KINETIC CHARACTERIZATION OF CATALYSIS BY THE CHEMOTAXIS PHOSPHATASE CHEZ:
MODULATION OF ACTIVITY BY THE CHEYp SUBSTRATE*
Ruth E. Silversmith1, Matthew D. Levin3, Elmar Schilling1,3, and Robert B. Bourret1
1Department of Microbiology and Immunology, University of North Carolina,
Chapel Hill, NC 27599-7290 and 3Department of Physiology, Development, and Neuroscience,
University of Cambridge CB2 3EJ, United Kingdom
Running title: Kinetics of CheZ phosphatase
Address correspondence to: Ruth E. Silversmith, Department of Microbiology and Immunology,
University of North Carolina, Chapel Hill, North Carolina 27599-7290, Tel. 919 966-2679; Fax.
919-962-8103; E-mail: silversr@med.unc.edu
3Current address: Department of Hematology and Oncology, Regensburg University Medical
Center, 93042 Germany
CheZ catalyzes the dephosphorylation of the response regulator CheY in the two-
component regulatory system that mediates chemotaxis in Escherichia coli. CheZ is a
homodimer with two active sites for dephosphorylation. To gain insight into cellular
mechanisms for the precise regulation of intracellular CheYp levels, we evaluated the
kinetic properties of CheZ. The steady state rate of CheZ-mediated dephosphorylation of
CheYp displayed marked sigmoidicity with respect to CheYp concentration and a k_cat of 4.9
s⁻¹. In contrast, the gain of function mutant CheZ 21IT with an amino acid substitution far
from the active site, gave hyperbolic kinetics and required far lower CheYp for half
saturation, but had a similar k_cat value as the wild type enzyme. Stopped flow fluorescence
measurements demonstrated a six fold faster CheZ/CheYp association rate for CheZ 21IT
(k.Ass = 3.4 x 10⁷ M⁻¹s⁻¹) relative to wild type CheZ (k Ass = 5.6 x 10⁶ M⁻¹s⁻¹). Dissociation of
the CheZ-CheYBeF₃ complex was slow for both wild type CheZ (k_diss = 0.040 s⁻¹) and CheZ 21IT
(k_diss = 0.023 s⁻¹) and, when taken with the k_ass values, implied K_d values of 7.1 nM and 0.68
nM, respectively. However, comparison of the k_diss and k_cat values implied that CheZ and
CheYp are not at binding equilibrium during catalysis and that once CheYp binds, it is
almost always dephosphorylated. The rate constants were collated to formulate a kinetic
model for CheZ-mediated dephosphorylation that includes autoregulation by CheYp and
allowed prediction of CheZ activities at CheZ
and CheYp concentrations likely to be present
in cells.
In the two-component regulatory system that mediates chemotaxis in Escherichia coli, the cell
continuously regulates the level of phosphorylation of the response regulator CheY in
response to an external chemical gradient (1-4). Intracellular levels of phosphorylated CheY
(CheYp), in turn, directly dictate cell swimming behavior. CheYp binds to the base of the
flagellum, thereby changing the direction of flagellar rotation from counterclockwise (causing
a forward run) to clockwise (causing a reorienting tumble). Because cells switch rapidly between
these two swimming behaviors as they sample their environment (5,6), it is essential that both
phosphorylation and dephosphorylation of CheY occur on a rapid time scale so that the
concentration of CheYp at any instant accurately reflects current environmental conditions.
Moreover, the highly cooperative relationship between intracellular CheYp concentration and the
probability of clockwise rotation (7) underscores the necessity of precise regulation of CheYp
levels. CheY is phosphorylated on Asp57 by its
cognate sensor kinase CheA at a rate that is
dictated by the rate of autophosphorylation of
CheA with ATP, which in turn is a function of the
activity state of coupled transmembrane receptors.
CheYp is dephosphorylated by the phosphatase
CheZ. The ability to perform chemotaxis is very
sensitive to intracellular CheZ activity as either a
modest increase or decrease of CheZ activity
disables chemotaxis (8-12). Much of the cellular pool of CheZ is associated with the large polar signaling complex (13-15), which also contains receptors, CheA, and the scaffolding protein, CheW. Colocalization of CheZ with the CheA kinase implies some futile cycling of CheY but ensures a uniform concentration of CheYp across the length of the cell (15-17).

CheZ is unrelated by amino acid sequence to other known classes of protein phosphatases and has a unique catalytic mechanism based on the introduction of additional catalytic elements into the CheYp active site for CheYp autodephosphorylation activity. CheZ is a homodimer with two CheYp binding sites, each binding site being composed of two independent surfaces from different domains of CheZ [Fig. 1, (18)]. The dominant domain of CheZ is a long four-helix bundle composed of a helical hairpin from each monomer with additional angled N-terminal helices at the non-hairpin end of the bundle. The C-terminus of each hairpin extends with a 32-residue flexible linker followed by a final amphipathic C-terminal alpha helix (C-helix). Like all response regulators, CheY has a β5α5 fold with the phosphorylated aspartate located within a highly conserved active site adjacent to a bound Mg2+ ion (19), essential for catalysis of all phosphotransfer events (20). In one interaction surface between CheY and CheZ, the α4β5α5 surface of CheY binds to the hydrophobic face of the CheZ C-helix. In the other interaction, the active site region on CheY interacts with a surface near the center of the four-helix bundle of CheZ. Within the latter interaction surface, the essential catalytic residue Gln147 from CheZ inserts into the CheY active site and is positioned to interact with a water molecule for in-line nucleophilic attack of the phosphoryl group, the same mechanism that has been proposed for CheY autodephosphorylation (21).

