Single-molecule detection of structural changes during Per-Arnt-Sim (PAS) domain activation

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The Per-Arnt-Sim (PAS) domain is a ubiquitous protein module with a common three-dimensional fold involved in a wide range of regulatory and sensory functions in all domains of life. The activation of these functions is thought to involve partial unfolding of N- or C-terminal helices attached to the PAS domain. Here we use atomic force microscopy to probe receptor activation in single molecules of photoactive yellow protein (PYP), a prototype of the PAS domain family. Mechanical unfolding of Cys-linked PYP multimers in the presence and absence of illumination reveals that, in contrast to previous studies, the PAS domain itself is extended by ≈ 3 nm (at the 10-pN detection limit of the measurement) and destabilized by $\approx 30\%$ in the light-activated state of PYP. Comparative measurements and steered molecular dynamics simulations of two double-Cys PYP mutants that probe different regions of the PAS domain quantify the anisotropy in stability and changes in local structure, thereby demonstrating the partial unfolding of their PAS domain upon activation. These results establish a generally applicable single-molecule approach for mapping functional conformational changes to selected regions of a protein. In addition, the results have profound implications for the molecular mechanism of PAS domain activation and indicate that stimulus-induced partial protein unfolding can be used as a signaling mechanism.

atomic force spectroscopy | photoactive yellow protein | receptor activation

Biological signaling starts with the activation of receptor proteins, in which stimuli trigger protein conformational changes that relay a signal to an associated signal transduction chain. A central question concerns the nature of the structural changes that activate a receptor protein. The mechanism of signaling by Per-Arnt-Sim (PAS) domain-containing proteins is of considerable interest, because these structural domains occur in many signaling proteins (1). In addition, incorrect signaling by PAS domains is associated with a range of diseases. Important examples are mutations in the human ERG potassium channel, which causes cardiac arrhythmias (2), and the involvement of hypoxia-inducible factors in myocardial and cerebral ischemia (3).

We investigate the photoinduced structural changes in the bacterial blue light receptor, photoactive yellow protein (PYP), a prototype for PAS domains (4). As shown in Fig. 1, PYP consists of two N-terminal α -helices (residues 1–25), followed by a typical PAS domain (1, 5) fold (residues 26–125) that contains a central six-stranded β -sheet flanked by α -helices (6). The protein exhibits photochemistry based on its *p*-coumaric acid chromophore (7). Blue light converts the initial pG state of PYP into the photoactivated pB state in milliseconds, whereas pB decays back to pG in ≈ 0.5 s (8). The pB state is believed to trigger negative phototaxis in purple bacteria (9).

The structural changes that occur upon pB formation involve partial unfolding of the receptor, indicating partial protein unfolding as a novel signaling mechanism (10). Although the crystal structure of a pB-like state exists (11), Xie *et al.* (12) have shown that the motion of PYP is greatly hindered in a protein crystal. Thus, the degree to which different regions of PYP

unfold in solution upon pB formation remains an open question. Previous solution spectroscopy experiments indicate that unfolding of the two N-terminal helices of PYP is involved (10, 13–15). Recent NMR measurements on a PYP mutant lacking the 25 N-terminal residues indicate that regions outside the central β -sheet also undergo a significant increase in flexibility (16). Interestingly, activation of LOV, a PAS domain blue-light receptor in plants, involves partial unfolding of a C-terminal helix immediately beyond the PAS domain core of the receptor (17), suggesting that the unfolding of N- or C-terminal helices is a general theme in PAS domain activation. Here, we use single-molecule force microscopy to probe the axis-dependent stability and structural changes in the PAS domain core of PYP that occur upon photoactivation.

Single-molecule pulling experiments involve the concomitant measurement of the distance between two attachment points and the force needed to extend the molecule (18–20). If the attachment points for pulling are well defined, such measurements intrinsically combine structural and energetic information. We used this approach to probe conformational changes in selected regions of PYP upon its activation.

