**Fatty acid and retinol-binding protein: Unusual protein conformational and cavity changes dictated by ligand fluctuations.**

G. P. Barletta, B. Corsico, and S. Fernandez-Alberti\*

Universidad Nacional de Quilmes/CONICET, Roque Saenz Peña 352, B1876BXD Bernal, Argentina

ABSTRACT

Lipid-binding proteins(LBPs) are cytosolic proteins responsible of the uptake, transport and storage of large variety of hydrophobic lipophilic molecules including fatty acids, steroids, and other lipids in the cellular environment. Among the LBPs, fatty acid binding proteins (FABPs) present preferential binding affinities for long-chain fatty acids. While most of FABPs in vertebrates and invertebrates present similar β-barrel structures with ligands accommodated in their central cavity, parasitic nematode worms exhibit additional unusual α-helix rich fatty acid- and retinol-binding proteins (FAR). Herein, we report the comparison of extended molecular dynamics simulations performed on the ligand-free and palmitic acid-bond states of the *Necator americanus* FAR-1 (Na-FAR-1) respect to other classical β-barrel FABPs. The α-helix fold encompasses a complex internal ligand-binding cavity with a remarkable conformational plasticity that allows reversible switching between distinct states. The size of the cavity is significantly affected by protein conformational changes. Besides, the ligand inside the cavity is not fixed but experience large conformational changes according to the cavity size dictated by the transient protein conformation. On the contrary, ligand binding on β-barrel FABPs shift the conformational equilibrium to a unique conformation placed in a well deep enough for the protein to be trapped fluctuating around it. The significantly more flexible Na-FAR-1 ligand-cavity can explain its larger ligand multiplicity respect to β-barrel FABPs.

## I. INTRODUCTION

Hydrophobic lipophilic molecules like fatty acids, steroids, retinoids and their derivatives participate in a large variety of functions within a cell, including energy storage, signaling and hormonal roles, and membrane permeability regulation among others. Their insolubility in water and their potential oxidative degradation require their coordinated transport and availability, protection and regulation throughout the hydrophilic intracellular environment. Lipid-binding proteins(LBPs) are a group of abundant cytosolic proteins that are responsible for these tasks throughout the aqueous environment inside numerous types of cells1.

Among the LBPs, fatty acid binding proteins (FABPs) present preferential binding affinities for long-chain fatty acids2,3,4,5,6. Despite their low sequence homology and their functional divergence relating to their particular lipid-binding preferences, they share a common tertiary structure7,8. They all have similar β-barrel structures that encase the bound fatty acid. The volume of the inner ligand-binding cavity is determined by the sidechains of the residues that define the molecular surface enclosing it. These residues vary between the different FABP types determining also the ligand specificity of the cavity. Various single point mutations performed on residues lining the cavity of different FABP types have been shown to modify their ligand specificity and affinity, and protein conformational stability9,10,11,12,13.

While FABPs can be found in vertebrates and invertebrates, unusual and unique classes of LBPs, the fatty acid- and retinoid-binding proteins (FARs) have been found exclusively in nematodes14. Parasitic worms possess limited lipid metabolism and depend on import of essential lipids from their host 15. FARs are secreted from the parasite into the host tissues and they have been proposed to play a role in the nutrient acquisition by the parasite and in reproduction in the male. Therefore, they are suitable targets for anthelmintic drugs and vaccine development16. For this purpose, a better knowledge of their structure-dynamics-function relationships is required.

Differently from the β-rich FABPs, FARs are highly helical proteins. While nematodes also produce β-barrel FABPs, the reasons why nematodes have specialized in the use of α-helix rich proteins remains unclear. At the present time, two orthologues FAR structures were solved, one from Necator americanus (Na-FAR-1 by protein nuclear magnetic resonance (NMR) and x-ray crystallography; PDB: 4UET and 4XCP, respectively)17, and another from Caenorhabditis elegans (Ce-FAR-7, by x-ray crystallography; PDB: 2W9Y)18. Both present similar overall α helix-rich structures with certain structural differences. Particularly, the size and shape of their internal cavities are different, denoting differences in their ligand selectivity. Na-FAR-1, in either its *apo*- and *holo*-conformations, presents a larger and more complex internal ligand-binding cavity.

