Gating of proton and water transfer in the respiratory enzyme cytochrome *c* oxidase

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved June 3, 2005 (received for review April 7, 2005)

The membrane-bound enzyme cytochrome *c* oxidase is responsible for cell respiration in aerobic organisms and conserves free energy from O_2 reduction into an electrochemical proton gradient by coupling the redox reaction to proton-pumping across the membrane. O_2 reduction produces water at the bimetallic heme a_3/Cu_B active site next to a hydrophobic cavity deep within the membrane. Water molecules in this cavity have been suggested to play an important role in the proton-pumping mechanism. Here, we show by molecular dynamics simulations that the conserved arginine/heme a_3 Δ-propionate ion pair provides a gate, which exhibits reversible thermal opening that is governed by the redox state and the water molecules in the cavity. An important role of this gate in the proton-pumping mechanism is supported by site-directed mutagenesis experiments. Transport of the product water out of the enzyme must be rigidly controlled to prevent water-mediated proton leaks that could compromise the proton-pumping function. Exit of product water is observed through the same arginine/propionate gate, which provides an explanation for the observed extraordinary spatial specificity of water expulsion from the enzyme.

proton translocation

Cytochrome *c* oxidase is the terminal member of the respiratory chains of mitochondria and many bacteria and is responsible for O_2 activation and reduction. Its important function as an energy-transducing proton pump has gathered both experimental and theoretical interest over almost three decades (1), but the molecular mechanism is still not understood. Interest has recently been focused on a possible involvement in this mechanism of the water molecules produced by O_2 reduction at the heme a_3/Cu_B site (2–5), and this raises the nontrivial question of how product water is removed from the enzyme. As in the aquaporins (6–10), water transfer in cytochrome *c* oxidase must be controlled in order not to create proton leaks along chains of water molecules being transported (11, 12).

^{17}O-labeled water molecules, the product of the reaction between reduced cytochrome *c* oxidase and $^{17}O_2$, were found coordinated to the Mg(Mn) ion bound to the protein within 8 ms after starting the reaction and before dilution with bulk water (13). The Mg(Mn) site is above the two heme groups in a domain rich in crystallographically detected water molecules (Fig. 1 and refs. 14–16). This important finding implies that the product water indeed takes a very specific route out of the heme a_3/Cu_B site, which is next to a hydrophobic cavity that is predicted to at least transiently hold several water molecules (2–4, 17, 18), although they have not been observed in the crystal structures. Hydrophobic cavities in proteins may indeed contain water that is sufficiently mobile to escape detection by x-ray crystallography but may be found by NMR spectroscopy (19). However, there is no obvious route in the static x-ray structures of cytochrome *c* oxidase by which the product water would be specifically transferred from the heme a_3/Cu_B center to the Mg(Mn) site, although a transfer pathway further out from the latter site has been described (20).

The water molecules in the hydrophobic cavity have been suggested to play a crucial role in transferring protons (derived from the negatively charged N side of the membrane) from a conserved glutamic acid residue (E242; numbering throughout according to subunit I in oxidase from bovine heart mitochondria), both to be consumed to form product water at the a_3/Cu_B site and to be pumped across the membrane (2–5). A redox state-dependent switch in the orientation of these water molecules, and thus in the destination of proton transfer, was proposed to explain this functional duality (5). Mutagenesis experiments have indicated (21) that the protons to be pumped are primarily transferred from E242 to the Δ-propionate group of heme a_3, which forms an ion pair with a conserved arginine, R438 (Fig. 1). Tsukihara *et al.* (14) challenged this view on the basis of the high stability of such an ion pair, and based on the crystal structure, protonation of the propionate would indeed seem very unlikely because of the nearby arginine cation. However, the x-ray models do not reveal dynamics and possible fluctuations in this structure: two features might actually tend to destabilize the ion pair, namely the strong hydrogen bond from a conserved tryptophan (W126) to the propionate O2D oxygen and the dipole moment of the water array in the cavity when pointing at the propionate (ref. 5 and Fig. 1). We decided to test this possibility by exploring the conformational space of the arginine/propionate pair by molecular dynamics (MD) simulations. The results show a remarkable redox state-dependent thermal dissociation of the pair, which may be of key importance both for the proton-pump mechanism and for transfer of product water out of the enzyme.

