

Supporting Information

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SI Materials and Methods

Cloning, Expression, and Purification of Proteins. H1-Tar and H1-2-Tar were initially cloned into pET28 as described previously (1). For binding experiments, N-terminally S-tagged soluble effectors were generated by subcloning from pET28 into pET29c using NcoI and XhoI restriction sites. All of the proteins were overexpressed in BL21(DE3) *Escherichia coli* cells under 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) induction for 16–20 h at 25 °C. Proteins were purified using an Ni-NTA gravity column and subsequently, on a Superdex 200 26–60 size exclusion column with gel-filtration elution buffer (GF buffer) of 20 mM Tris (pH 8.0), 150 mM NaCl, and 5–10% (vol/vol) glycerol.

In Vivo Activity of H1-Tar and H1-2-Tar. The data presented in Fig. S1 were originally reported in ref. 1 under the procedure as follows. *E. coli* cells, devoid of all other receptors, were transformed with the plasmid containing the gene of the effectors. Transformed cells were grown in terrific broth and induced with 2 μ M sodium salicylate for 1 h. The cells were washed and kept in buffer containing 10 mM potassium phosphate and 0.1 mM EDTA (pH 7.0). Cell tumbling frequency was measured after 5 min, during which time the cells adapt, with dark-field microscopy.

In Vitro CheY Phosphotransfer Assay. Assay mixtures consisted of 20 μ L 35% (vol/vol) glycerol solution of 2 μ M subunit concentrations of CheA and CheW each, 14.4 μ M effector subunit, 50 μ M CheY, and 2 μ L buffer composed of 62.5 mM Tris (pH 7.5), 625 mM KCl, 6.25 mM EDTA, and 125 mM MgCl₂ made up to volume with distilled water. The solution without CheY was incubated at 4 °C for 30 min and further incubated for 90 min after CheY addition. After this time, 5 μ L radioactive ATP solution (150 μ L aqueous solution containing 12 μ L 12 mM ATP and 1–7 μ L γ -³²P ATP; 0.250 mCi; Perkin-Elmer) was added to the protein solution to initiate the phosphotransfer reaction. After 30 s of exposure, the reaction was stopped with 25 μ L quenching buffer (3 $\times$ Coomassie blue, sodium dodecyl phosphate loading buffer, 50 mM EDTA). The sample was run on 4–20% (vol/vol) Tris-Gly gel for 2 h at 120 V to separate the components. After following the treatment described in ref. 2, the gel was placed in the cassette, and the film was visualized in an STORM phosphorimager after a minimum of 24 h of exposure. Densitometry with ImageJ software quantified the CheY band intensity after the background activity was removed by adjusting the brightness and contrast of the image.

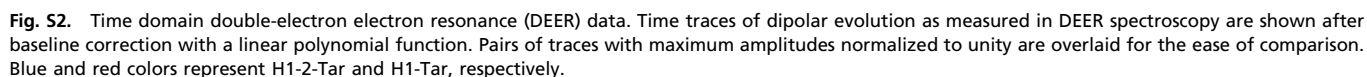
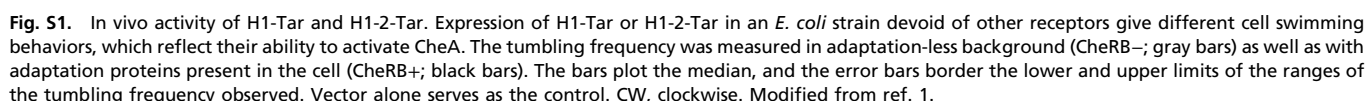
Pull-Down Experiments. H1-Tar and H1-2-Tar with an N-terminal S tag and a C-terminal His₆ tag were overexpressed from pET29c as described above. *E. coli* CheW and CheA were expressed from pET28 using the same procedure, and both carried N-terminal His₆ tags. After purification, the CheW His tag was readily removed by thrombin digestion, but CheA His-tag removal was not complete; hence, pull downs were performed with the S tag. S-protein agarose (60 μ L) was used to pull down S-tagged HAMP Tar fusions and associated molecules from a 100 μ L solution containing the three components in the subunit ratio of HAMP Tar:EcCheA monomer:EcCheW monomer of 6:1:1 (195 μ M HAMP Tar proteins:65 μ M EcCheA:70 μ M EcCheW). The S agarose was washed two times with buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 5% (vol/vol) glycerol, and 62.5 mM KCl before boiling with 2 $\times$ LDS sample buffer. The supernatant was then run on an SDS/PAGE gel,

which was Coomassie-stained, destained with a 25% (vol/vol) ethanol and 10% (vol/vol) acetic acid mixture, and dried before quantification (ImageJ). Molar ratios of bound CheA and CheW to effector were calculated from the intensities of the bands on SDS/PAGE gel measured with ImageJ software using the formula $((I_{\text{CheA/CheW}} - I_{\text{background with same area}}) / MW_{\text{CheA/CheW}}) / ((I_{\text{HAMP1-Tar/HAMP1-2-Tar}} - I_{\text{background with same area}}) / MW_{\text{HAMP1-Tar/HAMP1-2-Tar}})$.

