Receptor interactions through phosphorylation and methylation pathways in bacterial chemotaxis

(second messenger/feedback/excitation/memory/adaptation)

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ABSTRACT The effects of messages initiated by one receptor on the covalent modification of a second receptor were studied by use of a technique for rapidly separating the receptors. Methylation of the bacterial-chemotactic serine receptor increases as a result of aspartate binding to the aspartate receptor. The aspartate-induced methylation on the serine receptor is absent in a strain that lacks *cheA* and *cheW* genes and is not the result of physical interaction, such as the formation of heterodimers between the aspartate and serine receptors, or of alterations in the affinity of the serine receptor for the methyltransferase and the methylesterase. Serine-induced methylation of the serine receptor did not require *cheA* and *cheW*. A model is presented in which the receptor methylation level depends on the combination of (i) a ligand-induced conformational change on the receptor substrate of the methylation enzymes and (ii) an indirect cytoplasmic signal that operates through the methylesterase.

Organisms, tissues, and cells experience a large variety of stimulatory events and must not only respond to each one individually but also integrate their responses. Stimuli, such as light, hormones, or neurotransmitters, are sensed by transmembrane receptors that activate particular second-messenger systems (1). The second messengers are responsible for producing the appropriate response to the stimulus and coordinating the responses to different stimuli. For example, in bacterial chemotaxis a response regulator that integrates responses from a variety of bacterial chemotaxis receptors has been postulated as a device that controls output of the cells and provides short-term memory (2).

Bacterial chemotaxis is an attractive system for the investigation of second messengers because of the power of the available genetic and molecular biological tools. *Escherichia coli* and *Salmonella typhimurium* modulate their swimming behavior in reaction to changes in the concentrations of certain chemicals that act as attractants or repellents (3). Their motility pattern results from an integration of information obtained through a variety of receptors (4, 5). Aspartate and serine, for example, bind to specific cell-surface chemotaxis receptors, which produce signals that alter migration of the sensing bacterium (6–8). These receptors are methylated on specific glutamate residues by the CheR protein, a methyltransferase (9–12). The reaction is reversible, because hydrolysis of the methyl groups with the consequent production of methanol is catalyzed by the CheB protein, a methylesterase (13, 14). Both aspartate and serine increase the rate of methylation and decrease the rate of demethylation of the receptor to which they bind, *in vivo* and *in vitro* (15–19).

The theory of a response regulator suggested that there were two steps, a fast excitation step and a slow adaptation process (20). Methylation was identified with the adaptation

process (21–24), but the chemistry of the excitation was unknown until recently. *In vivo* experiments by Simon and co-workers (25, 26) strongly suggest that phosphorylation is involved in the second-messenger system of bacteria chemotactic signaling, as in that of eukaryotic cells (27). The protein that serves as a kinase in chemotaxis is the CheA protein, which had previously been identified as part of the signaling system between receptors and flagella, and the CheA protein is believed to modify the CheY protein, which is a strong candidate for the response regulator.

Previous work suggested a feedback signal generated at the receptor could inhibit the methylesterase (28, 29). However, direct investigation of the consequences of the regulation of the methylesterase for receptor methylation levels had not been attempted. The *cheA* and *cheW* genes are implicated in these feedback effects (30–32); these same genes had also been implicated in signaling from the receptor to the flagella. Such indirect effects could be produced through three possible mechanisms: (i) feedback through some cytoplasmic signal that reacted independently with either receptor, (ii) the presence of heterodimers in which a conformational change in an aspartate-receptor subunit induced a conformational change in a serine-receptor subunit, and (iii) an effect of differential binding of methyltransferase and/or methylesterase to the directly stimulated receptor, which could thereby alter the rates and levels of methylation of the other receptor. To establish the mechanism of the effect and its quantitative contribution to the excitation and adaptation systems required a method of separating two very similar receptors (33, 34) so that we could measure accurately the methylation level of each receptor. A technique that involved localized mutagenesis and that could be applied to other difficult-to-separate proteins was used to accomplish this task.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* strains RP437 (wild type for chemotaxis), RP4372 ($\Delta tar\text{-}tap$, *tsr*) and RP2898 ($\Delta cheA\text{-}cheW\text{-}tar\text{-}tap$) were obtained from J. S. Parkinson (University of Utah) (35).

