

# Determination of protein structural ensembles by hybrid-resolution SAXS restrained Molecular Dynamics.

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ABSTRACT. SAXS experiments provide low-resolution but valuable information about the dynamics of biomolecular systems, which could be ideally integrated into MD simulations to accurately determine conformational ensembles of flexible proteins. The applicability of this strategy is hampered by the high computational cost required to calculate scattering intensities from three-dimensional structures. We previously presented a hybrid resolution method that makes atomistic SAXS-restrained MD simulation feasible by adopting a coarse-grained approach to efficiently back-calculate scattering intensities; here, we extend this technique, applying it in the framework of metainference with the aim to investigate the dynamical behavior of flexible biomolecules. The efficacy of the method is assessed on the K63-diubiquitin, showing that the

inclusion of SAXS-restraints is effective in generating a reliable conformational ensemble, improving the agreement with independent experimental data.

## 1. INTRODUCTION

Biomolecules in solution can be characterized by a different extent of conformational dynamics depending on the specific system and experimental conditions<sup>1-3</sup>. While the dynamics of single domain proteins in native condition is generally limited to fluctuations around a well-defined structure, fully disordered proteins can only be described as statistical ensembles of conformations. In between these cases, multi-domain proteins connected by linker regions can populate multiple states generally characterized by a different size<sup>4</sup>.

Experimentally the characterization of conformational heterogeneity can be achieved by employing multiple solution techniques like nuclear magnetic resonance (NMR), Förster resonance energy transfer (FRET) and small angle X-ray scattering (SAXS)<sup>1,2</sup>. The latter has the advantage to be label free, to work with systems of any size and in essentially all experimental conditions<sup>5</sup>. An atomistic interpretation of scattering data could benefit from its combination with computational techniques, as Molecular Dynamics (MD) simulations, which could provide an accurate physical model to generate reliable conformational ensembles in agreement with SAXS data<sup>6</sup>. Common approaches employ SAXS to reweight conformational ensembles *a posteriori*, making use of statistically founded theoretical frameworks<sup>7-12</sup>. Recently, few methods in which SAXS experimental data are integrated into MD to drive conformational sampling have been proposed, nevertheless their application is hindered by the high computational cost required to calculate scattering intensities<sup>13-16</sup>.

In a previous work<sup>17</sup>, we developed a MD-based multi-resolution strategy to efficiently refine protein-DNA and protein-RNA complexes integrating SAXS experimental data with metainference<sup>18</sup>. According to this strategy, MD is run with full atomistic details, using standard atomistic force-field, while the back-calculation of SAXS intensities is performed in a coarse-grain fashion<sup>19</sup>, based on the Martini force field<sup>20</sup>. In the refinement protocol conformational averaging was not considered, under the assumption that a single structure, representing the most populated state of the system, could reliably reproduce all the measured experimental data used as restraints. In this work, we aim to further extend this approach to investigate the conformational space of biomolecules that can adopt multiple conformations in solution.

Here we applied our multi-resolution strategy to investigate the conformational ensemble of K63-linked diubiquitin (K63-Ub<sub>2</sub>). Diubiquitins represent an ideal test system as they are known to populate multiple conformational states due to the presence of a highly flexible linker connecting the C-terminal of the distal ubiquitin with either a lysine or the N-terminus methionine of the proximal domain (**Figure 1A**)<sup>21-27</sup>. In particular, the heterogeneity of K63-Ub<sub>2</sub> conformational space is supported by the presence of numerous crystallographic structures of this protein, free or in complex with diverse targets, displaying different degrees of opening and arrangements of the two subunits<sup>28-34</sup>. Furthermore, studies based on different biophysical techniques, including SAXS, NMR, cross-linking and FRET, support the hypothesis that K63-Ub<sub>2</sub> in solution populates a dynamic ensemble, including both extended and compact states<sup>26,27,35</sup>. This equilibrium between multiple states is considered critical in modulating the affinity of diubiquitin towards its biological partners<sup>26</sup>.

In the following, we present our SAXS-restrained all-atom M&M<sup>1,36,37</sup> simulation of K63-Ub<sub>2</sub>, performed with the hybrid resolution approach (hySAXS simulation), in comparison with an

unrestrained reference simulation, in which the same setting was used but for the inclusion of experimental data. Both conformational ensembles indicate an equilibrium between extended and compact conformations, but their assessment with independent experimental NMR paramagnetic relaxation enhancement (PRE) experiments<sup>27</sup> reveals that only the hySAXS restrained simulations can accurately describe the specific contacts responsible for the formation of compact states. All the methods described in this paper are freely available in the PLUMED-ISDB module<sup>38</sup> of the PLUMED library<sup>39</sup>, furthermore all the input files used are available on the PLUMED-NEST repository<sup>40</sup>, as plumID:19.057.