An understanding of how the conformational diversity of FARs contributes to their ligand multiplicity, varying the relative affinities for different hydrophobic lipophilic molecules, could enlighten their roles in parasitism and suggest possible targets for therapeutic interventions. Fluorescence-based ligand-binding assays and titration of Na-FAR-1 with sodium oleate followed using NMR reveal its high ligand multiplicity17, suggesting the more propensity of the α-helical fold to bind a larger variety and quantity of not only to FAs but also a broader range of lipid classes, such as phospholipids molecules, than the β-barrel fold. Besides, Na-FAR-1 ligand-binding induces substantial chemical shift changes for residues throughout the protein, indicating significant conformational changes that allow the structure to expand.

Herein we explore the structure-dynamics-function relationship of Na-FAR-1 using long molecular dynamics simulations in its *apo*- and *holo*-forms. We analyze its plasticity and the impact of the different conformations on the ligand-binding cavity volume. We were focused on the dynamics relationships between protein fluctuations, cavity changes and the enclosed ligand different conformations. A comparison of our results with those obtained from molecular dynamics simulations of the rat intestinal fatty-acid-binding protein (I-FABP) with the typical FABP β-barrel fold, and the orthologue Ce-FAR-7 is performed. Our analysis reveals that Na-FAR-1 encompasses a complex internal ligand-binding cavity with a remarkable conformational plasticity that allows reversible switching between distinct states according with the enclosed ligand different conformations.

**II. METHODS**

1. *Molecular Dynamics simulations*

Molecular dynamics simulations were performed for Na-FAR-1 and I-FABP, both in their *apo*- and *holo*- forms with palmitate in their binding pockets, and Ce-FAR-7 in its unligated form. These were carried out with AMBER 16 software package,19,20. Initial structures for each protein were obtained from the protein data bank21 (pdb id 4UET and 4XCP for *apo*- and *holo*-Na-FAR-117 respectively, 1IFB22 for *apo*-I-FABP, 2IFB23 and 1URE24 for *holo*-I-FABPs and 2W9Y18 for *apo*- Ce-FAR-7). Each protein was solvated with explicit water molecules in a rectangular periodic box large enough to contain the protein and 10 Å of solvent on all sides. Ions are added for charge neutralization. Periodic boundary conditions and particle-mesh Ewald(PME) sums were applied. The AMBER ff14SB25,26 force field and the TIP3P27 water model were used in all simulations. An initial minimization of the systems, consisting of 200-steps of steepest-descent and 800-steps of conjugate gradient, was followed by 400 ps of heating to reach the final temperature of 300K. During heating a harmonic constraint of 25.0 (kcal/mol)/Å2 was applied to the protein atoms. The time step was 2fs, and the SHAKE algorithm was employed to constrain bonds involving hydrogen atoms. A cutoff of 10 Å was applied to nonbonded interactions. Systems were equilibrated for XX ps at constant pressure reducing the constraints by XX (kcal/mol)/Å2 every 100 ps until all restraints were lifted. After that, the systems were equilibrated at the constant temperature of 300K using Andersen barostat and thermostat with a a γ collision frequency of 2 ps−1 during 12.2 ns. Finally, 3-μs production MD runs were performed, during which configurations were collected at 10-ps intervals.

1. *Principal Component Analysis*

Principal Component Analysis (PCA) is an extensively used statistical procedure to identify the essential dynamics from MD simulations28,29,30,31,32,33 and, thereby, facilitate the study of long time dynamics. For the sake of consistency, we briefly review PCA below.

Herein, PCA modes **Q***i* are 3*N* orthogonal eigenvectors obtained as columns of the eigenvector matrix **L**, that results after diagonalizing ()the covariance matrix of atomic fluctuations **C** whose elements are defined as:

(1)

where the sum goes over the *K* configurations stored during previously equilibrated MD simulations, is the mass-weighted internal displacement of Cartesian coordinate of the *ith* atom (*i*=1,…,*N*; *N*=number of residues in the protein(Cα) with mass *mi*, and the angular brackets represent the average obtained from the *K* configurations33. The elements of the diagonal matrix **Λ** represent the relative contribution of each PCA or essential mode (EM) to the overall fluctuation of the molecule. The eigenvectors are typically ordered according to descending eigenvalues, with the first PCA mode being the one with major contribution.

1. *Ligand-cavities: definition, volume and flexibility*

Ligand-cavities have been defined by visual inspection of the average of equilibrated MD structures and previous knowledge on each system. The complete list of residues lining the main ligand-binding cavity for each system is provided in Supplementary Materials (**Table S1**).

