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Inversion of a behavioral response in bacterial chemotaxis: Explanation at the molecular level

(flagella/polymorphism/*cheU* gene/response regulator)

SHAHID KHAN*, ROBERT M. MACNAB*, ANTHONY L. DEFRANCO†, AND D. E. KOSHLAND, JR.†

* Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520; and † Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT **Certain *cheU* mutants of *Salmonella* show inverted chemotactic behavior, being repelled by attractants and attracted by repellents. Such a dramatic change in behavioral pattern would seem at first glance to require drastic and complex alterations in the sensory processing system. In fact, the behavior can be explained by a simple shift in the level of a response regulator and the subtle effects of this shift on flagellar function. Flagella can exist in either a left-handed or a right-handed structure depending on applied torsion. Wild-type cells swim smoothly by counterclockwise rotation of a left-handed helical bundle and tumble when the motors briefly reverse to clockwise rotation (normal random motility). The *cheU* mutation causes a shift in response regulator level relative to the critical threshold value, resulting in extended clockwise operation so that the flagella are fully converted to the right-handed helical form. These cells therefore swim smoothly by clockwise rotation of a right-handed bundle and tumble when the motor briefly reverses to counterclockwise rotation (inverse random motility). Thus, tumbling is associated with brief reversals and not with a particular sense of rotation. A wild-type cell, with its steady-state response regulator level placing it initially in normal random motility, will swim smoothly on addition of attractant, whereas a *cheU* mutant with inverse random motility will tumble given the same stimulus. The phenomenon illustrates the profound behavioral consequences that can result from a single mutation in a key gene.**

Bacterial chemotaxis has emerged as a simple sensory and motor system, the study of which may provide useful clues for an understanding, in molecular terms, of more complex behavioral systems. The bacterial system is attractive because it is possible to analyze the components of the system—receptors, processing apparatus, and motors—with far more ease than in mammalian systems. Indeed, much progress has already been made in interpreting the bacterial system at the molecular level (1–5).

Recently, an intriguing set of *Salmonella* mutants, possessing inverted chemotactic responses, was described by Rubik and Koshland (6). Cells responded to attractants as though they were repellents, and vice versa. Moreover, this inverted behavior appeared to be the result of a single point mutation in the *cheU* gene. Thus, the alteration of a single protein changed a wild-type cell, which swam toward attractants such as serine and away from repellents such as phenol, into a mutant that swam away from serine and toward phenol.

Previous studies had established that unstimulated bacteria randomly alternate between swimming and tumbling behavior. The cells achieve chemotactic migration because of selective suppression of tumbles by positive attractant gradients (7, 8) or negative repellent gradients (9). In experiments with tethered cells, tumble suppression (smooth swimming) has been identified with counterclockwise (CCW) rotation of the flagella, and tumbling with clockwise (CW) rotation (10).

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A wide variety of chemotactic phenomena have been explained by a response (tumble) regulator model (2, 8, 9) in which the fluctuations of the regulator relative to a critical threshold level, X_{crit}, are presumed to determine bacterial behavior. Since only one of the many gene products that are vital to the sensory transduction system was altered in the inverted behavior mutants, compelling arguments could be made against simple inversion of the sensory or motor logic (6). What, then, could be the basis of the inverted behavior?

One clue to this dilemma was the finding of Macnab and Ornston (11) that a central feature of tumbling in peritrichous bacteria is the mechanically induced interconversion between the normal flagellar structure, which is a left-handed (LH) helix, and a quite distinct structure, the curly helix, which is right-handed (RH). This introduces another logical element (LH/RH) into the system. They emphasized that tumbling occurs during the process of transition between normal and curly structures. Moreover, they predicted that cells that completely converted all of their filaments to the curly structure as a result of sustained CW rotation would then swim smoothly and might give inverted chemotactic responses.

A second clue arises from the observation that the *cheU* gene maps (12) in the same locus as the *flaQ* gene (nonflagellate mutant phenotype) (13) and hence it may be at the interface between the sensory and mechanical components of the system (6). If so, an alteration in this gene product could alter the relationship between the response regulator level X and the critical value X_{crit} for reversal of rotation, without changing the basic logic of the response regulator.

