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Methamphetamine binds to α-synuclein and causes a conformational change which can be detected by nanopore analysis

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ABSTRACT

α-Synuclein is an intrinsically disordered protein of 140 amino acids which is abundant in dopaminergic neurons. Misfolding and aggregation of α-synuclein leads to the formation of Lewy bodies inside the neurons which is the hallmark of Parkinson's disease and related dementias. Here we show by nanopore analysis that the recreational drug, methamphetamine, binds to the N-terminus of α-synuclein and causes a conformational change which cannot be detected by circular dichroism spectroscopy. The results suggest a mechanism for the psychoactivity of methamphetamine as well as an increased incidence of Parkinson's disease amongst users of the drug.

1. Introduction

Parkinson's disease (PD) and Lewy body dementia (LBD) are two of a number of related neurodegenerative diseases which include, Alzheimer's disease (AD) Huntington's disease (HD), the "prion" diseases such as Creutzfeldt–Jakob Disease (CJD), and amyotrophic lateral sclerosis (ALS) [1]. In all cases, post-mortem analysis of brain tissue shows the presence of amyloid fibrils, plaques or Lewy bodies which consist of protein aggregates. Surprisingly, the proteins show no obvious sequence or structural homology; e.g. α-synuclein in PD and LBD; Aβ, which is derived from amyloid precursor protein (APP), in AD; hungtingtin protein in HD; prion protein in CJD; and superoxide dismutase in ALS. The fact that the proteins are not in their native conformations has led to the hypothesis that they are all "protein misfolding diseases" [2–4].

In PD and LBD, for example, the fibrils and Lewy bodies are composed mostly of aggregates of α-synuclein (αS) which has adopted a β-sheet structure [5–8]. The process of aggregation is not well understood but presumably occurs through oligomerization of the intrinsically disordered protein to form soluble protofibrils which then accumulate as insoluble aggregates [9–11]. The aggregates are found in Lewy bodies, synapses and axons of dopaminergic neurons, but how this leads to neurodegeneration is not well understood. As is the case with AD, there is good evidence in PD that the soluble intermediates are the direct cause of toxicity rather than the aggregates themselves [12–16]. Three theories have been presented.

First, misfolded proteins are normally sequestered or neutralized by cellular defense mechanisms which include the chaperone, proteasome and/or autophagosome responses. Thus one pathogenic possibility is that these responses are affected during PD such that normal protein turnover, which is essential for cell survival, cannot function [15,17,18]. A second possibility is based on the recent observation that αS functions as a ferrireductase which binds both Cu(II) and Fe(III) to produce Fe(II) with NADH as the cofactor. The Fe(II) is required by tyrosine hydroxylase to produce L-DOPA providing a direct link to the survival of dopaminergic neurons [19,20]. As well, Cu(II) and Fe(II) can both produce lethal reactive oxygen species (ROS) so that, again, misfolding of αS could lead to an increase in these metal ions and cellular toxicity [21]. Third, a very recent paper demonstrates that in vivo, αS exists as a stably-folded, mostly α-helical tetramer [22]. The authors propose that the tetramer must be destabilized as a prerequisite to refolding and aggregation. Whatever the mechanism, the presence and subsequent misfolding of αS plays a central role.

An emerging technique for studying protein folding and conformation is nanopore analysis [23]. Briefly, nanopores are naturally-occurring toxins that punch holes in lipid membranes and allow the passage of small molecules and proteins [24–28]. When a voltage is applied across the pore, a current will flow which is dependent

