Evaluating the Catalytic Contribution from the Oxyanion Hole in Ketosteroid Isomerase

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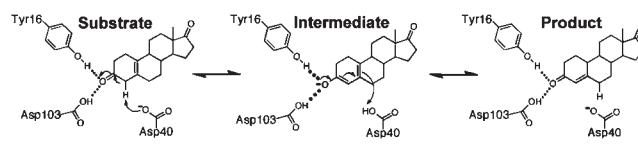
S *Supporting Information*

ABSTRACT: Prior site-directed mutagenesis studies in bacterial ketosteroid isomerase (KSI) reported that substitution of both oxyanion hole hydrogen bond donors gives a 10^5- to 10^8-fold rate reduction, suggesting that the oxyanion hole may provide the major contribution to KSI catalysis. But these seemingly conservative mutations replaced the oxyanion hole hydrogen bond donors with hydrophobic side chains that could lead to suboptimal solvation of the incipient oxyanion in the mutants, thereby potentially exaggerating the apparent energetic benefit of the hydrogen bonds relative to water-mediated hydrogen bonds in solution. We determined the functional and structural consequences of substituting the oxyanion hole hydrogen bond donors and several residues surrounding the oxyanion hole with smaller residues in an attempt to create a local site that would provide interactions more analogous to those in aqueous solution. These more drastic mutations created an active-site cavity estimated to be $\sim$650 Å^3 and sufficient for occupancy by 15–17 water molecules and led to a rate decrease of only $\sim$$10^3$-fold for KSI from two different species, a much smaller effect than that observed from more traditional conservative mutations. The results underscore the strong context dependence of hydrogen bond energetics and suggest that the oxyanion hole provides an important, but moderate, catalytic contribution relative to the interactions in the corresponding solution reaction.

Understanding the mechanisms that enzymes use to accelerate chemical reactions has been a central goal of biochemistry. The now-standard tools of site-directed mutagenesis and X-ray crystallography have contributed enormously to our understanding of enzyme action, identifying residues important for catalysis and placing them within the context of the active site. Nevertheless, quantitative understanding of the mechanisms by which enzymes achieve their rate enhancement remains limited.

Hydrogen bonding to groups that develop charge in the course of a reaction is a prevalent feature of enzyme active sites, and the classic example is stabilization of the incipient oxyanion in the oxyanion hole of serine proteases.[1–4] Nevertheless, experimental studies of the catalytic contribution from oxyanion hole hydrogen bonds are limited. For most serine proteases, the oxyanion hole hydrogen bonds are mediated by backbone amides and therefore cannot be readily mutated. Subtilisin was used in classic site-directed mutagenesis studies by Wells, as one of the oxyanion hole hydrogen bond donors is an Asn side chain,

Scheme 1. Mechanism of KSI-Catalyzed Isomerization

but the other oxyanion hole hydrogen bond donor, a backbone amide, could not be investigated.[3,4] Further, it is tempting to interpret rate effects from simple mutations as reporting on the contribution of individual residues to catalysis. However, different mutations can give different effects, and minimal or "conservative" mutations may introduce unfavorable effects beyond removal of a functional group, thereby exaggerating the apparent energetic benefit of the enzymatic group.[5–7]

KSI catalyzes the double bond isomerization of steroid substrates that involves formation of a dienolate intermediate within an active-site oxyanion hole composed of the side chains of Tyr16 and Asp103 and thereby allowing both oxyanion hole hydrogen bond donors to be probed by mutagenesis (Scheme 1).[8] We report herein the effect of fully removing the oxyanion hole hydrogen bond donors and also surrounding residues in an effort to replace the oxyanion hole with an aqueous-like environment. The results suggest a surprisingly small contribution of the oxyanion hole, relative to interactions in the corresponding solution reaction.

