Understanding β-strand mediated protein-protein interactions using peptidomimetics: tuning binding affinity of intrinsically disordered sequences by covalent backbone modification

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Abstract: A significant challenge in chemical biology is to understand and modulate protein-protein interactions (PPIs). Given that many PPIs involve a folded protein domain and a peptide sequence that is intrinsically disordered in isolation, peptides represent powerful tools to understand PPIs and templates for PPI modulator development. Using the interaction between small ubiquitin-like modifiers (SUMO) and SUMO-interacting motifs (SIMs), here we show that *N*-methylation of the peptide backbone can effectively restrict accessible peptide conformations, predisposing them for protein recognition. Backbone *N*-methylation in appropriate locations results in faster target binding, and thus higher affinity, as shown by fluorescence anisotropy, relaxation-based NMR experiments, and computational analysis. We show that such higher affinities occur as a consequence of an increase in the energy of the unbound state, and a reduction in the entropic contribution to the binding and activation energies. Thus, backbone *N*-Methylation may represent a useful modification within the peptidomimetic toolbox to probe β-strand mediated coupled binding and folding interactions.

Introduction

Protein-protein interactions (PPIs) drive and regulate many biological functions.([1-3]) Understanding the molecular mechanism by which protein-protein interactions occur is central to understanding function.([4]) Such mechanistic understanding can support development of: tools that can modulate PPIs to act as probes of healthy and disease states of biological processes, and drug discovery efforts.([5-8]) Many PPIs employ short peptides for recognition([9, 10]); aside from serving as templates for designing PPI inhibitors ([11-16]), a feature of short recognition peptides is that they are often intrinsically disordered([17]) and undergo disorder-order transitions, e.g., to form α-helices([18]) or β-strands([19]) on PPI formation. The association binding kinetics of coupled folding and binding are influenced by the larger hydrodynamic radius, flexibility and folding propensity of IDPs in comparison to folded proteins, alongside the abundance of exposed charged residues which are often associated with encounter complex formation.([17, 20]) IDPs in their apo form can also populate a bound-folded state to support a conformational selection binding mechanism. Whilst IDPs that populate a helical bound conformation can be easily evidenced by NMR chemical shifts, β-strand conformations are more challenging to characterise; a propensity to form extended structures is normally captured by NMR, but the extended ensemble typically contains numerous conformations, only some of which are binding-competent. The synthetic accessibility of peptides makes them ideal tools to study biomolecular mechanisms; modifications that alter the conformational landscape of a disordered peptide can be used to bias the energy landscape to probe binding mechanisms in a systematic way.([21, 22])

N-methylation of backbone amides has been shown to improve the affinity, interaction specificity, solubility, membrane permeability, and proteolytic stability of peptides.([23, 24]) However, these studies have generally focused on cyclic peptides. There are far fewer reports on *N*-methylation of linear peptides, and even fewer for peptides solely composed of L-amino acids.([25-27])([28]) *N*-methylation can restrict the conformations accessible to a peptide, as it disfavours backbone conformations in the bottom-left quadrant of the Ramachandran plot, which includes the α-helical region, φ ≈ -60° and ψ ≈ -50°.([29, 30]) However, it also allows access to alternative conformations by lowering the difference in stability between *cis*/*trans* amide rotamers.([31, 32]) Nonetheless, the precise manner in which *N*-methylation can be used to alter the backbone conformational preferences of linear peptides, in particular understanding what determines the extent to which *N*-methylation favours more-extended structures, is less explored. *N*-methylation also changes the hydrogen-bonding capabilities of peptides. In turn, this could alter the ability of a peptide to bind to protein targets, but it can also improve the physical properties of peptides; for instance, reducing propensities to self-assemble into amyloid-like structures,([33, 34]) and susceptibilities to certain proteases.([35]) Thus, in this work, we sought to explore the extent to which backbone *N*-methylation might serve as a tool to inform on and modulate IDP binding mechanism; using the interaction between small ubiquitin-like modifiers (SUMO) and SUMO-interacting motifs (SIMs) as a model to explore the effects of *N*-methylating a SIM peptide, we show that whilst backbone modification of some sites abrogates binding, at others it increases the peptide–protein association rate (k_{on}) resulting in increased binding affinity. For the latter, this behaviour can be rationalized as follows: *N*-methylation restricts the accessible peptide conformations, in effect predisposing them for target recognition. This is achieved by raising the energy of the unbound state, and decreasing the activation energy (entropy) required for binding. Such an approach may therefore represent an effective method by which to enhance protein binding affinity of disordered regions, and design peptidomimetic inhibitors of β-strand-mediated PPIs.