Abbreviations: αS, α-Synuclein; PD, Parkinson's disease

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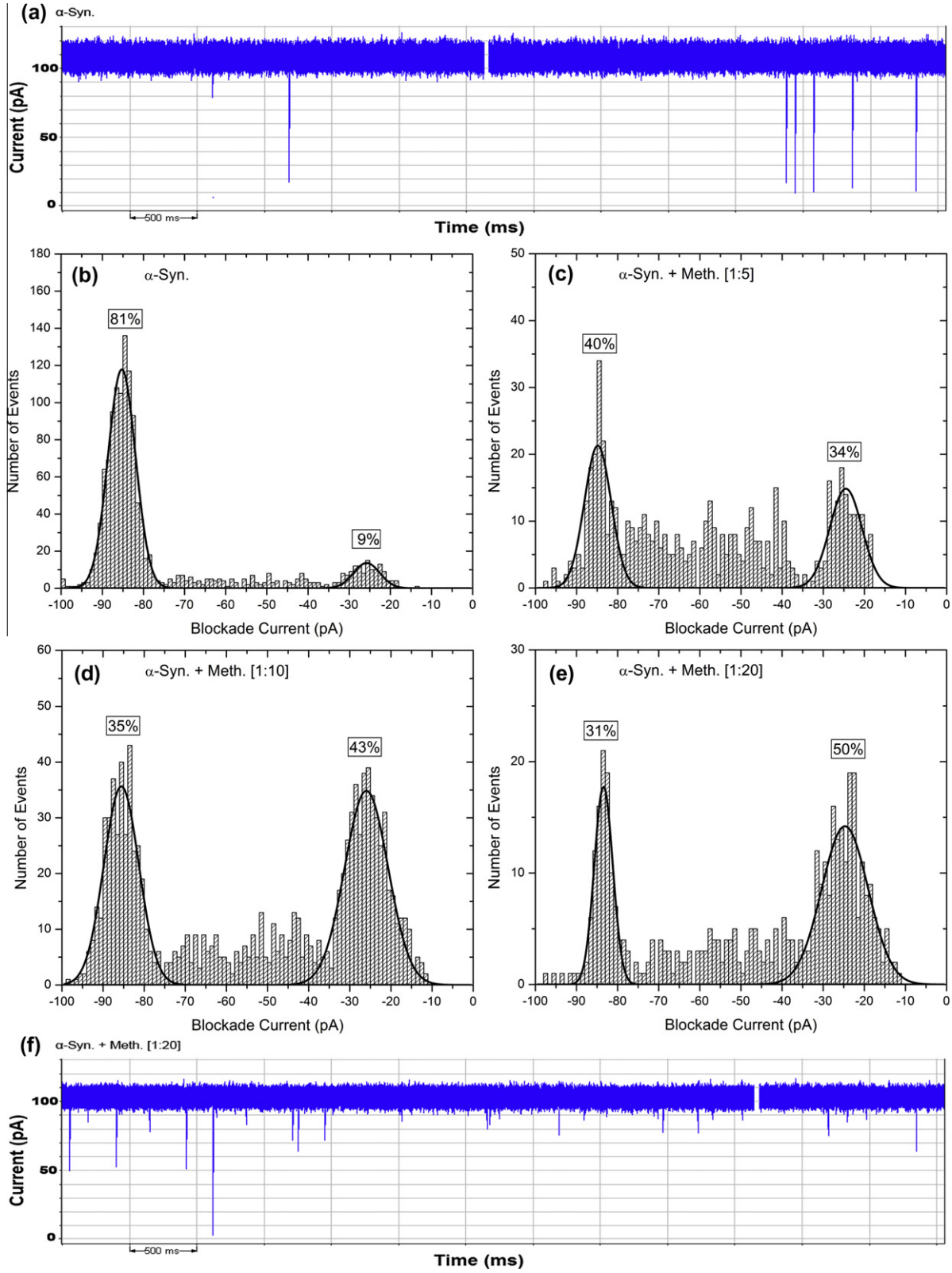

Fig. 1. Nanopore analysis of the interaction of methamphetamine with full length αS. (a, f) Typical current traces for αS and αS with 20 μM methamphetamine. (b–e) Blockade current histograms for αS with 0, 5, 10, 20 μM methamphetamine, respectively.

on the dimensions of the pore and the supporting electrolyte. However, if a large molecule passes through the pore (called translocation), or interacts with the pore, but does not pass through (called a bumping event), the current will be reduced. The current blockade (I) and time of blockade (T) represent the "signature" of a single molecule and can be measured by a conventional patch clamp apparatus. When a molecule changes conformation, the values of I and T will change. After measuring thousands of events, histograms of

