

Elucidation of a PTS–Carbohydrate Chemotactic Signal Pathway in *Escherichia coli* Using a Time-resolved Behavioral Assay

Renate Lux,* V. Ranjit N. Munasinghe,[†] Fred Castellano,* Joseph W. Lengeler,[‡] John E. T. Corrie,* and Shahid Khan*[§]

*Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461; [†]National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom; and [‡]Fachbereich Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany

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Chemotaxis of *Escherichia coli* toward phosphotransferase systems (PTSs)–carbohydrates requires phosphoenolpyruvate-dependent PTSs as well as the chemotaxis response regulator CheY and its kinase, CheA. Responses initiated by flash photorelease of a PTS substrates D-glucose and its nonmetabolizable analog methyl α -D-glucopyranoside were measured with 33-ms time resolution using computer-assisted motion analysis. This, together with chemotactic mutants, has allowed us to map out and characterize the PTS chemotactic signal pathway. The responses were absent in mutants lacking the general PTS enzymes EI or HPr, elevated in PTS transport mutants, retarded in mutants lacking CheZ, a catalyst of CheY autodephosphorylation, and severely reduced in mutants with impaired methyl-accepting chemotaxis protein (MCP) signaling activity. Response kinetics were comparable to those triggered by MCP attractant ligands over most of the response range, the most rapid being $11.7 \pm 3.1 \text{ s}^{-1}$. The response threshold was $<10 \text{ nM}$ for glucose. Responses to methyl α -D-glucopyranoside had a higher threshold, commensurate with a lower PTS affinity, but were otherwise kinetically indistinguishable. These facts provide evidence for a single pathway in which the PTS chemotactic signal is relayed rapidly to MCP–CheW–CheA signaling complexes that effect subsequent amplification and slower CheY dephosphorylation. The high sensitivity indicates that this signal is generated by transport-induced dephosphorylation of the PTS rather than phosphoenolpyruvate consumption.

INTRODUCTION

Chemotaxis of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* provides a paradigm for mechanistic analysis of how phosphorylation circuits mediate sensory responses (Bray, 1998). The motility of these bacteria consists of an alternating pattern of swimming runs and tumbles. A counterclockwise (CCW) rotating flagellar bundle drives swimming runs. Clockwise (CW) rotation of a presently undetermined number of flagella leads to bundle breakup, generating tumbling events that randomize cell orientation (Macnab and Ornston,

1977). Chemotactic migration is effected primarily by an increase of swimming runs up positive gradients (Berg and Brown, 1972). The bacteria use a temporal gradient-sensing mechanism. The chemotactic response to a sudden change in chemoeffector concentration consists of a subsecond excitation phase followed by slower adaptation back to prestimulus behavior (Macnab and Koshland, 1972).

The central chemotaxis circuit consists of the response regulator protein CheY, its kinase (CheA), and a catalyst of its autophosphatase activity (CheZ). CheY shuttles between methyl-accepting chemotaxis protein (MCP) complexes and flagellar motors. The MCP family of transmembrane chemoreceptors processes re-

[§] Corresponding author. E-mail address: skhan@aecom.yu.edu.

sponses to attractants (oxygen, amino acids, and periplasmic sugar-binding proteins) as well as to repellents (leucine, weak acids/bases, extremes of pH, and temperature). The autophosphorylating histidine kinase CheA, together with the linker CheW, is stably associated with the MCPs, forming signaling complexes (Gegner *et al.*, 1992). Analogous histidyl-aspartyl "two-component" phosphorelays and type I chemoreceptors mediate signal transduction processes in a wide range of species (Appleby *et al.*, 1996; Stock and Surette, 1996). CheA phosphorylates CheY on an aspartyl residue (Sanders *et al.*, 1989). Phosphorylated CheY (CheY.P) dissociates rapidly from the receptors (Schuster *et al.*, 1993) and binds to flagellar motors, enhancing CW rotation (Welch *et al.*, 1993). Configurational changes in MCPs triggered by binding of attractant ligands to the MCP periplasmic domain inhibit CheA activity, generating a positive, CCW motor response that promotes smooth-swimming. Withdrawal of MCP attractant ligands or addition of repellent ligands stimulates CheA kinase, generating negative, CW motor responses (Larsen *et al.*, 1974), hence tumbling. The response to amino acid attractants is exquisitely sensitive (Segall *et al.*, 1986). Aggregation of MCP signaling complexes may amplify individual ligand-MCP associations to achieve this high sensitivity (Bray, 1998).

Chemotaxis toward carbohydrates uses two pathways. In one pathway, carbohydrates use periplasmic binding protein components of the ATP-binding cassette transporters that bind MCPs when complexed with their sugar ligands. This association triggers smooth-swim responses independently of transport into the cell (Hazelbauer and Adler, 1971). The second pathway uses the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase systems (PTSs), in which chemotaxis is inextricably linked to transport of the substrate (Lengeler and Jahreis, 1996, and references therein). PTSs consist of membrane-bound and substrate-specific Enzyme II (EII) complexes that accept phosphate from a cytoplasmic donor phosphorelay to phosphorylate the substrate as it is transported. The relay consists of Enzyme I (EI), a PEP-dependent histidine kinase, and a phosphohistidine carrier protein (HPr). EI and HPr are common to all EIIs, of which there are at least 15 in *E. coli* (Postma *et al.*, 1996).

The chemotactic response range and threshold of PTS substrates has been characterized by swarm agar and capillary assays. The positive, response thresholds varied with the K_m values of the substrate for its EII, regardless of the specific substrate-EII combination examined. Neither binding of a PTS substrate to its EII nor intracellular accumulation and subsequent metabolism of its phosphorylated form can by themselves trigger a chemotactic response (Adler *et al.*, 1973;

Adler and Epstein, 1974; Lengeler, 1975; Lengeler *et al.*, 1981; Pecher *et al.*, 1983).

The role of the MCP-Che circuitry during PTS-dependent chemotaxis has been explored. Responses to PTS stimuli and their subsequent adaptation do not depend on MCP methylation as established by study of bacteria tethered by a single flagellum to glass coverslips (Niwano and Taylor, 1982). Gutted strains lacking all Che proteins except trace amounts of CheZ (Abouhamed *et al.*, 1998) responded to the PTS substrate mannose only during plasmid-based expression of CheA, CheW, and CheY, but the response was CW instead of CCW (Rowell *et al.*, 1995). EI, but not EI.P, inhibited CheA autophosphorylation *in vitro*; however, half-maximal inhibition was obtained at an EI/CheA ratio fivefold greater than that present in the cell (Lux *et al.*, 1995).

Thus, inhibition of CheY phosphorylation by CheA attributable to accumulation of unphosphorylated EI during transport could underlie PTS chemotaxis but may be supplemented or modulated in important ways by additional processes. Changes in PEP levels, which also occur during transport (Lowry *et al.*, 1971), could play this role. PEP was found to stimulate CheA autophosphorylation *in vitro* at physiological (1 mM) concentration (Lux *et al.*, 1995). Furthermore, because the pyruvate generated from PEP feeds via acetyl CoA into the tricarboxylic acid cycle, transport of PTS substrates is likely to affect levels of acetyl.AMP metabolites via acetyl CoA, as well as tricarboxylic acid cycle intermediates such as fumarate. These have recently been implicated in CheY acetylation (Ramakrishnan *et al.*, 1998) and control of motor switching (Montrone *et al.*, 1996; Prasad *et al.*, 1998), respectively.

