Published in final edited form as:
Anal Chem. 2013 December 3; 85(23): . doi:10.1021/ac403173p.

Hydrogen Exchange – Mass Spectrometry Measures Stapled Peptide Conformational Dynamics and Predicts Pharmacokinetic Properties

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Abstract

Peptide drugs have traditionally suffered from poor pharmacokinetic properties due to their conformational flexibility and the interaction of proteases with backbone amide bonds. “Stapled Peptides” are cyclized using an all-hydrocarbon cross-linking strategy to reinforce their α-helical conformation, yielding improved protease resistance and drug-like properties. Here we demonstrate that Hydrogen Exchange-Mass Spectrometry (HX-MS) effectively probes the conformational dynamics of Stapled Peptides derived from the survivin-borealin protein-protein interface and predicts their susceptibility to proteolytic degradation. In Stapled Peptides, amide exchange was reduced by over five orders-of-magnitude versus the native peptide sequence depending on staple placement. Furthermore, deuteration kinetics correlated directly with rates of proteolysis to reveal the optimal staple placement for improved drug properties.

Keywords

helix; stapled-peptide; protein folding; deuterium

Peptides represent an important class of therapeutic agents despite poor pharmacokinetic properties that result from rapid *in vivo* proteolysis. Since proteases only degrade peptides when unraveled, “Stapled Peptides” have been developed to limit proteolytic cleavage by locking peptides into an α-helical shape using an optimized cross-linking chemistry.[1, 2] This reinforced secondary structure imparts enhanced pharmacokinetic properties while mimicking the structure found at the interface of many protein-protein interactions. Stapled Peptides have also been shown to penetrate cells and disrupt intracellular protein-protein interactions, including many of the most important molecular targets of modern pharmaceutical development.[3–9] The Aurora B chromosomal passenger complex is one such target of interest for anticancer drug discovery. Within the complex, survivin binds to borealin via α-helical protein-protein interactions in a leucine-zipper motif, making this an attractive target for therapeutic intervention using an α-helical Stapled Peptide drug (Figure

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SUPPORTING INFORMATION AVAILABLE

Synthetic methods and characterization of Stapled Peptides; and experimental procedures for protease resistance, HX-MS studies, and CD spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

CONFLICT OF INTEREST DISCLOSURE

Indicated authors are employees and shareholders of Aileron Therapeutics, Inc.

1).[10, 11] We truncated the 39-amino acid helical region of borealin that binds to survivin and substituted varying positions opposite the binding interface with hydrocarbon crosslinks in place of main chain i→i+4 hydrogen bonds and then evaluated the conformational flexibility of the resulting 22-residue Stapled Peptides using hydrogen-deuterium exchange mass spectrometry (HX-MS).

HX between peptide backbone amide hydrogen atoms and deuterium in D_2O solvent offers a sensitive measure of folding dynamics and the conformational stability of proteins and peptides.[12–15] Amide hydrogen atoms that are protected from solvent have slower exchange rates relative to conformationally flexible peptides. Short peptides with no structure in solution are usually totally deuterated within seconds unless there is secondary structure involving hydrogen bonds that protects the backbone amide hydrogens from exchange.[16] Here we report that HX-MS analysis is a sensitive method to assess Stapled Peptide conformational rigidity. HX is very sensitive to staple type and placement, with complete exchange varying from under ten seconds to over three hours, a difference of over five orders of magnitude. Furthermore, the HX kinetics closely correlate with protease susceptibility, and accurately predict the best staple placement for optimal protease resistance.

The borealin Stapled Peptides shown in Table 1 were subjected to H-D exchange by dilution in 10 mM sodium citrate (pD 7.0) in D_2O, allowed to exchange for 0 min, 10 sec, 1 min, 10 min, 60 min, or 180 min and then acid-quenched to suppress H-D exchange during LC-MS analysis.[17] The HX-MS results are shown in Figure 2A for the linear (#1), unstapled (#5), single-stapled (#6), and double-stapled peptides (#7). Like most proteins under physiological conditions, the borealin peptides followed EX2 exchange kinetics rather than EX1 kinetics.

