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Divalent cations induce a compaction of intrinsically disordered myelin basic protein

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ABSTRACT

Central nervous system myelin is a dynamic entity arising from membrane processes extended from oligodendrocytes, which form a tightly-wrapped multilamellar structure around neurons. In mature myelin, the predominant splice isoform of classic MBP is 18.5 kDa. In solution, MBP is an extended, intrinsically disordered protein with a large effective protein surface for myriad interactions, and possesses transient and/or induced ordered secondary structure elements for molecular association or recognition. Here, we show by nanopore analysis that the divalent cations copper and zinc induce a compaction of the extended protein *in vitro*, suggestive of a tertiary conformation that may reflect its arrangement in myelin.

Introduction

Myelin basic protein (MBP) is essential for the development and stability of central nervous system myelin, and is a candidate autoantigen in multiple sclerosis, a human disease that is characterized by active degradation of the myelin sheath [1,2]. The designation "MBP" usually refers to the "classic" 18.5 kDa isoform which is a peripheral membrane-associated protein found in cell processes and compact myelin [3–6]. This isoform is extremely positively-charged (+19 at neutral pH), with combinatorial post-translational modifications generating a series of *charge components* named C1–C8 – briefly, C1 is the most cationic and the least modified, and C8 is the least-charged and most modified, being primarily deiminated (conversion of arginine to citrulline) [2,3,6]. The C1 component predominates in healthy myelin, whereas the proportion of C8 component (deiminated MBP) is increased in myelin of multiple sclerosis patients [2].

The three-dimensional conformation of 18.5 kDa MBP in myelin remains unknown. A three-dimensional C-shaped model of the structure has been proposed based on single particle electron microscopy and molecular modeling of highly-conserved sequence segments [7,8]. It was subsequently realized that all isoforms of MBP are intrinsically disordered proteins, and that the wide variety of surface, protein, or small ligand interactions would result in major conformational changes, *viz.*, "induced folding" [3,9]. In all associations that have so far been investigated structurally (phospholipid membrane, Ca^{2+}-calmodulin, actin), the 18.5 kDa MBP isoform has demonstrated a partial induced disorder-to-order transition, including several α-helical molecular recognition fragments [5,6]. These spectroscopic studies have provided evidence of induced ordered secondary structure, but no information on tertiary conformation.

Ever since the first molecular studies of MBP in the early 1970s, there has been the question of whether the protein holds two membrane leaflets together as a monomer, or as a dimer. The observation that one-dimensional ^{13}C solid-state NMR spectra of the protein do not change dramatically with increasing protein-to-lipid ratio (unpublished results of M.A.M. Ahmed, G. Harauz, V. Ladizhansky), supports a monomer model, which suggests that the protein must fold over to be able to interact with two separate membrane leaflets. This idea is supported by studies on other intrinsically disordered proteins that exhibit higher-order interactions despite lacking a compact tertiary structure, such as the microtubule-assembling protein tau which has been shown to exhibit a "double-hairpin" or "paperclip" arrangement in solution [10,11].

In this report, we demonstrate the stabilization by zinc and copper of a tertiary conformation in 18.5 kDa recombinant murine MBP (rmMBP) (Fig. 1). The divalent cation Zn^{2+} occurs in high proportions in myelin and is also believed to stabilize it [12,13], and it has been shown that the protein binds $Hg^{2+} > Cu^{2+} > Zn^{2+} > Mg^{2+} > Cd^{2+} > Co^{2+}$ in decreasing order of affinity [14]. Here, we use nanopore analysis to study MBP compaction by Cu^{2+} and Zn^{2+}. Briefly, nanopores are naturally-occurring toxins which create holes in lipid membranes and allow the passage of small molecules and proteins [15–18]. When a voltage is applied across the pore, a current will flow which is dependent on the dimensions