Mutant screens have been invaluable for analysis of chemotactic signal pathways, but PTS chemotactic mutants with associated defects in cellular metabolism may have been lethal and thus difficult to isolate. Biochemical data reveal molecular interactions, but assessment of their role in the intact cell requires behavioral assays. Behavioral capillary or swarm plate assays measure chemotactic response range but not kinetics, whereas flow cell-based assays of tethered bacteria measure adaptation, but only for responses that last several seconds. None of these assays permits quantification of the timing and amplitude of chemotactic signals. Photolysis of photolabile (caged) precursors by near-UV flash irradiation provides a potent means for *in vivo* perturbation, which in conjunction with appropriate time-resolved assays has been exploited for detailed mechanistic analysis of signaling pathways (Somlyo *et al.*, 1988; Corrie *et al.*, 1993; Ogden and Capiod, 1997). In a genetically characterized organism such as *E. coli*, this approach may also be coupled with analysis of mutants to temporally isolate, map, and characterize relatively ill-defined pathways, as illustrated here for the PTS chemotactic sig-

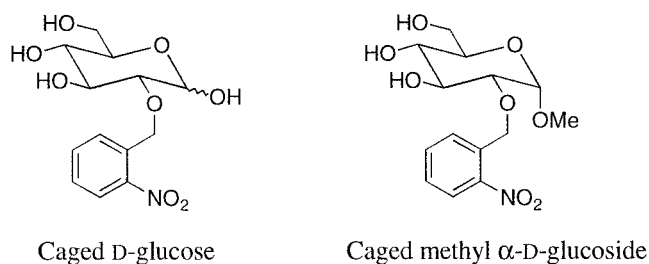


Figure 1. Chemical structures of the caged sugars used in this study.

naling pathway. Processing of PTS-dependent signals was time-resolved by coupling photolysis of caged precursors of D-glucose (D-glc) and its nonmetabolizable analog methyl α -D-glucopyranoside (Me α -glc) (Figure 1) to computer-assisted motion analysis of the response (Khan *et al.*, 1993, 1995). Available data indicate that chemotactic responses triggered by these sugars are representative of responses obtained for all PTS substrates studied thus far (Adler *et al.*, 1973; Lengeler *et al.*, 1981).

The temporal resolution of this assay revealed that MCP signaling complexes are also used by PTS substrates to relay chemotactic signals with rapidity and sensitivity comparable to amino acid attractants. The high sensitivity renders implausible the idea that the signal to the MCP complexes derives from perturbation of PEP or associated metabolite levels, but may be reconciled with interaction of PTS Enzyme I with the MCP-associated CheA.

MATERIALS AND METHODS

Caged Compounds

A caged fluorophore, the 1-(2-nitrophenyl)ethyl ether of 8-hydroxypyrene-1,3,6-trisulfonic acid (caged HPTS) was used to estimate the magnitude of the concentration jumps achieved in flash photorelease assays to within 20% (Jasuja *et al.*, 1999). Caged L-aspartate [aspartic acid β -(2,6-dinitrobenzyl) ester] and caged L-serine [N-1-(2-nitrophenyl)ethoxycarbonyl-L-serine] were used to measure excitation responses to aspartate and serine, attractant ligands for the major MCPs Tar and Tsr, respectively. The synthesis and photochemical properties of the caged HPTS, L-aspartate, and L-serine reagents have been described (Khan *et al.*, 1993; Jasuja *et al.*, 1999).

The synthesis and photochemical properties of caged D-glc [2-O-(2-nitrobenzyl)-D-glucose] used in this study have been described (Corrie, 1993), and caged Me α -glc was prepared by modification of the caged D-glc synthesis, as described below. A solution of methyl 3,4,6-tri-O-acetyl-2-O-(2-nitrobenzyl)- α -D-glucopyranoside (Corrie, 1993) (0.53 g, 1.16 mmol) in methanol (5.1 ml) was treated with 2 M aqueous NaOH (1.88 ml). After 1 h at room temperature, the solution was stirred with MeOH-washed Dowex 50 (Sigma, Dorset, United Kingdom; H⁺ form; 3.41 g) to neutralize the alkali, and then filtered. The filtrate was evaporated under reduced pressure, and the residue was flash-chromatographed (MeOH:CHCl₃, 6:94 vol/vol ratio; Merck, Dorset, United Kingdom; 40–63 μ m silica gel). Pure fractions recovered from chromatography were combined and evaporated under reduced pressure, and the residue was dissolved in water and lyophilized to give caged Me α -glc [methyl 2-O-(2-

nitrobenzyl)- α -D-glucopyranoside] as a pale yellow foam (0.27 g, 70%); (Found: [FAB mass spectrometry] [M + Na]⁺ 352.1020. [C₁₄H₁₉NO₈ + Na]⁺ requires M⁺, 352.1020); UV: λ_{max} (H₂O)/nm 265 (ϵ 5300 M⁻¹cm⁻¹); δ_{H} (400 MHz; D₂O; acetone standard) 8.09 (1 H, d, J 8.0 Hz, ArH-3), 7.72–7.78 (2 H, m, ArH), 7.53–7.63 (1 H, m, ArH), 5.06 (2 H, ABq, J 13.2 Hz, ArOCH₂) 4.87 (1 H, d, J_{1,2} 3.7 Hz, H-1), 3.85 (1 H, dd, J_{6,6'} 12.3 Hz, J_{5,6} 2.2 Hz, H-6), 3.73 (1 H, dd, J_{5,6'} 4.6 Hz, H-6') superimposed on 3.73 (1 H, t, J_{2,3} = J_{3,4} 9.6 Hz, H-3), 3.61 (1 H, ddd, J_{4,5} 9.6 Hz, H-5), 3.50 (1 H, dd, H-2), 3.40 (1 H, t, H-4), 3.36 (3 H, s, OMe).

The release rate of the glucoside upon flash photolysis was inferred from the decay rate of the photochemically generated *aci*-nitro intermediate, as described for the caged D-glc (Corrie, 1993). As for the caged D-glc, flash photolysis (pH 7.0, 20°C, 150 mM Na phosphate) showed biphasic decay, with rates of 97 and 7 s⁻¹ and relative amplitudes of 2.6:1. To determine the product quantum yield, Q_P, of photolysis, aliquots (0.5 ml) of a solution of caged Me α -glc and caged D-glc (each 50 μ M) with dithiothreitol (2 mM) in 10 mM sodium phosphate (Sigma) (pH 7.0) were exposed for varying times (8–24 s) to light from a xenon arc lamp (Photochemical Research Associates, London, Ontario, Canada) that passed through a Hoya Optics (Fremont, CA) U340 filter before illuminating the cell. The irradiated samples were kept at room temperature overnight to allow the anomers of the residual caged D-glc to reequilibrate (Corrie, 1993) and were analyzed by reversed-phase HPLC [Merck Li-chrosphere RP8 column (catalog no. 50832); mobile phase 10 mM sodium phosphate, pH 7.0, plus 25% MeOH (vol/vol); flow rate 1.5 ml min⁻¹; UV detection at 254 nm]. Caged Me α -glc eluted at 15.6 min and caged D-glc eluted as a double peak at 6.6 and 7.5 min. The extents of conversion for caged Me α -glc and caged D-glc were 35.1–68.7 and 39.6–75.3%, respectively (means of three determinations at each time point), with caged D-glc converted 1.07-fold more efficiently than caged Me α -glc. The Q_P for caged D-glc is 0.63 (Corrie, 1993), and the value for caged Me α -glc was therefore 0.59.

Growth Media and Chemicals

Bacteria were grown at 35°C in Luria broth (plus 10 mM D-glc and 2.5 mM CaCl₂) for P1 transduction, and in tryptone broth for behavioral assays. Tryptone swarm agar (0.35%) plates were used for motility selection, and minimal medium (Adler, 1973) swarm agar (0.27%) plates (Difco) were used for diagnosing phenotypes.

The bacteria were washed thrice and resuspended in motility buffer before experiments (10 mM sodium/potassium phosphate, pH 7.0, 10 mM potassium chloride, 0.1 mM EDTA, 5 mM lithium lactate, 125 μ M methionine). For flash photorelease assays, this buffer also contained 5 mM dithiothreitol. Stocks of lactate, methionine, and dithiothreitol were stored at -20°C and added to the motility buffer before experiments. D-glc, Me α -glc (< 0.01% D-glc contamination), L-aspartate, and L-leucine were purchased from Sigma (St. Louis, MO).