Materials and Methods

MD simulations were carried out essentially as described in ref. 5 but starting from a 50-Å diameter sphere of the bovine cytochrome *c* oxidase structure (ref. 14 and Protein Data Bank ID code 1v54) centered at the interface between the heme groups and including most of subunits I and II (6,353 atoms). Partial charges for the metal centers were derived mainly by the semiempirical PM3 and ZINDO/1 methods, but density functional theory methodology also was tested. Such variations in charge parameterization of the metal centers did not significantly change the results (5). Aspartic acid 364 and glutamic acid 242 were protonated, and all heme propionates were deprotonated, as suggested on the basis of electrostatic calculations (22). Four water molecules were modeled into the hydrophobic cavity, and an OH^- ligand was added to Cu_B (ref. 5 and Fig. 1). Heme a_3 was in its ferric state throughout. In other simulations, we have transferred the electron from heme *a* into the ferryl form of heme a_3, or into the so-called P_M state of the a_3/Cu_B site, with similar results (unpublished data). Simulations were performed by using the Amber99 force field in HYPERCHEM (version 7.1,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: MD, molecular dynamics.

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www.pnas.org/cgi/doi/10.1073/pnas.0502873102

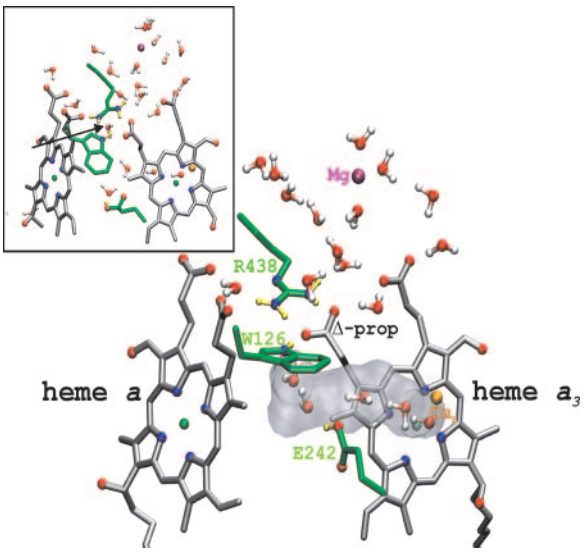

Fig. 1. Active site of cytochrome *c* oxidase. The irregular gray structure is the hydrophobic cavity between the heme a_3/Cu_B site and heme *a*, into which four water molecules have been modeled. All other water molecules are from the crystal structure (Protein Data Bank accession code 1v54). The modeled hydroxide ligand of Cu_B also is shown. The key residues R438, W126, E242 (in green; hydrogen atoms in yellow), and the Δ-propionate of heme a_3 are indicated. Heme iron is shown in green, and oxygen and nitrogen atoms are shown in red and blue, respectively. (*Inset*) Snapshot of exit of water (arrow) from the cavity via the open arginine/propionate gate. Color coding is as in the main figure. This figure was prepared by using the VMD program (33).

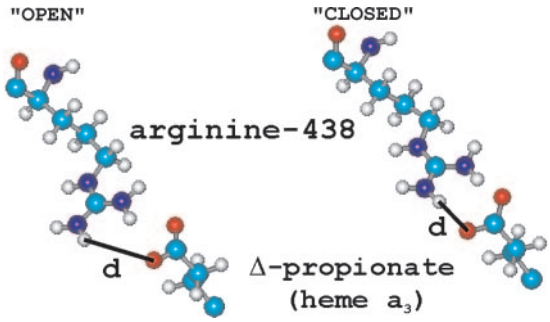

Fig. 2. Two states of the arginine/propionate ion pair. The mean equilibrium structure is on the right with a short distance, marked d, between the 2HH2 hydrogen of the arginine and the O2D oxygen of the propionate. The open gate is shown on the left.

