Dimerization Is Required for the Activity of the Protein Histidine Kinase CheA That Mediates Signal Transduction in Bacterial Chemotaxis*

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The histidine protein kinase CheA plays an essential role in stimulus-response coupling during bacterial chemotaxis. The kinase is a homodimer that catalyzes the reversible transfer of a γ-phosphoryl group from ATP to the N-3 position of one of its own histidine residues. Kinetic studies of rates of autophosphorylation show a second order dependence on CheA concentrations at submicromolar levels that is consistent with dissociation of the homodimer into inactive monomers. The dissociation was confirmed by chemical cross-linking studies. The dissociation constant (CheA2 ↔ 2CheA; KD = 0.2–0.4 μM) was not affected by nucleotide binding, histidine phosphorylation, or binding of the response regulator, CheY. The turnover number per active site within a dimer (assuming 2 independent sites/dimer) at saturating ATP was approximately 10/min. The kinetics of autophosphorylation and ATP/ADP exchange indicated that the dissociation constants of ATP and ADP bound to CheA were similar (KD values ~ 0.2–0.3 mM), whereas ATP had a reduced affinity for CheA-P (KD ~ 0.8 mM) compared with ADP (KD = ~ 0.3 mM). The rates of phosphotransfer from bound ATP to the phosphoaccepting histidine and from the phosphohistidine back to ADP seem to be essentially equal (kcat = ~ 10 min⁻¹).

The histidine protein kinase CheA is a central component of the system that mediates receptor signaling in bacterial chemotaxis (for reviews see Refs. 1 and 2). The protein purified from Escherichia coli or Salmonella typhimurium catalyzes the transfer of the γ-phosphoryl group from ATP to one of its own histidine residues, His48 (3–5). CheA forms a 2:2:2 complex with an 18,000 molecular weight protein, CheW, and the signaling domain of membrane chemoreceptors (6). Within this complex CheA autophosphorylation is regulated by the signaling state of the receptor (7, 8). Whether CheA is in a complex with CheW and receptor or alone in solution, the phosphoryl group is rapidly transferred from His48 to an aspartate residue, Asp57, in the chemotaxis response regulator protein, CheY (4, 9, 10). In its dephosphorylated state CheY binds to CheA (11). Phosphorylation of CheY reduces its affinity for CheA (11) and increases its affinity for the FliM protein at the flagellar basal structure (12). There is strong evidence to support the hypothesis that CheY-P binding to FliM causes a tumbling motor response (reviewed in Ref. 2).

Genetic and biochemical data indicate that CheA is composed of at least four structurally and functionally distinct domains (1, 13, 14). A phosphoaccepting domain at the N terminus (H box-containing or H domain) is coupled via a protease sensitive linker sequence to a CheY binding domain which is in turn linked to an ATP binding domain (catalytic or P domain). A fourth domain at the C terminus of the protein is required for formation of ternary complexes with CheW and the receptor. The kinase domain is homologous to corresponding domains in a large family of histidine protein kinases that function in signal transduction to provide phosphoryl groups for a second family of proteins with regulator domains that are homologous to CheY (15, 16). Although the activity of each histidine kinase is modulated by a different sensory input and each regulator domain effects a different response, the chemistry of autophosphorylation and phosphotransfer is conserved. In the case of CheA (17, 18) as well as the osmosensory kinase, EnvZ (19), and the nitrogren regulatory kinase, NRII (20), it has been shown that autophosphorylation can occur in trans with the kinase domain of one monomer catalyzing the phosphorylation of a histidine residue in another monomer. Evidence has been presented that NRII cannot catalyze the cis transfer of phosphate from ATP to histidine by an intramolecular reaction (20).