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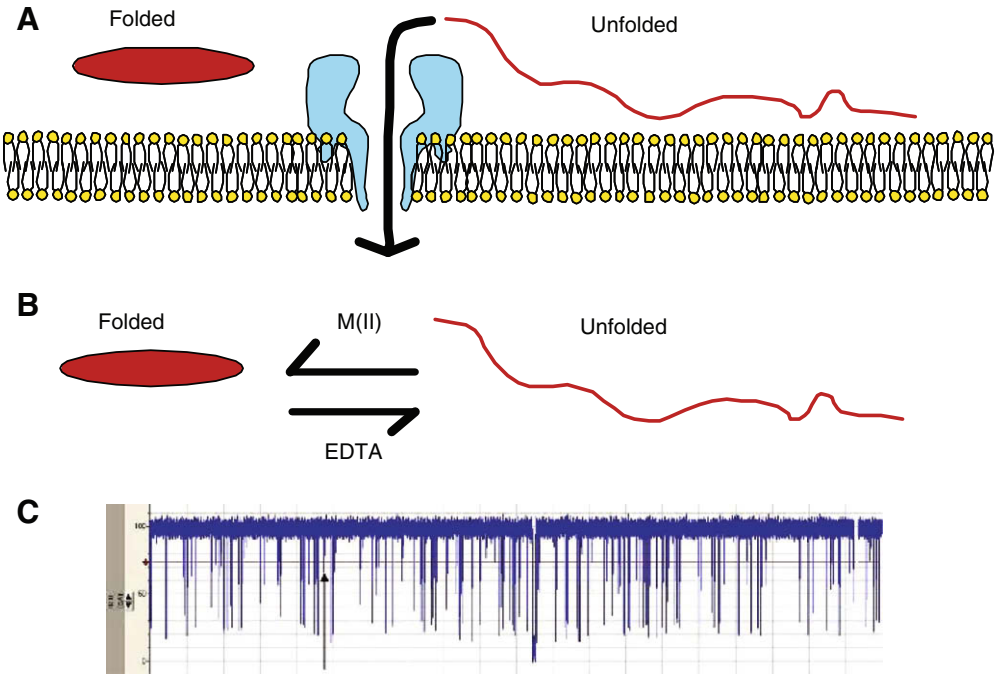

Fig. 1. (A) The unfolded or intrinsically disordered MBP can translocate the α-hemolysin pore embedded in a lipid bilayer. A weak association of MBP with the lipid bilayer may facilitate diffusion of the protein to the vicinity of the pore. (B) Upon binding divalent metal ions, the protein folds into a more compact conformation which can no longer translocate. (C) Current trace after adding rmC1 to the *cis*-side of the pore. The open pore current is −100 pA and each spike represents an event. The arrow points to a rare bumping event while the majority of events are presumed to be translocations under these conditions.

of the pore and the supporting electrolyte. However, if a large molecule passes close to the pore, called a bumping event, or passes through the pore, called translocation, 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. This technique has been used to study the folding of various proteins [19–22], including Zn-finger proteins in the presence and absence of metal ions [23], and is used here to study the effects of Zn^{2+} and Cu^{2+} on different variants of 18.5 kDa rmMBP.

Materials and methods

The recombinant murine unmodified 18.5 kDa isoform rmC1 and its pseudo-deiminated isoform rmC8, both hexahistidine-tagged, were over-expressed in *Escherichia coli* and purified as previously described [24,25]. We have also constructed untagged forms of rmC1 and rmC8, referred to henceforth as UT-rmC1 and UT-rmC8 (unpublished results of G.S.T. Smith, V.V. Bamm, G. Harauz). Plasmids that were confirmed by sequencing to encode the UT-rmC1 and UT-rmC8 proteins were transformed into BL-21-CodonPlus (DE3)pLysS cells (Stratagene), which were grown in M9 minimal media, essentially as routinely done for the hexahistidine-tagged variants. Cell lysates were centrifuged at 25,000 *g* for 45 min. The supernatant was filtered through a 0.8/0.2 μm membrane filter, and loaded onto a pre-charged 5 mL CM-HighTrap FF column (GE Healthcare Bio-sciences). The column was washed first with lysis buffer (6 M urea, 80 mM glycine, 1% Tween-20, pH 9.5), and subsequently with an ion exchange buffer (6 M urea, 80 mM glycine, pH 9.5). The protein was eluted with a 0–0.2 M NaCl gradient in ion exchange buffer at a flow rate of 1 mL/min. Pure protein fractions were pooled, dialyzed sequentially against first buffer and finally double-distilled water to remove salts, and then lyophilized. Using HPLC, the UT-rmC1 and UT-rmC8 preparations were determined to have a purity of >96%, commensurate with results obtained with the hexahistidine-tagged counterparts.