Previous studies reported that the *Pseudomonas putida* (pKSI) and *Comamonas testosteroni* (tKSI) double mutations that remove the Tyr and Asp hydrogen bond donors in the oxyanion hole result in a 10^5- and 10^8-fold decrease in activity, respectively (pKSI, Y16F/D103L; tKSI, Y16F/D103A; pKSI numbering is used throughout for simplicity).[9,10] The large rate reductions observed in the mutagenesis experiments and additional spectroscopic and computational studies have led to the suggestion that the oxyanion hole provides a major contribution to KSI catalysis.[11–13] Nevertheless, the conservative mutations replace the oxyanion hole hydrogen bond donors with bulky, hydrophobic side chains that could lead to suboptimal solvation of the incipient oxyanion in the mutants, thereby potentially exaggerating the energetic benefit of the hydrogen bond compared to water-mediated hydrogen bonds in solution.[7,14]

Indeed, Kraut et al. reported that, whereas the conservative Y16F mutation alone results in a $\sim$$10^5$-fold effect, mutation of
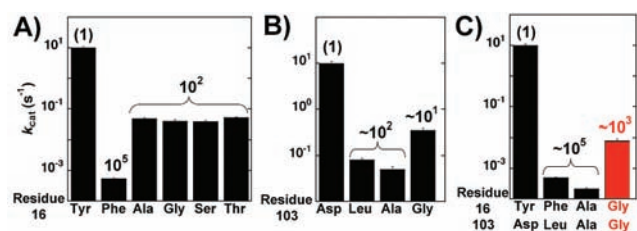
Figure 1. Effects of oxyanion hole hydrogen bond donor mutations on pKSI activity (k_{cat}) using the substrate 5(10)EST: (A) effects of Tyr16 mutations replotted from Kraut et al.,[7] (B) effects of Asp103 mutations, and (C) effects of full ablation of oxyanion hole hydrogen bond donors. Values are from Table S1. A comparison of k_{cat}/K_M values reveals a similar trend (Table S1).

Y16 to the less conservative Ala, Gly, Ser, Thr leads to a much smaller rate reduction of only $\sim 10^2$-fold (Figure 1A).[7] The Y16F mutation was suggested to replace Y16 with a hydrophobic environment, whereas mutation of Y16 to smaller residues appears to replace Y16 with a water-filled cavity that provides aqueous-like solvation of the oxyanion.[7] Thus, the catalytic contribution from the oxyanion hole relative to the nonenzymatic solution reaction might be smaller than previously ascribed from the seemingly conservative site-directed mutagenesis.[9,10]

Mutations of the D103 oxyanion hole hydrogen bond donor lead to a rate decrease of $\sim 10^2$- and $\sim 10^3$-fold for pKSI and tKSI, respectively (pKSI, D103L; tKSI, D103A).[9,10] These mutations again might not create sufficient space for water to replace the enzymatic hydrogen bond donor and could similarly exaggerate the energetic benefit of the enzymatic hydrogen bond donor compared to a water-mediated hydrogen bond.

We set out to determine whether less conservative oxyanion hole mutations would be less disruptive to KSI catalysis, presumably because hydrogen bonds to water could be restored, and we tested this model by mutating residues surrounding the oxyanion hole to create a local site that might provide interactions more analogous to those in aqueous solution.

Our results with the substrate 5(10)estrene-3,17-dione [5(10)EST] for the pKSI D103A and D103L mutations are similar to the previously reported ~ 200-fold rate decrease for the pKSI D103L mutation using the substrate 5-androstene-3, 17-dione (5-AND) (Figure 1B and Table S1).[9] We primarily used 5(10)EST to ensure that a chemical step is rate limiting and that K_M reflects binding.[15] Similar results for oxyanion hole mutations were obtained using the steroid substrate, 5-AND, and a one-ring substrate, 3-cyclohexen-1-one (Supporting Information). In contrast to the results with D103A and D103L, mutation to a smaller Gly residue at this position gave only a 28-fold rate decrease (Figure 1B); the smaller Gly might open more space and reduce or eliminate unfavorable interactions introduced from the seemingly conservative mutations.