Here we report an investigation of the phosphotransfer reactions catalyzed by purified CheA in the presence and the absence of CheY. Using 32P NMR we show that the site of phosphorylation on the histidine side chain is at the N-3 nitrogen rather than the N-1 nitrogen as had previously been reported (5). At micromolar concentrations CheA has been shown to be predominantly in a homodimeric form (21). Here we show that at submicromolar concentrations the monomer predominates (KD = 0.2–0.4 μM). A kinetic analysis of the rate of autophosphorylation under these conditions indicates a second order dependence on CheA concentration consistent with an intermolecular rather than an intramolecular autophosphorylation mechanism. Adenine nucleotides and CheY do not appear to affect the dissociation constant of the CheA dimer. Our results are consistent with the notion that dimer formation is an essential feature of CheA function in bacterial chemotaxis.

MATERIALS AND METHODS

Protein Purification—All proteins were derived from S. typhimurium genes expressed at high levels in E. coli as described previously. CheY (22), CheW (23), and CheA (15). CheA was purified from E. coli DH5α containing pME128 (pUC13 with a PstI–EcoRI CheA gene fragment...
that lacks the first translational start site in CheA) by the same protocol for CheA. Protein concentrations were determined by the BCA assay (Pierce) using bovine serum albumin as a standard. Values are expressed as monomer concentrations.

$^{31}P$ NMR of CheA–P Autophosphorylation of CheA was carried out by dialyzing 20–30 mg of CheA against 4 litters of buffer containing MgATP (50 mM Tris-HCl, pH 8.5, 20 mM KCl, 5.0 mM MgCl$_2$, 0.10 mM 1,4-dithiothreitol, 1.0 mM ATP) overnight at 4 °C. The bulk ATP was removed from the NMR sample by dialysis against the same buffer without ATP (3 × 4 liters). The sample was then dialyzed against buffer containing EDTA (50 mM Tris-HCl, pH 8.5, 20 mM KCl, 0.10 mM 1,4-dithiothreitol, 10 mM EDTA). After dialysis, the CheA samples were concentrated to 1 ml using an Amicon Centricon with a molecular weight cutoff of 30,000. Deuterium oxide (20%) served as a source of deuterium. The $^{31}P$ NMR spectra were obtained on a Bruker WM-250 spectrometer. Reactions were initiated by the addition of CheA and monitored in a liquid scintillation spectrometer. At isotopic equilibrium, the label will be distributed between ATP and ADP (to facilitate subsequent detection by UV; this addition had no effect on the distribution of label). ATP and ADP were separated by thin layer chromatography on polyethyleneimine-cellulose in 1 M acetic acid, 4% LiCl (4:1) (8). Spots corresponding to ATP and ADP were visualized by UV light (254 nm), excised, and assayed for radioactivity in a liquid scintillation spectrometer. At isotopic equilibrium, the label will be distributed between ATP and ADP in proportion to their chemical concentrations and from this the fraction of equilibrium attained (F) at any time (t) can be calculated (24). Plots of ln(1−F) versus time were linear, indicating that there was no loss of enzyme activity during the course of the experiment.

Exchange rates ($V_{ex}$) were calculated by the equation:

$$V_{ex} = -\frac{\ln(1-F)}{t} \frac{[ATP][ADP]}{[ATP][ADP]}$$

(1) Eq. 1

The results were analyzed in terms of the reactions described in Table II. It is assumed that rates of phosphotransfer are slow compared with nucleotide binding and dissociation. Initial estimates for dissociation constants for ATP and ADP were obtained by fitting subsets of the data to the Michaelis-Menten equation by nonlinear regression. Intercept replots were used to derive estimates of $K_{ex}$ (the maximal rate of exchange), $K_{ATP}k_i/k_f$ and $K_{ADP}k_i/k_f$. Slope replots were used to obtain estimates of $K_{ATP}$ and $K_{ADP}$. These estimates were entered into the program MINSO (MicroMath Scientific Software, Salt Lake City, UT), which refines parameters using non-linear least squares analysis. The data were then fit to the following relationship, which was simply derived from the reaction scheme in Table II:

