

pH Dependence of CheA Autophosphorylation in *Escherichia coli*

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Chemotaxis by cells of *Escherichia coli* and *Salmonella typhimurium* depends upon the ability of chemoreceptors called transducers to communicate with switch components of flagellar motors to modulate swimming behavior. This communication requires an excitatory pathway composed of the cytoplasmic signal transduction proteins, CheA_L, CheA_S, CheW, CheY, and CheZ. Of these, the autokinase CheA_L is most central. Modifications or mutations that affect the rate at which CheA_L autophosphorylates result in profound chemotactic defects. Here we demonstrate that pH can affect CheA_L autokinase activity in vitro. This activity exhibits a bell-shaped dependence upon pH within the range 6.5 to 10.0, consistent with the notion that two proton dissociation events affect CheA_L autophosphorylation kinetics: one characterized by a pK_a of about 8.1 and another exhibiting a pK_a of about 8.9. These in vitro results predict a decrease in the rate of CheA_L autophosphorylation in response to a reduction in intracellular pH, a decrease that should cause increased counterclockwise flagellar rotation. We observed such a response in vivo for cells containing a partially reconstituted chemotaxis system. Benzoate (10 mM, pH 7.0), a weak acid that when undissociated readily traverses the cytoplasmic membrane, causes a reduction of cytoplasmic pH from 7.6 to 7.3. In response to this reduction, cells expressing CheA_L, CheA_S, and CheY, but not transducers, exhibited a small but reproducible increase in the fraction of time that they spun their flagellar motors counterclockwise. The added presence of CheW and the transducers Tar and Trg resulted in a more dramatic response. The significance of our in vitro results, their relationship to regulation of swimming behavior, and the mechanisms by which transducers might affect the pH dependence of CheA autokinase activity are discussed.

Cells of the bacterium *Escherichia coli* sense changes in concentrations of certain amino acids and sugars by means of integral membrane chemoreceptors called transducers. Increased binding of attractant molecules to the periplasmic domain of the transducers increases the probability that the flagellar motors rotate counterclockwise (CCW). In vitro the transducer, in response to such binding and in concert with the cytoplasmic protein CheW (18 kDa), inhibits the autophosphorylation of the histidine protein kinase, CheA_L (76 kDa). This decreases the rate at which CheY (13 kDa) is phosphorylated via CheA_L-CheY phosphotransfer. Phosphorylated CheY readily autodephosphorylates, and another protein, CheZ (34 kDa), markedly accelerates this process. Thus, the level of phosphorylated CheY reflects the relative rates of CheY phosphorylation and dephosphorylation. CheY, when phosphorylated, binds to the flagellar motor switch (36), an interaction that enhances the probability of clockwise rotation. The default setting of the flagellar motor, i.e., that in the absence of phosphorylated CheY, is for CCW rotation. Therefore, reducing the level of phosphorylated CheY by decreasing the rate of CheA_L autophosphorylation results in increased CCW rotation. For reviews on bacterial chemotaxis, see references 2, 6, 8, 19, 31, and 32.

The gene *cheA* encodes two proteins that share the same reading frame, CheA_L and its shortened form, CheA_S (30). Thus, CheA_S (66 kDa) is identical to CheA_L except that it lacks the N-terminal 97 amino acids, which include the autophosphorylation site (His-48) (12, 16). The lack of this N-

terminal domain means that, unlike CheA_L, CheA_S cannot autophosphorylate. The shortened protein can, however, transphosphorylate CheA_L (33, 39).

Whereas wild-type cells of *E. coli* sense some sugars and amino acids as attractants, they sense weak acids, e.g., benzoate, as repellents (34). Addition of benzoate decreases the probability that the flagellar motors rotate CCW. This response is mediated by a reduction in cytoplasmic pH (15, 24, 28). In contrast, mutant cells that express the aspartate receptor, Tar, but not the more abundant serine receptor, Tsr, respond to such a reduction in the opposite way, i.e., by increasing CCW rotation (15, 24, 28, 34). These observations raise the following questions. How does cytoplasmic pH influence the direction of flagellar rotation? Why do the transducers, Tsr and Tar, mediate opposite responses to the same internal stimulus? Since increased CCW rotation results from decreased levels of phosphorylated CheY, it is likely that cytoplasmic pH influences any one of a number of processes that determine levels of phosphorylated CheY. For example, cytoplasmic pH might affect (i) the rate of CheA autophosphorylation, (ii) the rate of CheA-CheY phosphotransfer, (iii) the rate of CheY dephosphorylation (e.g., by affecting CheZ), or (iv) the ability of phosphorylated CheY to interact with the flagellar switch.

Distinguishing among these and other possibilities represents a difficult and complicated task. We have chosen, therefore, to approach this problem by first investigating simple model systems, both in vitro and in vivo. In vitro, we have measured the activity of purified CheA_L in the absence of all other chemotaxis proteins and found its autophosphorylation to be sensitive to pH in the range between 6.5 and 10.0, reaching maximal activity at about pH 8.5. This sensitivity resulted primarily from pH effects on the rate of phosphotrans-