Although the catalytic roles of the CheZ C-helix and the central region of the four-helix bundle have been elucidated, the function of the N-terminal helices and the non-hairpin region of the bundle is still not understood. However, there is some evidence that this region may be involved in regulation of CheZ activity. Many of the CheZ mutants that display a "gain of function" phenotype (Che', CCW flagellar rotation) (9,11) map to this region of CheZ, which is distal from the characterized catalytic surfaces. Several of the gain of function mutants display modest (2-5x) enhancements of CheZ activity when tested in a gel based assay monitoring the loss of radioactivity from [32P]CheYp (9,11). Analysis of pre-steady state time courses that monitor the CheZ-induced shift in the equilibrium between CheYp and CheY suggested that CheZ catalytic activity exhibits positive cooperativity with respect to CheYp concentration. In contrast, the gain of function mutant CheZ 54RC displays enhanced phosphatase activity at low CheYp concentrations, suggesting that the substitution resulted in a constitutively activated state (22). These observations suggest CheZ activity might be regulated in a manner that could potentially play an important role in control of CheYp levels in the cell.

Here, we further explore the fundamental kinetic properties of catalysis by CheZ to acquire a more quantitative understanding of CheZ activity and possible regulation. With a standard enzymological approach, we measured CheZ catalytic activity at steady state as a function of substrate (CheYp) concentration to assess the presence of regulation by CheYp and determine kinetic constants. To complement the enzymology, we characterized the kinetics of association and dissociation of the CheZ·CheYp complex using CheYp-dependent fluorescence changes in CheZ covalently labeled with the badan fluorophore. Results from measurements made on both wild type CheZ and the gain of function mutant CheZ 211T were combined to formulate a kinetic model for CheZ-mediated dephosphorylation that includes positive cooperativity with respect to CheYp. The model was used in computer simulations to predict CheZ activities at concentrations of CheZ and CheYp present in the cell.

**EXPERIMENTAL PROCEDURES**

*Site directed mutagenesis and protein purification* - The mutants cheZ 211T and cheZ 21IT 214FC were constructed using the Quikchange Mutagenesis Kit (Stratagene) by introduction of the codon for Thr21 into a pRS3 plasmid encoding either wild type CheZ or CheZ 214FC (8). Purification of CheZ (wild type, 211T, 214FC, or 211T 214FC) was carried out according
to published procedures (23) with 2 mM DTT present during purification of CheZ proteins containing the cysteine substitution at position 214. Unlike wild type CheZ and CheZ 21IT, both CheZ 214FC and CheZ 21IT 214FC exhibited detectable proteolysis of the C-terminal 42 residues (8) after the standard purification procedure. These proteins were therefore subjected to an additional high resolution ion exchange step (MonoQ, GE Biosciences), which separated full length from proteolyzed CheZ2 (24). CheY (wild type, 23ND, 59NR, and 113AP) was purified according to published procedures (25).

Kinetics of CheZ-catalyzed dephosphorylation of CheY- The rate of dephosphorylation of CheY in the presence or absence of CheZ was measured using the EnzChek P_i Assay (Invitrogen) adapted to a 96-well plate format from a previously described cuvette based assay (23). Wild type CheY was mixed with wild type CheZ (0, 50 nM, or 3.0 μM) in a total volume of 46 μL in buffer containing 50 mM Hepes, pH 7.0, 10 mM MgCl_2, and 1 mg/mL BSA in a flat bottomed polyethylene 96 well plate. At 30 s intervals, 4 μL of 50 mM monophosphoimidazole (MPI; synthesized according to (26)) was added and mixed by repetitive pipetting to give a final concentration of 4 mM MPI. After the designated reaction time (22-30 minutes at room temperature) the reactions were quenched with 150 μL of a solution containing 250 mM Tris, pH 7.5, 25 mM EDTA, 310 μM 2-amino-6-mercapto-7-methylpurine riboside (MESG) and 1.4 U/mL purine nucleoside phosphorylase (PNP). The high EDTA concentration stops both phosphorylation and dephosphorylation reactions and the assay kit components MESG and PNP convert the P_i into a product with a detectable absorbance. After 15 minutes incubation, the absorbances at 360 nm were read with a plate reader (Molecular Devices SpectraMax M2e). The absorbance values were converted into P_i release rates (μM P_i/s) using an empirically determined extinction coefficient derived from a standard P_i curve. All rates were corrected for the small amount of background P_i (introduced from the MPI preparations) deduced from controls lacking CheY. Preliminary experiments demonstrated that the rates were linear for at least one hour. For experiments comparing the activities of wild type CheZ and CheZ 21IT, CheY 113AP was used as the substrate due to its enhanced autophosphorylation rate (27). This was manifested in higher rates of P_i release in the presence of excess CheZ, which allowed a larger range of dephosphorylation rate measurements at low CheYp concentrations.

For a subset of the enzyme kinetic experiments, duplicates of each reaction condition were included and the absorbance values averaged. In these cases, the duplicates were in excellent agreement and use of the averaged values did not significantly affect the curve fit to the data relative to using single readings. Therefore, for most of the experiments, single reactions of each CheZ/CheY concentration condition were used in order to limit the number of samples so that the reactions could be quenched within thirty minutes. The results of multiple (3-4) independent experiments for each CheZ and CheY combination were averaged to get the final kinetic values. To measure time courses, reactions were scaled up in volume keeping all components at the same concentrations used in the single time point analysis. After initiation of the reaction by addition of monophosphoimidazole, 50 μL aliquots were removed at various times, placed in a new well of the microtiter plate, and assayed for P_i as described above.

For analysis of the dephosphorylation rate data, consideration was made of the following equilibrium:

\[
\text{CheY}_{\text{free}} \rightleftharpoons \text{CheYp} \quad (\text{Eqn. 1})
\]

where \( k_{\text{phosph}} \) is the pseudo-first order rate constant for phosphorylation at 4 mM MPI and \( k_{\text{dephosph}} \) is the first order rate constant for dephosphorylation. First, \( k_{\text{phosph}} \) was determined using the slope of the linear relationship between the rate of P_i release and CheY concentration in the presence of excess CheZ (3 μM). Under these conditions, dephosphorylation occurs rapidly, so that CheY is mostly unphosphorylated (CheY_{free}) and the measured rate of P_i release \( = k_{\text{phosph}} [\text{CheY}_{\text{total}}] \) (i.e. phosphorylation is rate limiting). Next, the concentration of CheYp present at steady state for each sample was calculated using the following relationships, derived from equation 1:
\[ \text{rate of } P_i \text{ release} = \frac{[\text{CheY}_\text{free}] \times k_{\text{phosph}}}{[\text{CheY}_p] \times k_{\text{dephosph}}} \]  
(Eqn. 2)

Solving for \([\text{CheY}_\text{free}]\),
\[ [\text{CheY}_\text{free}] = \frac{\text{rate of } P_i \text{ release}}{k_{\text{phosph}}} \]  
(Eqn. 3)

and,
\[ [\text{CheY}_p] = [\text{CheY}_{\text{total}}] - [\text{CheY}_\text{free}] \]  
(Eqn. 4)

Finally, the rate of \(P_i\) release due only to CheZ was determined for each data point by subtracting the amount of \(P_i\) release expected in the absence of CheZ (derived from the linear relationship between \(P_i\) release and CheYp concentration in absence of CheZ) from the \(P_i\) release rate in the presence of CheZ.