Cloning of *tar_S*-S517C and *tsr_E* onto a Single Plasmid, pSK140, and Functionality Test. The *EcoRI* fragment of the plasmid pAB100 (33) containing the *tsr_E* (*E. coli* serine receptor) gene was ligated into the *EcoRI* site of plasmid pFK139 (36), which encodes *tar_S*-S517C (*S. typhimurium* aspartate receptor with Ser-517 $\rightarrow$ Cys mutation) and ampicillin resistance, to create plasmid pSK140. Strains transformed with this plasmid and control plasmids were tested for their ability to exhibit chemotaxis on swarm plates (37). Colonies were inoculated into the center of 0.35% agar plates containing Vogel–Bonner citrate medium (38) supplemented with 1% glycerol and histidine, methionine, leucine, threo-

nine, and thiamine (each at 50 $\mu\text{g}/\text{ml}$) and, in some cases, the attractants 100 μM aspartate or 100 μM serine; either ampicillin (100 $\mu\text{g}/\text{ml}$) or tetracycline (8 $\mu\text{g}/\text{ml}$) was also in the medium. After an initial lag, the rate of swarming, as measured by the increase in diameter per hr, was linear. The plasmid pSK140 conferred chemotaxis toward both aspartate and serine on a strain (RP4372) that was defective in both traits. Plasmids pFK139 and pAB100 conferred chemotaxis toward only aspartate or serine, respectively (data not shown).

Separation and Measurement of Methylation of Receptors. Bacteria were grown to an OD_{650} of 0.7–1.2 units at 30°C in Vogel–Bonner citrate medium supplemented with 1% glycerol and histidine, methionine, leucine, threonine, and thiamine (each at 50 $\mu\text{g}/\text{ml}$) with ampicillin (250 $\mu\text{g}/\text{ml}$) or tetracycline (8 $\mu\text{g}/\text{ml}$) if required. The cells were collected by centrifugation at $5000 \times g$ for 10 min, washed twice with the same medium and supplements—except that the concentration of methionine was 5 $\mu\text{g}/\text{ml}$, and then resuspended at an OD_{650} of 1.0 in the same medium containing an additional 350 μCi of L-[methyl- ^3H]methionine per ml (85 Ci/mmol; 1 Ci = 37 GBq). The cultures were incubated at 30°C for 30 min before attractant addition.

At various times after attractant addition, duplicate 200- μl aliquots were precipitated in 1 ml of ice-cold 10% trichloroacetic acid. The pellet from one aliquot was extracted into 40 μl of 2 $\times$ sodium dodecyl sulfate (SDS) buffer (750 mM Tris-HCl, pH 8.8/20% glycerol (wt/vol)/0.004% bromphenol blue/4% SDS/1% 2-mercaptoethanol), whereas the other aliquot was extracted into 40 μl of a detergent solution containing 1% Zwittergent 3-12, 700 mM Tris-HCl (pH 8.0), 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, and 1 mM *o*-phenanthroline. The mixtures were then frozen in liquid nitrogen and stored at -80°C .

Polypeptides encoded by *tar_S-S517C* can be crosslinked by disulfide linkages to dimers in the presence of an oxidative catalyst (36), so 5 μl of a 50 mM copper *o*-phenanthroline solution was added to each Zwittergent 3-12-solubilized sample, which was then incubated for 10 min at 37°C. Seventeen microliters of 4 $\times$ SDS buffer (250 mM Tris-HCl, pH 6.8/40% glycerol/0.008% bromphenol blue/8% SDS, containing no 2-mercaptoethanol) was then added to each sample, which was subsequently frozen in liquid nitrogen and stored at -80°C .

To determine the level of base-labile methylation in receptor proteins and to separate the crosslinked *tar_S-S517C* from the *tar_E* all SDS-solubilized samples were boiled 3 min and analyzed on Laemmli 7.5% polyacrylamide gels (39). The gels were stained with Coomassie blue and destained in 10% acetic acid (vol/vol) to visualize the proteins. The region of the gel of each lane containing the monomeric receptor proteins (50–80 kDa) and that containing the dimeric receptor protein (>100 kDa) were cut into 2.5-mm slices. Base-labile methylation in the slices was measured by the method of Chelsky *et al.* (40). Recovery of counts in the crosslinked Zwittergent 3-12-solubilized samples was 60–90% of that in the SDS-solubilized samples. Therefore, analysis of the Zwittergent 3-12-solubilized samples was used to determine the ratio of dimer/monomer cpm, whereas the data obtained from the more reproducibly recovered SDS-solubilized samples were used to determine the total number of methyl-group cpm. By examining the methylation level of the aspartate receptor extracted from whole cells that lacked the serine receptor and treated with copper *o*-phenanthroline, we could show that $60.5 \pm 4.8\%$ (SD) of the aspartate receptor was in the crosslinked form. The presence or absence of 1 mM aspartate and/or 1 mM serine and the level of receptor methylation did not affect the extent of dimer formation. Crosslinking of the aspartate receptor is not influenced by the presence of the serine receptor. Therefore, the cpm above

background in the dimer band were divided by 0.6 to obtain aspartate-receptor methylation, and the remaining cpm were attributed to the serine receptor.

