Identification of Single Amino Acid Chiral and Positional Isomers Using an Electrostatically Asymmetric Nanopore

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ABSTRACT: Chirality is essential in nearly all biological organizations and chemical reactions but is rarely considered due to technical limitations in identifying L/D isomerization. Using OmpF, a membrane channel from Escherichia coli with an electrostatically asymmetric constriction zone, allows discriminating chiral amino acids in a single peptide. The heterogeneous distribution of charged residues in OmpF causes a strong lateral electrostatic field at the constriction. This laterally asymmetric constriction zone forces the sidechains of the peptides to specific orientations within OmpF, causing distinct ionic current fluctuations. Using statistical analysis of the respective ionic current variations allows distinguishing the presence and position of a single amino acid with different chiralities. To explore potential applications, the disease-related peptide β-Amyloid and its D-Asp isoform and a mixture of the icatibant peptide drug (HOE 140) and its D-Ser mutant have been discriminated. Both chiral isomers were not applicable to be distinguished by mass spectroscopy approaches. These findings highlight a novel sensing mechanism for identifying single amino acids in single peptides and even for achieving single-molecule protein sequencing.

INTRODUCTION

Amino acid isomerization can be induced by post-translational modifications or asymmetric synthesis.1−5 Identifying these isomers in a peptide at a single-molecule level is highly desirable in early disease diagnosis and chiral impurity analysis but challenging due to technical limitations. Mass spectrometry (MS)6 approaches are not easy due to identical mass between chiral isomers. While liquid chromatography-tandem MS is available, only a few chirality-containing peptides are determined based on limited L-/D-type amino acid enzymatic cleavage positional mismatch. Alternatively, circular dichroism is a method based on the differential absorption of circularly polarized light and allows the identification of chiral isomers. This approach could be further improved toward the single-molecule level, based on confining the polarized light into the single-molecule scale.7 However, developing an accessible method for identifying peptides containing chiral amino acids remains cumbersome. The chirality-containing peptides break the sidechain zig-zag alignment,8 which usually lacks the chiral center of the whole molecule. Therefore, the circular dichroism-based approaches are ineffective in identifying such stereo distortion. Thus, no single-molecule technique is available that easily distinguishes chiral amino acids and even positional isomers in single peptides. This lack of an easy method is likely a bottleneck for the discovery of more biological and chemical processes.

Protein nanopores have already been utilized to identify single amino acids9−12 and protein−protein interactions13 by amperometric recognition. Peptides with post-translational modifications are recognized by nanopore-based approaches, to the extent that the sensing principle relies on volume exclusion.9,14 Moreover, the inter-molecule interaction between the target peptides and the nanopore interface can lead to characteristic ionic current patterns for discriminating amino acids with similar volumes.15,16 The nanopore-based single molecule approach has demonstrated the possibility of sensing chiral molecules by designing a specific chiral environment, but the generalization of such a strategy toward peptide-containing chiral amino acid is lacking.17−19 Previous nanopore-based tools remain insufficient in identifying amino acids in peptides with identical volumes. The only difference between chiral isomers is the opposite orientation of the sidechains.

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To identify the sidechain orientation, herein, we use OmpF, a bacterial outer-membrane channel, as a chiral biosensor. The crystal structure of OmpF shows that the narrowest size of the constriction zone (CZ) is about 0.7 nm, one of the smallest CZs reported among the silent protein channels. The size is close to that of a single amino acid and promises to serve an enhanced stereo confinement. Notably, the CZ is composed of a positively charged ladder (K16, R42, R82, and R132) and a negatively charged pocket (D113, E117, and D121) on opposite sides of the CZ (Figure 1a), which creates a lateral electrostatic asymmetry in the CZ, the chirality directs the sidechain orientation during the pore—analyte interaction, which in turn determines the characteristic single-molecule current pattern used for identification purposes. The specific amplitudes of the current blockades and the respective residence time allow us to identify chiral and positional isomers. As examples for potential applications, we identified d-Asp in disease-related β-Amyloid mutants and an icatibant drug (HOE140) mixture with one of the impurities consisting of d-Ser.

