Active Site Interference and Asymmetric Activation in the Chemotaxis Protein Histidine Kinase CheA*

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(Received for publication, August 8, 1996, and in revised form, September 12, 1996)

The histidine protein kinase CheA is a multidomain protein that mediates stimulus-response coupling in bacterial chemotaxis. We have previously shown that the purified protein exhibits an equilibrium between inactive monomer and active dimer (Surette, M., Levit, M., Liu, Y., Lukat, G., Ninfa, E., Ninfa, A., and Stock, J. (1996) J. Biol. Chem. 271, 939–945). We report here a study of the kinetics of phosphorylation of the isolated phosphoacceptor domain of CheA catalyzed by the isolated catalytic domain of the protein. The reaction fits Michaelis-Menten kinetics ($K_m = 0.26 \text{ mM}$ for ATP and $0.10 \text{ mM}$ for phosphoacceptor domain; $k_{\text{obs}} = 17 \text{ min}^{-1}$).

The catalytic domain exhibits the same equilibrium between inactive monomers and active dimers as the full-length CheA protein. Thus, CheA dimerization is an intrinsically intrinsic property of this domain, independent of any other portion of the molecule and is required for its catalytic activity. In equimolar mixtures of full-length CheA and catalytic domain, homodimers and heterodimers are formed in equal concentration, indicating that all of the determinants for the dimerization are localized entirely on the catalytic domain. An analysis of the kinetics of phosphorylation catalyzed by CheA-catalytic domain heterodimers indicates half of the sites reactivity. The rate of CheA phosphorylation within this heterodimer is over 5-fold greater than that observed in CheA homodimers. The dramatic increase in activity within this asymmetric dimer raises the possibility that CheA activation by receptors involves a mechanism that directs catalysis to one active site while preventing interference from the other.

Bacterial signal transduction networks that regulate motility and gene expression generally involve two central enzymatic components, histidine protein kinases and phosphoaccepting response regulators (1, 2). Sensory information controls the rates of kinase autophosphorylation at specific histidine residues. These phosphoryl groups are subsequently transferred to a specific aspartate residue in the response regulator proteins, causing a conformational change in the regulator that leads to the generation of a response. The most intensively investigated signaling network of this type is the system that mediates chemotaxis responses in Escherichia coli and Salmonella typhimurium (3). The chemotaxis histidine kinase, CheA, and response regulator, CheY, that mediate chemotaxis in these species are found in all motile eubacterial and archaeobacterial strains that have been examined and are clearly specialized for sensory motor regulation.

The CheA protein is composed of at least four functionally and structurally distinct domains (Fig. 1). An N-terminal phosphoaccepting domain of approximately 130 residues that contains the site of histidine autophosphorylation, termed the P1 or H domain, is connected to a domain of approximately 70 residues that binds CheY, termed the P2 or Y domain, which is in turn linked to a catalytic, C, domain of approximately 250 residues that binds ATP and catalyzes the phosphorylation of the N-terminal H domain. Finally, a distinct C-terminal regulatory, R, domain functions to mediate regulatory interactions between CheA and membrane receptor-transducer proteins. The H and Y domains have been isolated as independently folding units, and their structures have been determined by NMR methods (4–7). Both are globular. The H domain is essentially an $\alpha$-helical bundle with the phosphoaccepting histidine side chain extending from the solvent-exposed surface of one helix. The Y domain is an open faced $\alpha$/$\beta$ sandwich. The H and Y domains are attached to one another and to the C domain by flexible linker sequences, and it is apparent that the two globular domains are free to move in solution like balls on a chain. No structural information is available concerning the C and R domains, although it has been shown that the R domain can be cleaved away without dramatically affecting the activity of the CheA catalytic domain (8).

Purified CheA is in equilibrium between an inactive monomeric form and an active homodimer ($K_d = 0.3 \pm 0.1 \mu M$) (9). Phosphorylation can occur in trans within the dimer, with the C domain of one subunit catalyzing the phosphorylation of the H domain of the other subunit (10, 11). It has not been determined whether the inactivity of CheA monomers is due to the lack of a phosphoaccepting H domain in trans or due to an intrinsic requirement of C domain dimerization for catalytic activity.

