Dynamic map of protein interactions in the *Escherichia coli* chemotaxis pathway

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Protein–protein interactions play key roles in virtually all cellular processes, often forming complex regulatory networks. A powerful tool to study interactions in vivo is fluorescence resonance energy transfer (FRET), which is based on the distance-dependent energy transfer from an excited donor to an acceptor fluorophore. Here, we used FRET to systematically map all protein interactions in the chemotaxis signaling pathway in *Escherichia coli*, one of the most studied models of signal transduction, and to determine stimulation-induced changes in the pathway. Our FRET analysis identified 19 positive FRET pairs out of the 28 possible protein combinations, with 9 pairs being responsive to chemotactic stimulation. Six stimulation-dependent and five stimulation-independent interactions were direct, whereas other interactions were apparently mediated by scaffolding proteins. Characterization of stimulation-induced responses revealed an additional regulation through activity dependence of interactions involving the adaptation enzyme CheB, and showed complex rearrangement of chemosensory receptors. Our study illustrates how FRET can be efficiently employed to study dynamic protein networks in vivo.

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Introduction

The chemotaxis pathway in *Escherichia coli* senses gradients of attractants and repellents to direct cellular movement toward favorable environments. Being a comparatively simple system with few components, chemotaxis in *E. coli* is an excellent model for the detailed quantitative study of signaling networks (Sourjik, 2004; Wadhams and Armitage, 2004). The sensory complex is formed by ligand-specific membrane-associated receptors (Tsr, Tar, Trg, Tap and Aer), histidine kinase CheA and coupling protein CheW. Ligand binding to receptor homodimers alters the autophosphorylation activity of the receptor-associated CheA. The kinase subsequently donates the phosphoryl group to response regulator CheY, which diffuses to flagellar motors and modulates their rotation. Dephosphorylation of CheY is catalyzed by the phosphatase CheZ. Adaptation is exerted by methyltransferase CheR and its antagonist, methylsterase CheB. CheB consists of a C-terminal catalytic domain and an N-terminal CheY-like regulatory domain, which is subject to activatory phosphorylation by CheA. Adaptation enzymes tune the ability of receptors to activate CheA by adjusting the level of receptor methylation on four specific glutamate residues in an activity-dependent manner, thereby returning kinase activity to a constant steady-state level under conditions of continuous stimulation. Two of the four glutamates at the methylation sites are originally translated as glutamines, which are functionally equivalent to methylated glutamates, and are converted to glutamates by the deamidase activity of CheB. Immuno- and cryo-electron microscopy have shown that thousands of chemoreceptors are organized in polar and lateral clusters, to which all other chemotaxis proteins localize, forming a large sensory machinery (Sourjik, 2004; Wadhams and Armitage, 2004). Allosteric interactions between receptors in clusters appear to play a central role in the amplification and integration of chemotactic signals (Li and Weis, 2000; Gestwicki and Kiessling, 2002; Sourjik and Berg, 2002b, 2004; Lai et al, 2005). To obtain a comprehensive view of the network dynamics in living cells, we tested all intracellular protein interactions in the pathway and their dependence on chemotactic stimulation.
by fluorescence resonance energy transfer (FRET). We further quantified concentration dependence and kinetics of stimulation-induced changes in protein interactions. Our results provide a holistic picture of the pathway and of its intracellular dynamics, and demonstrate the efficiency of the FRET-based interaction mapping approach.

Results
FRET mapping of protein interactions
FRET allows the detection of intracellular interactions of fluorescently labeled proteins non-invasively by the energy transfer from an excited donor to an acceptor fluorophore (Wouters and Bastiaens, 2001). The transfer efficiency depends on the distance between the fluorophores as \( R_0^2/(R_0^6 + R^6) \), with the Förster radius \( R_0 \)—at which the energy is transferred with 50% efficiency—being around the size of a typical protein. Such steep dependence on spacing and short characteristic distance make the energy transfer highly specific for proteins that are part of the same complex, either interacting directly or binding to a common scaffolding protein. High distance selectivity, however, can also impair FRET in a protein complex where the spacing between fluorescent labels is above the critical distance, approximately \( 2 \times R_0 \). In our interaction screen, we used cyan and yellow fluorescent proteins (CFP and YFP, respectively) as a donor–acceptor pair with \( R_0 \approx 49 \text{ Å} \), approximately the same size as a fluorescent protein monomer (Tsien and Miyawaki, 1998; Sourjik and Berg, 2002a). To reduce the chances of false negatives due to the large distance or unfavorable orientation of proteins in the complex and to find pairs with strongest FRET efficiency for subsequent investigation of stimulation dependence, we constructed a library of both N- and C-terminal fusions of CFP and YFP to all chemotaxis proteins, the aspartate receptor Tar and the serine receptor Tsr (Supplementary Table SI). E. coli CheA is endogenously expressed from two alternative start codons, yielding a long and a short variant, CheA\(_L\) and CheA\(_S\), respectively (Smith and Parkinson, 1980). To independently analyze both versions of CheA, we made separate fusions to CheA\(_{98–655}\) (CheA\(_S\)) and to CheA\(_{1098–1171}\), a mutant of CheA\(_S\) that does not express CheA\(_S\) (Sanatinia et al, 1995). Expression of full-length fusions was verified by immunoblot analysis (data not shown). Fusions with the fluorophore at the N terminus of receptors or CheB were omitted from further analysis because of failed membrane insertion or very low expression levels. Fusions to CheY, CheZ, CheR and CheB were fully functional, as tested by their ability to complement respective null mutants for chemotaxis-driven swarming in soft agar (Figure 1A), whereas fusions to the core components of the signaling complex—Tar, CheA and CheW—did not promote swarming. Nevertheless, all fusions localized to the chemosensory clusters (Figure 1B) as expected (Sourjik and Berg, 2000; Shiomi et al, 2002; Banno et al, 2004; Kentner et al, 2006).