### RESULTS AND DISCUSSION

Previous studies revealed that OmpF has a cationic selectivity and showed strong interaction with Arginine at negative bias voltage. For the proof-of-concept demonstration, a cationic model peptide N-Arg-Arg-Gly-Arg-Asp (Mol-1) in Van der Waals representation. The monomer on the right highlights the heterogeneous inner tunnel surface with loop L3 highlighted in magenta. (b) Schematic representation of the zig-zag alignment of each amino acid sidechain of Mol-1 in bulk (undertint color), together with arrows representing their reorientation, upon entering OmpF (dark color) during the simulations. For the calculation of the sidechain orientation, the angle was averaged along the channel axis from 0 to 10 Å, accordingly (see Figure S6a). Blue spheres represent arginine sidechains, and red sphere represents the aspartic acid sidechain. The backbone numbering denotes the position of the respective amino acid, starting from the N-terminus. The profile of the electrostatic potential is depicted in Figure S1. (c) Each sidechain orientation of Mol-1, determined quantitatively. An angle of 0° indicates that the sidechain points toward the acidic region, while it is oriented toward the basic ladder at 180°. (d) Typical nanopore-based amperometric readout of Mol-1. The colored panel shows a zoomed-in single-molecule event induced by Mol-1 interaction with OmpF. The current drop ∆I/I₀ and residence time t are the two parameters being analyzed. The red star denotes the enlarged events (orange panel) in the original trace. (e) The peptide sequences of Mol-1, 2, 3, and 4 are shown on the left. For the results on the right, a final concentration of 7.6 μM for Mol-1 and 3 and 3.7 μM for Mol-2 and 4. The color histograms depict the current drop ∆I/I₀ and residence time t measured at a bias voltage of −50 mV in 1.0 M KCl, 10 mM Tris, and 1 mM EDTA at pH 8.0. At least 1000 single-molecule events have been analyzed per peptide. Moreover, the peak values of the distributions have been normalized to one with this maximum value shown in black color. More detailed histograms are delineated in Figure S4.
reaching the CZ, all arginine sidechains are oriented toward the negatively charged pocket, and the aspartic acid sidechain faces toward the positively charged ladder (Figure 1c). Arg\(^{3}\) needs to reorient from the opposite orientation of ca. 135° against the backbone stiffness toward this region. In contrast, Arg\(^{4}\) is more flexible to orient from 100° since the preceding Gly\(^{3}\) has no sidechain. For detailed \textit{in silico} analysis, see also Supporting Note 1. To this end, we have concluded that each sidechain reorients in response to the lateral electrostatic field in the CZ.

The second series of experiments were devoted to elucidating what degree the backbone stiffness prohibits the sidechain orientation. The peptide \(\_{\text{N}}\text{Arg-Arg-Ala-Arg-Asp} (\text{Mol-2})\) was designed by substituting Gly\(^{3}\) with Ala\(^{3}\), to manipulate the stiffness of the peptide backbone by adding a methyl group. Performing single-molecule analysis, Mol-2 yielded \(\Delta I/I_0\) decreased to 61.1 \(\pm\) 0.9\% and the residence time stayed almost the same, 46.8 \(\pm\) 0.7 \(\mu\)s. The salt-bridge created between the C-terminus of Mol-2 and the OmpF vestibule gets stronger than that of Mol-1 (Figure S5). Arg\(^{3}\) orients from approximately 50 to 90°, toward the arginine ladder (Figure S6). To show the effect of the sidechain orientation, the positions Asp\(^{5}\) and Arg\(^{4}\) in the abovementioned peptides have been switched to invert the sidechain orientations, maintaining the zig-zag form. Thus, two peptides have been synthesized, that is, \(\_{\text{N}}\text{Arg-Arg-Gly-Asp-Arg} (\text{Mol-3})\) and \(\_{\text{N}}\text{Arg-Arg-Ala-Asp-Arg} (\text{Mol-4})\). The \(\Delta I/I_0\) for Mol-3 was 59.4 \(\pm\) 1.6\%, and the \(t\) value was 47.2 \(\pm\) 0.3 \(\mu\)s. However, for Mol-4, we obtained 60.6 \(\pm\) 0.3\% for \(\Delta I/I_0\) and a \(t\) value of 47.6 \(\pm\) 0.2 \(\mu\)s. Therefore, switching the positions of Arg\(^{3}\) and Asp\(^{5}\) at the C-terminus induces sidechain orientations inversely aligned, compared to Mol-1 and Mol-2, decreasing the \(\Delta I/I_0\). Overall, by analyzing the current blockades, Mol-1 has the best sidechain, matching the lateral electrostatic field in the CZ of OmpF. The electrostatic force at the CZ is enhanced in lower electrolyte concentration.\(^{24}\) In this work, we decreased the electrolyte concentration from 1.0 to 0.5 M and the \(t\) for Mol-1 rose by 38\% (Figure 2a). Applying the picture of sidechains reorientation in the asymmetry CZ, we designed chirality-containing peptides and positional isomers (Mol-5 to 9). Placing \(\beta\)-Arg at the 1st position (Mol-5), the residence time \(t\) increases to 112.3 \(\pm\) 33.0 \(\mu\)s (Figures 2b,c and S6). Placing \(\beta\)-Arg at the 2nd position (Mol-6), \(t\) increased to 137.9 \(\pm\) 37.0 \(\mu\)s. The orientation of each sidechain in the CZ is depicted schematically in Figure 2d, in accordance with the dynamics (Figure S8). For both Mol-5 and Mol-6, the calculated salt-bridge interactions got stronger than those for Mol-1, leading to longer \(t\) (Figure S9).

