‡Pantheceo A/S, Danish Science Park, Bage Allé 3, DK-2970 Harsholm, Denmark, and the **Center for Genomics and Bioinformatics, Karolinska Institutet, Stockholm SE-17177, Sweden

Peptide nucleic acid (PNA) is a DNA mimic with promising properties for the development of antisense agents. Antisense PNAs targeted to Escherichia coli genes can specifically inhibit gene expression, and attachment of PNA to the cell-permeabilizing peptide KFKFFKFKFFK dramatically improves antisense potency. The improved potency observed earlier was suggested to be due to better cell uptake; however, the uptake kinetics of standard or modified PNAs into bacteria had not been investigated. Here we monitored outer and inner membrane permeabilization by using chemical probes that normally are excluded from cells but can gain access at points where membrane integrity is disturbed. Membrane permeabilization was much more rapid in the presence of peptide-PNA conjugates relative to the free components used alone or in combination. Indeed, peptide-PNAs permeabilized E. coli nearly as quickly as antimicrobial peptides. Furthermore, as expected for outer membrane-active compounds, added MgCl₂ reduced cell-permeabilization. Concurrent monitoring of outer and inner membrane permeabilization indicated that passage across the outer membrane is rate-limiting for uptake. The enhanced cell-permeation properties of peptide-PNAs can explain their potent antisense activity, and the results indicate an unanticipated synergy between the peptide and PNA components.

Antisense agents are designed to inhibit gene expression through sequence-specific nucleic acid binding, and this normally requires the formation of 10 or more base pairs. Oligonucleotides of such lengths are typically too large for efficient passive cellular uptake by diffusion across lipid bilayers (1), and cellular outer membranes can pose additional barriers. Recently we introduced antisense peptide nucleic acids (PNAs)² as antisense agents for bacterial applications. The early experiments indicated very encouraging sequence specificity against reporter genes; however, uptake appeared to be limited by bacterial cell barriers (2, 3). This limitation is not surprising as Escherichia coli and other Gram-negative bacteria have outer and inner bilayer membranes. In addition, the outer membrane contains a lipopolysaccharide (LPS) layer that stringently restricts the entry of foreign molecules (4). Nevertheless, there are opportunities to overcome these cellular barriers and even exploit membrane charge characteristics to enhance delivery.

PNA is a DNA mimic with (standard) nucleobases attached to a pseudo-peptide backbone (see Fig. 1) (5). The PNA molecule provides improved hybridization affinity and specificity, chemical and enzymatic stability, and low toxicity. Antisense PNAs have outperformed DNA analogues in diverse systems (2, 6). The main limitation to wider applications in vitro appears to be poor cellular uptake; however, there are several recent examples showing improved delivery and antisense effects for PNAs attached to carrier peptides. For delivery into eukaryotic cells, PNAs have been attached to a peptide derived from the third helix domain of the Drosophila antennapedia transcription factor (7) and to a nuclear localization signal peptide (8). For delivery into bacteria, we have attached PNAs to a range of cell-permeabilizing peptides and found that this greatly improves antisense effects in E. coli (9). In particular, the KFKFFKFKFFK peptide (10) provides potent bactericidal effects when attached to an antisense PNA targeted to the essential acpP gene (9).

The KFKFFKFKFFK peptide was assumed to improve antisense effects by improving PNA uptake properties. Previous studies involving mutant strains and cell-permeabilizing agents show that the E. coli outer membrane LPS layer is a major barrier against PNA uptake into E. coli (11). The KFKFFKFKFFK peptide-PNAs were designed to overcome this barrier by providing a membrane-active domain (9). This is a reasonable delivery strategy because such cationic peptides are likely to be attracted to negatively charged portions of the LPS, and the uncharged peptide residues and PNA itself could aid passage across the hydrophobic membrane interior (12). Furthermore, the charge gradient across the cell membrane could help drive cationic peptides into cells (13). Although such mechanistic details are not yet established for membrane-active peptides, improved permeation potentially could enable efficient delivery of peptide-PNAs, and this could explain the improved antisense potencies for these agents (9).

If the improved antisense potencies observed for peptide-PNAs are indeed due to outer cell membrane permeabilization, the membrane activity should be measurable using small molecule indicators of membrane integrity (see Fig. 2). Here we show that membrane permeabilization by the peptide-PNA conjugate is higher than for free PNA or free peptide and similar to natural cationic antimicrobial peptides. The improved cell permeation properties of peptide-PNAs can explain their potent antisense effects, and the results reveal an unanticipated synergism between the two linked components.