When receptor demethylation was being examined, non-radioactive methionine (final concentration, 250 $\mu\text{g}/\text{ml}$) was added as a chase to the cultures that had been incubated for 30 min with radioactive methionine as described in the first paragraph of this section. The specific activity of the methionine was thereby reduced 50-fold. Receptor methylation was determined at various time points before and after attractant addition. Seven minutes of incubation with the nonradioactive methionine preceded the addition of attractant to ensure that the *S*-adenosylmethionine pools were equilibrated.

RESULTS

Stimulation of Methylation of the Serine Receptor During an Aspartate Response Requires the Presence of the Aspartate Receptor. To examine whether a response generated by one receptor affected the methylation of a second receptor required a special technique for measurement of the methylation level of each receptor separately. Two-dimensional polyacrylamide gels did not separate the aspartate and serine receptors sufficiently to allow for accurate quantification of their methylation levels. To make a clean separation of the two receptors, a cysteine residue was introduced by localized mutagenesis into the aspartate receptor (36). Cysteine residues were introduced at certain nonconserved positions without any effect on signaling properties of the receptor and, after cell rupture, such receptors could be crosslinked by oxidation through disulfide-bond formation (36). As a result, the aspartate receptor had the electrophoretic mobility on SDS/polyacrylamide gels of a protein twice the molecular weight of the closely homologous serine receptor, which could not form disulfide crosslinks. As described in the previous section, this technique allowed clean separation and simple calculation of the methylation of each receptor. In addition, overproduction of the receptors enabled examination of the indirect effects over a reasonable time frame by extending the time required for adaptation to stimuli.

The level of receptor methylation during various attractant responses was investigated in strain RP4372, lacking both aspartate and serine receptors, which was transformed with a plasmid encoding both receptors. As shown in Fig. 1, addition of aspartate increases the methylation level of both the aspartate and the serine receptors. Addition of serine likewise increases methylation on its own receptor and on the aspartate receptor. The increase in receptor methylation in response to 1 mM serine ceases after 10 min, the time required for adaptation. In cells that lack the aspartate receptor, aspartate addition has no effect upon the methylation of the serine receptor and vice versa; thus, the methylation change requires receptor stimulation. When that stimulation occurs, methylation of the receptor not directly stimulated increases during a response initiated by the other receptor in a wild-type strain. The directly stimulated receptor experiences a much greater change in methylation level.

Stimulation of Methylation of the Serine Receptor During an Aspartate Response Requires CheA and/or CheW Proteins. There have been previous suggestions that the cytosolic products of the *cheA* and *cheW* genes were responsible for the inhibition of methylesterase that occurs during a chemotactic response (28, 31) and for the generation of the chemotaxis second messenger (35). Therefore, we questioned whether these genes were also required for the aspartate-induced methylation of the serine receptor. The above experiment was repeated with a strain that lacked the CheA and CheW proteins (Fig. 2). In this strain there is no increased methylation of the serine receptor in response to 1 mM

FIG. 1. (A) Aspartate-receptor methylation in RP4372-pSK140 (overproducer of the serine and aspartate receptors) was measured at various times with or without chemoattractants, as described. In the presence of the serine receptor, methylation on the aspartate receptor increases during a response to 1 mM serine, but this increase stops at ≈ 10 min. (B) Serine-receptor methylation was measured under the same conditions. In the presence of the aspartate receptor, methylation on the serine receptor increases during a response to 1 mM aspartate.

aspartate. The increase in total receptor methylation in the cell induced by aspartate is due to methylation on the aspartate receptor alone, in contrast to the results obtained when the CheA and CheW proteins were present. Thus the strain lacking CheA and CheW exhibits only direct ligand-induced effects with no indirect effects.

Attractant-Induced Methylation Changes Are Not Additive. Table 1 compares the increase in cellular receptor methylation produced when cells are simultaneously presented with 1 mM aspartate and 1 mM serine with the sum of the methylation responses to 1 mM aspartate or 1 mM serine added separately. When the CheA and CheW proteins are

FIG. 2. Serine receptor methylation in RP2898 (Δ *cheA-cheW-tar-tap*)-pSK140 (overproducer of the serine and aspartate receptors) was measured with or without chemoattractants, as described. In the absence of *cheA* and *cheW*, serine receptor methylation is unaffected by 1 mM aspartate.

