Fast peptide exchange on major histocompatibility complex class I molecules by acidic stabilization of a peptide-empty intermediate

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Abstract

The cell biology and biochemistry of peptide exchange on major histocompatibility complex class I (MHC-I) proteins are of great interest in the study of immunodominance, which requires iterative optimization of peptide affinity, and cross-presentation of pathogen and tumor antigens, in which endogenous peptides are exchanged for exogenous ones. Even though several methods exist to catalyze peptide exchange on recombinant MHC-I proteins, the cellular conditions and mechanisms allowing for peptide exchange in vivo remain unclear. Here, we demonstrate that low pH, as present in endosomes, indeed triggers peptide exchange, and we dissect the individual steps of the exchange reaction. We find that low pH stabilizes the peptide-empty forms of MHC-I that occur as intermediates of the exchange reaction, and that is synergizes with dipeptides and with disulfide-mediated stabilization of MHC-I.

Keywords

crosspresentation, fluorescence anisotropy, major histocompatibility complex class I molecules, peptide exchange

1 | INTRODUCTION

Major histocompatibility complex class I molecules (MHC-I) bind intracellular peptides in the endoplasmic reticulum (ER) and present them on the cell surface, allowing for immune surveillance by CD8+ T cells. Peptide loading onto MHC-I in the ER involves iterative optimization, with low-affinity peptides exchanged for those with higher affinity, usually aided by the chaperones tapasin or TAPBPR (transporter associated with antigen processing binding protein-related). Peptide exchange is also thought to be central to cross-presentation, where in the endocytic pathway of professional antigen-presenting cells, peptides previously loaded onto

Abbreviations: A2, human MHC-I allotype HLA-A*02:01; A24, human MHC-I allotype HLA-A*24:02; dsA2, disulfide-stabilized A2; dsA24, disulfide-stabilized A24; dsMHC-I, disulfide-stabilized MHC-I; ER, endoplasmic reticulum; MD, molecular dynamics; MHC-I, major histocompatibility complex class I molecule(s); nanoDSF, nanoscale differential scanning tryptophan fluorimetry; TAPBPR, transporter associated with antigen processing binding protein-related; wtA2, wild-type A2; wtA24, wild-type A24; β2m, beta-2 microglobulin.

Classification: Biological Sciences/Immunology and Inflammation (or Biochemistry).
MHC-I are replaced by exogenous peptides in order to activate cytotoxic T cells against tumors. The molecular mechanisms and cellular conditions mediating peptide exchange, especially in the absence of exchange chaperones (as occurs in tumors that downregulate them), with tapasin-independent allotypes, and during cross-presentation, are not well understood. Of key interest is the role of pH, since the low pH of endocytic compartments has been postulated to destabilize the peptide-MHC-I complex to enhance peptide exchange during cross-presentation.

Peptide exchange on recombinant MHC-I is also of technological interest since they are used in the study of antigen-specific CD8+ T cell populations. Peptide exchange allows for the rapid generation of large numbers of peptide-MHC-I complexes for the analysis of antitumor and antiviral immune responses and for safety screening of recombinant T cell receptors.

We and others have identified several techniques for accelerating peptide exchange, among them the use of dipeptides that catalyze rapid peptide exchange at room temperature and pH 7. Here, we show that acidic pH accelerates peptide exchange by stabilizing a peptide-free intermediate of the reaction.