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** To whom correspondence should be addressed. Tel.: 46-8-728-63-85; Fax: 46-8-32-39-50; E-mail: liam.good@ghg.ki.se.

1 The abbreviations used are: PNA, peptide nucleic acid; LPS, lipopolysaccharide; NPN, N-phenyl-1-naphthylamine; ONPG, o-nitrophenyl-ß-D-galactoside.
EXPERIMENTAL PROCEDURES

Materials—The PNAs and PNA-peptides used in this study were synthesized using the tBoc protection strategy as described previously (9, 14). The peptide KFFKKFKFFK was synthesized by a standard, solid-phase tBoc strategy (15). Polymyxin B sulfate, N-phenyl-1-naphthylamine (NPN), o-nitrophenyl-β-D-galactoside (ONPG), and melittin were from Sigma Chemical Co. Nitrocefin was from Oxoid Ltd. (Basingstoke, Hampshire, United Kingdom). E. coli Cell Preparation—E. coli bacteria of wild type strain K12 were obtained from the Coli Genetic Stock Center (Yale University, New Haven CT), and E. coli strain ML-35p was provided by Prof. Robert I. Lehrer (Center for Health Sciences, Los Angeles, CA). Cells were grown in LB medium to 0.4 A\text{\textsubscript{600}} and harvested by centrifugation at 3000 × g, and suspended in 10 mM HEPES buffer (pH 7.2) or 10 mM sodium phosphate buffer (pH 7.4) to a concentration of 10^6 cells/mL. Cells were grown in LB medium to 0.4 A\text{\textsubscript{600}}, rinsed once using centrifugation, and then resuspended in 10 mM HEPES buffer (pH 7.2) or 10 mM sodium phosphate buffer (pH 7.4) to an A\text{\textsubscript{600}} of 0.5.

NPN Outer Membrane Permeabilization Assay—Two methods were used to assess outer membrane permeabilization. The NPN access assay was performed essentially as described previously (16). Briefly, E. coli cells were grown, harvested, and suspended as described above. NPN was added to a concentration of 10 mM into quartz cuvettes containing 2 mL of cell suspension. The permeating agents were added, and the sample was mixed by inversion of the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a Shimadzu RF-1501 spectrofluorometer with slit widths set to 1 nm and excitation and emission wavelengths set to 350 and 450 nm, respectively.

Nitrocefin Outer Cell Barrier Permeabilization Assay—Outer membrane permeability was determined essentially as described previously (17). Briefly, E. coli strain ML-35p cells, which carry a plasmid-borne β-lactamase gene, were prepared as described above. The permeabilization assays were carried out using 96-well microtiter plates with wells containing 100 μL of 5 mM HEPES (7.4), 5 mM carbonyl cyanide m-chlorophenylhydrazone, 20 μg/mL nitrocefin, and cells were added to 0.1 A\text{\textsubscript{600}}. Following addition of the permeabilizing substances, nitrocefin cleavage was monitored by light absorption measurements at 500 nm.

Inner Membrane Permeabilization Assay—Permeabilization of the inner membrane was assessed by measuring the access of ONPG to the cytoplasm essentially as described previously (18) using E. coli ML35p cells prepared as described above. Briefly, ONPG was added to a concentration of 100 μg/mL, and substrate cleavage in the presence of permeabilizers by β-galactosidase was monitored by light absorption measurements at 485 nm.

RESULTS

Outer Membrane Permeabilization—The PNA, peptide, and peptide-PNA conjugates used here (Fig. 1) are noninhibitory to cell growth at the concentrations used in the cell permeabilization experiments, and the PNAs show sequence-specific antisense effects (9). To test the ability of different PNA constructs to permeabilize the E. coli outer membrane, the hydrophobic fluorescent probe NPN was used as an indicator of membrane integrity (Fig. 2). NPN has a low fluorescence quantum yield in aqueous solution but fluoresces strongly in the hydrophobic environment of a biological membrane. Normally NPN is excluded from the outer membrane LPS layer but can enter at points where membrane integrity is compromised. PNA, peptide, or peptide-PNA was added to a suspension of E. coli ML35p cells in the presence of NPN, and all compounds were found to permeabilize the membrane to NPN but at different rates (Fig. 3A). Free PNAs showed a slow permeabilization rate, and the free peptide KFFKKFKFKFK showed only a slightly higher permeabilization rate. Simultaneous addition of free PNA and free peptide also showed a slow permeabilization rate similar to that for free peptide. In contrast, the peptide-PNA conjugate showed much higher activity than the free components, approaching the activity of polymyxin B in this assay. Similar results were obtained for E. coli K12 (data not shown).

