Commentary

SCAM Feels the Pinch

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The paper by Movileanu et al. (2001) in this issue of *The Journal* describes a test of the applicability of the substituted-cysteine-accessibility method (SCAM; Akabas et al., 1992; Akabas et al., 1994) to the problem of locating the narrowest region of a channel lumen. Movileanu et al. (2001) use unusually large sulfhydryl-directed reagents to probe the wide bore channel formed by Staphylococcal α-hemolysin. The structure of the membrane-associated form of this protein has been solved to high resolution (Song et al., 1996). It is a mushroom-shaped heptameric complex that inserts in the membrane of a susceptible cell with the wide cap on the extracellular side, or on the cis side of an artificial planar lipid membrane, and with the stem traversing the bilayer (see Figure 1 in Movileanu et al., 2001). The entrance to the pore at the extracellular or cis end is 30 Å wide. The pore immediately narrows to ∼20 Å, and then widens into a large vestibule 46 Å across. The pore again narrows to ∼16 Å, its major constriction, and then continues to its intracellular or trans end as an irregular cylinder, which is roughly 20 Å wide. Bayley and co-workers (Movileanu et al., 2001) demonstrate that they can locate the narrowest constriction of the pore by applying SCAM with large enough reagents.

The reagents were 2-pyridyl disulfide derivatives of polyethylene glycol (PEG) of average molecular masses: 1,000, 1,800, 2,500 and 5,000 D. They react to form mixed disulfides with cysteine in which the PEG moiety is attached to the cysteine sulfur. If these reagents were unhydrated spheres, their diameters would be ∼16, 19, 21, and 27 Å; all but the first too large to pass through the α-hemolysin pore. However, elongated configurations must be prevalent because all but the 5-kD reagent pass through the pore.

Cysteine-substituted α-hemolysin mutants were synthesized in vitro by coupled transcription and translation, purified, and incorporated into artificial planar bilayers. The reactions of the cysteines with the PEG reagents were monitored by their effects on the conductance of the pore, which were irreversible by washing but reversible by reduction. The basis for locating the narrowest constriction of the channel is simply that, all other things being equal, the rate of reaction of a reagent added to one side of the membrane with a cysteine on the near side of the constriction will be faster than the rate of reaction with a cysteine on the far side of the constriction. However, all other things are seldom equal.

Numerous factors can influence the reaction rate of a cysteine in a pore (Wilson and Karlin, 2001). There are two kinds of processes: (1) the transfer of reagent to and from the vicinity of the cysteine, which determines the local concentration of the reagent; and (2) the reaction of local reagent with the cysteine (Pascual and Karlin, 1998b). The transfer rate constants depend on steric hindrance and electrostatics along the pathway to the cysteine, whereas the local reaction rate constant depends on local steric hindrance and electrostatics. The reaction considered here with mercaptopyridine derivatives, like the reaction with methanethiosulfonate derivatives, takes place exclusively with the deprotonated cysteine sulfhydryl (Roberts et al., 1986). Hence, the local reaction rate depends on the sulfhydryl pK_a and the local pH, which also depends on local electrostatic interactions.

All of the factors influencing the overall reaction rate are likely to be different at different locations in the pore. How then can reaction rates be used to locate a constriction in the pore? The trick is to normalize the rates so that all factors cancel except for the effect of the constriction. The reagents, of course, must be large enough that their transfer rate constants are significantly lowered by the constriction, and a large enough number of substituted cysteines must be tested to bracket the constriction and to establish a pattern of reactivity on either side of it.[1]

Bayley and co-workers (Movileanu et al., 2001) normalized the rate constants for the reactions of the 1.8-, 2.5-, and 5-kD reagents by the rate constants for the reactions of the 1-kD reagent. The larger the reagent, the

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[1] The effect of steric hindrance along the pathway to a cysteine will depend on whether the concentration of reagent in the vicinity of the target cysteine is at equilibrium with the reagent in the medium or is in a steady state. When transfer rates are greater than the local reaction rate, reagent in a pore closed at one end, or reagent added to both sides of a pore open at both ends, will be at equilibrium with reagent in the medium. In contrast, reagent added to one side only of a pore that is open at both ends (the condition here) will be in a steady state. In the steady state, but not in the equilibrium state, the local concentration of reagent will be sensitive to steric hindrance along the pathway.

more its rate of reaction with a cysteine on the far side of the narrowest constriction in the pore will be slowed; and if all other factors affecting the rate constants were nearly the same for all the reagents, then the normalized rates should be ~1 with cysteines on the near side of the constriction and much <1 with cysteines on the far side of the constriction.

Bayley and co-workers (Movileanu et al., 2001) tested seven substituted cysteines, located from one end of the pore to the other. The rate constants were determined for the reactions of each of these cysteines with each of the four PEG reagents added on the cis side of the membrane and separately with the reagents added on the trans side of the membrane. The seven cysteines, starting from the cis end of the pore and progressing to the trans end, were at sequence positions 8 (cis end), 106, 111, 113, 117, 135, and 129 (trans end). Their locations in the lining of the pore are known from the high resolution structure. The narrowest constriction in the pore is around residue 113.

