

Multicomponent analysis: formation of a quarternary signal transduction complex



Abstract

Biacore's SPR technology was used to study the formation and presumed function of complexes between various components in the chemotactic signalling system in *E. coli*. Binding of chemotactic signalling molecules to the receptor regulates the activity of the protein kinase, cheA, through a coupling protein, cheW. CheA is responsible for phosphorylation of the response regulator, cheY, and the receptor modulator, cheB. By immobilizing cheY on the sensor chip surface, a quarternary complex could be formed involving cheY, cheA, cheW and the aspartate receptor, Tar. The complex was stabilised in the presence of ATP and aspartate.

Introduction

The chemotactic response in *E. coli* is mediated through the receptor-regulated activity of the histidine protein kinase, cheA, which phosphorylates the response regulator, cheY (determining the direction of flagellar rotation) and the receptor modulator, cheB (providing receptor adaptation to prevailing conditions).

Regulation of cheA by the receptor requires the coupling protein, cheW. CheA phosphorylates cheY in a two-step mechanism, involving autophosphorylation of a histidine residue in cheA followed by transfer of the phosphate group to cheY.

Using this technique, Simon and coworkers [1,2] have investigated complex formation between various components of the bacterial chemotactic signalling molecules, and also tested the effect of signal substances and phosphorylation conditions on the complexes. Biacore's SPR technology gives an immediate measure of complex formation, and different combinations of the system components can easily be tested in a relatively short time. The results demonstrate that an active quarternary signal transduction complex can be formed on the sensor chip surface, but only if the components are mixed and added in a prescribed order. These studies emphasise the potential of Biacore's SPR technology in studying the detailed mechanism of complex signal transduction systems.

Materials and methods

Sensor Chip CM5, 2-(2-pyridinyl-dithio)ethaneamine (PDEA) and amine coupling kit were obtained from Biacore AB, Sweden. Proteins cheA, cheY and cheW and Tar-containing vesicles were purified from *E. coli* as described elsewhere [3]. The modified protein, cheY51YC was expressed in a DcheY mutant strain containing the plasmid pRBB40. 51YC (provided by R. B. Bourret).

Immobilization of cheY

Immobilization of cheY through primary amine groups did not bind cheA injected over the sensor chip surface. The mutant protein, cheY51C, in which a single cysteine residue is introduced in a position remote from the active site [4,5], was therefore immobilized by thiol coupling chemistry [6]. This mutation did not affect the chemotactic function of cheY as assayed on swarm plates (R. B. Bourret, personal communication).

The sensor chip was equilibrated with a flow of HBS buffer (10 mM HEPES pH 7.4 containing 150 mM NaCl and 0.005% Tween 20) at 5 μ l/min, then activated by injecting 10 μ l of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide + 0.05

M N-hydroxysuccinimide (EDC/ NHS) followed by 45 μ l 50 mM PDEA in 0.1 M sodium borate pH 8.5. Excess NHS-esters on the surface were deactivated with a pulse of 2 μ l 1 M ethanolamine-HCl pH 8.5. The flow rate was reduced to 2 μ l/min and cheY51YC (45 μ l at 22 μ g/ml in 50 mM formic acid pH 2.5) was injected. Excess reactive sulfhydryl groups were finally deactivated by injecting 20 μ l 0.1 M cysteine in 50 mM formate buffer pH 4.5 containing 0.1 M NaCl.

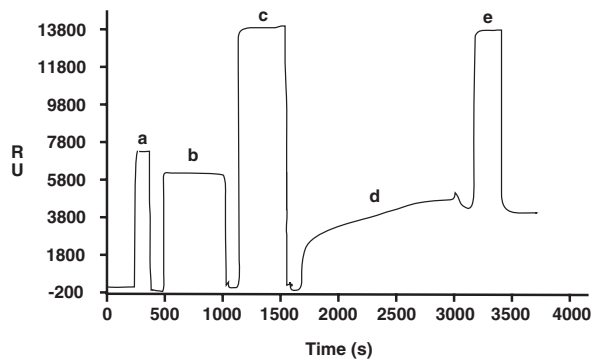
Fig. 1 shows a typical immobilization sensorgram.