Table 1. Increases in methylation in response to combined stimuli

Attractant (1 mM)	Receptor	Receptor methyl groups per 10 ⁸ cells, pmol	
		Wild type	<i>cheA cheW</i>
Serine	Serine	9.69	4.48
Serine + aspartate	Serine	10.00	4.51
Aspartate	Aspartate	12.39	1.85
Serine + aspartate	Aspartate	11.31	2.44
Serine + aspartate	Serine + aspartate	21.31	6.94
Calculated sum of serine and aspartate responses	Serine + aspartate	29.63	7.05

Methylation above background was measured after 10 min of an attractant response, as described. The calculated sum of serine and aspartate responses is the sum of total additional receptor methylation obtained during separate responses to 1 mM aspartate and 1 mM serine.

present (RP4372-pSK140) the methylation increase is less than the sum of the increases from 1 mM aspartate or 1 mM serine alone. In contrast, in the strain that lacks CheA and CheW (RP2898-pSK140) the response to 1 mM aspartate and 1 mM serine is additive. Thus the feedback system is responsible for the loss in additivity, whereas, as expected when each receptor is acting independently, additivity is observed. The reason for the lack of additivity is clear from Fig. 3A, in which saturation of the feedback system is evident (see below). Because complete inhibition of serine receptor demethylation occurs with 1 mM serine alone, it is clear that further addition of aspartate can have no additional effect.

FIG. 3. (A) The decrease in serine-receptor methylation in RP4372-pSK140 was measured with or without chemoattractants during an unlabeled methionine chase, as described. Serine-receptor demethylation is completely inhibited during the response to 1 mM serine and accelerates thereafter. Serine-receptor demethylation is partially inhibited by 1 mM aspartate. (B) The decrease in aspartate-receptor methylation was measured with or without 1 mM aspartate. Aspartate-receptor demethylation is completely inhibited during an aspartate response.

Demethylation of the Serine Receptor Is Partially Inhibited During an Aspartate Response. An increase in methylation of the serine receptor during an aspartate response may be due to either an increase in the rate of methyl esterification catalyzed by the methyltransferase or a decrease in hydrolysis of glutamic methyl ester catalyzed by the methylesterase. To test whether 1 mM aspartate inhibits demethylation of the serine receptor, a pulse-chase experiment was performed as described. Receptor methylation was measured at various time points after attractant addition. The serine-receptor methylation in this experiment is shown in Fig. 3A. Serine at 1 mM completely inhibits serine-receptor demethylation during the response (which stops after 8 min), whereas 1 mM aspartate is only partially effective in inhibiting demethylation. Demethylation of the aspartate receptor is completely inhibited by 1 mM aspartate (Fig. 3B). Because the loss of methyl groups by pulse chase affects only those methyl groups previously added to the receptor, the effect must be an inhibition of the esterase. The effects are greater upon the demethylation of the directly stimulated receptor than on those receptors not directly stimulated.

DISCUSSION

Evidence for an Indirect Cytoplasmic Feedback Effect. The results reported here and previously clearly establish that covalent modification of chemotaxis receptors is regulated by (i) the direct effects of a ligand-induced conformational change on the receptor to which the ligand is bound (18, 36) and (ii) a feedback effect mediated by cytosolic components of the chemotaxis second-messenger system. Stimulation of the CheR-catalyzed methylation and inhibition of the CheB-catalyzed demethylation of the initiating receptor are demonstrable in cell-free systems where they directly depend upon receptor conformational changes induced upon effector binding (18). In our studies we show that stimulation of one receptor can raise the methylation level of another receptor; the general inhibition of methylesterase during an attractant response can completely account for this indirect effect.

Two alternative explanations of the indirect effect can be eliminated. (i) The first hypothesis is that there are heterodimers—i.e., one serine receptor peptide and one aspartate receptor peptide in the same dimer—that affect each other by cooperative interpeptide interactions. That explanation has been eliminated by the finding that heterodimers do not exist (41). The fact that inhibition of the esterase requires the presence of CheA and/or CheW further indicates that this inhibition is not a result of direct protein-protein interactions between receptors or merely an artifact of receptor overproduction. (ii) The other hypothesis is that an indirect effect could be explained on the basis of a sequestration of transferase or esterase on the directly stimulated receptor, thus affecting the methylation of the alternate receptor. However, our findings—that both receptors are increased in their methylation and that the result critically depends on CheA and CheW—eliminate the second hypothesis. These findings not only provide quantitative support for an indirect cytoplasmic effect but also provide *in vivo* evidence that a phosphorylation pathway, as indicated by the *in vitro* studies of Simon and co-workers (25, 26) is the best candidate for the second-messenger system in bacterial chemotaxis. The decrease in esterase activity on attractant addition fits with the work of Simon and co-workers (26) and Stock *et al.* (43), which indicates that the CheA protein phosphorylates the esterase and activates it.

