Mechanical Mapping of Single Membrane Proteins at Submolecular Resolution

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Supporting Information

ABSTRACT: The capacity of proteins to carry out different functions is related to their ability to undergo conformation changes, which depends on the flexibility of protein structures. In this work, we applied a novel imaging mode based on indentation force spectroscopy to map quantitatively the flexibility of individual membrane proteins in their native, folded state at unprecedented submolecular resolution. Our results enabled us to correlate protein flexibility with crystal structure and showed that α-helices are stiff structures that may contribute importantly to the mechanical stability of membrane proteins, while interhelical loops appeared more flexible, allowing conformational changes related to function.

KEYWORDS: Single molecule, flexibility, atomic force microscopy, bacteriorhodopsin, membrane proteins, elasticity

The capacity of proteins to function relies on a balance between molecular stability and structural flexibility,¹ which is attained by the secondary structure and the intramolecular interactions of proteins.²,³ The flexibility of proteins and protein substructures is difficult to be quantitatively estimated. B-factors derived from crystallographic analysis or thermal fluctuations from neutron scattering measurements represent an indirect measurement of the flexibility of protein domains, but do not provide a quantitative value for the stiffness of each protein structure.⁴ Alternatively, dynamic force spectroscopy (DFS) has been used to probe the mechanical stability of proteins by pulling from specific sites and determining the forces required for protein unfolding.⁵–⁸ Nevertheless, DFS does not measure the compliance of protein subdomains to small deformations in their native, folded state. Recent advances in atomic force microscopy (AFM) imaging have allowed quantitative investigation of the flexibility of nanometer-size structures and membrane proteins.⁹–¹¹ However, the correlation between structure and flexibility of protein domains has not been possible due to the lack of simultaneous submolecular resolution in topography and mechanical mapping. Here we applied a novel AFM imaging mode based on indentation force spectroscopy to map the flexibility of individual membrane proteins in their native environment with unprecedented submolecular resolution. Our results enabled us to correlate protein flexibility with atomic structure and showed that protruding α-helices are rigid structures contributing importantly to the mechanical stability of membrane proteins, while interhelical loops appeared more flexible, allowing conformational changes related to function.

We studied the light-driven proton pump bacteriorhodopsin (bR), a seven-helix transmembrane protein that forms two-dimensional arrays in the plasma membrane of Halobacterium salinarum, forming the so-called purple membranes.¹² Bacteriorhodopsin is structurally similar to other rhodopsins and to G-protein coupled receptors and is one of the best-studied membrane proteins.¹³ The structure and mechanical resistance upon unfolding of bR has been previously determined by combining high-resolution AFM imaging and DFS.⁷,¹⁴ These works showed that the mechanical stability of bR upon unfolding was determined by helices and loops that unfolded sequentially. Early attempts to characterize the flexibility of membrane proteins in their folded state used AFM in contact mode at different compression forces allowing a first approach to estimate the flexibility of protein domains.¹⁵–¹⁸ However, the actual applied force was difficult to control and lead only to qualitative results. We allowed purple membranes to absorb on freshly cleaved mica for 20 min and then rinsed them extensively with imaging buffer (10 mM Tris-HCl, 150 mM KCl at pH 7.4). Immobilized membranes were scanned in PeakForce mode, which consists of oscillating the sample in the vertical direction with an amplitude of tens of nanometers and at a frequency of 2 kHz (Figure 1).¹⁹ The vertical piezo movement resulted in cycles of approaching and retracting traces that lead to force—distance curves in which the tip made intermittent contact with the sample surface. Topography information was obtained from the height correction per force, "peak" of force, while the slope of the contact region determined the stiffness of the sample at each pixel. Examples of the resulting images of topography and stiffness are shown in Figure 1, panels A and B, respectively. Three force curves obtained at three different