## 2. THEORY AND METHODS

### 2.1 Metainference

Metainference allows integrating experimental data with prior information, generally represented by a molecular mechanic force field<sup>18</sup>, taking into account the effect of conformational averaging and other sources of errors. In the case of Gaussian noise, the metainference energy can be written as<sup>41</sup>  $E_{MI} = E_{FF} + \frac{k_B T}{2} \sum_{i=1}^{N_d} \sum_{r=1}^{N_r} \frac{[d_i - \lambda f_i(\mathbf{X})]^2}{(\sigma_{r,i}^B)^2 + (\sigma_{r,i}^{SEM})^2} + E_\sigma$ , where  $E_{FF}$  is the energy of the force field,  $k_B$  the Boltzmann constant,  $T$  the temperature,  $d_i$  the set of  $N_d$  experimental data,  $f_i(\mathbf{X}) = \frac{1}{N_r} \sum_{r=1}^{N_r} f_i(X_r)$  is averaged over the  $N_r$  replicas,  $f_i(X_r)$  is the forward model used to predict observable  $i$  from conformation  $X_r$ ,  $\sigma_{r,i}^{Bias}$  is an uncertainty parameter that describes random and systematic errors,  $\sigma_{r,i}^{SEM}$  is the standard error of the mean related to the conformational averaging and  $E_\sigma$  is an energy term that accounts for normalization of the data likelihood and error priors. Monte Carlo sampling is used to sample the uncertainty  $\sigma_{r,i}^{Bias}$  and optionally a scaling parameter

$\lambda$  that relates experimental and back-calculated data (as in the case of SAXS experiment): these parameters are inferred during the simulation along with the model of the system. Importantly, if only one replica is considered, metainference becomes equivalent to the Inferential Structure Determination approach<sup>42</sup>; conversely, if  $\sigma_{r,i}^{Bias} = 0$  (i.e. in absence of data and forward model errors) it is equivalent to the replica-averaged MaxEnt modelling<sup>43</sup>.

Metainference can be combined with metadynamics to accelerate the exploration of the conformational space<sup>36,44</sup>. In particular, it was proposed to apply it in combination with parallel bias metadynamics<sup>45</sup> (PBMetaD), which allows the use of many collective variables (CVs) applying multiple low-dimensional bias potentials and therefore reducing the risk of missing slow degrees of freedom. In M&M multiple copies of the simulation are run in parallel, where all the replicas use the same conditions and force field and share the bias potential as in the case of multiple-walkers method<sup>46</sup>. The coupling of metainference and metadynamics is given by the calculation of the average forward model  $f_i(\mathbf{X})$ , where each replica contributes differently to the average with a weight  $w(X_r)$  depending on the bias potential  $V_{PB}$  according to:  $w(X_r) =$

$$e^{-\frac{V_{PB}(CV(X_r),t)}{k_B T}}.$$

## 2.2 Hybrid-resolution SAXS-driven metainference simulations

Given a coarse grain representation of a molecule of  $N$  atoms as a collection of  $M$  beads, each comprising a variable number of atoms, if the form factors  $F(q)$  of the beads are known the scattering intensities can be approximated as:

$$I(q) = \sum_{i=1}^M \sum_{j=1}^M F_i(q) F_j(q) \frac{\sin(qR_{ij})}{qR_{ij}},$$

where  $R_{ij}$  indicates the distance between the center of mass of beads  $ij$  and with the sum running over the number of beads. The complexity is therefore reduced from  $O(N^2)$  to  $O(M^2)$ . The form factors  $F(q)$  for custom beads can be computed adopting the Single Bead Approximation averaging over multiple structures<sup>19</sup>. Alternatively, form factors for beads based on Martini force field<sup>20</sup> are available<sup>47</sup> and were previously implemented in the PLUMED-ISDB module<sup>17,38</sup>.

Recently, we have implemented a hybrid multi-resolution strategy to perform full atomistic MD simulations in which SAXS intensities, computed at a coarse-grain level based on the Martini force field, are used as restraints within the metainference framework<sup>17</sup> (see **Figure 1A**). The virtual positions of the Martini beads are computed on-the-fly and are used in combination with Martini form factors<sup>47</sup> for SAXS calculations. The computational efficiency of this strategy can be further improved using a multiple time-step protocol, where the metainference bias is applied only every few time steps<sup>48</sup>. In our previous work, we demonstrated the reliability of the hybrid resolution approach for single-replica simulations in which two protein-nucleic acids complexes were refined against SAXS data. Here we extended the described approach to multi-replicas M&M simulations, with the aim to exhaustively explore the conformational space of flexible biomolecules, able to populate multiple conformational states.

### 2.3 Computational details of the simulations

K63-Ub<sub>2</sub>, for which both SAXS and PRE experimental data are available<sup>27,35</sup>, was used as a test system. As a starting model for the simulations, we used the chains B and C of PDB 2ZNV<sup>32</sup>: the K63R and D77 mutations in distal and proximal ubiquitin, respectively, were maintained to be coherent with SAXS measurements. MD simulations were performed with GROMACS 2018<sup>49</sup>, PLUMED 2<sup>39</sup> and the PLUMED-ISDB<sup>38</sup> module, using the Amber ff03w force field<sup>50</sup> with

TIP4P/2005 water model<sup>51</sup> and scaled protein-water Lennard-Jones parameters (amber03ws)<sup>52</sup>. The choice of this force field, that was specifically designed to increase molecules solvation, avoiding collapsed states and nonspecific protein-protein interactions, was guided by the fact that we expect an equilibrium between open and compact states of K63-Ub<sub>2</sub> with only transient inter-domain contacts. The system was solvated in a periodic dodecahedron box, initially 1.2 nm larger than the protein in each direction, and neutralized. After an initial energy minimization to a maximum force of 100 kJ/mol/nm, the solute was equilibrated under NVT condition at the temperature of 300 K for 50 ps using the Berendsen thermostat<sup>53</sup>; then Berendsen barostat was used to equilibrate the system in the NPT ensemble to the target pressure of 1 atm for 200 ps. The equilibration phase was followed by an initial MD simulation of 100 ns, from which a pool of well-equilibrated conformations was extracted to be used as starting models for the subsequent runs. During the production runs in the NPT ensemble, the md integrator was employed with a time step of 2 fs; the temperature was maintained at 300 K using the Bussi thermostat<sup>54</sup> and the pressure was controlled with Parrinello-Rahman barostat<sup>55</sup>. Bonds were constrained with the LINCS algorithm<sup>56</sup>, using a matrix expansion of the order of 6 and 2 iterations per step. Electrostatic was treated by using the particle mesh Ewald scheme<sup>57</sup> with a short-range cut-off of 0.9 nm and a Fourier grid spacing of 0.12 nm; van der Waals interaction cut-off was set to 0.9 nm.

