

## A Free-energy-based Stochastic Simulation of the Tar Receptor Complex

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We recently developed a stochastic-based program that allows individual molecules in a cell signalling pathway to be simulated. This program has now been used to model the Tar complex, a multimeric signalling complex employed by coliform bacteria. This complex acts as a solid-state computational cassette, integrating and disseminating information on the presence of attractants and repellents in the environment of the bacterium. In our model, the Tar complex exists in one of two conformations which differ in the rate at which they generate labile phosphate groups and hence signal to the flagellar motor. Individual inputs to the complex (aspartate binding, methylation at different sites, binding of CheB, CheR and CheY) are represented as binary flags, and each combination of flags confers a different free energy to the two conformations. Binding and catalysis by the complex are performed stochastically according to the complete set of known reactions allowing the swimming performance of the bacterium to be predicted.

The assumption of two conformational states together with the use of free energy values allows us to bring together seemingly unrelated experimental parameters. Because of thermodynamic constraints, we find that the binding affinity for aspartate is linked to changes in phosphorylation activity. We estimate the pattern of Tar methylation and effective affinity constant of receptors over a range of aspartate levels. We also obtain evidence that both the methylating and demethylating enzymes must operate exclusively on one or other of the two conformations, and that sites of methylation of the complex are occupied in sequential order rather than independently. Detailed analysis of the response to aspartate reveals several quantitative discrepancies between simulated and experimental data which indicate areas for future research.

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### Introduction

Components of cell signalling pathways, including protein kinases, protein phosphatases and transmembrane receptors, are often physically associated in compact clusters of molecules which may be attached to the cell membrane or cytoskele-

ton (Mochly-Rosen, 1995). Rather than allowing signals to diffuse haphazardly in the cell to the next molecule in the pathway, these signalling complexes operate as computational units, each receiving one or more inputs, processing the signals internally, then generating one or more specific outputs. Signalling complexes provide an intermediate level of organisation analogous to the integrated circuits and microprocessors used in the construction of large electronic circuits. They could help us make sense of the seemingly impenetrable jungle of molecular interactions that characterises even the simplest forms of cellular communication.

The cluster of proteins associated with the chemotactic receptor of coliform bacteria, here called the Tar complex, is particularly well understood and illustrates many features found in larger

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Abbreviations used: E, receptor complex; Ep, phosphorylated receptor complex; E\*, active receptor complex; E-B, receptor complex plus CheB; E-R, receptor complex plus CheR; [EEEE], Tar receptor with 0-methyl groups; [EEEm], Tar receptor with one-methyl group, [EEEmEm], [EEEmEmEm], [EmEmEmEm], Tar receptors with two, three, and four methyl groups, respectively.

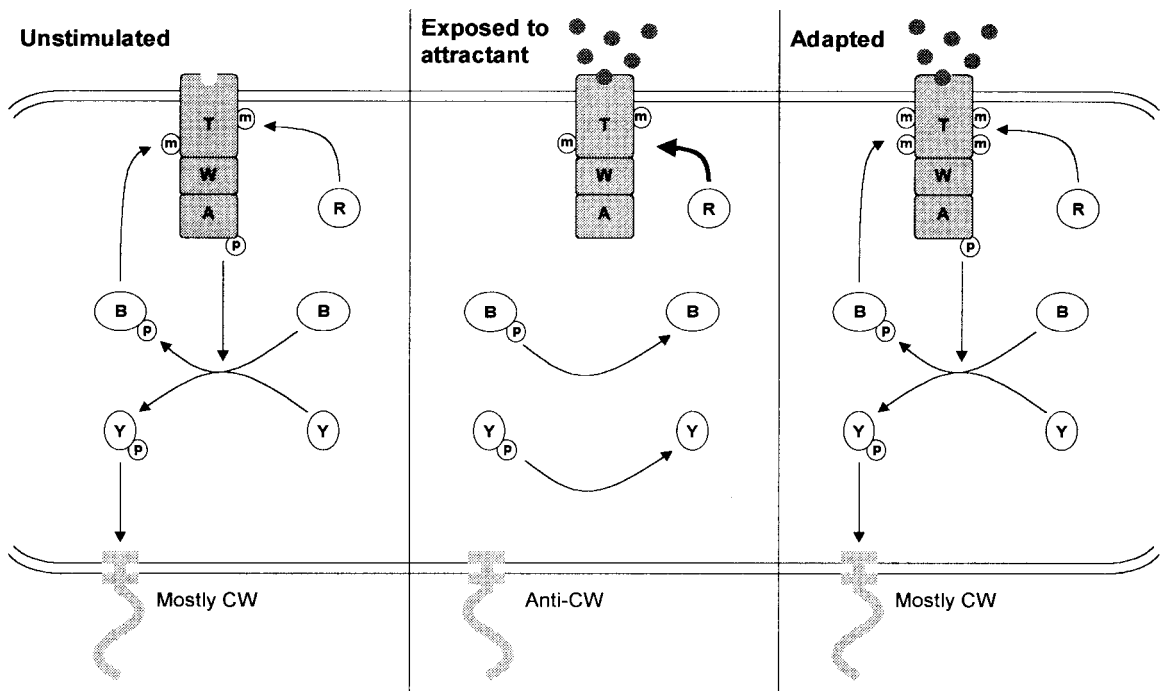
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eukaryotic complexes. The complex is built around a dimeric transmembrane receptor (Tar) that monitors the following extracellular stimuli: (i) the concentration of aspartate in the surrounding fluid (Clarke & Koshland, 1979); (ii) the concentration of maltose, through a specific interaction with the maltose binding protein (Mowbray & Koshland, 1987); (iii) nickel and cobalt ions, through the nickel binding protein NikA acting as repellents (De Pina *et al.*, 1995); (iv) ambient pH (Khan *et al.*, 1995); and (v) ambient temperature (Nara *et al.*, 1996). On its cytoplasmic domain, Tar is associated with a cluster of proteins through which it generates a signal sent to the flagellar motors, causing them to turn in a clockwise direction and hence generate a chaotic tumble.

Proteins associating with the Tar complex include the autophosphorylating protein kinase CheA, the transducing protein CheW, the methylating enzyme CheR and perhaps the protein phosphatase CheZ. A small highly charged protein CheY binds transiently with the Tar complex and is phosphorylated at a rate that depends on the level of activity of that complex. The phosphorylated product CheYp then diffuses to the flagellar motor where it interacts with the switch complex,

changes the direction of rotation of the motor, and hence modifies the swimming behaviour of the cell. A second diffusible cytosolic protein, CheB, which also interacts transiently with the Tar complex, removes the methyl groups added by CheR at a rate that depends on the level of stimulation. From a physiological standpoint, therefore, the Tar complex greatly simplifies the aspartate pathway, reducing it to a single freely diffusing species (CheY), a protein complex concerned with the stimulus (the Tar complex) and a second protein complex concerned with the behavioural response (the flagellar motor). This signalling pathway is illustrated in Figure 1 and reviewed by Parkinson (1993), Eisenbach (1996) and Stock (1994).

In a recent analysis of the mechanism of adaptation in coliform bacteria, Barkai & Leibler (1997) proposed a simple but elegant model that gives "exact" adaptation. That is, it returns to its precise initial state following exposure to a wide range of chemoattractant concentrations. Their model, based on an earlier suggestion by Asakura & Honda (1984), assumes that Tar complexes can exist in either an "active" or "inactive" conformation at any moment in time (the activity, here, referring to its ability to phosphorylate CheY).



**Figure 1.** Signalling pathway responsible for bacterial chemotaxis. In the absence of chemoattractants, the receptor complex, composed of Tar, CheW and CheA, is active in promoting the autophosphorylation of CheA (Gegner *et al.*, 1993). As discussed in the text, recent studies suggest that the receptor complex may exist in even higher-order structures (Bray, 1998; Liu *et al.*, 1997). Phosphoryl groups are transferred from CheA to CheY, forming phosphorylated CheY, which binds to FliM in the flagellar switch complex, increasing the probability that the motor will rotate in a clockwise direction. Attractants, such as the amino acid aspartate, bind to chemotaxis receptors and reduce the activity of the ternary complex. The rate of production of phosphorylated CheY is thereby decreased, causing the motor to revert to its native anticlockwise rotation state. In the continued presence of attractants, the chemotaxis receptors become more highly methylated as a result of: (i) the increased local activity of the methyltransferase CheR; (ii) the reduced local activity of the methylesterase CheBp; and (iii) the reduced global concentration CheBp, caused by a reduction in phosphate flux from CheA to CheB.