Hypercube, Gainesville, FL), allowing movement of all amino acids surrounding the cavity, Cu_B and its ligands, the heme Δ-propionates and Δ-methyl groups, arginines 438 and 439, all crystallographic water molecules immediately above the hemes, and those added to the cavity (a total of 360 atoms). The distance between the Cγ atom of E242 and the Δ-methyl carbon of heme a_3 was constrained to 7 Å by a harmonic force constant of 7 kcal/mol·Å^2 to prevent the E242 side chain from flipping away from the hydrophobic cavity (5). The structure was temperature-equilibrated and energy-minimized before the simulations.

Proton translocation by wild-type and W164F mutant cytochrome *c* oxidase from *Paracoccus denitrificans* (W126 in the bovine enzyme) incorporated in phospholipid vesicles was measured by using the O_2-pulse method, essentially as described in ref. 23, in 100 mM KCl medium supplemented with 15 μM horse heart cytochrome *c*, 5 mM potassium ascorbate, and 1 μM valinomycin (pH 7.0–7.4). Known amounts of O_2 were added as microliter injections of pure water equilibrated with air at 25°C, and the pH change was calibrated with small aliquots of anaerobic 1 mM HCl.

Results and Discussion

Our rationale was to simulate the dynamics near the active site in conditions that mimic functional states of the enzyme as closely as possible. The catalytic cycle of cytochrome *c* oxidase may be characterized as including four elementary reaction steps, in each of which an electron at heme *a* (derived from cytochrome *c* through the Cu_A center) is transferred to the a_3/Cu_B site, accompanied by pumping of a proton across the membrane and by uptake of a substrate proton to the a_3/Cu_B site from the inside of the membrane (24). The basic conditions were chosen such as to simulate the dynamics before and after electron transfer. Consequently, when heme *a* was reduced, the a_3/Cu_B site was oxidized (ferric/cupric). Alternatively, heme *a* was oxidized, in which case Cu_B was reduced. In some experiments, we positioned the pumped proton to various sites above the heme groups in this latter redox state (with reprotonation of E242), but this positioning did not affect the organization of the water molecules to form a proton transfer path from E242 to the binuclear site (5) and was therefore not included in this study. Strictly speaking, this model applies only for one of the four elementary steps in the cycle, namely the one where the ferric/cupric a_3/Cu_B site receives an electron, but this is sufficient for the present purposes. However, we also have modeled electron transfer into the so-called F and P_M states of the binuclear site (see *Materials and Methods*), with results very similar to those with the basic model reported here.

The first impression from the simulations was temporal rigidity. Interatomic distances generally fluctuated <0.5 Å around equilibrium positions during typical simulations of 50–200 ps each at 300 K. Even the large number of crystallographic water molecules "above" heme a_3 (Fig. 1) showed little or no translation. These simulations (the current compounded time is 2.5 ns for each redox state) have confirmed that the orientation of the water molecules in the cavity depends strongly on redox state (5). When heme *a* is reduced and the a_3/Cu_B site is oxidized, these water molecules are consistently orientated to direct protons from E242 to the Δ-propionate of heme a_3. In this state, proton transfer from E242 to the a_3/Cu_B site is not supported by the orientation of the water molecules, which occurs only when the electron has been moved to the a_3/Cu_B site.

Against this background a considerable thermal fluctuation stood out as exceptional. The 2HH2–O2D hydrogen–oxygen distance of the R438-propionate pair fluctuated reproducibly and reversibly to transient maxima of >4 Å from the equilibrium position of ≈2 Å (Figs. 2 and 3*A*). The time between initiation of opening and completion of closure was 0.4–0.8 ps, with an "open time" (defined as a distance >4 Å) of ≈0.1 ps. Interestingly, quantum chemical simulations with bacteriorhodopsin (25) indicate that this time is sufficient for transfer of a proton by the Grotthuss mechanism (12) across a prearranged water array. The fluctuation occurred with a probability of $\approx 10^{-3}$ at 300 K (≈4 kcal/mol) and with about a 10-fold increase in frequency at 450 K. Its most remarkable property was a strong redox-state dependence: it occurred only in the redox state where the water molecules in the cavity are orientated for proton transfer from E242 to the propionate (Fig. 3) and failed entirely (at 300 K), even in the appropriate redox state, if these water molecules were removed (Fig. 3*B*, yellow trace), if W126 was "mutated" to phenylalanine (Fig. 3*B*, blue trace), or if the partial charges of the indole group of W126 were made equal to zero (not shown). Clearly, both the water dipole orientation and the hydrogen bond from W126 are required to destabilize the ion pair, as anticipated. The mutation corresponding to W126F had an interesting effect on proton-pumping, as measured in vesicle-