The data were fit using nonlinear regression (Prism software) directly to the Hill equation,
\[ v = \frac{V_{\text{max}} [\text{CheY}_p]^n}{(K_{0.5}^n + [\text{CheY}_p]^n)} \]  
(Eqn. 5)

with \(V_{\text{max}}, K_{0.5}\) (the concentration of CheYp necessary for half maximal velocity) and \(n\) (the Hill coefficient) as floating variables.

**Labeling CheZ with the badan fluorophore**

Fractions from the Mono Q salt gradient elution that contained only full length CheZ 214FC or CheZ 21IT 214FC were pooled and immediately supplemented with 1 mM TCEP [(tris-2-carboxyethyl) phosphine hydrochloride; Pierce] to keep Cys214 reduced. The badan (6-bromoacetyl-2-dimethylaminonaphthalene; Invitrogen) was prepared fresh as a 25 mM stock solution in dimethylformamide. The badan solution was added to the CheZ (100 \(\mu\)M) in five equal aliquots at five minute intervals while stirring to give a final concentration of 1 mM badan. The reaction was placed in the dark for 2 hours at room temperature, quenched by the addition of 10 mM DTT, and clarified by centrifugation to remove a small amount of insoluble matter generated during the reaction. The clarified sample was then chromatographed on G-75 Sephadex in 50 mM Tris, pH 7.5, 0.5 mM EDTA, 10% (v/v) glycerol, 2 mM DTT to separate the badan-labeled CheZ from unreacted badan. The protein concentration of badan-labeled CheZ (*B-CheZ or *B-CheZ 211T) was determined using a Bradford assay (Biorad Protein Assay Kit) with unlabeled CheZ as a standard. The concentration of badan in the labeled protein was determined by the absorbance at 392 nm (\(\varepsilon = 12.9 \text{ mM}^{-1} \text{cm}^{-1}\)) (28). Labeling ratios were 1.2 mol/mol and 0.7 mol/mol for *B-CheZ and *B-CheZ 211T, respectively.

**Fluorescence measurements**

Steady state fluorescence measurements of *B-CheZ or *B-CheZ 21IT were made on a Perkin Elmer LS-50B spectrophotometer with a circulating water bath to maintain cuvette temperature at 20 °C. For emission scans, the excitation wavelength was 392 nm and the emission was scanned from 400-600 nm. For time courses and equilibrium titrations, the excitation wavelength was 392 nm and the emission wavelength was 500 nm for *B-CheZ and 470 nm for *B-CheZ 211T. Excitation and emission slit widths were adjusted to optimize the signal/noise ratio. For equilibrium titration of *B-CheZ or *B-CheZ 21IT with CheYBeF3 or CheY 23NDBeF3, badan-CheZ was diluted to 0.1 \(\mu\)M in buffer containing 50 mM Hepes, pH 7.0, 10 mM MgCl2, 10 mM NaF, and 0.1 mM BeCl2. Repetitive additions of small volumes of CheY were made and the fluorescence monitored continuously. Subsequent additions were made only after the fluorescence had stabilized as a result of the previous addition. The final intensities were corrected for the dilution due to the addition of CheY. For measurement of emission spectra and equilibrium titrations, a 1 x 1 cm cuvette was used (sample volume 1.5 mL) with magnetic stirring. The cuvette chamber was left open during experiments to eliminate the sensitivity of the fluorescence signal to opening and closing of the cuvette chamber.

*B-CheZ·CheYBeF3 dissociation time courses were monitored at 20 °C using a rapid mixing accessory (Applied Photophysics RX-2000) in conjunction with the Perkin Elmer LS-50B spectrofluorimeter. In these experiments, one syringe of the rapid mixer contained *B-CheZ (1.0 \(\mu\)M) and CheY (2.0 \(\mu\)M) in buffer containing 50 mM Hepes, pH 7.0, 10 mM MgCl2, 0.10 mM BeCl2, and 10 mM NaF. The other syringe contained unlabeled CheZ (20 \(\mu\)M; either wild type or CheZ 211T to match the CheZ used in the complex) in the same buffer. Mixture of the two solutions results in recovery of the fluorescence of
uncomplexed *B-CheZ at a rate that reflects dissociation of CheYBeF$_3$ from the badan-CheZ-CheYBeF$_3$ complex. For each CheZ, six to eight repetitive time courses were recorded with data points taken every 100 ms. Data from each time course were fit to an equation describing a single exponential decay using nonlinear regression (Prism) and the first order rate constants of the repetitive shots were averaged. The resultant rate constants from two to three independent experiments for both wild type CheZ and CheZ 21IT were then averaged.