Figure 1  Functionality and localization of YFP fusions. (A) Complementation assay for chemotactic swarming on soft agar plates. Plates were inoculated with wild-type cells (top), the respective mutant containing the fusion (bottom right) and the mutant without the fusion (bottom left). The Tar fusion was tested in the strain UU1250, which lacks all receptor genes, and CheA\(_L\) and CheA\(_S\) fusions in the strain VS166, which lacks the entire cheA gene. Respective mutants expressing Tar, CheA and CheW fusions did not swarm at any induction level tested (plate shown contained 50 \( \mu \text{M} \) IPTG), other fusions were expressed at 50 \( \mu \text{M} \) (CheR–YFP and YFP–CheY), 40 \( \mu \text{M} \) (CheY–YFP), 20 \( \mu \text{M} \) (CheZ fusions, YFP–CheR) or without (CheB–YFP) IPTG. Although the CheB–YFP construct only partially complemented the \( \Delta \text{cheB} \) mutant even without induction when expressed from the pTrc promoter, complementation was nearly 100% for the same construct expressed under tighter control of the arabinose promoter (not shown). (B) Localization to clusters in the wild-type strain RP437. IPTG inducer levels were 5 \( \mu \text{M} \) (CheZ–YFP and CheB–YFP), 10 \( \mu \text{M} \) (CheA–YFP, CheA\(_S–\)YFP), 20 \( \mu \text{M} \) (Tar–YFP, YFP–CheA\(_L\) and YFP–CheA\(_S\)) or 50 \( \mu \text{M} \) (CheW, CheY and CheR fusions; YFP–CheZ).
FRET mapping of protein interactions was made by acceptor photobleaching (Figure 2A; Supplementary Figure S1A). For simplicity, all protein pairs were tested in the same wild-type E. coli strain RP437 (Parkinson and Houts, 1982). To avoid false negatives that could arise from competitive binding of native proteins, negative results were confirmed in respective knock-out mutants. For pairs including YFP–CheR or CheB–YFP, the respective catalytic mutants, YFP–CheR\(^{D154A}\) and CheB\(^{S164C}\)-YFP (Barnakov et al, 2002; Shiomi et al, 2002), were also tested, as expression of the enzymatically active fusions influences the receptors’ methylation level and activity.

After the identification of positive FRET pairs, the dependence of these interactions on chemotactic stimulation was examined using a flow assay (Sourjik et al, 2007), whereby cells were attached to a coverslip in a flow chamber and changes in the YFP/CFP ratio were recorded in response to a stepwise addition and subsequent removal of 1 mM \(\varepsilon\)-methyl-\(\lambda\)-aspartate (MeAsp), a non-metabolizable analog of the Tar-specific attractant aspartate (Figure 2B; Supplementary Figure S1B). This analysis enabled us to picture the entire network of protein interaction in the chemotaxis pathway, with 19 positive FRET pairs being identified out of the 28 possible protein combinations, not counting the identical interactions of CheA\(_D\) and CheA\(_S\) separately (Figure 2C; Supplementary Table SII). Most pairs showed FRET even in the absence of all other chemotaxis proteins, arguing for direct interactions (Figure 2C, solid lines), whereas other combinations showed FRET only in the presence of either CheA or receptors (Figure 2C, dotted and dashed lines, respectively), indicating their dependence on a common binding partner. Additionally, several interactions are direct but depend on the kinase activity of CheA (see below).

In most cases where four possible combinations of N- and C-terminal fusions could be tested, interactions were observed with all of these combinations (Supplementary Table SII). A notable exception was CheR, for which only the N-terminal fusion showed interactions with other proteins, although both fusions localized to receptor clusters (Figure 1B). The only previously reported interactions that were not detected in the initial screen were those between Tar and CheW or CheA, which was expected because of the large distance between the C terminus of Tar and the binding site for both CheA and CheW at the signaling domain. A truncated Tar\(^{1–425}\)-CFP construct, with CFP being positioned closer to the signaling domain, indeed showed FRET in combination with CheW–YFP, though not with YFP–CheW or with N-terminal fusions to CheA and CheA\(_S\) or to a CheA\(_S^{509–655}\) fragment, which corresponds to the CheW homologous receptor-binding P5 domain of CheA.

