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METHODS AND APPLICATIONS

Development of in vivo HDX-MS with applications to a TonB-dependent transporter and other proteins

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Abstract

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) is a powerful tool that monitors protein dynamics in solution. However, the reversible nature of HDX labels has largely limited the application to in vitro systems. Here, we describe a protocol for measuring HDX-MS in living *Escherichia coli* cells applied to BtuB, a TonB-dependent transporter found in outer membranes (OMs). BtuB is a convenient and biologically interesting system for testing in vivo HDX-MS due to its controllable HDX behavior and large structural rearrangements that occur during the B12 transport cycle. Our previous HDX-MS study in native OMs provided evidence for B12 binding and breaking of a salt bridge termed the *Ionic Lock*, an event that leads to the unfolding of the amino terminus. Although purified OMs provide a more native-like environment than reconstituted systems, disruption of the cell envelope during lysis perturbs the linkage between BtuB and the TonB complex that drives B12 transport. The in vivo HDX response of BtuB's plug domain (BtuBp) to B12 binding corroborates our previous in vitro findings that B12 alone is sufficient to break the *Ionic Lock*. In addition, we still find no evidence of B12 binding-induced unfolding in other regions of BtuBp that could enable B12 passage. Our protocol was successful in reporting on the HDX of several endogenous *E. coli* proteins measured in the same measurement. Our success in performing HDX in live cells opens the possibility for future HDX-MS studies in a native cellular environment.

IMPORTANCE

We present a protocol for performing in vivo HDX-MS, focusing on BtuB, a protein whose native membrane environment is believed to be mechanistically

important for B12 transport. The in vivo HDX-MS data corroborate the conclusions from our previous in vitro HDX-MS study of the allostery initiated by B12 binding. Our success with BtuB and other proteins opens the possibility for performing additional HDX-MS studies in a native cellular environment.

KEYWORDS

BtuB, hydrogen-deuterium exchange, mass spectrometry, outer membrane proteins

1 | INTRODUCTION

Hydrogen-deuterium exchange (HDX) is a powerful biophysical technique that monitors protein dynamics. Information on solvent accessibility and conformational fluctuations of backbone amide protons is obtained without the need for mutations or synthetic labels.[1–3] In combination with mass spectrometry (MS), the HDX-MS technique has been applied to a variety of soluble and membrane proteins. Notably, the method allows one to study a protein's dynamics and thermodynamics, information potentially with residue-level resolution.[4–8] However, the reversible nature of the deuterium label demands rapid sample work-up under conditions carefully selected to limit the loss of the label prior to MS analysis, a process termed "back exchange". This process has limited the application of HDX-MS to in vitro systems. Here, we report an in vivo application of HDX-MS and demonstrate the potential of the technique to probe complex biological systems whose function may depend on being in a native cellular environment.

BtuB, the *E. coli* outer membrane (OM) vitamin B12 transporter, is a well-studied member of the TonB-dependent transporter family.[9–11] BtuB is a convenient and biologically interesting protein for testing in vivo HDX-MS due to its controllable HDX behavior and the enigmatic conformational rearrangements involved in pore formation during the B12 transport cycle. Despite intense study, key aspects of the transport mechanism remain unknown, most notably the conformational changes involved in pore formation and the coupling to the TonB complex.[9,12] Recent studies have found evidence that BtuB undergoes different rearrangements in living cells compared with reconstituted systems, especially those employing detergents.[13,14]

Our previous HDX-MS study of BtuB, conducted in a partially purified OM, provided evidence for B12 binding-induced allostery in the luminal domain related to the unfolding of the amino-terminal region.[15] While studying OM proteins (OMPs) in native OMs is a considerable advance over reconstituted systems, OMs still lack important features that are only present in an intact cellular context. The transport function of BtuB may rely on lipopolysaccharide, an energized inner membrane (IM), or peptidoglycan,[13,14,16–18] yet all of these are disrupted upon cell lysis. Additionally, the sarkosyl treatment employed to remove the IM from our previous OM preparations[15] may have introduced additional perturbations to the BtuB dynamics.[14,15,19]

Here, we conducted HDX-MS on BtuB in live *E. coli*. HDX labeling was initiated by transferring *E. coli* overexpressing BtuB into deuterated growth media. Our post-HDX lysis and purification conducted under HDX quench conditions (e.g., 2°C, pH 2.5) limited back exchange to manageable levels. The in vivo HDX data for BtuB's plug domain (BtuBp) are similar to our prior data obtained in OMs, particularly the B12 binding-induced unfolding of the amino terminus.[15] Finally, we discuss the conditions necessary for performing HDX-MS in vivo on other proteins and provide examples of endogenous *E. coli* proteins whose HDX was observed in the same measurements.