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† Deceased. This paper is dedicated to her memory.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Chemotaxis gene(s) expressed ^a	Source or reference
AJW29	HCB696(pMPC3)	<i>tar, trg, trpP-cheA, lacP-cheY</i>	This study
AJW31	HCB696(pAJW20)	<i>tar, trg, trpP-cheA98MI, lacP-cheY</i>	This study
AJW32	HCB696(pAJW21)	<i>tar, trg, trpP-cheA98MI cheW tar', lacP-cheY</i>	This study
AJW40	HCB696(pAJW24)	<i>tar, trg, trpP-cheA98MI cheW, lacP-cheY</i>	This study
AJW47	HCB722(pAJW20)	<i>trpP-cheA98MI, lacP-cheY</i>	This study
AJW48	HCB722(pMPC3)	<i>trpP-cheA, lacP-cheY</i>	This study
AJW49	HCB722(pAJW24)	<i>trpP-cheA98MI cheW, lacP-cheY</i>	This study
AJW70	HCB696(pAJW103)	<i>tar, trg, trpP-cheA cheW, lacP-cheY</i>	This study
AJW71	HCB722(pAJW103)	<i>trpP-cheA cheW, lacP-cheY</i>	This study
AJW248	HCB433 $\Delta(tar-cheZ)2286$	<i>cheA, cheW</i>	RP5135 \times HCB433 \rightarrow Eda ⁺ Che ⁻
AJW253	AJW248(λ DFB19)	<i>cheA, cheW, lacP-cheY</i>	This study
HCB433	$\Delta(tsr)7021 \Delta(trg-100 zdb::Tn5$	<i>tar, tap, cheA, cheW, cheR, cheB, cheY, cheZ</i>	35
HCB440	$\Delta(tsr)7021 \Delta(cheA-cheY)::XhoI$ (Tn5) ^b	<i>trg</i>	7
HCB696	HCB440(λ AJW3)	<i>trg, tar, lacP-cheY</i>	This study
HCB722	$\Delta(tsr)7021 trg::Tn10 \Delta(cheA-cheY)::XhoI(Tn5)^b$ (λ DFB19)	<i>lacP-cheY</i>	36
RP5135	$\Delta(tar-cheZ)2286$	<i>tsr, trg, cheA, cheW</i>	J. S. Parkinson, University of Utah

^a Of the set *tsr, trg, tar, tap,* and *cheAWRBYZ*.

^b Although *cheZ* is present, it is not expressed at detectable levels.

fer from ATP to CheA_L and not from alterations in the affinity of CheA for ATP. In vivo, we have measured the pH response of cells which express different combinations of signaling components, including transducers, CheA_L, CheA_S, CheW, and CheY. We found that cells which expressed CheA_L, CheA_S, and CheY, but not CheW or transducers of any type, responded to internal pH changes, albeit weakly. The presence of both CheW and the transducers Tar and Trg significantly increased the amplitude of this response. Our in vitro and in vivo results raise the possibility that CheA_L can function as a pH sensor whose activity is influenced by changes in cytoplasmic pH.

MATERIALS AND METHODS

Chemicals. Restriction enzymes and other DNA reagents were obtained from American Allied Biochemical (*EcoRI* and *PstI*), Bethesda Research Laboratories (*ClaI*, *MluI*, *PstI*, and T4 DNA ligase), Boehringer Mannheim (*PvuII* and *SauI*), and New England BioLabs (*Bsu36I* and *EcoRV*). Synthetic sodium L-aspartate, [γ -³²P]ATP, and BioLite cocktail were purchased from ICN Pharmaceuticals Inc. Ampicillin, kanamycin sulfate, tetracycline hydrochloride, isopropyl- β -D-thiogalactoside (IPTG), sodium benzoate, peroxidase-conjugated goat anti-rabbit immunoglobulin G, and 4-chloronaphthol were purchased from Sigma Chemical Co. Tryptone and agar were from Difco Laboratories. A bicinchoninic acid protein assay kit was purchased from Pierce. Chromaphor protein stain was from Promega. Other chemicals were reagent grade.

Bacterial strains and plasmids. All strains were isogenic derivatives of *E. coli* K-12 and are listed in Table 1. Strain HCB722 was described previously (37). Of all the genes that encode the transducers and the cytoplasmic chemotaxis proteins, only *cheY* is expressed by cells of this strain. This gene is carried on the temperate phage λ DFB19 (Fig. 1) (37); it is under the control of *lacP*, which permits variable expression by the addition of the inducer IPTG. Strain HCB696 is identical to strain HCB722 except that it also expresses *tar* and *trg*. *tar* is carried on the phage λ AJW3 (Fig. 1) (37), which is identical to λ DFB19 except that it also expresses *tar* under control of its native promoter (positioned such that expression proceeds opposite that of *cheY*); *trg* is at its native site in the chromo-

some. Transformants of strains HCB722 and HCB696 that express various combinations of CheA_L, CheA_S, and CheW from multicopy plasmids were constructed by the methods of Silhavy et al. (27). In contrast to these transformants, cells of strain AJW253 express all three proteins (CheA_L, CheA_S, and CheW) from single chromosomal copies of *cheA* and *cheW*. Like the HCB722 transformants, these cells express *cheY* from the temperate phage λ DFB19. Generalized transductions involved the use of phage P1kc (27).

All plasmids used in this study were derivatives of pDV4 (20) and are shown in Fig. 1. The following alleles are under the control of the tryptophan promoter, *trpP*, of *Serratia marcescens*: *cheA cheW*, on plasmids pDV4 and pAJW103; *cheA98MI cheW*, on plasmids pAJW21 and pAJW24; *cheA*, on plasmid pMPC3; *cheA98MI*, on plasmid pAJW20; and *cheA521*, on plasmid pMPC4. Expression from *trpP* occurs in the absence of its inducer, 3- β -indoleacrylic acid; thus, inducer was not added to either growth medium or swarm agar plates. Plasmids (pDV4, pAJW103, and pMPC3) that encode the wild-type *cheA* allele express CheA_L and CheA_S in a ratio of about 3:1 (16). Plasmids (pAJW21, pAJW24, and pAJW20) that encode the allele *cheA98MI* express CheA_L but not CheA_S. The lack of CheA_S expression results from a mutation (constructed by H. Sanatinia, University of Utah) that changes the CheA_S translation initiation codon ATG to the noninitiation codon ATA. This results in the conservative change Met-98 \rightarrow Ile-98, which does not significantly alter the levels of CheA_L expression as measured by immunoblot analysis (data not shown). Although the mutant protein, CheA98MI, possesses reduced kinase activity relative to that of the wild-type protein, cells that express CheA98MI exhibit chemotactic behavior similar to that of wild-type cells (reference 25 and data not shown). The plasmid (pMPC4) that encodes the allele *cheA521* expresses CheA_S but not CheA_L. This allele carries a nonsense (amber) mutation between the CheA_L and CheA_S translational start sites; therefore, translation of CheA_L terminates prematurely (22). This mutation does not significantly alter the levels of CheA_S expression as measured by immunoblot analysis (data not shown).