A combination of the concepts of LH–RH helical interconversion and the response regulator model leads to the hypothesis shown in Fig. 1. Two swimming modes are shown—at one extreme the normal mode with CCW rotation of an LH helix (CCW–LH) and, at the other the inverse mode with CW rotation of an RH helix (CW–RH). Thus, smooth swimming could be caused by either an abnormally high or an abnormally low level of response regulator. At less extreme levels, swimming would be punctuated by tumbles, giving rise to random (normal) or random (inverse) motility. In some intermediate range pure tumbling would be expected.

This hypothesis was used to explore the inverted chemotactic behavior of the *cheU* mutants, which we show in this paper to derive from a very strong steady-state bias to CW rotation.

MATERIALS AND METHODS

Bacterial Strains. The strains used are all derivatives of wild-type *Salmonella typhimurium* LT2. ST1 is the result of a motility selection (14). The *cheU* mutants, ST120, ST134, and ST155, were obtained by diethyl sulfate mutagenesis of a *his*,

Abbreviations: CCW, counterclockwise; CW, clockwise; LH, left-handed; RH, right-handed; FPA, *p*-fluorophenylalanine.

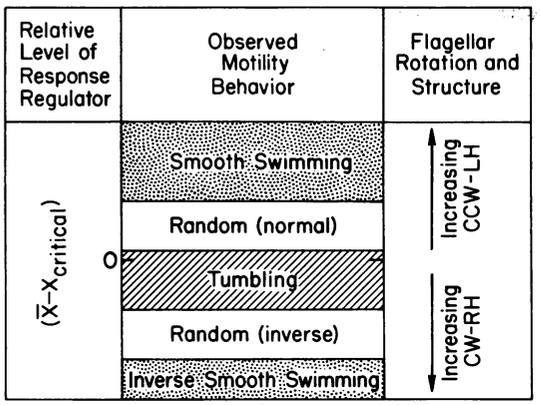

FIG. 1. Scheme to describe motile behavior of *Salmonella* in terms of relative response regulator level ($\bar{X} - X_{crit}$), sense of flagellar rotation (CCW/CW), and helical structure (LH/RH) of the flagellar filaments. The inverse smooth zone is shown narrower to conform with experimentally observed asymmetry.

thy auxotroph (ST23) (14). SL4041 is a tumbling *cheX* mutant obtained by Vary and Stocker (15). Cells were grown at 37° in nutrient broth or in minimal citrate medium (16) plus glycerol (1%, vol/vol), histidine (20 μg ml^{-1}) and thymine (100 μg ml^{-1}).

Microscopy. Flagellar filaments were observed by high-intensity dark-field light microscopy (11, 17). Video records were made with a Dage/MTI 650 silicon intensifier target television camera and a Panasonic NV8030 recorder. The suitability of this camera for recording flagellar filaments was first demonstrated by Hotani (personal communication).

Tethering Procedures. Cells were blended for 45 s in an Eberbach 8580 semi-micro container by a Waring blender 1120 and incubated with shaking at 35° to allow partial recovery of flagellation. After 25 min, chloramphenicol was added (to 100 μg ml^{-1}) to the cells, which were then spun down (1600 × *g*, 10 min) and resuspended in tethering medium (10 mM potassium phosphate, pH 7.5/0.1 mM potassium EDTA/67 mM NaCl) plus chloramphenicol at 100 μg ml^{-1}. Cells were tethered to microscope coverslips coated with antifilament antibody (18) and observed in a flow cell (19). The media were drawn from reservoir tubes maintained at 35 ± 0.5°.

***p*-Fluorophenylalanine (FPA) Treatment.** FPA was added to log-phase cells which were then deflagellated by blending for 45 s. After growth for 4 hr at 35°, cells had fully recovered flagellation. At 1.6 μg ml^{-1} FPA, *ca* 50% of the regrown flagella were curly, and at 5 μg ml^{-1}, they were all curly. These FPA concentrations are far below those required (*ca* 0.1–1 mg ml^{-1}) in previous procedures (11). Cells prepared by the present method are more vigorous, probably because of reduced FPA concentration and incubation time.