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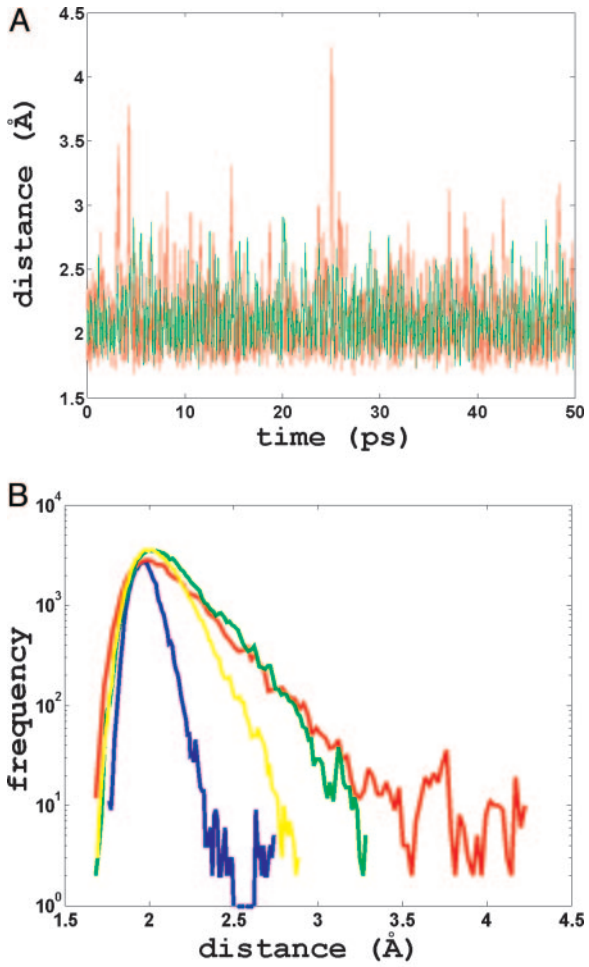

Fig. 3. Fluctuations of the arginine/propionate pair. (*A*) MD simulations at 300 K monitoring the 2HH2–O2D distance over time. Green traces are from simulations with heme *a* oxidized and Cu_B reduced; all others are with heme *a* reduced and Cu_B oxidized. (*B*) Plots of the frequency (probability, inverse energy) at which a certain distance occurs. Yellow trace, all water molecules were removed from the hydrophobic cavity; blue trace, the residue W126 was changed to phenylalanine. The red and green traces are from simulations with heme *a* reduced/Cu_B oxidized, and heme *a* oxidized/Cu_B reduced, respectively (see also *A*).

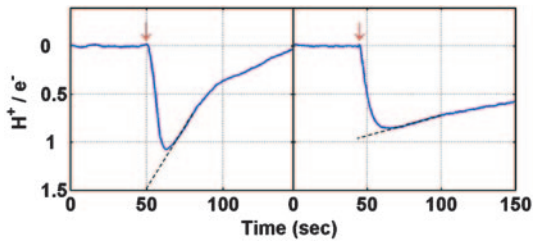

Fig. 4. Proton-pumping by the W164F mutant and wild-type enzyme from *P. denitrificans*. Calibrated oxygen pulses (arrows) were given to anaerobic suspensions of wild-type (*Left*) and mutant enzyme (*Right*; W164F corresponds to W126F in the bovine enzyme structure). For conditions, see *Materials and Methods*. Note that oxidation of ascorbate yields ejection of 0.5 H^+/e^- and should be subtracted. Linear back-extrapolation of the protonic decay has been drawn to aid the eye (dashed line).

incorporated cytochrome *c* oxidase from *P. denitrificans*. The ultimate reductant in the experiments of Fig. 4 is ascorbate, the oxidation of which itself releases 0.5 H^+/e^-. Thus, proton translocation amounted to ≈1.0 and 0.5 H^+/e^- for wild-type and mutant enzyme, respectively, which supports a role of this tryptophan in the proton-pumping mechanism.