Protein purification and immunoblotting. CheA was purified as described previously (39). Over 95% of the purified CheA was CheA_L as determined by Coomassie blue staining of

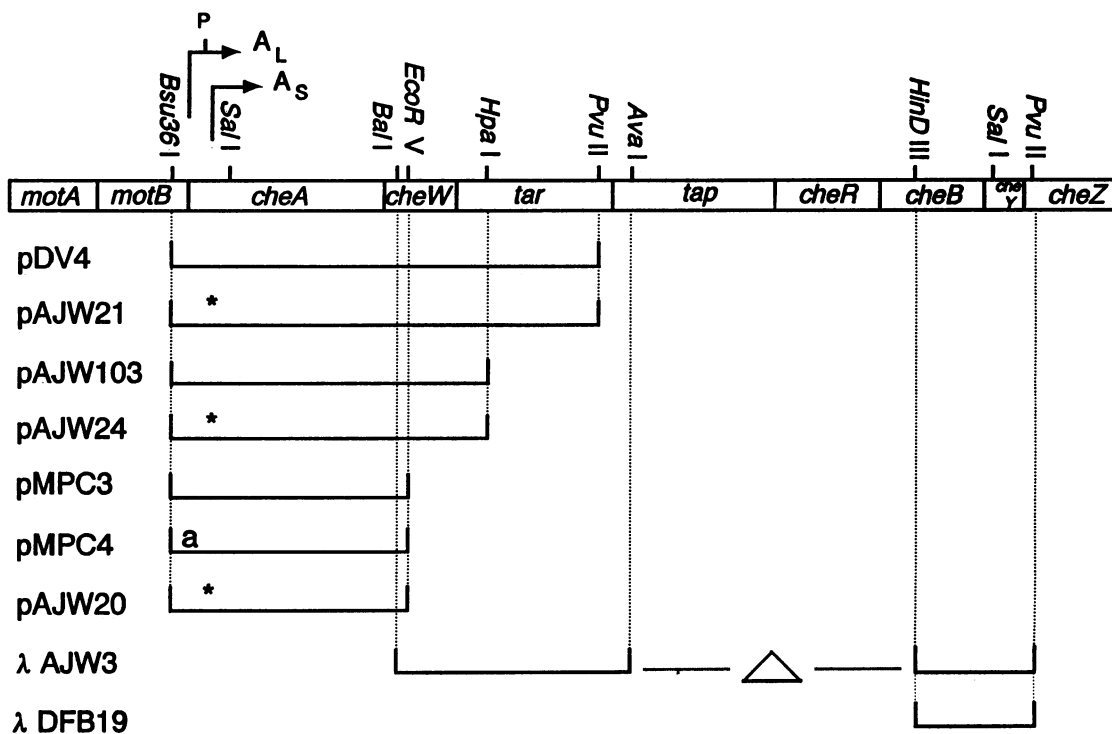


FIG. 1. Portions of the *mocha* and *meche* operons encoded by plasmids and phages used for this work. Endpoints are indicated by restriction sites, which were either present in the native sequence or prepared with synthetic linkers. Arrows indicate the locations of translational initiation sites for CheA_L and CheA_S; P indicates the approximate location of the codon for the phosphorylation site in CheA_L. Also shown are the approximate locations of *cheA* mutations: a, amber mutation that results in expression of CheA_S only; *, ATG→ATA missense mutation that results in the expression of CheA_L 98MI only.

polyacrylamide gels. Immunoblot analyses (11) used affinity-purified rabbit anti-CheA antibodies.

CheA phosphorylation. For single-time-point analyses, CheA (final concentration, 6 μ M) was mixed with 50 μ M [γ -³²P]ATP (about 2,000 cpm pmol^{-1}) in 50 mM TKMD buffer (50 mM Tris HCl, 50 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol) at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, or 9.5. The reactions, performed at room temperature, were terminated after 2 min by the addition of 3 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (18). Samples were subjected to SDS-10% PAGE at 23°C in a Hoefer SE600 protein gel apparatus under the conditions described by Laemmli (18). Radioactivity was measured by autoradiography (60 min) and by counting with a Betascope 603 blot analyzer (Betagen, Mountain View, Calif.).

To determine the stability of phosphorylated CheA_L, 50 μ M [γ -³²P]ATP was added to purified preparations of CheA_L (final concentration, 6 μ M) at room temperature buffered in 50 mM TKMD at either pH 7.0 or 8.0. After 2 min, the ATP was absorbed by addition of activated charcoal, which was removed 15 min later by filtration. The zero time point was defined as the end of the filtration, and aliquots were removed at 15-min intervals and mixed immediately with 3 \times SDS-PAGE sample buffer. Gels were run, and the level of CheA phosphorylation was quantified as described above.