Bacterial Strains

The bacterial strains used in this work are listed in Table 1. Mutant strains used for analysis of PTS-mediated chemotactic responses were constructed in *E. coli* K12 strain JW1184-1. Mutations were moved via P1 transduction (Arber, 1960) as modified (Lengeler, 1975).

Behavioral Assays

Flash Photorelease Assays. Flash photorelease assays were performed using shuttered (30 ms duration), near-UV (330–380 nm) epi-illumination, as described previously (Khan *et al.*, 1993). Video records were digitized at 30 frames/s (VP320 digitizer, Motion Analysis Inc., Santa Rosa, CA) and analyzed off-line (SunSparc2 workstation, ExpertVision version 1.4 motion analysis software; M. Motion Analysis, Santa Rosa, CA) using frame-to-frame rate of change of

Table 1. Strains

Strain	Relevant genotype	Source, derivation, or reference
JWL184-1	<i>MgIP⁻ mal⁺ GalP63</i>	Lengeler <i>et al.</i> (1981)
RP2859	$\Delta(\textit{cheRcheB})2241$	Parkinson and Houts (1982)
RP4971	$\Delta(\textit{cheB})63$	J.S. Parkinson (unpublished data)
RP5099	<i>zea::Tn10-3</i>	J.S. Parkinson (unpublished data)
RP9351	$\Delta(\textit{cheW-tap})2217 \Delta(\textit{cheZ})67-25 \textit{zea}::\textit{Tn10-3}$	Liu and Parkinson (1989)
JLV37-115	as JWL184-1 but <i>cheZ</i>	A.P. Vogler (unpublished data)
JLV92	as JWL184-1 but <i>ptsH5</i>	Grübl <i>et al.</i> (1990)
LJ120	as K-12 but $\Delta(\textit{ptsG}) \text{Cam}^r$	K. Jahreis (unpublished data)
JWL191	as JWL184-1 but <i>ptsI191 malA1</i>	Lengeler <i>et al.</i> (1981)
TP2865	$\Delta(\textit{crr}) \text{Kan}^r$	Lévy <i>et al.</i> (1990)
LLR103	as JWL184-1 but $\Delta(\textit{crr}) \text{Kan}^r$	P1(TP2865) \times JWL184-1 to Kan^r
LLR104	as JWL184-1 but $\Delta(\textit{ptsG}) \text{Cam}^r$	P1(LJ120) \times JWL184-1 to Cam^r
LLR413	as RP4971 <i>zea::Tn10-3</i>	P1(RP5099) \times RP4971 to Tet^r
LLR414	as RP2859 <i>zea::Tn10-3</i>	P1(RP5099) \times RP2859 to Tet^r
KLR202	as JWL184-1 but $\Delta(\textit{cheW-tap})2217\Delta(\textit{cheZ})67-25 \textit{zea}::\textit{Tn10-3}$	P1(RP9351) \times JWL184-1 to Tet^r
KLR203	as JWL184-1 but $\Delta(\textit{cheB})63 \textit{zec}::\textit{Tn10-3}$	P1(LLR413) \times JWL184-1 to Tet^r
KLR204	as JWL184-1 but $\Delta(\textit{cheRcheB})2241 \textit{zea}::\textit{Tn10-3}$	P1(LLR414) \times JWL184-1 to Tet^r

direction (*rcd*) and linear speed (*spd*) operators. The instrumentation, software, and operators have been described (Khan *et al.*, 1993, 1995). The population *rcd* responses were fitted using routines available in Sigmaplot (Jandel Scientific Inc., San Rafael, CA). Excitation response rates, k_{ex} were determined from single exponential fits. When a lag was evident, as in near-threshold responses, the response half-time, $t_{1/2}$ ($= \ln 2/k_{\text{ex}}$), was determined by a logistic fit.

Flow Cell Assays. A continuous laminar flow cell (Berg and Block, 1984) was used for tethered cell experiments. Cells were sheared (21-gauge needles) and tethered on flagellar antibody-coated coverslips essentially as described (Khan *et al.*, 1993). Buffer exchange was effected via an eight-way valve positioned close to the inlet of the flow cell. Continuous flow was maintained at 0.24 ml/min. Flow cell wash-in/wash-out kinetics were determined by exchange of buffers containing the hydrophilic chromophore HPTS (Molecular Probes, Eugene, OR).

Wash-in of buffer containing an attractant (D-glc or L-aspartate) or repellent (L-leucine) stimulus was maintained for 4 min followed by flow-in of buffer for an equivalent time. A sample was subjected to three to five such exchange cycles per experiment. Transition times (i.e., time period from flow-in to the first post-stimulus reversal) (Berg and Tedesco, 1975) were taken as a measure of adaptation. These were determined from video playback off-line using a stopwatch. They depended on the duration between successive jumps. For intervals <2 min per wash-in/wash-out cycle, transition times decreased with successive jumps, implying that this duration was not sufficient to attain a metabolic steady state (Lowry *et al.*, 1971; Weigel *et al.*, 1982).

RESULTS

Rapid Processing of PTS Chemotactic Signals

Excitation responses to step stimuli of D-glc were time-resolved by computerized motion analysis of swimming cell responses to flash photolysis of caged D-glc. In strain JWL184-1, which lacks periplasmic binding protein-mediated chemotaxis toward D-glc, photorelease of D-glc (4–40 μM) elicited rapid excitation responses (Figure 2). There was no detectable response in *ptsI* or *ptsH* mutant strains lacking EI (Figure 2,

inset) or HPr, respectively, consistent with results from capillary assays (Lengeler *et al.*, 1981). Responses of the mutants to photoreleased aspartate were similar to those measured for other *E. coli* strains (Jasuja *et al.*, 1999). These results implied that the PTS machinery mediated the responses observed in the JWL184-1 parent strain.

Extracellular Photorelease of Nanomolar Glucose Elicits Detectable PTS Chemotactic Responses

A general concern with use of caged compounds is that secondary products of the photolysis reaction may not be chemically or biologically inert. A further concern for this particular application was that the response might be due to photolysis of caged D-glc that had permeated into the cytoplasm. The control experiments with the mutant strains alleviated these concerns, but left open the possibility that the wild-type (JWL184-1) responses were due to PTS-specific uptake and subsequent intracellular photorelease of caged D-glc. Responses to intracellular photorelease should be independent of rates of PTS transport, provided the substrate has equilibrated between the extracellular and intracellular phases. Therefore, EII mutants with impaired rates of glucose transport were tested to determine whether responses were due to intracellular photorelease. Experimental cultures were incubated with caged glucose for times sufficient to allow its equilibration between the extracellular and intracellular phases (30 min). Longer incubation times did not measurably increase response strength. Mutant strains LLR103 and LLR104 lacked the soluble cytoplasmic (EIIA^{Glc}) or the transmembrane (EIIBC^{Glc}) component of the D-glc-specific EII, respectively.