To better understand the interactions of the H and C domains within CheA dimers we have undertaken a detailed analysis of the kinetics of phosphorylation of a fragment of CheA composed solely of H domain by a fragment of CheA that contains only the C domain. The results indicate that the C domain exists in an equilibrium between an active dimer and an inactive monomer with a $K_d$, essentially identical to the $K_d$ for dissociation of intact CheA dimers. Thus, the dimerization of CheA depends entirely on dimerization of the C domain, and C domain dimerization is required for kinase activity. An analysis of the kinase activities of heterodimers of CheA in association with fragments of CheA that contain the C domain indicates that CheA dimers exhibit half of the sites reactivity. Apparently, the interaction of an H domain with one of the active sites of a catalytic domain dimer precludes interaction of a second H domain with the other active site.

MATERIALS AND METHODS

*This work was supported by National Institutes of Health Grant AI20980 (to J. B. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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were grown at 37°C in 6 liters of LB, 100 
confirmed by sequencing. 

Expression and Purification of H Domain—

Expression and Purification of C Domain—

Phosphorylation of H domain catalyzed by C domain and phosphotransfer from phospho-H domain to CheY. Aliquots of H domain (final concentration, 10 μM) plus 0.20 mM [γ-32P]ATP were incubated for 10 min either alone (lane 1) or in the presence of 2.0 μM C domain (lane 2), 2.0 μM CheA, lane 3, or 2.0 μM CheA (lane 4). CheY was then added, to a final concentration of 10 μM, to half of each reaction mixture, and after 30 s the reactions were terminated with SDS sample buffer. Lane 6, H domain plus C domain plus CheY; lane 7, H domain plus CheA, plus CheY; lane 8, H domain plus CheA plus CheY. The samples were subjected to 15% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27). A band of protein migrating above CheA corresponds to cross-linked CheA dimers.

RESULTS

Phosphoaccepting Activity of H Domain—It has previously been shown that a fragment of CheA containing only the C domain is able to phosphorylate a fragment containing only the H domain (13). This activity is at least 10-fold greater than the rate of H domain phosphorylation catalyzed by the intact CheA protein or by a naturally occurring variant of CheA termed CheAS produced from an alternative site of translational initiation corresponding to Met<sub>his</sub> in the full-length CheA protein (Fig. 2). Presumably, the H and/or Y domains in CheA and CheAS interfere with the ability of the free H domain to interact with the active site in the catalytic domain. As has been observed previously (13), the phospho-H domain acts as an efficient CheY phosphodonor in the complete absence of the CheY binding domain (Fig. 2, compare lanes 6 and 8).

At concentrations of purified C domain in the micromolar range the dependence of the rate of phosphorylation on H domain concentration exhibited Michaelis-Menten kinetics with an apparent K<sub>m</sub> for H domain of approximately 100 μM (Fig. 3). The fact that the x axis intercept in the inset to Fig. 3, which corresponds to K<sub>a</sub>, is the same at saturating and subsaturating ATP concentrations indicates that the affinity of C domain for H domain is unaffected by ATP binding. At saturating ATP, the estimated K<sub>obs</sub> for H domain phosphorylation is approximately 17 min<sup>-1</sup>. This is almost twice the k<sub>obs</sub> for CheA autophosphorylation, approximately 9.0 min<sup>-1</sup>, ob-
Fig. 3. Rate of H domain phosphorylation catalyzed by C domain as a function of H domain concentration. Rates were measured using a coupled ATPase assay (see “Materials and Methods”) in the presence of 2.0 μM C domain and 2.0 mM ATP. The simulated curve was obtained using a $K_m$ of 104 μM and a $k_{cat}$ of 17.0 min$^{-1}$. The inset shows results from experiments performed in the presence of 2.0 mM (○) and 0.20 mM ATP (●) plotted according to the method of Hanes (28).