For kinetic studies, CheA (final concentration, 1 μ M) in 50 μ l of 5 mM TEDKM buffer (5 mM Tris HCl [pH 7.5], 50 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EDTA) was rapidly mixed with 50 μ l of a concentrated buffer solution of the desired pH (see below) containing between 50 and 500 μ M [γ -³²P]ATP (specific activity, between 2,000 and 20,000 cpm

pmol^{-1}). Aliquots (10 μ l) were removed at various times and mixed with an equal volume of SDS-EDTA sample buffer (4% SDS, 50 mM EDTA, 25 mM Tris HCl [pH 6.8], 20% sucrose, 10% β -mercaptoethanol). Samples were subjected to SDS-10% PAGE with the conditions of Laemmli (18), except that Chromaphor protein stain (Promega) was added to the buffer in the upper electrode compartment, so that protein staining was accomplished during the electrophoresis run. Chromaphor permits immediate visualization of protein bands without the use of acidic staining and destaining procedures that promote hydrolysis of the CheA phosphohistidine bond (5, 40). Gel slices containing CheA were placed in BioLite cocktail (ICN) and analyzed by scintillation counting. At the following pH values, the indicated buffer combinations were used: pH 6.5 and 7.0, 150 mM Tris HCl in the presence or absence of 50 mM morpholineethanesulfonic acid (MES); pH 7.5, 7.7, 8.0, 8.5, 8.75, and 9.1, 150 mM Tris HCl; pH 9.5 and 10.0, 150 mM Tris HCl in the presence or absence of 50 mM bicarbonate. Each of these buffer solutions also contained 5 mM MgCl₂, 50 mM KCl, and ATP at the desired concentration. Final pH values were determined by measuring the pHs of test solutions obtained by mixing the same volumes of 5 mM TEDKM (in the absence of CheA) and the same concentrated buffer solutions as used for the measurements of phosphorylation. Preliminary experiments indicated that Tris and several other buffers, e.g., *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and morpholinepropanesulfonic acid (MOPS), moderately inhibit CheA autophosphorylation at concentrations of 50 and 150 mM. Therefore, we included 150 mM Tris in all reactions, even those performed at pH values at which Tris has limited buffering capacity, e.g., pH 6.5, 9.5, and 10.0. At these pH

values, we obtained comparable results in the presence and absence of the second buffer component, i.e., MES or bicarbonate, which did have significant buffering capacity. Neither of these second buffer components appeared to inhibit autophosphorylation. Since the longest time required to measure the full course of the autophosphorylation reaction was 60 min, control experiments were performed to show that such an extended incubation at pH values ranging from 6.5 to 10.0 did not significantly affect the catalytic activity of CheA.

Tethered-cell assays. Cells were grown in tryptone broth at 30°C to mid-exponential phase and harvested, washed, and tethered as described by Wolfe et al. (38), except that IPTG was present throughout growth. Rotational behavior was recorded on videotape and analyzed by computer (3). Computations of bias (mean $[\pm$ standard error of the mean]) of the fraction of time that cells rotate CCW) were based on measurements spanning 3 min for each of 8 to 24 cells. Mean values were computed over a time span of 40 s and plotted every 4 s. Only cells exhibiting a measured bias of less than 0.95 were scored, by eye, for their responses to L-aspartate or benzoate. The responses to benzoate of a subset of these cells were also quantified by computer.

RESULTS

In vitro autophosphorylation of CheA_L and the effects of pH.

We performed a series of in vitro experiments using purified preparations of wild-type CheA_L. This protein was purified to near homogeneity, as judged by SDS-PAGE, and confirmed as CheA by Western immunoblotting using anti-CheA antibody (39).

To obtain an initial assessment of the effect of pH upon CheA_L autophosphorylation, we added [γ -³²P]ATP to purified preparations of CheA_L buffered across a range of pH values from 6.5 to 9.5. At the conclusion of a 2-min labelling period, each reaction was terminated by the addition of 3× SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by autoradiography. The level of phosphorylated CheA_L observed at this single time point increased about sevenfold from a minimum at pH 6.5 to a maximum at pH 8.0. Above pH 8.0, the level of phosphorylation decreased such that at pH 9.0 the level was about half the maximum level. Qualitatively similar results were obtained with purified CheA_L98MI (data not shown).

To determine whether our results were due to differential degradation of the CheA_L phosphohistidine at different pH values, we assessed the stability of phosphorylated CheA_L. When incubated for 30 min at either pH 7.0 or 8.0, greater than 85% of the phosphorylated CheA_L remained (data not shown). Thus, our results cannot be due to instability of the phosphorylated form of CheA_L. These results are consistent with previous work on CheA (13, 40) and on other proteins which contain phosphohistidine (1, 14, 35).

To better characterize the pH-sensitive component of the autophosphorylation reaction, we monitored the appearance over time of phosphorylated CheA_L after mixing purified preparations of CheA_L with various concentrations of [γ -³²P]ATP at a series of pH values: 6.5, 7.0, 7.5, 7.7, 8.0, 8.5, 8.75, 9.1, 9.5, and 10.0. At various time points, aliquots were withdrawn, mixed with 3× SDS-PAGE sample buffer, and then subjected to SDS-PAGE. Gel slices containing CheA_L were excised, and the radioactivity in each slice was measured by scintillation counting. CheA_L autophosphorylation followed an exponential time course that we analyzed to determine the apparent first-order rate constant at each ATP concentration (Fig. 2). At each pH value tested, the dependence of the