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regions of the sample surface, mica, lipid and protein, are shown in Figure 1C. The difference in the slope of each curve provides a first signature of the elasticity of the probed surface, revealing the steepest slope on curves obtained on the stiffest surface, mica. It is noteworthy the coincidence between the approach and retraction traces, which revealed negligible dissipation upon deformation, reflecting the purely elastic response of the sample at the probing frequency. The lipid region had an average thickness of 2.8 \pm 0.2 \text{ nm}, while bR patches had a thickness of 6.4 \pm 0.2 \text{ nm}. The thickest region in the topography (\sim 16 \text{ nm}) reflected a folded patch where two or more layers of bR were present (star in Figure 1A,B). The stiffness image (Figure 1B) clearly reflected the different mechanical properties of the various regions, being mica the stiffer region (brightest color). The average (\pm standard deviation) stiffness of the lipid bilayer was 61 \pm 4 \text{ pN/nm}, while that of bR was 79 \pm 8 \text{ pN/nm}. It is remarkable that, even though the lipid bilayer was few nanometers thinner than bR, its stiffness was unambiguously detected to be lower than the protein surface (Figure 1D, lower panel). This suggests that the hard underlying substrate did not affect significantly the determination of the mechanical properties for a 2.8 \text{ nm} thick lipid bilayer film at the applied forces (\sim 250 \text{ pN}). Indeed, the contribution of a hard substrate on the determination of the elastic properties of thin layers has been shown to be <25\% for deformations \sim 20\% of the layer thickness.\textsuperscript{20} Thus, although a slight overestimation of the lipid stiffness was expected, the deformation applied on bacteriorhodopsin patches (0.5—1 \text{ nm}, 8—17\% of the total thickness) fell well below this limit suggesting only a minor if at all effect on the measured stiffness.\textsuperscript{21} The multilayered membrane region appeared even more compliant than the lipid with an average value of 31 \pm 3 \text{ pN/nm}, probably because the various layers were not firmly supported and may bend under load. Another possible source of uncertainty in the reliable determination of membrane stiffness were torsion forces experienced by the tip during scanning. We only observed this possible effect when scanning very steep height differences at elevated rates (e.g., between mica or lipid and the folded membrane in Figure 1B, scan direction from right to left), but not on moderate height changes (e.g., between lipid and bR in Figures 1 and 2). Minimization of torsion forces was possible given the short time of actual contact between the tip and the sample (\sim 50 \text{ ms}) during the oscillation cycle; torsion forces would be expected at relatively high velocities (few \text{ \mu m/s}) and important height gradients (tens of nm per nm). Moreover, high-resolution imaging in contact mode on the same sample and very similar scan range is recorded at typically 6 \text{ Hz} (\sim 2 \text{ \mu m/s}) without detectable torsion effects. Hence, under the acquisition conditions (\sim 250 \text{ nm/s}) used here, the feedback loop was not challenged. Furthermore, the high-degree of 3-fold molecular symmetry of the bR molecules as shown here was strong indirect evidence that friction forces, resulting in torsion, were negligible. Recent measurements using torsional harmonic AFM cantilevers have provided relevant information about the flexibility of bacteriorhodopsin proteins, showing differences between the cytoplasmic and extracellular surfaces\textsuperscript{11} and are in quantitative agreement with our results. However, the resolution achieved by the authors did not allow distinguishing different mechanical features within protein substructures.

Given the high-force sensitivity and the feedback control on the applied force of PeakForce imaging, we were able to acquire high-resolution images of the cytoplasmic surface of purple membranes; an example is shown in Figure 2. The characteristic bR trimer arrangement in a hexagonal lattice (a = b = 62.5 \text{ Å}; \gamma = 120\degree) was clearly resolved. Notice the sharp border delimiting the bR and lipid regions and the single molecule defects in the protein lattice due to missing bR proteins, a proof of real single molecule resolution.\textsuperscript{22} In the stiffness image (Figure 2B), the bR and lipid regions had two marked stiffness values. In agreement with the overview analysis, the bR patch appeared significantly stiffer than the lipidic region. The average stiffness of bR in Figure 2B was 74 \pm 30 \text{ pN/nm}, which is in agreement with the
Raw images of topography and stiffness (B) were used for high-resolution imaging. Remarkably, the molecular defects observed in the topography appeared as more compliant forces applied while loops were more easily deformed. It is important to note that the reported stiffness values reflect the resistance of protein subdomains to vertical forces. Nevertheless, average estimates of the flexibility of bR have been determined from neutron scattering measurements at different temperatures of the thermal fluctuations of individual bR atoms from nonsupported samples, obtaining values of 14 and 280 pN/nm for the solvent-exposed loops and the protein core, respectively. These results suggested stiff α-helices and compliant interhelical loops, as suggest our results. Moreover, the magnitude of the stiffness was in quantitative agreement with our values. Although B-factors reported from electron crystallography analysis are only an indirect measure of protein flexibility, as they report about the positional stability of a protein moiety in the 3D-crystal, they were also in relatively good agreement with our results with α-helices having lower B-factors (being stiffer) than interhelical loops (Figure 3E). However, the high B-factor values of E–F loop would suggest this as the most flexible one, in contrast to our results. Early work using high-resolution imaging allowed a qualitative mapping of the flexibility of cytoplasmic surface of bR, concluding also that E–F loop was the most flexible loop, as it showed the highest values in standard deviation (SD) maps.