The linear peptide had the fastest HX exchange due to its conformational flexibility and exposure of backbone hydrogen atoms to D_2O solvent. The “unstapled” peptide has two α-methylated linker residues that contribute to its helicity and this modestly reduced its conformational flexibility, though complete HX still occurred in less than one minute. Significant backbone protection was afforded by stapling in the middle of the peptide, with complete exchange requiring around an hour of exposure to D_2O. The double-stapled peptide showed dramatic enhancement of conformational rigidly, as evidenced by its sluggish rate of HX, requiring over three hours of exposure to D_2O to exchange most of the backbone hydrogen atoms.

The equilibrium conformation of the peptides was measured by far-UV circular dichroism (CD) spectroscopy and compared to the full-length borealin peptide helix as a control to gauge the percent helical content. CD studies were performed under buffer and pH conditions identical to those in the HX-MS study. As shown in Figure 2B, all of the borealin peptides have a CD signature typical of an α-helix, with double minima near 208 and 222 nm and a maximum near 195 nm. The CD spectra indicate that helical content was increased upon stapling, particularly upon double-stapling, from around 50% to over 95%.[18] This result is consistent *in silico* conformational simulations on related stapled p53 peptides.[19]

We next investigated the *in vitro* proteolytic stability of these peptides as a predictor of their *in vivo* pharmacokinetic properties. The peptides were exposed to trypsin then sampled at time points of 0, 5, 30, 60, and 120 minutes and then acid-quenched prior to MS analysis. Because multiple trypsin cleavage sites adjacent to lysine and arginine residues are present throughout the borealin peptide sequence, the linear peptide was very susceptible to degradation and had an *in vitro* half-life of approximately 4.8 min (Figure 2C). The Stapled Peptides were dramatically more resistant to protease digestion, with half-lives 10- to 40-

fold longer than the linear peptide. Remarkably, more than 90% of the double-stapled peptide remained intact after 5.0 h of exposure to trypsin.

To correlate the conformational dynamics and stability of the borealin peptides to protease resistance and pharmacokinetic properties, we compared deuterium incorporation for all the peptides in Table 1 (Figure 3A). Deuterium exchange in the Stapled Peptides varied considerably and was strongly dependent on the staple position. While protease stability did not correlate with helical content as measured by CD spectroscopy (Figure 3B), resistance to proteolysis was strongly correlated with HX (Figure 3C).

Conformational dynamics depend critically upon the staple position. Interestingly, two of the Stapled Peptides exhibited faster HX and proteolysis than the others, despite comparable equilibrium helicity. These fast-exchanging Stapled Peptides (#2 and #3) had staples near the N-terminus, whereas peptides #4 and #6 exhibited slower exchange and proteolysis, and had staples in the middle of the peptide or closer to the C-terminus. Peptides #3 and #7 showed comparable levels of helicity as measured by CD (85% and 95%, respectively) but dramatically different exchange and proteolysis kinetics, suggesting the C-terminal staple position in #3 may enhance equilibrium helical content but not improve conformational rigidity. These structure-dynamics relationships were neither predicted a priori, nor suggested by CD measurements, as CD spectroscopy only measures the average conformation of dynamic secondary structures.

Our results support the conclusion that Stapled Peptides' improved pharmacokinetic properties derive from their slower conformational dynamics and reduced proteolytic susceptibility relative to native peptide sequences. Importantly, HX kinetics correlate with improved protease resistance much better than static "snapshots" of equilibrium helical content by CD spectroscopy. Furthermore, we demonstrate that HX-MS is generally applicable to measuring conformational flexibility and predicting the proteolytic stability of peptide drugs. Automated HX-MS instrumentation and the ability to quickly and efficiently synthesize larger and more diverse peptide libraries using parallel synthesis techniques may enable HX-MS to be employed as high throughput screening tool for important drug targets.[20, 21] Currently we are using affinity selection-mass spectrometry for mixture-based binding assessment to take advantage of concurrent emerging advancements in combinatorial chemistry.[22, 23] The combination of HX-MS and mixture-based binding assays may accelerate the discovery of Stapled Peptide drugs with improved target binding and pharmacokinetic properties for a range of intracellular protein-protein interactions, and this effort is the subject of continuing investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Prof. Robert S. Hodges and Dr. Brooke Hirsch at the University of Colorado, Denver, for circular dichroism analysis; Kelly Barhite for experimental assistance; and Eric Smith for editorial support. Partial support of this work was provided by funding from the National Institutes of Health (R01-GM086507 and R01-GM101135 to JRE) and the Waters Corporation (JRE).