Two metadynamics multi-replicas simulations were performed: 1) a metainference simulation, consisting of 32 replicas, in which metainference was used to enforce the agreement with SAXS data according to the hybrid approach (hySAXS) and 2) an unrestrained simulation, consisting of 8 replicas, in which similar settings of simulation (1) were used but without the inclusion of experimental restraints. PBMetaD was performed in combination with well-tempered metadynamics<sup>58</sup> and the multiple-walker scheme<sup>46</sup>, where Gaussians with an initial height of 1.0

kJ/mol were deposited every 0.4 ps using a bias factor of 30. Four CVs were biased: two of them (hydContacts and polContacts) counts the number of the hydrophobic and polar contacts between the two ubiquitin domains, the other two (TICAcv1 and TICAcv2) are the results of the linear combination of numerous angles as determined by a Time-lagged Independent Component Analysis<sup>59</sup> (TICA) performed on the initial 100 ns MD simulation (see Supplementary Information for more details). The width of the Gaussians was determined with the dynamically-adapted gaussian approach<sup>60</sup>, using a time window of 4 ps to estimate CVs fluctuations and setting as minimum values for the width 0.01, 0.05, 0.01 and 0.01 for hydContacts, polContacts, TICAcv1 and TICAcv2, respectively.

Experimental SAXS intensities for K63-Ub<sub>2</sub> are available in the SASDCG7<sup>35</sup> entry of the SASDB database<sup>61</sup>. For the hySAXS simulation, a set of 11 representative SAXS intensities at different scattering vectors, ranging between 0.06 Å<sup>-1</sup> and 0.16 Å<sup>-1</sup> and equally spaced, were included as restraints. These representative intensities were extracted from the experimental data, where a 21-point running average was performed to reduce the influence of experimental noise. Metainference was applied every 10 steps, using a single Gaussian noise per data-point and sampling a scaling factor between experimental and calculated SAXS intensities with a flat prior between 0.5 and 1.5.

For the hySAXS simulation, each replica was evolved for 250 ns, resulting in a total simulation time of 8 μs; for the unrestrained simulation, 750 ns per replica were run, for a total of 6 μs. Convergence was assessed using the block analysis procedure, in which free-energy profiles are computed over different blocks of simulations and lastly, the weighted average error along the free energy profile is computed as a function of the block length. In **Figure S1**, the free-energy profiles and the block average analysis are reported, showing that both simulations converged with comparable errors. As a preliminary control, we checked the RMSD of the single Ub domains. In

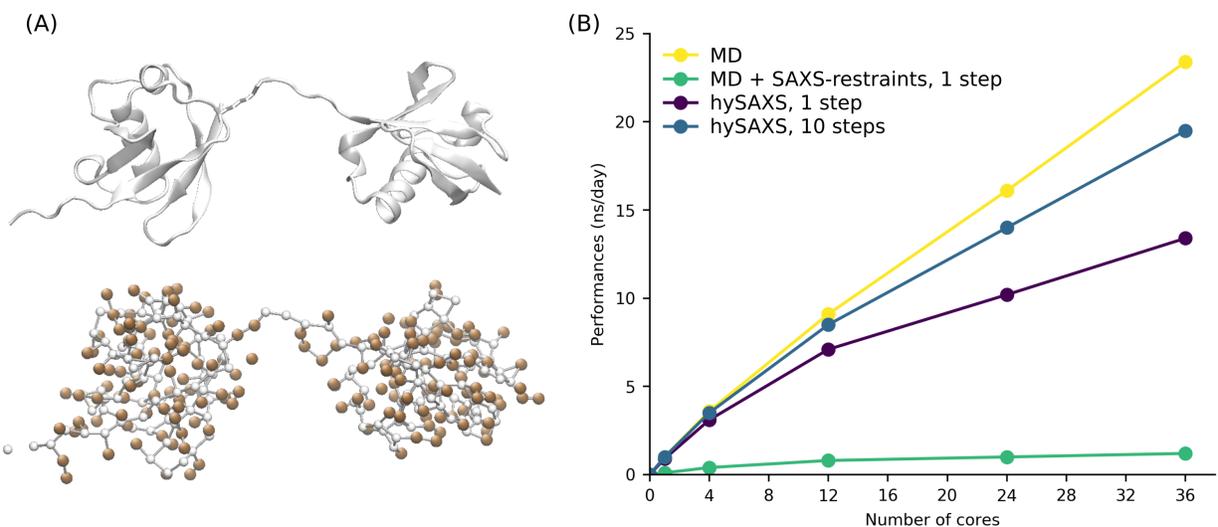
both simulations, the Ub domains are well folded. The comparison of RMSD distribution in the two simulations (**Figure S2**) showed lower RMSD values for the hySAXS ensemble with respect to the unrestrained one: that could be due to the shorter simulation time per replica as well as to a protective effect of the SAXS restraints against some destabilization resulting from the use of amber03ws.