$$V_{ex} = \frac{k_f k_i [CheA]_{total}}{k + k_f [ATP][ADP]}$$

(2) Eq. 2

Chemical Cross-linking of CheA—Reactions were carried out at 23 °C in 25 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 5.0 mM MgCl$_2$, with CheA at the indicated concentrations. Dithiobis(succinimidyl propionate) (5.0 mg/ml in Me$_2$SO) was added to a final concentration of 0.20 mg/ml. After 2 min the reactions were quenched with the addition of Tris-HCl and lysine to final concentrations of 50 mM and 10 mM, respectively. Aliquots containing 185 ng of CheA from each reaction were analyzed on 8% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose (0.45 μm, Micron Separations Inc, Westboro, MA). Protein was detected by Western blotting using rabbit polyclonal to CheA. $^{33}P$-labeled goat anti-rabbit antisera (DuPont NEN) was used as secondary antibody, and the blots were quantitated using a Molecular Dynamics PhosphorImager.

RESULTS

CheA Autophosphorylation Occurs at the N-3 Position of Histidine—In the initial report showing that the phosphorylated residue in CheA is His$^{48}$, it was concluded that the modification occurred at the N-1 position (5). The chromatographic procedures used in this analysis would not, however, have been expected to resolve the N-1 and N-3 isomers. The rate of hydrolysis of CheA–P as a function of pH is consistent with the modification being at the N-3 rather than the N-1 position (4), and the rates of hydrolysis of phosphohistidines in homologous bacterial histidine kinases (25–27) have given similar results. Moreover, using more refined chromatographic procedures it was demonstrated that the phosphohistidine in the related kinase PhoM is the N-3 isomer (27). In order to conclusively determine the site of phosphorylation in CheA, the $^{31}P$ NMR spectrum of CheA–P was determined. The results (Table I)
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TABLE I

| Phospho-derivative                  | Chemical shift | Reference |
|-------------------------------------|----------------|-----------|
| 1-Phosphohistidine                  | -5.70          | 39        |
|                                     | -5.7           | 40        |
|                                     | -6.01          | this work |
| 3-Phosphohistidine                  | -4.67          | 39        |
|                                     | -4.8           | 40        |
|                                     | -5.01          | this work |
| 1,3-Phosphohistidine                | -4.3           | 39        |
|                                     | -4.3           | 40        |
|                                     | -4.85          | this work |
|                                    | 1,3-Phosphohistidine | -4.73 this work |
| Phospho-CheA                         | -4.86          | this work |
| CheA phosphopeptide                 | -4.73          | this work |

The conditions used were as follows: pH 9.3, D_2O, room temperature (39); pH 8.0, D_2O, room temperature (40); pH 8.5, 5.0 mM Tris-HCl, D_2O, 4°C except for the CheA phosphopeptide, which was done at 22°C (this work). In the report by Gassner et al. (39), the authors used an earlier convention for reporting chemical shifts, which were presented as positive values. These have been converted to negative values here for consistency.

The results we have obtained concerning the effects of adenine nucleotides binding on the kinetics of autophosphorylation and ADP/ATP hydrolysis to NADH oxidation in an ATP-regenerating system using phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. At sufficiently high concentrations of CheY, CheA is dephosphorylated as fast as it is phosphorylated, and the rate-limiting step is CheA autophosphorylation. Under these conditions the dependence of CheA-mediated ATPase activity on CheA concentration at saturating ATP and CheY indicates a second order dependence at low CheA concentrations with an apparent K_D for dimer dissociation of 0.2 μM (Fig. 2), which is close to the value we estimated from directly assaying CheA autophosphorylation in the absence of CheY (Fig. 1). These results lend further support to the conclusion that CheA exists in equilibrium between an inactive monomer and an active dimer. We have previously shown that CheY binds with high affinity to the dephosphorylated form of CheA (11). Because the ATPase assay was performed with saturating CheY, the fact that essentially the same apparent K_D for CheA dimer dissociation was obtained by this procedure as when autophosphorylation rates were measured in the complete absence of CheY indicates that CheY binds equally to both the monomeric and dimeric forms of CheA.