2 | RESULTS

2.1 | Low pH accelerates peptide exchange on HLA-A*02:01 and HLA-A*24:02

It has been previously proposed that low-affinity peptides are released from MHC-I at low pH values of 5.5 – 7 (corresponding to conditions in endolysosomal compartments) and exchanged for high-affinity peptides. To test this proposition with recombinant proteins, we expressed the human MHC-I heavy chains HLA-A*02:01 (in the following termed A2) and HLA-A*24:02 (A24) in Escherichia coli and folded them in vitro with their light chain beta-2 microglobulin (β2m) and various low- and high-affinity peptides (see Table S3 for a list of peptides). First, to validate the experimental system for monitoring peptide exchange, we used an established exchange technology, that is, dipeptide-catalyzed peptide exchange, where the complex of MHC-I with a “leaving” peptide is incubated with an excess of “incoming” peptide and a high concentration of catalytic dipeptide, leading to peptide exchange. We monitored exchange by fluorescence anisotropy (Figure 1a,b), which measures the exchange rate constant (kex):

\[ L + M_{\text{empty}} \xrightleftharpoons[k_{\text{ex}}]{k_{\text{off}}} ML \]

(with M = MHC-I, L = leaving peptide, I = incoming peptide). Next, without dipeptide, we tested different pH values. Lowering the pH from 8 to 6 triggered a dramatic increase in the exchange rate for both allotypes, with up to 100-fold acceleration (Figure 1c-f, Table S1). Decreasing the pH any further was not feasible, since at pH 5.5, β2m dissociates from the heavy chain.

2.2 | Small effects of low pH on peptide association

To address the mechanistic details of this acidic acceleration of peptide exchange, we first assumed that peptide exchange consists of two steps, that is, the dissociation of the pre-bound (“leaving”) peptide, described by the dissociation rate constant k_{off}, and the association of the incoming peptide, described by the association rate constant, k_{on}, as follows:

\[ M_{\text{empty}} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} ML \]

Measuring the k_{on} separately from the k_{off} requires an empty receptor, and since the binding site of empty wild type MHC-I is natively unfolded and binds peptide only slowly (if at all), we used empty disulfide-stabilized MHC-I (dsMHC-I) molecules, which have a disulfide bond linking the α1 and α2 helices of the peptide-binding groove, conformationally stabilizing the F pocket region in the absence of peptide in a structure that is close to the peptide-bound state. We first validated the use of dsA2 and dsA24 by repeating the exchange experiments, and we found that they exchange peptides significantly faster than their wild type (wt) counterparts and that they, importantly, show a similar dipeptide-mediated and acidic acceleration of peptide exchange (Figure S1; Table S1). Disulfide stabilization did not significantly alter the affinity of A2 toward peptides and dipeptides (Tables S1 and S2).

We now measured the association rates (k_{on}) of the fluorescently labeled peptides to the empty dsA2 and dsA24 molecules. Due to the very high association rate of peptide to dsA24, the data have limited value for that allotype; but for A2, decreasing the pH from 8 to 7 only doubled the k_{on} (Figure 2a,b; Table S1, Figure S5); when the pH was lowered further, from 7 to 6, the k_{on} remained the same (dsA24) or even decreased (dsA2). These results suggest that the acidic acceleration of peptide exchange is not caused by an increase in the peptide association rate.
2.3 Small effects of low pH on peptide dissociation rate and equilibrium binding affinity

Therefore, we next sought to determine whether low pH destabilizes the complex of MHC-I with the leaving peptide, promoting peptide dissociation. First, we reasoned that an increased $k_{\text{off}}$, at a rather stable $k_{\text{on}}$, should be accompanied by an increase in the equilibrium dissociation constant ($K_D$), since

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}}.$$  \hspace{1cm} (3)

We, therefore, measured the $K_D$ by peptide titration with steady-state anisotropy and found that the pH did not significantly affect the peptide binding affinity of the complexes (Figure 2c,d). Second, another readout of peptide binding affinity to MHC-I is the thermal stability of the peptide-MHC-I complexes, to measure it, we used nanoscale differential scanning fluorimetry (nanoDSF), which quantifies protein unfolding by the change in the fluorescence emission of tryptophan indoles and generates a thermal denaturation curve, the midpoint of which corresponds to the melting temperature ($T_m$). We found that there was little influence of the pH on the $T_m$ of high-affinity peptide complexes with wtA2 or wtA24, whereas for medium-affinity peptides, there was a slight stabilization at low pH (Figure 2e). Third, we directly measured the dissociation of a fluorescently labeled leaving peptide from dsA2 at different pH values and found a two-fold increased $k_{\text{off}}$.
at pH 6, compared to pH 8 and 7 (Figure 2f). Taken together, these data suggest that any pH-induced changes in the peptide dissociation rate or the peptide/MHC complex stability are subtle, and that they are not responsible for the strong acidic acceleration of peptide exchange.