### 3. RESULTS AND DISCUSSION

To evaluate our hySAXS approach, after assessing its computational performances in comparison with conventional MD simulations and atomistic SASX restraints, we tested its ability to improve the agreement of MD with experimental SAXS data in comparison with a state-of-the-art force-field (unrestrained simulations). As a model system, we employed K63-Ub<sub>2</sub> for which independent data are available to validate our results.

#### 3.1 The hySAXS approach is computationally efficient.

In **Figure 1B** we compared the performances of i) a conventional atomistic MD simulation (yellow); ii) all-atom metainference simulations where SAXS restraints with atomistic forward model were included every step (green); iii) all-atom hySAXS simulations where SAXS restraints were included every step (purple) or every 10 steps (blue). The use of the hybrid approach significantly improved the performances of SAXS-driven MD simulations compared to the ones adopting atomistic scattering evaluation. This gain can be further increased using a multiple time-step protocol (**Figure 1B**, blue line), in which the restraint is applied every few time steps. This strategy is well justified in the case of SAXS data, which are characterized by slow temporal fluctuations, and allows to approach the performances of conventional MD simulations.



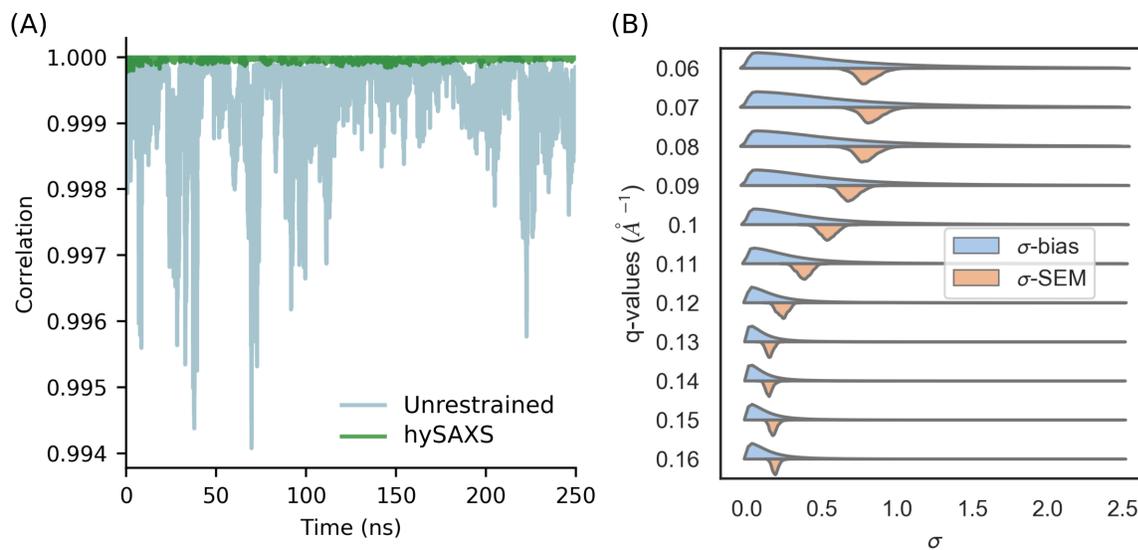
**Figure 1.** (A) K63-Ub<sub>2</sub> (2473 atoms) showed as cartoon representation (top) or highlighting the centers of the 328 Martini beads (bottom), colored in white and orange for backbone and sidechain, respectively. (B) Performances, as a function of the number of cores, estimated on Intel Xeon E5-2697 2.30 GHz for a single replica of K63-Ub<sub>2</sub> in water.

### 3.2 Monitoring hySAXS simulation.

To evaluate on-the-fly the effectiveness of SAXS restraints, we monitored the correlation between back-calculated and experimental data as a function of the simulation time (**Figure 2A**) comparing hySAXS to an unrestrained simulation. The comparison revealed a better agreement in the hySAXS simulation (**Figure 2A**), confirming the efficacy of the restraints. This is supported by other statistical properties, including the sum of square deviation and the slope/intercept of the linear fit (**Figure S3**).

We also monitored the intensity of experimental restraints, which depends on the square sum of the uncertainty parameters  $\sigma_{r,i}^{Bias}$  and  $\sigma_{r,i}^{SEM}$  (cf. Theory and Methods). To this aim, we computed the distribution across the hySAXS ensemble of both  $\sigma_{r,i}^{Bias}$ , which is associated with experimental

and forward model inaccuracies, and  $\sigma_{r,i}^{SEM}$ , i.e. the standard error of the mean over the replicas. We observed a broader distribution of the sampled parameter  $\sigma_{r,i}^{Bias}$  with respect to  $\sigma_{r,i}^{SEM}$  (**Figure 2B**), with greater uncertainties associated with smaller scattering angles (where indeed the global conformation mostly influences SAXS profiles). The values of  $\sigma_{r,i}^{SEM}$  are always within the range sampled by  $\sigma_{r,i}^{Bias}$ , indicating that the two sources of error comparably contribute to the restraint weight and suggesting that the number of replicas (which concurs in determining the magnitude of  $\sigma_{r,i}^{SEM}$ ) is sufficient.