Site-Directed Spin Labeling. Selected residues in the cysteineless HAMP Tar proteins were mutated to cysteine using Quikchange PCR protocol (Stratagene). Cys-substituted effector molecules with N-terminally His₆ tag were overexpressed as above. The proteins were bound to an Ni-NTA affinity column and then reacted with cysteine-specific nitroxide S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonylthioate spin label (MTSL) (Toronto Research Chemicals Inc.) on column for 3–4 h at room temperature and subsequently, 4–6 h at 4 °C before elution with buffer of 25 mM Hepes (pH 8.0), 250 mM Imidazole (pH 8.0), 500 mM NaCl, and 10% (vol/vol) Glycerol. After elution, samples were subjected to size-exclusion chromatography (Superdex 200 26–60) for additional purification and removal of unreacted MTSL contaminants. On-column spin labeling was adopted to minimize interdimer disulfide bond formation. Although cross-linking was limited by fast on-column exchange of reducing agents for spin label, we could not avoid it entirely.

PDS Measurement. Because HAMP Tar molecules are homodimers, one Cys substitution generates two spin labels per dimer. PDS was used to measure the distance distribution between these two spin labels as previously described (1, 3). In brief, $\sim$ 100 μ M spin-labeled dimer was prepared in GF buffer with 35% (vol/vol) glycerol. The dipolar evolution at 17.35 GHz was measured on a home-built 2D Fourier-transform-ESR instrument using four-pulse double-electron electron resonance with a 16-ns pump pulse. The baseline of the time domain data was corrected with a linear polynomial function. Double-electron electron resonance data were then converted to distance distribution between spin pairs with Tikhonov regularization (4) followed by maximum entropy refinement (5). The distance distributions are normalized to unity for the ease of comparison.

CW-ESR Data Collection and Analysis. CW-ESR data on the same spin-labeled samples were collected at three different temperatures (4 °C, 20 °C, and 30 °C) on a Bruker Elexsys E500 EPR instrument at 9.4 GHz with 100 kHz modulation frequency and 1.6 G modulation amplitude. Two components (one broad, representing a near rigid limit, and one sharper, representing faster motion) were observed at all temperatures, although their relative intensities did change. The fractions of these two components were determined by double integration of the experimental spectrum. A third minor component from a very small fraction of free MTSL does not affect estimates for the two major fractions. The spectra were analyzed using NLSL software (6) and its recent versions on the MATLAB platform. Initially, the spectral component corresponding to the broad signal (Fig. S3) was simulated and fit corresponding to very slow rotational diffusion with D_r of $\sim$ 0.8–1 $\times$ 10⁷ s^{−1}). This signal was then removed from the experimental spectrum by spectral subtraction, leaving just that of the more mobile component. Then, the motional parameters for this component (Table S3) were estimated by NLSL simulations using the MOMD model (6).



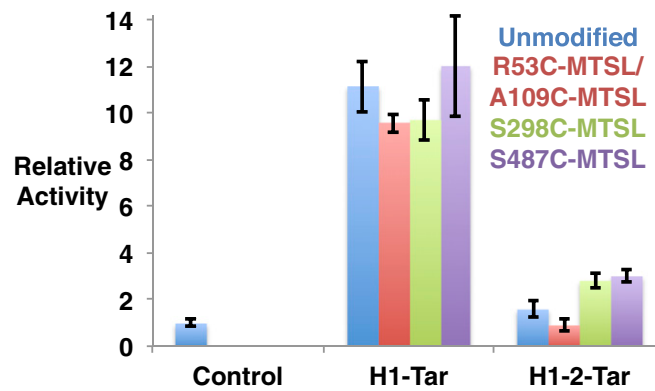


Fig. S3. Constancy of signaling properties of mutated and spin-labeled effectors. Mutations and spin labels used in the ESR study do not alter the signaling properties of the effectors as measured in the CheY phosphotransfer assay. SEMs, calculated from at least three independent experiments ($3 \leq n \leq 4$), are presented as the error bars.