2 | RESULTS

The primary challenge in conducting HDX-MS in vivo is obtaining peptides from a live sample while minimizing loss of the deuterium label to back exchange. Sample purification, which had occurred during the period between cell harvesting and initiation of HDX, is now conducted post-HDX labeling and under quench conditions. This necessitates optimization of the protocol to strike a compromise between sample quantity, purity, and back exchange. Our in vivo HDX-MS protocol provides a solution to this challenge for the OMP BtuB (Figure 1a).

HDX was initiated by transferring a culture of *E. coli* overexpressing BtuB into a deuterated growth medium (22°C, pD_{read} 6.8). A carbon source was included during HDX labeling to prevent starvation, although HDX was insensitive to the addition of LB medium, most likely because of our short, sub-10 min labeling time

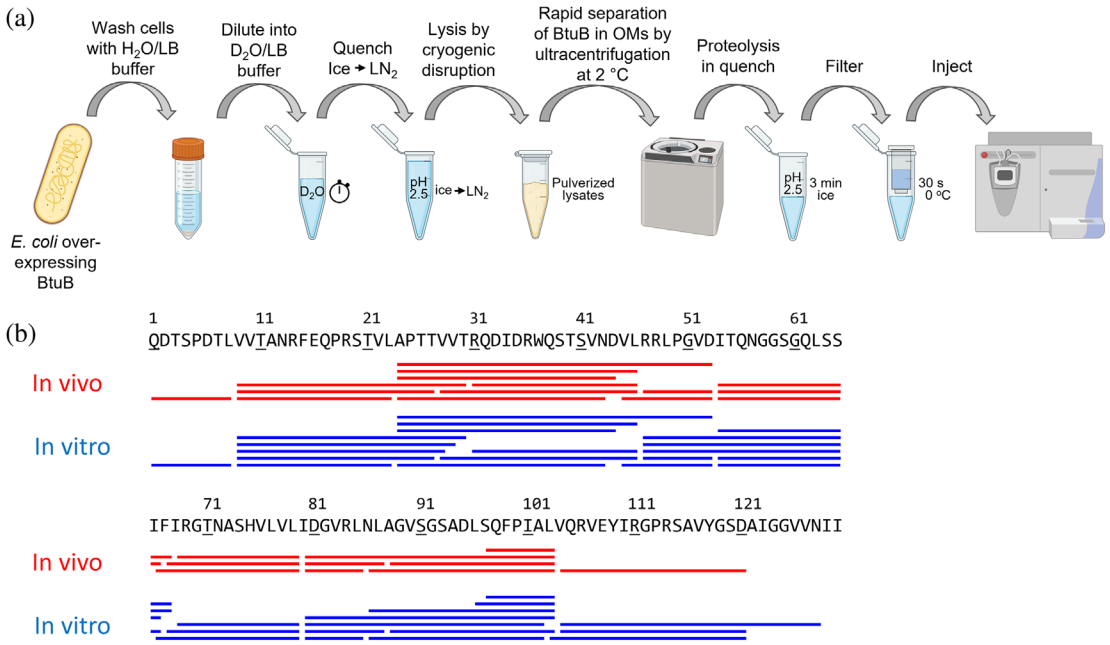

FIGURE 1 Experimental workflow and sequence coverage for in vivo HDX-MS on BtuB. (a) In the in vivo HDX-MS protocol, living *E. coli* cells overexpressing BtuB are diluted in a D_2O buffer supplemented with a carbon source. After quenching, cells are lysed using cryogenic disruption, and OMs containing BtuB are separated by ultracentrifugation at pH 2.5, 2°C. Figure created with BioRender. (b) In vivo measurements observe 25 peptides (red) with 93% coverage of BtuBp, while previous in vitro measurements observe 35 (blue) with 98% coverage of BtuBp.[15]

(Figure S1). After labeling, HDX was quenched, cells were lysed by cryogenic disruption, and the lysate was resuspended in the quench buffer and ultracentrifuged at 100,000*g*, 2°C for 20 min. This centrifugation time is shorter than typically used for membrane proteins but it was sufficient to obtain BtuB samples suitable for MS analysis.