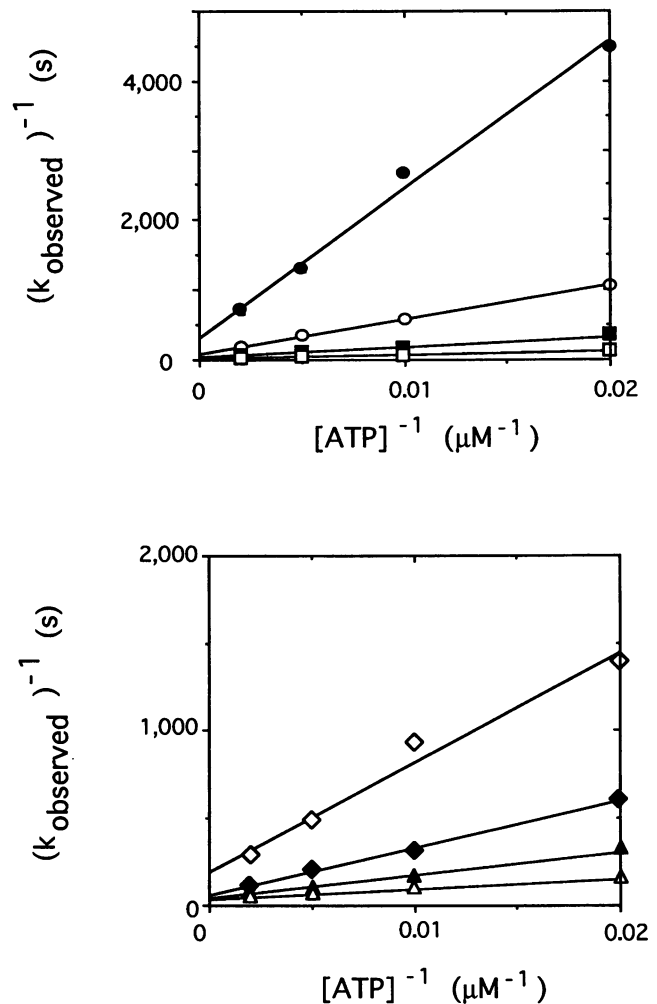


FIG. 2. Effects of ATP concentration and pH on the rate of formation of phospho-CheA_L. Reaction time courses were monitored (see Materials and Methods). Double-reciprocal plots are shown for reactions performed at pH 7.0 (●), 7.5 (○), 8.0 (■), 8.75 (□), 9.1 (△), 9.5 (▲), 9.5 (◆), and 10.0 (◇). Values shown are averages of three independent determinations at each pH. Lines are linear least-squares fits to the data.

observed rate constant ($k_{2,observed}$) on the concentration of ATP exhibited saturation kinetics. Thus, CheA autophosphorylation can be modelled as a reaction of the type $A + B \rightleftharpoons C \rightarrow D$. Doing so yields the following equation:



For each pH tested, we plotted the reciprocal of the observed rate constant versus the reciprocal of the ATP concentration (Fig. 2). From these data, we extrapolated both the apparent rate constant for phosphotransfer from ATP to CheA_L ($k_{2,apparent}$, derived as the reciprocal of the y-axis intercept) and the affinity of CheA for ATP ($K_D = k_{-1}/k_1$, where k_{-1}/k_1 represents the reciprocal of the extrapolated x-axis intercept). The apparent K_D exhibited little sensitivity to pH (Fig. 3A). In contrast, the apparent rate constant for the phosphotransfer step was quite sensitive to pH, increasing over 40-fold as the

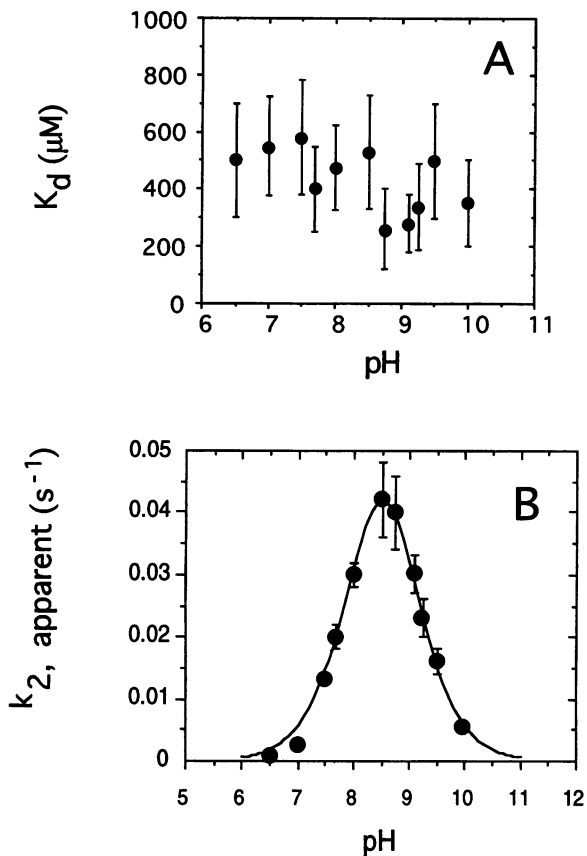
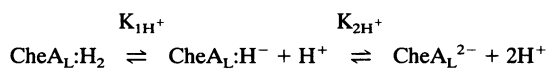