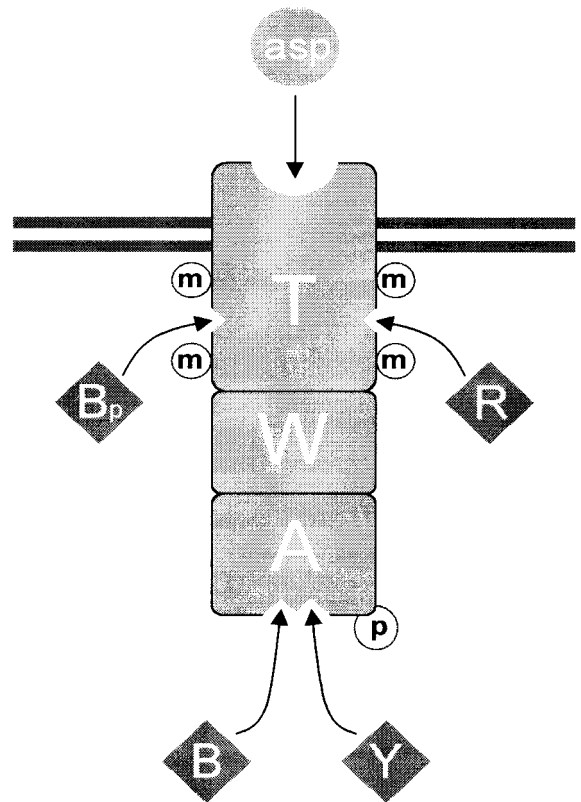
Aspartate binding reduces the probability that the receptor complex will be in an active conformation, in effect lowering the free energy of the inactive conformation relative to the active conformation. Methylation increases the activity of the receptors by stabilising the active conformation, ultimately restoring the pre-stimulus concentration of receptor complexes in the active conformation. Exact adaptation is achieved with the key assumption that demethylation is dependent solely upon the activity of the receptors, thus ensuring that for any equilibrium state, where the net rate of receptor methylation is zero, the activity of the receptors, and therefore the swimming behaviour of the cell, will be constant.

Here, we have extended the work of Barkai & Leibler (1997) to build a detailed and physically realistic computer model of the Tar complex that incorporates all of the chemical steps known to participate in its response to aspartate. The model employs recently developed software to represent individual molecules and molecular complexes as separate units interacting stochastically according to experimentally determined rate constants (Morton-Firth, 1998; Morton-Firth & Bray, 1998). Each complex in the population is represented in the computer as a discrete object that flips rapidly between two alternative conformations. The substructure of the complex and its precise composition are ignored in these simulations. The association of the complex with aspartate and other signalling molecules, as well as the state of methylation of key residues in the Tar molecule, are represented internally by binary flags. In combination, these flags control the probability of occupation of the two conformations (Figure 2). A key feature of this new approach is that wherever possible we relate rate constants and binding constants to changes in free energies of the different states, thereby obtaining a unified picture of the transformations undergone by the Tar complex.

## Results

### Conformational dependence of methylation and demethylation

Barkai & Leibler (1997) demonstrated that adaptation will be "robust" (exact over a wide range of starting conditions) only if the adapting modification (in this case, methylation) changes with the activity of the receptor. In their analysis, they proposed that the necessary dependence could be obtained if the phosphorylated form of CheB (CheB<sub>p</sub>), the species with highest demethylating activity, works only on the active conformation of the receptor. This is consistent with experiments showing that changes in receptor conformation caused by ligand binding can change the accessibility of methylation sites to CheB (Stock & Koshland, 1981). In our model, accordingly, we allow CheB<sub>p</sub> to bind receptors in an active conformation only.



**Figure 2.** Illustration of binding sites and methylation states for the chemotaxis receptor complex, composed of Tar receptors, CheW and CheA molecules. Receptor dimers can be in one of five methylation states (with zero, one, two, three or four methyl groups), and can also bind ligand, phosphorylated CheB and CheR; CheA dimers can be phosphorylated, and bind CheB and CheY. In addition, the receptor can associate with a chemoattractant or chemorepellent (in this case, aspartate) and the complex is either in an inactive or active conformation (data not shown). The diagram is not meant to represent the actual composition of the complex, nor the positions of binding sites on its surface.

Previous models have considered CheR binding to active and inactive receptors with different affinities, and sometimes assume that CheR works at saturation, so the rate of methylation by CheR will always be constant. However, we found in a series of exploratory simulations that if CheR works at saturation, adaptation cannot be exact. For a wild-type bacterium exposed to 1 mM aspartate, we predict a deviation of 6-7% from the pre-stimulus CheY<sub>p</sub> concentration after adaptation if CheR works at saturation. Furthermore, Michaelis-Menten kinetics require the concentration of substrate to be significantly higher than the  $K_M$  of the enzyme for saturation effects, which does not hold for the experimentally measured  $K_M$  of 4.2  $\mu$ M for CheR with the receptor (Simms & Subbaramaiah, 1981), and a total receptor complex concentration of 5  $\mu$ M (Gegner *et al.*, 1992). Therefore, to satisfy the requirement for activity-dependent methylation, CheR in our model is allowed to bind to

inactive receptor complexes only. Further support for this assumption comes from evidence that attractant binding changes the accessibility of Tar to CheR (Stock & Koshland, 1981).

### Constraints on activation probabilities

The activity of a receptor complex is determined by its ligand occupancy and methylation state (Borkovich *et al.*, 1989). In our computer model, this gives rise to ten probabilities of receptor complex activation, corresponding to ten free-energies of active relative to inactive conformation: five for non-ligand-bound receptors having zero to four methyl groups and five for aspartate-bound receptors having zero to four methyl groups. Here, we will refer to receptors with different levels of methylation as [EEEE], [EEEEm], [EEEmEm], [EEEmEmEm], and [EmEmEmEm] following the usage of Borkovich *et al.* (1992). Note that the brackets here signify composition, since the precise sequence of methylation of the Tar molecule is not known. Calculations reveal important constraints that must be imposed on these probabilities.

Firstly, receptors in the zero methylation state cannot be further demethylated. Therefore if CheBp works only on active conformations, then logically zero methylated receptors should be entirely in the inactive conformation. That is,  $p \sim 0.0$  for [EEEE]. For similar reasons, the conformational dependence of CheR implies that fully methylated receptors, which cannot be further methylated, will be fully active. That is,  $p \sim 1.0$  for [EmEmEmEm]. These limits also make sense from the standpoint of signalling efficiency, since they ensure that the full range of methylation (from [EEEE] to [EmEmEmEm]) corresponds to the full range of signal strength (from  $p = 0$  to  $p = 1$ ).

Secondly, measurement of CheA phosphorylation activation by Tar at different levels of methylation gives ratios of activity for [EEEE], [EEEmEm], and [EmEmEmEm] of approximately 0:0.5:1.0 (Borkovich *et al.*, 1992). Thus we take  $p = 0.5$  for unoccupied receptor with two methyl groups (the average level of methylation in an unstimulated, wild-type bacterium).

Thirdly, earlier work from the same laboratory showed that addition of aspartate to [EEEmEm] decreases phosphorylation to approximately 25% of its initial rate (see Table 4 in Borkovich & Simon, 1990). Hence we assign  $p = 0.125$  for the activity of occupied receptor with two methyl groups.

Finally, following adaptation to high levels of aspartate, the average methylation level of Tar rises from two methyl groups to three methyl groups (Stock, 1994). Since by definition these two states have the same activity, we assign  $p = 0.5$  to occupied receptor with three methyl groups.