Cavity volumes are calculated using our previously developed method34, particularly suited to measure changes in cavity volumes due to small atomic coordinate displacements in the direction of specific predefined directions of protein structural displacements. Following our previous works we make use of the Volume Gradient Vector (**V**ol), defined as the vector of partial derivatives of the cavity volume in the basis of PCA modes , that is,

(2)

Within the frame of the quasi-harmonic analysis approximation35, the variation of the potential energy of a protein in the direction of **V**o is defined as

(3)

with

(4)

being , *kB* the Boltzmann constant and T the absolute temperature (300K). *X* represents a relative displacement in the direction of **V**ol. Therefore, we consider as measure of flexibility of the cavities.

## III. RESULTS AND DISCUSSION

1. *α-helix rich FARs*

While most of FABPs presents a β-barrel folding, FARs reveals an unusual α-helical fold. In the case of Na-FAR-1, it consists of a wedge-shaped structure composed of 11 helices with different lengths that enclose an internal ligand-binding cavity. The overall ligand-binding conformational change involve a global RMSD of 1.14 Å between conformers, calculated from the all-atom (or alpha carbon? Yo pondria alpha carbon en todos) superposition of averaged *apo-* and *holo-*structures obtained from the corresponding equilibrated MD simulations(see **Figure 1(a)** and **(b)**). The main structural distortions upon ligand binding are localized on helices αX, αXX… αX-αX loop. Among these SSE, αX, αXX… αX-αX loop have shown the largest root mean square fluctuations (RMSF) (see **Figure S1**) during our MD simulations, particularly residues X, XX, XXX present the largest relative flexibility. Which of them lines the surface of the internal cavity? We need a conexion between SSE(or residues) with the largest RMSF and those that line the surface of the cavity.

A further inspection of the internal ligand-binding cavity can be seen in **Figures 1(c)** and **(d)** where the distributions of cavity volumes, calculated over the set of structures collected during the equilibrated MD simulations of *apo-* and *holo-*Na-FAR-1, are shown. Their average values are 1353 ±254 Å3 and 1397±266 Å3 respectively. These values differ from the corresponding 940 Å3 and 2170 Å3 calculated on the initial experimental structures17. As we have pointed out previously, we define internal cavities according to average structures obtained from our MD simulations. The distributions shown in **Figures 1(c)** and **(d)** are the result of the protein thermal fluctuations that can involve different conformational changes throughout the 3-μs of MD simulations. Fluctuations of helices that form the cavity introduce relatively small protein structural rearrangements that can lead to significant changes on the internal cavity size36. Histograms shown in **Figures 1(c)** and **(d)** reveal that internal cavity can duplicate its volume due to protein fluctuations. While the distribution of volume cavities for *apo-*Na-FAR-1 corresponds to a Gaussian distribution that can be associated to thermal fluctuations around a unique protein conformation, this is not the case for *holo-*Na-FAR-1.

Volume cavity changes can be associated to protein fluctuations. Therefore, in order to elucidate this feature, MD simulations were analyzed in terms of PCA. The 1st and 2nd PCA modes of *apo-* and *holo-*Na-FAR-1are shown in **Figure 2(a)** and **(b)**.Descripcion del movimiento que involucra c/u y su relacion con los SSE que forman las paredes de la cavidad. **Figure 3(a)** and **(b)** shows the projection of the set of MD snapshots of *apo-* and *holo-*Na-FAR-1 onto their corresponding 1st and 2nd PCA modes. Thermal fluctuations of *apo-*Na-FAR-1 are revealed as gradual combinations of both modes without showing significant prevalence of structural distortions in any specific direction. That is, *apo-*Na-FAR-1 does not visit any new conformation that persist a significant amount of time during the MD simulation. On the contrary, we can observe that *holo-*Na-FAR-1 actually evidence the existence of three different conformers: two stable conformers presenting structural distortions mainly in both senses of the direction of the 1st PCA mode (conformers A and B (ponerles A y B en las indicaciones de las figuras), and a third conformer C in the direction of the 2nd PCA mode. Descripcion de los 3 conformers y sus diferencias, RMSD entre ellos y cuales son los SSE en que mas se diferencian y su relacion con la cavidad. Therefore, the distribution of internal cavity volumes shown in **Figure 1(d)** can be interpreted as the contribution of three different conformations explored by *holo-*Na-FAR-1 during the MD simulation. **Figure 4(a)** shows the distribution of cavity volumes for each of them (poner conformer A, B y C). While two of the *holo-*Na-FAR-1conformers (A y B?) enclose relative small internal cavities with average volumes of 1130±126Å3 and 1211±150Å3, the other conformer (C?) presents a large cavity of 1568±222Å3. These results indicate that *holo-*Na-FAR-1 presents a remarkable conformational plasticity that drives a complex internal cavity dynamics. The three identified conformers are in dynamical equilibrium connected by conformational changes involving the two lowest PCA modes. **Figure 4(b)** shows the evolution in time of the cavity volume displaying the different contributions of each of the three conformers. Reversible interconversions between them can be observed during the MD simulation.