Our protocol generated peptides with a sequence coverage comparable to that obtained in vitro (Figure 1b and Table S1). Although the presence of endogenous *E. coli* proteins increased the level of background signals, we were able to obtain nearly full coverage of BtuBp, including the overlapping series of amino-terminal peptides that exhibited the greatest changes in HDX upon B12 binding in vitro. Mass spectra representative of our signal-to-noise levels are displayed in Figure S2.

As expected, back exchange levels for HDX in vivo were systematically higher than those in vitro, mostly due to an increase in post-quench sample handling time. Nevertheless, the HDX response of BtuBp to B12 binding was still confidently measured and consistent with our previous findings in vitro. In this article, we continue the use of our prior nomenclature for describing peptides and regions[15]; regions are capitalized while the sequence endpoints are denoted in the subscript, with corresponding peptides in parentheses, for example, BtuB's amino terminus is covered by Region_{3-8} (Peptide_{1-8}).

2.1 | The in vivo allosteric response of BtuBp to B12 binding

The B12 binding-induced stability changes seen throughout BtuBp qualitatively agreed with previous in vitro observations, exhibiting similar trends in HDX acceleration or slowing (Figure 2). The similarity included our primary finding related to the *Ionic Lock*, a conserved salt bridge between Arg14 and Asp316.[14,20] We[15] and others[14] found that the binding of B12 results in breakage of the *Ionic Lock*, unfolding of the *Ionic Lock* region, which exposes the Ton box, thereby allowing the Ton box to bind TonB. We continued to find in vivo that the peptides covering the *Ionic Lock* region ($\text{Peptides}_{9\text{-}23,\ 9\text{–}27,\ \text{and}\ 9\text{–}30}$) exhibited the greatest response to B12 binding, a ~ 1,000-fold acceleration in HDX, agreeing with our in vitro HDX-MS measurements (Figure 3a and S5.3–5.4).

B12 binding also caused a slight destabilization of the amino terminus of the Ton box and the substrate binding loop 3 (SB3), as indicated by the acceleration of HDX for Region_{3-8} (Peptide_{1-8}) and Region_{82-87} ($\text{Peptides}_{80-85\ \text{and}\ 80-87}$), respectively (Figures 2 and S5.19). Again, this behavior matched our in vitro HDX findings. Furthermore, we observed B12 binding-induced stabilization of SB2, as indicated by a slight slowing of HDX for $\text{Region}_{68\text{-}79}$ ($\text{Peptide}_{66\text{-}79,\ 67\text{–}69,\ \text{and}\ 68\text{–}79}$; Figures 2 and S5.16–5.18). BtuBp's amino-terminal helices were