This disagreement suggests caution in interpreting the significance of B-factors and AFM SD maps as quantitative indicators of protein flexibility. Indeed, being the most protruding structure, without the stabilization of interprotein or lipid mediated interactions, it is reasonable that the E–F loop would show higher fluctuations, resulting in higher B-factors from crystallography analysis and higher SD values from AFM topography averages, even if being stiffer than other structures. A similar effect may

Figure 3. Correlation of averaged images with structure. High-resolution images of topography (A) and stiffness (B). Individual trimers are encircled. 3-fold symmetrized correlation average topograph (C) and stiffness (D) calculated from 13 bacteriorhodopsin trimers from A and B and overlaid with the atomic structure. Individual loops are labeled. False color ranges were 1.5 nm in A and C, and 39–109 pN/nm in B and D. (E) Lateral view of the atomic structure colored by B-factors (top) of each cytoplasmic loop, and cross-sectional profiles (bottom) of topography (black) and stiffness (± standard error of the mean, red) along the arrows shown in D. The red dashed line shows the average stiffness of the lipidic region in D. Asterisks reflect statistical significance difference in stiffness by Student’s t-tests analysis between protruding α-helices and the center of interhelical loops.

Supporting Information). The relative stiffness of the various elements of individual bRs was analyzed in the symmetrized stiffness average, which provided higher signal-to-noise ratio (Figure 3D). For comparison, we chose the atomic structure at 3.0 Å resolution of bR in the purple membrane packing obtained from electron crystallography of 2D crystals because its analysis was performed on bR in lipid bilayers very similar to the sample we used. We overlaid the structure with the topography and the stiffness images to correlate the secondary structure of the protein with its topography and mechanical properties (Figure 3C,D). We focused on the three cytoplasmic loops and α-helices of bR by plotting the cross sections of topography and stiffness along each loop (Figure 3E). On the one hand, the topographical profiles correlated well with the atomic structure, reflecting the low degree of deformation applied during imaging. Although the topography profile of A–B loop is in agreement with its atomic structure reflecting a short α-helix A and α-helix B sticking further out of the membrane surface, it appeared to be lower than C–D loop, which is in contrast to the atomic structure. This disagreement, however, was explained by the fact that A–B loop was found to be the most compliant loop, compared with C–D and E–F loops and thus resulted in the most deformed structure at the applied imaging forces. On the other hand, the stiffness profiles showed no direct correlation with topography but a clear correlation with secondary structure, even if tip shape effects were expected at this level of resolution. The stiffest regions of the three cross sections coincided with the α-helices that protruded importantly from the lipid bilayer plane (80–100 pN/nm), while more compliant regions correlated with the interhelical loops (70–90 pN/nm). The lower stiffness of the short α-helices (A,D,E) may reflect the contribution of the fluid surrounding lipids. These results suggested that α-helices were able to resist important compression forces applied while loops were more easily deformed. It is important to note that the reported stiffness values reflect the resistance of protein subdomains to vertical forces. Nevertheless, average estimates of the flexibility of bR have been determined from neutron scattering measurements at different temperatures of the thermal fluctuations of individual bR atoms from nonsupported samples, obtaining values of 14 and 280 pN/nm for the solvent-exposed loops and the protein core, respectively. These results suggested stiff α-helices and compliant interhelical loops, as suggest our results. Moreover, the magnitude of the stiffness was in quantitative agreement with our values. Although B-factors reported from electron crystallography analysis are only an indirect measure of protein flexibility, as they report about the positional stability of a protein moiety in the 3D-crystal, they were also in relatively good agreement with our results with α-helices having lower B-factors (being stiffer) than interhelical loops (Figure 3E). However, the high B-factor values of E–F loop would suggest this as the most flexible one, in contrast to our results. Early work using high-resolution imaging allowed a qualitative mapping of the flexibility of cytoplasmic surface of bR, concluding also that E–F loop was the most flexible loop, as it showed the highest values in standard deviation (SD) maps. This disagreement suggests caution in interpreting the significance of B-factors and AFM SD maps as quantitative indicators of protein flexibility. Indeed, being the most protruding structure, without the stabilization of interprotein or lipid mediated interactions, it is reasonable that the E–F loop would show higher fluctuations, resulting in higher B-factors from crystallography analysis and higher SD values from AFM topography averages, even if being stiffer than other structures. A similar effect may
occur in helix F. The highest stiffness of E–F loop we observed, compared to A–B and C–D loops, is actually reasonable taking into account that it is the only cytoplasmic loop that might present an interaction between amino acids Lys159 and Glu161, which may stabilize the structure.2