Adopting the above  $p$  values as benchmarks, we can construct a list of free-energy changes for the other conformational states of the receptor (Figure 3). Free energies of activation for the two

methylated receptor, obtained from the values of  $p = 0.5$  and  $p = 0.125$  using the formula given in Methods and Calculations, are calculated as 0.0 kcal/mol and 1.18 kcal/mol, respectively. Note that the difference between these values is close to the value of 1.21 kcal/mol predicted from the  $K_d$  values (see Methods and Calculations). For reasons just given, occupied receptor with three methyl groups has a free energy change value 0.0 kcal/mol, so unoccupied receptor with three methyl groups must be  $-1.18$  kcal/mol ( $p = 0.874$ ).











Other values are assigned from these values with the additional constraints that  $p$  values for [EEEE] must be close to zero and for [EmEmEmEm] must be close to 1.0. In every case, moreover, the difference between ligand unoccupied and ligand occupied receptor must be 1.18 kcal/mol. So as to give specific values we have also adopted a quantal change of free energies of 1.18 kcal/mol for steps in methylation, corresponding to the effect of going from [EEEmEm] to [EEEmEmEm]. These latter figures are clearly arbitrary, however, and may have to be revised in the light of future data.

### Comparison with experimental observations

Incorporation of the free-energy values from Figure 3 into our stochastic model of the Tar complex allows us to predict the response of a bacterium to a range of aspartate concentrations. The change in swimming behaviour in response to the addition of saturating aspartate predicted by the stochastic simulation accurately matches experimental data within the limits of experimental and stochastic error. As shown in Figure 4, the simulated bias rises to a maximum immediately upon exposure to aspartate, and then falls asymptotically to its prestimulus value (Stock, 1994). Note that the experimental values in Figure 4(a), and the simulation in Figure 4(b) represent values averaged over of a large number of bacteria, whereas the simulation in Figure 4(c) shows the statistical fluctuations expected in the swimming of individual bacteria.

The duration of the response to aspartate predicted by the stochastic program used in this study (StochSim, see Methods and Calculations) is proportional to the amount of receptor bound, as in real bacteria. Experiments performed using methylaspartate, a non-metabolised derivative of aspartate, demonstrate this relationship (Spudich & Koshland, 1975). Taking into account the difference in receptor binding affinity between aspartate and methylaspartate, the duration of the response measured in these experiments agrees with that predicted by StochSim over four orders of magnitude in aspartate concentration, an impressively wide range (Figure 5).

The adaptational response shows an accurate return to pre-stimulus swimming behaviour over a wide range of aspartate concentrations (see Figure 4). At concentrations of aspartate less than

Non-ligand bound			Ligand bound		
Species	$p$	$\Delta G$ (kcal/mol)	Species	$p$	$\Delta G$ (kcal/mol)
	0.017	2.37		0.003	3.55
	0.125	1.18		0.017	2.37
	0.500	0.00		0.125	1.18
	0.874	-1.18		0.500	0.00
	0.997	-3.55		0.980	-2.37

**Figure 3.** Estimated free energy changes. Symbols represent the Tar receptor in different states of methylation and ligand occupancy (simplified from Figure 2);  $p$  is the probability of being in the active conformation and  $\Delta G$  is the associated free energy change (here given in kcal/mol) for the inactive-to-active transition. The basis for the estimates is given in the text.

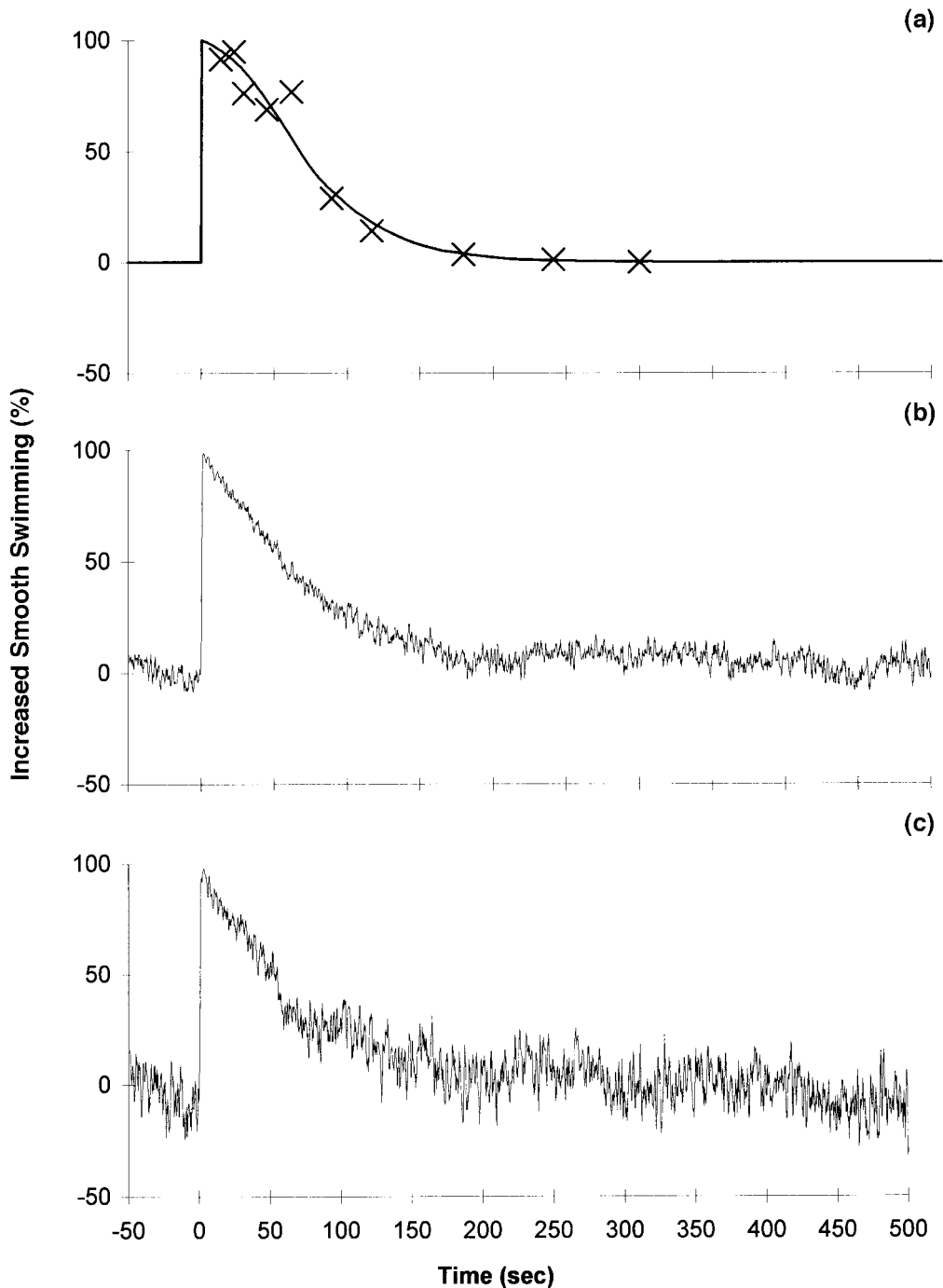
5  $\mu\text{M}$ , the predicted concentration of CheY $_p$  after adaptation is within 1% of the prestimulus value while at higher concentrations of aspartate the adaptation error rises to 2%. This value is well within the error due to stochastic fluctuations in CheY $_p$  concentration and certainly not measurable by current experimental techniques (Stock, 1994). However, it is interesting from a mechanistic standpoint to ask how it arises, and we return to this question below.

Each simulation generates an extremely large body of data relating to the receptor complexes in the bacterium over many thousands of time steps. We examined, for example, the detailed changes in the methylation of receptors as they adapted to different levels of aspartate. This revealed that although there was a net shift towards higher methylation levels (from an average of two to an average of three methyl groups per Tar, in agreement with experiment) the population was highly heterogeneous (Figure 6(a) and 6(b)). There was also a transient reduction in the effective  $K_d$  value for aspartate following stimulation due to a temporary decrease in the proportion of active receptors (Figure 6(c)). Many other parameters could in principle be followed. The amount of quantitative information in these simulations far exceeds anything yet measured experimentally, although where measurements have been made there is

excellent quantitative agreement, as in the dose response to aspartate, the time-course of response to a stepwise addition of attractant, and the duration of adaptation.