We next considered both oxyanion hole hydrogen bond donors together. We mutated both pKSI residues to either Ala or Gly. The overall pKSI Y16A/D103A structure (PDB ID 3T8N) was the same as that previously determined for wild-type pKSI (PDB ID 1OPY), with a root-mean-square deviation of 0.3 Å for the backbone atoms and no indication of structural rearrangements introduced by the mutations (Figure S1). With 5(10)-EST, the Y16A/D103A double mutation led to a 45 000-fold decrease in activity, similar to the $\sim 10^5$-fold effect previously reported for Y16F/D103L using 5-AND (Figure 1C).[9] However,

the more drastic Y16G/D103G double mutation decreased activity less, by only 1200-fold (Figure 1C, red bar, and Figure S2). The K_M values for the oxyanion hole mutants were within 2-fold of that for wild-type KSI, suggesting that the mutations do not affect ground-state binding and indicating nearly identical rate effects on k_{cat} and k_{cat}/K_M (Table S1).

The ~ 40-fold smaller decrease in activity for the double glycine mutant compared to the double alanine mutant is not the result typically expected for more extensive and less conservative mutation, but the result is consistent with the model in that opening more space creates a water-filled cavity that replaces the enzymatic oxyanion hole hydrogen bond donors. Thus, the $\sim 10^3$-fold rate reduction for KSI without enzymatic oxyanion hole hydrogen bond donors suggests that the oxyanion hole may provide a significantly smaller catalytic contribution than the 10^5- to 10^8-fold suggested from site-directed mutagenesis experiments.[9,10] Alternatively, the double Gly mutation could introduce fortuitous interactions, e.g., from ordered water molecules that mitigate some of the deleterious effect. To further test these models, we performed additional tests in pKSI and in the related tKSI.

If the Y16G/D103G double mutant were to render the oxyanion hole effectively water-like, additional mutations that open space would have little effect on activity. In contrast, if structured water molecules stabilize the incipient oxyanion in the Y16G/D103G double mutant, then these additional cavity mutations would be expected to disrupt the positioned waters, thereby decreasing activity. The extensive mutagenesis necessary to "excavate" the oxyanion hole is likely to disrupt folding or positioning for many enzymes, so there could be multiple origins of additional deleterious effects. Fortunately, KSI is amenable to the extensive mutagenesis necessary for in-depth investigation of oxyanion hole catalysis.

The pKSI oxyanion hole hydrogen bond donors (red) are surrounded by two hydrogen-bonding residues, Tyr57 and Tyr32 (blue), and a constellation of hydrophobic residues (black and green, Figure 2A). We mutated the active-site tyrosine residues and multiple surrounding hydrophobic residues to create space. Tests to ensure the stability of these mutants are described in the Supporting Information. Remarkably, the additional mutations led to little change in activity (Figure 2B) compared to the Y16G/D103G double mutant (Figure 2B, red bar). The similar activity for mutants with additional surrounding groups removed supports the model that mutation of Tyr16 and Asp103 to smaller Gly residue replaces the oxyanion hole with an effectively solution-like site.

We next tested if analogous mutations leads to similar results in the related tKSI. The homologous pKSI and tKSI enzymes have the same oxyanion hole hydrogen-bonding residues, but the surrounding residues are different, so it is unlikely that there would be the same coincidental effects from mutations that might reposition groups or give prepositioned water molecules within the oxyanion hole.

Previous studies using the 5-AND steroid substrate reported an enormous rate reduction of 10^8-fold for Y16F/D103A tKSI.[10] We determined that mutation of both tKSI oxyanion hole hydrogen bond donors to Ala led to a 10^5-fold decrease in activity (Figure S3). Mutation of both oxyanion hydrogen bond donors to Gly again led to a less severe effect of $\sim 10^4$-fold (Figures S3 and S4). Mutation of the neighboring Tyr57 to Phe and Ala increased or led to little further change in the oxyanion hole background (Figures S3–S5). The similar effects
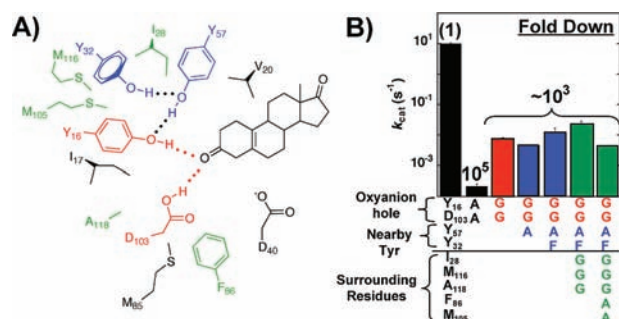
Figure 2. "Carving out" the pKSI oxyanion hole. (A) Schematic representation of oxyanion hole hydrogen bond donors (red), tyrosine network (blue), and surrounding residues (green and black). The substrate is also shown in black. (B) Rate effects from ablating neighboring side chains in the oxyanion hole mutant background. Values are from Table S1. Bars and residues are colored according to panel A.

of ~10^3-fold in pKSI and tKSI from mutations to create space in the oxyanion hole (Tables S1–S5) suggest that the smaller rate effects are not from coincidental effects introduced upon mutation.