2.4 Small salt effect on kinetic and equilibrium constants

We next sought to gain more information on the nature of the rate-determining step for peptide exchange. We reasoned that decreasing the pH might influence either
the inter-molecular electrostatic interactions such as attraction\textsuperscript{36,37} or repulsion of the peptide; this would alter $k_{\text{on}}$ or $k_{\text{off}}$, which we had largely excluded above. Alternatively, a pH decrease might influence MHC-I conformation or conformational dynamics, making the MHC-I molecule more susceptible to peptide binding and thus leading indirectly to a faster binding of peptide. We further reasoned that we might be able to differentiate between these two explanations by using a high salt concentration, since salt disrupts long-range electrostatic ligand-receptor interactions more strongly than it disturbs protein conformational changes.\textsuperscript{38,39}

We, thus, repeated the peptide exchange, binding affinity, and association rate determination experiments for dsA2 and dsA24 in the presence of 500 mM NaCl. While the equilibrium binding affinities were generally not affected (Figure S3a,b), the exchange rate of A2 decreased by about factor 10 with high salt, but the pH dependence remained (Figure 3a,b). For A24, the effect of salt was not significant. Interestingly, association rates were also not much affected by high salt (Figure S3c,d), suggesting that in the binding of peptides to A2 and A24, electrostatic attraction of the peptide by the protein does not play a significant role. We conclude that while allotype-specific differences exist, the high-salt experiments give no convincing evidence for an involvement of peptide association or dissociation steps in limiting the rate of peptide exchange, but instead point to an effect of the pH on the conformation, or the conformational dynamics, of the molecule.

Taken together, the results so far suggest that pH changes do not affect peptide association, peptide dissociation, or equilibrium affinity of peptide binding to an extent large enough to explain the dramatic acceleration of peptide exchange with lower pH that is seen in Figure 1c–f, and that we need to investigate the conformational change of MHC-I itself.

### 2.5 Evidence for an unstable intermediate

The peptide-empty intermediate of the exchange reaction ($M_{\text{empty}}$ in Equation 2) is accessible to us in the form of the peptide-free disulfide-stabilized A2 or A24 molecules. We thus measured their thermostabilities by nanoDSF, and we found that both empty dsMHC-I are significantly (10–12°C) stabilized against denaturation by low pH (Figure 4a). We therefore decided to expand our model by assuming that the intermediate of the exchange reaction, that is, the peptide-empty MHC-I, exists in an equilibrium between a native empty form that can rapidly bind peptide ($M_{\text{receptive}}$) and a partially unfolded empty form that does not bind peptide ($M_{\text{non-receptive}}$). Such an

![Figure 3](https://example.com/figure3.png)

**Figure 3** Evidence for long-range peptide-MHC-I interaction with dsA2 but not dsA24. Exchange reaction with 60 nM of the high affinity peptide-MHC-I (a) dsA2/ILKEPVHGV and (b) dsA24/TYASNTSTL with 20 nM incoming FITC peptides in citrate-phosphate buffer at pH 7 and 8, with or without 500 mM NaCl. Values and repeats are in Table S1.
equilibrium has been postulated by us and others:

\[
ML \xrightarrow{k_{\text{off}}} M_{\text{receptive}} \xrightarrow{k_{\text{on}}} ML
\]