The experimentally observed FRET efficiency for individual combinations could be indicative of the relative binding affinity of the respective fusions but also depends on the distance between protein termini in the complex and relative
orientation of the fluorophores. It is always lower than the theoretical FRET efficiency for the respective donor–acceptor pair and depends on the expression levels of the donor and acceptor, due to the contribution of the autofluorescent cell background to the overall cyan signal, and also because in most cases the acceptor is not in sufficient excess to saturate all donor molecules.

**Stimulation-induced changes in protein interactions**

Nine FRET pairs were responsive to chemotactic stimulation, five of which clearly correspond to direct protein interactions (black circles in Figure 2C; Supplementary Table SIII). Among them, CheY–CheZ (Sourjik and Berg, 2002b) and receptor–receptor (Vaknin and Berg, 2006) interactions were previously detected using *in vivo* FRET, along with an additional direct stimulation-dependent interaction between CheY and FliM (Sourjik and Berg, 2002a). The stimulation dependence of the other interactions involving CheY, CheB and CheZ (grey circles in Figure 2C) could be indirect (see Discussion). Stimulation-induced changes in interactions involving CheY or CheB were phosphorylation dependent, and were not observed for non-phosphorylatable mutants CheY<sup>D357A</sup> or CheB<sup>S164C,S164C</sup>, whereas receptor–receptor FRET responses are believed to reflect a change in the spacing between receptor dimers upon ligand binding (Vaknin and Berg, 2006, 2007, 2008). Consistent with that, stimulation-induced fractional changes in FRET were strongest for interactions that involved CheY and weakest for interactions that involved receptors (Supplementary Table SIII).

To determine the sensitivity of interactions to stimulation, we recorded response amplitudes across a range of MeAsp concentrations (Figure 3). Although the absolute values of FRET efficiency depend on several parameters, relative changes in FRET allow direct quantification of stimulus effects on the concentration of the FRET complex or on the conformational changes in this complex. Measurements were made with a minimal set of pathway components in the background. Strain VS116, which does not express any chemotaxis genes, was used to measure receptor–receptor interactions that involved receptors (Supplementary Table SIII). FRET between CheB<sup>S164C</sup> and Tar or CheA responded to CheZ–CheY (black circles), CheA<sub>S</sub>–CheB<sup>S164C</sup> (red squares), Tar–CheB<sup>S164C</sup> (green diamonds), and FliM–CheY (yellow triangles) fusion pairs in the strain VS153, and Tar–Tar (magenta open circles) and Tar–Tsr (pink open squares) in the strain VS116 (RhC). Cells were equilibrated in the buffer before each stimulation. Response amplitudes were calculated as described in Materials and methods and normalized to the maximal response upon stimulation with 1 mM MeAsp. Smooth curves are fits to the data using a multisite Hill model. Absolute values of stimulation-dependent and total FRET and protein expression levels are summarized in Supplementary Tables SIII and SIV, respectively. Source data is available for this figure at www.nature.com/msb.

![Figure 3](image-url) **Figure 3** Dose–response curves for direct stimulation-dependent interactions. Responses to steps of MeAsp for CheZ–CheY (black circles), CheA<sub>S</sub>–CheB<sup>S164C</sup> (red squares), Tar–CheB<sup>S164C</sup> (green diamonds), and FliM–CheY (yellow triangles) fusion pairs in the strain VS153, and Tar–Tar (magenta open circles) and Tar–Tsr (pink open squares) in the strain VS116 (RhC). Cells were equilibrated in the buffer before each stimulation. The characteristic times, τ, (Figure 4) for all interactions involving CheY, the response kinetics was apparently determined by dephosphorylation of phospho-CheY. CheY–CheZ FRET decayed with τ~0.33 s (Figure 4A), consistent with the in vitro rate of CheZ-stimulated CheY dephosphorylation estimated before (Sourjik and Berg, 2002a). CheY–CheA and CheY–FliM FRET showed slower increase and decay kinetics, respectively (Figure 4B and C), which corresponded to the autoprophosphorylation rate of phospho-CheY in the absence of CheZ. Similarly, decay in the interaction of CheB with Tar (Figure 4D) presumably reflected kinetics of CheB dephosphorylation. The characteristic time, τ~2.7 s, and the derived first-order dephosphorylation rate constant, 1/τ~0.37 s<sup>−1</sup>, were close to the previously measured in vitro rate constant for CheB dephosphorylation, 0.35 s<sup>−1</sup>, at the room temperature (Stewart, 1993). Small response amplitude of the CheB–CheA FRET pair precluded reliable measurement of its kinetics, but it is likely to be similar to that of the CheB–Tar pair. Response kinetics of Tar–Tar and Tar–Tsr binding to the C terminus of receptors and not to CheA. On the other hand, the CheA<sub>S</sub>–CheB<sup>S164C</sup> FRET response was still present, albeit much weaker, in the strain DK1. FRET was also observed between a CheA<sub>S</sub>–CheB<sup>DS66C</sup> fusion, which cannot bind receptor clusters, and fusions to CheB<sup>S164C</sup> or to the N-terminal CheB domain, CheB<sup>1-134</sup>.