A pair of surface-exposed residues were mutated to Cys, and the resulting PYP mutants were linked into a linear chain (polyPYP) via disulfide bonds to allow measurements of the distance between the two amino acid residues (see *Materials and Methods*). Similar chemical-linking strategies have recently been reported in studies of polyGFP by Dietz *et al.* (21), polyubiquitin by Carrion-Vazquez *et al.* (20), polyE2lip3 (an all- β protein) by Brockwell *et al.* (22), and polylysozyme by Yang *et al.* (23). Single polyPYP assemblies were stretched by using an atomic force microscope (AFM) to measure the unfolding length (ΔL) and unfolding force (ΔF) for individual PYP molecules. When an external force is applied to the ends of the polyPYP chain, each PYP molecule experiences a force distributed across the region of the protein located between the two Cys side chains. Thus, the choice of the positions of the Cys mutations defines the orientation of the force vector across individual proteins and localizes the region of the protein to which the force-extension measurement is sensitive. The initial position of the two Cys residues is known from the crystal structure, and the experimental results report changes in the distance and force between the two Cys residues along this well defined coordinate.

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Abbreviations: PAS, Per-Arnt-Sim; PYP, photoactive yellow protein; MD, molecular dynamics; SMD, steered MD.

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Results and Discussion

The multimerization of the 48/85 mutant of PYP was analyzed by SDS/PAGE and MALDI-TOF MS, revealing that the resulting samples contained polyPYP with >10 subunits (Fig. 2). The disulfide-linking reaction occurred spontaneously in solution in the presence of air. PolyPYP with 10 subunits formed readily in 8 h. The specificity of the linkage is defined by the positions of Cys mutations on the PYP. The PYP orientations can be parallel or antiparallel relative to each other, but this is unlikely to have

Force–Extension Curves in the Dark and Under Illumination. Representative force–distance curves (Fig. 3) measured for polyPYP in the dark (Fig. 3, black curves) and under saturating (supporting information) continuous blue light illumination (Fig. 3, blue curves) show a characteristic sawtooth pattern of protein ruptures (19, 20, 24). The distance between dips and the height of each peak represent ΔL and ΔF , respectively, for the unfolding of the PAS or PAS–Chr regions of PYP into an extended amino acid chain. The data show that photoexcitation reduces both the extensible length, ΔL , and mechanical stability, ΔF , of these two regions in PYP. These structural changes are reversible. Inter-

a 48/85

Force (pN)

Extension (nm)

b 36/128

Force (pN)

Extension (nm)

ΔF

ΔL

Fig. 3. AFM force vs. distance curves (pulling rate 1 $\mu\text{m/s}$) for the unfolding of single polyPYP molecules along the 48/85 axis (a) and the 36/128 axis (b) in the dark (in black) and during continuous blue light illumination (in blue).

which is relayed (*iv*) to the N-terminal helices (13, 14) by the side chain of Asn-43 (34). Future studies are needed to reveal how partial PAS domain unfolding contributes to signaling and whether this mechanism operates more widely in the PAS domain family.

Materials and Methods

Cys-Directed PYP Multimerization. An N-terminal histidine-tagged version of PYP from *Halorhodospira halophila* was overproduced in *Escherichia coli* by using the pQE80 plasmid (Qiagen) and purified by Ni-affinity chromatography (33). To ensure maximal purity of the protein, the PYP sample bound to the Ni resin was washed extensively using three different washing buffers with the following order: (i) 50 mM NaPO₄/50 mM NaCl, pH 7.2; (ii) 50 mM NaPO₄/200 mM NaCl, pH 7.2; (iii) 50 mM NaPO₄/200 mM NaCl/2 mM imidazole, pH 7.2. Each washing buffer used at least three column volumes. Elution of PYP was performed with 50 mM NaPO₄/200 mM NaCl/100 mM imidazole, pH 7.2. The Cys mutants of PYP were obtained by mutagenic PCR using the QuikChange kit (Stratagene) and confirmed by DNA sequencing. Protein purification was performed in the absence of reducing agents. PYP mutants were concentrated to $\approx$ 5 mg/ml using Centricons (Millipore) and then injected into a dialysis cassette (Pierce, Rockford, IL). The multimerization reaction was carried out overnight in the presence of a constant stream of air bubbling through the solution in a buffer consisting of 50 mM NaPO₄ and 200 mM NaCl, pH 8.0. Exposure of the PYP mutants to air resulted in their spontaneous multimerization into polyPYP. The longest PYP multimers were selected by size exclusion chromatography in 50 mM NaPO₄/50 mM NaCl, pH 7.2, and concentrated in the same NaPO₄ buffer. The final PYP concentration of the polyPYP sample used for AFM experiments was $\approx$ 0.1–0.5 mg/ml.