RESULTS

Direct Visualization of Flagellar Operation in the *cheU* Mutants. To test the idea that the *cheU* mutants might exhibit inverse swimming, flagellar operation was studied by high-intensity dark-field microscopy (11, 17, 20) and extensive videotape recordings were made. Swimming cells were seen to possess a flagellar bundle or, in many cases, groups of sub-bundles. Whenever cells slowed down sufficiently, the bundled filaments were in a short-wavelength "curly" form, like that seen transiently in tumbling wild-type cells (11). By flash photography, we confirmed that vigorously swimming cells also use the curly form (Table 1). Swimming with curly bundles was especially evident in high-viscosity medium (Fig. 2). No example of a cell swimming with normal bundles was noted for the three *cheU* strains (ST120, ST134, and ST155) that exhibited inverted chemotactic behavior. Other *cheU* mutants (ST203, ST213, and ST221), reported (6) as being smooth swimming and lacking inverted responses, showed the normal waveform.

Table 1. Comparison of normal and inverse swimming in *Salmonella*

Strain	Wavelength of flagella in bundle,* μm	Swimming speed,† μm s^{-1}
ST1 (wild-type)	2.36 (0.03)‡	34.3 (1.7)‡
ST120 (*cheU*)	—	17.3 (0.6)
ST134 (*cheU*)	1.19 (0.02)	20.6 (1.0)
ST155 (*cheU*)	—	20.0 (0.8)

* During a period of continuous illumination and recording, a shutter was quickly inserted, and opened for 2 ms. Data are means of 15 bundles.

† Speed measurements from video records of cells at 25° swimming on a microscope slide. Data are means of 10 vigorous cells during a 1 s interval of uninterrupted swimming.

‡ Standard errors (SEM) are given in parentheses.

Swimming by means of curly bundles looks somewhat different from normal swimming. Many cells fail to align their long axes with the direction of travel. Furthermore, the swimming speed is about one-half the normal value (Table 1), as might be expected from a helix with one-half the wavelength of the normal helix, but roughly the same pitch angle.

When the inverted mutants tumbled, bundle dispersal occurred accompanied by normal–curly polymorphic transitions, much as has been described previously (11, 20) both for wild-type and for tumbling mutants such as SL4041 (*cheX*). The curly waveform, however, was much more prevalent in the tumbling of *cheU* cells. Detached filaments from the *cheU* mutants were normal, emphasizing that the curly form in motile cells is mechanically induced by viscous torsion.

Right-Handedness of the Curly Form. Analysis of heteromorphous filaments (Fig. 3) demonstrates that the curly waveform observed in the *cheU* mutants is RH, as was found in other studies of the curly form generated during tumbling (11), at abnormal pH, or in flagellar mutants (22, 23). Values of wavelength (Table 1) and pitch angle (data not shown) agree with previous studies (11, 23).

A Novel Tethering Technique. To test these hypotheses tethering experiments were necessary, and this requires attachment by a single flagellar filament (18). Since the *cheU* strains are insensitive to catabolite or temperature repression of flagellation, a novel tethering technique was developed, involving deflagellation by blending and subsequent limited regrowth, arrested at a suitable time point by chloramphenicol. Regrowth for 25 min at 35° gave optimal tethering characteristics. Electron microscopy showed that these cells had on average 1.6 flagella, usually 2 μm or shorter, whereas unblended cells had 5–10 flagella, often as long as 10 μm.

No significant difference was observed between the rotational behavior of ST1 tethered after deflagellation and partial regrowth, and SL3625 (a leaky *fla* mutant) tethered directly. The presence of short, free filaments in blended cells and the use of chloramphenicol therefore do not appear to affect cell rotational behavior appreciably.

Rotational Analysis of *cheU* Mutants. According to the predictions of Macnab and Ornston (11), swimming by means of an RH helical bundle requires prolonged CW rotation. Tethering of the *cheU* mutants by the above procedure revealed (Table 2) that, as predicted, they spend a very large proportion of their time in CW rotation compared with wild-type cells or even tumbling mutants such as SL4041.