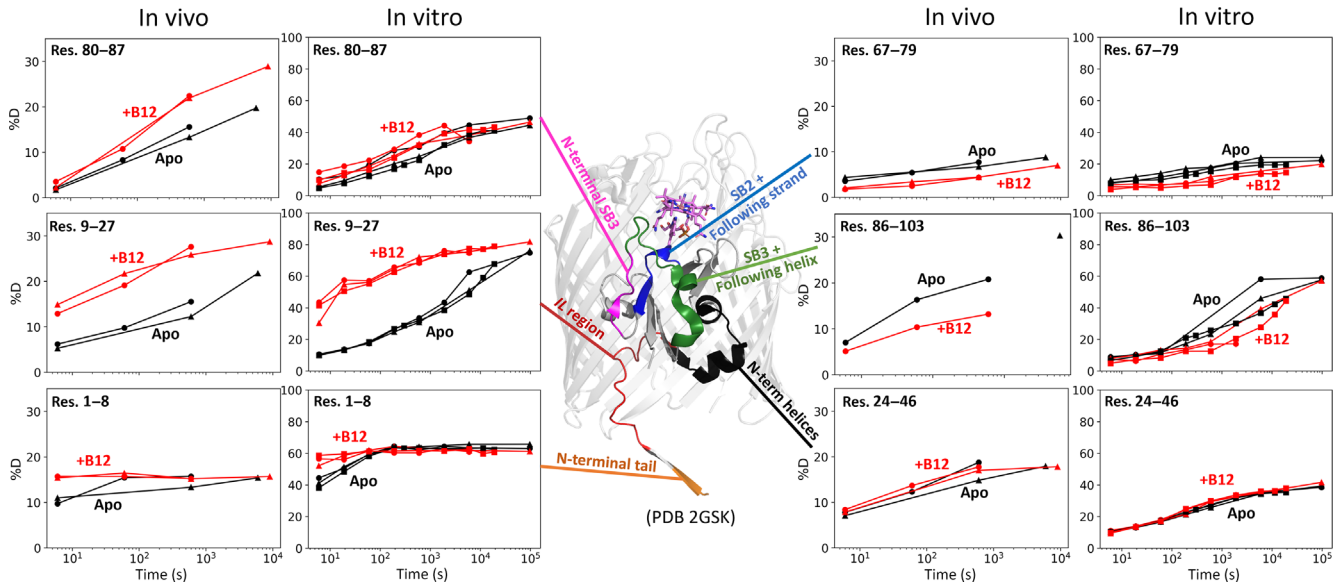

FIGURE 2 Effects of B12 binding on BtuBp's HDX measured in vivo and in vitro. Sides: Uptake plots showing comparisons between biologically duplicated in vivo data (circles and triangles) and previously reported triplicated in vitro data[15] (circles, triangles, and squares). Center: Each peptide is associated with a colored region on the BtuB structure. Incomplete uptake curves are shown in cases where not every time point within a biological replicate was confidently measured.

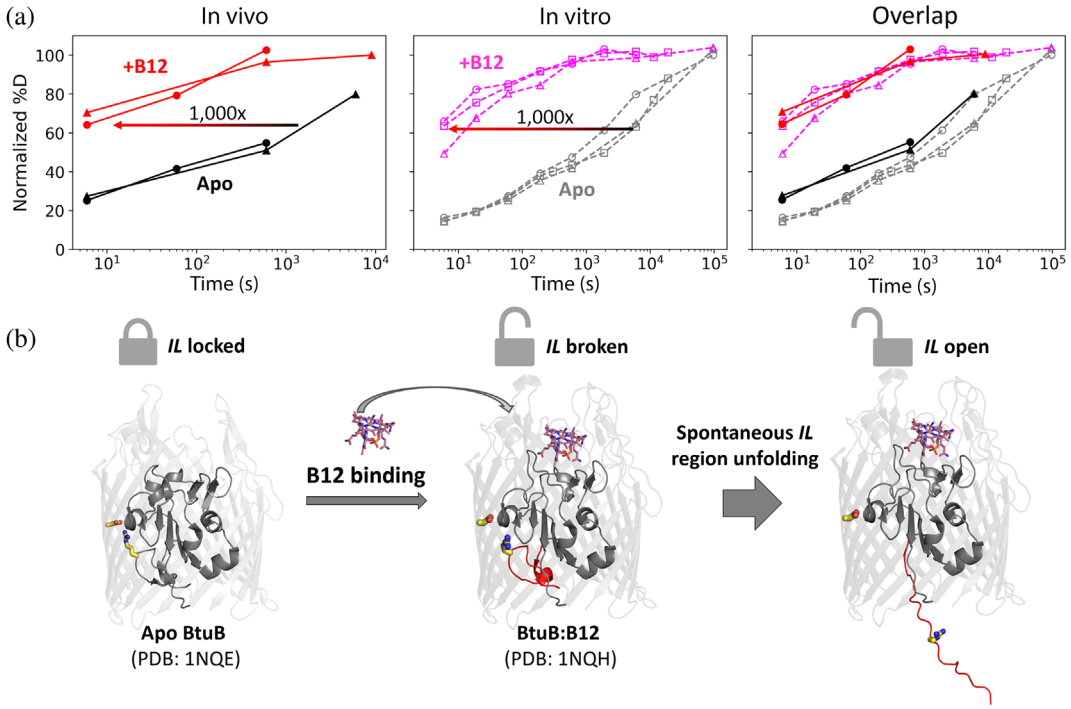

FIGURE 3 B12 binding induces unfolding of the *Ionic Lock* region of BtuBp in vivo similarly to in vitro. (a) Uptake plots for $Peptide_{9–23}$ for (Left) biologically duplicated in vivo data (solid lines; circles and triangles) (Apo: black; B12-bound state: red) and (Middle) previously reported triplicated in vitro data[15] (broken lines; circles, triangles, and squares) (Apo: gray; B12-bound state: magenta). Deuteration levels are normalized to account for back exchange levels. Right: overlapped uptake curves of in vivo and in vitro uptake curves. (b) Model of BtuBp's conformational change upon B12 binding. The left and right states are observed in the HDX-MS as apo and B12-bound states, respectively. The structure on the right is modeled per HDX-MS data. The middle plot is a crystal structure of BtuB in a B12-bound state and is not observed in HDX-MS. Sidechains for the *Ionic Lock* residues are shown as yellow sticks. B12 is shown as magenta sticks. The *Ionic Lock* region is colored red. *Source*: Adapted from Zmyslowski et al.[15]