In contrast to the predicted adaptational response, the rapid "impulse" response to a short and briefly applied pulse of aspartate reveals significant discrepancies. Experimentally, if an impulse of aspartate is supplied at low concentration, the bias of wild-type cells increases sharply almost to 1.0, falls to a value below the pre-stimulus bias, and then returns slowly to base-line (Segall *et al.*, 1986). This "overshoot" phenomenon is usually explained by saying that the methylation of the receptor complex increases during the brief pulse of aspartate, so that immediately after withdrawing the stimulus, the receptor complex activity will be greater than pre-stimulus activity, leading to the overshoot in bias. Our simulations of the impulse response however exhibit no significant overshoot.

A second discrepancy concerns the sensitivity of the response to very low concentrations of aspartate. The model shows a response to aspartate at concentrations greater than about 100 nM (Figure 7) and exhibits a gain (change in bias divided by fractional change in receptor occupancy) of 0.9. Recent measurements using caged aspartate indicate the minimum threshold of detec-

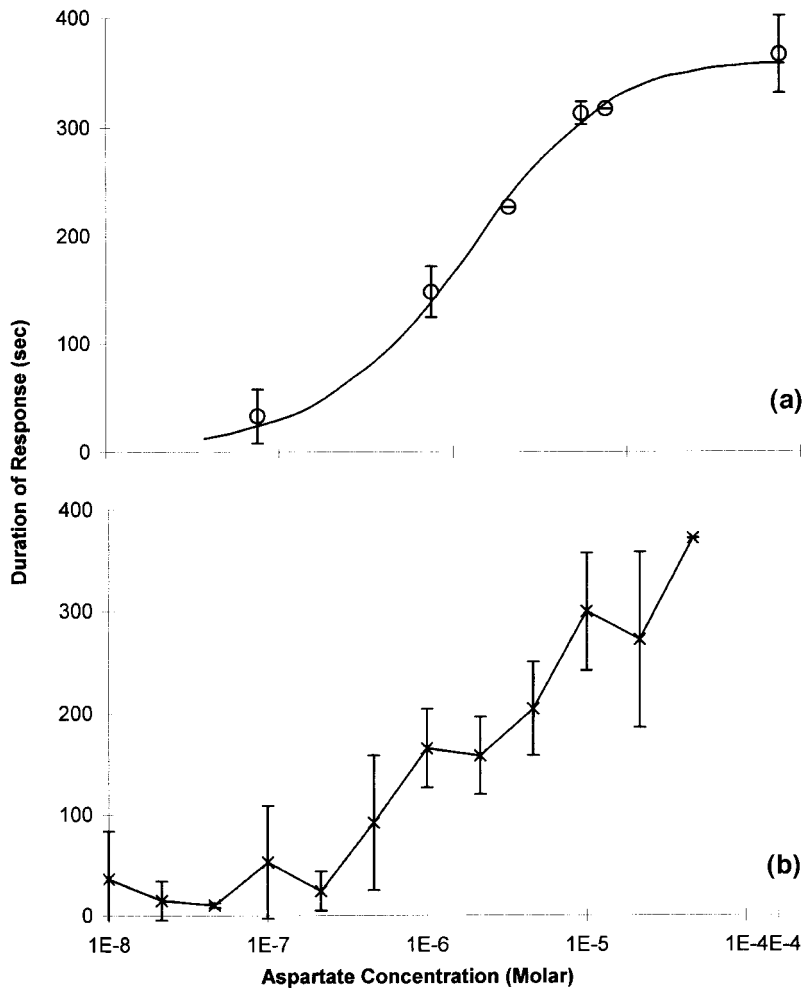


**Figure 4.** Time-course of adaptation, experimental and simulated. The increase in smooth swimming behaviour (bias) is measured as a percentage of maximum. Aspartate (1 mM) is added at 0 seconds. (a) Experimental data taken from Stock (1994). (b) The mean swimming behaviour predicted by five simulations. (c) Swimming behaviour predicted in a single simulation. In (b) and (c), the data have been smoothed by calculating a 1.0 second running average.

tion occurs for a 0.2% change in receptor occupancy, which corresponds to an aspartate concentration of 6 nM (R. Jasuja *et al.*, unpublished results) and a gain of 35 in a background concentration of 6 nM aspartate (S. Khan, personal communication). The effect of this large discrepancy is also visible in time-courses showing the response

to low concentrations of aspartate, where the experimentally measured bias might approach 1.0 (Segall *et al.*, 1986), whereas the bias predicted from the model does not change significantly.

The possible origin of these above differences between simulation and experiment are discussed opposite.



**Figure 5.** Duration of the adaptation response to different concentrations of aspartate. (a) Experimental data taken from Berg & Tedesco (1975), in which they measured the time between exposure of 17 bacteria to methylaspartate and the restoration of anticlockwise rotation. For comparison with aspartate data, the equivalent concentration of aspartate has been calculated to achieve the same level of Tar occupancy based on dissociation constants of  $160 \mu\text{M}$  and  $1.2 \mu\text{M}$  for methylaspartate and aspartate, respectively (Biemann & Koshland, 1994; Mesibov *et al.*, 1973). (b) Duration of the response predicted by Stoch-Sim. Each value represents the time taken for the bias to fall to within 0.001 of the pre-stimulus value, averaged over three simulations.