As expected from the discussion above, the rate constants of a given reagent with the different cysteines, in some cases with near neighbors, differed by orders of magnitude. The rate constants of the three larger reagents normalized by the rate constants for the 1-kD reagent, however, were more regular. With reagent on the cis side (as in Figure 1 of Movileanu et al., 2001; we will take this as up), the normalized rate constant fell off below 117 for the 1.8-kD reagent and below 106 for the 2.5- and 5-kD reagents. There was no detectable reaction of the 5-kD reagent below 106. With reagent added on the trans side (we will take this as down), the normalized rates fell off above 111 for the 1.8-kD reagent, above 117 for the 2.5-kD reagent, and above 113 for the 5-kD reagent. There was no detectable reaction of the 5-kD reagent above 113.

The implications are different with the different reagents. The results with the 5-kD reagent indicated that there is a rate-limiting constriction between 106 and 113. The results with the 2.5-kD reagent indicated that there is a constriction between 106 and 117. The results with the 1.8-kD reagent did not bracket the constriction.

There is another way for us to normalize the rate constants: at each cysteine, we divide the rate constant for reagent added from the cis side by the rate constant for reagent added from the trans side. The basis for this normalization is the assumption that once a reagent molecule gets to a cysteine, the path by which it got there will not affect the local reaction rate (Wilson and Karlin, 1998). Thus, by our dividing the rate constants for reagent added to the one side by the rate constant for reagent added to the other side, we factor out all local influence on the rate constants. Only the different rates of transfer to the cysteine remain. If we carry out this normalization (by dividing the cells in Table II by

TABLE I
Rate Constant for Reagent Added to the Cis Side Divided by
Rate Constant for Reagent Added to the Trans Side

	T129C (trans)	L135C	T117C	M113C	E111C	S106C	K8C (cis)
1-kD	0.81	0.33	0.83	0.89	0.94	0.46	2.6
1.8-kD	0.18	0.084	0.97	0.84	1.3	0.87	49
2.5-kD	0.12	0.13	0.2	0.59	2.4	1.2	53
5.0-kD	<0.3	<0.1	<0.03	<1	ID*	>1.3	>39

*ID, indeterminate.

the cells in Table I of Movileanu et al., 2001), we get the results shown in Table I.

These ratios of rates reflect the steady-state reagent concentration at each cysteine after reagent was added to the cis side divided by the steady-state reagent concentration at the same cysteine after reagent was added to the trans side. For all four reagents, there is an obvious change in the ratios between S106 and K8. There was a significantly higher concentration of reagent at K8 for reagent added on the cis side than for reagent added on the trans side and vice versa for S106. After reagent is added to the cis side, the concentration at K8 is likely to be close to the bulk concentration in the medium on the cis side. After passing through the constriction between K8 and S106, however, the reagent is likely to be rapidly diluted in the large cavity; consequently, the steady-state reagent concentration is higher at K8 than at S106. Conversely, reagent coming from the trans side dilutes rapidly as it passes the constriction between S106 and K8 into the cis-side extramembranous medium; consequently the steady-state reagent concentration is higher at S106 than at K8. Thus, the ratios of rates are consistent with the minor constriction seen in the crystal structure.

With regard to the major constriction around M113, the ratios for the two larger reagents indicate a dividing line between significantly higher concentration from the cis side and significantly higher concentration from the trans side around M113. Thus, looked at two ways, the data of Movileanu et al. (2001) conform with reasonable fidelity to the high resolution structure of the pore.

Gates are channel obstructions that move, and SCAM has been used to locate gates in a voltage-gated K$^+$ channel (Liu et al., 1996, 1997) and in an acetylcholine receptor channel (Wilson and Karlin, 1998, 2001). Liu et al. (1997) determined the ratio of the rate constants for the reactions of charged methanethiosulfonate reagents with cysteines substituted in the S6 segment of the *Shaker* K$^+$ channel in the open and closed states of the channel. The reagents were added only to the cytoplasmic side of the membrane, and the ratio of the rate constants reflected a combination of changes in accessibility from the cytoplasmic end of the pore and possible changes in the local environment of the target cys-

teine. With additional clever experiments involving the trapping of Cd^{2+} ions, the combined results could be interpreted with little ambiguity to locate the gate.

In the acetylcholine receptor, the region of the gate was accessible to small, charged methanethiosulfonate reagents from either side of the membrane, and it was possible to determine the ratio of rate constants both with respect to the side of application and with respect to the state of the gate (Wilson and Karlin, 1998). In this ratio of ratios, the influence of local structural changes was eliminated, leaving only the ACh-induced changes in transfer rates to the target cysteine from the two sides. The effect of opening the gate was small when the cysteine was between the gate and the end of the channel to which reagent was added and was large when the cysteine was on the other side of the gate. The occluded region forming the gate in the resting state was located within a stretch of three or four residues at the cytoplasmic end of the channel. In the desensitized state, the occluded region of the channel expands to include a stretch of seven to nine residues (Wilson and Karlin, 2001).

SCAM requires some clues about the structure being probed. In the first instance, these clues guide the selection of residues to be mutated to cysteine. SCAM can provide rigorous tests of the assumed structures (Lu and Miller, 1995; Kuner et al., 1996; del Camino et al., 2000). It can also provide information not readily available by other means about structural dynamics (Kellenberger et al., 1996; Larsson et al., 1996; Yang et al., 1996), channel blocker sites (Kirsch et al., 1994; Pascual et al., 1995; Xu et al., 1995; Gross and MacKinnon, 1996; Pascual and Karlin, 1998a), and electrostatics (Stauffer and Karlin, 1994; Cheung and Akabas, 1997; Pascual and Karlin, 1998b; Wilson et al., 2000). Until the 3-D structures of all channel proteins are known, and even after, SCAM and other methods that probe surface properties will continue to help us understand how these proteins work.

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