Oligomerization of the Phosphatase CheZ Upon Interaction with the Phosphorylated Form of CheY

THE SIGNAL PROTEIN OF BACTERIAL CHEMOTAXIS*

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Earlier studies have suggested that CheZ, the phosphatase of the signaling protein CheY in bacterial chemotaxis, may be in an oligomeric state both when bound to phosphorylated CheY (CheY–P) (Blat, Y., and Eisenbach, M. (1994) Biochemistry 33, 902-906) or free (Stock, A., and Stock, J. B. (1987) J. Bacteriol. 169, 3301–3311). The purpose of the current study was to determine the oligomeric state of free CheZ and to investigate whether it changes upon binding to CheY–P. By using either one of two different sets of cross-linking agents, free CheZ was found to be a dimer. The formation of the dimer was specific, as it was prevented by SDS which does not interfere with cross-linking mediated by random collisions. The dimeric form of CheZ was confirmed by sedimentation analysis, a cross-linking-free technique. In the presence of CheY–P (but not in the presence of non-phosphorylated CheY), a high molecular size cross-linked complex (90–200 kDa) was formed, in which the CheZ:CheY ratio was 2:1. The size of the oligomeric complex was estimated by fluorescence depolarization to be 4–5-fold larger than the dimer, suggesting that its size is in the order of 200 kDa. These results indicate that CheZ oligomerizes upon interaction with CheY–P. This phosphorylation-dependent oligomerization may be a mechanism for regulating CheZ activity.

Bacteria such as Escherichia coli or Salmonella typhimurium use chemotaxis to navigate toward favorable environments and retreat from non-favorable ones (1). The sensory information from the receptors is integrated by a cytoplasmic signal transduction network of chemotaxis proteins (see for review, Refs. 2–5) and transmitted to the flagella by the signaling molecule CheY (6, 7). CheY interacts with the switch-motor complex at the base of the flagellum (6–10) and changes the direction of rotation from the default direction, counter-clockwise (9–17) to clockwise (12, 15, 17), and thereby causes the cell to reorient (18). The clockwise causing activity of CheY is regulated by phosphorylation (19). The phosphorylation level is determined by the kinase CheA and the phosphatase CheZ (20–22). The activity of the kinase, CheA, is modulated by chemotactic stimuli via the membrane chemotaxis receptors and the chemotaxis protein CheW (23–25). On the other hand, regulation of CheZ activity by chemotactic stimuli has not been demonstrated.

In a previous study we found that the binding of CheZ to phosphorylated CheY (CheY–P) is 2 orders of magnitude higher than to non-phosphorylated CheY, and that several molecules of CheZ can bind to a single CheY–P molecule (26). Earlier observations indicated that CheZ can be in two oligomeric forms, 115 and >500 kDa, as estimated by size-exclusion chromatography (27) (the molecular size of the monomer is 23.9 kDa (27)). The observations of both studies taken together suggest that the oligomeric state of CheZ is modulatory. Here we examine this possibility and demonstrate that CheZ is a dimer which oligomerizes upon interaction with CheY–P. The possibility that this phosphorylation-dependent oligomerization is a regulation mechanism for CheZ activity is investigated in a subsequent work (28).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The overexpression of nonlabeled and radiolabeled E. coli CheZ (CheZS)1 and of radiolabeled E. coli CheY (CheYS) was carried out in strain RP437, wild type for chemotaxis (29), carrying the plasmid pRL22 (30). Nonlabeled CheY S was overexpressed in strain EW30 (26) which contains a deletion from cheA to cheZ and carries the plasmid pRL22(ΔPvulI). For the overexpression of S. typhimurium CheF (CheF S) we constructed the plasmid pEWS5 as follows. CheF S was ampliﬁed from total DNA of strain ST1 (31) by polymerase chain reaction (PCR) using the primers 5’-CCGAATTCATGGCGGATATCAAGACG-3’ and 5’-CCGGATCTCTAGATGCCCCATTTCATTCAAGG-3’, which contained added EcoRI and BamHI sites, respectively, at their 5’ end. The ampliﬁed fragment was digested by EcoRI and BamHI, and ligated with pBTac1 (Boeringer Mannheim) predigested by EcoRI and BamHI. The resultant plasmid, pEWS5, overexpressed CheF S under the control of tac promoter. The plasmid pEWC1 used for the overexpression of S. typhimurium CheZ214FC, was constructed similarly to the construction of pEWS5 except that the primers 5’-CCGAATTCATGACCACTCTTACTCAAGCC-3’ and 5’-CCGGATCTCTTAAAGCAGGACTGTCCAGCAGGTC-3’ were used for the PCR. CheYS and CheYS214FC were overexpressed in RP1091 (RP437ΔcheY-chochz) (14) and RP3098 (RP437Δ(fliC-fihA) (32)), respectively.