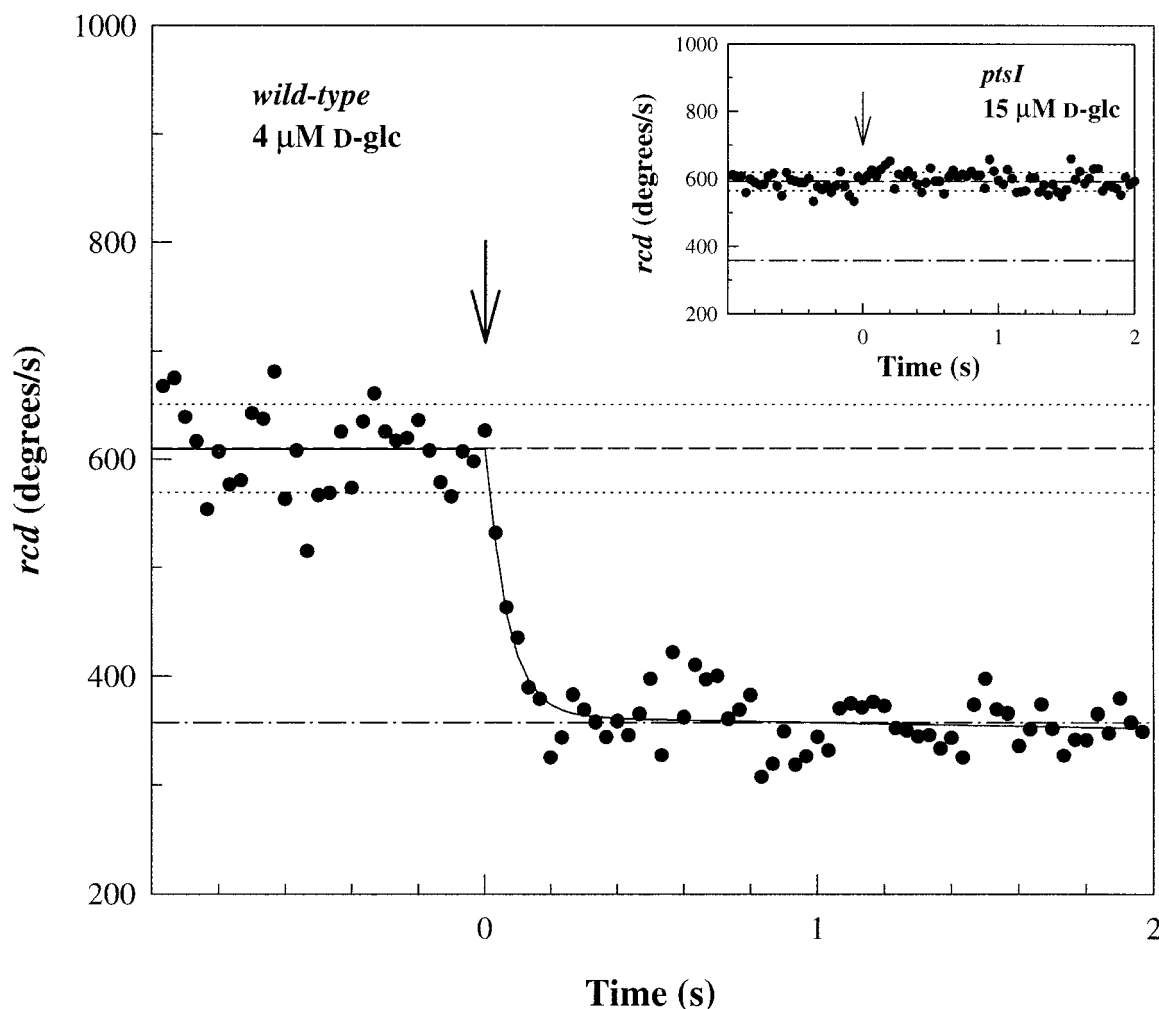


Figure 2. PTS-dependent excitation response to D-glc photorelease. Excitation response of strain JWL184-1 (wild type for PTS chemotaxis) to photorelease of $4 \pm 0.9 \mu\text{M}$ D-glc . The saturation response is well-fit by a single exponential ($k_{\text{ex}} = 14.9 \pm 0.9 \text{ s}^{-1}$). Inset: Nonresponsivity of mutant strain JWL191 (*ptsI*) lacking EI to $15 \mu\text{M}$ D-glc photorelease. Arrows denote photolyzing flash. The prestimulus population *rcd* (see MATERIALS AND METHODS) means (dashed lines) \pm frame-to-frame SD (dotted lines) are shown. Dashed-dotted lines indicate the *rcd* value for complete smooth-swimming.

They responded to D-glc photorelease, but response thresholds were an order of magnitude greater than the nanomolar threshold seen for JWL184-1 (see below). These increased thresholds were commensurate with low-affinity transport of D-glc by the mannose EII (Lengeler *et al.*, 1981) or EIIA^{Nag}/EIIB-C^{Glc} chimeras (Vogler *et al.*, 1988). Thus, the observed responses were due to transmembrane transport of extracellularly photoreleased D-glc .

Saturation smooth-swim responses obtained on photorelease of $0.8 \mu\text{M}$ D-glc (Figure 3A) had single exponential form but a slower rate (k_{ex}) than those observed on release of $4 \mu\text{M}$ D-glc . At $0.04 \mu\text{M}$, the response just failed to saturate and also deviated from a single exponential (Figure 3B). A still fivefold lower concentration

elicited responses close to the detection threshold (Figure 3C). Such threshold responses were characterized by a marked lag phase. This indicated that at low concentrations the PTS signal pathway contained more than one rate-limiting process. Furthermore, these data showed importantly that the PTS chemotactic response had a nanomolar detection threshold rather than the micromolar level determined by capillary assays (Adler and Epstein, 1974; Lengeler *et al.*, 1981).

The response threshold for Me $\alpha\text{-glc}$ was 40-fold higher than that for D-glc , consistent with the lower affinity of the D-glc PTS for Me $\alpha\text{-glc}$. Photorelease of $8 \mu\text{M}$ Me $\alpha\text{-glc}$ evoked a slower response (Figure 3D) than that for a comparable concentration jump of photoreleased D-glc (Figure 2). Its form deviated

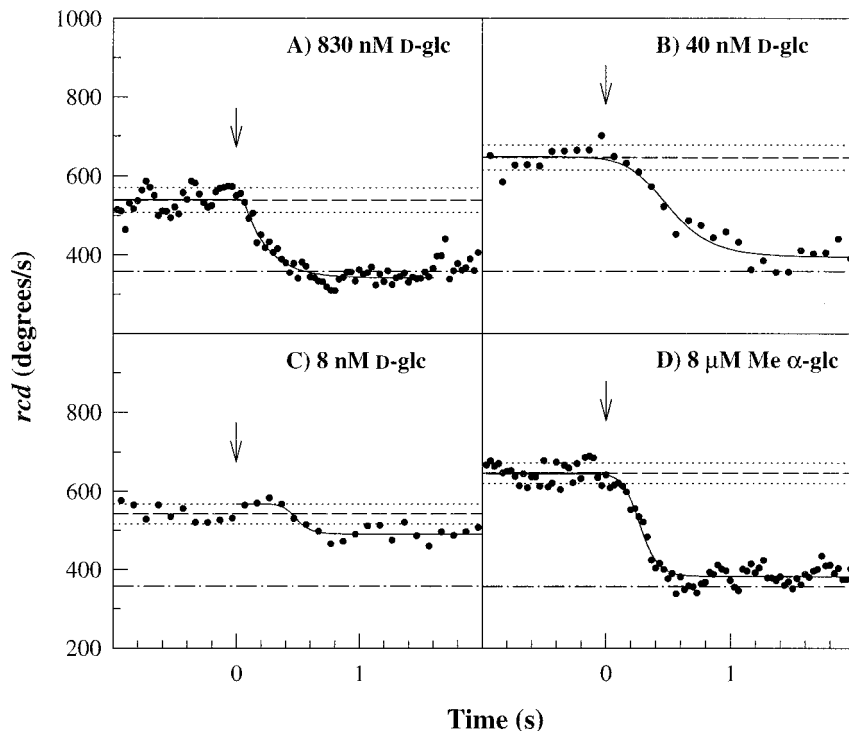


Figure 3. PTS chemotactic response sensitivity. Wild-type JWL184-1 responses. (A) $0.83 \pm 0.09 \mu\text{M}$ D-glc; $k_{\text{ex}} = 4.9 \pm 0.4 \text{ s}^{-1}$. (B) $40 \pm 3 \text{ nM}$ D-glc. (C) $4 \pm 0.3 \text{ nM}$ D-glc. (D) $8 \pm 0.2 \mu\text{M}$ α -methyl glucoside. Note the lag preceding the *rcd* decrease, evident in c and less so in b and d. $t_{1/2}$ values were ~ 500 ms for b and c and 250 ms for d, respectively. Arrows and reference lines are as in Figure 2.

from a single exponential and was similar to that seen for 50 nM D-glc photorelease. Responses to photorelease of higher concentrations followed single exponential excitation kinetics, whereas responses to lower concentrations showed a lag that increased with decreasing concentrations. This concentration dependence was qualitatively similar to that observed for D-glc. In contrast, single exponential kinetics characterized the responses to amino acid attractants down to the smallest measurable values (Jasuja *et al.*, 1999).