where \( k_{\text{den}} \) and \( k_{\text{ren}} \) are the rate constants for the transitions to (den = denaturation) and from (ren = renaturation) the unfolded empty state. We then assumed that the pH influences the concentration of unfolded and folded equilibrium such that at pH 6, there is more \( M_{\text{receptive}} \) leading to the higher denaturation temperatures seen in Figure 4a. To test this assumption in a mathematical model, we performed a kinetic simulation of an exchange reaction with all known parameters, and we were able to recapitulate the results of Figure 1e by assuming a tenfold decrease of \( k_{\text{ren}} \) between pH 8 and pH 7, and again between pH 7 and pH 6, without assuming any pH-mediated changes to \( k_{\text{on}} \) or \( k_{\text{off}} \) (Figure 4c,d). We conclude that the simplest applicable model predicts a pH-dependent reversible equilibrium between two states of the peptide-empty intermediate of the exchange reaction.

To model the dynamic behavior of A2 and A24 during the intermediate unbound state, we performed molecular dynamics (MD) simulations, using as start structures published crystal structures with the bound peptide coordinates removed. To mimic the dominant protonation states, the ionizable side chains located in the binding groove were assumed to be protonated at pH 6 (Glu55, Glu63, Asp77, His70, His74, and His114 residues in HLA-A*02:01 vs. Glu55, Glu63, Asp74, His70 and His74 in...
HLA-A*24:02) and adopting the standard ionization states at pH 8 (acidic residues charged, histidine neutral), which represents the extremes of possible protonation states of residues in the binding groove at the two pH values. During the simulations (up to 750 ns), the $\alpha_1$ and $\alpha_2$ helices that form the peptide binding groove exhibited enhanced fluctuations compared to the simulations in the presence of bound peptide at both pH values. No unfolding of the structures was observed. However, at pH 6, both allotypes adopted more open grooves than at pH 8 (Figure 4b). In the closed forms seen at pH 8, the binding grooves are too narrow to accommodate an incoming peptide, whereas open states with a groove that is wider, or closer to the bound state, may still allow productive peptide binding. Hence, the MD simulations suggest that the non-receptive empty form may correspond to a structure with a closed binding groove that needs to re-open in order to bind the peptide. The dominance of the closed state observed in the MD simulations at pH 8 explains the dramatic reduction of the peptide exchange at pH 8 vs. pH 6.

2.6 Acidic acceleration is synergistic with dipeptide-mediated peptide exchange

Finally, we put this model to the test by examining its synergy with dipeptide-mediated peptide exchange. We previously demonstrated that dipeptides of appropriate sequence accelerate peptide dissociation ($k_{\text{off}}$, by displacing the termini of the peptide,\textsuperscript{11}) and association ($k_{\text{on}}$, by keeping the F pocket region in a folded state\textsuperscript{42}). We reasoned that if low pH accelerated exchange by a different mechanism, for example, conformational stabilization of the intermediate, then the two should be strongly synergistic. Indeed, when comparing the effect of pH and dipeptide on peptide exchange for wtA2, we found that they synergized in accelerating peptide exchange (Figure 5). We conclude that the data are consistent with a model in which dipeptides and low pH accelerate peptide exchange by separate mechanisms.

3 DISCUSSION

The kinetics of peptide binding and exchange are central to the dynamic process of peptide selection and optimization in live cells, which co-determines immunodominance and is thus essential to vaccine design.\textsuperscript{25,33,43–46} Peptide exchange is especially interesting in cross-presentation, where professional antigen-presenting cells present exogenous peptides on MHC-I\textsuperscript{6,47,48}; this process may not rely on peptide-empty MHC-I molecules escaping from the ER but involve the exchange of ER-bound peptides generated from endocytosed material in the endosomes.\textsuperscript{15,48–51} Since ER-loaded and tapasin-optimized peptide/MHC-I complexes are stable on the timescale of minutes to hours, researchers have asked whether the acid environment of endosomes might accelerate the peptide exchange for crosspresentation.\textsuperscript{6,7,15,49} We show here that at pH 6, peptide exchange on A2 and A24 is indeed significantly accelerated. Peptide association kinetics has been especially difficult to study, since the empty forms of class I that were available had slow binding kinetics and are assumed to be in a molten globule state.\textsuperscript{20,32,52,53} The advent of disulfide-stabilized empty molecules has made such association studies possible.\textsuperscript{8} Thus, we have been able to test the recent proposition—from MD simulations—that peptide binding to A24 is strongly supported by electrostatic attraction\textsuperscript{54}; this is not supported by our data, which show only a mild decrease in the association rate at high
salt for A2, and none for A24. So, while binding (and dis-