FIG. 3. Effect of pH on kinetic constants that define the interaction of CheA with ATP. (A) Plot of kinetically determined K_D of the CheA · ATP complex versus pH. (B) pH dependence of the apparent rate constant for phosphotransfer from ATP to CheA_L. Values of $k_{2, \text{apparent}}$ were determined from the y-axis intercepts of double-reciprocal plots (Fig. 2 and data not shown). Error bars represent the standard errors of the mean. The solid line results from calculations described below and indicates the pH dependence of $k_{2, \text{apparent}}$ expected for the detailed scheme described below. The best agreement between the calculated and the experimental values was obtained by using $\text{p}K_{1\text{H}^+} = 8.1$ and $\text{p}K_{2\text{H}^+} = 8.9$. Calculations at a series of different $\text{p}K_{1\text{H}^+}$ and $\text{p}K_{2\text{H}^+}$ values indicated that the uncertainty associated with the reported values is ± 0.3 . In making these calculations, we assumed that two distinct proton dissociation events determine the fraction of CheA that is present in a form capable of acquiring the γ -phosphoryl group of ATP, one that increases the probability of phosphorylation (governed by the proton dissociation constant $K_{1\text{H}^+}$) and one that decreases the probability of phosphorylation (governed by the proton dissociation constant $K_{2\text{H}^+}$):



Thus, $\text{p}K_{1\text{H}^+}$ represents the $\text{p}K_a$ of the group responsible for CheA_L activation, and $\text{p}K_{2\text{H}^+}$ represents the $\text{p}K_a$ of the group responsible for CheA_L deactivation. In this scenario, only the singly protonated form (CheA_L:H⁻) reacts with ATP to generate phosphorylated CheA_L at an appreciable rate such that $k_{2, \text{apparent}} = k_2 \times$ (the fraction of CheA_L present in the singly protonated form [CheA_L:H⁻]). Expressing this relationship in terms of the two proton dissociation equilibria (9, 24) gives the following equation:

$$k_{2, \text{apparent}} = k_2(1 + 10^{-\text{pH} + \text{p}K_{1\text{H}^+}} + 10^{\text{pH} - \text{p}K_{2\text{H}^+}})^{-1}$$

To obtain estimates of the values of $\text{p}K_{1\text{H}^+}$ and $\text{p}K_{2\text{H}^+}$, we systematically substituted different combinations of the two dissociation constants into the last equation and then calculated $k_{2, \text{apparent}}$ at a series of 40 different pH values between 6 and 11.

pH increased in the range between 6.5 and 8.5 and decreasing 10-fold in the pH range 8.5 to 10.0 (Fig. 3B). Our quantitative analysis of the pH dependence of CheA_L autophosphorylation is in accordance with the qualitative description provided previously by Hess et al. (13).

In vivo behavior of gutted cells that express CheY and the transducers Tar and Trg. We transformed cells of strain HCB696, which express CheY and the transducers Tar and Trg, with plasmids that express CheA_L, CheA_S, and CheW (pAJW103); CheA_L and CheW (pAJW24); CheA_L, CheW, and a large partially functional fragment of Tar (pAJW21); CheA_L and CheA_S (pMPC3); and CheA_L only (pAJW20). These transformations yielded strains AJW70, AJW40, AJW32, AJW29, and AJW31, respectively. After growth in the presence of either 25 or 100 μM IPTG to induce the expression of *cheY*, cells of each strain were tethered and their individual biases were determined (Table 2, top).

To ascertain the ability of cells to respond to changes in intracellular pH, we exposed these tethered cells to benzoate. *E. coli* cells normally maintain a cytoplasmic pH of about 7.6 (23, 29, 41). The addition of benzoate (10 mM) at pH 7.0 has been shown to cause a decrease in pH of about 0.3 U (29). Cells that expressed transducers and CheY together with either CheA_L, CheA_S, and CheW (strain AJW70) or CheA_L and CheW (strains AJW32 and AJW40) responded to additions of both L-aspartate and benzoate. Cells that expressed CheA_L, CheA_S, and CheW (strain AJW70) exhibited the strongest responses to both L-aspartate and benzoate; i.e., their responses had the largest amplitude and longest duration. Figure 4A demonstrates the response to the addition and removal of benzoate of one such cell. Cells that expressed transducers, CheA_L, and CheA_S, but not CheW (strain AJW29), did not respond to L-aspartate. Surprisingly, these cells did respond to benzoate, albeit weakly and not uniformly across the entire sample tested. Figure 4B and C demonstrate the response to the addition and removal of benzoate of two such cells. Each cell exhibited a large unstimulated bias (about 0.95 and 0.90, respectively). Following the addition of benzoate, however, each shifted its bias to 1, i.e., shifted it to CCW rotation exclusively. Upon the removal of benzoate, the bias of each cell returned to the lower level. Because cells that expressed CheA_L only (strain AJW31) exhibited very large unstimulated biases (i.e., they spun almost exclusively CCW) (Table 2, top), we were unable to determine whether they responded to either L-aspartate or benzoate.

In vivo behavior of gutted cells that express CheY but not the transducers Tar and Trg. We next transformed cells of strain HCB722, which express CheY but not transducers, with plasmids that express CheA_L, CheA_S, and CheW (pAJW103); CheA_L and CheW (pAJW24); CheA_L and CheA_S (pMPC3); and CheA_L only (pAJW20). These transformations yielded strains AJW71, AJW49, AJW48, and AJW47, respectively. Cells of these strains were grown, tethered, and analyzed as described above, except that a single IPTG concentration (100 μM) was used to induce the expression of *cheY*. Although they did not respond to L-aspartate, cells of strains that expressed CheA_L and CheA_S (AJW48) or CheA_L, CheA_S, and CheW (AJW71) responded to benzoate by increasing their bias, i.e., by increasing CCW rotation (Table 2, bottom). Cells that expressed all three proteins (strain AJW71) exhibited the strongest response, one which was similar in amplitude and duration to that exhibited by cells (strain AJW70) which also expressed transducers (Table 2, top). Because cells which expressed either CheA_L and CheW (strain AJW49) or CheA_L alone (strain AJW47) exhibited very large unstimulated biases, we were unable to test their responses to either stimulus.