Force Spectroscopy of Single polyPYP Molecules. New gold-coated, silicon nitride AFM cantilevers (Bio-Levers; Olympus) were treated in a UV/Ozone cleaner (Novascan, Ames, IA) for 30 min and washed in chloroform. The spring constants (6.4 pN/nm and 22 pN/nm) of the two types of cantilevers used in the experiments were calibrated by applying the equipartition theorem to the thermal noise spectrum (35). A 100- μ l drop of the polyPYP sample was applied to a clean glass slide. All data were collected

using an AFM instrument (MFP-ID; Asylum Research, Santa Barbara, CA) on top of an inverted Nikon microscope. Light from a 452-nm blue laser (Melles-Griot, Carlsbad, CA) was focused with a $\times$ 10 objective to excite PYP at 1,000 mW/cm². UV/visual light absorbance measurements of polyPYP under identical conditions indicated that this light intensity results in nearly 100% photoconversion of the pG state to the pB state (supporting information). Only force–distance curves containing at least three consecutive rupture events were selected for analysis. The last rupture event was rejected because it usually represents detachment of the polyPYP molecule from the glass surface or tip of the cantilever. The short distance between rupture events in 48/85 PYP does not allow reliable worm-like chain analysis. Hence, the unfolding lengths for 48/85 and 36/128 data were analyzed in the same manner by measuring the distances between consecutive dips in the force–extension curves. Measuring the distances between consecutive peaks yielded similar unfolding length distributions.

SMD Simulations. SMD simulations (25) of PYP were performed with the NAMD program (26) and the CHARMM27 (36) force field. The structure of PYP (Protein Data Bank ID code 2PHY) was solvated and equilibrated in water at 300 K for 1 ns. We simulated the single PYP stretching by fixing the C α of residue 48 (or 36) and applying external forces to the C α of residue 85 (or 125). We applied the forces by restraining residue 85 (or 125) to a harmonic potential and moving at a constant velocity in the direction connecting 48–85 (or 36–125). PYP was stretched at 0.1 Å/ps for 1 ns. We repeated the simulations three times along the 48/85 and 36/125 axes and obtained similar results.