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 [19,22,23]. 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. Five microliters of this solution was added to one side of the membrane and the current was monitored until stable pore insertion was achieved. The protein (10 μL of 1 mg/mL) was added to the *cis*-side of the pore with the positive electrode on the *trans*-side (Fig. 1A). 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. The lifetime data was obtained by fitting each blockade duration distribution with a single exponential function [19,22,23].

Results and discussion

The α-hemolysin pore assembles into the membrane and gives rise to an open pore current of about −100 pA when the applied

Table 1
Blockade currents (I) and times (T) for rmC1 at different voltages. The subscripts 1 and 2 refer to bumping and translocation events, respectively.

Voltage (mV)	I_1 (pA)	I_2 (pA)	T_1 (ms)	T_2 (ms)
−50	−11[a]	−30	0.15	0.11
−100	ND[b]	−65	ND	0.15
−150	ND	−105	ND	0.55

[a] The estimated errors are ±1 pA for I and ±10% for T.
[b] ND, not determined because the number of events was too small.

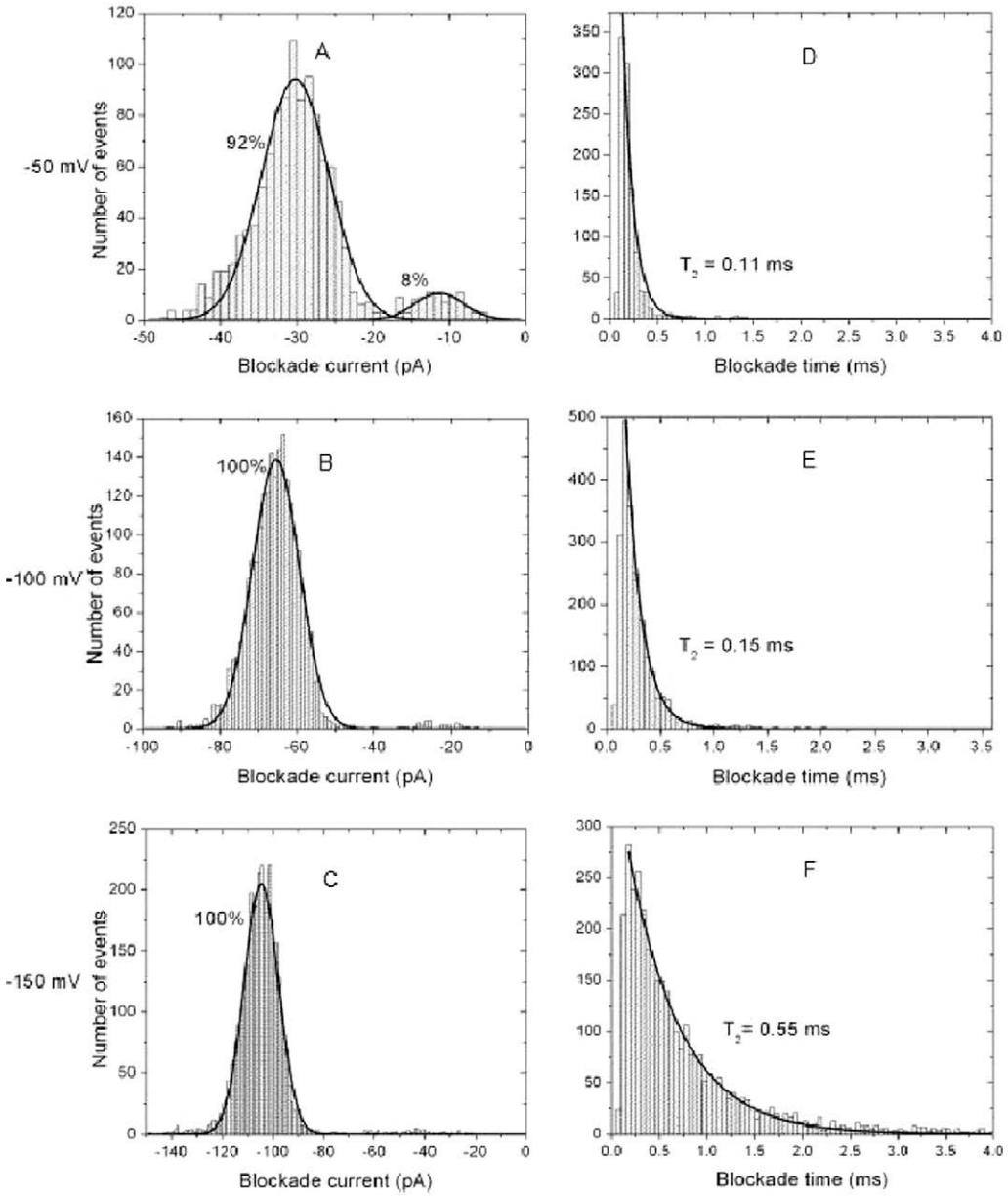

Fig. 2. Current blockade histograms for rmC1 at (A) −50 mV, (B) −100 mV, and (C) −150 mV. The peaks can be adequately fit with a Gaussian distribution to yield the current blockade, *I*. The corresponding histograms of blockade times are shown in panels (D–F) which can be fit with a single exponential function to yield the half life, *T*.

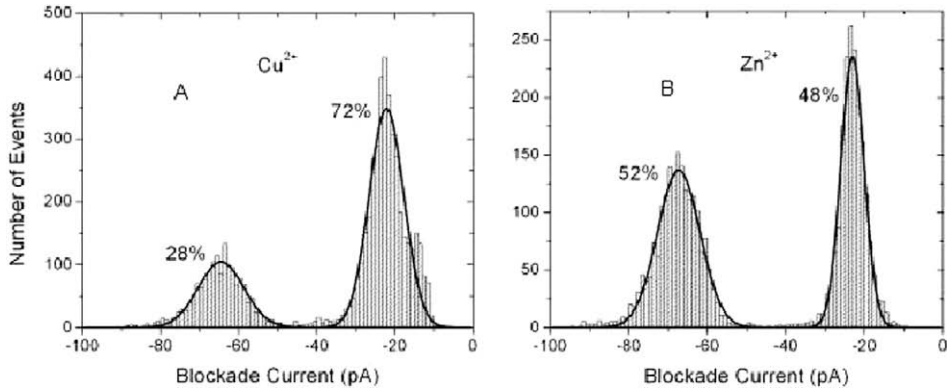

Fig. 3. Current blockade histograms for rmC1 at −100 mV after addition of (A) 0.1 mM Cu^{2+}, or (B) 0.1 mM Zn^{2+}. A large proportion of bumping events is now apparent.

voltage is 100 mV. As shown in Fig. 1C, upon addition of 10 μL of 1 mg/mL rmC1 to the *cis* (or vestibule) side of the membrane, a large number of current blockades are observed. Most of the events have *I* values (currents) ranging from −50 to −80 pA which are caused by translocation of the protein through the pore, but there are also some events at about −20 pA which are due to the mole-