PTS Chemotactic Signals Are Transmitted via the MCP-Che Phosphorelay

PTS chemotaxis requires CheA and CheY (Rowell *et al.*, 1995). This might be because the chemotactic signal generated by PTS substrates is relayed to the flagellar motors via the Che phosphorylation cascade. Alternatively, phosphorylated CheY may be required for PTS chemotactic signal reception by flagellar motors. To distinguish between these possibilities, excitation responses of a *cheZ*-negative mutant to D-glc photorelease were measured. The mutant response (Figure 4A) was dramatically slowed by comparison to the wild-type response. It was comparable to responses of *cheZ* mutants to serine photorelease (Khan *et al.*, 1993). Thus, a decrease in CheY.P levels is implicated in the mechanism by which the PTS signal effects motor re-

sponse. This signal cannot act via CheZ, because *cheZ* mutants still respond.

How might the PTS and Che phosphorelays communicate? Decrease in CheY.P levels may be brought about by inhibition of CheA kinase activity or by direct interaction of the PTS signal with CheY.P. In the former alternative, PTS signal processing should be affected by deletion of the MCPs, because the activity of soluble CheA is negligible relative to MCP-bound CheA (Gegner *et al.*, 1992; Schuster *et al.*, 1993). Unfortunately, mutant strains with the MCPs deleted have a smooth-swimming phenotype (Khan *et al.*, 1993). Alternatively, therefore, mutant strains with altered MCP-bound CheA activity were examined. Deletion of the linker CheW or the MCP-modifying enzymes, the methyltransferase CheR and/or the methylesterase CheB, are known to impair MCP signaling triggered by amino acids. These deletions also affected PTS chemotactic signaling.

CheW is required for interaction of the MCPs with CheA. Hence, *cheW cheZ* double-deletion mutants have close to wild-type swim-tumble bias (Figure 4B). This double mutant did not respond to photorelease of up to 0.5 mM D-glc or 0.5 mM serine or, in tethered cell assays, concentration jumps up to 1 mM serine. Because *cheZ* mutants responded strongly to 50 μM D-glc photorelease (Figure 4A), the lack of response in the double mutant must be

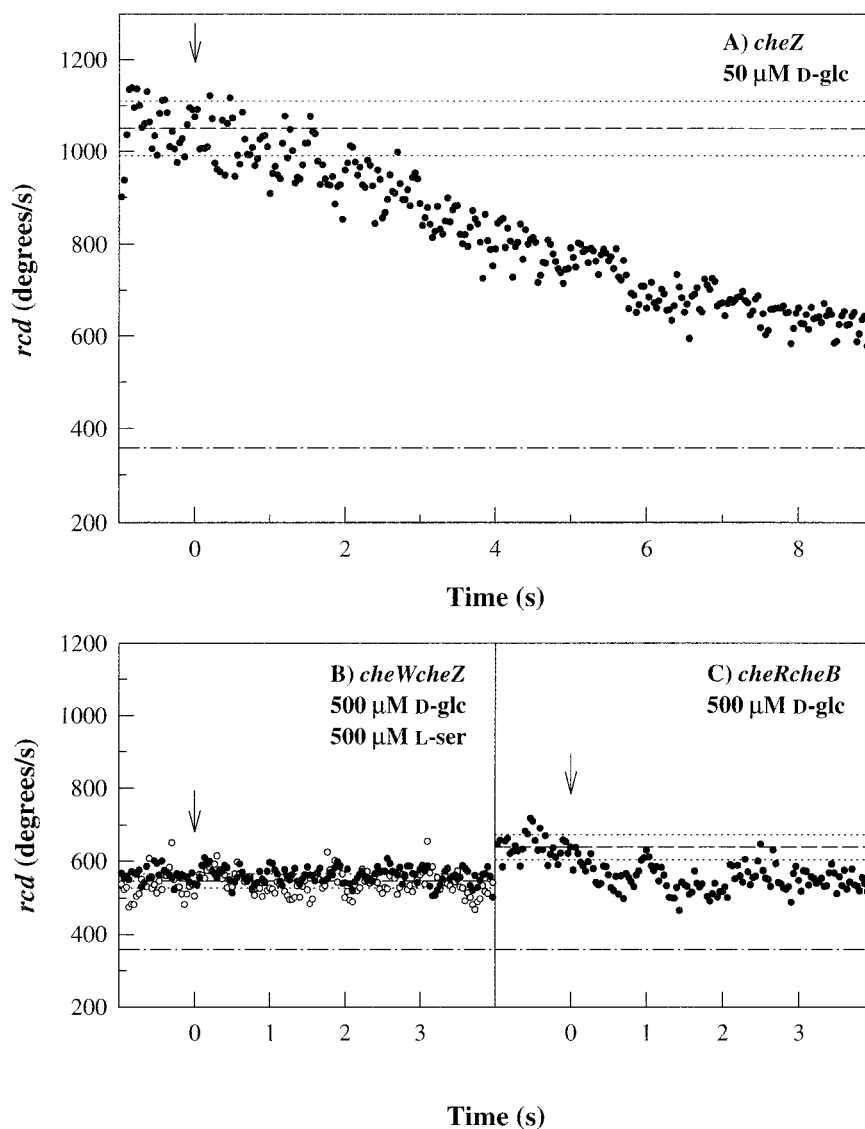


Figure 4. PTS-dependent excitation responses of *che* mutant strains. (A) *cheZ* mutant JLV37-115; 50 μM D-glc photorelease. (B) *cheW cheZ* mutant (KLR202); 500 μM photorelease of L-serine (\circ) and D-glc (\bullet). (C) *cheR cheB* (KLR204) mutant; 500 μM D-glc photorelease. Arrows and reference lines are as in Figure 2.

due to the *cheW* deletion. These data are consistent with the report that CheW is required, in addition to CheA and CheY, to enable gutted strains to respond to the PTS substrate mannose (Rowse *et al.*, 1995). In the latter case, CheW is needed for interaction of the PTS signal with CheA. The present experiments show that the physiologically relevant inhibition of MCP-bound CheA activity by PTS signals also requires CheW.

In tethered cell assays, *cheB* mutants could be transiently driven into complete CCW rotation by step increases of attractant amino acids ($\leq 20 \mu\text{M}$ serine; $\leq 400 \mu\text{M}$ aspartate). These abnormally large increases were presumably needed to overcome the reduced sensitivity (Segall *et al.*, 1986) and extreme CW bias of *cheB* mutants. D-glc concentration jumps up to 1 mM failed to elicit a measurable response.

Similarly, photorelease of 0.5 mM D-glc elicited barely detectable responses from *cheB* mutants (Lux, unpublished results) or *cheR cheB* mutants, which had close to wild-type swim-tumble bias (Figure 4C). This reduced sensitivity was not due to increased MCP methylation levels, because elevation or reduction of these levels by aspartate (1 mM) or leucine (10 mM), respectively (Springer *et al.*, 1979), was without effect. It may result, therefore, from the absence of the deaminase activity of CheB rather than its methyltransferase activity. In any case, the absence of CheB places MCP-CheA complexes in a conformation that interferes not only with transmembrane signaling by periplasmic ligands but also interaction with the cytoplasmic PTS chemotactic signal.