To measure the kinetics of association of *B-CheZ or *B-CheZ 21IT with phosphorylated CheY 59NR, a SPEX Fluorolog-3 spectrofluorimeter equipped with a Jobin Yvon Horiba F-3009 Microflow stop flow device (dead time ~ 5 ms) was used. For these experiments, one syringe contained 0.4 μM badan-CheZ (*B-CheZ or *B-CheZ21IT) in reaction buffer (50 mM Hepes, pH 7.0, 10 mM MgCl$_2$) and the other syringe contained CheY 59NR (2-60 μM) in reaction buffer supplemented with 20 mM phosphoramidate (synthesized according to (29)). The experiments were carried out at room temperature (22-23 °C) or at a constant temperature of 23 °C using a circulating thermostatted water bath. For each CheY concentration, 4-13 time courses were recorded by repetitive shots and data were recorded at 10 ms intervals for *B-CheZ reactions and 2 ms intervals for *B-CheZ 21IT reactions. The data (the first 300 ms for *B-CheZ and 60 ms for *B-CheZ 21IT) were fit to a single exponential decay using nonlinear regression (Prism). CheY 59NRp binds efficiently to CheZ but is resistant to its phosphatase activity (23) and was used in these experiments to avoid the toxicity of beryllium. Computer simulations- Rate equations for the reaction scheme presented in the Discussion were derived and encoded in a C++ program employing Euler's method as the numerical integrator. Each simulation was run for 60 s of simulated time to reach steady state. Inputs to the program are the amounts of unphosphorylated CheY and CheZ and a set of rate constants for the reactions; outputs of the program are the concentration of CheYp and the rate of phosphate release at time 60 s, given an external source of phosphoryl groups in excess. The simulations were performed on a PowerPC G4 running Mac OS X version 10.4.9.

**RESULTS**

Steady state kinetic analysis of dephosphorylation of CheYp mediated by wild type CheZ indicates positive cooperativity- The rate of P$_i$ release in reactions containing various amounts of CheY (0 to 5 μM), 4 mM monophosphoimidazole, and either 0, 50 nM or 3 μM CheZ were measured and plotted as a function of total CheY concentration (Fig. 2A). Rates of P$_i$ release in the absence of CheZ reflect CheY autodephosphorylation and rates in the presence of 3 μM (excess) CheZ represent the fastest rate of P$_i$ release possible at each CheY concentration, i.e. the rate of CheY autophosphorylation. Analysis of the raw data as described in Experimental Procedures first involved calculation of the concentration of CheYp present under each steady state reaction condition (Fig. 2B), which finally led to the relationship between CheZ activity and CheYp concentration (Fig. 2C). This relationship displayed marked sigmoidicity, suggesting the presence of positive cooperativity. Fitting the data from three independent experiments to the Hill equation gave a Hill coefficient of 2.1 ± 0.3 (see Figure 2C for sample curve fit), a K$_{0.5}$ value of 0.8 ± 0.1 μM and a k$_{cat}$ of 4.9 ± 0.1 s$^{-1}$. Several control experiments were performed to ensure that the rates measured at low CheYp were not an artifact of the experimental process due to the small magnitudes of P$_i$ release rates in the presence and absence of CheZ. First, time courses of P$_i$ release were carried out for reactions containing 0.2, 0.4 and 0.6 μM CheY (±50 nM CheZ) for 80 minutes rather than the standard 30 minutes. The time courses were linear over the entire time course, eliminating the possibility that a lag phase contributed to low activity. Furthermore, the longer time points (with larger differences in absorbance in the presence and absence of CheZ) gave the same specific activity as those measured in the single time point experiment, thus excluding low absorbances as a source of error. Second, the concentration of bovine serum albumin in the reaction buffer was varied between 0, 0.10, 0.50, and 1 mg/mL with no change in results, implying that the low
concentrations of CheY present in these reactions were not adsorbed on to the surface of the microtiter plate.

**Comparative kinetic analysis for gain of function mutant CheZ 21IT indicates loss of cooperativity.** Cells containing CheZ 21IT are incapable of chemotaxis due to constitutively counterclockwise flagellar rotation, consistent with an increase in CheZ phosphatase activity (30). Ile21 is located on the N-helix, close to the bottom of the four helix bundle and more than 40 Å from the active site (Fig. 1). Preliminary experiments for kinetic analysis of CheZ 21IT showed that at 50 nM CheZ 21IT (the same concentration of CheZ used in the wild type CheZ experiment), the rate of Pi release at low CheY concentrations (0.1 - 0.4 μM) was the same as that for 3 μM (excess) CheZ while V_max was approximately unchanged (data not shown). Therefore, at low CheY concentrations, CheZ 21IT was dephosphorylating CheY as fast as it was being phosphorylated, precluding determination of accurate rates. Therefore, for analysis of CheZ 21IT, we used the CheY 113AP mutant whose autophosphorylation is six fold faster but has similar CheZ sensitivity as wild type CheY (27). These features should give higher rates of Pi release in the presence of excess CheZ and therefore a larger window in which to assess activity due to limiting amounts of CheZ. As predicted, the data demonstrated a slope for P_i release from CheY 113APp (Fig. 2D) as observed with wild type CheYp, with only modest changes in other kinetic parameters. Kinetic parameters derived from three independent measurements gave a Hill coefficient of 2.2 ± 0.2, K_0.5 of 0.3 ± 0.1 μM and k_cat of 3.5 ± 0.3 s^{-1}. In contrast, CheZ 21IT displayed apparently hyperbolic kinetics with enhanced activity relative to wild type CheZ at low CheY concentrations but had a similar k_cat as wild type CheZ (Fig. 2D). The kinetic parameters for CheZ 21IT substitution is to eliminate the positive cooperativity observed with wild type CheZ while enhancing activity at low CheYp concentrations. This suggests that the positive cooperativity displayed by wild type CheZ depends on structural features present at the nonhairpin end of the four helix bundle (Fig. 1).

**Monitoring CheZ/CheY complex formation using badan-CheZ.** Previous studies exploited two CheY derivatives to monitor the interaction of CheZ and CheYp without the complication of catalytic removal of the phosphoryl group. CheY 59NRp binds to CheZ but is resistant to its phosphatase activity due to a salt bridge between CheY Arg59 and CheY Glu89, which is essential for CheZ activity (23,31). CheYBeF_3 is a stable derivative of CheYp that has proven to be an excellent structural and functional analogue of CheYp (18,19). CheZ binds both CheY 59NRp and CheYBeF_3 with sufficiently high affinity that there is essentially quantitative complex formation when both proteins are present at 200 nM, indicating a submicromolar K_d value (23).