Placing \(d\)-Arg at the 4th position (Mol-7) yields a \(\Delta I/I_0\) of 76.9 \(\pm\) 3.1\% and \(t\) of 64.9 \(\pm\) 0.3 \(\mu\)s. Since the \(d\)-Arg\(^{4}\) and \(l\)-Asp\(^{5}\) sidechains face toward the same side in bulk, upon entering the CZ of OmpF, the lateral electrostatic field tears
them apart toward the opposite channel walls, against the stiffness of the peptide backbone. The analysis of the sidechain orientation dynamics revealed that Arg\textsubscript{2} fails to orient toward the negatively charged pocket (Figures 2d and S8). As this conformation is energetically less favorable, Mol-7 resides in the CZ for a shorter time than Mol-1. Considering that Mol-8 contains both D-Arg\textsubscript{2} and D-Arg\textsubscript{4} (Figure 1d), the measurements resulted in a ΔI/Γ\textsubscript{0} of 73.7 ± 9.1% and t of 104.9 ± 23.0 μs. As expected, the sidechain of D-Arg\textsubscript{2} orients from 135 to 10°, while the D-Arg\textsubscript{4} and L-Asp\textsubscript{5} sidechains remain in the same orientation as in Mol-7 (Figures 2d and S8). For D-Asp\textsubscript{5} at the C-terminus (Mol-9), the ionic current ratio ΔI/Γ\textsubscript{0} decreased to 73.7 ± 3.6% and the residence time t to 61.3 ± 0.1 μs. This relatively short t can be attributed to L-Arg\textsubscript{4}D-Asp\textsubscript{5} that requires additional energy to orient them differently against the backbone stiffness, similar to the case of D-Arg\textsubscript{4}L-Asp\textsubscript{5} (Mol-7 and Mol-8).

In short, the measurements of the residence times and reductions in the ionic flux revealed a sensitivity of both parameters for D-amino-acid-containing peptides. Ionic current recordings, using a single OmpF protein, can detect the presence of chiral amino acids including positional isomers. The presence of D-Arg at various positions in the model peptides Mol-5 to Mol-7 caused variations in ionic current...
blocksages and residence times. Note that each monomer of the trimeric OmpF pore is assembled from a single polypeptide, creating a heterogeneous sensing environment. The lateral electrostatic field at the CZ enforces the reorientation of the peptide sidechains. As a control, we used an OmpF triple mutant (R42E, R82E, and R132E) which eliminates the strong asymmetric lateral charge distribution at the CZ. This OmpF triple mutant did not allow for chiral discrimination, which further proves the importance of the lateral electrostatic field (Figure S10). This is markedly different from the commonly used pore-forming toxins which are self-assembled from repeats of monomers. Wild type aerolysin, as an example, is not capable of detecting if a peptide contains chiral isomers (Figure S11).

As a possible application, we characterized disease- and drug-relevant peptide chirality-containing isomers. The Amyloid β peptide (Aβ) is a crucial indicator for the progression of Alzheimer’s disease. Its D-Asp1 mutation causes unusual aggregates in vivo. To measure this critical segment of β-Amyloid, N-Arg-Ala-Glu-Phe-Arg-His-Asp (Mol-10) and its isomer D-Asp1 (Mol-11), OmpF-based nanopore assay was performed. Contrary to Mol-10 hardly interacting with OmpF, Mol-11 interacts with the OmpF nanopore, with typical current blockades being counted (Figure 3a). The ratio ΔI/I0 from Mol-11 has a value of 82 ± 9 μs, and t is 219 ± 89 μs. We ascribe this finding to the fact that the sidechain of Arg3 faces toward the negatively charged pocket in the presence of D-Asp1, altering the orientation of the subsequent latter sidechains (Figure S12). A varying concentration of Mol-11 in the fixed high interference concentration of Mol-10 of 56.2 μM shows that event counts of the D-Asp1 mutant Mol-11 can be linearly fitted to the increasing concentration ratio of [Mol-11]/[Mol-10] (Figure S13a). Therefore, OmpF could achieve identification of the rare species of the chirality mutant from the mixture. Moreover, our results demonstrate that the OmpF report of Mol-11 from the mixture is independent of the Mol-11 concentration (Figure S13b), and the events from Mol-11 are distinguishable from Mol-10. The OmpF identification of the D-Asp1 isomeric of β-Amyloid (Mol-11) from the WT β-Amyloid (Mol-10) would facilitate the qualitative evaluation of the isomers in pathogenesis and disease progression.