sociation) kinetics of peptides to MHC-I are allotype-

specific in detail, we do not find evidence for long-range interactions between receptor and ligand in the association process.

This also means that the acidic acceleration of peptide exchange that we describe here is not caused by an electrostatic charge that would attract the peptide. Likewise, we exclude effects on peptide dissociation and on the equilibrium constant. This leaves as the simplest explanation a model of a peptide-empty intermediate of the reaction that exists in two states, only one of which is peptide-receptive, and whose equilibrium is shifted with changing pH (Equation 4).

Such an unstable empty intermediate is not a leap of faith. Peptide-empty class I molecules were always known to be conformationally unstable, both in vitro and in cells, and this showed in MD simulations. From such simulations, we can assume that there are not two empty forms but an ensemble or continuum of many states; but a simulation with two states already gives sufficient approximation to the data (Figure 4c). A calculation demonstrates that there is enough time during an exchange reaction for an MHC-I protein to partially unfold: a typical association rate of 10 nM peptide to dsA2 is $2 \times 10^7 \text{M}^{-1} \text{min}^{-1}$ (Figure 2a), giving a collision frequency of 0.2 min$^{-1}$ and a half-time of 3.5 min for the productive binding of exogenous peptide to empty A2. Thus, during an exchange reaction, the binding site may remain empty for several minutes until an incoming peptide molecule binds productively. The model in Equation 4 assumes that at acidic pH, a larger proportion of the peptide-free intermediate of the exchange reaction is in a peptide-receptive state ($M_{\text{receptive}}$) than at neutral pH. This is strongly supported by our finding that if the pH is lowered, the $k_{\text{ex}}$ increases until it comes close to the $k_{\text{on}}$, suggesting that a substantial portion, if not all, of MHC-I molecules is available for productive peptide bind-

ing (Figure S4), that is, that peptide dissociation is not a rate-limiting step, and that $M_{\text{receptive}} \gg M_{\text{non-receptive}}$ at pH 6.

Why, then, are the $k_{\text{on}}$ values at pH 8 and pH 6 barely different from each other? This apparent paradox is explained by a thought experiment: in fluorescence anisotropy, the fastest association rate will always dominate the initial rate determination, and so one always measures the $k_{\text{on}}$ to the $M_{\text{receptive}}$ if it is present in the mixture.

For us, the structure of the $M_{\text{receptive}}$ and $M_{\text{non-receptive}}$ states is especially interesting. We and others have found that in empty MHC-I, the F pocket region is especially conformationally flexible. When the two heli-