Binding experiments

Binding experiments were performed at a constant flow rate of 5 μ l/min in TEMK buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA). Protein concentrations were 250 μ g/ml cheA, 200 μ g/ml cheW and 200 μ g/ml Tar vesicles. Component mixtures were allowed to stand for 1 minute before injection to permit complex assembly. The sensor chip was regenerated after each binding experiment with 2 μ l 6 M guanidinium hydrochloride followed by extensive washing with TEMK buffer.

Figure 1

Figure 1
Sensorgram showing immobilization of cheY51YC to Sensor Chip CM5.
a Activation with NHS/EDC.
b Introduction of reactive SH groups with PDEA.
c Deactivation of excess NHS-esters with ethanolamine.
d Immobilization of cheY51YC.
e Deactivation of excess reactive SH groups.
See text for further details.



Results

Fig. 2 shows a sequence of attempts to bind components of the chemotaxis signalling system to immobilized cheY. Binding of cheA alone to cheY was observed, with estimated rate constants $k_{\text{ass}} = 368 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{diss}} = 1.14 \times 10^{-5} \text{ s}^{-1}$ ($K_D = 3 \times 10^{-8} \text{ M}$). No further components bound during subsequent injections of cheW, Tar or a mixture of cheW and Tar. However, if cheA, cheW and Tar were mixed prior to injection over cheY, more material was bound than can be accounted for by cheA alone. This result indicated that a multiple complex was binding to cheY on the sensor chip surface. In a series of binding tests using different combinations and sequences of components, multiple complex formation was only observed when all three components were mixed prior to injection, leading to the conclusion that a quaternary complex of cheY, cheA, cheW and Tar is formed on the sensor chip surface. Addition of 0.5 mM ATP after binding components to immobilized cheY had no effect on the binary cheY/cheA

complex, but markedly destabilized the quaternary cheY/ cheA/cheW/Tar complex (Fig. 3). The effect was specific to ATP, since addition of a variety of other nucleotides (ADP, cAMP, GTP, GDP, dUTP and dTTP) had no effect on the stability of the complex.

ATP-induced dissociation of the quaternary complex was prevented by including 1 mM aspartate in the ATP pulse (Fig.4). Aspartate alone had no effect. Binding of aspartate to the receptor is known to inhibit the kinase activity of cheA [7]. Addition of ATP had no effect on a complex formed with the mutant protein, cheA48HQ, which lacks the histidine autophosphorylation site, or with cheY51YC57DA, which lacks phosphotransferase recipient ability (not shown). Taken together, these results show that ATP-induced destabilization of the quaternary complex on the sensor chip surface reflects receptor-regulated phosphorylation of cheY by cheA.

Figure 2 Top left
Binding of separate and mixed components to immobilized cheY51YC. CheA alone (a) bound to the surface, but subsequent injection of cheW (b), Tar (c), or a cheW/Tar mixture (d) resulted in no further binding. When all three components were premixed before injection (e), the amount bound was several-fold greater than with cheA alone.

Figure 3 Bottom left
Addition of 0.5 mM ATP to cheA bound to immobilized cheY51YC (a) has no effect. However, addition of ATP causes marked dissociation of the quaternary complex Tar/cheW/cheA/cheY (b).

Figure 4 Top right
Addition of 1 mM aspartic acid without (a) or with (b) 0.5 mM ATP had no significant effect on the stability of the surface-bound quaternary Tar/cheW/cheA/cheY complex. Dissociation was induced only by addition of ATP alone (c).

Figure 5 Bottom right
Quaternary complex containing fully demethylated Tar does not respond to addition of ATP (a). Methylation of the receptor is required for ATP-induced dissociation (b).