In order to further understand the effect of higher conformational plasticity of *holo-*Na-FAR-1 respect to *apo-*Na-FAR-1 on the ligand binding, the dynamics of the ligand within the cavity has been explored. For this purpose, ligand structural fluctuations have been analyzed using PCA. **Figure 5(a)** shows the projection of the ligand structures, obtained throughout the MD simulation, onto its 1st and 2nd PCA modes. Two distinctive ligand conformations represented by the structural distortions in both senses of the direction of the 1st PCA mode can be observed. They correspond to the bent and stretched conformations shown in **Figure 5(b)**. As can be seen in **Figure 5(c)**, the ligand fluctuates between them, being the stretched conformation associated with large cavity volumes while the bent one is observed within smaller cavity volumes(**Figure 5(d)**). That is, far from being fixed within the cavity, the ligand experience large conformational changes associated to changes of cavity volume.

The relationship between the different *holo-*Na-FAR-1 conformers, with their corresponding associated changes in the internal cavity volume, and the different ligand conformations can be analyzed by depicting the distribution of distances between the extremes of the palmitate molecule (see **Figure 6**). We can observe that the stretched palmitate conformation is associated to the *holo-*Na-FAR-1 conformer (A?)with the largest internal cavity, while the bent conformation is mainly present on the other two conformers (B y C?). Since the three *holo-*Na-FAR-1 conformers are in dynamics equilibrium during the MD simulation (see **Figure 4(b)**), the ligand changes its conformation accordingly to the corresponding changes in the cavity sizes associated to each protein conformational change.

Finally, MD simulations have been performed on the orthologue Ce-FAR-7 in its *apo*-conformation. **Figure 7(a)** shows the average structure obtained from the corresponding equilibrated MD simulation. The RSMD between average *apo-*Na-FAR-1 and *apo*-Ce-FAR-7 is XX Å. In agreement with *apo*-Na-FAR-1, **Figure 7(b)** shows that the distribution of its internal cavity volume can be associated to protein fluctuations around a unique conformation characterized by a free energy landscape with a relatively deep well.

1. *β-barrel FABPs*

While FARs exhibit α-helix rich folds, most FABPs presents a typical FABP β-barrel fold. In order to understand how the different folds impact on the protein properties associated to the transport of a variety of ligands with different shapes and sizes, MD simulations have been performed on the rat intestinal fatty-acid-binding protein (I-FABP) in its *holo* and *apo* forms. The ligand-binding conformational change involves a structural distortion with a RMSD=1.00 Å. The average internal ligand-cavity is significant smaller than Na-FAR-1, being 605±145 Å3 and 926±85 Å3 for *apo*-I-FABP and *holo* I-FABP respectively (see **Figure 8**). We can observe that the distribution of cavity volumes for *apo*-I-FABP can be associated to the contribution of different conformations explored during the MD simulation. On the contrary, ligand-binding funnels *holo*-I-FABP onto a unique rigid state. These results are in good agreement with previous NMR measurements performed on human L-FABP37 and rat I-FABP38 that describe ligand binding as a transition of the protein structure from a slightly more disordered and flexible *apo*-state to a more ordered *holo*-state. This increased mobility and discrete disorder in the *apo*-state may facilitate the entry of the ligand into the cavity.

PCA allows the identification of the different *apo-*I-FABP conformers and their corresponding effect on the volume of the internal cavity (see **Figures 9(a)** and **(b)**). Four different conformers, associated to different combinations of structural distortions in the directions of the 1st and 2nd PCA modes, have been identified. Two of them (A and B?) are associated to smaller cavity volumes than the other two(C and D?). **Figures 9(c)** shows that *apo*-I-FABP experiences multiple conformational changes throughout the MD simulation, indicating a relatively low energy barrier between its states.

Ligand binding seems to shift the conformational equilibrium of I-FABP to a unique conformation with a sufficiently deep well to ensure that a significant fraction of protein molecules are trapped fluctuating in it. This feature is also confirmed by performing MD simulation on another *holo*-I-FABP obtained by NMR (see **Figure 10(a)**). The resulted distribution of cavity volumes, shown in **Figure 10(b)**, indicates a Gaussian distribution corresponding to fluctuations around a unique minimum in the protein conformational space.