ces are covalently connected with a disulfide bond between residues 84 and 139, conformational fluctuations decrease (as proposed by MD simulations), and recombinant MHC-I remain soluble in their peptide-free form, suggesting that F pocket conformational stabilization is a main task of the bound peptide in maintaining MHC-I stability. The substantial increase of $k_{\text{on}}$ and $k_{\text{ex}}$ that we observe with dsMHC-I over wtMHC-I (Table S1) thus formally demonstrates that conformational stabilization of a peptide-free intermediate accelerates peptide association. However, acidic acceleration is not identical to disulfide stabilization. We know that because low pH leads to a considerable $k_{\text{ex}}$ increase even for the dsMHC-I (Figure S1e,f). Importantly, this mechanistic difference also applies to the wtMHC-I (i.e., it is not an artifact of the dsMHC-I proteins), since low pH and dipeptides (which stabilize the F pocket like the disulfide bond) are synergistic in exchange acceleration (Figure 5). The observation that disulfide stabilization does not work for all MHC-I allotypes (S. Sp., unpublished) agrees with this observation, further suggesting that other regions of MHC-I may need to be stabilized. Still, after all, we do not yet know which exact conformational rearrangement accompanies the $M_{\text{receptive}} \leftrightarrow M_{\text{non-receptive}}$ transition of Equation 4. It might be one of the movements of the binding site observed previously, or else it might involve a movement of the $\alpha_3$ domain, or to $\beta_2\text{m}$.

The maximal peptide exchange rate that can be achieved at low pH is tenfold higher for A24 than for A2 (Table S1). According to our data, the simplest explanation is that the peptide-free intermediate of A24 is proportionally more in the $M_{\text{receptive}}$ state. This explanation is supported by our observation that the peptide-empty form of A24 has a much higher $T_m$ ($\Delta T_m = 7.5^\circ\text{C}$ at pH 8) than empty A2 (Figure 4a). We have shown earlier that MHC-I allotypes differ in the conformational stability of the empty form, and that this correlates with dependence on the conformational chaperone, tapasin. However, A2 and A24 share a low tapasin dependence, which suggests that the response of empty MHC-I to acidic conditions, as encountered in the endosomes, is governed by a set of rules that are as yet unknown. It will be exciting to uncover them, and to correlate them with cross-presentation in living cells, where the low pH of endosomes is known to be very important for the recycling of endocytosed MHC-I to the surface.

4 METHODS

4.1 Production of MHC-I heavy chains and $\beta_2\text{m}$

MHC-I heavy chains and $\beta_2\text{m}$ were produced in *E. coli*, as described previously. Briefly, proteins were
expressed in *BL21(DE3)pLysS* using pET series plasmids (Novagen). Inclusion bodies containing expressed proteins were harvested by sonication in lysis buffer followed by washing in detergent buffer and wash buffer and solubilizing the protein in 8 M urea buffer (8 M urea, 50 mM HEPES pH 6.5, and 100 μM β-mercaptoethanol). Proteins were stored at −80°C until used for in vitro folding.

### 4.2 In-vitro folding and purification of MHC-I

All MHC-I proteins were folded and purified as previously described. Briefly, wtA2, dsA2, wtA24 and dsA24 molecules, heavy chains (1 μM) and β2m (2 μM) diluted in a folding buffer composed of 0.1 M Tris pH 8.0, 500 mM L-Arginine-HCl, 2 mM EDTA, 0.5 mM oxidized glutathione and 5 mM reduced glutathione with 10 μM low-affinity peptides or with 10 mM of dipeptide (GM for A2, GF for A24). The folding reaction was then incubated at 4°C for 1 week, followed by concentrating folded proteins with a 30 kDa cutoff membrane filters (Vivaflow-200; Sartorius). Folded MHC-I monomers were purified with size exclusion chromatography using AKTAGo HiLoad 26/600 Superdex-200 pg gel filtration column (Cytiva). All MHC-I folded monomers were stored at −80°C until further use.

### 4.3 Thermal stability measurements

Thermal stability was measured using nanoDSF by dissolving MHC-I complexes in in citrate-phosphate buffer, pH 7.6 at the concentration of 100 μg/ml. Each capillary was loaded with 10 μl of test samples either in duplicates or triplicates. Experiments were carried out using the Prometheus NT.48 (Nano Temper Technologies). The temperature gradient was set to an increase of 1°C/min in a range from 20 to 80°C. *Tm* values were calculated by the PR. ThermControl v2.1 software from the first derivative of the fluorescence at F330 and plotted using GraphPad prism.