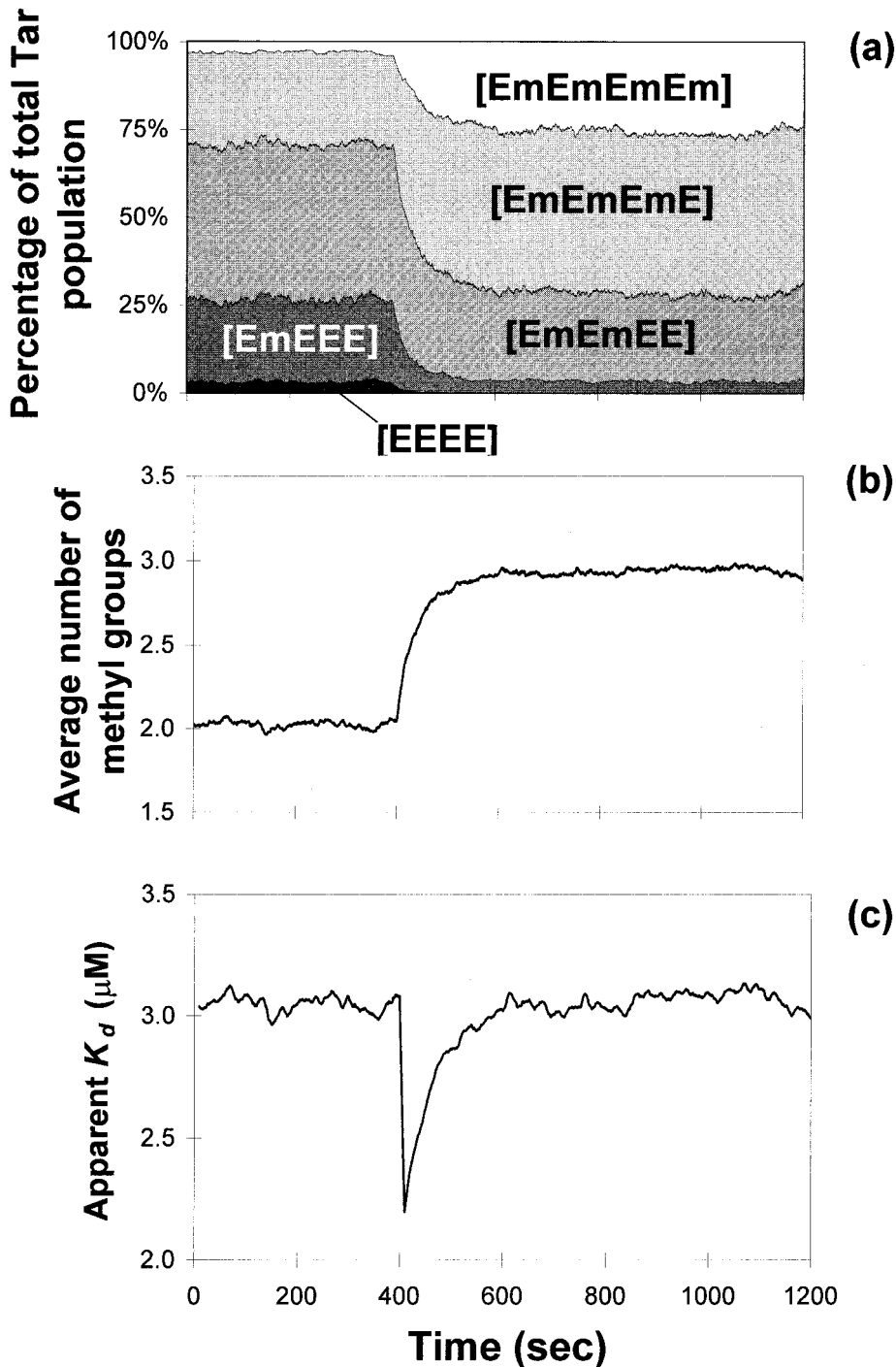
### Adaptation error

Although the simulated adaptation is sufficiently accurate to reproduce experimental findings it does show a small residual error. For example, if we simulate exposure to  $100 \mu\text{M}$  aspartate and allow adequate time for adaptation to take effect, then there remains a difference between pre-stimulus and post-stimulus CheYp concentrations of about 2%. The origins of this error are of interest from a mechanistic standpoint and can be readily dissected by means of the program. We find two sources of error: (i) those that affect the level of activation of the receptor complex, E, and (ii) those that affect the rate of phosphorylation of CheY.

The concentration of active ternary complex,  $E^*$ , shows a residual adaptation error within 2% after the addition of various concentrations of aspartate. This error results from the formation of the complexes E-B and E-R, which creates a small pool of receptor complexes which cannot bind CheR and CheB respectively (in that E-B cannot bind CheR, and E-R cannot bind CheB). Because of this small

pool, the rates of methylation and demethylation are not precisely proportional to the numbers of inactive and active receptor complexes. An error in adaptation is introduced in this manner that will be more noticeable the larger the change in aspartate concentration.

With regard to the second source of adaptation error, one might expect that if  $E^*$  adapts exactly after exposure to aspartate, the amount of phosphorylated receptor complex (on which the swimming behaviour depends) will also return to its pre-stimulus concentration. However, this is not necessarily the case. Phosphorylation occurs only on activated receptor complexes to produce  $E^*p$  whereas the dephosphorylation reactions that remove this phosphoryl group work on both active and inactive species (that is,  $Ep + E^*p$ ). Thus, if we replace the phosphotransfer reactions to CheY and CheB by a unimolecular reaction to dephosphorylate  $Ep$ , we find that the lower the concentration of  $Ep$ , the more exact the adaptation becomes. The reason for the loss of co-ordination between  $E^*$  and  $Ep$  concentrations become apparent with elementary analysis (see the Appendix).

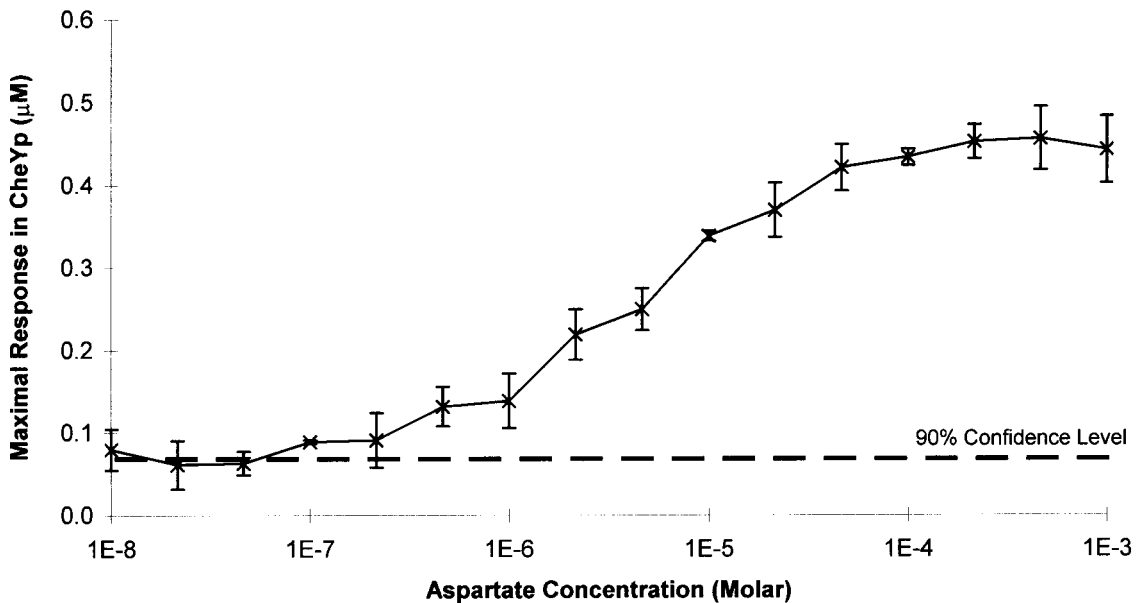


**Figure 6.** Changes in methylation of Tar following exposure to aspartate. Simulations show the response to a saturating concentration of aspartate (added at 400 seconds). (a) Distribution of different methylation levels, expressed as percentage of the total population of Tar receptors. This is a stacked area graph in which the value for each methylation level is represented by the height of its band. For example, the proportion of [EEEE] prior to stimulus is seen from the chart to be about 70-25%, or 45% of the total Tar population. (b) Average number of methyl groups per Tar receptor. (c) The apparent  $K_d$  value for aspartate.

### Local versus global adaptation

The mechanisms responsible for adaptation can be classified into two distinct categories: those that promote the adaptation of an individual receptor to its bound ligand and those that allow heterologous

receptors, not associated with ligand, to adapt under the indirect influence of the bound receptors. In our simulation, and in agreement with experimental evidence (Bourret *et al.*, 1991), CheR participates in local adaptation alone. CheBp, on the other



**Figure 7.** Predicted maximal response of CheYp to increasing concentrations of aspartate. Each point represents the mean of three simulations. The maximal response is taken as the minimum concentration of CheYp in the five seconds following the addition of aspartate. The horizontal line indicates the stochastic error in CheYp concentration in the absence of aspartate (calculated as the 90% confidence interval for CheYp over time). A maximal response below this line (corresponding to a threshold value of about 100 nM aspartate) cannot be differentiated from stochastic fluctuations in CheYp concentration.

hand, diffuses freely in the cytoplasm, binds to receptors in their active conformation and hydrolyses their methyl ester groups (Stock & Koshland, 1981). A reduction in phosphoryl transfer from Ep caused by ligand binding will thus lower the amount of Bp in the cell, and cause a consequent lowering in the rate of demethylation. In principle, this change should affect all receptors, whether or not they are the ones actually associated with the stimulating ligand.