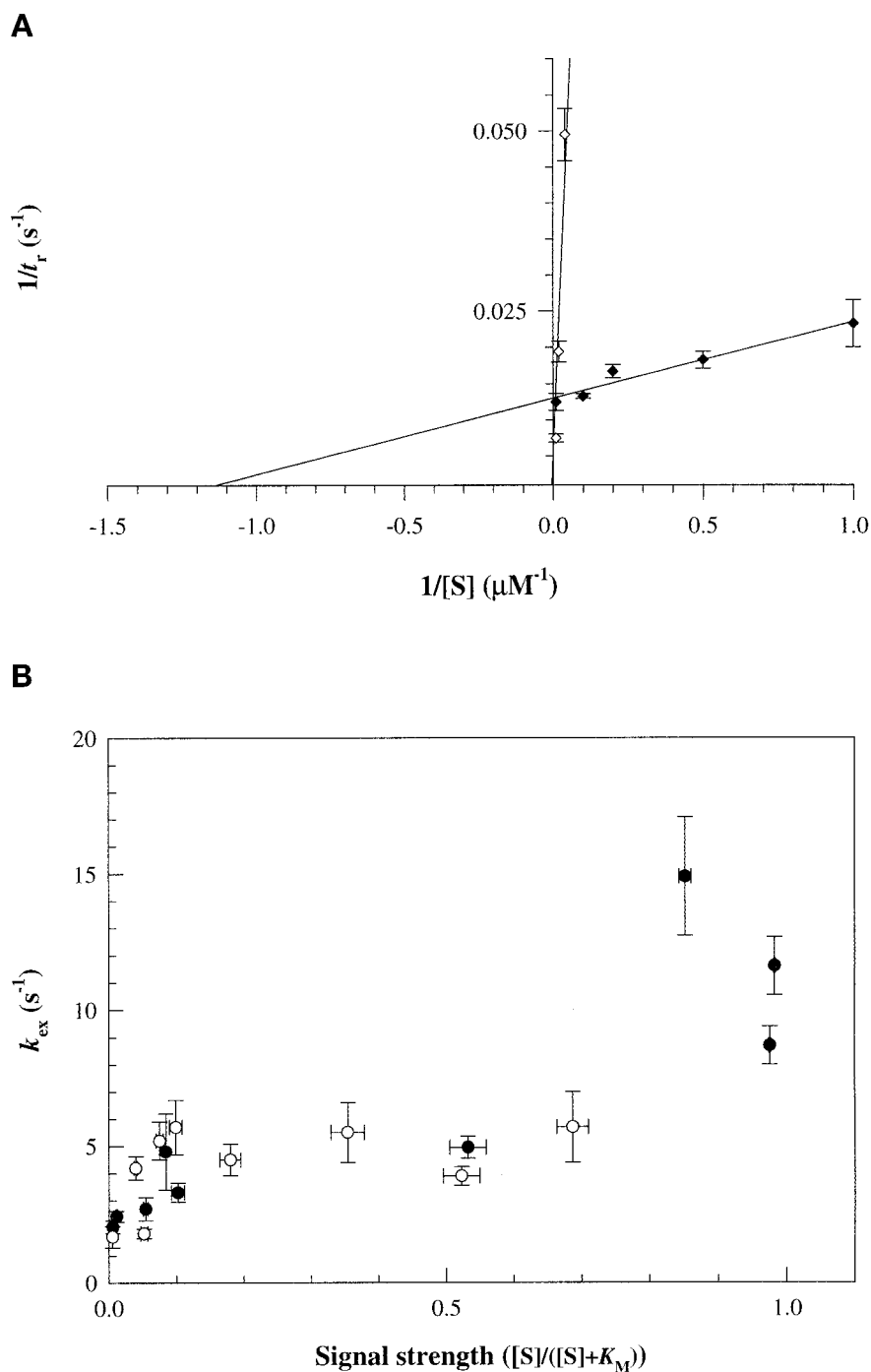


Figure 5. Adaptation and excitation kinetics of the positive PTS signal. D-gluc (closed circles); Me α -gluc (open circles). (A) Reciprocal plots of JWL184-1 tethered cell transition times, t_r (\pm SE), versus substrate concentration. Responses at other D-gluc or Me α -gluc concentrations were normalized by responses to 10 μ M D-gluc determined for each experiment. The latter had a mean t_r of 75 ± 1.8 s. Each data point typically represents results of two independent experiments (20–50 cells/experiment). (B) Excitation response rates, k_{ex} , determined from photorelease assays, plotted against signal strength.

PTS Chemotactic Excitation and Adaptation Kinetics Scale with Signal Strength

Complete smooth-swimming responses were obtained at 50–100 nM photoreleased D-gluc, but response rates continued to increase with D-gluc concentration. Cell densities in the experimental samples used for photorelease assays prevented measurement of chemotactic adapta-

tion because the extracellularly photoreleased D-gluc was rapidly depleted by PTS-mediated uptake. Therefore, adaptive transition times (t_r) (Berg and Tedesco, 1975) were measured in tethered cell assays to assess the signal strength obtained for concentration increases >100 nM.

Transition times increased with D-gluc or Me α -gluc concentration. An apparent K_m of 0.9 ± 0.5 μ M was

obtained for D-glc from reciprocal plots (Figure 5A). This strain has an apparent K_m for transport through EII^{Glc} of 5 μ M (Lengeler *et al.*, 1981). Values for other *E. coli* strains ranging from 3 to 20 μ M have been reported. The data for Me α -glc were consistent with the 40-fold difference in response thresholds determined in photorelease assays, as well as with differences between the D-glc and Me α -glc transport K_m values (Adler and Epstein, 1974; Stock *et al.*, 1982; Misset *et al.*, 1983; Grenier *et al.*, 1986).

The chemotactic K_m values were used to define signal strength. This was $\{[S]/([S] + K_m)\}$, where $[S]$ is photoreleased sugar concentration. Excitation response rates increased with signal strength. Responses at high signal strength (>0.8) had mean response rates ($11.7 \pm 3.1 \text{ s}^{-1}$) indistinguishable from those measured for the MCP attractant ligand aspartate (Jasuja *et al.*, 1999). Rates of responses to D-glc and Me α -glc were superimposable (Figure 5B).

Responses to Withdrawal of PTS Substrates Depend on Metabolic State

CW responses to withdrawal of D-glc were not observed under our standard buffer conditions, which contained lactate. Instead, a weak positive CCW response, whose duration did not depend on concentration, was occasionally observed (Figure 6). Weak CW responses were obtained during D-glc withdrawal in buffers lacking lactate; however, addition of lactate to such buffers also caused CCW responses, and its withdrawal caused CW responses. This suggested that the bacteria were partially deenergized in these buffers and that responses to both glucose and lactate under these conditions reflected changes in their energy levels. Negative CW responses to withdrawal of amino acid attractants or addition of repellents were obtained in tethered cell assays, as expected (Larsen *et al.*, 1974).