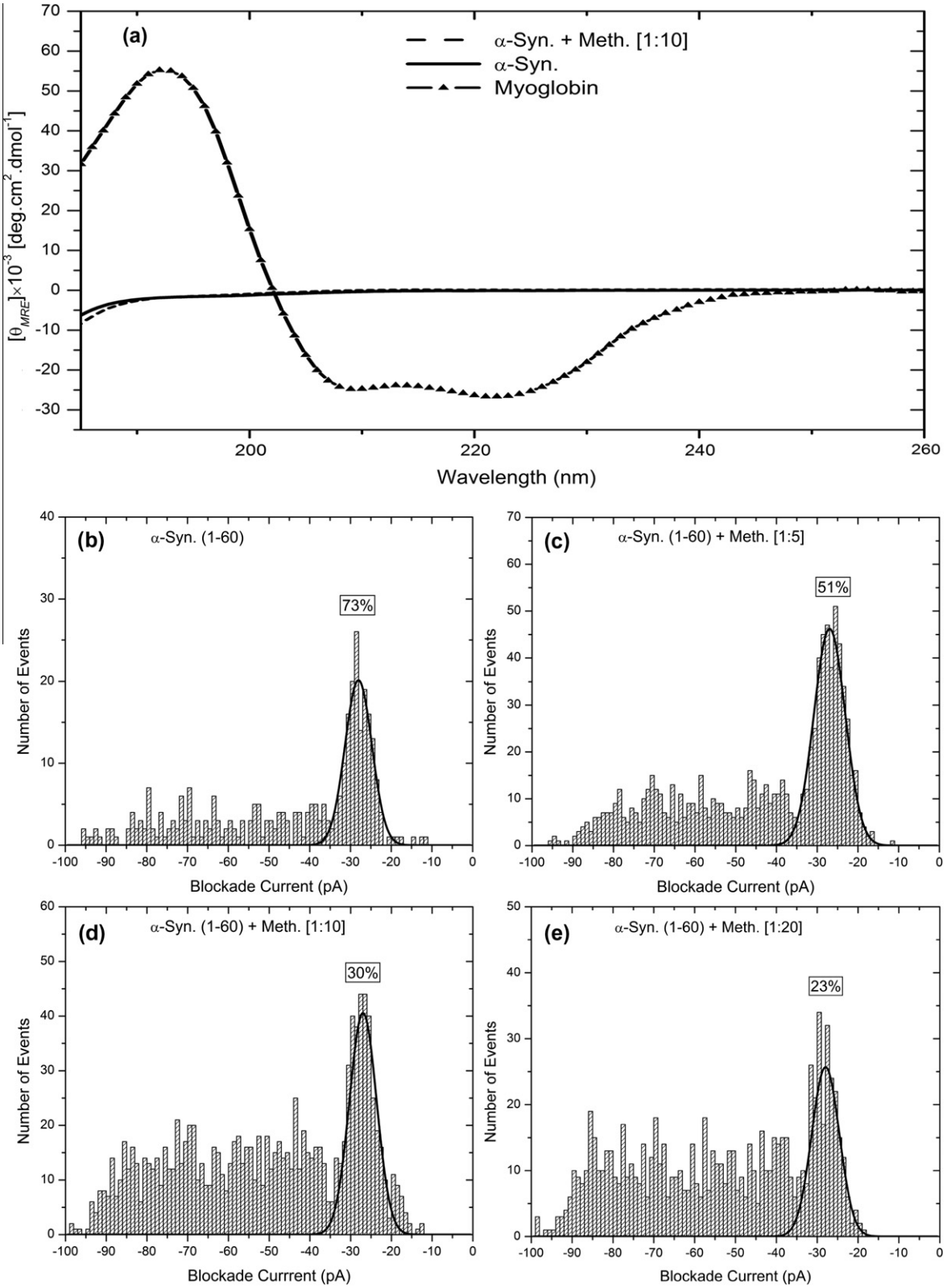

Fig. 2. (a) CD spectra for full length αS with and without 10 μM methamphetamine. For comparison the spectrum for myoglobin is also shown. (b–e) Blockade current histograms for truncated αS(1–60), with 0, 5, 10, 20 μM methamphetamine, respectively.

blockade current (I) and blockade time (T) are plotted from which average values of I and T can be derived. We have used this technique to study the folding of various peptides and proteins [29–32], including zinc-finger proteins in the presence and absence of metal ions [33], prion-derived peptides in the presence of Cu(II) [34] and Myelin Basic Protein in the presence of divalent metal ions [35]. It is ideally suited for studying peptides such as αS which can adopt multiple conformations since each molecule is interrogated individually. It is also very sensitive since in theory a single molecule can be detected. Therefore, nanopore experiments can be performed at