In order to further probe the interaction between CheZ and CheYp for the purposes of evaluating how this high affinity affects the overall kinetic mechanism of CheZ catalysis, we sought a method to directly monitor time courses for both association and dissociation of the CheZ-CheYp complex. Badan is a fluorophore whose emission is highly sensitive to its local environment and was thus a candidate for use as a probe to monitor the CheZ/CheYp interaction. Position 214 of CheZ (phenylalanine in wild type CheZ) is the C-terminal residue of the C-helix and is adjacent to the α_4β_5α_5 binding surface of CheYBeF_3 in the CheZ-CheYBeF_3 structure (Fig. 1) (18,32). The emission spectra of both *B-CheZ and *B-CheZ 21IT (λ_ex = 392 nm) underwent substantial degrees of quench (~40-50% of maximal intensity) upon binding CheYBeF_3 (Fig. 3A), indicating that these labeled CheZs could serve as useful probes for monitoring CheZ/CheYp binding events. However, while the fluorescence emission spectra of the complexed *B-CheZ and *B-CheZ 21IT were very similar, there was a substantial difference in the emission spectra of the derivatized CheZs in their uncomplexed forms. The emission spectrum of *B-CheZ (λ_max = 500 nM) was red shifted more...
than 30 nm relative to *B-CheZ 21IT (λ_max = 470 nM). This suggests that the fluorophores, located on the C-helix of CheZ, experience different environments in the wild type versus the gain of function CheZ 21IT. Emission spectra of *B-CheZ complexed to CheY 59NRp or wild type CheYp (using phosphoramidate as a phosphodonor) were very similar to the CheZ-CheYBeF_3 spectrum (data not shown). Control experiments that varied the order of addition of components demonstrated that the emission spectrum characteristic of complex formation (Fig. 3A) depended on the presence of all components: CheZ, CheY, Mg^{2+}, and either BeF_3 or phosphoramidate. *B-CheZ displayed catalytic properties similar to wild type CheZ (Fig. 2) when its activity was measured as a function of wild type CheYp (data not shown). The relationship was sigmoidal with K_0.5 ~1.4 μM and k_cat ~ 3 s^{-1} (cf Fig. 2C).

Equilibrium titrations of the fluorescence of *B-CheZ with CheYBeF_3 (Fig. 3B) demonstrated the expected high affinity concluded from other studies. At 0.1 μM *B-CheZ, approximately equimolar amounts of CheYBeF_3 were required to saturate the change in fluorescence, indicating that nearly all of the added protein was bound. A similar titration using *B-CheZ 21IT also displayed essentially quantitative binding (Fig. 3B). Similar fluorescence results were obtained when badan was covalently attached to Cys210 in the CheZ derivative 210DC indicating that neither the phenylalanine to cysteine substitution nor the badan derivatization at position 214 specifically disrupted an essential CheZ/CheYp interaction.

Association kinetics of B-CheZ and CheYp-Time courses monitoring the association of *B-CheZ or *B-CheZ 21IT with CheY 59NRp were recorded under pseudo-first order kinetic conditions using stopped flow fluorescence spectroscopy. For both CheZs, the time courses fit well (average R^2 = 0.98 for *B-CheZ and 0.96 for *B-CheZ 21IT) to equations describing a single exponential decay (see Fig. 4A for sample time courses and curve fits). Plots of the pseudo-first order rate constants (k_assoc) versus CheY 59NRp concentration were linear (Fig. 4B), indicating a rate limiting step that was a bimolecular process. The slopes of the lines gave the bimolecular rate constants. The value for *B-CheZ (5.64 ± 0.02 x 10^6 M^{-1}s^{-1}; average and standard deviation of two independent measurements) is well within the window typically observed for protein/protein association events (33). Strikingly, the k_assoc value for *B-CheZ21IT was six-fold faster (3.36 ± 0.00 x 10^7 M^{-1}s^{-1}). This enhanced k_assoc value likely contributes to the shift of the kinetic profile of CheZ 21IT to lower CheYp concentrations relative to wild type CheZ (Fig. 2D) and may also explain the loss of cooperativity (see Discussion). The observation that the pseudo-first order rate constant was proportional to CheYp concentration indicated that the rate limiting step in association of wild type CheZ and CheZ 21IT with CheYp is the association of the first binding surface; association of the second surface must ensue rapidly. This experiment does not distinguish the order of binding events for CheYp to the two binding surfaces of CheZ.

Dissociation kinetics of the CheZ-CheYBeF_3 complex- While continuously monitoring fluorescence, addition of 0.1 μM CheYBeF_3 to 0.1 μM *B-CheZ or 0.1 μM *B-CheZ 21IT resulted in a time dependent quench due to formation of the CheZ-CheYBeF_3 complex. After the change in fluorescence had stabilized, addition of a 20-fold excess of unlabeled CheZ resulted in a time dependent increase in fluorescence until the initial fluorescence had been recovered (data not shown). Under these conditions, the CheYBeF_3 that dissociates from the complex will nearly always reassociate with unlabeled CheZ so that the rate of recovery of the fluorescence of unbound *B-CheZ reflects the rate of dissociation of the CheZ-CheYBeF_3 complex. To rigorously measure the rate of dissociation, the CheZ-CheYBeF_3 complex was mixed with excess CheZ using a rapid mixing apparatus and the time courses were recorded. The time courses for dissociation fit well to single exponential decays (R^2 > 0.99, Fig. 5) and the deduced k_diss values from five to six successive shots through the apparatus were averaged for each experiment. The k_diss values (average of two or three independent experiments) were 0.040 ± 0.004 s^{-1} for the wild type CheZ-CheYBeF_3 complex and 0.023 ± 0.001 s^{-1} for the CheZ 21IT-CheYBeF_3 complex. Taking the measured k_assoc and k_diss values for calculation of K_d = k_diss/k_assoc gives K_d values of 7.1 nM and 0.68 nM for binding of CheYBeF_3 to wild type CheZ and CheZ 21IT, respectively. The analogous experiment using CheY 59NRp and *B-CheZ gave
a dissociation rate constant of 0.13 s\(^{-1}\) (data not shown). The increase in rate relative to the CheYBeF\(_3\) experiment likely reflects the fact that autodephosphorylation (k \(\sim 0.04\) s\(^{-1}\)) (34) and/or modest CheZ-mediated dephosphorylation is likely followed by rapid dissociation of the CheZ-CheY 59NRp complex.