We conclude that dissociation of the salt bridge is triggered by hydration of the propionate, and that the redox state dependence is mediated by the orientation of the water molecules in the cavity, from which this hydration takes place. Dissociation of the ion pair (Fig. 2) is expected to raise the pK_a of the propionate substantially (and to lower the pK_a of the arginine), which may transiently allow the transfer of the pumped proton from E242 to the propionate (5, 21). This, in turn, may raise the electron affinity of the a_3/Cu_B site, allowing fast electron transfer from heme *a* (26, 27) and trapping the proton at the propionate. Alternatively, protonation of the propionate is transient and coupled to deprotonation of the arginine, which may release its proton to some other site above the heme groups. In this scenario, proton exchange between the propionic acid and the deprotonated arginine follows with restoration of the ion pair. After switching of the water array toward the a_3/Cu_B site and reprotonation of E242 from the N side, this second proton will be transferred to the a_3/Cu_B site, producing the equivalent of water (protonating an oxygenous ligand), with release of the pumped proton toward the positively charged P side of the membrane by electrostatic repulsion (5). The arginine/propionate pair might thus function as a gate in the mechanism that controls proton access from the N and the P sides of the membrane, respectively.

The W126F mutation decreased the overall pumping efficiency, but only by 50% (Fig. 4). Even though arginine/propionate dissociation was not observed with the W126F mutant enzyme by MD simulations on the 0.1- to 0.2-ns time scale (Fig. 2*C*), it may well occur on the much slower time scale of enzyme turnover, therefore resulting in only partial decoupling of the pump mechanism.

If product water would exit the cavity through the open arginine/propionate gate, it would be funneled precisely to the Mg(Mn) domain, as observed experimentally (ref. 13 and Fig. 1). To test this idea, we added a fifth water molecule to the cavity or removed the crystallographic water above the ion pair. In both cases, MD simulations showed gate-opening and exit of a water molecule by this route (Fig. 1 *Inset*). With an "overload" of water molecules in the cavity, the redox-state dependence is overridden and the gate opens also with heme *a* oxidized and the a_3/Cu_B site reduced. This observation emphasizes the importance of a correct balance in the dynamics of removal of product water, because gate-opening in this redox state could allow backflux of protons from the outside directly into the a_3/Cu_B site. Ferguson-Miller and Mills (28) have mutated the equivalent of R438 in cytochrome *c* oxidase from *Rhodobacter sphaeroides* (R481) to a lysine. The phenotype is interesting in the current context because it shows normal proton-pumping efficiency but apparently a decreased rate of proton leakage from the P side into the a_3/Cu_B site. It is possible, therefore, that the gate discussed here is more tightly shut when the arginine is replaced by a lysine, the charge density of which is much higher. MD simulations on this mutant enzyme might shed further light on this situation.

Without water in the cavity, there was no discernible water diffusion in the opposite direction from the domain above the hemes. In this case, the gate remained closed (Fig. 3*B*), which once more underlines the notion that ion pair dissociation is triggered specifically "from below" by the water molecules in the cavity. Finally, it may be noted that an arginine/propionate ion pair has previously been suggested to gate water transfer in cytochrome P450 (29).

Thermal fluctuations are fundamental to the function of biological macromolecules, especially molecular motors and energy transducers (30–32). The reversible redox-state-dependent dissociation of the arginine/propionate ion pair

described here may play a fundamental role as the long-sought gate and protonic switch in the proton-pumping mechanism of cytochrome *c* oxidase. It is interesting that water, the product of the reaction at the a_3/Cu_B site, may acquire a key role in the proton-pump mechanism before being expelled, but how proton and water transfer are coordinated is not yet understood and requires further study. Strict control of both proton and water transfer is necessary in the respiratory enzyme to secure its function as a primary energy transducer in biology. This work gives a hint of how this may be achieved by a specific thermal fluctuation in the atomic structure.

We thank Gerhard Hummer, Régis Pomès, and Arieh Warshel for valuable comments and Vivek Sharma for help with the figures. This work was supported by grants (to M.W.) from the Sigrid Juselius Foundation and the Academy of Finland (Program 44895).