Tethering experiments were also used to examine chemo-

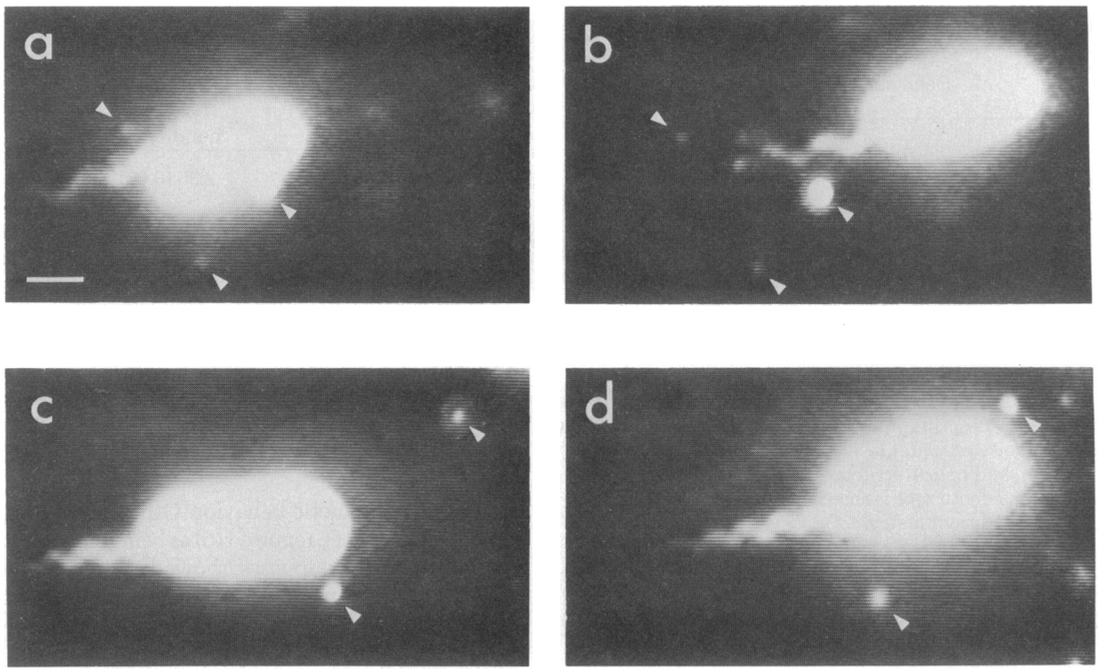

FIG. 2. Normal and inverse swimming in *Salmonella*. The cells are seen to have moved from left to right in the 1.3 s between images (stationary reference points, arrows). (*a,b*) Wild-type (ST1) swimming in normal CCW–LH mode. (*c,d*) *cheU* mutant (ST120) swimming in inverse CW–RH mode; note curly bundle. Cells are in 3% Methocel-100 to reduce swimming speed and enhance bundle formation. Bar = 2 μm.

tactic responses in the *cheU* mutants. The data (Table 3) show that chemotactic stimulation gave rotational responses of the conventional sense, namely enhancement of CCW rotation upon addition of attractants, and suppression of CCW rotation upon addition of repellents. ST155, which among the inverted *cheU* mutants exhibits most CCW rotation in an unstimulated state, gave a distinct response to a single attractant stimulus (0→10 mM serine), an appreciable fraction (25%) of cells being fully driven into CCW rotation and the remainder exhibiting oscillation between CCW and CW rotation. In the case of ST120 and ST134, simultaneous stimulation with serine and aspartate was needed to get an observable response, and no cell exhibited extended CCW rotation. This agrees with the observation that attractants cause ST120 and ST134 to tumble (6). The suppression of CCW rotation upon addition of repellents was readily demonstrated with ST155, but not with ST120 and ST134, on account of their low steady-state CCW incidence. A convincing indirect demonstration was the immediate cancellation of a CCW attractant response upon addition of the repellent phenol (Table 3).

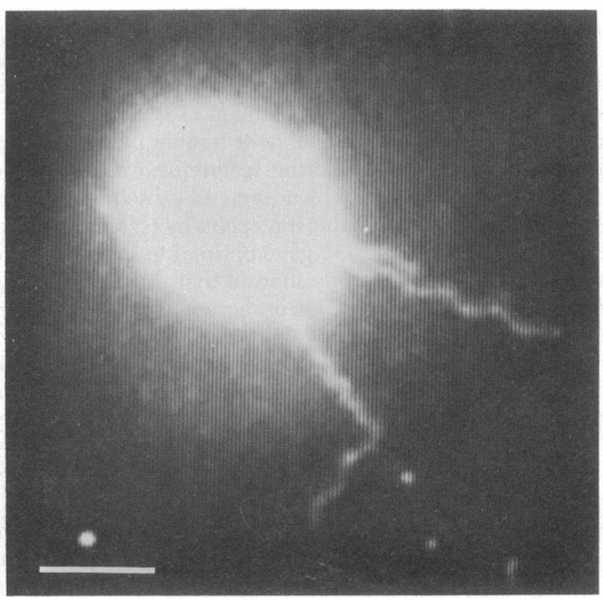

FIG. 3. Partially relaxed heteromorphous filament on *cheU* mutant (ST120) cell. A simple geometrical argument (11, 21) proves that the two waveforms in such a structure must be of opposite handedness; the normal waveform is known to be LH (20), and so the curly form must be RH. Bar = 5 μm.