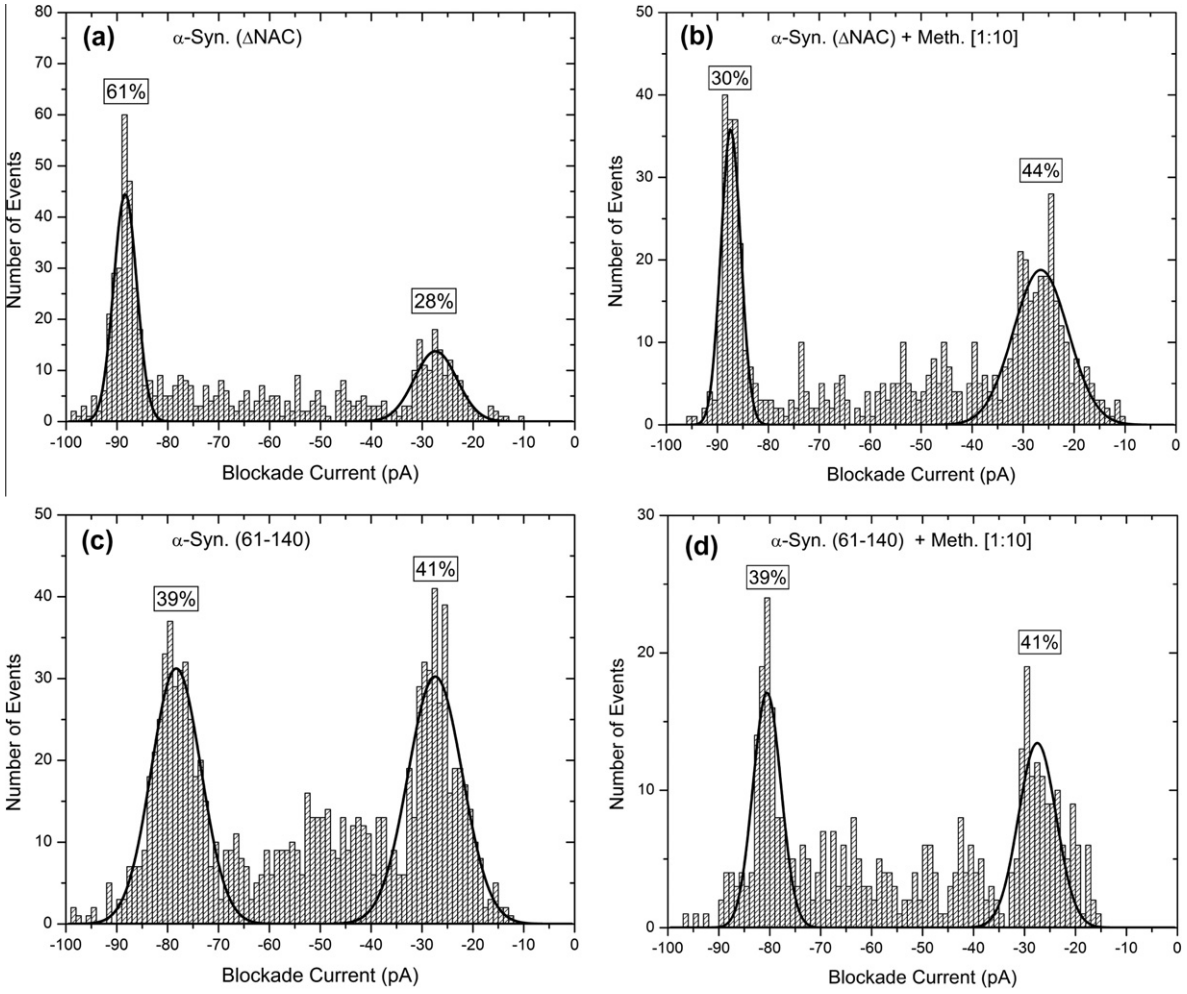

Fig. 3. (a, b) Blockade current histograms for αS(ΔNAC) with 0 μM and 10 μM methamphetamine. (c, d) Blockade current histograms for αS(61–140) with 0 μM and 10 μM methamphetamine.

sufficiently low concentrations that aggregates only form slowly, if at all. Thus there are several advantages compared to conventional structural techniques such as NMR or X-ray crystallography [23].