1. Wikström, M. (2004) *Biochim. Biophys. Acta* **1655,** 241–247.
2. Riistama, S., Hummer, G., Puustinen, A., Dyer, R. B., Woodruff, W. H. & Wikström, M. (1997) *FEBS Lett.* **414,** 275–280.
3. Hofacker, I. & Schulten, K. (1998) *Proteins Struct. Funct. Genet.* **30,** 100–107.
4. Zheng, X., Medvedev, D. M., Swanson, J. & Stuchebrukhov, A. A. (2003) *Biochim. Biophys. Acta* **1557,** 99–107.
5. Wikström, M., Verkhovsky, M. I. & Hummer, G. (2003) *Biochim. Biophys. Acta* **1604,** 61–65.
6. Fujiyoshi, Y., Mitsuoka, K., de Groot, B. L., Philippsen, A., Grubmüller, H., Agre, P. & Engel, A. (2002) *Curr. Opin. Struct. Biol.* **12,** 509–515.
7. de Groot, B. L., Frigato, T., Helms, V. & Grubmüller, H. (2003) *J. Mol. Biol.* **333,** 279–293.
8. Jensen, M., Tajkhorshid, E. & Schulten, K. (2003) *Biophys. J.* **85,** 2884–2899.
9. Burykin, A. & Warshel, A. (2003) *Biophys. J.* **85,** 3696–3706.
10. Chakrabarti, N., Roux, B. & Pomès, R. (2004) *J. Mol. Biol.* **343,** 493–510.
11. Nagle, J. F. & Morowitz, H. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 298–302.
12. Agmon, N. (1995) *Chem. Phys. Lett.* **244,** 456–462.
13. Schmidt, B., McCracken, J. & Ferguson-Miller, S. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 15539–15542.
14. Tsukihara, T., Shimokata, K., Katayama, Y., Shimada, H., Muramoto, K., Aoyama, H., Mochizuki, M., Shinzawa-Itoh, K., Yamashita, E., Yao, M., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100,** 15304–15309.
15. Ostermeier, C., Harrenga, A., Ermler, U. & Michel, H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10547–10553.
16. Svensson-Ek, M., Abramson, J., Larsson, G., Törnroth, S., Brzezinski, P. & Iwata, S. (2002) *J. Mol. Biol.* **321,** 329–339.
17. Olkhova, E., Hutter, M. C., Lill, M. A., Helms, V. & Michel, H. (2004) *Biophys. J.* **86,** 1873–1889.
18. Cukier, R. I. (2004) *Biochim. Biophys. Acta* **1656,** 189–202.
19. Yu, B., Blaber, M., Gronenborn, A. M., Clore, G. M. & Caspar, D. L. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 103–108.
20. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., *et al.* (1998) *Science* **280,** 1723–1729.
21. Puustinen, A. & Wikström, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 35–37.
22. Kannt, A., Lancaster, C. R. D. & Michel, H. (1998) *Biophys. J.* **74,** 708–721.
23. Backgren, C., Hummer, G., Wikström, M. & Puustinen, A. (2000) *Biochemistry* **39,** 7863–7867.
24. Bloch, D., Belevich, I., Jasaitis, A., Ribacka, C., Puustinen, A., Verkhovsky, M. I. & Wikström, M. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 529–533.
25. Lee, Y.-S. & Krauss, M. (2004) *J. Am. Chem. Soc.* **126,** 2225–2230.
26. Verkhovsky, M. I., Jasaitis, A. & Wikström, M. (2001) *Biochim. Biophys. Acta* **1506,** 143–146.
27. Pilet, E., Jasaitis, A., Liebl, U. & Vos, M. H. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 16198–16203.
28. Mills, D. A. & Ferguson-Miller, S. (2002) *Biochim. Biophys. Acta* **1555,** 96–100.
29. Oprea, T. I., Hummer, G. & Garcia, A. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2133–2138.
30. Frauenfelder, H., Sligar, S. G. & Wolynes, P. G. (1991) *Science* **254,** 1598–1603.
31. Oster, G. (2002) *Nature* **417,** 25.
32. Réat, V., Patzelt, H., Ferrand, M., Pfister, C., Oesterhelt, D. & Zaccai, G. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4970–4975.
33. Humphrey, W., Dalke, A. & Schulten, K. (1996) *J. Mol. Graphics* **14,** 33–38.