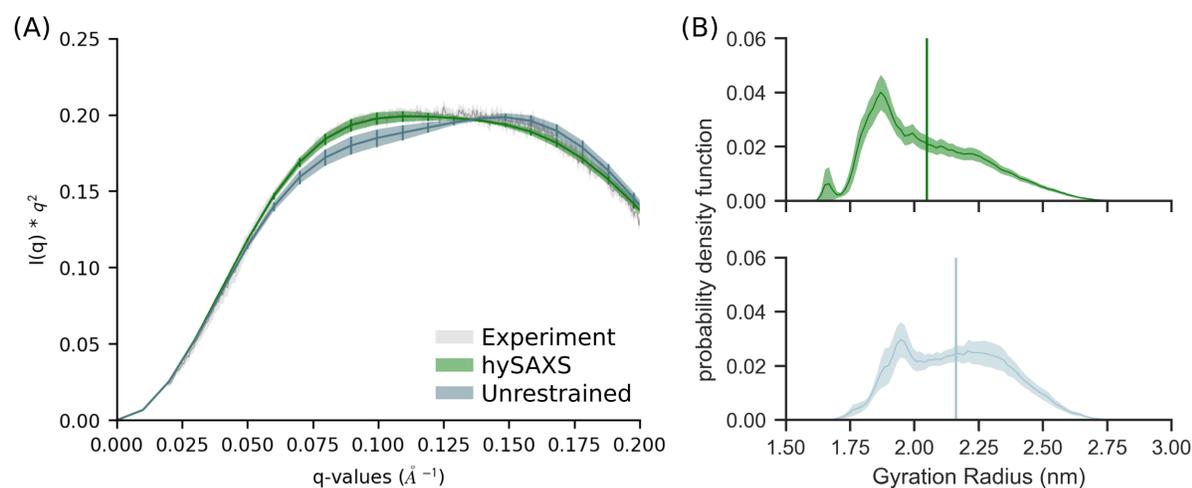
**Figure 2.** (A) Correlation, as a function of the simulation time, between experimental and back-calculated SAXS intensities, averaged over the replicas. The intensities considered are the ones used as restraints in the hySAXS simulation. (B) Probability density functions of the uncertainty parameters  $\sigma^{Bias}$  and  $\sigma^{SEM}$  (expressed in a.u.) for the 11 scattering angles considered.

### 3.3 Comparison of the resulting conformational ensembles.

The agreement with experimental SAXS data was eventually evaluated considering the whole conformational ensembles sampled within the unrestrained or hySAXS simulations. To this aim, we needed to estimate a scaling factor  $\lambda$  that relates experimental and calculated data. This value could in principle be determined by comparing the intensities at  $q = 0$  scattering angle, but since  $I(0)$  cannot be measured in SAXS experiments, we chose the  $\lambda$  that minimizes the  $\chi^2$  (computed over 19  $q$ -values in the range 0.02-0.20  $\text{\AA}^{-1}$ ) between hySAXS and experimental intensities. We observed that hySAXS provides a better match with experimental data (as confirmed by the  $\chi^2$ , 0.44 and 3.6 for the hySAXS and unrestrained simulations respectively), while the unrestrained ensemble strongly deviates from the experimental profile, showing a shape that is indicative of an over-sampling of extended conformation (**Figure 3A**). Importantly, our conclusions are independent upon the choice of the scaling factor, indeed hySAXS simulation provides a better agreement with experiments also when choosing a  $\lambda$  that minimizes the  $\chi^2$  of unrestrained intensities (**Figure S4**).

Accordingly, we noticed a remarkable effect of SAXS restraints on the inter-domain dynamics, as shown by the comparison of the probability density function of the gyration radius and the minimum inter-domains distance (**Figure 3B and S5**). Both the ensembles populate a wide range of gyration radius values (spanning from 1.5 to 3.0 nm), in agreement with the observation that K63-Ub<sub>2</sub> exists in a dynamic ensemble comprising both extended and compact states. Nevertheless, the hySAXS ensemble prefers more compact conformations, resulting in an average gyration radius of  $2.05 \pm 0.03$  nm, in contrast with the one of  $2.16 \pm 0.05$  nm obtained for the unrestrained ensemble. Altogether, our results support the idea that the unrestrained ensemble

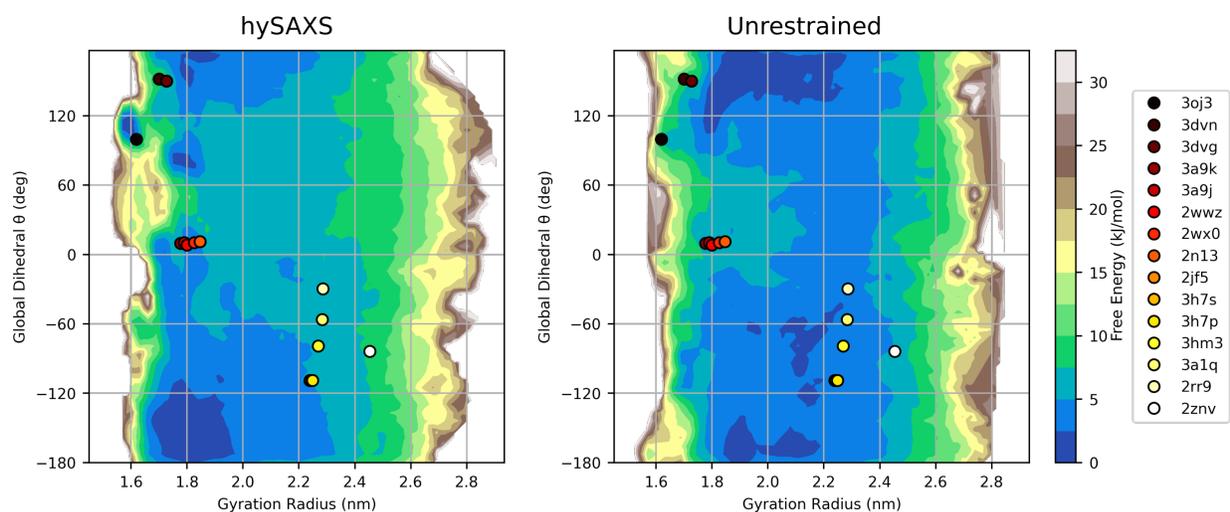
over-estimates the population of extended conformations while the hySAXS ensemble better reproduces the correct balance between compact and open states. This propensity towards extended states for the unrestrained ensemble could be explained by the use of the amber03ws, a force-field that was specifically designed to prevent the over-stabilization of compact states for IDPs but that has some known limitations with well-folded biomolecules<sup>62</sup>. Importantly, here we showed that SAXS restraints could be effectively used to contain this trend.