The functional tests described above assume that the mutations open space for water and that there are no structural rearrangements that enhance function. We were unable to obtain diffractable crystals of the Gly oxyanion hole mutants, but we were able to solve the crystal structure of tKSI Y16A/Y57F/D103A (PDB ID 3T8U, Table S6). The overall structure obtained at 2.5 Å resolution is nearly identical to that of wild-type tKSI (PDB ID 8CHO; 2.3 Å),[16] with a root-mean-square deviation of 0.4 Å for backbone atoms. The electron density map for the tKSI Y16A/Y57F/D103A structure, contoured at 1.0σ, shows well-defined density for the modeled atomic positions of the residues surrounding the oxyanion hole (Figure 3A), with modeled side-chain positions nearly superimposable with wild type. Thus, the X-ray crystallographic results suggest the mutations do not induce structural rearrangements (Figure S6). Removal of the oxyanion hole residues generates a cavity of ~150 Å^3 (Figure 3B), sufficient to accommodate 4–5 waters.[17] The subsequent mutations of surrounding groups in tKSI and pKSI presumably allow access of additional water molecules with space sufficient to accommodate 15–17 waters in the most severe mutants.[17] As activity is increased for multiple variants relative to that of the Ala oxyanion hole mutants, these mutations presumably do not cause deleterious active-site rearrangements.

The results herein indicate that mutations that ablate both oxyanion hole hydrogen bond donors and open space for water result in ~10^3- to ~10^5-fold smaller rate decreases in pKSI and tKSI compared to the more conservative Y16A/D103A and Y16F/D103A mutations (Table S1 and ref 10). The variation in rate effects with different mutations underscores that hydrogen bond energetics and mutagenesis experiments are highly context dependent. In the nonenzymatic solution reaction, water can rearrange to solvate the incipient oxyanion (Figure 4A). Wild-type KSI has two oxyanion hole hydrogen bond donors positioned to hydrogen bond with the oxyanion (Figure 4B). "Conservative" mutations replace the enzymatic hydrogen bond donors with a hydrophobic environment that desolvates the localized negative charge in the transition state relative to aqueous solution (Figure 4C, gray shading), whereas water in the carved-out
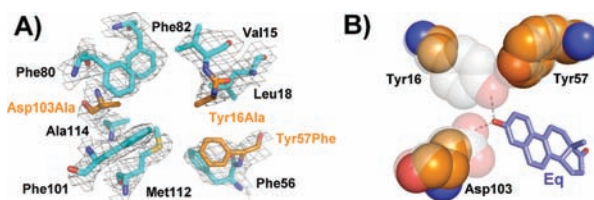

Figure 3. Crystal structure of tKSI Y16A/Y57F/D103A reveals a cavity in place of the oxyanion hole. (A) Sigma-A weighted $2F_o - F_c$ electron density for active-site residues (contoured at 1.0σ). Side chains of the mutated residues are colored orange. (B) Space-filling representation of the oxyanion hole residues. Wild-type oxyanion hole hydrogen bond donors and Tyr57 are transparent to show the mutant cavity.
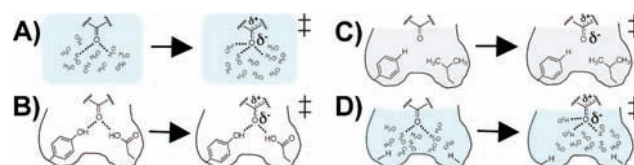

Figure 4. Schematic models for the effects of oxyanion hole mutations on KSI catalysis. (A) Water molecules rearrange in the solution nonenzymatic reaction to provide stabilization of the oxyanion from two or three hydrogen bonds. (B) The wild-type KSI oxyanion hole has two positioned hydrogen bond donors that stabilize the incipient oxyanion. (C) Conservative mutation of the oxyanion hole hydrogen bond donors ablates enzymatic hydrogen bonds and introduces a neighboring hydrophobic environment. (D) Less-conservative, smaller mutations ablate the enzymatic hydrogen bonds but create a cavity that allows water to solvate the incipient oxyanion. Figure adapted from Kraut et al.[7]

mutant arranges to stabilize the localized negative charge, as also occurs in solution (Figure 4D).