Using the ATPase assay we have found that CheA activity is severalfold higher in the presence of potassium and ammonium cations compared with reactions containing only sodium cations (NH_4^+ > K^+ > Na^+). This effect is observed at high or low concentrations of CheA. Thus, monovalent cations appear to affect the autophosphorylation reaction directly rather than acting to alter the CheA monomer-dimer equilibrium. CheA autophosphorylation has a pH optimum near 8.4 (9, 29). The spectrophotometric assays reported here were performed mostly at pH 7.5. Experiments conducted at higher pH also indicated that this pH effect was on CheA autophosphorylation rather than CheA dimerization or ATP binding. The ATPase activity at 1.0 μM CheA was also unaffected by addition of up to 20 μM CheW. CheW has been reported to form a weak complex with CheA (21), but it does not appear that this interaction has any effect on CheA activity independent of its involvement in the formation of the ternary complex with the receptors.

In the foregoing kinetic analyses we have assumed that the two active sites within a CheA dimer function independently. Tawa and Stewart (28) have reported that CheA autophosphorylation proceeds as a simple exponential to a level of phosphorylation of 1 phosphohistidine per monomer, so it is apparent that phosphorylation at one site does not, at least to a first approximation, alter the rate at the second site. The results we have obtained concerning the effects of adenine nucleotide binding on the kinetics of autophosphorylation and ADP/ATP exchange also show no indication of linkage between active sites. Nevertheless, during catalysis CheA must undergo a large conformational change associated with altered interactions of the H and C domains to allow autophosphorylation and subsequent phosphotransfer to CheY, and it is not clear that the current level of kinetic analyses would have revealed cooperative interactions between active sites that may be associated with these conformational transitions.

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termed CheAS that begins at Met98 and therefore lacks the domain that produces an N-terminally truncated variant of CheA. Genes contain a secondary translational start site in murium cheA. The domain of CheA S retains an ability to catalyze the phosphorylation of CheA proteins with an intact phosphoaccepting domain. Thus, CheAS would stimulate kinase activity through the formation of CheA-CheAS heterodimers.

Effect of CheA Dimerization on Phosphotransfer from CheA-P to ADP—The transfer of phosphoryl groups from CheA-P to ADP can be readily assayed by first phosphorylating CheA with γ-32P-labeled ATP, isolating the 32P-labeled CheA-P, and then measuring the loss of label upon addition of ADP. A kinetic analysis of this reaction performed by Tawa and Stewart (28) indicated that two processes were involved. Whereas the majority of the CheA-P rapidly transferred its phosphoryl group to ADP, a distinct slow phase was noted that appeared to be due to a small fraction of CheA-P that slowly converted from an ADP-unreactive to an ADP-reactive form. From our results we would predict that this slow form should correspond to monomeric CheA-P, which must be converted to dimer before it can transfer its phosphoryl group to ADP. To test this possibility we have measured the time course of CheA-P diphosphorylation as a function of CheA-P concentration (Fig. 3). As predicted, the fraction of CheA-P in the slowly reactive form does in fact depend on CheA-P concentration. A quantitative analysis of our results gives an estimated Kd for CheA-P of 0.2 μM. Thus, CheA phosphorylation does not appear to significantly affect the monomer-dimer equilibrium.

Effects of CheAS on CheA Activity—The E. coli and S. typhimurium cheA genes contain a secondary translational start site that produces an N-terminally truncated variant of CheA termed CheA S that begins at Met98 and therefore lacks the domain of CheA that contains the phosphoaccepting histidine, His48 (30, 31). It has previously been shown that the kinase domain of CheAS retains an ability to catalyze the phosphorylation of CheA proteins with an intact phosphoaccepting domain (17, 18). Thus, CheAS must be competent to form dimers with CheA, and we would predict that at submicromolar concentrations of CheAS where a significant fraction of the protein is monomeric, the addition of micromolar concentrations of CheA S would stimulate kinase activity through the formation of CheA-CheAS heterodimers. This supposition was verified using the ATPase assay to measure kinase activity. At low CheA concentrations, the addition of relatively high concentrations of CheAS caused an increase in autophosphorylation activity, and no concentration dependence of CheA was observed over a range of concentrations (0.1–5 μM) where CheAS was present at 5 μM (Fig. 2). CheAS would also be expected to have an activating effect on CheA-P phosphotransfer by forming CheAS-CheA-P heterodimers and catalyzing phosphotransfer to ADP. When the effect of CheAS was examined it was found to cause a dramatic decrease in the amount of the slow phase component (Fig. 3).