**Figure 3.** (A) Kratky plot comparing the experimental curve with the ones calculated (via atomistic approach) from the hySAXS and the unrestrained conformational ensembles. (B) Distribution of the gyration radius in the hySAXS (green) and in the unrestrained (light blue) conformational ensembles. The vertical bars indicate the average back-calculated gyration radius, the shade indicates the standard error, computed via block-average analysis.

Lastly, in **Figure 4**, we reconstructed 2d-free energy landscapes in a space defined by the  $C\alpha$ -gyration radius and a global dihedral angle  $\theta$  (used also as metadynamics CVs, see **Figure S6**), that describes the relative orientation of the two ubiquitin domains. Interestingly, the coordinates in this space of the available K63-Ub<sub>2</sub> PDB structures mostly fall in regions characterized by low

free-energy according to both hySAXS and unrestrained simulations, indirectly supporting the reliability of the employed force field. The inspection of the 2d-fes revealed that in the two ensembles the Ub domains can reorient freely when extended but prefer different Ub-Ub orientation in compact conformations. The contact map analysis (**Figure S7**) confirms the absence of highly stable inter-domains contacts, supporting the idea that numerous different interfaces are accessible, and shows that in the two simulations diverse groups of residues are preferred for inter-domain interactions, where the major differences concern the residues of distal ubiquitin (the majority of contacts are engaged by distal residues 42-49 according to hySAXS and by residues 6-11 according to the unrestrained simulation). Based on this observation, we hypothesized that SAXS restraints could help in sampling more reliable protein-protein interfaces. To test this hypothesis, we proceeded by validating our conformational ensemble against PRE data.



**Figure 4.** 2d-free energy surface for K63-Ub<sub>2</sub>, derived by the hySAXS (left panel) and the unrestrained (right panel) ensembles, as a function of the C $\alpha$ -gyration radius and the global dihedral angle  $\theta$  (see Supplementary **Text S1** and **Figure S6**). The coordinates of the available PDB structures in this space are plotted with points (colored from red to white, from more compact

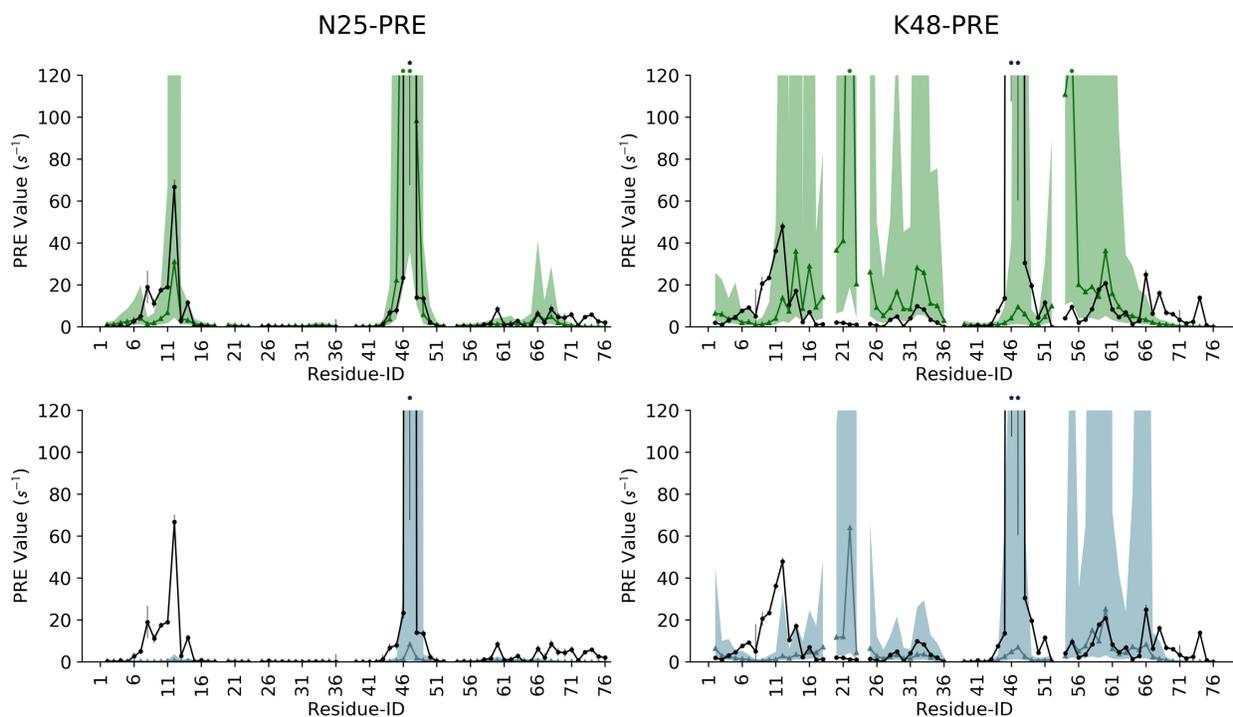
to extended conformations). To make the  $C\alpha$ -gyration radius comparable with the one back-calculated from the PDB structures, only residues 1-72 of the two ubiquitin domains were considered.