These and other aspects of the energetics of hydrogen bonding are complex. Hydrogen bonds donated by the enzyme are expected to be energetically favorable relative to hydrogen bonds donated by water as the enzymatic groups have lower pK_a values than water and are thus stronger hydrogen bond donors.[18,19] In addition, the enzymatic hydrogen bonding groups are pre-positioned to favor hydrogen bonding in the transition state, whereas water molecules must rearrange to optimize interactions in the transition state.[14,20] However, in the carved-out mutants and in solution, three hydrogen bonds might form with the oxyanion. Hydrogen bond energetics are also sensitive to the surrounding environment, and a favorable process that involves charge accumulation in aqueous solution can be unfavorable in other environments.[14,21]

Enzymes catalyze reactions relative to the nonenzymatic reaction in water. Replacing the oxyanion hole hydrogen bond donors with a water-filled cavity provides an important though crude estimate of the catalytic contribution from the oxyanion hole hydrogen bonds compared to the analogous reaction in water in which there are water-mediated hydrogen bonds. Prior results have indicated that wild-type KSI provides a 10^{11}–10^{12}-fold rate increase above the acetate-catalyzed isomerization of 5(10)EST (Figure 5).[14,22,23] KSI with the oxyanion hole and surrounding residues removed provides an ~10^9-fold rate increase compared to the acetate-catalyzed reaction, only ~10^3-fold less than wild-type KSI. These comparisons strongly suggest that the oxyanion hole provides an important, but moderate, catalytic contribution and focus us on the task of uncovering how binding
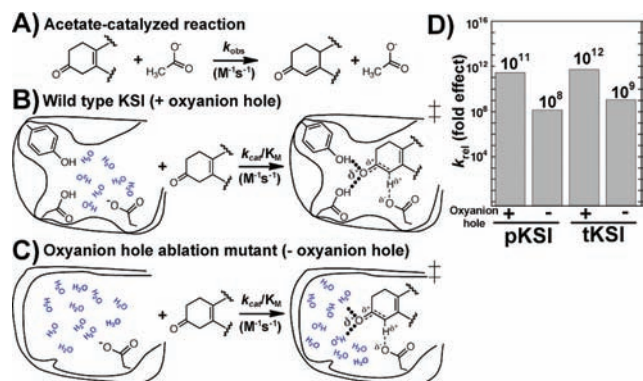
Figure 5. Determining the catalytic contribution from the oxyanion hole in KSI. (A) The second-order solution reaction of a KSI substrate with acetate ion. (B) The second-order reaction of wild-type KSI with the same substrate. (C) The second-order reaction of the KSI oxyanion hole ablation mutant. (D) Comparison of the second-order rate constant with the second-order nonenzymatic acetate-catalyzed reaction rate constant to measure the overall rate increase for KSI relative to the solution reaction that uses the same general base functionality. Comparison of reaction from the KSI E·S complex relative to the acetate-catalyzed reaction is given in Figure S7.

interactions and general base catalysis can provide the remaining substantial catalytic contributions.[24]

The $\sim10^3$-fold rate reduction for full ablation of the KSI oxyanion hole is similar to the rate effects previously reported for mutation of the Asn oxyanion hole hydrogen bond donor to Ala, Leu, Thr, and Gln in subtilisin, a serine protease.[3,4] While the effect from ablating all oxyanion hole hydrogen-bonding groups in subtilisin could be larger than the effects reported for single mutations, perhaps the oxyanion hole is no longer utilized in the mutants such that the overall modest effect of the oxyanion hole is general. Support for the latter model arises from the observation of incipient oxyanions pointed away from serine protease oxyanion holes[25,26] and the similar mutational effects of the oxyanion hole Asn of subtilisin and Kex2 to neutral residues versus the presumably negatively charged Asp residue.[4,27] It will be fascinating to ascertain the generality versus idiosyncrasies of oxyanion hole and other catalytic contributions between different enzymes by using extensive mutagenesis akin to that carried out herein.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. Experimental methods, Figures S1—S7, and Tables S1—S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