### 4.4 Peptide binding, exchange, and dissociation

Peptide binding and exchange was performed using fluorescence anisotropy as previously described. To assess peptide binding by fluorescence anisotropy, 10 nM fluorochrome-labeled peptide NLVPK*FITC*VATV or QYTPVS*K*FITC*LF (GeneCust) and 30 nM of purified folded empty dsA2 or dsA24 were used unless otherwise mentioned. The FITC subscript denotes the lysine residue whose side chain was modified with fluorescein, taking care that labeling the peptide did not interfere with binding to MHC-I. Peptide binding was measured at room temperature (22–24°C) in citrate-phosphate buffer pH 6, pH 7, and pH 8 in a total reaction volume of 100 μl.

For peptide exchange studies, 10 nM of NLVPK*FITC*VATV or QYTPVSK*FITC*LF (GeneCust) was added to 30 nM of purified MHC-I/peptide complexes of wt or ds A2 or A24, respectively. Where indicated, the dipeptides GM, GL and GF (in the single-letter amino acid code; by GeneCust) were added at 10 mM final concentration.

For peptide dissociation studies, 10 nM of NLVPK*TAM*-RAVATA was added to 30 nM of purified empty dsA2, followed by the addition of 10 μM NLVPMVATV after 1 hr. Association was tracked for the first hour, followed by dissociation for 6 hr following addition of NLVPMVATV.

All kinetic measurements were performed with Tecan Infinite M1000 PRO (Tecan) multimode plate reader by measuring anisotropy (FITC *λem* = 494 nm, *λex* = 517 nm). Data were plotted using GraphPad Prism.

### 4.5 Kinetics simulations

Kinetics simulations were done in Microsoft Excel with 10,800 steps and a time increment of 1 s.

### 4.6 Molecular dynamics simulations

MD simulations were performed starting from the coordinates of A2 (pdb entry pdb5d2n) and A24 (pdb entry pdb2bck). For the simulations in the absence of bound peptides, the coordinates of the bound peptides were removed. The disulfide bridge to generate the disulfide-stabilized variants was introduced by in silico substitution of residues 84 and 139 by Cys residues. Protonation states of acidic and basic groups were calculated using the Amber18 package. Proteins were solvated in octahedral boxes with explicit TIP3P water molecules keeping a minimum distance of 10 Å between protein atoms and box boundaries. The ion concentration was adjusted to 0.1 M with explicit sodium and chloride ions. The parm14SB force field was used for the proteins and
peptides. The simulation systems were energy minimized (5,000 steps) after solvation followed by heating up to 310 K in steps of 100 K with position restraints on all heavy atoms of the proteins. Subsequently, positional restraints were gradually removed from an initial 12 kcal-mol\(^{-1}\)Å\(^{-2}\) to 0.5 kcal-mol\(^{-1}\)Å\(^{-2}\) within 0.5 ns followed by a 1 ns unrestrained equilibration at 310 K. All production simulations were performed at a temperature of 310 K and a pressure of 1 bar. The hydrogen mass repartition option of Amber was used to allow a time step of 4 fs. Unrestrained production simulations for up to 750 ns were performed. Trajectory analysis was performed using the cpptraj module of the Amber18 package.

**AUTHOR CONTRIBUTIONS**

**Ankur Saikia**: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); validation (lead); writing – original draft (equal). **Andries Hadeler**: Data curation (supporting); formal analysis (supporting); investigation (supporting). **Pranathi Prasad**: Formal analysis (supporting); writing – original draft (equal). **Martin Zacharias**: Data curation (supporting); formal analysis (supporting); investigation (supporting); software (lead); visualization (lead); writing – review and editing (supporting). **Sebastian Springer**: Conceptualization (equal); project administration (lead); resources (lead); supervision (lead); validation (supporting); writing – original draft (supporting); writing – review and editing (lead).

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**CONFLICT OF INTEREST**

The authors declare that no competing interests exist.

**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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