1. *Relative flexibility of the ligand-cavities*

The different FARs and FABPs analysed in this study have shown ligand-cavities with different shapes whose dynamics is subject to the corresponding protein plasticity. In order to analyse which LBP fold encompass a more flexible cavity and, therefore, a cavity that can contribute to a larger ligand multiplicity, we calculate the variation of the potential energy of each LBP in the direction of **∇Vo** (see **section II.C**). Results are shown in **Figure 10(a)**. We can observe that the internal cavity of *apo-*Na-FAR-1 results the most flexible one, followed by *holo-*Na-FAR-1 and *apo*-I-FABP. *apo*-Ce-FAR-7 and the two *holo*-I-FABPs present relatively more rigid cavities. While either *holo-*Na-FAR-1 and *apo*-I-FABP encompass cavities with different sizes according to the transient protein conformation, **Figure 10(b)** and **(c)** display the analysis of the corresponding individual conformers. We can observe that, in both cases, each conformer result relatively more rigid than the average shown in **Figure 10(a)**, indicating that their individual contributions introduce an additional component to the overall flexibility of the cavity. These results indicate a propensity of Na-FAR-1 to bind not only fatty acids but also a broader range of lipid classes such as phospholipids. This feature is in agreement with previous fluorescence experiments performed on Na-FAR-1 and Ce-FAR-717.

## IV. CONCLUSIONS

Protein fluctuations−cavity changes relationships have been explored on different α-helix rich FARs and β-barrel FABPs using long equilibrated MD simulations of either *apo*- and *holo*- states. We found a significantly flexible Na-FAR-1 ligand-cavity that can explain the observed larger ligand multiplicity of α-helix FARs respect to β-barrel FABPs.

We have reported to different ligand-binding strategies Particularly, *holo*-Na-FAR-1 presents a remarkable conformational plasticity that drives a complex internal cavity dynamics involving different states. The size of the cavity is significantly affected by protein conformational changes. Besides, the ligand also changes its conformation accordingly to them. That is, far from being fixed within the cavity, the ligand experience large conformational changes between a bent and stretch conformation according to the size of the cavity dictated by the transient protein conformation. On the contrary, ligand binding on I-FABPs seems to shift the conformational equilibrium to a unique conformation with a sufficiently deep well to ensure that a significant fraction of protein molecules are trapped fluctuating in it. In this way, α-helix FARs and β-barrel FABPs seems to follow two different strategies for ligand-binding. While the formers involve a *holo*-state with high plasticity experience conformational changes that significant impact on the cavity volume and embedded ligand conformations, the latter experience an inverse ligand-modulated disorder-order transition leading to a *holo*-state with restricted motional freedom.

The flexibility of protein cavities can impact on functional aspects like ligand affinities and binding promiscuities. The present work can encourage the development of drugs that rigidize the cavity of Na-FAR-1, reducing its ligand multiplicity and, therefore, the efficiency to play its biological function like transport and storage of large variety of hydrophobic lipophilic molecules in the parasitic nematode worms.

**Figure Captions**

**Figure 1.** Averaged **(a)** *apo-* and **(b)** *holo-*structures for Na-FAR-1 obtained from the corresponding equilibrated MD simulations, indicating the main secondary structure elements (SSE) and the encompass ligand cavity. Distribution of ligand cavity volumes, calculated over the set of structures collected during the equilibrated MD simulations of **(c*)*** *apo*- and **(d)** *holo*-Na-FAR-1.

**Figure 2.** 1st (red?) 2nd (blue?) PCA modes of **(a)** *apo-* and **(b)** *holo*-Na-FAR-1.

**Figure 3.** Projection of the set of MD snapshots of **(a)** *apo-* and **(b)** *holo-*Na-FAR-1 onto their corresponding 1st and 2nd PCA modes.

**Figure 4.** **(a)** Distribution of cavity volumes for each of the conformers (A, B and C) of *holo*-Na-FAR-1 during the MD simulation; **(b)** Evolution in time of the cavity volume displaying the different contributions of each of the three A, B and C conformers.

**Figure 5.** **(a)** Projection of the palmitate structures, obtained from the set of MD snapshots of *holo-*Na-FAR-1, onto its 1st and 2nd PCA modes; **(b)** Distribution of cavity volumes according to the conformation of the ligand encompassed in it; **(c)** Evolution in time of the cavity volume displaying the different conformations adopted by the ligand.