Polymyxin B and other cationic antimicrobial peptides are believed to compete for magnesium ion binding sites within the LPS layer as part of their membrane-permeabilizing activities (13). If PNAs permeabilize E. coli cells by a mechanism similar to polymyxin B, the activity should be limited by added Mg^{2+} ions. To test this possibility, excess MgCl\textsubscript{2} (1 mM) was added and found to dramatically limit membrane permeabilization by peptide-PNA molecules (Fig. 3B). This suggests that competition for the Mg^{2+} binding sites within the LPS layer is an important step in the uptake process. However, it is also possible that excess magnesium ions reduce the electrostatic attraction between the positively charged PNA construct and negatively charged bacterial outer membranes.

Concurrent Measurements of Outer and Inner Membrane Permeabilization—To corroborate the results from the NPN assay and enable simultaneous monitoring of outer and inner cell membrane permeabilization, two chromogenic reporter molecule systems were used at the same time (Fig. 2). Permeabilization of the outer membrane was monitored using nitrocefin as a probe. Nitrocefin is normally excluded by the outer cell membrane, but if able to pass this barrier it can be cleaved by β-lactamase localized within the periplasmic space. Cleavage results in a color change from yellow to red, and this can be used to monitor outer membrane permeabilization. In a similar

![Figure 1. Chemical structure of peptide nucleic acid and cell barrier probes used in this study.](http://www.jbc.org/)

![Figure 2. Illustration of an E. coli cell and the permeabilization assays used in this study.](http://www.jbc.org/)
magnesium ions on NPN fluorescence increases. The entry into the hydrophobic outer membrane.

A

monitored. Enhanced uptake and fluorescence is caused by NPN entry

B

coli ML35p cells were incubated with NPN, and NPN fluorescence was monitored. Trials in the presence of 1 mM MgCl₂ (open circle, ○), peptide-PNA #1900 (○), and polymyxin B (○). B, peptide-PNA permeabilization of E. coli outer membrane and the effect of added magnesium ions. Peptide-PNA #1900 was added at 1 mM, and NPN fluorescence was monitored in the absence (○) or presence (×) of added magnesium ions (10 mM).

way, permeabilization of the inner membrane was monitored using the β-galactosidase substrate ONPG as a probe. In E. coli strain ML35p, which lacks lac permease, ONPG can be cleaved by β-galactosidase localized within the cytoplasm, and this results in the appearance of a yellow color (18). With these two reporter systems, it is possible to monitor permeabilization of both the outer and inner E. coli membranes.

The results for outer membrane permeabilization in this assay were similar to those observed with the NPN fluorescence assay described above. Free PNA and free peptide when used separately or in combination showed little membrane activity, whereas the peptide-PNA conjugate permeabilized the outer membrane more rapidly. In this assay the peptide-PNA permeabilized cells at a rate that was similar to melittin but slower than polymyxin (Fig. 4A). Similar results were seen for PNA #1901, which contains a different PNA sequence (9) (data not shown). As seen in the NPN assay, the peptide-PNA conjugate was much more membrane-active than the free components. Therefore, both assays indicate a surprisingly high membrane activity of the peptide-PNA conjugate.

The inner membrane also was permeabilized much more rapidly by the peptide-PNA conjugates than by free PNA or free peptide used separately or in combination (Fig. 4B). Furthermore, comparison of the results for outer and inner membrane permeabilization indicates that the membrane activities for peptide-PNA conjugates resemble that of melittin with inner membrane permeabilization closely following outer membrane permeabilization. In contrast, polymyxin B, which is highly active against the outer membrane (18), showed relatively weak inner membrane activity (Fig. 4B). Because outer and inner cell membrane monitoring was concurrent, it is possible to comment on the relative rates of permeabilization of the two membranes (18). The similar rates observed for outer and inner membrane permeabilization, characterized by 50% probe conversion after ~5 min, indicate outer membrane passage as rate-limiting for peptide-PNA uptake as also appears to be the case for free PNA, free peptide (Figs. 3 and 4), and most other foreign compounds (4).