Figure 1. Overview of the interaction between the M-IR2 region of RanBP2 ($SIM_{2705-2717}$), and hSUMO-1_{18-97} (SUMO) and the effects of backbone modification on the strength of interaction. (a) Lowest energy structure of the NMR-derived structural ensemble for SIM/SUMO (PDB ID, 2LAS), highlighting key interactions (identified using Arpeggio)(*36*) between the parent SIM peptide and SUMO. (b) Differences in potency for variant SIM peptides relative to the parent SIM peptide as determined in fluorescence anisotropy competition assays; hatched bars highlight variants for which precise IC_{50} values could not be measured; see ESI Fig. S1 for conditions and titration data. (c) Sites of *N*-methylation for which detailed NMR analyses were performed (Figs. 2-5).

Results and Discussion

Position-dependent effects of *N*-methylation on binding affinity

As a model β-strand-mediated PPI to explore the effect of *N*-methylation on peptide conformation and protein binding, we chose the interaction between human SUMO-1_{18-97} (SUMO) and the SIM motif found in the M-IR2 region of RanBP2. Our chosen SIM sequence – Ac-Asp-Asn-Glu-Ile-Glu-Val-Ile-Ile-Val-Trp-Glu-Lys-Lys-NH_2 (herein referred to as the 'parent SIM peptide') – was taken from Namanja *et al.*,(*37*) who modified the wild-type RanBP2 M-IR2 $SIM_{2705-2717}$ sequence to make it more amenable to biophysical study. This SIM peptide is intrinsically disordered in the absence of a binding partner,(*38*) but binds to SUMO through β-augmentation with low micromolar affinity.(*37*) Key non-covalent interactions present in the bound complex (Fig. 1a) include hydrogen bonds from SUMO to the backbone of SIM residues Glu2709, Ile2711, and Val2713, and side chain H-bonds and π interactions to SIM Trp2714. Hydrophobic packing of isoleucine and valine side chains from SIM along the SUMO binding cleft also contributes to binding affinity; previously, we conducted an alanine scan on this sequence and identified a contiguous stretch of hot-spot residues from Val2710 to Trp2714 (VIIVW; ΔΔG ≥ 4.2 kJ/mol for each residue).(*38*) This corresponds to the (V/I)X(V/I)(V/I) or (V/I)(V/I)X(V/I/L) consensus motif commonly found in SIMs.(*37, 39*) In addition, Glu2709 was just below the threshold for being classified as a hot-spot residue.

On this basis, we performed a systematic backbone *N*-methyl scan for all 13 residues of the parent SIM peptide. Peptides were prepared using Fmoc-based solid phase peptide synthesis (see the Supporting Information for procedures and characterization) and their

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relative binding affinities to SUMO were estimated using a fluorescence anisotropy competition assay.(*38*) *N*-methylation at six sites (Me-Asp2705, Me-Asn2706, Me-Glu2707, Me-Ile2708, Me-Ile2712, Me-Lys2717) led to similar or slightly improved inhibitory potency to the parent SIM peptide (Fig. 1b; Fig. S1, Table S1). By contrast, *N*-methylation at five sites (Me-Val2710, Me-Ile2711, Me-Ile2713, Me-Trp2714, and Me-Lys2716) led to significantly diminished inhibitory potency, and the remaining two sites (Me-Ile2709, and Me-Glu2715) exhibited moderately reduced potency. Significantly, changes in inhibitory potency were observed both within and outside the consensus hot-spot region.

Effects of *N*-methylation on the unbound peptide

We next sought to rationalise the changes in potency observed as a consequence of *N*-methylation. For some variants (e.g., Me-Ile2711 and Me-Val2713), disruption of binding could reasonably be attributed to loss of key backbone hydrogen bonds between SIM and SUMO, and/or the steric hindrance conferred by the methyl group disrupting adjacent interactions (Fig. 1a). For the remaining variants, however, the observed changes in IC_{50} could not be readily explained based on changes to hydrogen bonding interactions or possible steric clashes between the variant peptide and the protein. We therefore considered that changes in binding affinity may instead arise from changes in backbone conformation (which can in turn affect side-chain conformations) or changes in the electron density of amide bonds. With a selection of peptides from across the *N*-methylated SIM series (Fig. 1c), this was investigated using solution NMR methods and computational analyses for the unbound peptides.