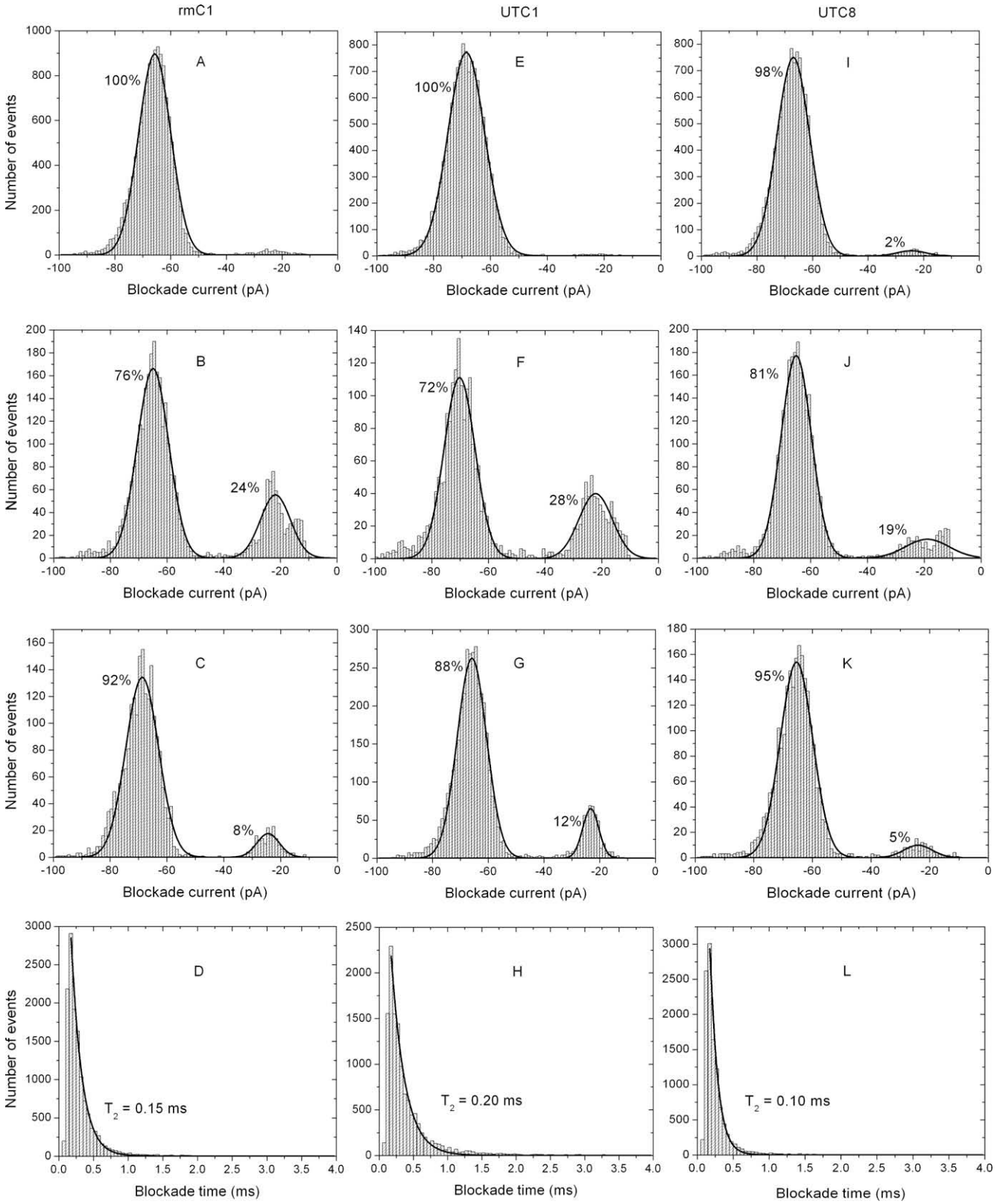

Fig. 4. Current blockade histograms for (A) rmC1, (B) rmC1 with 0.02 mM Cu^{2+}, and (C) rmC1 with 0.2 mM Zn^{2+}, (E) UT-rmC1, (F) UT-rmC1 with 0.02 mM Cu^{2+}, and (G) UT-rmC1 with 0.2 mM Zn^{2+}, (I) UT-rmC8, (J) UT-rmC8 with 0.02 mM Cu^{2+}, and (K) UT-rmC8 with 0.2 mM Zn^{2+}. The corresponding blockade time histograms for panels (A), (E), and (I) are shown in panels (D), (H), and (L), respectively.

cule bumping into the pore and then diffusing away. The very large number of events (about 100/s) that we observe for MBP was very surprising since, at this concentration, most proteins of this size yield on average about 10 events/s. This number tends to be even lower for basic proteins such as MBP because they have to diffuse to the pore against the voltage gradient (unpublished results of B. Krasniqi and J.S. Lee). A possible explanation for this exceptional behaviour is that MBP is weakly bound to the membrane through ionic interactions which facilitates two-dimensional diffusion to the pore. In contrast, other basic proteins must undergo three-dimensional diffusion against the voltage in order to reach the pore.