■ REFERENCES

(1) Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331–358.
(2) Robertus, J. D.; Kraut, J.; Alden, R. A.; Birktoft, J. J. *Biochemistry* **1972**, *11*, 4293–4303.
(3) Bryan, P.; Pantoliano, M. W.; Quill, S. G.; Hsiao, H. Y.; Poulos, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3743–3745.
(4) Wells, J. A.; Cunningham, B. C.; Graycar, T. P.; Estell, D. A. *Philos. Trans. R. Soc. London A* **1986**, *317*, 415–423.
(5) Kraut, D. A.; Carroll, K. S.; Herschlag, D. *Annu. Rev. Biochem.* **2003**, *72*, 517–571.
(6) Fersht, A. R. *Structure and Mechanism in Protein Science*, 2nd ed.; W.H. Freeman and Co.: New York, 1999.
(7) Kraut, D. A.; Sigala, P. A.; Fenn, T. D.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1960–1965.
(8) Pollack, R. M. *Bioorg. Chem.* **2004**, *32*, 341–353.
(9) Choi, G.; Ha, N. C.; Kim, S. W.; Kim, D. H.; Park, S.; Oh, B. H.; Choi, K. Y. *Biochemistry* **2000**, *39*, 903–909.
(10) Thornburg, L. D.; Goldfeder, Y. R.; Wilde, T. C.; Pollack, R. M. *J. Am. Chem. Soc.* **2001**, *123*, 9912–9913.
(11) Cleland, W. W.; Frey, P. A.; Gerlt, J. A. *J. Biol. Chem.* **1998**, *273*, 25529–25532.
(12) Zhao, Q.; Abeygunawardana, C.; Talalay, P.; Mildvan, A. S. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8220–8224.
(13) Feierberg, I.; Aqvist, J. *Biochemistry* **2002**, *41*, 15728–15735.
(14) Kraut, D. A.; Sigala, P. A.; Pybus, B.; Liu, C. W.; Ringe, D.; Petsko, G. A.; Herschlag, D. *PLoS Biol.* **2006**, *4*, 501–519.
(15) Pollack, R. M.; Bantia, S.; Bounds, P. L.; Koffman, B. M. *Biochemistry* **1986**, *25*, 1905–1911.
(16) Cho, H. S.; Choi, G.; Choi, K. Y.; Oh, B. H. *Biochemistry* **1998**, *37*, 8325–8330.
(17) Tsai, J.; Taylor, R.; Chothia, C.; Gerstein, M. *J. Mol. Biol.* **1999**, *290*, 253–266.
(18) Hine, J. *J. Am. Chem. Soc.* **1972**, *94*, 5766–5771.
(19) Stahl, N.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108*, 4196–4205.
(20) Warshel, A. *J. Biol. Chem.* **1998**, *273*, 27035–27038.
(21) Shan, S. O.; Herschlag, D. *Methods Enzymol.* **1999**, *308*, 246–276.
(22) Schwans, J. P.; Kraut, D. A.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14271–14275.
(23) Zeng, B.; Pollack, R. M. *J. Am. Chem. Soc.* **1991**, *113*, 3838–3842.
(24) Wilde, T. C.; Blotny, G.; Pollack, R. M. *J. Am. Chem. Soc.* **2008**, *130*, 6577–6585.
(25) Bobofchak, K. M.; Pineda, A. O.; Mathews, F. S.; Di Cera, E. *J. Biol. Chem.* **2005**, *280*, 25644–25650.
(26) Simon, L.; Goodman, J. M. *J. Org. Chem.* **2010**, *75*, 1831–1840.
(27) Brenner, C.; Bevan, A.; Fuller, R. S. *Curr. Biol.* **1993**, *3*, 498–506.