We further determined kinetics of responses to a saturating stimulus of attractant for most direct stimulation-dependent interactions. All kinetics could be well fitted by an exponential decay function, yielding characteristic response times, τ (Figure 4). For all interactions involving CheY, the response kinetics was apparently determined by dephosphorylation of phospho-CheY. CheY–CheZ FRET decayed with τ~0.33 s (Figure 4A), consistent with the in vitro rate of CheZ-stimulated CheY dephosphorylation estimated before (Sourjik and Berg, 2002a). CheY–CheA and CheY–FliM FRET showed slower increase and decay kinetics, respectively (Figure 4B and C), which corresponded to the autoprophosphorylation rate of phospho-CheY in the absence of CheZ. Similarly, decay in the interaction of CheB with Tar (Figure 4D) presumably reflected kinetics of CheB dephosphorylation. The characteristic time, τ~2.7 s, and the derived first-order dephosphorylation rate constant, 1/τ~0.37 s<sup>−1</sup>, were close to the previously measured in vitro rate constant for CheB dephosphorylation, 0.35 s<sup>−1</sup>, at the room temperature (Stewart, 1993). Small response amplitude of the CheB–CheA FRET pair precluded reliable measurement of its kinetics, but it is likely to be similar to that of the CheB–Tar pair. Response kinetics of Tar–Tar and Tar–Tsr.
pairs had characteristic response times of 1.1 and 1.7 s, respectively (Figure 4E and F). However, monoexponential function did not perfectly fit the data, indicating biphasic response kinetics, which was consistent with the biphasic response observed in dose–response measurements (Figure 3).

**Discussion**

Altogether, our FRET analysis of protein interactions in the chemotaxis pathway of *E. coli* identified 19 positive FRET pairs out of the 28 possible protein combinations, with 9 pairs being responsive to chemotactic stimulation. The obtained map of protein interactions is in good agreement with previous biochemical data and localization analyses (Sourjik, 2004; Wadhams and Armitage, 2004), and offers a better insight into the pathway regulation.

**Core of the sensory complex**

The core of the chemosensory complex is formed primarily by the receptor–receptor interactions, and is further stabilized by the binding of CheA and CheW (Maddock and Shapiro, 1993; Kim et al., 1999; Ames et al., 2002; Kentner et al., 2006). This ternary complex is stable on the time scale of signaling and adaptation, with characteristic equilibration time being 12 min for CheA and CheW, and more than 30 min for receptors (Schulmeister et al., 2008). We observed energy transfer among most fusions to the core proteins. Receptor–receptor FRET results primarily from the interactions between homodimers, rather than from an intradimeric energy transfer, as FRET signals were similar for the Tar–Tsr and Tar–Tar pairs (Supplementary Table SII). This is consistent with previous reports (Vaknin and Berg, 2006, 2007, 2008) and suggests that receptors are tightly packed in the cluster, with distances between the C termini of different dimers being shorter that the respective intradimeric distances. We further observed FRET for the CheA–CheA, CheA–CheW and CheW–CheW pairs. CheA–CheA FRET was largely intradimeric, as the strength of the signal was not affected by the lack of all other chemotaxis proteins (Supplementary Table SII). This could mean that CheA dimers are less densely packed in the sensory complexes than receptor dimers, consistent with a high ratio of receptors to CheA in the active complexes (Levit et al., 2002). Energy transfer efficiency was similar for all combinations of CheA fusions, indicating similar distances between all termini in the dimer. FRET between CheA and CheW was also independent of the other proteins and thus reflects the expected direct interaction between the two. FRET between two CheW fusions required receptors, suggesting that CheW fusion proteins come into proximity by binding to receptor oligomers and possibly also to CheA dimers.

Interactions of receptors with CheW and CheA were the only two established protein interactions in the chemotaxis pathway that were initially not detected in our assay. This was not surprising because the CheA and CheW-binding site is ~210 Å away from the receptors’ C terminus (Kim et al., 1999), meaning that the distance between the fluorophores for these pairs is above the critical range for energy transfer between CFP and YFP (~100 Å). Using a truncated Tar^{1-425}–CFP
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construct, FRET was indeed detected in combination with CheW–YFP. However, no signal was obtained with YFP–CheW or with the N-terminal fusions to CheA, CheA or the P5 fragment of CheA (CheA509–655). As all of these fusions localize to receptor clusters (Figure 1B), and fusions to CheA and the P5 fragment have been previously shown to bind to receptors in the absence of CheW (Kentner et al., 2006), it appears that only the C terminus of CheW is positioned in sufficient proximity to the Tar1425 fluorophore for FRET. Tar–CheA interaction was thus the only false negative in our screen.