The structural nature of α-helices and interhelical loops provides a possible explanation to our results. Forces applied on interhelical loops only find resistance from the intrinsic elasticity and the arched shape of the polypeptide chain, while forces applied to α-helices would require deformation of a similarly resistant polypeptide chain with a highly curved structure stabilized by a network of hydrogen bonds and hydrophobic interactions. This interpretation is in agreement with works showing that the mechanical stability of proteins is regulated by inter- and intramolecular interactions.27–29 The observed properties can also be interpreted from simple structural viewpoints; while loops resist the applied force carrying bending stresses, protruding α-helices may carry compression stresses, being thus more efficient in supporting applied forces, similarly as how different arch shapes in architectural constructions carry stresses. The flexibility of interhelical loops suggests a relationship with conformational changes of bR associated with function. In fact, a recent work using high-speed AFM showed that the E–F loop underwent an outward displacement of 0.69 ± 0.15 nm when bR was irradiated with 532 nm green light.30 From our results, assuming a similar stiffness of the E–F loop in the outward direction, we estimated the elastic energetic cost upon conformational change of the E–F loop to be 5.2 k_BT. This energy is ~5% of the energy of a green photon and provides additional information about the energy spent during the bR photocycle.

In this work, we applied a new imaging mode based on force spectroscopy that allowed us to map the flexibility of membrane proteins at submolecular resolution. Our data supports the hypothesis that interhelical loops of bacteriorhodopsin are compliant structures providing structural flexibility to allow functions involving conformational changes, while protruding α-helices are rigid structures providing molecular stability. Although this behavior might be specific of bacteriorhodopsin, future experiments using the proposed approach may allow it to be generalized to other membrane proteins. The applied methodology brings a new perspective to the relation between structure, function, and mechanics of membrane proteins and opens an avenue for future studies of the quantitative mechanical properties of materials at nanometer resolution.