Three *N*-methylated SIM variants with ΔΔG values close to that of the parent SIM peptide (Me-Ile2708, Me-Ile2712, Me-Lys2717) and two variants with positive ΔΔG values (Me-Val2710 and Me-Lys216) were selected (Fig. 1c) to interrogate the determinants of binding affinity, relative to the parent SIM peptide. Using one-dimensional ^{1}H NMR experiments, we ruled out changes in the population of the *cis* isomer at the *N*-methylated amide bond and changes in peptide oligomeric state as drivers of the observed changes in potency (Figs. S2).

Simulated annealing calculations of *N*-methylated peptides indicated that methylation of backbone amide bonds could restrict the accessible *phi* (φ) and *psi* (ψ) angles rendering the α-helical region of Ramachandran space inaccessible (Fig. 2a). As the parent SIM sequence has been shown to lack stable secondary structure in the absence of a binding partner,(*38*) it is possible that some of the differences in binding affinity observed could be explained by changes to the secondary structure propensity of *N*-methylated SIM variants. NMR chemical shifts of backbone nuclei (Hα, Cα and Cβ) can be used as reporters of even small changes in secondary structure of disordered proteins/peptides,(*40*) and α-like or β-like chemical shifts are indicative of an increase in the population of those secondary structures. Therefore, the backbone and side chain chemical shifts of parent SIM and its *N*-methylated variants were assigned using ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H NOESY spectra and natural abundance ^{1}H-^{13}C HSQC spectra.

As anticipated, the backbone chemical shifts of the parent SIM were consistent with those for a fully unstructured (random coil) peptide (Fig. S3). Comparison of chemical shifts within the *N*-methylated region of each variant, relative to parent SIM, is complicated by the fact that *N*-methylation will increase the electron density of the associated amide bond, due to the electron-donating character of the methyl group. In the absence of any structural changes, we expect the chemical shift of neighbouring H_A atoms (i.e., H_A of the *N*-methylated residue and H_A of the preceding residue) to be shifted downfield when compared to parent SIM chemical shifts. This is what is observed for Me-Val2710, Me-Ile2712, Me-Lys2716 and Me-Lys2717 (Fig. 2b and Fig. S4). Excluding atoms whose chemical environment is directly impacted by the introduction of the *N*-methyl group (i.e., atoms within

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six bonds of the methyl carbon), the measured backbone chemical shifts of all five *N*-methylated variants differed very little from the shifts of the parent SIM, indicating that no long-range secondary structural elements (e.g., extended, β-rich structure) had been detected for these unbound peptides. In further support of this conclusion, we observed no NOE's indicative of helical or strand conformations.

Figure 2. Conformational analysis of *N*-methylated peptides: (a) Ramachandran plots of an *N*-methylated residue (*i*) and the preceding residue (*i*-1) in a peptide, as a function of the repulsion energy as calculated by XPLOR-NIH (see SI Materials and Methods). (b) Hα or Cα secondary

chemical shifts for SIM and *N*-methylated variant peptides. Propensity for β-strand/α-helix is shown in red/blue bars respectively with threshold for significant propensity denoted by dashed grey line. Chemical shift values around the methylation site are shown as open bars. (c) ^{1}H -^{1}H NOESY strips of the Me-Ile2708 variant peptide (5 °C, 500 μM peptide, 20 mM sodium phosphate, pH 7.4, 0.02% NaN$_3$, mixing time 500 ms) highlighting the E2706-H$_\beta$ to E2709-H$_N$ NOE.