Among the interactions in the sensory core, only the Tar–Tar and Tar–Tsr interactions showed stimulation dependence. The decrease in the energy transfer between the C-terminal fusions to receptors upon attractant stimulation is believed to be caused by attractant-induced conformational changes in the receptor complexes rather than by their dissociation (Vaknin and Berg, 2006, 2007, 2008). No stimulation dependence was observed for the other FRET signals, suggesting that there are no major rearrangements in the packing or stoichiometry of the sensory core with stimulation. Furthermore, lack of any detectable response to attractant for the intradimeric CheA FRET indicates that the stimulation-induced conformational changes in this dimer—which are believed to regulate kinase activity—have to be subtle. These conclusions, though, have to be taken with caution as fusions to the sensory core components were not fully functional.

Interactions involving CheZ and CheY

CheZ and CheY were previously shown to localize to receptor clusters through interaction with CheA (Sourjik and Berg, 2000; Cantwell et al., 2003), and we confirmed both of these interactions. CheA–CheY and CheA–CheZ interactions were observed even in the absence of all other proteins, and for fusions to both CheA and CheA. Direct interaction between CheZ and CheA contradicted previous reports that only CheA is able to bind CheZ (Cantwell et al., 2003), but was confirmed by the localization of CheZ–YFP to receptor clusters in a strain expressing CheA, but not CheA or CheY (Supplementary Figure S2). The degree of CheA-mediated CheZ localization was weaker than in the wild type, which presumably explains why this interaction was missed in the previous fluorescence-microscopy based study (Cantwell et al., 2003). However, FRET signals for the CheZ interactions with C-terminal CFP fusions to both forms of CheA were comparable, possibly indicating differences in CheZ binding to free and cluster-associated CheA. N-terminal CFP fusion to CheA indeed showed much stronger FRET than the N-terminal fusion to CheA, but this might reflect shorter distance between fluorophores in the latter case.

The CheY–CheZ pair also showed FRET, which apparently has two components—one due to the direct interaction between the two proteins and the other mediated by CheA. Phosphorylation-dependent FRET between CheY and CheZ was previously observed (Sourjik and Berg, 2002b), even in mutant strains where CheZ or CheY did not bind to CheA (Vaknin and Berg, 2004; VS, unpublished data). This phosphorylation-dependent interaction between CheY and CheZ could only be observed for the C-terminal CheZ fusions, consistent with the crystal structure of the phospho-CheY complex with CheZ (Zhao et al., 2002). On the other hand, a weak FRET signal between CheY and CheZ was observed for both C- and N-terminal CheZ fusions in the CheA–CheW strain (Supplementary Table SII). As in this strain CheA is largely inactive and therefore CheY is expected to be unphosphorylated, FRET is likely to be explained by the binding of CheY and CheZ to CheA, which brings them in proximity. No CheY–CheZ FRET was observed in the absence of CheA at the used expression levels.

Consistent with it being a dimer, CheZ also showed FRET with itself even in the absence of all other chemotaxis proteins. Additionally, positive FRET signals were observed for CheW–CheY, CheY–CheB, CheY–CheY and CheZ–CheB pairs. For all of these pairs, energy transfer was not observed in the absence of CheA and presumably results from the proximity of respective fusion proteins when bound to the latter. Indeed, the CheW–CheY interaction was not seen in the strain expressing CheAM57, which lacks CheY-binding P2 domain and therefore cannot bind but is still able to phosphorylate CheY. For the CheY–CheY pair, FRET was still observed in the CheA125 strain, but the energy transfer could also take place at the CheZ dimer or at the flagellar motor.

All of the direct interactions that involve response regulator CheY—with the kinase, phosphatase and flagellar motor—exhibited stimulation dependence. CheY phosphorylation decreased its affinity to CheA, and increased the affinity toward CheZ and FliM, in agreement with previous biochemical studies (Li et al., 1995; McEvoy et al., 1999). However, the directionality of the CheA–CheY FRET response was inverted in the presence of CheZ, which can be explained by a strong binding of phospho-CheY to CheZ, which is itself associated with CheA. Apparently, the increase in FRET caused by association of phospho-CheY with CheA-bound CheZ outbalances the FRET decrease caused by dissociation of phospho-CheY from CheA, consistent with a previous report that described changes in the localization of CheY to chemosensory clusters (Vaknin and Berg, 2004). CheW–CheY and CheY–CheY FRET also showed dependence on CheY phosphorylation, presumably as a result of the change in CheY affinity for CheA. However, we cannot exclude the possibility that the CheY–CheY interaction is direct but requires CheA activity.