Stability of Curly Form and Polymorphic Transitions. The incidence of CCW rotation in tethered cells of ST120 and ST134 is so low that one would expect a smoother swimming phenotype than is actually observed [see data of Rubik and Koshland (6)]. To examine whether the intrinsic instability of the curly helix was causing or prolonging erratic motility, cells were deflagellated and permitted to grow curly flagella by the presence of FPA (24). These FPA-treated cells were actively motile and swam somewhat more smoothly than untreated cells, but still tumbled quite frequently, indicating less intrinsic stability to the curly helix bundle than the normal CCW-LH bundle.

We next examined whether normal-to-curly transitions might significantly affect the observation of rotation of tethered cells. To estimate the length of free filament, we viewed tethered cells from the side by coating the edge of a cover slip with antibody. All rotating tethered cells appeared to be quite close (<0.5 μm) to the edge surface. The rotation associated with a normal-to-curly transition for a filament of 0.5 μm is *ca* 0.6 turns. The elastic torsional difference between CCW and CW rotation for a 0.5-μm filament rotating at 10 Hz is *ca* 0.2 turn (25). Between polymorphic transitions and elastic distortion there is therefore the possibility of damping to the extent of about 0.8 turn, which might mask brief intervals in the CCW mode.

DISCUSSION

The results above lead to the following conclusions: (*i*) Smooth swimming can be achieved by CW rotation of flagella in the RH helical structure as well as by the normal CCW–LH mode.

Table 2. Unstimulated behavior of tethered cells of *Salmonella*

Strain	Motility phenotype	Location of defect	Nonreversing (CW only) cells, %*	Fraction of time in CCW rotation, %		Duration of CCW interval, s (reversing cells only)†
				Reversing cells only†	All cells	
ST1	Wild-Type	—	0	87.5 (8.6, 2.0)	87.5	4.06 (3.05, 0.70)
SL4041	Tumbling	*cheX*	12	8.5 (9.3, 2.1)	7.5	0.60 (0.61, 0.14)
ST155	Inverse	*cheU*	70	3.5 (8.5, 2.0)	1.1	0.28 (0.32, 0.07)
ST120	Inverse	*cheU*	84	1.9 (3.4, 0.8)	0.3	0.13 (0.09, 0.02)
ST134	Inverse	*cheU*	92	1.5 (2.5, 0.6)	0.1	0.14 (0.13, 0.03)

* Fraction that showed no CCW rotation in a 60 s observation period, in a population containing at least 20 reversing cells.
† Cell means, based on samples of 20 vigorously rotating cells, recorded for 60 s each. Standard deviations and standard errors are given in parentheses.

(*ii*) Tumbling occurs as a result of the instability of the flagellar bundle caused by brief reversals of rotation, and can be observed for both CCW → CW and CW → CCW transitions. (*iii*) Wild-type and inverted chemotactic behaviors can both be explained by a single model involving different relative levels of a response regulator and an understanding of LH vs. RH filament stability. (*iv*) The same overall swimming patterns (smooth, random) can derive from different circumstances at the molecular level. These conclusions and their implications for behavioral systems in general are discussed below.

Smooth Swimming Can Derive from CW Rotation. Normal smooth swimming is known to result from CCW rotation (10) of LH helical flagella (11, 20, 26). Macnab and Ornston (11) showed that reversal to CW rotation, by placing the flagellar filaments under RH torsion, caused a progressive conversion of the filaments to an RH helical structure (curly), during which time the cells tumble. They predicted that, if CW rotation were to last long enough to fully convert all filaments to the RH structure, smooth swimming should result; and indeed they observed brief periods of such swimming—e.g., with a tumbling mutant in high viscosity medium. The prediction has now been confirmed by utilizing the *cheU* mutants.