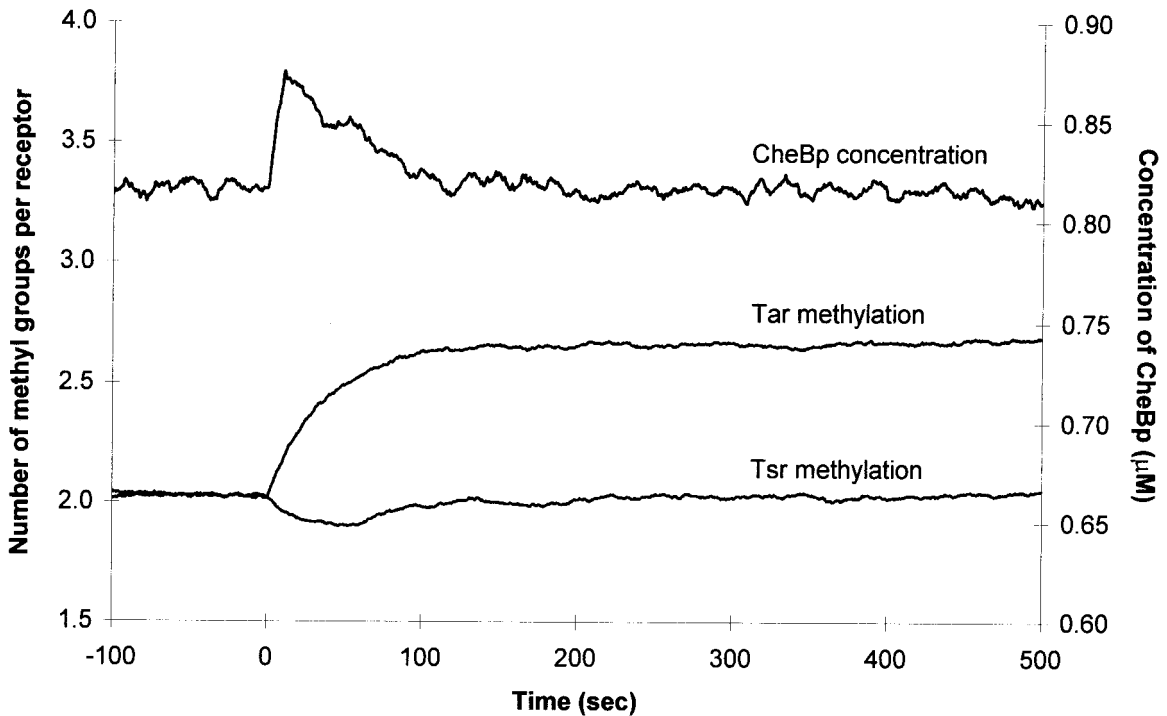
We have analysed the changes in methylation of Tar and Tsr in response to the addition of aspartate predicted by simulation. As expected, aspartate binding increases the methylation level of aspartate-sensitive receptors (Tar), which represent approximately 50% of the total receptor population (Ninfa *et al.*, 1991). Unexpectedly, however, the methylation level of non-aspartate-sensitive receptors (mainly Tsr) shows a fall rather than the expected rise, before returning to pre-stimulus levels (Figure 8). This result can be explained by observing that the cytosolic concentration of CheBp rises immediately after aspartate addition. The binding of aspartate to Tar causes an immediate fall in receptor complex activity and lowers its binding affinity for CheBp, thereby causing a release of free CheBp into the cytoplasm. At this stage, the reduction in CheBp formation by phosphotransfer from CheAp ensures that the rise of CheBp is small and short-lived. We will see in the Discussion that this pattern

might be changed if we assume conformational coupling between receptors.

### Ordered versus independent methylation

There has been a great deal of uncertainty over whether the four sites of methylation in Tar are methylated in a preferred order or independently. Early experimental evidence suggested that methyl groups are added by CheR and removed by CheB in a preferred order (Stock & Koshland, 1981). Later evidence seemed to contradict this theory: for example, Kehry *et al.* (1984) found that after the removal of attractant, there was a sharp increase in demethylation of sites which had been methylated before the addition of attractant, which seems inconsistent with a "last in, first out" pattern of methylation. Similarly, data reported by Terwilliger *et al.* (1986), demonstrated that all four sites undergo methylation and demethylation in the presence and absence of attractant.

In order to examine this question, we performed a series of test simulations in which methylation was assumed to be either (i) non-sequential, with each methylation site having an independent probability of being occupied, or (ii) sequential, with potential sites of methylation being occupied in turn. Under the first set of conditions, we found that the system fails to adapt exactly, and deviates by up to 50% of the pre-stimulus activity after



**Figure 8.** Global and local changes in methylation. The addition of  $10\mu\text{M}$  aspartate results in changes in the concentration of CheBp and associated changes in the number of methyl groups bound to Tar and the non-aspartate sensitive receptor, Tsr. The curves represent the average of three simulations (ten seconds running average for the concentration of CheBp).

exposure to attractant. By contrast, the second, non-sequential model gave exact adaptation over a wide range of stimulus conditions.

## Discussion

The simulation strategy used in our program was strongly influenced by our view of how signal complexes work. Software objects representing individual receptor complexes were equipped with sets of binary flags representing sites of covalent modification or binding sites. Each complex in the program flipped between one of two conformations with a probability determined by its current combination of binary flags. During execution of the program, discrete time-slices were simulated in each of which two molecular species (each a molecule or complex involved in chemotaxis) were selected at random. Interactions between the selected species (such as binding or catalysis) were determined using a look-up table containing the probability of their reaction and a random number generator, as described (Morton-Firth, 1998; Morton-Firth & Bray, 1998). Any individual complex could, if required, be traced throughout the course of a simulation as it passed through multiple states. The performance of the entire set of molecules in the bacterium was automatically recorded and could be displayed in different formats.

Our model provides an effective way to examine the behaviour of the aspartate detection system.

The program was fast enough to simulate the behaviour of all of the chemotactic signalling molecules in an individual bacterium over a period of several minutes of biological time on a modest desktop computer. Furthermore, because of its modular structure, the program was also transparent, allowing every possible state of the complex to be represented and all interactions included. For example, competition between CheB and CheR for binding to the Tar receptor and the conformational dependence of methylation and phosphorylation, were included explicitly in the model. Although some of these detailed interactions have been modelled previously, conventional simulations using multiple differential equations have difficulty in dealing with all of these manifold possibilities in combination.

## Free-energy changes

Our experience with this program led us to appreciate the central importance of conformational changes in the function of the receptor complex, and the usefulness of free-energy values in cataloguing these changes. Methylation, ligand occupancy and association with modifying enzymes, all result in changes in the free energy values of the two conformational states. The performance of the complex (its rate of phosphorylation, methylation and binding affinity for ligand) can all be derived from these free-energy values. Although direct measurements of free-energies have not been

made, we were able to estimate the free-energy differences between cognate active and inactive conformations for a range of states. Free-energy changes provide a convenient "currency" with which to relate the different actions of the complex. We believe that free-energy values will become increasingly useful as data on this and other signal complexes accumulates.

### $K_d$ values

Our use of free-energy values allowed us to correlate the change in activity associated with ligand binding to the binding affinity of that ligand. Furthermore, we found that we could make the simplifying assumption that the two conformations each had a fixed affinity constant for aspartate regardless of their level of methylation or association with Che proteins. The effective  $K_d$  for aspartate of an individual receptor then depended solely on the occupancy of the two states (that is, the free-energy changes between the two) and agreed with the relatively few measurements that have been made. Furthermore, precise estimates of  $K_d$  values could be made over the entire range of exposure to aspartate and used to calculate changes in occupancy, needed for precise measurement of gain. Note that we found no need to include a second site for aspartate binding on the Tar dimer. Experimental data suggests that in *Salmonella typhimurium* (Biemann & Koshland, 1994) and perhaps in *Escherichia coli* (R. Jasuja, personal communication) the Tar receptor has a second, lower affinity binding site for aspartate. However, we were able to obtain a sufficiently large range of values with a single site.

### Phosphotransfer rates

In our analysis we found a small source of adaptation error due to the dynamics of phosphorylation of CheY. As shown in the Appendix, this error arises from the accumulation of receptor complexes in their phosphorylated state, Ep. We can reduce this error, therefore, by minimising the population size of Ep at equilibrium. One approach is to increase the rate of association of CheY and Ep above the diffusion-limited rate constant of  $10^6 \text{ M}^{-1}\text{s}^{-1}$ , which is typical for protein-protein interactions (Northrup & Erickson, 1992). Another tactic is to assume that CheY binds to unphosphorylated receptor complex (Stewart, 1997) and that the resultant complex can undergo autophosphorylation with almost immediate phosphotransfer to CheY before dissociation. Simulations implementing this reaction exhibit further reductions in Ep concentration and increased accuracy in CheYp adaptation. In effect, binding of CheY primes the unphosphorylated receptor complex, allowing a more rapid removal of phosphate when autophosphorylation occurs, ensuring that association of CheY and Ep does not limit the rate of phosphotransfer.