For Me-Ile2708, the H$_A$ chemical shift pattern surrounding the *N*-methylated peptide bond differs for both the *trans* and *cis* isomers; the residue before the *N*-methylated amide is significantly more upfield than in the other SIM variants, while the residue after the *N*-

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methylated amide is more downfield (Fig. 2b), suggesting the possibility of a turn-like collapse.(*41, 42*) An NOE between N2706-H$_A$ and E2709-H$_N$ was also observed for the *trans* isomer of Me-Ile2708 (Fig. 2c), indicating some localized structuring of residues at the *N* terminus of this variant. However, we note that the *N* terminus does not participate in SIM/SUMO recognition and thus it is difficult to predict how such ordering will affect binding.

Overall, our NMR analysis on the unbound peptides strongly indicates that restriction of the available conformational space as a consequence of *N*-methylation does not induce significant changes in secondary structure. Thus, the observed changes in affinity likely arise from altered binding kinetics or differences in the bound SIM/SUMO structure.

Binding kinetics of the SIM/SUMO interaction from relaxation-based NMR

To determine more accurately the thermodynamic parameters for binding and investigate the binding kinetics of the parent SIM and its *N*-methylated variant peptides, NMR-relaxation based methods were used (Figs. 3 and 4). Initially, a series of ^{1}H-^{15}H NMR spectra were acquired using ^{15}N-enriched SUMO in the presence of natural abundance ^{14}N-SIM peptides. For the parent SIM peptide and for variants with similar values of ΔG$_{bind}$ (Me-Ile2708, Me-Ile2712, Me-Lys2717), peptide binding to SUMO was observed in the slow exchange regime on the chemical shift timescale, giving rise to two sets of ^{1}H-^{15}N resonances for residues in the SIM-binding pocket (helix α1 and strand β2 of SUMO), corresponding to the bound and unbound species (Fig. 3a). For these tighter-binding variants (Me-Ile2708, Me-Ile2712, Me-Lys2717), bound-state chemical shift differences relative to SUMO/parent SIM (Δω) were only observed for residues expected to lie in proximity to the *N*-methylated SIM residue (Fig. 3b and Fig. S5-S6,(*37*) indicating that these peptides bind SUMO in the canonical binding conformation. Non-overlapping exchange cross-peaks were evident for a subset of residues when the ^{15}N magnetizations of the bound and unbound states were mixed following t1 (^{15}N) evolution (ZZ-exchange spectroscopy). Global fitting of the intensities of auto and cross peaks as a function of mixing time to McConnel equations (Fig. 3c, Fig. S7-S10; SI for methods) gave apparent first-order association k_{on}^{app} and first-order dissociation k_{off} rates for binding, which were converted to k_{on} and dissociation equilibrium constants (K_D). For the parent, Me-Ile2708, Me-Ile2712 and Me-Lys2717 peptides, ^{15}N ZZ-exchange data at 5 °C fitted well to a 2-state bound-unbound model, yielding K_D values in the low micromolar range (Table 1). These confirm a significant increase in affinity for the *N*-methylated series (e.g. ΔΔG$_{Me-Ile2712 \text{ vs parent}}$ ~ 2 kJ mol^{-1}). For this set of four peptides, k_{off} values were similar (4-8 s^{-1}) suggesting that k_{on} is the primary cause of changes in K_D. Indeed, a positive correlation was observed between these two parameters (see Fig. 5a). The observed faster association rates, in the absence of any changes in the charge of the variant SIM peptides, could suggest that *N*-methylation at these locations conformationally predisposes these peptides for SUMO binding.

Figure 3. (a) ^{1}H-^{15}N HSQC spectra of ^{15}N-SUMO bound to parent SIM (black) or Me-Ile2712 (blue). Two sets of resonances corresponding to unbound (state A) and bound (state B) SUMO are observed. The same spectral region in the ^{15}N ZZ-exchange experiment with 100 ms mixing time is shown for Me-Ile2712. Exchange cross-peaks describing the flow of magnetization from state A to B (and vice versa) are labelled accordingly. (b) Combined ^{1}H-^{15}N chemical shift differences for SUMO residues when in complex with the parent SIM vs when in complex with the Me-Ile2712 SIM variant. SUMO residues which could not be confidently assigned are shown in black, while the remaining residues are coloured according to the magnitude of the chemical shift difference, relative to the standard deviation (σ) of the dataset (< 1σ, grey; 1-2σ, yellow; ≥2σ, pink; a cartoon representation of the SIM/SUMO complex coloured using the same colour scheme is shown on the right). (c) Measured intensities (dots) for the auto and cross-peaks from the ^{15}N ZZ-exchange experiment for Me-Ile2712 shown in (a) as a function of mixing time. Solid lines represent fits to the simple 2-state model shown in (a).