**Figure 6.** Distribution of distances between the extremes of the palmitate molecule for each of the conformers (A, B and C) of *holo*-Na-FAR-1 during the MD simulation.

**Figure 7.** **(a)** Averaged structure of *apo*-Ce-FAR-7 obtained from the equilibrated MD simulation; **(b)** Distribution of of its internal cavity volume, calculated over the set of collected MD structures.

**Figure 8.** Averaged **(a)** *apo-* and **(b)** *holo-*structures for I-FABP obtained from the corresponding equilibrated MD simulations, indicating the main secondary structure elements (SSE) and the encompass ligand cavity. Distribution of ligand cavity volumes, calculated over the set of structures collected during the equilibrated MD simulations of **(c*)*** *apo*- and **(d)** *holo*- I-FABP.

**Figure 9.** **(a)** Projection of the set of MD snapshots of *apo-*I-FABP onto its 1st and 2nd PCA modes; **(b)** Distribution of cavity volumes for each of the *apo-*I-FABP conformers (A, B, C and D) during the MD simulation; **(c)** Evolution in time of the cavity volume displaying the different contributions of each of the four conformers A, B, C and D conformers.

**Figure 10.** **(a)** Potential energy change in the direction of **∇V**ol for each **(a)** protein structure, **(b)** *holo-*Na-FAR-1 conformer, (c) *apo*-I-FABP conformer.