Figure 2

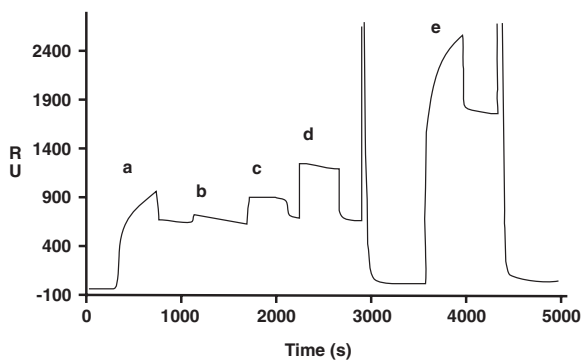


Figure 4

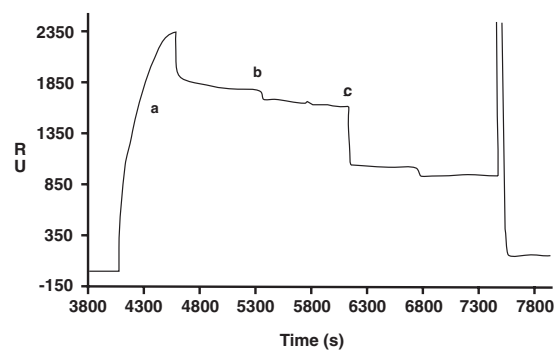


Figure 3

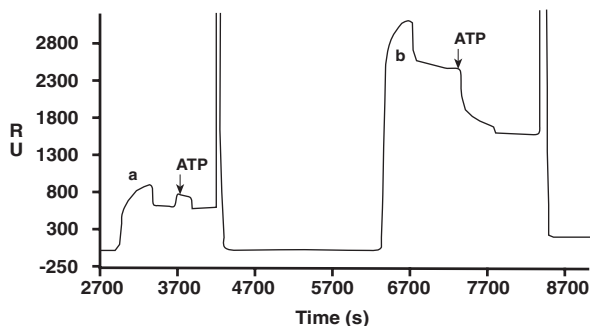
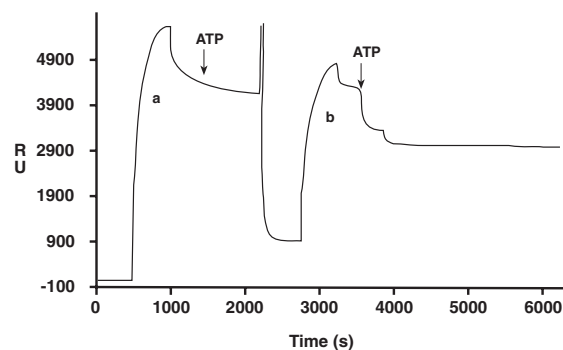


Figure 5



References

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The significance of the complex destabilization as part of the signal transduction mechanism is further supported by experiments showing that the response to ATP is influenced by the level of methylation of Tar. Methylation at four residues in the cytoplasmic domain of the receptor is implicated in adaptation to new ligand concentrations [8], and increasing methylation levels increase the ability of the receptor to stimulate phosphorylation of cheY [7,9]. Complexes formed on the sensor chip surface with fully demethylated Tar are insensitive to the addition of ATP (Fig. 5).

Discussion

These studies show that dissociation of the quarternary cheY/cheA/cheW/Tar complex is correlated with conditions that favour phosphorylation of cheY, and responds to well-defined regulatory aspects of the signal transduction mechanism. CheA binds on its own to cheY, and is able to phosphorylate cheY at a measurable rate in solution [4,10]. However, addition of ATP has no effect on the stability of the binary cheA/cheY complex on the sensor chip surface. Autophosphorylation of cheA occurs through an intermolecular reaction in cheA dimers, and the regulation of autophosphorylation by receptor and

cheW requires intact cheA dimers [11]. If the affinity between cheA monomers is significantly weaker than between cheA and cheY (measured as 3×10^{-8} M in this study), cheA alone may bind to immobilized cheY as inactive monomers. Tar is also a dimer [12], and formation of the active quarternary complex may require that cheA dimers are stabilized in solution by binding to the Tar/cheW complex before binding to immobilized cheY. This analysis of the assembly, structure and dynamics of the quarternary signal transduction complex is made feasible by the direct monitoring of complex formation in real-time. The general nature of the techniques used suggests that a similar approach should prove useful for other receptor signalling systems as well. The formation of multi-component signal transduction complexes with biological activity on the sensor chip surface offers a new tool in the detailed study of the specific interactions that regulate inter- and intracellular communication.

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