TABLE 2. Bias and response of strains that express CheY and subsets of CheA_L, CheA_S, CheW, and Tar

Strain	Expression ^a of:					Bias at the following IPTG concn ^b :		Response ^c to:	
	CheA _L	CheA _S	CheW	CheY	Tar			Aspartate	Benzoate
						25 μM	100 μM		
AJW70	+	+	+	+	+	0.90 ± 0.03 (22)	0.35 ± 0.08 (20)	+++	+++
AJW32	+ ^d	—	+	+	+ ^e	0.53 ± 0.11 (17)	0.78 ± 0.07 (20)	++	++
AJW40	+ ^d	—	+	+	+	0.99 ± 0.01 (24)	0.74 ± 0.10 (20)	++	+
AJW29	+	+	—	+	+	0.86 ± 0.08 (13)	0.83 ± 0.04 (17)	—	++
AJW31	+ ^d	—	—	+	+	0.98 ± 0.02 (14)	0.95 ± 0.02 (19)	0	0
AJW71	+	+	+	+	—		0.62 ± 0.07 (18)	—	+++
AJW49	+ ^d	—	+	+	—		0.97 ± 0.03 (12)	0	0
AJW48	+	+	—	+	—		0.77 ± 0.03 (16)	—	++
AJW47	+ ^d	—	—	+	—		0.99 ± 0.01 (20)	0	0

^a +, expressed; —, not expressed: CheY from *lacP*, Tar from its native promoter, and CheA_L, CheA_S, and CheW from *trpP*.

^b Mean ± standard error of the mean (number of cells) of the fraction of time a cell spins CCW following growth in the presence of IPTG at the concentration specified.

^c The strongest response (CCW) by cells at either induction level to the addition of 10 μM aspartate or 10 mM benzoate (pH 7.0), based on the fraction of cells that respond and the amplitude and duration of those responses. 0, not detectable because the bias is ~1; —, no response; +, weak response; ++, moderate response; +++, strong response.

^d CheA_L98ML.

^e A large N-terminal fragment of Tar that retains some function.

To verify these observations, we tested cells of another strain (AJW253) that, in addition to CheY, expressed CheA_L, CheA_S, and CheW. In contrast to the cells described above, these cells retained *cheA* and *cheW* in their usual chromosomal locations. Thus, both genes were present in single copy. These cells also responded to benzoate but not to L-aspartate (data not shown).

DISCUSSION

Here, we have demonstrated that the rate of CheA_L autophosphorylation in vitro is sensitive to pH in the range between 6.5 and 10.0, yielding maximal activity at about pH 8.5. This sensitivity results primarily from pH effects on the rate of ATP-to-CheA_L phosphotransfer and not from alterations in the affinity of CheA for ATP. These results are most simply explained by the existence of two distinct proton dissociation events which influence the ability of CheA_L to interact productively with ATP. The dissociation of a proton from some, as yet unknown, protein moiety with a pK_a of ~8.1 would enhance the ability of CheA to acquire the γ-phosphoryl group of ATP, while the dissociation of a proton from another group with a pK_a of ~8.9 would diminish this ability.

In the absence of additional information, it is not possible to assign these pK_a values to specific groups of the CheA_L protein. However, it is tempting to speculate that the titratable group which possesses a pK_a of ~8.1 may be the imidazole of His-48, the site of CheA autophosphorylation (12). Although the imidazole nitrogen of isolated histidine possesses a pK_a of 6.2, it is not unusual for the pK_a of such a moiety, when incorporated into protein, to be shifted several units by surrounding amino acids (9). In the protonated form, the imidazole nitrogens of His-48 would be less capable of nucleophilic attack on the γ-phosphoryl group of ATP. Thus, the rate of autophosphorylation would be markedly enhanced by raising the pH to a level that resulted in deprotonation of the histidyl ring. The nature of the second titratable group, which when deprotonated markedly diminishes the rate of autophosphorylation, is unclear. However, it is possible that the protonated form of this second group could facilitate phosphorylation of His-48 by maintaining an appropriately charged environment at the phosphorylation site.

It must be emphasized that we have no direct evidence

indicating that either of the kinetically defined, protonatable groups is attached to His-48 or is located in the vicinity of the CheA_L kinase and/or ATP-binding sites. It is conceivable that the kinase active site of CheA could be affected indirectly by protonation and deprotonation of groups not directly involved in catalysis of the phosphotransfer reaction. Regardless of the specific nature and location of these groups, however, it is clear that our in vitro results predict that decreasing the cytoplasmic pH from 7.6 to 7.3 (as expected in response to the addition of 10 mM benzoate buffered at pH 7.0) would significantly decrease the rate of CheA autophosphorylation. Previous work (4, 21) has shown that CheA autophosphorylation represents the rate-limiting step in generating phosphorylated CheY in the presence of physiologically relevant concentrations of CheA, CheY, and ATP. Therefore, such a decrease in cytoplasmic pH would be expected to lower the concentration of phosphorylated CheY, thereby causing an increase in the probability of CCW rotation of cells expressing CheA and CheY. Conversely, an increase in cytoplasmic pH from 7.3 to 7.6 would be expected to decrease the probability of CCW rotation.

Such results were obtained in vivo. Cells that expressed CheA_L, CheA_S, and CheY, but not transducers or CheW, responded, albeit weakly, to a decrease in cytoplasmic pH by increasing CCW rotation of their flagellar motors and to an increase in pH by decreasing CCW rotation. These results indicate that the transducers, CheW, CheR, CheB, and CheZ are not absolutely required for response to changes in internal pH. Some of these components, however, likely influence this sensitivity. For example, the expression of CheW greatly enhanced the amplitude and duration of the response such that it resembled that exhibited by cells which express all the chemotaxis proteins except the transducer Tsr.