The CW–RH, or "inverse," mode of smooth swimming was demonstrated in various ways: (*i*) direct visualization of the curly helical bundle with half the normal wavelength, (*ii*) establishment from heteromorphous filaments that the curly structure is RH, (*iii*) CW rotation of tethered cells, (*iv*) lowered swimming velocity, and (*v*) increased stabilization of the curly form by FPA treatment or increased viscosity of the medium.

Tumbling Occurs Because of Brief Motor Reversals. Originally, tumbling was described as a consequence of CW flagellar rotation (10). From this description, prolonged CW rotation should yield prolonged tumbling. In fact, as described above, prolonged rotation in either direction produces smooth swimming. Tumbling must result from brief motor reversals, with the flagella in transition between LH and RH structures (11), which causes the bundle to fly apart (20). Thus, tumbling in a wild-type cell, which swims smoothly in the normal CCW–LH mode, is a consequence of brief CW rotation, whereas tumbling, for cells which swim smoothly in the inverse CW–RH mode, results from brief CCW rotation.

This leads to the more complex scheme of swimming and tumbling shown in Fig. 1. Wild-type cells, swimming in the CCW–LH mode and tumbling as a result of brief reversals to CW rotation will execute a random walk pattern which, for convenience, we call "normal random." However, another way of generating a random walk is by swimming in CW–RH mode and tumbling as a result of brief reversals to CCW rotation. This behavior, which will prevail whenever the response regulator has a mean steady-state level $\overline{X}_{ss}$ well below the critical value X_{crit}, we call "inverse random." Both behavioral patterns are similar—alternate swimming and tumbling.

If reversals occur too frequently, the individual flagella never have the opportunity to form a stable bundle, and constant tumbling results. This will occur whenever $\overline{X}_{ss}$ is close to X_{crit}, because $\overline{X} - X_{crit}$ will then frequently fluctuate through zero. The tethered-cell data indicate that the interval of reversed rotation needed to generate a tumble is shorter in the inverse mode, probably because a curly bundle is more easily destabilized; thus, Fig. 1 has been drawn asymmetrically.

Explanation of Inverted and Normal Chemotactic Behavior. The inverted behavior of the *cheU* mutants can now be explained simply by postulating a change in the relative values of the response regulator at steady-state ($\overline{X}_{ss}$) and the critical value (X_{crit}) compared to wild type. Repellents and attractants can then alter the regulator level in the mutants in the same manner as in the wild type, but with quite different behavioral consequences.

The specific explanations of the inverted mutant (6) and wild type behaviors are given in Fig. 4. In each case, attractants increase the $\overline{X}$ level and repellents decrease it. Because the nongradient values of $\overline{X}$ are quite different, the behavioral responses are quite different. All three classes shown are superficially similar in the absence of a gradient, exhibiting random motility, but sudden increases of attractants move the wild type from normal random to smooth, the inverted mutants from inverse random to tumbling, and the partially inverted

Table 3. Chemotactic responses of tethered cells of *Salmonella*

Stimulus	ST1 (wild-type)	ST155 (*cheU*)	ST120 & ST134 (*cheU*)
(a) None (see Table 2)	Predominantly CCW	Predominantly CW	Predominantly CW
(b) L-serine (0→10 mM)	CCW only	Enhancement of CCW	Slight enhancement of CCW
(c) L-serine (0→10 mM) + L-aspartate (0→10 mM)	CCW only	CCW only, or marked enhancement of CCW	Enhancement of CCW
(d) Phenol (0→10 mM)	CW only	CW only	CW only
(e) Phenol [as in (d)], given during response to serine + aspartate [as in (c)]	Cancellation of CCW	Cancellation of CCW	Cancellation of CCW