**REFERENCES**

(1) Frauenfelder, H.; McMahon, B. Prog. Natl. Acad. Sci. U.S.A. 1998, 95 (9), 4795–4797.
(2) Gunasekaran, K.; Nussinov, R. J. Mol. Biol. 2007, 365 (1), 257–273.
(3) Kumar, S.; Ma, B.; Tsai, C.-J.; Wolfsön, H.; Nussinov, R. Cell Biochem. Biophys. 1999, 31 (2), 141–164.
(4) Zaccari, G. Science 2000, 288 (5471), 1604–1607.
(5) Rief, M.; Gautel, M.; Oesterhelt, F.; Fernandez, J. M.; Gaub, H. E. Science 1997, 276 (5315), 1109–12.
(6) Marszalek, P. E.; Lu, H.; Li, H.; Carrion-Vazquez, M.; Oberhauser, A. F.; Schulten, K.; Fernandez, J. M. Nature 1999, 402 (6757), 100–103.
(7) Oesterhelt, F.; Oesterhelt, D.; Pfeiffer, M.; Engel, A.; Gaub, H. E.; uuml; ill; D. J. Science 2000, 288 (5463), 143–146.
(8) Zhang, X.; Halvorsen, K.; Zhang, C.-Z.; Wong, W. P.; Springer, T. A. Science 2009, 324 (5932), 1330–1334.
(9) de Pablo, P. J.; Schaap, I. A. T.; MacIntosh, F. C.; Schmidt, C. F. Phys. Rev. Lett. 2003, 91, 9.
(10) Ivanovska, I. L.; de Pablo, P. J.; Ibara, B.; Sgalari, G.; MacIntosh, F. C.; Carrascosa, J. L.; Schmidt, C. F.; Wuite, G. J. L. Proc. Natl. Acad. Sci. U.S.A. 2004, 101 (20), 7600–7605.
(11) Dong, M.; Husale, S.; Sahin, O. Nat. Nanotechnol. 2009, 4 (8), 514–7.
(12) Oesterhelt, D.; Stoeckenius, W. Proc. Natl. Acad. Sci. U.S.A. 1973, 70 (10), 2853–2857.
(13) Hirai, T.; Subramaniam, S.; Lanyi, J. K. Curr. Opin. Struct. Biol. 2009, 19 (4), 433–439.
(14) Muller, D. J.; Kessler, M.; Oesterhelt, F.; Miller, C.; Oesterhelt, D.; Gaub, H. Biophys. J. 2002, 83 (6), 3578–3588.
(15) Muller, D. J.; Fotiadis, D.; Engel, A. FEBS Lett. 1998, 430 (1), 105–111.
(16) Muller, D. J.; Schabert, F. A.; Buldt, G.; Engel, A. Biophys. J. 1995, 68 (5), 1681–1686.
(17) Scheuring, S.; Ringler, P.; Borgnia, M.; Stahlberg, H.; Muller, D. J.; Agre, P.; Engel, A. EMBO J. 1999, 18 (18), 4981–4987.
(18) Scheuring, S.; Seguin, J.; Marco, S.; Legy, D.; Breton, C.; Robert, B.; Rigaud, J.-L. J. Mol. Biol. 2003, 325 (3), 569–580.
(19) Adamick, J.; Berquand, A.; Mezzenga, R. Appl. Phys. Lett. 2011, 98, 19.
(20) Dimitriadis, E. K.; Horkay, F.; Maresca, J.; Kachar, B.; Chadwick, R. S. Biophys. J. 2002, 82, 2798–2810.
(21) Domke, J.; Radmacher, M. Langmuir 1998, 14 (12), 3320–3325.
(22) Koutsos, V.; Manias, E.; Tenbrinke, G.; Hadziioannou, G. Europhys. Lett. 1994, 26 (2), 103–107.
(23) Stark, M.; Möller, C.; J. Müller, D.; Guckenberger, R. Biophys. J. 2004, 80 (6), 3009–3018.
(24) Fechner, P.; Boudier, T.; Mangenot, S.; Jaroslawski, S.; Sturgis, J. N.; Scheuring, S. Biophys. J. 2009, 96 (9), 3822–3831.
(25) Mitsuoka, K.; Hirai, T.; Murata, K.; Miyazawa, A.; Kidera, A.; Kimura, Y.; Fujiyoshi, Y. J. Mol. Biol. 1999, 286 (3), 861–882.
(26) Bicout, D. J.; Zaccari, G. Biophys. J. 2001, 80 (3), 1115–1123.
(27) Cao, Y.; Yoo, T.; Zhuang, S.; Li, H. J. Mol. Biol. 2008, 378 (5), 1132–1141.
(28) Cao, Y.; Yoo, T.; Li, H. Proc. Natl. Acad. Sci. U.S.A. 2008, 105 (32), 11152–11157.
(29) Knowles, T. P.; Fitzpatrick, A. W.; Meehan, S.; Mott, H. R.; Vendruscolo, M.; Dobson, C. M.; Welland, M. E. Science 2007, 318 (5858), 1900–1903.
(30) Shibata, M.; Yamashita, H.; Uchihashi, T.; Kandori, H.; Ando, T. Nat. Nanotechnol. 2010, 5 (3), 208–212.