### 3.4 Validation and analysis of the Ub-Ub interfaces.

PRE experiments from NMR are particularly suited to provide information about inter subunit distances in multi-domain proteins. In these experiments, after conjugation of a specific residue with a paramagnetic probe, PRE can be measured for the other domain, where PRE values are proportional to the inverse sixth power of the distance between the paramagnetic center and the nuclei. Due to this functional form, PRE data are extremely sensitive to closed states even if sparsely populated<sup>63</sup>. Therefore, a comparison of the conformational ensemble against PRE is particularly indicated to validate the Ub-Ub interfaces of the compact states and their relative population.

Liu and co-workers previously acquired inter-subunit PRE data for K63-Ub<sub>2</sub>, conjugating the paramagnetic probe on residues N25 or K48 of the distal ubiquitin, after N25C/K48C mutations, and detecting many large PRE for some residues of the proximal unit<sup>27,64</sup>. We back-calculated the same PRE values from our hySAXS and unrestrained conformational ensembles, approximating the paramagnetic center–nuclei distances with the distances between the  $C\beta$  atom of N25 or K48 and all the amide hydrogens of the proximal ubiquitin. To account for this approximation, we evaluated an error of  $\pm 3\text{\AA}$  on the estimation of these distances, which finally gave us an estimation of the minimum/maximum PRE values. We observed that experimental N25-PRE is in good agreement with the ones calculated from the hySAXS ensemble (**Figure 5, upper-left panel**), suggesting that the compact interfaces are correctly sampled in our hySAXS run. Conversely, the

unrestrained ensemble fails to reproduce N25-PRE for the proximal unit residues 8-14 (**Figure 5, lower-left panel**). Both the hySAXS and the unrestrained ensembles correctly identify the regions where high K48-PRE are detected (**Figure 5, right panels**), nevertheless in both cases we observe a significant over-estimation of the PRE involving the residues 20-23 of the proximal unit.



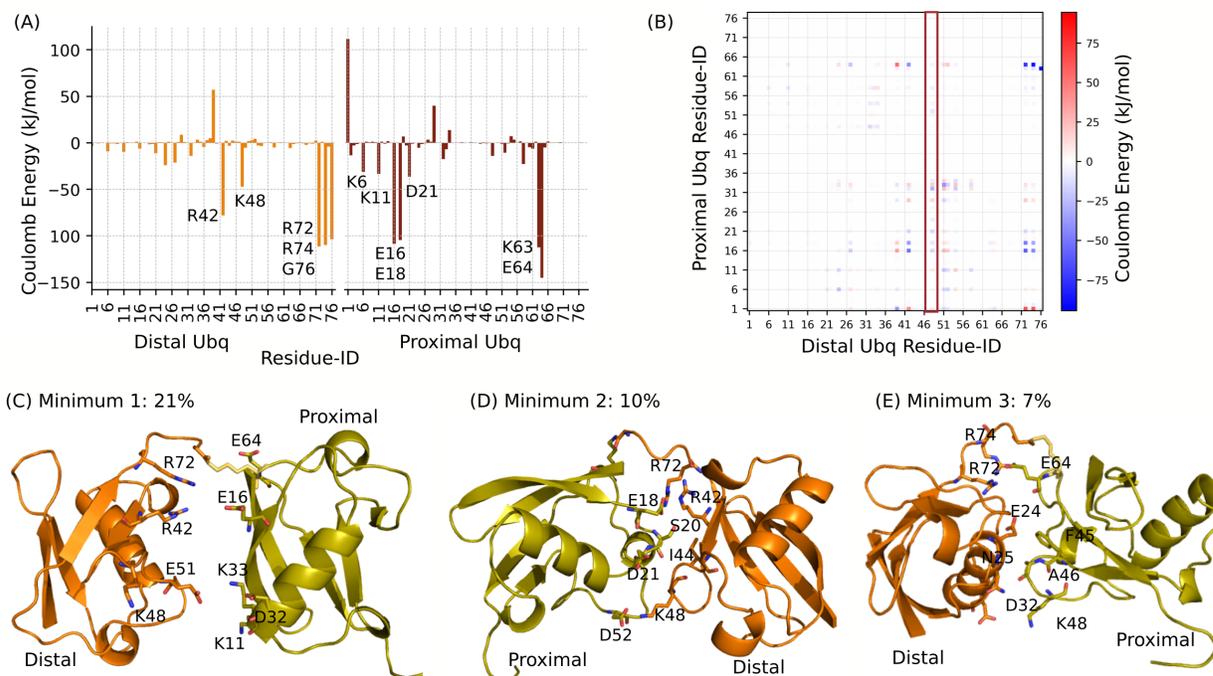
**Figure 5.** Comparison of experimental (black line) and back-calculated inter-subunit PRE for the residues of K63-Ub<sub>2</sub> proximal ubiquitin, with the paramagnetic probe conjugated at N25C (left panels) or K48C (right panels) of the distal ubiquitin. The area between the minimum/maximum back-calculated PRE values, considering a  $\pm 3\text{\AA}$  error on the estimation of probe-nuclei distances, is colored with green or light-blue shades, for hySAXS and unrestrained ensembles, respectively. The respective back-calculated PRE, without distance correction, is shown with green and light blue lines. PRE values higher than 120  $s^{-1}$  are indicated with a star on the top of the graph.