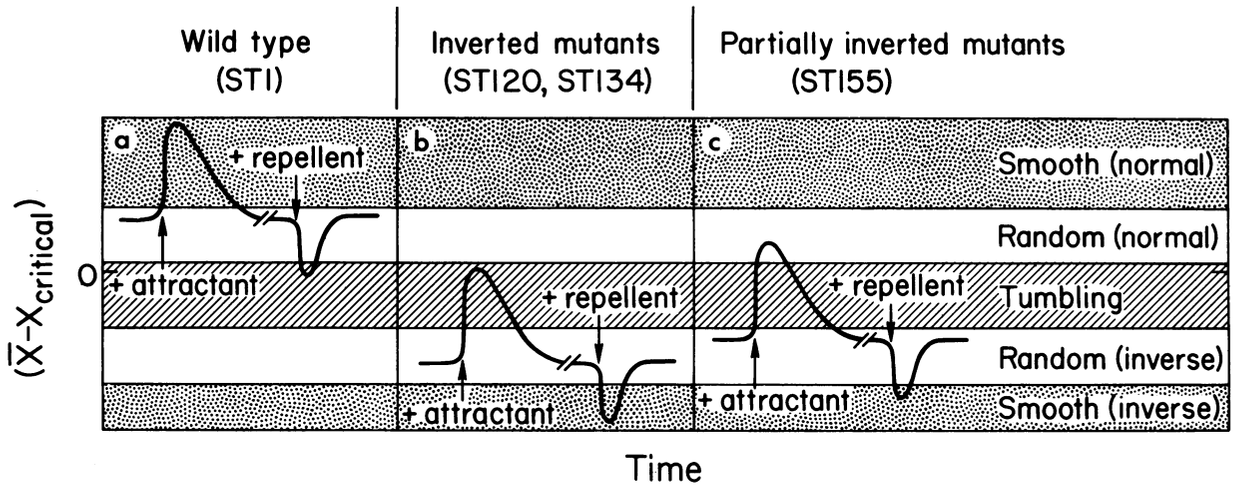

FIG. 4. Model for chemotactic response of wild-type and mutant cells. The observations (6) on inverted-behavior mutants are explained by identical changes in the average response regulator concentration, $\bar{X}$, by assuming that the relative value ($\bar{X}_{ss} - X_{crit}$) is different in the mutants. The instantaneous value, X, is presumed to fluctuate around the average value, $\bar{X}$, in a Poissonian manner. In each case $\bar{X}$ increases on addition of attractants and decreases on addition of repellents before adapting back to its original level. (*a*) In wild type, attractant increases $\bar{X}$ from normal random to smooth zone, whereas repellents decrease it to tumbling zone. (*b*) Inverted mutants have $\bar{X}_{ss}$ initially in an inverse random zone in which an increase in $\bar{X}$ causes tumbling, whereas a decrease causes smooth swimming. (*c*) Partially inverted mutant has $\bar{X}_{ss}$ in upper part of inverse random zone, so that a sudden increase moves it to normal random zone. This would be interpreted as no change in behavior, as was observed (6). Adaptation leads to drop in $\bar{X}$ through tumbling zone to inverse random, explaining the observed (6) delayed response.

mutant from inverse random to normal random (no apparent change in motility pattern). The smooth *cheU* mutants (6) are readily explained by an ($\bar{X}_{ss} - X_{crit}$) level so high that the bacteria are permanently smooth swimming.

All the *cheU* mutant behavior can thus be explained by the normal logic of the response regulator system operatir g with different initial values of $\bar{X}_{ss} - X_{crit}$. Since the mutations affect a protein at the interface between the sensory and mechanical aspects of the system (6), it is tempting to conclude that X_{crit} rather than $\bar{X}_{ss}$ is altered. However, it is sufficient to postulate that the mutation in the *cheU* gene alters the relative relationship between $\bar{X}_{ss}$ and X_{crit}. The inverted behavior then follows logically from the displacement into different zones of flagellar bundle stability.

Implications for Other Sensory Systems. These data have important implications for sensing systems in regard to genotype vs. phenotype. A phenotype of smooth swimming in bacteria cannot categorically be stated to result from CCW rotation. Nor can a bacterium with random swimming in a nongradient situation be assumed to be normal. The relative displacement of its response regulator level coupled with the mechanical properties of the flagella can thus produce deceptively normal behavior in some circumstances yet bizarre inverted behavior in others.

This finding may have important implications for other types of cells. The output of any cell, such as an electrical signal or the secretion of a hormone or antibody, involves events which are frequently as complex as tumbling and smooth swimming in bacteria. Hence a phenotypic inversion of response might well be observed in other systems without its involving a change in sensing or in processing of the initial signal but involving instead changes in the affinity of the response regulator for the final component at the output end of the system. The present results therefore emphasize the importance, for a proper understanding of sensory processes, of describing behavior in terms of molecular events.

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