Equilibrium binding of \(*B\)-CheZ and \(*B\)-CheZ 21IT to CheY 23ND- CheY Asn23 is located on the \(\alpha_1\) helix and interacts with the CheZ four-helix bundle. Accordingly, CheY 23ND is partially resistant to CheZ phosphatase activity due to diminished binding to CheZ (35). Interestingly, when CheZ phenotypic suppressors of CheY 23ND were originally isolated, many were also gain of function mutants, i.e. they conferred a Che- phenotype due to counterclockwise flagellar rotation for strains containing wild type CheY. CheZ 21IT was isolated independently from screens for gain of function mutants (9) and for CheY 23ND suppressors (30). We measured equilibrium binding of CheYBeF\(_3\) or CheY 23NDBeF\(_3\) to \(*B\)-CheZ or \(*B\)-CheZ 21IT (Fig. 6). At 0.1 \(\mu\)M \(*B\)-CheZ, addition of sub-stoichiometric quantities of CheY 23NDBeF\(_3\) resulted in modest fluorescence changes. In contrast, the response of \(*B\)-CheZ 21IT to the same titration was of greater magnitude (Fig. 6, inset). Plotting the degree of fluorescence changes as a function of CheY 23NDBeF\(_3\) concentration showed that for \(*B\)-CheZ, the binding was decreased from the quantitative binding of wild type CheYBeF\(_3\) under the same conditions. However, for CheZ 21IT the binding was essentially quantitative. Therefore, CheZ 21IT compensates for the defective binding of CheY 23ND, presumably through the enhanced association rate demonstrated above. This result provides an explanation for why the same CheZ mutants were identified in both genetic screens.

**DISCUSSION**

**Fundamental enzymatic properties of the CheZ phosphatase**- Here we determined several fundamental kinetic parameters that describe CheZ-catalyzed dephosphorylation of its physiological substrate, CheYp. Measurement of enzyme activity as a function of substrate concentration revealed strong positive cooperativity with respect to CheYp. Independent measurement of the association and dissociation rates of CheZ and CheYp implicated a dissociation constant of 7.1 nM, markedly lower than intracellular concentrations of either protein (36). However, because \(k_{\text{diss}}(0.040\) s\(^{-1}\)) << \(k_{\text{cat}}(4.9\) s\(^{-1}\), CheZ and CheYp are not expected to be in binding equilibrium during catalysis and nearly every binding event will result in dephosphorylation of CheY.

Regulation of CheZ activity by CheYp: positive cooperativity- The observation here of sigmoidicity—an indicator of positive cooperativity—in the relationship between steady state CheZ activity and CheYp concentration is in agreement with an earlier study that used a pre-steady state kinetic approach (22). This finding implicates a mechanism by which the cell could temper CheZ phosphatase activity when intracellular CheYp concentrations are low or, conversely, maximize activity when CheYp levels are high. Computer simulations have predicted that such CheYp-induced activation of CheZ would increase the robustness of the chemotaxis system towards cell-to-cell variation in chemotaxis proteins (37,38) and implicate a role for CheZ in adaptation, the ability of the cell to return CheYp levels toward prestimulus levels after the stimulus is removed (39).

Here, we further demonstrated that the kinetics of the gain of function mutant CheZ 21IT did not display positive cooperativity and had markedly enhanced phosphatase activity, but only at low CheYp concentrations (subsaturating for wild type CheZ). Cells containing CheZ 21IT cannot mediate chemotaxis because of constitutively counterclockwise flagellar rotation and consequent inability to tumble (30), a result of low CheYp. Thus, it appears that for successful chemotaxis, there is an absolute requirement for reduction of CheZ phosphatase activity at low CheYp levels. The independent observation here that CheZ 21IT has an enhanced association rate with CheYp relative to wild type CheZ provides a plausible explanation for the higher activity at low CheYp. The faster association would result in higher occupancy of CheZ 21IT with CheYp relative to wild type CheZ when CheYp is limiting. Furthermore, the absence of positive cooperativity for CheZ 21IT implicates a role for the non-hairpin end of the CheZ four helix bundle in regulation of CheZ catalytic activity.
**Possible kinetic basis for positive cooperativity:** Positive cooperativity is most often a result of communication between active sites within an oligomeric enzyme (40). The Hill coefficient of ~2 measured here is consistent with communication between the two active sites within the CheZ dimer. We used mathematical modeling to assess the compatibility of our kinetic data with a model whereby the second CheYp binds CheZ with higher affinity than the first. The simulation included rate equations for each reaction in the scheme,

![Diagram of reaction scheme]

and the simulation target was the uncorrected raw kinetic data (as in Figure 2A), to avoid potential error introduced by estimation of CheYp concentrations. Starting with a model of noninteracting CheZ active sites and using measured rate constants, the simulation predicted enzymatic activity for 50 nM CheZ that changed over a similar range of CheYp concentrations as measured experimentally (Fig. 7). However, as expected, this model did not predict sigmoidicity, but instead predicted rates that were consistently higher than the experimental rates at low CheYp (Fig. 7, inset). The output of the simulation proved to be quite sensitive to modest changes in $k_{on}$ (with the curves shifting to the lower CheYp as $k_{on}$ increased) but was relatively insensitive to changes in $k_{off}$ (data not shown). In contrast to the noninteracting sites model, a reasonable fit to the experimental kinetic data was achieved with $k_{on1} < k_{on2}$ (Fig. 7). The $k_{on1}$ and $k_{on2}$ values used in the simulation ($9.2 \times 10^{5} \text{ M}^{-1}\text{s}^{-1}$ and $3.4 \times 10^{7} \text{ M}^{-1}\text{s}^{-1}$, respectively) (Fig. 7) are consistent with the measured wild type CheZ/CheYp association rate ($5.6 \times 10^{6} \text{ M}^{-1}\text{s}^{-1}$) because the measured rate reflects fluorescence quenching from association of both the first and second CheYp molecules and would be expected to be intermediate in magnitude between $k_{on1}$ and $k_{on2}$. Furthermore, the measured $k_{ass}$ for CheZ21IT, when used as a value for $k_{on2}$ (Fig. 7) gave a reasonable fit to the experimental data, consistent with the possibility that the CheZ21IT dimer may associate with both CheYp molecules at a similar rate as the second CheYp for wild type CheZ. A model with $k_{on1} < k_{on2}$ implies that $k_{on1}$ is somehow suppressed for wild type CheZ. This suppression may be relieved by binding the first CheYp for wild type CheZ or by the CheZ 21IT mutant for binding both CheYp molecules.