The increased rate of nitrocefin or ONPG conversion also could be attributed to β-lactamase or β-galactosidase escape from cells rather than to probe entry. However, this seems unlikely because the enzymes are much larger than the chromogenic probes and permeabilizing peptides used here. Furthermore, neither β-lactamase nor β-galactosidase leakage in the presence of cell-permeabilizing peptides was associated with the permeabilization assays performed here (17, 18). Nevertheless, to ensure that such leakage did not confound the results, we treated E. coli with peptide-PNA at 1 μM for 30 min and measured β-lactamase and β-galactosidase activity in the supernatant. No increase was observed relative to untreated cells (data not shown), suggesting that conversion of the chromogenic probes occurs mainly inside cells.
Concentration Dependence—To gain insight into mechanistic aspects of the peptide-PNA membrane activities, the peptide-PNA was used in the nitrocefin outer membrane permeabilization assay at a range of concentrations. The time response to addition of the conjugate at concentrations between 0.03 and 10 \( \mu M \) indicates that relatively large changes in permeabilization rates occurred with midrange concentration changes (1–5 \( \mu M \)) (Fig. 5). The nonlinear dose response, although modest, may reflect some form of intermolecular cooperative activity between peptide-PNA conjugates during cell entry as observed for certain antimicrobial peptides (19). In this case, however, further analyses are needed to explore this aspect of cell entry by peptide-PNAs.

DISCUSSION

PNA antisense effects in eukaryotic and prokaryotic cells can be dramatically improved by the attachment of so-called “carrier” peptides to the PNA (7, 9). For \( E. coli \) applications, attachment of the cell-permeabilizing peptide KFFKFKFKFKK increased antisense potency against \( E. coli \) RNA targets by up to 2 orders of magnitude (9). Although it was proposed that the improved antisense effects observed for peptide-PNAs over “naked” PNAs were due mainly to improved cell permeation, this was not established experimentally. Here we have begun to characterize the kinetics of cell permeation by standard PNA and peptide-PNA conjugates and report studies using small chemical probes as indicators of outer and inner membrane permeabilization (Figs. 1 and 2). The results show that peptide-PNAs are indeed membrane permeabilization reagents. Peptide-PNAs showed permeabilization rates much higher than that of the free peptide and similar to natural antimicrobial peptides. Therefore, the results support the contention that cell entry is a limiting factor for antisense PNA potency.

Concurrent monitoring of outer and inner membrane permeabilization indicated that peptide-PNA activity at the inner membrane closely followed that of the outer membrane for both standard PNAs and peptide-PNAs. The results are consistent with an earlier study comparing the antisense effects of standard PNAs in mutant and permeabilized cells (11), showing that it is predominantly the outer membrane that limits PNA entry. Therefore, while the kinetics of permeabilization were much faster for peptide-PNAs, permeation of the outer membrane appears to remain rate-limiting for cellular uptake. To explain the improved cell uptake of peptide-PNAs across \( E. coli \) cell membranes we can envision a self-promoted uptake pathway as proposed for certain cationic antimicrobial peptides (13). In this proposed pathway, the peptide-PNAs would contact the outer membrane through electrostatic attraction and compete for Mg\(^{2+}\) binding sites between LPS molecules. This competition would disrupt the integrity of the outer cell membrane, providing opportunities for the membrane charge gradient to force positively charged conjugates into cells. At this time, such details remain speculative.

For antimicrobial applications, the linked peptide potentially could improve PNA efficacy in two ways. As a carrier domain, the KFFKFKFKFKK peptide can increase antisense effects by improved cellular uptake (9). As well, by acting as a permeabilizing domain the peptide could kill cells by causing cell leakage (Figs. 3 and 4). Both effects are potentially useful in antimicrobial development, and the results shown here indicate that peptide-PNAs can possess a surprisingly high level of membrane activity.

Although the peptide-PNA was expected to show more membrane activity than free PNA, the observation that the conjugate is more membrane-active than the free peptide was unexpected. This enhanced activity suggests that both portions of the conjugate contribute to membrane activity in a synergistic manner. One possibility is that the uncharged PNA residues provide or enhance amphipathic features that help perturb membranes.

In summary, PNAs can permeabilize both the outer and inner \( E. coli \) cell membranes, and these activities can be significantly enhanced by conjugation to the cell-permeating peptide KFFKFKFKFKK. The improved cell permeabilization observed for peptide-PNA conjugates appears to explain the improved antisense potencies of such conjugates. The mechanistic basis for the peptide-PNA intramolecular synergism observed is not understood; however, competition for Mg\(^{2+}\) binding sites within the outer membrane LPS layer and some form of synergism between the peptide and PNA components appear to be important for efficient permeation. The high membrane activity exhibited by peptide-PNA conjugates raises prospects for practical applications of such antisense PNAs and opens additional possibilities to explore in peptide-PNA design.

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