Several studies suggest that environmental factors are important for the development of Parkinson's disease. For example, Paraquat is common pesticide that has been implicated as a potential neurotoxicant in PD [36]. Similarly, it is known that the psychoactive effects of methamphetamine are caused by dopamine release and recently it has been reported that methamphetamine addicts have a higher incidence of PD [37,38]. Furthermore, overexpression of αS within neurons of the substantia nigra induced by amphetamine derivatives is related to the occurrence of intracellular inclusions [39,40]. Here we demonstrate by nanopore analysis that methamphetamine causes a conformational change in αS. It is known that α-synuclein binds dopamine and we propose that methamphetamine binds to the protein and causes release of dopamine. If this proves to be correct then there is a direct link not only between methamphetamine and its psychoactivity but also between methamphetamine abuse and the increased incidence of PD.

2. Materials and methods

α-Synuclein and the truncated peptides were purchased from rPeptide (Bogart, GA, USA). They were dissolved in water at concentration of 1 mg/ml. (+)-Methamphetamine hydrochloride solution at 1 mg/ml, analytical standard for drug analysis, was purchased from Sigma–Aldrich.

Nanopore analysis of these isoforms in a buffer of 1 M KCl, 10 mM HEPES–KOH, pH 7.8 was performed as described in detail previously [23,33,34]. Briefly, a lipid bilayer was painted onto a 150 μM aperture in a teflon perfusion cup which separated two buffer compartments of volume 1.5 mL. α-Hemolysin was purchased from Sigma–Aldrich and diluted in buffer to a final concentration of 1 μg/mL. 5 μL of this solution was added to one side of the membrane and stable pore insertion was confirmed by an increase in the current to 100 pA. The protein (6 μL, 14.5 μL, 8.5 μL and 12 μL of αS (1–60), αS, αS (61–140) and αS (ΔNAC) peptide solutions of 1 mg/mL, respectively) was added to the *cis*-side of the pore with the positive electrode on the *trans*-side. The final concentration of protein at the *cis*-side was 1 μM. The experiments were carried out at 22 ± 1 °C with an applied potential of −100 mV at a band width of 10 kHz using an Axopatch 200B amplifier (Axon Instruments) under voltage clamp conditions. The blockade current populations were obtained by fitting the blockade current distribution with the Gaussian function [23,33,34].

The circular dichroism (CD) spectra of αS were recorded on an Applied Photophysics π*-180 spectrophotometer at 22 ± 1 °C. Peptide solutions used for the CD measurements were made in 10 mM potassium phosphate buffer at pH 7.8 at 0.5 mg/ml. The spectrophotometer was calibrated with a solution of (1S)-(+)-10-camphorsulfonic acid. Corrections to the record spectra were made by