Fig. 4. Steady state rates of H domain phosphorylation as a function of ATP concentration. The coupled ATPase assay (see “Materials and Methods”) was used to measure the rate of H domain phosphorylation at ATP concentrations varying from 0.050 to 2.0 mM in the presence of 2.0 μM C domain and 200 μM H domain. The simulated curve was obtained using the predicted values for a $K_m$ for ATP of 0.26 mM and $k_{cat}$ of H domain phosphorylation at 200 μM H domain and saturating ATP of 14.0 min$^{-1}$. The inset shows results from the experiments performed in the presence of 200 μM (●), 50 μM (○), and 20 μM H domain (□) plotted according to the method of Hanes (28).

The apparent $K_m$ for ATP in the phosphorylation of H domain by C domain is approximately 0.26 mM, close to the $K_m$ of 0.33 mM obtained with CheA under similar conditions (9). This value is independent of the concentration of H domain (Fig. 4, inset).

These quantitative measures of rates of H domain phosphorylation were obtained in a coupled assay under steady state conditions where H domain phosphorylation was rate-limiting (i.e. sufficient CheY was added, 25 μM, to ensure a relatively rapid dephosphorylation of phosphohistidine groups and subsequent hydrolysis of phospho-CheY). In the absence of CheY, the initial rate of ATP hydrolysis was the same as the steady state rate obtained in its presence (data not shown). In parallel experiments it was also shown that the initial rate of H domain phosphorylation measured by assaying production of $^{32}\text{P}$-la-

beled H domain directly was the same as the ATPase activity measured in the presence of CheY. To accurately assay the rate using radiolabeled ATP, it is necessary to directly measure the specific activity of [$γ\text{--}^{32}\text{P}$]ATP. Using thin layer chromatography (9), we have found that the portion of [$γ\text{--}^{32}\text{P}$]ATP in our commercially obtained material is generally only about 50% of the total radioactivity present. This type of contamination could explain previously reported differences between CheA autophosphorylation rates measured by $^{32}\text{P}$ labeling in the absence of CheY and ATPase activity measured in the presence of CheY (14).

C Domain Dimerization—At micromolar concentrations, purified C domain eluted during molecular sieve chromatography with an apparent molecular weight of 58,000. This is the value predicted for a globular dimer. Cross-linking studies performed at different concentrations of C domain indicated a $K_D$, of approximately 0.21 μM (Fig. 5), essentially the same as that exhibited by homodimers of full-length CheA (9). This result indicates that the determinants for CheA dimerization are localized entirely to the C domain. If this were the case one would expect that in a mixture of equal concentrations of C domain and CheA there should be equal amounts of CheA homodimers, C domain homodimers, and CheA-C domain heterodimers. This prediction was confirmed by size exclusion chromatography of an equimolar mixture of CheA and C domain, where it was shown that 36% of the C domain eluted as a heterodimer with CheA while 64% eluted at a position corresponding to the C domain homodimer (Fig. 6).