In order to demonstrate that the events represent translocations, the experiment was repeated at −50 and −150 mV. In Fig. 2(A–C), the results are presented as histograms of blockade current in which the type 2 events are translocations, and the infrequent type 1 events are bumpings. The corresponding blockade time histograms for the translocation events are shown in Fig. 2(D–F), and the values of I and T are summarized in Table 1. In general, two conditions must be met for an event to be considered a translocation. First, the value of I_2 (the translocation current) must be roughly proportional to the applied voltage because the volume of the pore occupied the protein is independent of voltage, and thus the blockade current is simply proportional to voltage [19]. Second, for a very basic protein such as MBP (+19) the value of T_2 (the translocation time) is expected to increase exponentionally with voltage [20]. Thus, we are confident that the protein is translocating through the pore.

The effect of adding 0.1 mM Cu^{2+} or Zn^{2+} on the blockade current histograms at −100 mV is shown in Fig. 3(A, B). In the presence of Cu^{2+}, the proportion of translocations is reduced from about 98 to 28%, and there is a corresponding increase in the bumping events to 72%. For Zn^{2+} the effect is less pronounced with 52% translocations and 48% bumping events. The total number of events did not change significantly. Therefore, in this buffer, the metal ions are not causing aggregation, as they would for this protein dissolved in phosphate buffer [26]. As controls, it was shown that (a) Mg^{2+} has no effect on the proportion of translocations, (b) addition of EDTA restores the event profiles to 100% translocation, and (c) there is no adverse effect on the pore or the open pore current at these concentrations of metal ions (data not shown). The simplest explanation is that Cu^{2+} and Zn^{2+} cause folding or compaction of the protein into a conformation that is too large to pass through the pore. Previous work has suggested that a collagen triple helix, a helix-turn-helix, or a two-stranded β-sheet [15,17,20] can all translocate through the pore. On the other hand, a folded zinc-finger [23] and certain four-stranded structures (unpublished results of J.S. Lee) cannot. Thus, the conformation adopted by MBP in the presence of divalent metal ions is probably more complex than a simple fold between two different regions of the protein (such as a "hairpin"), with the data presented here supporting a more complicated conformation (such as a "paperclip") [10,11]. It is also worth noting that the translocation times (for the fraction which still translocates) in the presence of Cu^{2+} and Zn^{2+} are 0.19 ± 0.05 and 0.17 ± 0.05 ms, respectively, which is not significantly different from the value of 0.15 ± 0.03 ms in the absence of metal ions (data not shown). The blockade currents also remain unchanged. Thus, it would appear that there are no intermediate folded conformations which are still capable of translocating.

Fig. 4 shows the effect of lower concentrations of Cu^{2+} and Zn^{2+} on rmC1 as well as UT-rmC1 and UT-rmC8. As expected for rmC1, at 0.02 mM divalent metal ions there are fewer bumping events than at 0.1 mM, and Cu^{2+} is more effective than Zn^{2+}. For UT-rmC1 the results are very similar, demonstrating that the His_6-tag has little effect on the tertiary folding of the protein *per se*. For UT-rmC8, it is clear that the divalent metal ions are less effective at causing folding of the protein. As well, the translocation time is reduced compared to rmC1 and UT-rmC1, presumably because rmC8 is less positively-charged (+13 at neutral pH).

In conclusion, we have shown by nanopore analysis that a more compact structure of 18.5 kDa MBP, which can no longer translocate, is formed upon addition of Cu^{2+} and Zn^{2+}. A recent independent study has also suggested an induced folding of native MBP by zinc ions, as ascertained by synchrotron circular dichroic spectroscopy [27]. This higher-order topology of MBP may represent how it stabilizes compact myelin multilayers *in vivo*, and is another example of induced folding of an intrinsically disordered protein by ligand association [9].

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