### Methylation reactions

Our simulations provide fresh insight into the control over methylation and demethylation reactions. In agreement with Barkai & Leibler (1997), we found that exact adaptation requires that the demethylation reaction catalysed by CheBp works only on receptors in their active conformation. Moreover, we found that the opposing reaction of methylation, catalysed by CheR, must also be conformation sensitive, in this case working on the inactive state.

We also addressed the question of whether CheR transfers methyl groups to the four methylation sites on the Tar receptor in a strict sequence or randomly. Comparison of these two methylation strategies in test simulations clearly demonstrated that sequential methylation gives the most accurate adaptation over a range of aspartate concentrations.

We deduced that the failure to achieve exact adaptation with random methylation occurs because the overall rate of methylation of a receptor complex is no longer dependent solely upon the proportion of receptors that is in the active conformation. With each methylation event, the number of sites available for methylation falls, changing the maximal velocity of CheB and CheR. At equilibrium, therefore, when the net rate of methylation is zero, the activity, which is dependent on these maximal velocities, will vary according to the amount of receptor complex in each methylation state. By contrast, a model in which each methyl group is added in turn allows each methylation to occur at the same rate and to be influenced to the same degree by the conformation of the receptor. Exact adaptation can therefore be achieved.

It is relevant to note in this context, that the simulations reveal considerable fluctuations in numbers of methyl sites occupied over time. In a population of receptors with an average level of methylation of two methyl groups, for example, there will always be some receptors with zero, one, three or four methyl groups. Because of this heterogeneity, it is easy to understand why earlier experiments using radioactive pulses failed to show the last-in-first-out pattern expected of sequential methylation (Kehry *et al.*, 1984; Terwilliger *et al.*, 1986).

### Discrepancies with experiment

In our simulation of global adaptation we observed a transient decrease in methylation of heterologous receptors following an aspartate stimulus (see Figure 8). This decrease was traced to a sudden increase in cytosolic CheBp caused when this protein dissociated from Tar receptors upon aspartate binding. However, there is some experimental evidence that the methylation level of heterologous receptors actually rises rather than falls in this situation (Sanders & Koshland, 1988). The

**Table 1.** Chemical reactions used in the complete model of bacterial chemotaxis

Description	Reaction	$k_f$	$k_r$	Notes
Demethylation	$E_x^* + Bp \rightleftharpoons E_x^* Bp$	$1 \times 10^6$	1.25	Bp only binds E in an active conformation (Borczuk <i>et al.</i> , 1986; Sanders & Koshland, 1988; Stock & Koshland, 1981). With the lack of relevant experimental data, the dissociation constant of E-Bp is assumed to be the same as <i>in vitro</i> measurements of the dissociation constant of E-R, 2.5 $\mu\text{M}$ (Wu <i>et al.</i> , 1996) with a correction to take account of only 50% receptor activity in an unstimulated environment. Rate of association assumed to be diffusion limited.
	$E_x Bp \rightarrow E_{x-1} + Bp$	0.155		Rate of demethylation by phosphorylated CheB is $1.1 \times 10^{-1} \text{ s}^{-1}$ (converted from net rate of demethylation given by Lupas & Stock, (1989). However, not all CheB is phosphorylated so the rate must be adjusted accordingly (R. B. Bourret, personal communication; R. C. Stewart personal communication); simulations predict approximately 75% of CheB is phosphorylated.
Methylation	$E_x + R \rightleftharpoons E_x R$	$5 \times 10^6$	1.0	R only binds E in an inactive conformation (Shapiro <i>et al.</i> , 1995; Stock & Koshland, 1981; Terwilliger <i>et al.</i> , 1986). The concentration of R is much lower than E (see Table 2) and receptors are localised to the cell pole (Maddock & Shapiro, 1993), suggesting most R is bound to E.
	$E_x^* R \rightarrow E_{x+1} + R$	0.819		Rate of methylation calculated by equating the net rate of demethylation and methylation; in the absence of aspartate, receptors contain two methyl groups (Stock & Koshland, 1981; Terwilliger <i>et al.</i> , 1986).
Autophosphorylation	$E^* \rightarrow E^*p$	15.5		R.C.S., personal communication.
CheY reactions	$Y \rightleftharpoons Yp$	$1.24 \times 10^{-3}$	$4.5 \times 10^{-2}$	Autophosphorylation and autodephosphorylation (Lukat <i>et al.</i> , 1991; Stewart, 1997).
	$Yp \rightarrow Y$	14.15		Represents dephosphorylation of Yp by CheZ using bimolecular rate constant of $1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Lukat <i>et al.</i> , 1991) and CheZ dimer concentration of 14.15 $\mu\text{M}$ (Matsumura <i>et al.</i> , 1990).
CheY phosphotransfer	$Ep + Y \rightarrow EpY$	$5 \times 10^6$		Transfer of the phosphate group to CheY is very rapid (Stewart, 1997). Rates of association are likely to be rate-limiting in the cell. Here, chosen to be faster than the diffusion-limited rate constant of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ due to the speed of the overall phosphotransfer reactions (R.C.S., personal communication).
	$EpY \rightleftharpoons E + Yp$	20	$5 \times 10^6$	Dissociation constant of E-Yp is 4 $\mu\text{M}$ (Li <i>et al.</i> , 1995).
	$EY \rightleftharpoons E + Y$	7.5	$5 \times 10^6$	Dissociation constant of E-Y is 1.5 $\mu\text{M}$ (Stewart, 1997).
CheB reactions	$Bp \rightarrow B$	0.35		Stewart 19933a).
CheB phosphotransfer	$Ep + B \rightarrow EpB$	$5 \times 10^6$		Transfer of the phosphate group to CheB is very rapid (Stewart, 1997). Rates of association are likely to be rate-limiting in the cell. Chosen to be faster than the diffusion-limited rate constant of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ due to the speed of the overall phosphotransfer reactions (R.C.S., personal communication).
	$EB \rightleftharpoons E + B$	16	$5 \times 10^6$	Dissociation constant of E-B is 3.2 $\mu\text{M}$ (Li <i>et al.</i> , 1995).
	$EpB \rightleftharpoons E + Bp$	16	$5 \times 10^6$	Dissociation constant assumed to be the same as E-B.

E represents the receptor complex (Figure 4), which can exist in an active ( $E^*$ ) or inactive (E) conformation; a subscript indicates a particular methylation state (0, 1, 2, 3 or 4); Pr(active) indicates the probability that the receptor complex is in an active conformation. Further information is available on <http://aslan.home.ml.org/exptdata.htm>

**Table 2.** Initial concentrations used in the complete model of bacterial chemotaxis

Species	Concentration ( $\mu\text{M}$ )	Notes
E (aspartate sensitive, Tar)	2.5	Concentration of MCP monomer is 10 $\mu\text{M}$ (Gegner <i>et al.</i> , 1992); concentration of Tar monomer is 5 $\mu\text{M}$ (Ninfa <i>et al.</i> , 1991).
E (Tsr, Tap, Trg, Aer)	2.5	
R	0.235	Simms <i>et al.</i> (1987)
B	2.27	Simms <i>et al.</i> (1985)
Y	18	Zhao <i>et al.</i> (1996)

latter result could be explained if binding of aspartate to Tar receptors caused a concomitant decrease in activity of non-aspartate binding receptors such as Tsr, a form of adaptational "crosstalk" (Hazelbauer *et al.*, 1989).

A possibly related issue is that our model of bacterial chemotaxis failed to predict the relatively high gain observed experimentally in this signalling pathway (Khan *et al.*, 1993; Segall *et al.*, 1986; Spiro *et al.*, 1996). This was not a simple matter of adjusting a few rate constants, and no amount of tweaking of our current model increased gain by the necessary order of magnitude. To resolve this discrepancy we anticipate that future models will need to invoke novel mechanisms of signal amplification (Alon *et al.*, 1998).

An example of such a mechanism is the recently proposed spread of ligand binding signals across neighbouring receptor complexes (Bray *et al.*, 1998). The chemotactic receptors of *E. coli* have been found to associate in a cluster at one end of the cell (Maddock & Shapiro, 1993), and evidence has been obtained for cross inhibition between adjacent receptors (Gardina & Manson, 1996). Preliminary calculations and simple simulations indicate that the spread of conformation in a cluster of receptors could increase the size of the signal to a given stimulus and therefore in principle generate a larger gain (M. Levin & D. Bray, unpublished results).