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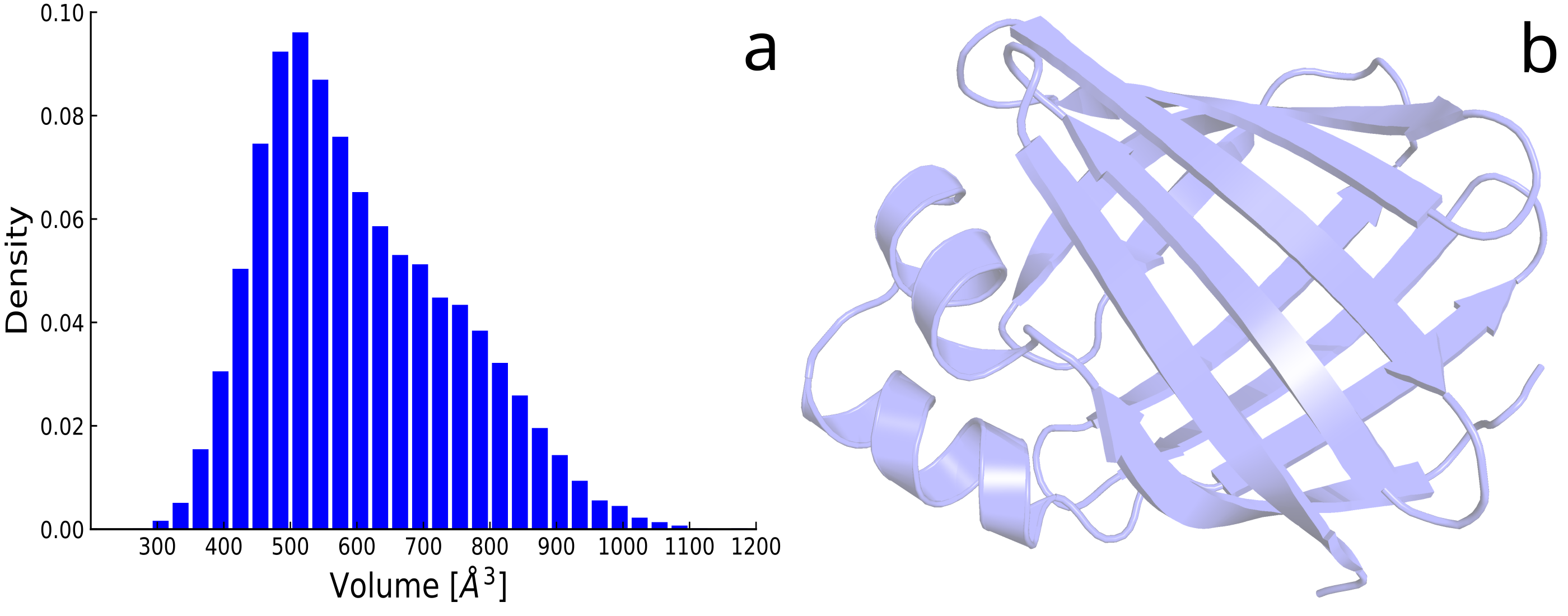
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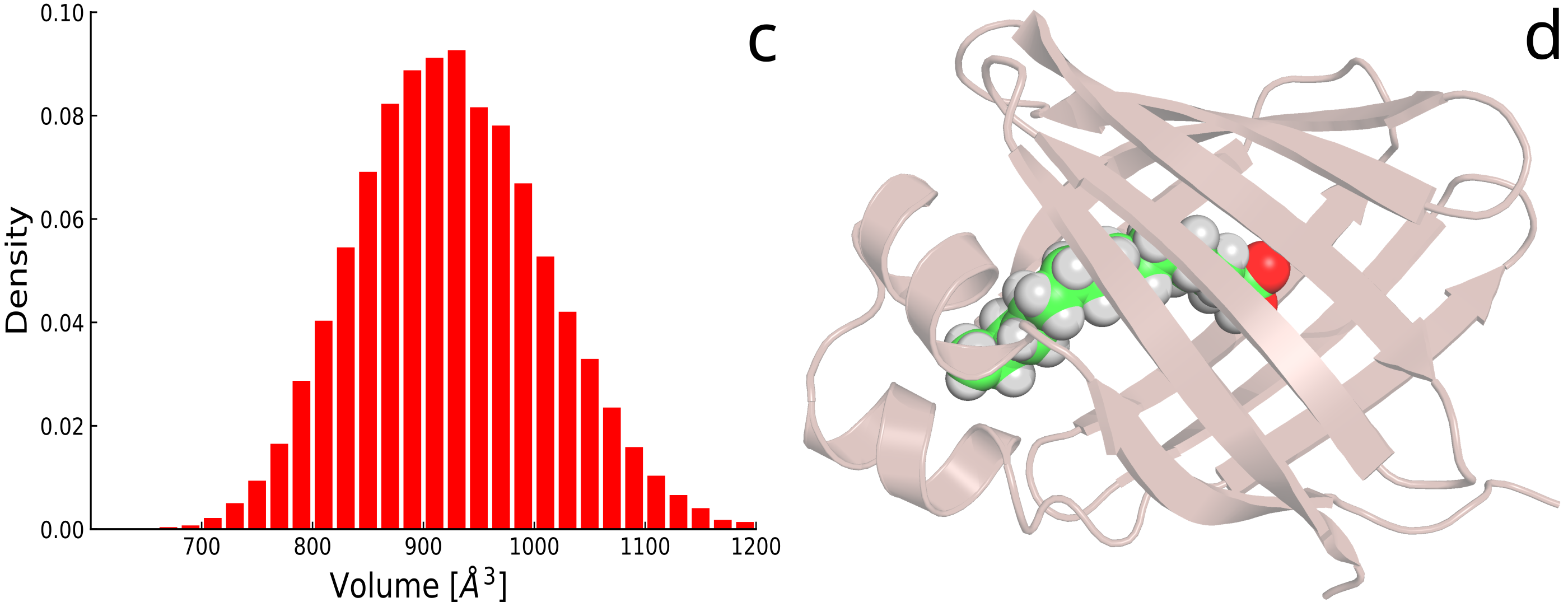
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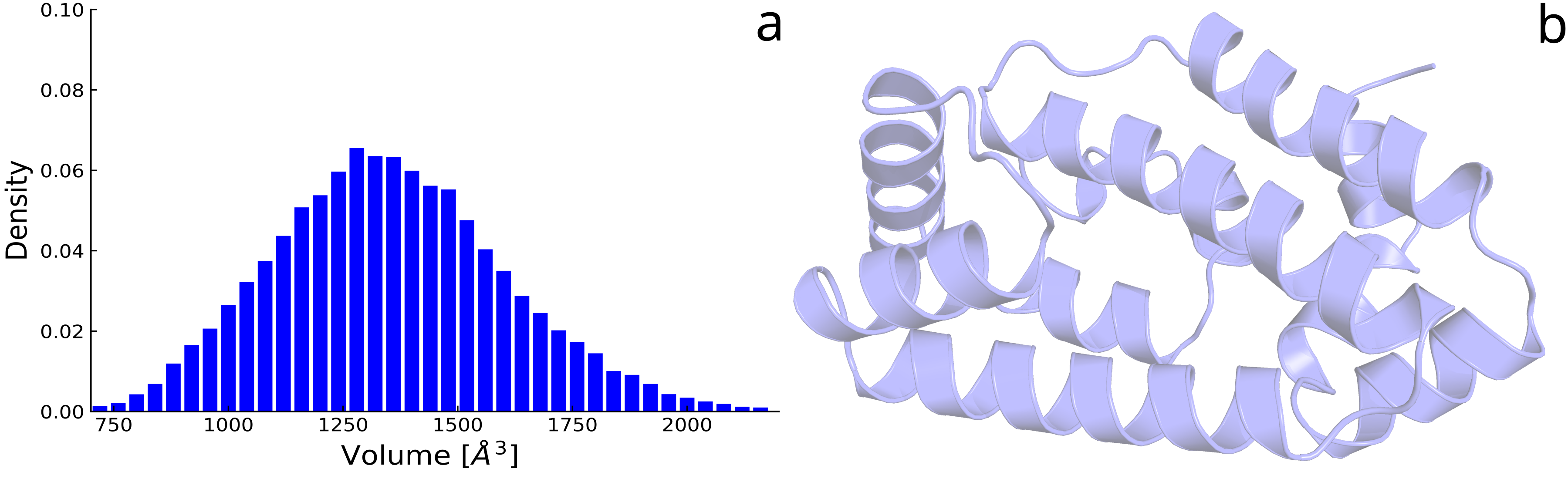
**Figures**