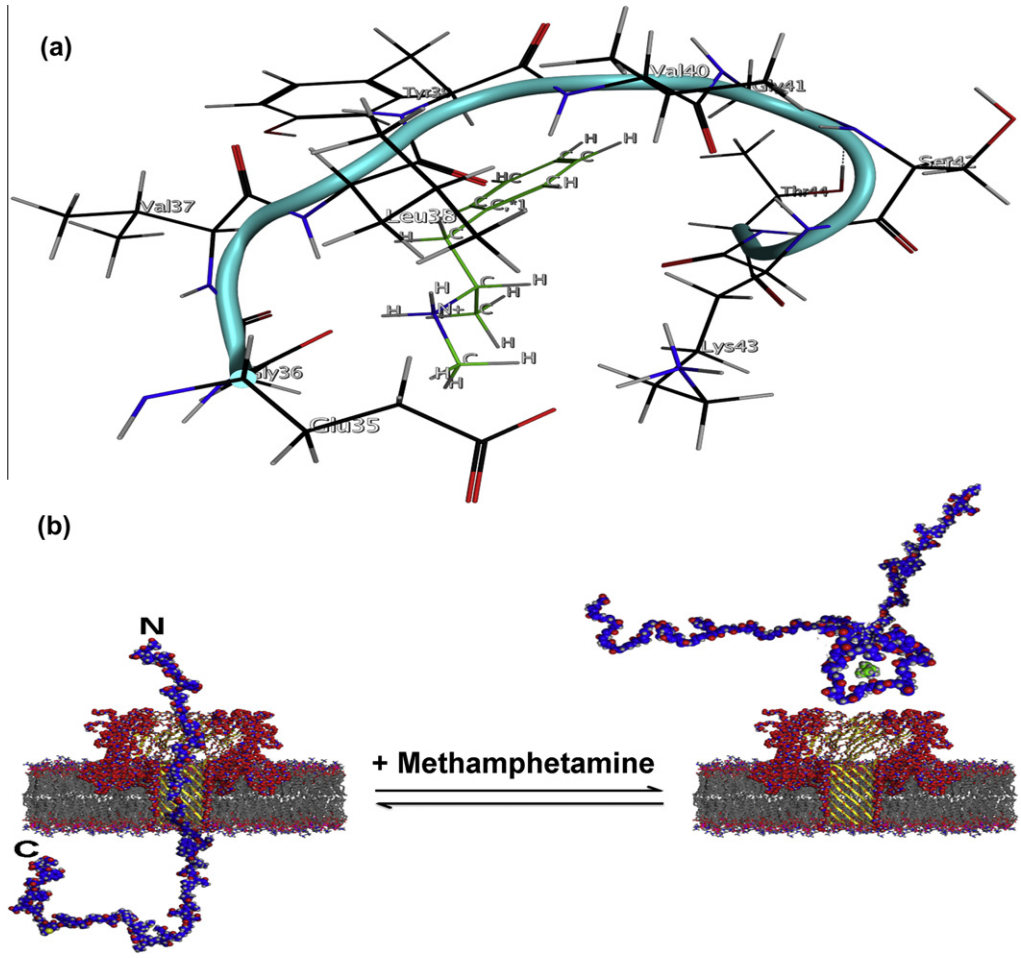

Fig. 4. (a) Proposed binding site for methamphetamine in the N-terminal region of αS. (b) Full length αS can readily translocate the α-hemolysin pore from the C-terminal end which carries a net negative charge. In the presence of methamphetamine, a loop is formed which causes bumping events to be more likely.

subtracting the spectrum of the buffer or the spectrum of the buffer and the ligand. The mean residue ellipticity (θ_{MRE}) is calculated to normalize the ellipticity. The mean residue ellipticity (θ_{MRE}) was reported in deg cm^2 dmol^{-1} [41]. In order to allow for the comparisons between the CD spectra of αS with a typical spectrum of α-helix, the CD spectrum of horse myoglobin (PCDDBID: CD0000047000) was used [42,43]. The unit of myoglobin spectra, delta epsilon, was converted to the mean residue ellipticity [41].

The 3D structure of αS (PDB ID: 1XQ8) together with the protonated form of the 3D conformer of (+)-methamphetamine (PubChem CID: 10836) were used for the docking simulation which was performed using molecular operating environment (MOE) from the Chemical Computing Group, Inc. as described previously [44]. Briefly, the target peptide, αS(1–60), was subjected to an MMFF94 energy minimization protocol until the root mean square of the conjugate gradient reached <0.05 kcal mol^{-1} Å^{-1}. The MOE-Dock default parameters were used for the docking simulation. The MMFF94s was selected as the force field [44]. Refinement and Rescoring 2 were set up to the Forcefield and Alpha HB, respectively. A global docking was performed with the target peptide. The docking was carried out for 30 iterations. The docking interaction with the lowest energy (−50 kcal/mol) is shown in Fig. 4a.

3. Results and discussion

A typical current trace for αS covering about 6s is shown in Fig. 1a and most of the events have large current blockades. After collecting events for about 30 min a current blockade histogram can be derived which is shown in Fig. 1b. The major peak is at −85 pA and, as discussed previously, this is due to translocation of the protein since the time of the events decreases with increasing applied voltage [23]. The minor peak at −25 pA is due to bumping events which represent about 9% of the total number of events. The effect of increasing methamphetamine concentrations are shown in Fig. 1c–e. At 5 μM the translocation peak is decreased and the % events in the bumping peak increases to 34%. There are also a large number of events with intermediate current blockades which are difficult to characterize because they do not yield a clear Gaussian distribution. At 10 μM of drug the bumping events increase to about 43% and at 20 μM, there is a further increase to 50%. A typical current trace for 20 μM drug is shown in Fig. 1f which clearly shows a decrease in the proportion of translocation events compared to Fig. 1a. In these experiments, the concentration of αS is 1 μM so that the binding constant (Ka) is in the range of 10^5–10^6 M^{-1}. It would appear that the drug/αS complex can give rise to some translocation events because they are still observed even at the highest drug concentration. As well, because of the presence of intermediate peaks it is not possible to calculate a more accurate value for Ka.