No changes in energy transfer upon stimulation could be observed for the CheZ dimer, suggesting that it does not undergo any major structural rearrangements. Although oligomerization of CheZ upon binding phospho-CheY has been previously suggested (Blat and Eisenbach, 1996), later biochemical experiments (Silversmith, 2005) and our in vivo results seem to disprove this idea. Nevertheless, the CheZ dimer does undergo a modest change of conformation and activation (Blat et al., 1998; Silversmith, 2005) upon binding to phospho-CheY. This might result in an increased affinity to CheA and could explain a weak CheY-dependent increase in the energy transfer between CheZ and CheA with pathway activation. Alternatively, some increase in the CheA–CheZ FRET may be caused by CheZ binding to the CheA-bound phospho-CheY. In any case, higher local concentration of CheZ would result in an increased association of CheZ with CheA at the cluster, which has been reported to enhance phosphatase activity (Wang and Matsumura, 1996). Recent Brownian
demonstrating that CheR binding to receptors is not affected by again cannot be excluded that some of them are direct but involving CheB are presumably mediated by CheA, although it et al N-terminal domain of CheB (Li et al, 1995). Other interactions involving CheB are presumably mediated by CheA, although it again cannot be excluded that some of them are direct but require kinase activity.

All interactions of CheR were stimulation independent, demonstrating that CheR binding to receptors is not affected by stimulation. In contrast, Tar–CheB$^{S164C}$ FRET increased with kinase activation, indicating that association of CheB with the pentapeptide is phosphorylation dependent. The binding sites targeted by CheB and CheR overlap (Lai et al, 2006), and when the Tar–CheRD$^{D154A}$ fusion pair was expressed together with the non-fused CheB$^{S164C}$, a decrease in FRET was indeed observed with pathway activation, in agreement with a displacement of the CheR fusion by competitive binding of phospho-CheB$^{S164C}$ to the pentapeptide sequence.

Given the homology between CheY and the CheB N-domain, it was expected that phosphorylation of CheB would decrease its affinity for CheA, and such decrease in affinity was suggested based on the indirect evidence (Anand and Stock, 2002). However, we observed that the CheA–CheB$^{S164C}$ FRET increased with kinase activation. As the putative CheA-binding region in the CheB N-domain is buried in the interaction with the C-domain (Djordjevic et al, 1998a), it is conceivable that the phosphorylation exposes this region through release of the interdomain linkage and thereby increases the affinity for CheA. The response was still present in a strain lacking the receptor pentapeptide sequence or with a CheA fragment that does not associate with receptors, confirming that the effect does not depend on CheB binding to receptors. However, the response in these strains was much weaker, indicating that the association of CheB with adjacent receptor pentapeptides indeed facilitates the binding to CheA, possibly by raising the local CheB concentration.

**Levels of pathway regulation**

Recording FRET responses to stimulation also enabled us to distinguish two levels of activity-dependent changes in the pathway: stimulation-induced rearrangement of receptors in the sensory complexes, and downstream changes in the phosphorylation of CheY and CheB. Consistent with a previous report (Vaknin and Berg, 2007), cooperativity—represented by the Hill coefficient—is high for the phosphorylation-dependent FRET pairs, but low for the receptor–receptor responses. Sensitivity, as indicated by the MeAsp concentration eliciting a half-maximal response, is in a similar range for all protein pairs. A somewhat lower sensitivity for the response of FliM–CheY and CheA–CheY could be due to the slow dephosphorylation—and thus higher level—of phospho-CheY in the absence of CheZ. Tar–Tar responses were reported to exhibit lower sensitivity than CheY–CheZ at low levels of receptor modification, but higher sensitivity at high levels of modification. The approximately equal sensitivities in our measurements is presumably due to the intermediate level of receptor modification in the VS153 background, where receptors remain in the original half-modified state with two glutamates and two glutamines that mimic the behavior of methylated glutamates.

Interestingly, the interaction between Tar and the serine receptor Tar responds to MeAsp with a lower sensitivity than the Tar–Tar interaction. Similar observation was previously made at the level of kinase activity (Sourjik and Berg, 2004) and agrees with the prediction of allosteric models (Duke et al, 2001; Sourjik, 2004; Mello and Tu, 2005; Keymer et al, 2006) that homogeneous sensory complexes should be more sensitive to stimulation. Allosteric interactions are thus involved in signal processing already at the receptor level. Moreover, receptor pairs show a biphasic response in dose–response and kinetics measurements, indicating that either the same receptor complexes undergo two distinct stimulation-induced movements or rearrangements, or that two receptor sub-populations exist in cells, possibly corresponding to weakly and strongly clustered receptors. At present, we can only speculate about the molecular details of these ligand-induced receptor rearrangements at the cluster. Previously observed changes in homo-FRET between receptor fusions have been interpreted in terms of receptor movement in trimers of dimers (Vaknin and Berg, 2006, 2007, 2008), but changes in FRET might as well reflect movement of neighboring trimers or both. Because their kinetics are slower than changes in CheY phosphorylation—reflected by the phosphorylation-dependent interaction of CheY with CheZ—these rearrangements do not seem to directly represent the change in receptor conformation that regulates kinase activity but are rather induced by it.