Effect of C Domain Dimerization on Kinase Activity—We have previously shown that the autophosphorylation activity of CheA is completely dependent on its dimerization. One possible explanation for this is the requirement for an H domain in trans. Another possibility is that the C domain must be dimeric to be active. To test for the dependence of C domain dimerization on kinase activity, rates of H domain phosphorylation were examined as a function of C domain concentration. The kinase activity of the isolated C domain toward the H domain exhibits a concentration dependence in the submicromolar range that is consistent with an equilibrium between inactive monomers and active dimers. The apparent $K_D$ for C domain dissociation es-
that can interact independently with the two linked H domains. If each CheA homodimer has two symmetric active sites with the accessibility of free H domain for the kinase active and/or Y domains linked to the C domain in CheA interfere rate of C domain homodimers, presumably because the H domain phosphorylation is presumed to be caused by the partitioning of the C domain from active C domain homodimers into relatively inactive CheA-C domain heterodimers. The solid curve in Fig. 8 shows the decrease in activity that would be predicted from this effect assuming the heterodimer to be completely inactive. From this result we conclude that the CheA-C domain heterodimer, like CheA homodimer, has a very low activity toward free H domain. Thus, the presence of the H and Y domains on one subunit is sufficient to effectively inhibit the interaction of both potential kinase active sites with free H domain. Moreover, in a complementary experiment high concentrations of H domain (400 μM) caused a 30% inhibition of the rate of CheA phosphorylation in CheA-C domain heterodimers (Fig. 9). Free H domain had a much smaller inhibitory effect on CheA homodimer autophosphorylation, probably because the associated H domains compete with free H domain for accessibility at the site. These results indicate that in CheA dimers there is negative cooperativity between active sites. In other words, occupancy of one active site within a dimer by a substrate H domain precludes occupancy of the other active site. The effect of increasing concentrations of C domain on the rate of CheA autophosphorylation was examined in the absence of free H domain (Fig. 10). The results indicate a dramatic stimulation due to the formation of CheA-C domain heterodimers with over 5-fold higher autophosphorylation activity than CheA homodimers. Similar results were obtained by adding increasing concentrations of CheA to a fixed concentration of C domain (Fig. 11). When the latter experiment was performed with a fixed concentration of CheA, however, the results indicated that the CheA-CheA heterodimers had only about a 20% greater autophosphorylation activity than CheA in trans, one would predict that CheA-C domain heterodimers would have one active site (in CheA) that would phosphorylate only free H domain and one active site (in C domain) that would preferentially phosphorylate the linked H domain in trans. When rates of phosphorylation by CheA-C domain heterodimers were examined, however, dramatically different results were obtained. When increasing concentrations of CheA where added to a fixed concentration of C domain, a dramatic inhibition of H domain phosphorylation was observed (Fig. 8). This decrease in H domain phosphorylation is presumed to be caused by the partitioning of the C domain from active C domain homodimers into relatively inactive CheA-C domain heterodimers. The solid curve in Fig. 8 shows the decrease in activity that would be predicted from this effect assuming the heterodimer to be completely inactive. From this result we conclude that the CheA-C domain heterodimer, like CheA homodimer, has a very low activity toward free H domain. Thus, the presence of the H and Y domains on one subunit is sufficient to effectively inhibit the interaction of both potential kinase active sites with free H domain. Moreover, in a complementary experiment high concentrations of H domain (400 μM) caused a 30% inhibition of the rate of CheA phosphorylation in CheA-C domain heterodimers (Fig. 9). Free H domain had a much smaller inhibitory effect on CheA homodimer autophosphorylation, probably because the associated H domains compete with free H domain for accessibility at the site. These results indicate that in CheA dimers there is negative cooperativity between active sites. In other words, occupancy of one active site within a dimer by a substrate H domain precludes occupancy of the other active site. The effect of increasing concentrations of C domain on the rate of CheA autophosphorylation was examined in the absence of free H domain (Fig. 10). The results indicate a dramatic stimulation due to the formation of CheA-C domain heterodimers with over 5-fold higher autophosphorylation activity than CheA homodimers. Similar results were obtained by adding increasing concentrations of CheA to a fixed concentration of C domain (Fig. 11). When the latter experiment was performed with a fixed concentration of CheA, however, the results indicated that the CheA-CheA heterodimers had only about a 20% greater autophosphorylation activity than CheA in trans.
homodimers. This result is consistent with the observation that CheA homodimers have an activity toward free H domain similar to that of CheA homodimers (Fig. 2).

DISCUSSION

The histidine kinase CheA functions in bacterial chemotaxis to integrate sensory receptor inputs and relay this information to response regulators in the cytoplasm. The protein occupies a central position in the chemotaxis signal transduction network, and it has been shown that CheA can interact directly with at least four other Che proteins, CheW, CheY, CheB, and CheZ, as well as with chemotaxis receptors such as Tar and Tsr (15–19). The complex regulatory functions of CheA are reflected in the complex multidomain structure of the protein, with the central histidine kinase or C domain being flanked by domains responsible for mediating interactions between the kinase domain and other signal transduction components (3, 20). In order to better understand the mechanisms that regulate this core kinase activity, we have undertaken a detailed kinetic analysis of its activity in the absence of flanking regulatory regions. Using its activity in the absence of flanking regulatory regions. Using

\[ K_{m} = \text{Michaelis-Menten constant for free H domain and MgATP of approximately } 0.10 \text{ and } 0.26 \text{ mM, respectively and a } k_{\text{obs}}\text{ of } 17 \text{ min}^{-1}. \]