For the weak peptide binders (Me-Val2710 and Me-Lys2716), only the unbound SUMO resonances were observed in ^{1}H-^{15}N HSQC spectra, even at high SIM concentrations. This suggests that the population of the bound complex is small and/or binding does not take place in the slow chemical shift timescale. Thus, to investigate the binding kinetics of those variants, we used Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion that is sensitive to exchange between states with skewed populations on the millisecond timescale.(*43*) Large CPMG profiles for SUMO residues in the SIM-binding site were observed at 5 °C upon addition of Me-Val2710 or Me-Lys2716 (Fig. 4a and Fig. S11-S13). These profiles were absent for apo SUMO (Fig. S14), suggesting that the observed millisecond dynamics are due to transient SIM binding. Global fitting of CPMG data at 600 and 750 MHz to a 2-state model yielded excellent fits (Fig. 4 and Fig. S11-12). CPMG-derived chemical shifts of the transiently populated, bound state of SUMO in the presence of Me-Val2710 or Me-Lys2716 corelated well with those of the stable, parent SIM-bound state, with the exception of some residues in the SIM binding pocket (see Fig. 2b, S11b, S11b), suggesting that the bound state of SUMO in the presence of these SIM variants is not significantly different to that with parent SIM. However, k_{off} values for the weakly bound variants increased by more than 50-fold (~200-550 s^{-1}; Fig. 5b) – therefore, while the structure of SUMO in the bound state

complex is unchanged by *N*-methylation, *N*-methylation at residues Val2710 and Lys2716 either prevents or hinders the ability of the SIM peptide to adopt its canonical binding conformation, leading to fast dissociation and a lower affinity interaction. It is surprising that Me-Val2710 does not show improved binding affinity as the NH is not involved in hydrogen-bonding and methylation should restrict Ramachandran space to conformations that favour binding.(*44*) Therefore, it is likely that methylation adversely influences the accessible χ space of the isopropyl side chain of Val2710 (a hot-spot residue), making it incompatible with SUMO binding. In the case of Lys2716, we consider it more likely that methylation of this residue perturbs the allowable χ space for Trp2714.

Table 1. Binding affinities and rates for the binding of *N*-methylated SIM peptides to SUMO. K_D, k_{on}, and k_{off} values were determined for select SIM variants by NMR (using ^{15}N ZZ-exchange experiments for tighter binding variants, and CPMG experiments for weaker binding variants). NMR data was acquired in 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 2 mM DTT, 0.02% NaN$_3$, 5 °C.

Peptide	K_D at 5 °C (µM)	k_{on} (s^{-1}M^{-1}) ×10^5	k_{off} (s^{-1})
Me-Ile2708	14.0 ± 0.4	3.27 ± 0.09	4.58 ± 0.04
Me-Val2710	388 ± 7	5.36 ± 0.10	208 ± 1
Me-Ile2712	14.5 ± 0.9	4.10 ± 0.28	5.90 ± 0.08
Me-Lys2716	510 ± 40	10.9 ± 0.7	553 ± 17.9
Me-Lys-2717	13.6 ± 0.5	6.57 ± 0.27	8.95 ± 0.12
Parent SIM	33.6 ± 0.9	2.45 ± 0.07	8.22 ± 0.08

All together, our results suggest that the restriction of the conformational space available to *N*-methylated peptides can increase association kinetics which correlates with enhanced binding. Conversely, in some cases, if the modified peptide cannot adopt a stable binding conformation, *N*-methylation can favour dissociation which correlates with diminished binding.

Figure 4. (a) ^{15}N CPMG profiles of ^{15}N-SUMO in the presence of Me-Lys2716 at 750 MHz (purple) and 600 MHz (green). Experimental data are shown as dots, while fits to a 2-state model (see Figure 3a) are shown as solid lines. (b) Correlation of fitted ^{15}N $\Delta\omega$ values for the Me-Lys2716 bound SUMO with those experimentally observed for SUMO bound to parent SIM (Pearson correlation coefficient ~0.86).