At the downstream level of regulation, we observed the increased affinity of phosphorylated CheB to both its binding partners at the receptor cluster. Such dependence implies an additional enhancement of a negative feedback from the level of kinase activity to that of receptor activity, which is provided by CheB phosphorylation. This feedback is important to maintain robust output of the chemotaxis system under such perturbations as gene expression noise (Kollmann et al, 2005). Our results suggest that phosphorylation not only largely
enhances the methylsterase activity of CheB (Anand and Stock, 2002) but also raises the local concentration of active CheB in the vicinity of methylation sites. CheB activation by binding to the pentapeptide sequence of receptors might enhance the feedback even further (Barnakov et al., 2002). Consistent with its role in the feedback regulation, stimulation-induced changes in the CheB–Tar interaction showed slowest kinetics in the initial pathway response. The dynamic behavior of CheB and its phosphorylation-dependent competition with CheR and CheY in binding receptors and CheA (Li et al., 1995), respectively, may play an important role in determining response kinetics and pathway robustness. Indeed, a recent computational analysis suggested that strong binding of phosphorylated CheB to receptors and weak binding of phosphorylated CheY to CheA are important for robust adaptation in chemotaxis (Matsuzaki et al., 2007).

Conclusions

Despite previous reservations (Phizicky et al., 2003), our study demonstrates that FRET can be successfully used to systematically map and quantify all intracellular protein interactions in a signaling network, including transient interactions, with no false positives and nearly no false negatives. A strong advantage of FRET is that it enables systematic mapping of activity dependence of interactions and measuring response kinetics, and therefore yields a dynamic picture of the network. In addition to direct interactions, protein proximities—mediated by association with a common interaction partner—can be identified, facilitating the characterization of multiprotein complexes and their dynamics in vivo. FRET-based interaction mapping thus stands as a simple and reliable means for the investigation of other protein networks in bacteria or eukaryotes.

Materials and methods

Fluorescent protein fusions

Plasmids used to express fluorescent protein fusions are listed in Supplementary Table S1. With the exception of CheY, CheZ and FliM (Sourjik and Berg, 2002a,b), all fusions were constructed as described before (Kentner et al., 2006): the target gene was amplified by PCR and cloned into respective vectors pDK5, pDK66, pDK2 and pDK85, derivatives of pTrc99a (Pharmacia; pBR ori, pTrc promoter, AmpR) carrying eYFP (S65T, S202V) and cYFP (S65T, S202V) sequences which encode true monomeric versions of YFP and CFP, respectively (Zacharias et al., 2002). CFP fusions were subsequently moved to vector pDK79 (pACYC ori, pBAD promoter, KanR) and pDK198 and pDK203 were generated by transferring tar-cfp from pDK53 to plasmids pDK85 and pDK80, respectively. Mutations in cheB506D and cheZ534D were generated by PCR, using oligonucleotides encoding the respective amino-acid substitution. Immunoblotting with a monoclonal GFP-specific antibody (JL8; BD Biosciences) was used to confirm that fusions were expressed as full-length proteins with little degradation.

Expression levels of CFP from plasmid pDK2 (26 900 ± 1200 copies per cell) and CheY-YFP from pVS18 (39 400 ± 1400 copies per cell) at full induction were quantified by fluorimetry. Cell cultures were washed and concentrated threefold in tethering buffer, counted in a Neubauer chamber, diluted to the same concentration and lysed by sonication. CFP and YFP signals of the lysates were measured in a fluorimeter, and fluorescent protein numbers were calculated by comparison with a known amount of purified CFP/YFP, which was added to a lysate of control cells not expressing any fluorescent protein. Expression levels of the stimulus-dependent FRET fusion pairs (Supplementary Table SIV) could not be measured the same way, because in some cases the CFP signal was too low to the background. Therefore, CFP and YFP signals of the FRET pairs were quantified from fluorescence microscopy images by measurement of the integrated intensity of about 100–200 single cells, using ImageJ software (W Rasband; http://rsb.info.nih.gov/ij/). Cells expressing CFP from pDK2 and CheY-YFP from pVS18, respectively, at full induction were used to calibrate expression levels (CFP/YFP molecules per cell). To convert the absolute molecule numbers per cell to molar concentration, we calculated the cell volume (1.80 ± 10−13 l) from the average dimensions of an E. coli cell on fluorescence images.