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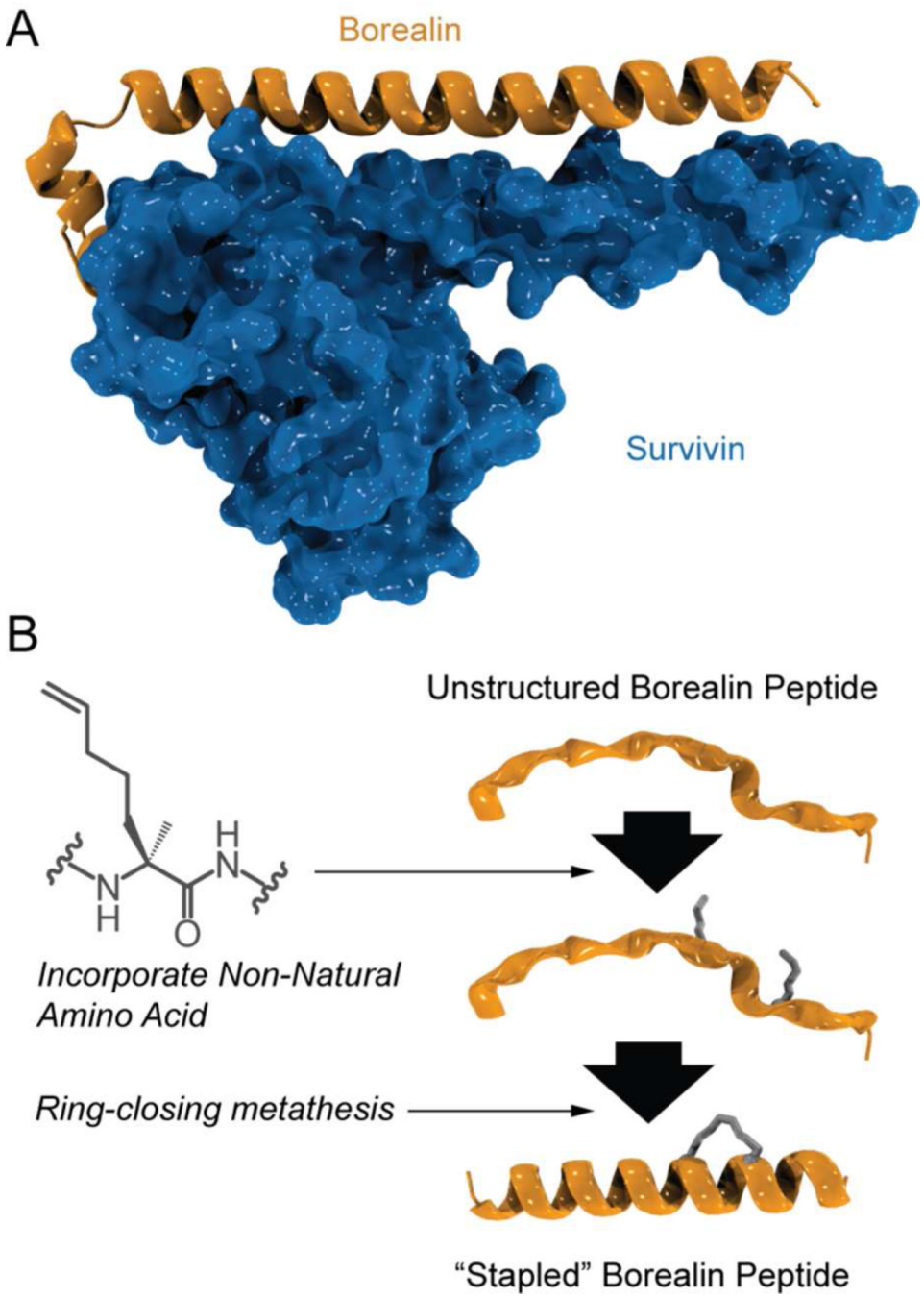

Figure 1.
(A) The 39-amino acid C-terminal helix following the Baculovirus IAP Repeat (BIR) domain of borealin (PDB# 2RAW) was truncated and (B) substituted with hydrocarbon crosslinks to bind survivin while imparting improved drug properties.