The model described above accounts for the initially paradoxical observation of positive cooperativity for an enzyme that binds extremely tightly to its substrate. In this situation, the occupancy of the enzyme with substrate is a function of $k_{ass}$ and $k_{cat}$ (not $K_m$ and $k_{cat}$) and with the stipulation that $k_{cat2} > k_{cat1}$, predicts sigmoidicity in active site occupancy. However, we have not ruled out the possibility that the positive cooperativity observed for CheZ is due to a higher $k_{cat}$ value (per site) when both active sites are occupied relative to when just one active site is occupied (40,41).

**Mechanism of CheZ/CheYp association and structural basis for enhanced $k_{ass}$ for CheZ 21IT:**

With two independent surfaces of interaction between CheZ and CheYp, association of the two proteins must occur in two sequential steps. Direct binding between CheYp and the isolated four-helix bundle was not detected in two independent studies (18,42) whereas CheYp binds to the isolated C-helix with a $K_d$ of 26 μM (43), making it probable that binding occurs first to the C-helix. The association kinetics between CheZ and CheYp demonstrated that the rate limiting step for both wild type CheZ and CheZ 21IT involves the initial association with CheYp. The CheZ 21IT substitution may therefore affect the function of the linker/C-helix region with the result of enhancing the ability of the C-helix to bind CheYp. Ile 21 is proximal to the site where the linker emanates from the four-helix bundle (Fig. 1), so a substitution at that position could affect the position and/or motion of the linker, which has a high degree of mobility independent of the four-helix bundle when CheYp is not bound (24). The observation that the emission of the badan fluorophore linked to the C-helix of CheZ 21IT was blue-shifted 30 nm relative to wild type CheZ (Fig. 3) is consistent with a direct effect of the CheZ 21IT substitution on the linker/C-helix. The
fluorescence emission maximum of badan shifts to higher wavelengths as the polarity of its environment increases (44), suggesting that CheZ 21IT puts the C-helix in a more hydrophobic environment than in the wild type derivative, possibly indicative of an additional interaction. The enzyme kinetic features of CheZ 21IT were qualitatively similar to another gain of function mutant, CheZ 54RC (22) which also displays enhanced activity at low CheYp concentrations. Therefore, CheZ 21IT, CheZ 54RC and, perhaps, other gain of function mutants may share an enhanced rate of association with CheYp. The clustering of these substitutions on the non-hairpin end of the four-helix bundle may implicate this region in regulation of the rate of CheYp association.

Prediction of CheZ occupancy with CheYp-
Our enzyme activity measurements were made at a CheZ concentration (50 nM) that is significantly lower than that found in the cell, which ranges from 2-20 μM CheZ monomer and 5-50 μM CheY, depending on cell type and growth conditions (12,36). Local concentrations of CheZ and CheYp are likely higher at the cell pole where the CheA kinase and much of the CheZ is localized. Simulations using the optimized kinetic constants (Fig. 7) predicted that the K₀.₅ value (the concentration of CheYp which gives half the maximal velocity) increases markedly with CheZ concentration (Fig. 8). Whereas the intracellular CheZ monomer:CheY ratio appears to be fairly constant at ~1:2.4 (36), the ratio of CheZ to total CheYp is less certain but computer simulations have predicted ratios ranging from ~1:1 to 2:1 (CheZ chain: CheY) during chemotaxis, depending on the stimulation state of the cell (38). Taking this estimation with the predictions of CheZ occupancy shown in Fig. 8, implies that CheZ would be subsaturated with CheYp under much of the window of CheYp concentrations expected during chemotaxis. This conclusion is consistent with the successful application of whole cell FRET between fluorescently labeled CheZ and CheYp as a monitor of intracellular CheYp concentrations (15,45). The simulation further predicts that the pool of CheYp bound to CheZ may be a significant fraction of total intracellular CheYp.

REFERENCES


FOOTNOTES

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Abbreviations: CheZ 21IT, isoleucine at position 21 changed to a threonine (this notation is used throughout the manuscript); *B-CheZ, CheZ 214FC with the badan fluorophore covalently linked to cysteine 214; *B-CheZ 21IT, CheZ 21IT 214FC with the badan fluorophore linked to Cys214; MPI, monophosphoimidazole; CheYBeF3, CheY with bound BeF3− and Mg2+ ions. K0.5 is the concentration of the CheYp substrate necessary for half maximal reaction velocity.

FIGURE LEGENDS

Fig. 1. The CheZ2(CheY·BeF3·Mg2+)2 ribbon structure (PDB 1KMI) (18) with features pertinent to this study marked. The two identical chains comprising CheZ2 are magenta and cyan, and CheY is gray. The ovals encase the two identical CheZ active site regions with essential catalytic residue Gln147 of each CheZ monomer shown in stick form in the color of its parent chain, BeF3− in red, and Mg2+ in yellow. The bottom circles encase CheZ Ile21 in the color of the parent chain and CheY Asn23 is orange. The orange star marks the location of the badan fluorophore (CheZ C-terminal residue 214); an identical site located on the CheZ C-terminus of the other CheZ chain (cyan) is not visible in this view of the complex. The 32 residue disordered linkers that were not visible in the crystal structure are sketched in as dotted lines. Because the co-crystal structure did not reveal the connectivity between the C-helix and the rest of the CheZ monomer, the designation of either C-helix as cyan or magenta is not certain.