A conformational spread mechanism could improve aspects of the performance of the model other than the gain. For example, it could also help to generate an overshoot following the application of a brief exposure to aspartate. As already mentioned, an overshoot was seen experimentally in early studies (Segall *et al.*, 1986), but here the size of the overshoot was undetectable under most conditions. However, if one ligand binding event causes a change of conformation of multiple receptors, then the number of receptors undergoing methylation will be increased by the same factor. Thus, the number of "excess" methylated receptors following a brief stimulus will be greater and could produce an overshoot of the required magnitude. Evidently, detailed examination of specific models will be needed to assess the strength of coupling between receptors and other parameters.

## Methods and Calculations

Simulations were performed using StochSim 1.0, a discrete, stochastic simulator, running under Microsoft Win-

dows 98 on a 400 MHz Pentium II PC with 128 MB of RAM. Details of the StochSim program and of the algorithms it employs, together with a discussion of the advantages of discrete stochastic modelling, are given elsewhere (Morton-Firth, 1998; Morton-Firth & Bray, 1998). The reactions given in Table 1 were simulated using the initial concentrations in Table 2, with a total cell volume of  $1.41 \times 10^{-15}$  l (Kuo & Koshland, 1989).

The model is based on the two-state model of chemoreceptor proteins (Asakura & Honda, 1984). Each receptor complex, E, contains two receptor molecules, two molecules of CheW and two molecules of CheA, and can be in either an active or inactive conformation. Possible interactions between receptor complexes due to their clustering on the surface of the bacterium (Maddock & Shapiro, 1993) have not been modelled, but are discussed here. The methylation state of the receptor complex is controlled by the methyltransferase CheR and the methyl-esterase CheB. CheB is activated by phosphotransfer from CheA and can bind only active receptor complexes, as proposed by Barkai & Leibler (1997; see Table 1). In contrast to the latter study, however, we assume that CheBp is free to diffuse away from the receptor complex that phosphorylates it and act on another receptor. This is supported by experiments with a constitutively active form of CheB, which lacks the normal inhibitory N-terminal domain and cannot interact with CheA. Cells carrying this modified CheB are able to adapt, presumably because the freely diffusing methyl-esterase is able to work on any receptors in their active conformation (for a review, see Bourret *et al.*, 1991).

CheR is not controlled directly, but we show here that in order to achieve accurate adaptation it must bind only inactive receptor complexes. There are no experimental data available to distinguish between the methylation state of the two receptor molecules in the Tar dimer. We therefore take the methylation state to be a property of the entire signalling complex rather than of the individual receptor monomer.

The Tar complex is assumed to exist in one of two conformational states, active and inactive. The probability of being in the active state ( $p$ ) depends on the binding of aspartate and on the level of methylation. We assume that the receptor complex only undergoes autophosphorylation when in the active conformation. Values of  $p$  for different states of the receptor complex were estimated in the course of this study (see Figure 3).

## $K_d$ values

We have assumed in our model that each of the two conformational states of the Tar receptor complex has a characteristic affinity for aspartate. The affinities of the two conformations do not change with methylation, but since the proportion of the two conformations will change, so too will the average affinity over time. Published values of the binding strength of aspartate to Tar vary over at least a factor of 10. Here, we have based

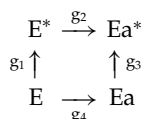
our  $K_d$  values on the value of 3.0  $\mu\text{M}$  for *E. coli* Tar with two methyl groups (that is, [EEEmEm]; Mowbray & Koshland, 1990). From Borkovich *et al.* (1992), we know that the ratio of activity of receptors with zero and four methyl groups respectively is approximately 1:7. Assuming that [EEEmEm] has equal amounts of the two conformations (see Figure 3), then the dissociation constant of the inactive conformation will be given by:

$$\frac{1}{2} \left( \frac{1}{K_d} + \frac{1}{7K_d} \right) = \frac{1}{3}$$

that is,  $K_d$  (inactive) = 1.7  $\mu\text{M}$  and  $K_d$  (active) = 12  $\mu\text{M}$ .

### Free energy calculations

For a receptor in the [EEEmEm] state we have the following equilibria:



Where  $g_1$ ,  $g_2$ ,  $g_3$  and  $g_4$  are the free energy changes. Note that for balance,  $g_1 + g_2 = g_3 + g_4$  or  $g_1 - g_3 = g_4 - g_2$ .

The value of  $g_4$  can be obtained from the  $K_d$  of binding of aspartate to the inactive conformation (1.7  $\mu\text{M}$ ) using the formula  $\Delta G = RT \ln K_d$  which at 37°C corresponds to  $g_4 = -8.19$  kcal/mol. Similarly, the value of  $g_2$  corresponds to the  $K_d$  for aspartate of the active species (12  $\mu\text{M}$ ) and hence  $g_2 = -6.98$  kcal/mol.

The difference between these two values ( $g_4 - g_2$ ) is therefore  $-1.21$  kcal/mol, which by the above equation must correspond to  $g_1 - g_3$ , the difference in free energies for the activation of receptor in its unoccupied and occupied state. The values for  $g_1$  and  $g_3$  can be calculated from the formula:

$$\Delta G = -RT \ln \left( \frac{p}{1-p} \right)$$

using estimated values of  $p$ , as described in the Results section.

### Binary flags

Each receptor complex is represented as a discrete software object within the computer simulation and has 12 associated binary attributes, or flags (see Figure 2). Most of these flags are modified during chemical reactions. Four are used to represent methylation sites on the receptor; four denote which cytoplasmic signalling proteins are bound and one is a phosphorylation flag. One flag indicates whether the complex is in the active or inactive conformation, and has a certain probability of being in one state or another depending on the state of other flags (namely the methylation and ligand occupancy flags). As ligand binding is several orders of magnitude faster than other reactions in the pathway, the flag indicating ligand occupancy is implemented similarly, i.e. it has a certain probability related to ligand concentration, of being in one state or another.

The 12th flag allows the influence of non-aspartate receptors on the flux of phosphate and methyl groups to be examined. One setting of this flag indicates that the complex contains the aspartate-binding receptor Tar. The other setting indicates that the complex contains one of the other receptors, Tsr, Trg, Tap or Aer. With the latter setting, the complex does not bind aspartate but the reac-

tions of methylation, phosphorylation and binding of CheB, CheR and CheY are fully operative. The value of this flag is assigned to each complex at the start of the simulation, according to the desired concentration of Tar, and cannot then be modified by reaction.

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## Appendix

Rate of formation of phosphorylated receptor complex =  $k_{\text{auto}}[E^*]$ , where  $k_{\text{auto}}$  is the rate constant for autophosphorylation of receptor complex and  $[E^*]$  is the concentration of unphosphorylated receptor complexes in the active conformation.

Rate of destruction of the phosphorylated receptor complex =  $k_{\text{dephosph}}([E^*p] + [Ep])$ , where  $k_{\text{dephosph}}$  is the rate constant for dephosphorylation of receptor complex,  $[E^*p]$  is the concentration of phosphorylated receptor complexes in the active conformation and  $[Ep]$  is the concentration of phosphorylated receptor complex in the inactive conformation. At equilibrium, rate of phosphorylation = rate of dephosphorylation:

$$\Rightarrow k_{\text{auto}}[E^*] = k_{\text{dephosph}}([E^*p] + [Ep]) \quad (\text{A1})$$

Also, the total receptor complex activity,  $A$ , is constant at equilibrium due to adaptation:

$$[E^*] + [E^*p] = A$$

Substituting equation (A2) into equation (A1):

$$[E^*p] + [Ep] = k_{\text{auto}}(A - [E^*p])/k_{\text{dephosph}} \quad (\text{A2})$$

As  $[E^*p] \rightarrow 0$ , the total concentration of phosphorylated receptor complex,  $[E^*p] + [Ep] \rightarrow \text{constant}$ .

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