**FIG. 2. The effect of CheA concentration on the kinetics of CheA autophosphorylation at steady state.** A coupled assay (see text) was used to measure the rate of CheA autophosphorylation at 2 mM ATP and CheA from 0.08 to 5 μM (open circles). The data were fit to a model that assumes that CheA is in equilibrium between inactive monomer and active dimer with apparent Kd of 0.17 μM (see text for details). The rate of CheA autophosphorylation was also determined in the presence of 5 μM CheAS (closed circles).

**FIG. 3. Kinetics of CheA-P dephosphorylation by ADP.** The time course for CheA-P dephosphorylation was determined at two concentrations of CheA-P: 0.25 μM (open circles) and 4 μM (open squares). The rate of dephosphorylation of 0.25 μM CheA-P was also determined in the presence of 3.75 μM CheAS (closed circles).

Kinetic Characterization of Kinase-catalyzed Exchange of Phosphoryl Groups from ATP to ADP—Previous results had indicated that CheA can catalyze isotope exchange between ATP and ADP (8, 32). These findings, along with the isolation of the phosphorylated enzyme (3) are consistent with a ping-pong mechanism (23) for the histidine kinase ATP/ADP exchange reaction. Our results indicated that the exchange reaction occurred over a broad pH range (6.5–9.5) with an optimum at pH 8.4, and in addition the reaction was stimulated by potassium. These observations are similar to results obtained by measuring rates of CheA autophosphorylation (9, 29). All exchange experiments reported here were performed at pH 8.4 in the presence of potassium. Isoxope exchange rates were measured at different concentrations of enzyme. The specific activity observed for CheA exhibited a concentration dependence (Fig. 4) that fits the pattern expected from an inactive monomer/active dimer equilibrium with a Kd for dimer dissociation of 0.4 μM. Steady state rates of isotope exchange between ATP and ADP were measured at 10 μM CheA where the protein is essentially all in its dimeric form (Fig. 5). For ping-pong reactions, reciprocal plots of rate versus varied substrate (ATP) at different fixed substrate (ADP) should be parallel. Dead-end inhibition by the fixed substrate results in deviations of the slopes and inhibition by the varied substrates results in an upward curvature as the line approaches the y axis. CheA showed dead-end inhibition by both ADP and ATP. Thus, the unproductive complexes of ADP bound to dephosphorylated enzyme and ATP bound to phosphorylated enzyme appear to significantly affect the kinetics. The data were fit to the isotope exchange rate equation for a ping-pong mechanism (see Equation 2), and values were obtained for constants that are functions of the binding constants of ATP and ADP to the phospho and dephospho forms of CheA as well as the rate constants for phosphotransfer from ATP to CheA (kP) and from CheA-P to ADP (kD) (Table II).
buffer components. According to our kinetic scheme, the V\textsubscript{max} additional information. This was obtained by measuring the constants and the forward and reverse rate constants requires \( k \) equals by CheA at concentrations ranging from 0.22 to 20 \( \mu \text{M} \) (at 211 \( \mu \text{M} \) \(^{14}\text{C}\)ADP and 535 \( \mu \text{M} \) ATP). The data are expressed as the observed rates of exchange (\( \mu \text{M} \) \(^{14}\text{C}\)ATP formed/min) divided by the enzyme concentration versus enzyme concentration. The data were fit to a model that assumes that CheA is in equilibrium between inactive monomer and active dimer.