unaffected by B12 binding, as indicated by the unchanged HDX levels for $Region_{26\text{-}46}$ ($Peptides_{24\text{-}43,\ 24\text{–}44,\ 24\text{–}46,\ 24\text{–}53,\ and\ 28\text{–}46}$; Figures 2 and S5.5–5.9). In summary, the in vivo measurements recapitulated all of the major B12 binding-induced changes in HDX observed in vitro.

Because endogenous TonB-ExbB-ExbD complexes were still present in our in vivo measurements, one may consider that the HDX reflected the time and ensemble average for BtuBp in a functional B12 transport cycle. Catching BtuB during the transport cycle, however, is unlikely due to the high molar excess of overexpressed BtuB molecules relative to naturally abundant TonB-ExbB-ExbD complexes.[21,22] Under our B12 concentration of 16 μM, we expect that most BtuB molecules were in the B12-bound state, as endogenous levels of TonB complex were likely insufficient to drive the transport cycle for a detectable fraction of the BtuB molecules.[21]

Additionally, TonB action during the BtuB transport cycle is thought to depend on cellular proton motive force (pmf).[17,23] We conducted in vivo HDX-MS on BtuB in the presence of CCCP, a pmf dissipator. BtuBp's HDX was insensitive to CCCP in the presence of B12 (Figure S3), arguing against a significant population fraction of BtuB being in the actively transporting state. Hence, we think that the dominant state of BtuB during our experiments was the B12-bound state. To further study the effect of pmf on BtuB's transport mechanism, it may be necessary to overexpress TonB, ExbB, and ExbD[18,24] to similar levels as BtuB.

All important HDX features observed in vivo matched the in vitro study in spite of the increased back exchange levels. This increase was determined from the maximum final deuteration levels of fully deuterated peptides (% D_{max}), e.g., the $\%D_{max}$ decreased from 78% (in vitro) to 28% (in vivo) for $Peptide_{9\text{-}23}$. To examine which steps were responsible for the additional loss of deuterium, we increased the ultracentrifugation time from 20 to 40 min. For $Peptide_{9\text{–}23,}$ this increase led to the $\%D_{max}$ falling from 28% to 22% (Figure S4), a level that is consistent with BtuBp being unfolded under quench conditions during ultracentrifugation, and having an intrinsic exchange rate of 77 min^{-1} at pH 2.5, 2°C. Similar results were observed in the peptides that became fully deuterated, indicating that the 20-min ultracentrifugation step was a significant source of back exchange in our experiments.

2.2 | In vivo HDX of endogenous proteins co-measured with BtuB

During our experiments targeting BtuB, we fortuitously identified peptides comprising 8% of the *E. coli* strain K12 proteome (346/4438 proteins, Table S2). As a result, we were able to obtain HDX data for several endogenous proteins, including GroEL, Histone-like DNA binding proteins (HU-α and HU-β), and Elongation factor Tu 2 (EF-Tu2) (Figure 4 and Table S2). HDX for these four proteins exhibited high reproducibility and was insensitive to B12 addition, as expected (Figure 4).

3 | DISCUSSION

HDX-MS has become a widely used tool for both soluble and membrane proteins.[4–8] The method, however, has mostly been limited to in vitro studies using purified components, with HDX in cells being detectable via nuclear magnetic resonance (NMR) spectroscopy.[25] Here we present a protocol for performing HDX-MS on an OMP in live *E. coli* cells.