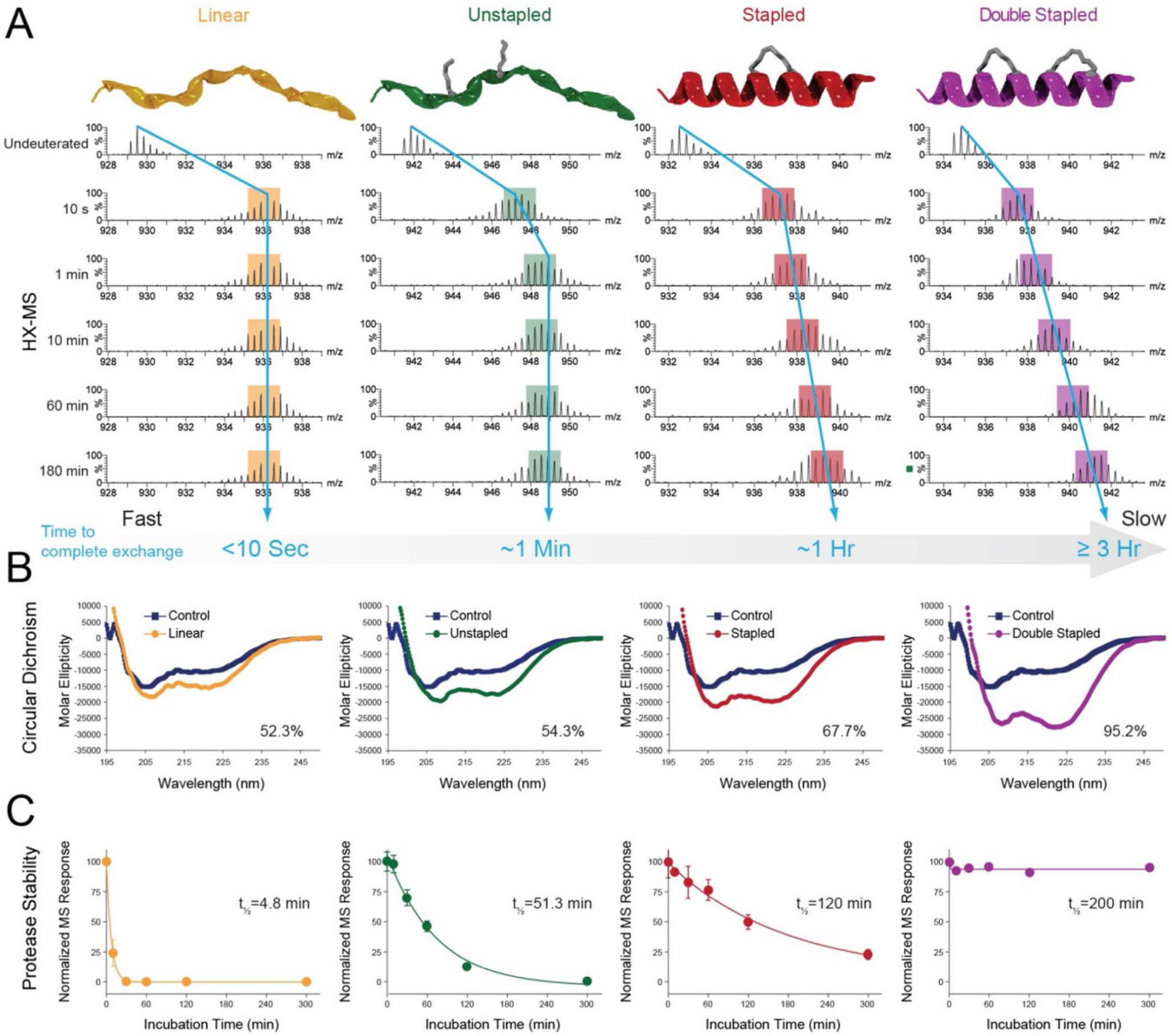

Figure 2.