DISCUSSION

Previous knowledge, summarized in INTRODUCTION, indicated that CheA could be inhibited and stimulated by unphosphorylated EI and PEP, respectively, and additionally identified further control points downstream in the Che signal pathway that could be affected by the PTS transport-induced drop in PEP levels. These possibilities were distinguished and the nature of the PTS signal clarified by time-resolved quantification of the excitation response. Mutant strains and rapid photorelease of PTS chemoattractants were used to dissect the pathway and characterize its sensitivity. Excitation and adaptation kinetics of PTS-mediated chemotactic responses triggered by photorelease of D-glc and the nonmetabolizable Me α -glc were compared to assess whether per-

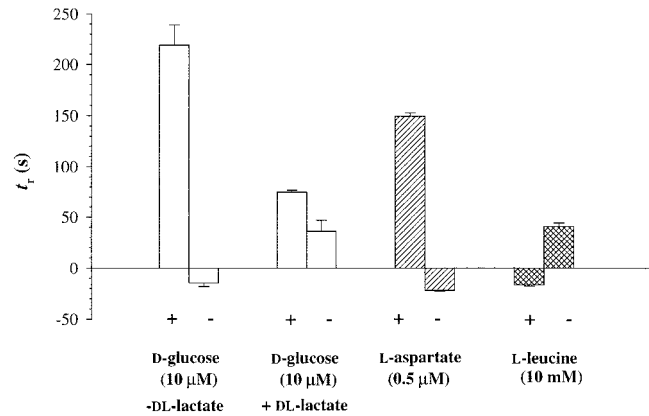


Figure 6. Tethered cell responses to D-glc withdrawal. Transition times, t_r (\pm SE), on addition (+) or withdrawal (-) are shown. Positive and negative values denote CCW and CW responses, respectively. Results obtained with a single experimental culture split into two halves, one energized by DL-lactate and the other not. Tethered cells lacking lactate were partially deenergized because they responded positively to its addition and negatively to its withdrawal. Similar results were obtained in additional experiments ($n > 5$ for each condition). Responses to addition or withdrawal of the attractant aspartate (0.5 μ M) or the repellent leucine (10 mM), periplasmic ligands for the MCPs Tar and Tsr, respectively, measured in separate experiments, are also shown. These responses were insensitive to the presence of lactate. In each case, 20–50 cell populations were analyzed.

turbation of metabolite levels affects response. The PTS signal was found to have nanomolar sensitivity for D-glc. It was processed with a rate comparable to signals generated by MCP attractants over most of the response range. These findings provide novel insight into the in vivo operation of the PTS phosphorelay during chemotaxis.

Coupling between the PTS and Che Phosphorelays

EI and HPr negative mutants did not respond to photorelease of D-glc. EIIA^{Glc} and EIIBC^{Glc} transport mutants had elevated response thresholds. Hence the responses observed in wild-type bacteria were due to extracellular photorelease of the sugars that required subsequent PTS-dependent transport to effect a chemotactic response.

The slower kinetics of the excitation response in *cheZ* mutants showed that the parameter sensed by flagellar motors was the decrease in CheY.P levels. Thus the PTS signal does not act via an independent pathway on a step affecting CheY.P binding to, or action on, the motor, nor does it affect CheZ-dependent dephosphorylation of CheY. *cheR cheB* and *cheW cheZ* mutants have impaired MCP-based signaling. These strains hardly responded to PTS-dependent chemotactic signals generated by D-glc photorelease. These observations, together with the kinetics of the

Table 2. PTS concentration and rate changes during chemotactic excitation

Response	[S] (nM)	$t_{1/2}$ (s)	v ($\mu\text{M}\cdot\text{s}^{-1}$)	ΔEI	ΔPEP
Close to threshold	10	0.5	2.5	0.25	0.0025
Close to saturation	50	0.34	12	0.45	0.005
Monoexponential kinetics	>200	<0.1	>40		

Fractional changes in PEP and phosphorelay components were computed from initial rates of transport, $v = \{[S] \cdot V_{\max} / ([S] + K_m)\} \mu\text{M}\cdot\text{s}^{-1}$; where [S] = extracellularly photoreleased glucose; K_m was determined from Figure 5A. The fractional change, $\Delta C = (v \cdot t_{1/2}) / C_T$; where C_T , the total concentration, is 5, 25–100, and 25–50 μM for [EI], [HPr], and [EIIA], respectively (Scholte *et al.*, 1982; Mattoo and Waygood, 1983), and 0.1–1 mM for [PEP] (Lowry *et al.*, 1971). Therefore, ΔHPr and $\Delta\text{EIIA}^{\text{Glc}}$ are one-fifth to one-twentieth the ΔEI . This assumes that the components are initially present almost entirely in their phosphorylated forms and that back reactions are negligible during the excitation time period (see RESULTS). The latter assumption is likely to be valid for $\Delta[\text{HPr}]$ and $\Delta[\text{EIIA}]$, but will provide an underestimate for $\Delta[\text{EI}]$ or $\Delta[\text{PEP}]$. V_{\max} , hence v , were expressed as rates of micromolar change in intracellular concentration per second ($\mu\text{M}\cdot\text{s}^{-1}$). Reported values expressed as $\mu\text{mol}\cdot\text{g}^{-1}$ dry wt $\cdot\text{min}^{-1}$ were converted using cytoplasmic volume/gram dry weight = (number of bacteria/gram dry weight) (volume/bacterium) = $(6 \times 10^{12}) (1.4 \times 10^{-12})$ ml/g = 8.4 ml/g. V_{\max} for PTS D-glc transport = 200 $\mu\text{M}\cdot\text{s}^{-1}$. In contrast, V_{\max} for aspartate transport <1 $\mu\text{M}\cdot\text{s}^{-1}$ (Kay, 1971). CheY.P dephosphorylation rate = $(k_c + k_-) [\text{CheY.P}] = (10 \text{ s}^{-1}) (2 \mu\text{M}) = 20 \mu\text{M}\cdot\text{s}^{-1}$ (Jasuja *et al.*, 1999). Thus, this will become the single rate-limiting process for PTS chemotactic signaling for 0.2 μM and greater concentration jumps.

cheZ mutant response, argued against the possibility that these signals acted directly to accelerate CheY.P dephosphorylation. They indicated instead that the signals inhibited the CheA kinase activity of MCP signaling complexes.

Thus, the mutant analysis provides evidence for a single PTS chemotactic signal that is transmitted via MCP signaling complexes to effect a decrease in CheY.P levels, hence CCW motor response.

Timing and Amplification in the PTS Chemotactic Signal Pathway

Rapidity, $k_{\text{ex}} = 4.4 \pm 0.9$ to $11.7 \pm 3.1 \text{ s}^{-1}$ over the 0.1 to 0.9 range of signal strength (Figure 5B), and high sensitivity, i.e. detection of $K_m/100$ concentration differences (10 nM for D-glc), constitute our two major findings regarding the physiology of the PTS chemotactic response. These properties constrain possibilities regarding the nature of the PTS signal.

Rapid, reversible histidine phosphorylations/dephosphorylations, with rates of 10^6 – $10^8 \text{ M}^{-1}\text{s}^{-1}$ (Anderson *et al.*, 1993; Meadow and Roseman, 1996), characterize the PTS phosphorelay. These rapid kinetics are consistent with structural data showing that the phosphorylatable histidine residues are located on exposed surface loops and do not require large conformational changes for accessibility (McEvoy and Dahlquist, 1997). Flux at steady state in the absence of substrate transport is much slower than the phosphotransfer rates. It is limited by the slow ($3.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ [Chauvin *et al.*, 1994]) dimerization of EI monomers, maintaining the PTS phosphoenzymes predominantly (>80%) in their phosphorylated form (Hoving *et al.*, 1981; Nelson *et al.*, 1986).

Transport results in redistribution of the PTS components toward their nonphosphorylated forms at a rate determined by the concentration of substrate. K_m

values determined for chemotactic adaptation provide further support for the premise that the substrate concentration dependence for chemotaxis reflects that for transport (Lengeler *et al.*, 1981). Adoption of this premise accounts simply for the shift from biphasic to single exponential decay kinetics. Given a maximal transport rate (V_{\max}) of $70 \pm 20 \mu\text{mol}\cdot\text{g}^{-1}$ dry wt $\cdot\text{min}^{-1}$ (Adler and Epstein, 1974; Stock *et al.*, 1982), PTS phosphotransfers will exceed the rate for CheY.P dephosphorylation at 200 nM extracellular D-glc (Table 2) and will not limit signaling for this and greater concentration jumps. This is consistent with the observation that single exponential excitation kinetics with a rate equal to that obtained for signaling by MCP periplasmic ligands are obtained over most of the response range, with a lag evident for 50 nM and lower D-glc concentration jumps (Figures 3 and 5B).