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- Taylor, B. L. & Zhulin, I. B. (1999) *Microbiol. Mol. Biol. Rev.* **63**, 479–506.
- Vincent, G. M. (1998) *Annu. Rev. Med.* **49**, 263–274.
- Semenzal, G. L. (2000) *Genes Dev.* **14**, 1983–1991.
- Pellequer, J.-L., Wagner-Smith, K. A., Kay, S. A. & Getzoff, E. D. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 5884–5890.
- Hefti, M. H., Francoijs, K. J., de Vries, S. C., Dixon, R. & Vervoort, J. (2004) *Eur. J. Biochem.* **271**, 1198–1208.
- Borgstahl, G. E. O., Williams, D. R. & Getzoff, E. D. (1995) *Biochemistry* **34**, 6278–6287.
- Hoff, W. D., Dux, P., Hård, K., Devreese, B., Nugteren-Roodzant, I. M., Crielgaard, W., Boelens, R., Kaptein, R., van Beeumen, J. & Hellingwerf, K. J. (1994) *Biochemistry* **33**, 13959–13962.
- Meyer, T. E., Yakali, E., Cusanovich, M. A. & Tollin, G. (1987) *Biochemistry* **26**, 418–423.
- Sprenger, W. W., Hoff, W. D., Armitage, J. P. & Hellingwerf, K. J. (1993) *J. Bacteriol.* **175**, 3096–3104.
- Lee, B. C., Pandit, A., Croonquist, P. A. & Hoff, W. D. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 9062–9067.
- Genick, U. K., Borgstahl, G. E., Ng, K., Ren, Z., Pradervand, C., Burke, P. M., Srajer, V., Teng, T. Y., Schildkamp, W., McRee, D. E., et al. (1997) *Science* **275**, 1471–1475.
- Xie, A., Kelemen, L., Hendriks, J., White, B. J., Hellingwerf, K. J. & Hoff, W. D. (2001) *Biochemistry* **40**, 1510–1517.
- Harigai, M., Imamoto, Y., Kamikubo, H., Yamazaki, Y. & Kataoka, M. (2003) *Biochemistry* **42**, 13893–13900.
- van der Horst, M. A., van Stokkum, I. H. M., Crielgaard, W. & Hellingwerf, K. J. (2001) *FEBS Lett.* **497**, 26–30.
- Imamoto, Y., Kamikubo, H., Harigai, M., Shimizu, N. & Kataoka, M. (2002) *Biochemistry* **41**, 13595–13601.
- Bernard, C., Houben, K., Derix, N. M., Marks, D., van der Horst, M. A., Hellingwerf, K. J., Boelens, R., Kaptein, R. & van Nuland, N. A. (2005) *Structure (London)* **13**, 953–962.
- Harper, S. M., Neil, L. C. & Gardner, K. H. (2003) *Science* **301**, 1541–1544.
- Bustamante, C., Macosko, J. C. & Wuite, G. J. L. (2000) *Nat. Rev. Mol. Cell. Biol.* **1**, 130–136.
- Carrion-Vazquez, M., Oberhauser, A. F., Fisher, T. E., Marszalek, P. M., Li, H. & Fernandez, J. M. (2001) *Prog. Biophys. Mol. Biol.* **74**, 63–91.
- Carrion-Vazquez, M., Li, H., Lu, H., Marszalek, P. E., Oberhauser, A. F. & Fernandez, J. M. (2003) *Nat. Struct. Biol.* **10**, 738–743.
- Dietz, H. & Rief, M. (2006) *Proc. Natl. Acad. Sci. USA* **103**, 1244–1247.
- Brockwell, D. J., Paci, E., Zinober, R. C., Beddard, G. S., Olmsted, P. D., Smith, D. A., Perham, R. N. & Radford, S. E. (2003) *Nat. Struct. Biol.* **10**, 731–737.
- Yang, G., Cecconi, C., Baase, W. A., Vetter, I. R., Breyer, W. A., Haack, J. A., Matthews, B. W., Dahlquist, F. W. & Bustamante, C. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 139–144.
- Fisher, T. E., Oberhauser, A. F., Carrion-Vazquez, M., Marszalek, P. E. & Fernandez, J. M. (1999) *Trends Biochem. Sci.* **24**, 379–384.
- Lu, H. & Schulten, K. (1999) *Protein Struct. Funct. Genet.* **45**, 453–463.
- Nelson, M. T., Humphrey, W., Gursoy, A., Dalke, A., Kale, L. V., Steel, R. D. & Schulten, K. (1996) *Int. J. Supercomput. Appl. High Perform. Comput.* **10**, 251–268.
- Lee, B. C., Croonquist, P. A., Sosnick, T. R. & Hoff, W. D. (2001) *J. Biol. Chem.* **276**, 20821–20823.
- Ohishi, S., Shimizu, N., Mihara, K., Imamoto, Y. & Kataoka, M. (2001) *Biochemistry* **40**, 2854–2859.
- van Brederode, M. E., Hoff, W. D., van Stokkum, I. H. M., Groot, M. L. & Hellingwerf, K. J. (1996) *Biophys. J.* **71**, 365–380.

30. Takeshita, K., Imamoto, Y., Kataoka, M., Tokunaga, F. & Terazima, M. (2002) *Biochemistry* **41**, 3037–3048.
31. Craven, C. J., Derix, N. M., Hendriks, J., Boelens, R., Hellingwerf, K. J. & Kaptein, R. (2000) *Biochemistry* **39**, 14392–14399.
32. Kort, R., Vonk, H., Xu, X., Hoff, W. D., Crielaard, W. & Hellingwerf, K. J. (1996) *FEBS Lett.* **382**, 73–78.
33. Xie, A., Hoff, W. D., Kroon, A. R. & Hellingwerf, K. J. (1996) *Biochemistry* **35**, 14671–14678.
34. Rajagopal, S., Anderson, S., Srajer, V., Schmidt, M., Pahl, R. & Moffat, K. (2005) *Structure (London)* **13**, 55–63.
35. Hutter, J. L. & Bechhoefer, J. (1993) *Rev. Sci. Instrum.* **64**, 1868–1873.
36. MacKerell, A. D. (1998) *J. Phys. Chem.* **102**, 3586–3616.