As the comparison with both N25 and K48-PRE supports the reliability of our hySAXS ensemble in sampling correct Ub-Ub interfaces, we hypothesized that the observed deviations could arise as a consequence of the introduction of the paramagnetic probe at the K48 site in PRE experiments, along with the K48C mutation. Indeed, while N25C mutation is more conservative, the replacement of a charged amino acid (K48C) could destabilize relevant inter-domains contacts. To support this hypothesis, we analyzed the energetic contributions of each residue to the interface formation. We found that, according to the hySAXS ensemble, K48 of proximal ubiquitin is important in stabilizing electrostatic interactions at the interface and that a part of these contacts are indeed engaged with the negatively charged D21 residue of distal ubiquitin, belonging to the region where the major deviations were observed (**Fig 6A,B**). Importantly, we verified that this is not the case for N25, where neither Coulomb nor Lennard-Jones interactions seem to play a major role in stabilizing the Ub-Ub interfaces (**Figure 6A and S8**).

In order to have a deeper insight into the sampled Ub-Ub interfaces, we analyzed the conformational minima identified by our hySAXS run. The pool of compact conformations (defined as the ones with  $C\alpha$ -gyration radius lower than 2.0 nm and accounting for the 57% of the conformational space) were clustered based on backbone RMSD with a cutoff of 6 Å. This procedure identified three main clusters, with populations of 21%, 10%, and 7%, respectively. As expected, these three conformational minima contain quite heterogeneous conformational states (**Figure S9**), supporting the idea that K63-Ub2 can transiently populate many different possible interfaces. Nevertheless, the inspection of both their structures and of the corresponding energy matrices (**Figure S9**) allowed us to characterize more in detail the interfaces and the contacts driving the inter-domain recognition (**Figure 6C-E**). We observed that in all the three minima the positive residues R42, R72 and/or R74 of distal ubiquitin engage electrostatic interactions with

negatively charged residues of the proximal domain (mainly E16-E64, E18 and E64 for minima 1, 2 and 3, respectively). In addition to these interactions further contacts characterize the different minima, again involving mainly charged residues (**Figure 6C-E**). While in the most populated minimum 1 hydrophobic interactions are almost absent, these are present in the other minima: in minimum 2 contacts between distal I44 and proximal S20 are observed, while in minimum 3 the interface is stabilized also by contacts between the aliphatic side-chains of distal residues E24-N25 and the proximal F45-A46.

Overall, our analysis revealed the involvement of many charged residues in the Ub-Ub interface and suggests that K63-Ub<sub>2</sub> prefer electrostatic interfacial contacts, being hindered by steric constraints to interact via the common I44/I36 hydrophobic patches, in line with previous reports<sup>65</sup>. Our results are in agreement with previous mutagenesis experiments concerning the E64 residue of the proximal unit, which plays a major role in both minima 1 and 3 interfaces. Indeed, it was reported that E64 is important for the stabilization of closed conformations, where an E64R mutation was shown to decrease the binding affinity toward ligands, known to bind the K63-Ub<sub>2</sub> closed states, via an entropically-driven mechanism. Herein, our results support the conformational selection mechanism proposed by Liu and coworkers<sup>27</sup> for K63-Ub<sub>2</sub> ligand recognition.



**Figure 6.** (A) Per-residue Coulomb energy obtained summing over the residue-residue energetic contributions for pairs of residues belonging to the two different Ub domains. Residues of distal and proximal ubiquitin are colored in orange and red, respectively; the lowest energy peaks are labeled. (B) Coulomb energy matrix reporting on the electrostatic interactions between the two domains. The column corresponding to the interactions engaged by distal K48 is highlighted in red. (C-E) Representative conformations extracted from the main minima of the compact state. Their population is reported and relevant residues for the interface are highlighted in sticks.

#### 4. CONCLUSIONS

In this work, we presented a hybrid-resolution MD-based strategy, useful to determine conformational ensembles providing an accurate interpretation of SAXS data. The proposed approach makes the inclusion of SAXS in MD simulations feasible in terms of computational efficiency without losing atomistic details and allows us to deal with highly flexible systems, aiding in the estimation of the population of the different existing conformational states.

To prove the efficacy of the method, it has been here applied to study the conformational ensemble of the multidomain protein K63-Ub<sub>2</sub>. Our results reveal that the inclusion of SAXS restraints can significantly influence the relative positioning of the different sub-units and the degree of protein extension improving the reliability of the conformational sampling, as supported by indirect validations and by quantitative comparison with independent experimental data.

## ASSOCIATED CONTENT

The following files are available free of charge.

**Supporting Information** (PDF). Supplementary Text 1: description of metadynamics Collective Variables. Figures: 1. Simulation convergence; 2. RMSD pdf of the single ubiquitin domains; 3. Statistical parameters to monitor hySAXS simulation; 4. Kratky plot; 5. Inter-domain minimum distance probability distribution; 6. Graphical representation of the global dihedral angle  $\theta$ ; 7. Contact map of the hySAXS and unrestrained ensemble; 8. Per-residue Lennard-Jones interface contribution; 9. Energy matrices and conformational ensembles for the hySAXS compact minima.

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## ABBREVIATIONS

K63-Ub<sub>2</sub>, K63-linked diubiquitin; MD, Molecular Dynamics; M&M, metadynamics metainference; PBMetaD, parallel bias metadynamics; CV, collective variable; SAXS, small angle X-ray scattering; PRE, paramagnetic relaxation enhancement; NMR, nuclear magnetic resonance; FRET, Förster resonance energy transfer; TICA, Time-lagged Independent Component Analysis.

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