From initial transport rates, estimated from concentration jumps producing threshold and saturation responses, respectively, it may be estimated that PEP levels will change imperceptibly (<0.2%) during chemotactic excitation (Table 2). It is difficult to imagine how such small decreases in PEP levels can be converted to appropriate changes in MCP signaling activity. PEP levels do drop over 1–3 min (Lowry *et al.*, 1971), in turn affecting transport rates (Weigel *et al.*, 1982), but these changes are too slow to be relevant for chemotactic signal processing. Furthermore, subsequent metabolism of D-glc might be expected to retard the drop in PEP levels induced by its transport. This will not occur for the nonmetabolizable Me α -glc. The superimposition of plots of excitation response rates versus chemotactic signal strength for both sugars (Figure 5B) therefore also argues against a role for perturbation of PEP levels in chemotactic signaling.

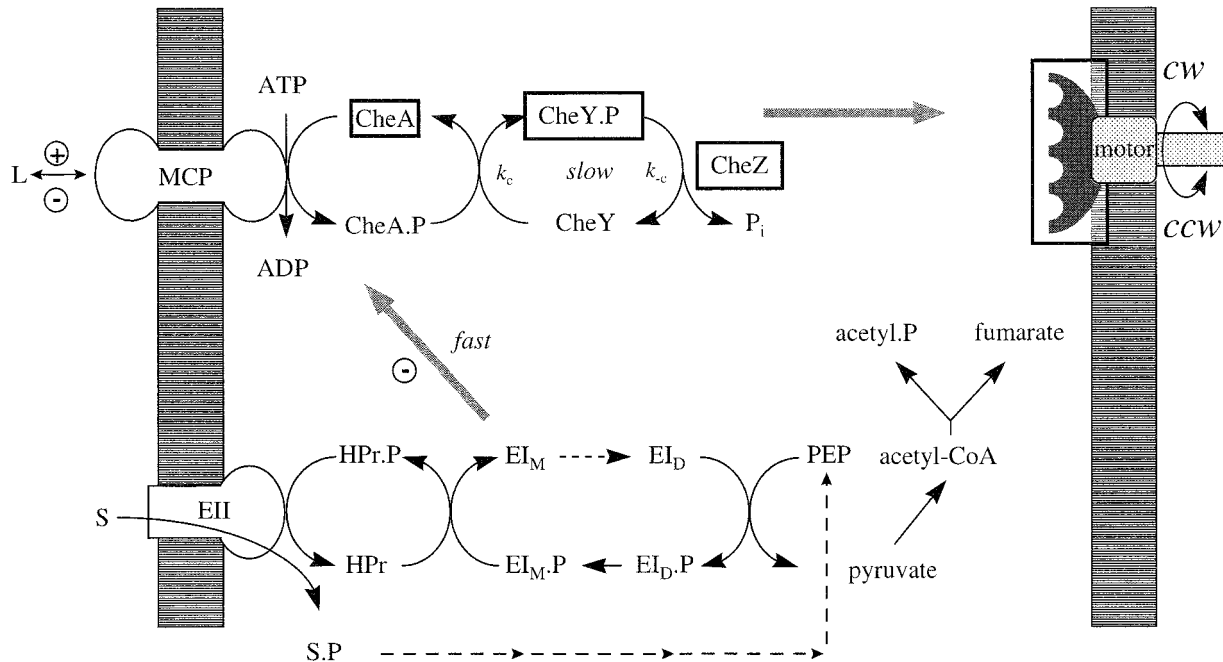


Figure 7. Properties of the PTS chemotactic pathway. The PTS is poised far from equilibrium because of the slow dimerization of EI monomers (EI_D to EI_M , dashed arrow), obligatory for phosphorylation by PEP. The positive chemotactic signal triggered by transport of PTS substrates (S) may originate either from a decrease in phosphorylation levels of the donor phosphorelay or from changes in metabolite levels caused by PEP consumption. It may act on CheA, CheZ, CheY.P, or the motor switch (boxed items). The present study sought to distinguish between these possibilities. The analysis of mutant responses showed that the PTS signal acted solely to inhibit MCP-bound CheA. The high response sensitivity argued that it is generated by the dephosphorylation of PTS phosphorelay components, most likely EI. PEP levels decrease little, and their subsequent restoration by metabolism of D-glc (dashed arrows, small arrowheads) does not affect signaling. The response kinetics implied that signaling (gray arrows) was limited, as for periplasmic MCP ligands (L), by CheY.P dephosphorylation (i.e., $k_c + k_{-c}$) over most of the PTS chemotactic response range if PTS dephosphorylation kinetics were determined by the transport rate, consistent with known transport and biochemical data. Rephosphorylation of the PTS phosphorelay on withdrawal of PTS substrates will be limited by EI dimerization and may not be rapid enough to stimulate CheA (+), in contrast to withdrawal of MCP attractant ligands. Evidence for such a signal was not obtained.

In contrast, large changes in EI levels will occur during chemotactic excitation. Fractional increases in concentration of the dephosphorylated forms of other PTS components, HPr and $EIIA^{Glc}$, which are present at an order of magnitude higher concentration, will be correspondingly less (Table 2). Ignorance of the basal leakage rate prevents an accurate estimate of the fractional increase in unphosphorylated [EI]; however, this will be substantial and will occur over times comparable to the excitation time, even for near-threshold responses. Macromolecular crowding, as suggested earlier (Lux *et al.*, 1995), and/or CheW–MCP association could increase the affinity of unphosphorylated EI for CheA, allowing substantial inhibition of CheA activity to be achieved. In addition, subsequent amplification will be necessary to transduce the corresponding change in CheA activity to the observed motile responses (see Discussion in Lux *et al.*, 1995).

Cessation of transport caused by substrate withdrawal will induce redistribution of the PTS com-

ponents toward their phosphorylated forms. This process will be limited by the slow EI dimerization and should therefore elicit, at best, a weak negative chemotactic signal. The CW response observed in tethered cells (Rowell *et al.*, 1995) may not be due to PTS-generated signals but rather to perturbation of cellular energy levels because it seems to require partial deenergization of the bacteria. In the presence of lactate, a negative response was not observed.

Changes in PEP levels may also be involved in adaptation (Lux *et al.*, 1995). The fact that both D-glc and Me α -glc have adaptation kinetics commensurate with the corresponding transport K_m values indicates that adaptation cannot result solely from reequilibration of PEP pools, nor can it be due solely to MCP methylation (Niwano and Taylor, 1982). Multiple adaptive processes may be operative. Assessment of their contribution over physiologically relevant time scales will require time-resolved analysis of small stimuli.

Concluding Comment

Why are chemotactic responses to PTS substrates coupled to transport, unlike responses to amino acids? The answer may lie in the fact that transport of sugars/carbohydrates is intimately related to cellular energy metabolism. The free-energy change resulting from cleavage of the PEP phosphate bond is one of the highest known (Atkinson and Morton, 1960). This is used via the PTS cascade to scavenge these compounds from the medium much more rapidly than amino acids (Table 2 legend), whose uptake does not immediately affect cellular physiology. Distinct machinery has therefore evolved to allow amino acids to effect rapid chemotactic responses. Coupling of the PTS and Che phosphorelays affords an elegant solution where rapid PTS phosphotransfer reactions, already in place for transport, provide a time-resolved readout of the extracellular substrate concentration. This readout is amplified and relayed to flagellar motors using mechanisms intrinsic to the MCP-Che machinery (Figure 7). ATP-driven ATP-binding cassette transporters also mediate high-affinity sugar transport (Boos and Lucht, 1996). In this case the solution is direct interaction of the periplasmic binding proteins with the MCPs, because perturbations of intracellular ATP levels are prohibitive. In both cases, the MCP signaling complex emerges as the key element in chemotactic signal processing, a role that may be related to its importance in signal amplification.

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