Fig. 4. Effect of enzyme concentration on the isotope exchange rate between ADP and ATP. The rate of exchange catalyzed by CheA at concentrations ranging from 0.22 to 20 \( \mu \text{M} \) (at 211 \( \mu \text{M} \) \(^{14}\text{C}\)ADP and 535 \( \mu \text{M} \) ATP). The data are expressed as the observed rates of exchange (\( \mu \text{M} \) \(^{14}\text{C}\)ATP formed/min) divided by the enzyme concentration (see “Materials and Methods”). ADP concentrations are as follows: ○, 31 \( \mu \text{M} \); ●, 61 \( \mu \text{M} \); □, 93 \( \mu \text{M} \); ▲, 123 \( \mu \text{M} \); □, 185 \( \mu \text{M} \).

Table II

| Reaction | Constant | Estimated value
|----------|----------|-------------------
| CheA \( \cdot ATP \rightarrow \) CheA\( \cdot P \cdot ADP \) | \( k_f \) | \( 7.0 \text{ min}^{-1} \)
| CheA \( \cdot P \cdot ADP \rightarrow \) CheA \( \cdot ATP \) | \( k_r \) | \( 7.3 \text{ min}^{-1} \)
| CheA \( \cdot ATP \rightarrow \) CheA \( \cdot P \cdot ADP \) | \( K_{ATP} \) | \( 0.274 \text{ mM} \)
| CheA \( \cdot P \) \( \leftrightarrow \) CheA \( \cdot P \cdot ADP \) | \( K_{ADP} \) | \( 0.240 \pm 0.052 \text{ mM} \)
| CheA \( + ADP \rightarrow \) CheA \( \cdot ADP \) | \( K_{ADP} \) | \( 0.160 \pm 0.059 \text{ mM} \)
| CheA \( + ATP \rightarrow \) CheA \( \cdot P \cdot ATP \) | \( K_{ATP} \) | \( 0.810 \pm 0.140 \text{ mM} \)

*The values for the following terms are derived directly from the isotope exchange experiments (see “Materials and Methods”: \( k_f, k_r, K_{ATP} \)). The values of \( k_f, k_r, K_{ATP} \) were calculated using the value of \( K_{ATP} \).

Fig. 5. Isotope exchange between ATP and ADP catalyzed by CheA. \( ^{14}\text{C}\)ADP and ATP were varied, and exchange rates were determined at 25 °C. CheA was at 10 \( \mu \text{M} \). The lines are drawn to fit the entire set of data for the rate equation (see “Materials and Methods”). ADP concentrations are as follows: ○, 31 \( \mu \text{M} \); ●, 61 \( \mu \text{M} \); □, 93 \( \mu \text{M} \); ▲, 123 \( \mu \text{M} \); □, 185 \( \mu \text{M} \).

To determine the values of the individual nucleotide binding constants and the forward and reverse rate constants requires additional information. This was obtained by measuring the \( V_{\text{max}} \) and \( K_m \) for CheA autophosphorylation using the ATPase assay: \( K_m = 0.33 \text{ mM} \) and \( V_{\text{max}} = 11.0 \text{ min}^{-1} \) in pH 7.5 reaction buffer and \( K_m = 0.27 \text{ mM} \) and \( V_{\text{max}} = 7.4 \text{ min}^{-1} \) in pH 8.4 reaction buffer (Fig. 6). The pH profile with maximum activity at pH 8.4 was observed for both reaction buffers. The higher \( V_{\text{max}} \) at pH 7.5 observed here is due to the different reaction buffer components. According to our kinetic scheme, the \( V_{\text{max}} \) equals \( k_f(CheA_{\text{total}}) \), and the \( K_m \) corresponds to \( K_{ATP} \). These relationships allow the determination of all the kinetic constants (Table II). The results indicate that \( k_f \) and \( k_r \) are approximately equal. Tawa and Stewart (28) came to a similar conclusion by directly comparing these rates. The values for these rate constants are relatively low (\( \sim 7 \text{ min}^{-1} \)). This slow phosphotransfer rate tends to argue in favor of a central assumption in our kinetic analysis, namely that nucleotide binding equilibria are attained much more rapidly than phosphotransfer catalysis. The apparent dissociation constants for ADP, approximately \( 0.2 \text{ mM} \), were not significantly affected by CheA phosphorylation and are similar to the apparent dissociation constant of ATP for the dephosphorylated enzyme. Phosphorylation of CheA decreases its affinity for ATP by almost a factor of three, indicating an unfavorable interaction between the \( \gamma \)-phosphoryl group of the nucleotide and the phosphohistidine moiety.