In general, an increase in bumping events can be attributed to the folding of a molecule into a more compact conformation which impedes translocation. For example, addition of Zn^{2+} to a Zn-finger peptide causes formation of a compact β-sheet/α-helix sandwich which can no longer translocate [33]. As expected, the transition

from a disordered to an ordered conformation is also accompanied by a large change in the CD spectrum [45]. As shown in Fig. 2a, the CD spectrum of αS is very weak and typical of an intrinsically disordered protein compared to myoglobin, for example, which is mostly α-helical. However, addition of methamphetamine does not cause a significant change in the CD spectrum of αS which implies that the drug is not inducing an α-helical or β-sheet conformation. On the other hand, formation of a simple loop or binding pocket (see Fig. 4) would not be expected to alter the CD spectrum but would effect the event profile; a result which emphasizes that nanopore analysis can detect subtle conformational changes which may not be amenable to spectroscopic techniques.

The αS protein can be divided into three regions; the N-terminus comprising amino acids 1–60 which can adopt an α-helical conformation when bound to anionic lipids; a central hydrophobic NAC domain (61–95) which is thought to be responsible for β-sheet formation and eventual fibrillization; and an acidic C-terminus which contains the binding site for dopamine [46,47]. In order to delineate the binding site of methamphetamine, several deletion mutants were studied. Fig. 2b–e shows the results for the N-terminal peptide αS(1–60). In the absence of drug (Fig. 2b) there is no clear translocation peak and about 73% of the events are bumpings. Upon addition of increasing concentrations of drug the proportion of bumping events decreases to 51%, 30%, and 23% in the presence of 5, 10 and 20 μM drug, respectively. Simultaneously, there is an increase in the proportion of events with higher blockade current and at 20 μM drug about 15% of the events have blockade currents above −80 pA, presumably due to translocations. From these results it is clear that methamphetamine binds to the N-terminus and causes a conformational change although it is not obvious why there are fewer bumping events in the presence of the drug in the case of αS(1–60) but more bumping events for the full length protein. One explanation is that the truncated peptide has a net charge of +4 whereas the full-length protein has a net charge of −9 (because the C-terminus is acidic). Therefore, αS(1–140) will be electrophoretically driven towards the pore whereas the interaction of αS(1–60) with the pore will be diffusion controlled. Moreover, it has been demonstrated previously that the dipole moment of a peptide can have a profound effect on the ratio of translocation to bumping events [32,48]. αS(1–140) has a large dipole moment because of the preponderance of negative charge on the C-terminus which will position the protein on the correct trajectory for translocation i.e. C-terminus toward the pore (see Fig. 4b). In contrast, inspection of the sequence of αS(1–60) shows that it will have a much smaller dipole moment because the charge is evenly distributed. Thus, in an unfolded state it is likely to straddle the pore giving rise to a typical bumping event.

An N-terminal binding site for methamphetamine was confirmed by investigating αS(ΔNAC) (Fig. 3a and b) for which there is an increase in bumping events on addition of drug and αS(61–140) (Fig. 3c and d) for which the drug has no apparent effect on the blockade current profile. A possible binding site in the N-terminus encompassing residues 35–44 (Fig. 4a) was identified by docking methamphetamine to unfolded αS(1–60) using molecular operating environment (MOE) from the Chemical Computing Group, Inc. [44]. The binding is stabilized in part by polar interactions between Glu35, Gly36, Tyr39 and Thr44, and the amine of the drug as well van der Waals interactions between Leu38, Val40 and Lys43 and the benzene ring of the drug. The peptide becomes folded around the drug forming a loop which is stabilized by ionic interaction between Glu35 and Lys43 (Fig. 4a). In order for the complex to translocate the loop would presumably have to unfold and so bumping events become more prevalent.

In conclusion, we have shown that methamphetamine binds αS in the N-terminal region causing a conformational change which can be detected by nanopore analysis but not CD. The interaction may be responsible for the psychoactivity of methamphetamine as well as the increased incidence of PD amongst drug users. In the long term, this technique will prove useful for assessing candidate drugs for treating PD.

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