The Direct and the Indirect Effects. A schematic illustration of the probable situation is shown in Fig. 4. The serine and the aspartate receptors interact with the CheA kinase (inhibiting it when there is an increase of attractant, activating it when there is a decrease). The kinase phosphorylates the

A. Steady State

B. Addition of Asp

FIG. 4. A model for the regulation of receptor methylation and flagellar rotation. (A) Interaction between the receptors and the proteins involved in intracellular signaling and receptor methylation when no ligand is bound and the cell is in an adapted state. The aspartate (R_A) and serine (R_S) receptors have a given level of methylation (Me) between i and $i + 1$ or j and $j + 1$ methyl groups per polypeptide, which is determined by the conformation of the ligand-free receptor. The rates of methyl-group addition by the methyltransferase and methyl-group removal by the methylesterase are equal. The more highly methylated receptors activate the CheA kinase to phosphorylate the CheY protein and the methylesterase, enhancing the functions of each. The phosphorylated CheY protein more effectively promotes clockwise (cw) flagellar rotation, whereas the phosphorylated methylesterase is more active in demethylation. The CheZ phosphatase reverses the CheA-catalyzed phosphorylations. (B) Effect of an aspartate response. Aspartate binds to the aspartate receptor and changes the receptor conformation so that receptor methylation is enhanced ($\oplus$ above the methyltransferase reaction arrow), while demethylation is strongly inhibited. The ligand-bound form of the receptor no longer activates the CheA kinase (dotted arrows from the aspartate receptor to the kinase) so the phosphorylation of the CheY protein and the methylesterase is diminished (kinase reaction arrows are dotted). The flagella rotate counterclockwise (ccw), and the methylesterase is no longer activated. Because the rate of methylation of the serine receptor is unaffected by the presence of aspartate and the rate of demethylation is diminished (dotted arrow from the methylesterase), the methylation level of the serine receptor increases. After adaptation (data not shown) the biochemical reactions will return the state to that of A, except that the ligand-bound aspartate receptor will have a different, higher steady-state level of methylation.

CheY protein to generate the response regulator, phosphorylated CheY, and also phosphorylates the esterase, thus activating it. Because the same intermediates are generated whether the response is initiated by the serine receptor or the aspartate receptor, the esterase will act on methyl groups on either receptor—hence the indirect feedback effect.

The reconstitution and individual receptor studies reported previously (18, 36) established that a direct effect of ligand

binding upon the conformation of the receptor alters receptor properties as a substrate for methyltransferase and methyl-esterase. There is also, as originally suggested by Dahlquist, Springer, and their co-workers (28, 29, 31, 32), an indirect feedback effect that operates through the cytoplasm. Our results quantify the extent of that effect, establishing that both the direct conformational effect and indirect cytoplasmic feedback exist and contribute approximately the same to the behavioral output. The methylation increase on the directly stimulated receptor is predominantly the consequence of the ligand-induced conformational change and secondarily the consequence of the indirect methyl-esterase effect. Changes in the methylation of the receptor not directly stimulated, on the other hand, result from the indirect methyl-esterase effect. After adaptation to an increased attractant concentration—i.e., the cessation of continuous smooth swimming—a longer than average period of tumbling normally ensues. This overshoot phenomenon (42) results from the overmethylation of those receptors not directly stimulated. Stimulation of demethylation during this overshoot would restore the methylation level of the receptors not directly stimulated to that existing before the increase in attractant concentration.

Why should the excitation system provide information back to the methylation system? The answer would seem to lie in the asymmetry of the time intervals needed for chemotaxis. When the bacterium moves in a favorable direction, it suppresses tumbling and should continue to move in a straight line as long as the gradient remains favorable. If the bacterium moves in the wrong direction and generates a tumbling signal, it should do so only long enough to try the new direction. Activation of the methyl-esterase by phosphorylation should provide that function. When the bacterium moves up a favorable gradient, the tendency to keep going forever is tempered slightly by inhibition of the methyl-esterase, which keeps the signal in a range that can respond to adverse changes in gradient.

In more complex organisms there is feedback between second-messenger systems and between receptors. In most of these cases it is not apparent why this phenomenon should exist. The results reported here suggest that common intermediates offer an opportunity to fine-tune the system for an optimum biological response.

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