A potential concern for conducting HDX on live cells is that D_2O may not permeate across the membrane bilayers and only the outward-facing components would be labeled. However, several studies have demonstrated that D_2O permeation across the bilayer[26] occurs within 1 ms at 23°C for 90% PC/10% cholesterol liposomes[27] and equilibrates within 200 ms at 22°C with dipalmitoylphosphatidylcholine.[28] Furthermore, HDX-NMR studies on Tom40 embedded in dimyristoylphosphatidylcholine liposomes observed labeling of both inward- and outward-facing residues.[29]

BtuB provides a convenient system for testing in vivo HDX-MS for two main reasons. First, as an OMP, BtuB can be highly overexpressed[15] and effectively separated from cytosolic proteins via ultracentrifugation. Together with preferential proteolytic cleavage, these three effects enrich MS signals from BtuBp above those from other proteins (Table S2). Second, the B12-induced changes in HDX with BtuBp are readily controllable and large enough to be observed even with high back exchange levels.

Despite the smaller differences in absolute deuteration levels due to increased back exchange, we continued to observe the ~1,000-fold HDX acceleration at the *Ionic Lock* region as a result of B12 binding (Figure 3a). Such quantitative agreement of B12-binding effects in vivo and in vitro corroborates our previous finding that the binding of B12 alone is sufficient to unfold the *Ionic Lock* region and enable the binding of TonB to the Ton box (Figure 3b). Furthermore, our in vivo HDX-MS data on BtuBp provide no evidence for the formation of a pore large enough for B12 passage, as we see no additional B12 binding-induced unfolding at other regions of BtuBp (Figures 2 and S5.5–5.25).