Fig. 2. Steady state dephosphorylation kinetics of CheZ. (A) Representative raw data with absorbances at 360 nM converted to P1 release rates for reactions carried out in the absence of CheZ (open circles), in the presence of 50 nM CheZ (closed squares), or 3 μM CheZ (closed triangles). (B) The data in panel A were corrected for the concentration of CheYp present at steady state under each reaction condition and replotted. Reactions carried out in the absence of CheZ (open circles) or presence of 50 nM CheZ (closed squares). The line represents the best fit to the rates in the absence of CheZ. (C) Final analysis represents the subtraction of the P1 release expected in the absence of CheZ (determined from the equation of the line shown in Panel B) from each rate measured in the presence of 50 nM CheZ. The results are shown for two independent trials. Trial 1 (closed squares) is the data shown in panels A and B. Trial 2 (open squares) is data from an independent experiment. The curve shown is the best fit to the Hill equation for the Trial 1 using nonlinear regression. Note different x-axis scales in panels A-C. (D) Final data analysis for representative experiment measuring P1 release kinetics of 50 nM wild type CheZ (closed squares) or 50 nM CheZ 21IT (closed triangles) with CheY 113APp as substrate. The curves represent the best fit to the Hill equation using nonlinear regression.

Fig. 3. Sensitivity of the fluorescence intensity of the badan fluorophore attached to the C-terminus of CheZ to complex formation with CheYBeF3. (A) Fluorescence emission spectra of badan-labeled CheZ in the presence and absence of CheYBeF3. The excitation wavelength was 392 nm. *B-CheZ (solid black line), *B-CheZ-CheYBeF3 (dashed black line), *B-CheZ 21IT (solid gray line), *B-CheZ 21IT-CheYBeF3 (dashed gray line). Spectra of uncomplexed *B-CheZ and *B-CheZ 21IT were normalized independently to 100% and spectra of their respective complexed forms were plotted relative to the uncomplexed form of the same CheZ. (B) Equilibrium titration of the fluorescence intensity of 0.1 μM badan-CheZ with CheY. *B-CheZ titrated with CheY in the presence (closed squares) and absence (open squares) of BeF3−. *B-CheZ 21IT titrated with CheY in the presence (closed triangles) and absence (open triangles) of BeF3−.
Fig. 4. Association kinetics of badan-CheZ and CheY 59NRp measured by stopped flow fluorescence. (A) Representative time courses (left) and curve fits (right) for association of *B-CheZ (top) and *B-CheZ 21IT (bottom) with CheY 59NRp. Note the different time scales. The concentrations of CheZ and CheY 59NRp were 0.2 μM and 4 μM, respectively. (B) Representative plots of k_{obs} versus the CheY 59NRp concentration for *B-CheZ (closed squares) and *B-CheZ 21IT (closed triangles). The lines represent linear regression fits of the data and the slopes are equal to the bimolecular rate constants for association (k_{ass}). Error bars represent the standard deviation from the mean of multiple sequential reaction time courses (n= 4-11) and are smaller than the size of the data point when not visible.

Fig. 5. Kinetics of dissociation of the badan-CheZ·CheYBeF₃ complex. Representative fluorescence time courses reflecting dissociation of (A) *B-CheZ·CheYBeF₃ and (B) *B-CheZ 21IT·CheYBeF₃ (note different time scales). For both panels, the data points are in gray and the curve fit for a single exponential process is shown as a dashed line. The insets are traces of the raw fluorescence data.

Fig. 6. Equilibrium titrations of *B-CheZ and *B-CheZ 21IT with CheY 23NDBeF₃. Fluorescence changes upon addition of incremental amounts of CheY 23NDBeF₃ to 0.1 μM *B-CheZ (squares) or *B-CheZ 21IT (diamonds) measured at equilibrium. The inset shows the raw data for the first several additions. Top: *B-CheZ with additions of 0.060 μM CheY 23NDBeF₃ at the times designated with a solid arrow. Bottom: *B-CheZ 21IT with additions of 0.020 μM CheY 23NDBeF₃ at times designated with an open arrow.

Fig. 7. Simulations of CheZ-catalyzed dephosphorylation of CheY. The computer program described in Experimental Procedures was used to simulate the raw kinetic data in Fig. 2A. For each CheZ concentration, simulations were performed to determine the total (auto- and CheZ-catalyzed) phosphate-release rate for initial CheY concentrations between 0 and 6 μM in increments of 0.1 μM. The experimental data are shown as symbols: excess CheZ (closed triangles); 50 nM CheZ (open diamonds); and no CheZ (open circles). The simulation outputs are shown as lines: excess CheZ (gray solid line); no CheZ (gray dashed line); non-cooperative simulation for 50 nM CheZ with k_{on1} = k_{on2} = 5.6 x 10^6 M⁻¹ s⁻¹ (black dashed line), cooperative simulation for 50 nM CheZ with k_{on1} = 9.2 x 10^5 M⁻¹ s⁻¹ and k_{on2} = 3.4 x 10^7 M⁻¹ s⁻¹ (black solid line). For all simulations, k_{cat1} = k_{cat2} = 4.9 s⁻¹, k_{off1} = k_{off2} = 0.040 s⁻¹, k_{p} = 0.17 s⁻¹, and k_{p} = 0.030 s⁻¹. Inset: expanded view of data for 50 nM CheZ at low CheY concentrations.

Fig. 8. Computer predictions of CheZ occupancy. For each CheZ concentration, simulations were performed to determine solely the CheZ-catalyzed phosphate-release rate as a function of total CheYp concentration (the total of free CheYp and the amount bound to CheZ) for initial CheY concentrations from 0 μM in increments of 1 μM (0.1 μM for 50 nM CheZ). Solid black line, 50 nM CheZ; dashed black line, 1 μM CheZ; solid gray line, 4 μM CheZ, and dashed gray line, 10 μM CheZ. The simulation for 50 nM CheZ gave a Hill coefficient of 1.7.
Figure 1
Figure 2

A. P release rate (µM/s)

B. P release rate (µM/s)

C. CheZ-dependent P release rate (µM/s)

D. P release rate (µM/s)
Figure 3

A

Normalized fluorescence (% uncomplexed intensity)

Wavelength (nm)

B

Fluorescence intensity

[CheY] (μM)
Figure 5
Figure 6
Figure 7
Figure 8