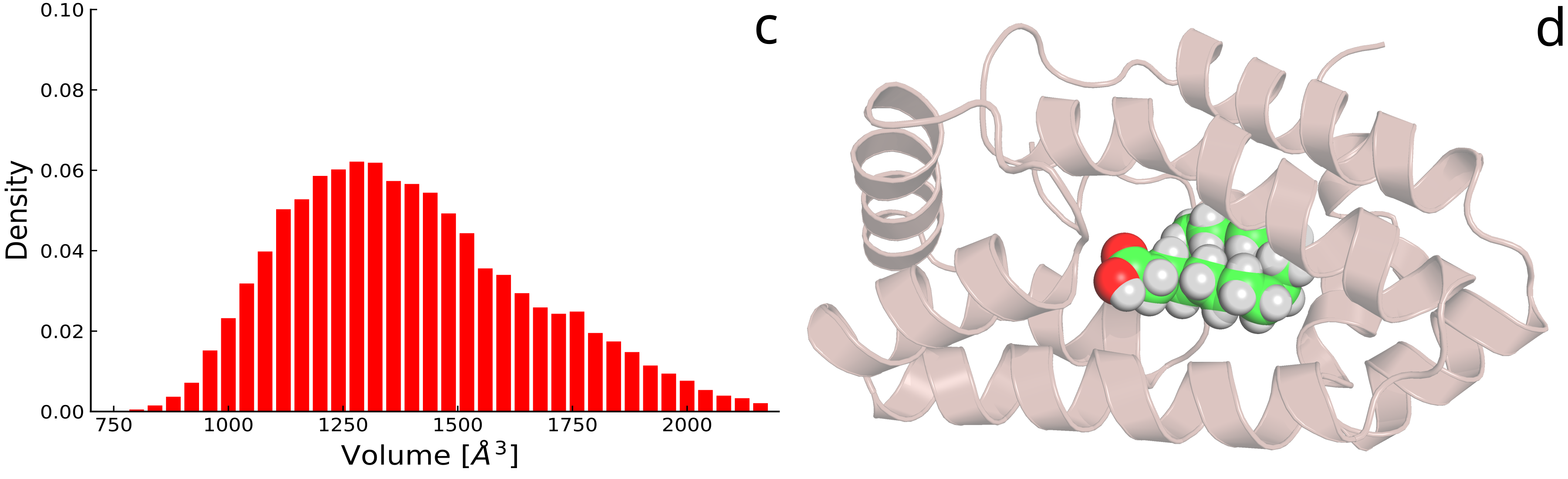
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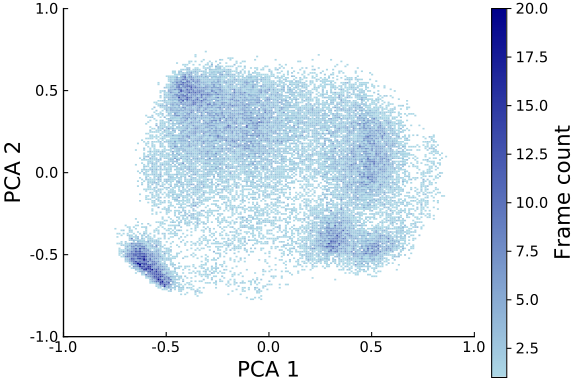
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