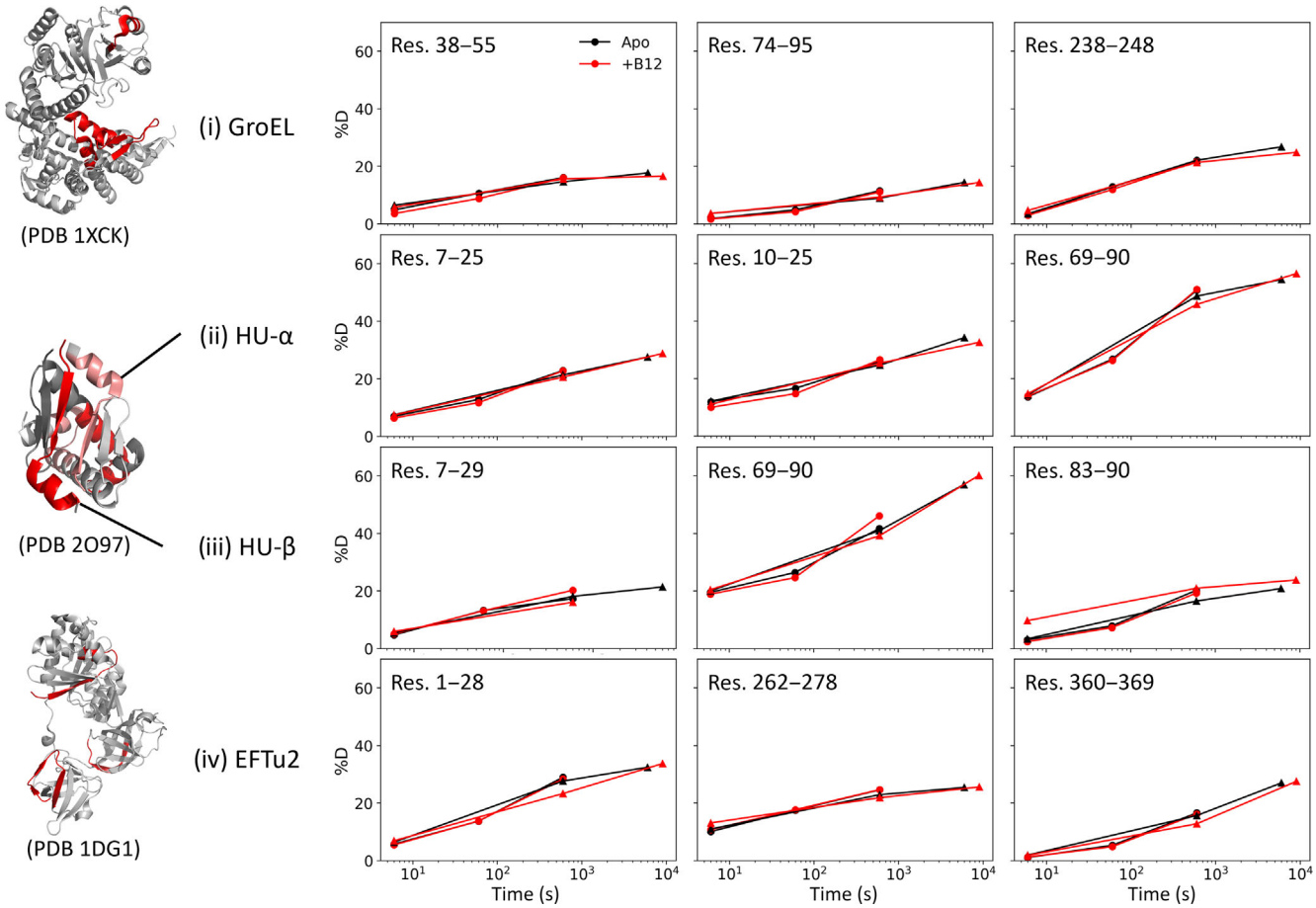

FIGURE 4 HDX of endogenous *E. coli* proteins measured in experiments targeting BtuB. Structures are shown for the following proteins, with respective numbers of assigned peptides and sequence coverage: (i) GroEL: 21 peptides, 46.9%; (ii) Histone-like DNA binding protein α chain (HU-α): 12 peptides, 67.8%; (iii) HU-β: 10 peptides, 88.9%; (iv) Elongation factor Tu 2 (EF-Tu2): 13 peptides, 41.4%. Uptake plots show biologically duplicated data (circles and triangles) in the absence (black) and presence (red) of B12. The locations of the peptides shown are highlighted in red (except for HU-α peptides which are in salmon) in the corresponding structures.

3.1 | HDX Protocol considerations

To provide sufficient material for MS, initiating HDX in concentrated cells is desirable. Caution should be taken to minimize cell death during HDX labeling. We recommend that HDX be conducted on slowly dividing cells in the presence of a carbon source to avoid starvation. A short HDX labeling period is recommended to avoid issues such as cell division and deoxygenation. In our experiments, 10 min is sufficient to fully label peptides covering the *Ionic Lock* region, although we present time points going out to 150 min. We expect minimal cell death despite the high cell density due to our short label time and inclusion of a carbon source.[30]

The main challenge for conducting in vivo HDX-MS is maintaining a low level of back exchange, occurring mainly during the purification of the quenched protein. To reduce back exchange, the post-label steps should be as short as needed to avoid subsequent column fouling and spectral crowding, that is, one should purify to the minimum level necessary for a system of interest. Sample isolation should be conducted under quench conditions (pH 2.5, 0°C) to reduce intrinsic exchange rates. Specialized liquid chromatography (LC) operating at sub-zero temperatures may also be employed to improve separation.[31,32] Ideally, in vivo HDX-MS should be performed without overexpression to preserve the relative protein concentrations, which may be possible with future optimization of the back exchange levels. To facilitate rapid isolation under quench conditions, we are in the process of developing a purification tag that functions at low pH.

Given our success in performing in vivo HDX-MS on BtuB, we expect that a similar protocol could be applied to other OMPs as they generally can be highly overexpressed[33] and rapidly separated from cytosolic proteins via ultracentrifugation. However, OMPs are not the only viable target for in vivo HDX-MS. During our experiments targeting BtuB, we measured HDX for highly expressed cytosolic proteins (Figure 4 and Table S2). These proteins appear to share the qualities of being abundant, easily digestible, and

pelletable due to their large sizes or association with large complexes. Therefore, we expect that our in vivo HDX-MS protocol can be applied to a variety of other proteins.

4 | CONCLUSION

We describe a new protocol conducting HDX-MS on the OMP BtuB in live *E. coli* cells. Critically, the protocol maintained a manageable level of back exchange while providing adequate signals. Our in vivo HDX-MS data corroborated previous findings that (a) B12 alone is sufficient to initiate *Ionic Lock* breakage in BtuB and the unfolding of its amino terminus; (b) B12 binding alone does not create a channel sufficient for B12 transport. Overall, our success with BtuB and other proteins in vivo opens the way for future HDX-MS studies of protein function and dynamics in a native cellular environment.

5 | MATERIALS AND METHODS

Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri) unless otherwise noted.