Our results clearly indicate that autophosphorylation of CheA is sensitive to pH in vitro. Although we cannot rule out the possibility that the alterations of motor bias observed in vivo may also involve changes in the stability and/or efficacy of phosphorylated CheY as it interacts with the motor, it is conceivable that pH sensitivity of CheA autophosphorylation directly enables changes of swimming behavior in response to changes in cytoplasmic pH for cells that express CheA and CheY but not transducers. This possibility raises interesting questions concerning the molecular mechanism underlying pH

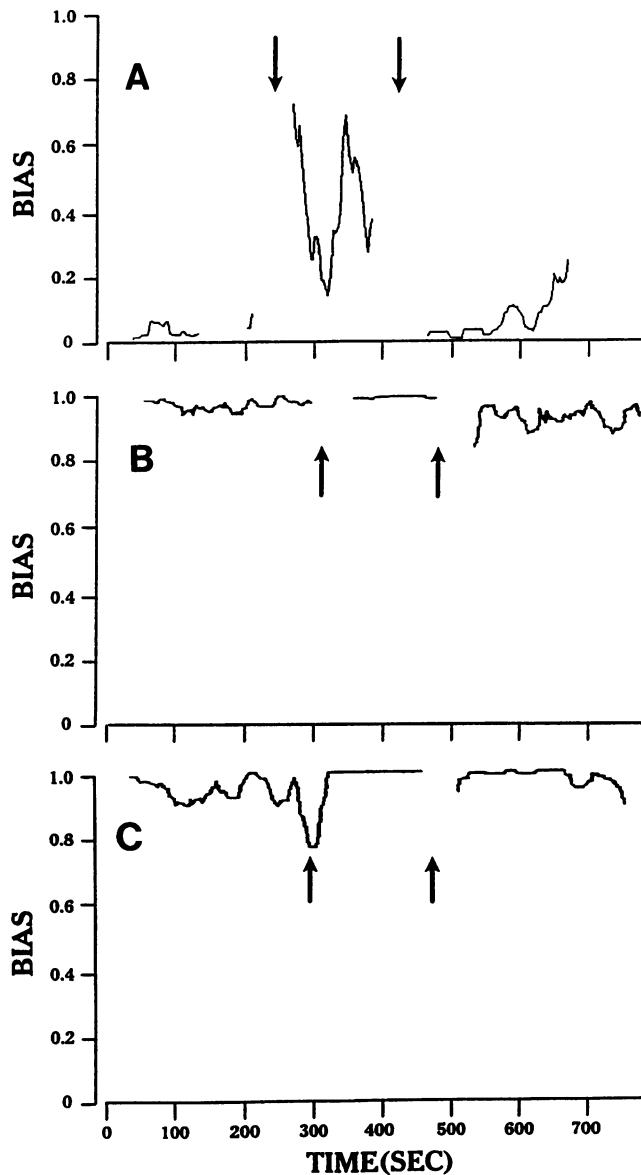


FIG. 4. Measurement of bias (fraction of time that tethered cells rotate CCW) of individual cells to which benzoate (10 mM, pH 7.0) was added (first arrows) and removed (second arrows). (A) Cell of a strain expressing CheA_L, CheA_S, CheW, Tar, Trg, and CheY (AJW70). (B and C) Two cells of a strain expressing CheA_L, CheA_S, Tar, Trg, and CheY but not CheW (AJW29). Cells were grown in the presence of 100 μ M IPTG to induce CheY. The breaks in the plots (which represent running averages) span intervals perturbed by buffer flow or during which the cell stopped spinning.

responsiveness of wild-type cells which have, in addition to CheA and CheY, CheW and a full complement of transducers: Tar, Tsr, Trg, and Tap. Krikos and coworkers (17) suggested that the sensor for chemotactic responses to pH resides in the cytoplasmic domains of the transducers Tar and Tsr. It is possible that, by virtue of the pH dependence of its rate of autophosphorylation, CheA could serve as a sensor of cytoplasmic pH in the absence of transducers and CheW and that the sensitivity of that sensor might be enhanced by their presence. How might transducers exert such an influence? They could possibly enable pH changes to exert a more

pronounced effect upon the rate of CheA autophosphorylation. In vitro, the formation of ternary complexes by transducers, CheA, and CheW (10) markedly enhances the autokinase activity of CheA (5). Thus, pH might influence the formation of these ternary complexes. Alternatively, it might affect the ability of the transducers to stimulate CheA activity within these complexes.

In most respects, the transducers Tar and Tsr appear to direct the autokinase activity of CheA via a common mechanism (5). However, one intriguing difference between these two transducers exists. Although it elicits a CCW response from cells that express Tar but not Tsr, benzoate evokes a clockwise response from cells that also express Tsr. One possible biochemical explanation for the differences could be that a decrease in cytoplasmic pH in the presence of Tsr results in the stimulation of CheA autophosphorylation, whereas the same pH change in the presence of Tar results in the inhibition of CheA autophosphorylation. Perhaps Tsr decreases the pH optimum of CheA autophosphorylation (by ~ 1.5 U) such that the relevant pH values (7.6 and 7.3) would fall on the basic limb of the bell-shaped profile of rate versus pH. Under such circumstances, a decrease in pH would cause an increased rate of CheA autophosphorylation and, thus, a clockwise response by the flagellar motor. Alternatively, pH could affect the coupling of Tsr and Tar to CheA in opposing ways; i.e., identical decreases in cytoplasmic pH might increase Tsr-CheA coupling yet decrease Tar-CheA coupling. In vitro investigations of the pH effect on transducer-CheA-CheW interactions will further define the molecular events underlying the bacterial chemotactic response to changes in cytoplasmic pH.

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