Increased affinity is due to a lower association activation barrier

To determine thermodynamic parameters for the SIM/SUMO interaction, we studied the temperature dependence of the NMR-derived exchange parameters(*45*) for the parent peptide, along with those for a strong and a weak binding N-Me variant – Me-Ile2712 and Me-Lys2716, respectively (Figs. S7, S8, S13). Van't Hoff analysis of the calculated binding affinities revealed SIM binding to be an entropically-driven process (Table 2 and Fig. 5c). In comparison to the parent SIM peptide, the entropy of Me-Ile2712 binding at 25 °C ($T\Delta S_{bind}$) increased by ≈15 kJ/mol, which was only partially compensated by ≈12 kJ/mol increase in enthalpy (ΔH_{bind}) (Fig. 5c, Table 2), leading to a more favourable free energy of binding (ΔG_{bind}). Thus, the increased affinity for Me-Ile2712, relative to the parent, is entropy driven. For Me-Lys2716, both ΔH_{bind} and $T\Delta S_{bind}$ were diminished, resulting in a smaller ΔG_{bind} and a weak affinity.

Eyring plots were obtained for the temperature-dependence of the k_{on} and k_{off} rates (Fig. 5d and 5e), from which the association/dissociation activation enthalpies ($\Delta H_{ass}^{\ddagger}/\Delta H_{diss}^{\ddagger}$), activation/dissociation entropies ($T\Delta S_{ass}^{\ddagger}/T\Delta S_{diss}^{\ddagger}$), and thus activation/dissociation free energies ($\Delta G_{ass}^{\ddagger}/\Delta G_{diss}^{\ddagger}$), could be determined. The association of SIM and its variants with SUMO has an enthalpic activation barrier in all cases (Table 2), while peptide dissociation has an enthalpic barrier for parent SIM and Me-Ile2712, and a smaller but entropic barrier for Me-Lys2716 (Fig. 5d, Table 2). At 25 °C, $T\Delta S_{ass}^{\ddagger}$ for Me-Ile2712 is more favourable by

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≈11 kJ/mol, which is compensated only by a ≈8 kJ/mol more unfavourable $\Delta H^{\ddagger}_{ass}$. Considering that the dissociation free energy barrier, $\Delta G^{\ddagger}_{diss}$, is practically identical for the parent and Me-Ile2712 peptides (≈64 kJ/mol), the slightly more favourable $T\Delta S^{\ddagger}_{ass}$ gives rise to the 3 kJ/mol decrease in ΔG_{bind} for Me-Ile2712 (Fig. 5c-e). Taken together, these data suggest that entropy-driven lowering of the association activation barrier arising from an increase in the free energy of the *N*-methylated peptide (relative to the parent SIM) represents a plausible explanation for the increased affinity for Me-Ile2712.

Figure 5. (a) Linear free energy relationship (LFER) plot depicting relationship between k_{on} and K_d for the binding of parent SIM (black), Me-Ile2708 (green), Me-Val2710 (red), Me-Ile2712 (blue), Me-Lys2716 (purple) or Me-Lys2717 (orange) to SUMO. (b) LFER plot depicting relationship between k_{off} and K_d for binding of SIM variants (colours as for (a)) to SUMO. (c) Van't Hoff plots of the temperature-dependence of binding affinities for parent SIM (black), Me-Ile2712 (blue) and Me-Lys2716 (purple); (d-e) Eyring plots for the association rate (d), k_{on}, and dissociation rate (e), k_{off}, for parent SIM (black), Me-Ile2712 (blue) and Me-Lys2716 (purple). For (c)-(e), experimental data-points are shown as dots and solid lines represent linear fits to the data.

Table 2: Binding thermodynamics for the SIM/SUMO interaction at 25 °C. Values were calculated by fitting the Van't Hoff or Eyring equations. Uncertainties represent the standard deviation of the fitted parameters, calculated in a Monte-Carlo fashion.