5.1 | Bacteria culture and protein expression

BtuB in the pAG1 vector was transformed into BL21-CodonPlus (DE3) *E. coli* competent cells. A single colony was used to inoculate 50 mL of LB media supplemented with 100 μg/mL Ampicillin (LB_{Amp}) and the culture was grown at 37°C overnight with agitation (225 rpm). The next day, 5 mL of the starting culture was inoculated into 500 mL of LB_{Amp} in a baffled 2.8 L Fernbach flask. The culture was grown at 37°C until the OD_{600} reached 0.6 and then induced at 37°C for 4 hours with 1 mM IPTG. Cells were harvested by centrifugation for 10 min at 3,625*g* at 20°C. Cells were washed and pelleted twice, each time with 50 mL of H_2O/LB buffer containing 50 mM NaPi, 150 mM NaCl, 1× LB, pH 7.2, to equilibrate cells in a pH-controlled environment for HDX labeling while providing enough carbon source to maintain cellular metabolism. Washed cells were resuspended in H_2O/LB buffer for a total volume of 5 mL and were immediately used for HDX labeling.

5.2 | Hydrogen-deuterium exchange

HDX was initiated by diluting 200 μL of the dense cell suspension into 800 μL of 1× LB medium made with D_2O (99.9% D, Sigma-Aldrich reference no. 151882), resulting in 80% D in the label reaction. The D_2O/LB buffers were at pD_{read} 6.8 and contained 50 mM NaPi, 150 mM NaCl, 1× LB, and were supplemented with (a) Apo: 1% dimethyl sulfoxide (DMSO); (b) B12: 1% DMSO, 20 μM B12, and 2 mM $CaCl_2$; (c) B12/CCCP: 1% DMSO, 5 μM CCCP, 20 μM B12, and 2 mM $CaCl_2$. For HDX experiments with B12 and CCCP, cells were incubated with 5 μM CCCP for 1 min at 22°C before labeling was initiated. HDX labeling was performed for 6 s to 150 min at 22°C and quenched via the addition of 500 μL of ice-chilled quench buffer (600 mM Glycine, pH 2.5). Quenched cell suspensions were immediately pelleted by centrifugation for 1 min at 6,000*g* at 2°C, re-suspended in 80 μL of ice-chilled quench buffer, and then flash-frozen into cell beads by slow drip into an LN_2-filled 2 mL Eppendorf Safe-Lock Tube (Eppendorf Catalog No. 022363379) containing one 7-mm milling ball. After pouring out the excess LN_2, frozen cell beads were stored at −80°C until lysis.

5.3 | Rapid separation of BtuB and LC–MS

Frozen cell beads from each quenched HDX reaction were cryogenically pulverized with a mixer mill (Retsch, MM 400) over 10 cycles, with each cycle consisting of 90-s grinding at 30 Hz followed by 3-min cooling in LN_2. Pulverized lysates were stored at −80°C until thawing by repeated trituration in ice-chilled quench buffer. The cell lysate was clarified by centrifugation for 4 min at 13,000*g*, 2°C. OM-embedded BtuB was separated by ultracentrifugation for 20 min at 100,000*g*, 2°C. The resulting pellet containing BtuB was re-suspended in 67 μL of an ice-chilled quench mixture consisting of 60 μL of 600 mM glycine, 2 M urea, pH 2.5, 2 μL of thrice-desalted 5 mg/mL porcine pepsin (Sigma-Aldrich, reference no. P6887) in 100 mM sodium citrate, 2.5 μL of 2.5 mM DDM, and 2.5 μL of a 300 mg/mL aqueous suspension of ZrO_2-coated silica (Sigma-Aldrich, reference no. 55261-U). Digestion with pepsin was carried out by 3 min incubation at 0°C. Solubilized peptides were obtained by filtering the resulting mixture through a cellulose acetate spin cup (Thermo Pierce, Waltham, Massachusetts, reference no. 69702) by centrifugation for 30 s at 13,000*g*, 2°C. The filtrate was flash-frozen in a dry ice/ethanol bath and stored at −80°C until injection onto the LC–MS system. The LC–MS system used and data analysis performed were described previously.[15] Data points that could not be fit by HDExaminer 3.1 were excluded.

AUTHOR CONTRIBUTIONS

Xiaoxuan Lin: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Adam M. Zmyslowski:** Conceptualization (equal); data curation (supporting); investigation (supporting); methodology (equal); validation (supporting); writing – review and editing (supporting). **Isabelle A. Gagnon:** Investigation (supporting). **Robert K. Nakamoto:** Conceptualization (supporting); investigation (supporting); validation (supporting); writing – review and editing (supporting). **Tobin R. Sosnick:** Conceptualization (supporting); funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); validation (supporting); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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