Strains and their growth

E. coli strains in this study (Supplementary Table S1) are derived from K12 strain RP437. Strains VS166 (AceA), VS167 (AceA Af/(tap-cheZ)) and DK1 (tar App Ache/cb-cheZ Δtsr) were made by in-frame deletion of cheA in strains RP437 and RP2893 (Δ(tap-cheZ)), and the tar pentapeptide sequence in the strain VS153 (Δ(che/cb-cheZ) Δtsr), respectively. Cells were grown in tryptone broth (TB; 1% tryptone and 0.5% NaCl) with added antibiotics. Ampicillin, chloramphenicol and kanamycin were used at final concentrations of 100, 35 and 50 µg/ml, respectively. Overnight cultures, grown at 30°C, were diluted 1:100 and grown at 34°C for about 4 h, to an OD660 of 0.45–0.5, in the presence of inducers isopropyl β-D-thiogalactoside (IPTG) and arabinose. Standard induction levels for FRET measurements were 0.01% arabinose and 50 mM IPTG, except for pDK58, pDK135, pDK159, pDK203 (20 µM IPTG) and pDK198 (100 µM IPTG). Cells were harvested by centrifugation (4000 r.p.m., 5 min), washed and resuspended in tethering buffer (10 mM potassium phosphate, 0.1 mM EDTA, 1 mM L-methionine, 67 mM sodium chloride, 10 mM sodium lactate, pH 7) prior to FRET measurements.

Chemotaxis tests shown in Figure 1A were performed on TB soft agar plates (1% tryptone, 0.5% NaCl and 0.3% agar) at 30°C overnight.

Fluorescence imaging

Images shown in Figure 1B were obtained by a Zeiss Axio Image.Z1 microscope, as described before (Kentner et al., 2006).

FRET measurements

Measurements were performed on a custom-modified Zeiss Axiovert 200 microscope. For acceptor photobleaching (Supplementary Figure S1A), cells were concentrated about 10-fold by centrifugation, resuspended in tethering buffer and applied to a thin agarose pad (1% agarose in tethering buffer). Excitation light from a 75 XBO lamp, attenuated by a ND60 (0.2) neutral-density filter, passed through a band-pass (BP) 436/20 filter and a 495DCSP dichroic mirror and was attenuated by a ND60 (0.2) neutral-density filter, passed through a band-pass (BP) 436/20 filter and a 495DCSP dichroic mirror and was recorded before and after bleaching of YFP by a 20-s laser pulse, and (both from National Instruments, Austin, TX). CFP emission was recorded before and after bleaching of YFP by a 20-s laser pulse, and FRET was calculated as the signal increase divided by the total signal recorded before and after bleaching of YFP by a 20-s laser pulse, and FRET was calculated as the signal increase divided by the total signal recorded before and after bleaching, as defined previously (Sourjik et al., 2007). FRET was scored as significant above 0.5% increase in CFP emission.

To measure concentration dependence of FRET responses to chemostimulation (Supplementary Figure S1B), cells were attached to a polylysine-coated coverslip and placed in a flow cell, which was kept under constant flow (500 µl/min) of tethering buffer by a syringe pump (Harvard Apparatus). By rapid exchange of the buffer reservoir, solutions of x-methyl-D,L-aspartate (MeAsp; Sigma) in tethering buffer were added or removed. Excitation was through a BP 436/20 filter and
reflected by a 455 dichroic mirror. Emission from a field of 300–500 cells passed through the 455 DC mirror, and was split by a 515 dichroic mirror into two signals, passing through a BP 485/40 cyan filter and a BP 535/30 yellow filter, respectively, on photon multipliers. FRET, defined as the fractional change in cyan fluorescence due to energy transfer, was calculated from changes in the ratios of yellow and cyan fluorescence signals, and the data were fit to a multisite Hill model with either one or two apparent dissociation constants (K0.5), as described before (Sourjik and Berg, 2002a; Sourjik et al., 2007), using KaleidaGraph 3.6 (Synergy Software, Reading, PA).

The same experimental procedure was used for kinetics measurements, except attractant was added at a higher flow rate, 1500 µl/min, and signal integration time was set to 0.03–0.3 s, depending on a FRET pair. The liquid exchange profile in the flow cell at this rate was determined using 100 nM fluorescein solution as a marker, and used to estimate the ramp profile for stimulation with 10 mM aspartate solution (Supplementary Figure S3). Aspartate rather than MeAsp was used, because it binds with >10 × higher affinity to Tar. Under these conditions, saturating attractant concentration for all FRET pairs was reached within 0.1 s, and the observed response times were therefore not limited by the rate of stimulation. FRET values were calculated from the ratios of yellow and cyan fluorescence signals as described above and normalized to the average pre-stimulus FRET value. Characteristic response time, τ, for different FRET pairs (Figure 4) was determined by fitting the data to a model of exponential decay, \( \exp(-t/t_\alpha) \), where \( t/t_\alpha \) is time after stimulation.

To estimate the stimulus-dependent fraction of the total FRET signal (Supplementary Table SIV), the relative change in the CFP signal in response to a saturating stimulus with 1 mM MeAsp was recorded (stimulus-dependent FRET), followed by subsequent acceptor bleaching (total FRET), using cells in the flow cell and the same microscopic setup as for the previously described acceptor bleaching measurements.

**Supplementary information**

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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