A second peptide, the so-called icatibant peptide drug HOE140, is a highly selective antagonist at the bradykinin B2 receptor. The sequence of HOE140 is N-Asp-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg (Mol-12), containing chiral amino acids. Early studies hinted at the fact that the peptide containing p-Ser isomer (Mol-13) is the most difficult to be probed among all the impurities, when using GC chromatography. Both the drug and its isomer were measured using the present OmpF protocol, and generated specific current distribution patterns of current blockades, as shown in Figure 3b–d. Characterizing the blockade events for Mol-12, we got 89 ± 17% for ΔI/I0 and 89 ± 19 μs for t. The presence of Mol-13 yielded a ΔI/I0 of 80 ± 11% and t value of 62 ± 28 μs. Comparing Mol-12 and Mol-13, the D-Asp1-L-Asp2 combination of Mol-12 at the N-terminus of the sequence nicely matches the electrostatic distribution in the CZ, similar to Mol-S, while the inverted orientation at D-Ser in Mol-13 leads to a decrease of the residence time of about 50%, compared to Mol-12. Additionally, an all L-type variant of HOE140 (Mol-14, Figure S14) was synthesized and sensed with OmpF to further prove the mechanism of the sidechain orientation in the CZ.

Finally, a mixture of Mol-12 and Mol-13 has been measured using OmpF with various molar ratios (Figure 3c,d). The distinct single molecule distribution pattern could be used for quantitatively and qualitatively determining the impurity of Mol-13 (Figures 3d and S15). The linear relationship between calibrated counts of Mol-13 and the concentration ratio of Mol-13 to Mol-12 is independent of the concentration of Mol-12 (Figures 3d and S17). It is noteworthy that by performing MS characterization (cf. Supporting Note 4), neither Mol-10 and Mol-11 nor Mol-12 and Mol-13 were identifiable (Figure 3a,b), suggesting the limitation in identifying the stereochemistry of amino acids, while the nanopore-based approach presented here is more applicable. Therefore, OmpF allows discrimination of chiral impurities from the icatibant drug, providing a single-molecule method for quality control of the peptide drugs.

**CONCLUSIONS**

In summary, the lateral electrostatic field in the CZ of OmpF leads to the sidechain reorientation during peptide translocation. The resulting changes in the current drop and residence times allow identification of peptides containing chiral amino acids and positional isomer at the single-molecule level. As for potential detection examples, we measured disease-related β-Amyloid and HOE140 impurities containing chiral amino acids. All peptides have been probed using OmpF based on electric recognition. One of the major concerns of OmpF-sensing peptides is the short residence time. Many speed-controlled approaches can potentially be utilized to boost the peptide sensing capability through nanopores, for example, forming peptide-DNA conjugation for enzyme-directed amino acid stepwise scanning. Moreover, we found strong indication that the chirality of the polar sidechain from serine (Mol-13) creates additional interactions with the residues in the CZ of OmpF (e.g., Thr112,122 and Asn101), thus from serine (Mol-13) creates additional interactions with the residues in the CZ of OmpF (e.g., Thr112,122 and Asn101), thus increasing the residence time to hundreds of milliseconds. This finding might further explain the possible discrimination of the presence of D-Ser and L-Tic containing polar and hydrophobic sidechains, respectively. In conclusion, using nanopores with the lateral electrostatic field can control the sidechain orientation. This can be employed to detect properties that cannot be distinguished using nanopores to preserve the symmetry. Similarly, a lateral polar or hydrophobic environment in the CZ might allow for identification of sidechain polarities or hydrophobicity properties. According to the structure and functions of each residue at the CZ, the enhanced laterally asymmetric effect can be achieved by introducing critical sensing residues (e.g., containing polar sidechains: Ser and Gln and hydrophobic sidechains: Phe and Trp) on the CZ. Lastly, an asymmetric CZ allows for high sensitivity to the sidechain orientation, bringing a new mechanism into proteomic studies. Along with the small confining size of the CZ in OmpF, which is compatible with the volume of 1–2 amino acids, asymmetries in the CZ provide additional opportunities for nanopore sensing in single-molecule proteomic studies which have not been explored so far.
ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c03923.

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Notes
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