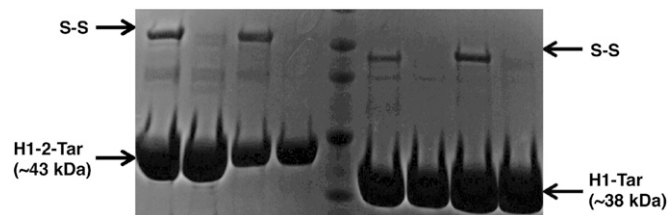


Fig. S4. Typical SDS/PAGE analysis of double-electron electron resonance (DEER)/CW-ESR samples. The samples for DEER/CW-ESR measurement reveal the existence of disulfide cross-links on SDS/PAGE gel. The disulfide links are reduced on DTT addition. Samples for S298C are shown as an example in all four states. Lane 1, H1-2-Tar in QQQQ state; lane 2, H1-2-Tar in QQQQ state with DTT; lane 3, H1-2-Tar in QEQE state; lane 4, H1-2-Tar in QEQE state with DTT; lane 5, molecular mass marker; lane 6, H1-Tar in QEQE state; lane 7, H1-Tar in QEQE state with DTT; lane 8, H1-Tar in EEEE state; and lane 9, H1-Tar in EEEE state with DTT.

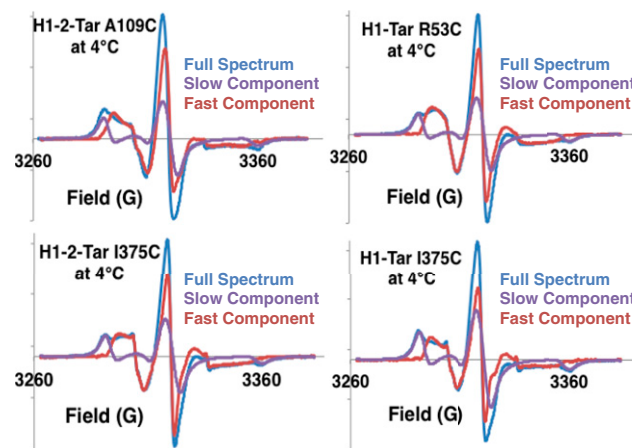


Fig. S5. Deconvolution of the CW-ESR spectra into two components. CW-ESR spectra (blue) are deconvoluted into slow (purple) and fast (red) motional components.

Table S1. Parameters of the distance distribution for each site at OE/OE adaptation state

Residue and HAMP Tar	$r_{\max}$ (Å)	FWHM (Å)
R53C/A109C		
H1-Tar R53C*	26.0	10.3
H1-2-Tar A109C [†]	24.2	2.9
E270C		
H1-Tar*	29.0	22.0
H1-2-Tar [†]	25.6	13.9
S487C		
H1-Tar*	29.2	8.4
H1-2-Tar [†]	25.1	7.9
S298C		
H1-Tar*	32.7	8.9
H1-2-Tar [†]	32.6	11.2
A312C		
H1-Tar*	28.6	7.6
H1-2-Tar [†]	28.3	7.6
A417C		
H1-Tar*	28.9	6.7
H1-2-Tar [†]	29.3	6.6
I375C		
H1-Tar*	28.7	6.6
H1-2-Tar [†]	29.0	11.5
A381C		
H1-Tar*	24.5	5.5
H1-2-Tar [†]	23.3	9.6

[†]H1-2-Tar.

Table S2. Comparison of the parameters of distance distributions at different adaptational states

Mutant and modification	$r_{\max}$ (Å)	FWHM (Å)
H1-Tar R53C		
QEQE	26.0	10.3
EEEE	25.2	5.9
H1-2-Tar A109C		
QEQE	24.2	2.9
QQQQ	22.2	3.3
H1-Tar S298C		
QEQE	32.7	8.9
EEEE	34.6	12.3
H1-2-Tar S298C		
QEQE	32.6	11.2
QQQQ	28.9	10.6
H1-Tar A417C		
QEQE	28.9	6.7
EEEE	29.3	6.2
H1-2-Tar A417C		
QEQE	29.3	6.6
QQQQ	29.2	5.9
H1-Tar I375C		
QEQE	28.7	6.6
EEEE	29.8	15.3
H1-2-Tar I375C		
QEQE	29.0	11.5
QQQQ	30.0	8.4

Maximum probable distance ($r_{\max}$) and full width at half-maximum (FWHM) are compiled for distance distribution at different regions in HAMP Tar proteins with different adaptational states.

Table S3. Motional parameters for the mobile components

Simulation parameters for the mobile components	4 °C		20 °C		30 °C	
	$\text{Log}(R_{\text{prp}})$	C_{20}	$\text{Log}(R_{\text{prp}})$	C_{20}	$\text{Log}(R_{\text{prp}})$	C_{20}
H1-2-Tar A109C	7.62	−1.6	7.70	−1.4	7.73	−0.93
H1-Tar R53C	7.66	−1.66	7.75	−1.12	7.87	−0.98
H1-2-Tar I375C	7.66	−1.1	7.78	−1.1	7.85	−1.02
H1-Tar I375C	7.64	−1.2	7.72	−1.1	7.82	−1.02

Mobility as $\text{log}(R_{\text{prp}})$ and ordering of the attached MTSL as C_{20} are listed for all of the mobile components at various temperatures as obtained from the simulation. $\text{Log}(R_{\text{f}})$ is set at 8.5 for all of the simulations.