HX-MS, CD and protease stability results for select borealin Stapled Peptides. (A) Conformational dynamics are probed by monitoring time-dependent mass increases using mass spectrometry. Typical HX-MS spectra of 3+ ions of the linear (#1), unstapled (#5), stapled (#6), and double-stapled (#7) borealin peptides (see Table 1 for sequences) show the time course of H-D exchange. Trend lines and time to complete exchange demonstrate HX rate differences of over five orders-of-magnitude from the native sequence to its double-stapled congener (B) Far-UV CD spectra were obtained in 10 mM sodium citrate, pH 7.0. The units of the ordinate are deg.cm^2·dmol^{-1}, and % helicity is calculated in comparison to the full-length native sequence. (C) Stability to proteolysis, expressed as half-life ($t_{½}$), was measured with a trypsin-to-peptide ratio of 1:100 at pH 7.0 (n = 2).

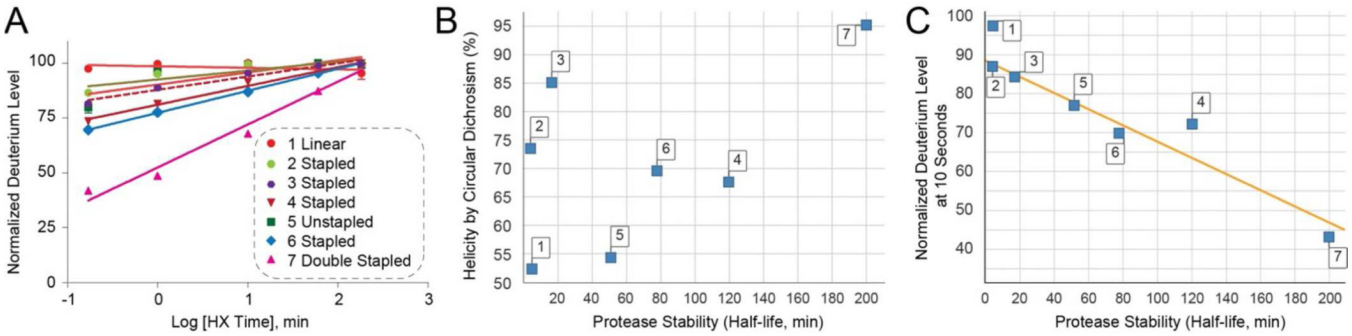

Figure 3.

(A) Estimation of the H-D exchange rates for borealin peptides from Table 1 as measured by HX-MS. The y-axis represents the number of exchanged amide hydrogen atoms normalized to the total number of exchangeable backbone amide hydrogens in the peptide, including those inside the “Staple” macrocycle, with exchange rates approximated as the inverse slope (1/min) of a linear trend line; (B) equilibrium helicity as measured by CD correlates poorly ($r^2 < 0.3$) with protease stability; (C) Protease stability shows a strong correlation with HX for Stapled Peptides ($r^2 = 0.92$).

Table 1

Stapled Peptides derived from the helical region of the borealin-survivin complex. A pair of olefin-containing non-natural amino acids (red X) was substituted at various i→i+4 positions opposite the binding interface and 'stapled' by ruthenium-catalyzed olefin metathesis. Peptide 5 is unstapled and the blue X (X) refers to the amino acids substitutions without olefin metathesis.

1	Linear	Ac-DFDREVEIRIKQIESDRQNLLK-NH_2
2	Stapled	Ac-XFDRXVEIRIKQIESDRQNLLK-NH_2
3	Stapled	Ac-DFDXEVEXRIKQIESDRQNLLK-NH_2
4	Stapled	Ac-DFDREVEXRIKXIESDRQNLLK-NH_2
5	Unstapled	Ac-DFDREVEXRIKXIESDRQNLLK-NH_2
6	Stapled	Ac-DFDREVEIRIXQIEXDRQNLLK-NH_2
7	Double-Stapled	Ac-DFDXEVEXRIXQIEXDRQNLLK-NH_2