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inability of monomeric CheA to autophosphorylate its associated phosphoaccepting domain. We show here, however, that the requirement for CheA dimerization is an intrinsic property of the C domain alone, independent of any requirement for transphosphorylation of associated H domains. Moreover, all of the determinants for dimerization seem to reside within the C domain, since the $k_d$ values for C domain dimer dissociation are essentially identical to the dissociation constants of CheA dimers or of CheA-C domain heterodimers. From the activities of heterodimers of different mutant CheA proteins it has previously been proposed that residues from both subunits within a dimer participate in formation of the active site (23). The dimerization requirement for CheA activity is consistent with this hypothesis.

The $k_{obs}$ for CheA homodimer autophosphorylation is 9.0 min$^{-1}$, the value obtained for the C domain homodimer is 17 min$^{-1}$, and that for the CheA-C domain heterodimer is approximately 60 min$^{-1}$. The relatively low activity of CheA homodimers is presumably due to interference between the two associated H domains. The associated CheY binding and regulatory (Y and R) domains could contribute to this interference. CheA-CheA heterodimers exhibit a $k_{obs}$ of 12 min$^{-1}$. Thus, despite the fact that more than half of the H domain is missing in CheA-C, the remaining H domain and the intact Y and R domains still interfere with kinase activity to almost the same extent as in CheA homodimers. The dramatically higher rate observed with the heterodimer compared with the C domain homodimer indicates that the associated H domain in CheA is better positioned to serve as a substrate than free H domain.

Assuming that CheA dimers are symmetric, there must be two active sites/dimer. The fact that C domain activity follows Michaelis-Menten kinetics indicates that the two sites are independent or that the protein exhibits half of the sites reactivity. The apparent interference between the two H domains in CheA homodimers suggests that H domain binding at one site may preclude the binding and phosphorylation of an H domain at the opposing site, i.e., CheA dimers may exhibit half of the sites reactivity. This mechanism is supported both by the fact that H domain is poorly phosphorylated by the heterodimer and by the inhibitory effects of high concentrations of H domain on heterodimer autophosphorylation. It has previously been shown that both H domains within a CheA homodimer are phosphorylated with kinetics that fit a single exponential function (14). This is not inconsistent with negative cooperativity or half of the sites reactivity, however, since the two sites could act sequentially to phosphorylate the two associated H domains.

CheA functions in chemotaxis in a ternary complex with CheW and receptor, and within this complex CheA autophosphorylation rates can be increased over 100-fold compared with values obtained with pure CheA under comparable conditions (24). One of our primary goals in studying the kinetics of purified CheA is to understand the mechanism of this activation. In evaluating the rates of various CheA constructs compared with rates obtained with CheA-CheW-receptor complexes it is necessary to compare absolute rates rather than -fold activation. In experiments where activation has been measured within complexes, the reported degree of activation depends both on CheA activity within the complex, which is generally optimized, and on the activity of pure CheA under the same conditions, which is generally far from optimal. Measurements of CheA autophosphorylation under conditions of CheA-CheW-receptor complex formation give values for $k_{obs}$ of up to 70 min$^{-1}$ (25). This value is close to the $k_{obs}$ of approximately 60 min$^{-1}$ that we have obtained for autophosphorylation within the C domain-CheA heterodimer. Because of incomplete complex formation, the $k_{obs}$ of 70 min$^{-1}$ is undoubtedly an underestimate. Nevertheless, the relatively high rate of autophosphorylation within the heterodimer suggests that the mechanism of CheA activation within the ternary complex may involve an asymmetric mechanism that restricts the relative positioning of the domains associated with the C domain and/or causes a conformational change in the C domain to favor catalysis by one active site without interference from the other.

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