Chemical cross-linking of the CheA dimer is concentration-dependent—Kinetic results where CheA activity was measured using several independent methods were all consistent with CheA being a dissociable dimer (\( K_D = 0.2-0.4 \mu \text{M} \)) that is inactive in its monomeric state. We were able to directly measure the CheA monomer-dimer equilibrium by examining the effect of CheA concentration on the formation of cross-links between monomers (Fig. 7). CheA at different concentrations was cross-linked using dithiobis(succinimidyl propionate), and the products were analyzed by electrophoresis in polyacrylamide gels under nonreducing conditions. Only two species were observed on cross-linking over a range of CheA concentrations (0.1–25 \( \mu \text{M} \)). The low molecular weight species corresponds to monomer with and without internal cross-links. The second species co-migrated with molecular mass standards of 210–240 kDa. This high molecular weight form corresponds to
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**Fig. 7. Dithiobis(succinimidyl propionate) cross-linking of CheA dimers as a function of CheA concentration.** The reactions were carried out with 0.1-20 μM CheA, and the extent of CheA dimer cross-linked was quantitated as described under “Materials and Methods.” The amount of dimer was normalized to the maximum amount of dimer observed under these cross-linking conditions (≈ 70% of the total CheA). The data represent the averages of two independent experiments. Error bars represent standard errors of the mean. The line represents a model that assumes CheA is in equilibrium between inactive monomeric and active dimeric forms with a K_D of 0.3 μM (see text).

The CheA histidine kinase of E. coli and S. typhimurium is a member of the large family of histidine protein kinases involved in bacterial signal transduction. In cells CheA is regulated in a complex with CheW and chemoreceptors so that its activity reflects the signalizing state of the receptor. To establish a foundation for understanding the mechanism of CheA regulation, we have characterized CheA activity using the purified enzyme. The kinetic results obtained provide information about the basic mechanism of histidine kinase autophosphorylation.

Using phosphorous NMR we have demonstrated that the site of CheA phosphorylation is at the N-3 position of histidine. This fits the hydrolysis data obtained for the CheA phosphoramide group under a variety of different conditions of pH (4). From hydrolysis data of the phosphohistidine in homologous bacterial histidine kinases (25–27), it seems likely that members of this family generally employ a mechanism involving phosphorylation at N-3 of the histidine imidazole side chain. The modified residue has been shown to co-chromatograph with 3-phosphohistidine in the case of the protein kinase PhoM (27).

Phosphohistidines generally have high phosphodonor potentials, and it has been previously shown that histidine protein kinases can catalyze an exchange of phosphoryl groups between ATP and ADP. Assuming a ping-pong mechanism, the kinetics of the exchange reaction and the kinetics of autophosphorylation can be used to estimate the binding constants of ADP and ATP for both the phospho- and dephosphoenzymes. The results indicate that ADP and ATP bind almost equally to dephospho-CheA. This suggests that the Mg(II) coordination may involve the α-β phosphates of the nucleotide rather than the β-γ phosphates. In this position it is doubtful that the bound metal would play a central role in the phosphotransfer reaction. This seems to be a general feature of protein kinases that work through a phosphohistidine intermediate. For example in nucleoside diphosphate kinase the α-β coordination has been directly demonstrated by x-ray crystallographic studies (33–35). Furthermore, the Myxococcus xanthus nucleoside diphosphate kinase efficiently autophosphorylates with ATP in the absence of magnesium (33).