Parent SIM	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
Binding (K_d)	113.1 ± 0.7	146.7 ± 2.8	-33.6 ± 1.0
Association (activation, k_{on})	164.8 ± 1.0	134.6 ± 1.0	30.2 ± 1.2
Dissociation (activation, k_{off})	52.0 ± 0.3	-11.9 ± 0.3	64.0 ± 0.4
Me-Ile2712			
Binding (K_d)	125. 2 ± 0.2	161.8 ± 2.0	-36.6 ± 2.0
Association (activation, k_{on})	172.5 ± 2.1	145.5 ± 2.1	27.5 ± 2.2
Dissociation (activation, k_{off})	50.2 ± 0.3	-14.7 ± 0.2	64.8 ± 0.4
Me-Lys2716			
Binding (K_d)	31.4 ± 0.2	52.5 ± 0.2	-21.1 ± 0.3
Association (activation, k_{on})	48.5 ± 0.2	13.0 ± 0.2	35.4 ± 0.3
Dissociation (activation, k_{off})	17.9 ± 0.1	-38.5 ± 0.1	56.5 ± 0.1

Conclusions

We have performed a systematic backbone *N*-methylation of a 13-residue SIM peptide and assessed the effects on SUMO binding using a combination of competition fluorescence anisotropy and relaxation-based NMR experiments. At seven positions in the sequence, binding was abrogated or adversely affected, whereas at six positions binding was unaffected or improved. In instances where binding was diminished, this could be readily ascribed to the methylated peptide being unable to adopt a stable bound conformation. In instances where methylation led to improved binding potency, this could be ascribed to faster binding to SUMO. Interestingly, this occurred for both hot-spot and flanking residues from the SIM peptide.

In setting these results within the context of potential molecular mechanisms of recognition, we note the following additional considerations. First, there are likely subtle effects, such as a small increase in hydrogen-bond accepting ability of the carbonyl that might be expected upon *N*-methylation of the peptide bond. In turn, this would be anticipated to increase binding enthalpy. In addition, we cannot exclude the possibility that increased hydrophobicity or differential solvation of the methylated peptides influences affinity. Nonetheless, the entropy driven increased k_{on} rates that we observe lead us to conclude that increased affinities are caused by restricting the accessible conformational space of the *N*-methylated peptides. We simulated Ramachandran plots, which show that *N*-methylation significantly limits the phi(ϕ)/psi(ψ) angles accessible to residues on either side of the methylation site, such that residues are limited to extended or turn-like conformations (i.e.,

excluded from α-helical space). However, NMR analyses suggest the unbound peptides do not adopt a defined conformation in the absence of SUMO. We contend that the overall ensemble of SIM conformers has a higher ground state energy and that this lowers the entropy of activation for SUMO binding (see free energy diagrams in Fig. 6a). We note that the polyproline-II conformation is somewhat intermediate between the α-helix and β-strand conformations,[46] and that *N*-methylation is known to restrict the conformational space of peptide backbones.[29, 30] Thus, whilst the methylated peptides cannot be considered as pre-organized for SUMO binding, the ensemble is expected to disfavour α-space and thus favour β-space localized around the *N*-methylated residue so is primed or predisposed towards SUMO recognition. Previously, pre-organization of a peptide that recognises its target through a bind-and-fold[20] mechanism (Fig. 6b) was shown not to enhance affinity for its target, because constraining limits "the number of ways to bind",[47] whilst for a peptide which recognises its target through conformational selection (Fig. 6b),[20] constraining should increase affinity.[13] Given *N*-methylation does not seem to induce a specific extended conformer and instead favours an ensemble of conformers that are compatible with binding, the effect observed here may represent a useful strategy to drive affinity enhancement for ligands which bind their target through conformational selection or bind-and-fold mechanisms. Modulating peptide binding by tuning the entropy of activation/binding represents an untapped approach for design of peptidomimetic ligands; our future studies will apply this strategy to other β-strand mediated PPIs to explore the generality of these observations and for development of therapeutically relevant PPI inhibitors.

Figure 6. (a) Potential free energy diagrams together with schematics that could explain the data presented in Table 2. Grey, green and magenta dashed lines represent ΔG_{bind}, $\Delta G_{ass}^{\ddagger}$ and $\Delta G_{diss}^{\ddagger}$ respectively and their values are given in kJ/mol at 25°C (note: we show the parent and Me-Ile2712 bound complexes as isoenergetic on the basis of their HSQC bound state spectra and $\Delta G_{diss}^{\ddagger}$); (b) schematic illustrates the extremes of conformational selection and bind-and-fold protein binding mechanisms.

Declarations of interest

The authors declare no competing financial interests.

Author contribution statement

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