We estimate that the turnover number of S. typhimurium CheA is approximately 10/min, and Tawa and Stewart (28) have reported slightly lower values for the E. coli enzyme. It seems likely that the phosphotransfer reactions require the movement of the histidine group from a relatively solvent-exposed position where it is free to bind to the active site of a phosphoaccepting regulatory protein such as CheY to a position within the active site of the kinase. The differences in ATP affinity for the phospho- and dephosphoenzymes corresponds to a difference in standard free energy of only about 1 kcal/mol, which argues strongly for the notion that the phosphohistidine group has relatively little access to the kinase active site where the nucleotide is bound. Exposure of the phosphohistidine is also evidenced by the well documented observation that the response regulators can generally accept phosphoryl groups from noncognate histidine kinases (16). It has been shown that the response regulators can independently function to catalyze the transfer of phosphoryl groups from phosphorylaminidoate to the phosphoaccepting aspartyl carboxylate (36). Moreover, there is essentially no affect of CheY binding to CheA on either the K_m for ATP or the turnover number for phosphotransfer.

CheA has previously been shown to exist in a dimeric state at μM concentrations (21). Here we show that this dimer reversibly dissociates into an inactive monomeric form. The K_D for dimer dissociation under physiological conditions was found to be 0.2–0.4 μM. The possibility of intersubunit phosphorylation between the kinase domain of one CheA subunit and the phosphohistidine domain of a second subunit has previously been demonstrated using heterodimers of CheA mutants (17, 18). Our data indicate that in the isolated enzyme, the intersubunit mechanism is preferred by CheA. It has previously been shown that for NRII, a CheA homolog involved in nitrogen regulation, histidine autophosphorylation also involves an intersubunit rather than an intrasubunit phosphotransfer mechanism (20). This aspect of histidine kinase function may therefore be a conserved feature for most members of the histidine kinase superfamily. In many respects, enzyme I involved in phosphoenolpyruvate:carbohydrate phosphotransferase systems is analogous to the CheA and the histidine kinase superfamily. This enzyme catalyzes the phosphorylation of a histidine at the N-3 position using phosphoenolpyruvate as a phosphodonor rather than ATP. Like CheA, the site of phosphorylation is in one domain, and the phosphoacceptor binding/dimerization functions are in a second domain (37). Furthermore, like CheA, the enzyme is inactive as a monomer and active as a dimer (38).

The CheA monomer-dimer equilibrium appears not to be affected by nucleotide binding, phosphorylation, or the binding of CheY or CheW. One might expect that the interactions of the phosphorylation site with the active site would contribute to the stability of the dimer. This may not be the case, however. Findings obtained from subunit exchange experiments with CheAΔ5, which lacks the site of histidine phosphorylation, indicate that this truncated protein is completely competent to function in the phosphorylation of a variant with a defective kinase domain (18). Moreover, deletion of the domain of CheA that couples the kinase to the chemoreceptors (with CheW) had no effect on kinase autophosphorylation (13). The finding that
dimerization is unaffected by ATP binding or by phosphorylation or even deletion of the phosphoaccepting histidine supports the notion that the phosphoaccepting histidine has very little contact with the kinase active site. Clearly more work has to be carried out to establish the nature of the interactions of the phosphorylation site with the active site and how these interactions contribute to dimer stability.

Most histidine kinases are receptors with a transmembrane topology similar to that of the chemoreceptors that function to regulate CheA. Genetic evidence suggests that at least in the case of EnvZ, receptor dimerization is sufficient to cause kinase activation. NRII is a stable dimer that like CheA is a cytosolic protein, but there is no evidence that NRII kinase activity is regulated. Activation of CheA by dimerization is insufficient to account for the activation of CheA when it is in a complex with chemoreceptors and CheW, however. In fact, the CheA-CheW-receptor ternary complex appears to be a stable 2:2:2 dimer, and it has been shown that disulfide cross-linking of receptor subunits within this structure does not preclude kinase regulation (41).

Acknowledgments—We thank Pam Lane and Tanya Tolstykh for help with preparation of proteins and members of the Stock lab for critical reading of the manuscript.

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