Protein Radiolabeling—Radiolabeling of CheYS and CheZ was carried out by inducing the expression of CheYS and CheZ in the presence of L-[35S]cysteine as described previously (26).

Protein Purification—The purification of CheYS (nonlabeled) and CheZS (nonlabeled and radiolabeled) was carried out on a Cibacron column followed by a G-50 (for CheY) or Sepharose CL-6B (for CheZ) column as described previously (26). Radiolabeled CheYS was purified as the non-labeled CheYS, only that smaller-scale columns (4 ml of Cibacron and 65 × 1 cm G-50) were used. CheYS was purified as CheYS.

1 The abbreviations used are: CheZE, E. coli CheZ; AcP, acetyl phosphate; CheYE, E. coli CheY; CheY S, S. typhimurium CheY; CheZS, S. typhimurium CheZ; DMS, dimethylsulfoxide; EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.
(26), except that the bacteria were grown on Luria broth and induced with 0.66 mM isopropyl-β-D-thiogalactopyranoside. CheZ214FC was purified as follows. RP1091 cells containing pEWC1 were grown at 35°C in 1.5 liter of Luria broth containing 100 μg/ml ampicillin. At OD600 = 0.4, overexpression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After 4 h, the cells were harvested by centrifugation, washed once by buffer A (20 mM NaH2PO4, pH 7.4, 2 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), resuspended in 35 ml of buffer A, and sonicated. Membranes and unlysed cells were removed by centrifugation at 165,000 × g for 60 min. The lysate was loaded on a 40-ml Sepharose CL-6B column pre-equilibrated with buffer A. The column was washed with 100 ml of buffer A, followed by 170 ml of buffer A containing 275 mM NaCl. CheZ was eluted from the column by a 250-ml linear gradient of 275–450 mM NaCl in buffer A. The CheZ-containing fractions were pooled and concentrated by ultracentrifugation through a 5-kDa cut-off membrane, using an Amicon chamber (model 52). The concentrated fractions were loaded on to a 20-ml hydroxylapatite column, pre-equilibrated with buffer B (10 mM NaH2PO4, pH 7.0, 2 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). The column was washed with 60 ml of buffer B, and CheZ was eluted with a 200-ml linear gradient of buffer B containing 10–200 mM NaH2PO4. The CheZ-containing fractions were concentrated to a volume of 2 ml and loaded on to a Sephadex G-150 column (50 × 1.5 cm), pre-equilibrated with a solution of Tris-HCl (50 mM, pH 7.9), KCl (200 mM), CaCl2 (1 mM), 2 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The CheZ-containing fractions were concentrated and stored in 10% glycerol at −80°C. 

Cross-linking—The reaction mixture (20 μl) for cross-linking contained NaCl (90 mM), KCl (10 mM), MgCl2 (5 mM), Tris-HCl (50 mM, pH 8.5) for cross-linking by dimethylsuberimidate (DMS) or 35 mM, pH 7.9, for cross-linking by 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), and CheZ (1.3 μg). CheY (20 μg), and acetyl phosphate (AcP, 18 mM) were also added as additional cross-linking reagents. The reaction was initiated by the addition of DMS (3 mg/ml) or EDC followed by NHS (66 and 13 mM, respectively), and allowed to proceed for 3 h (with DMS) or 40–60 min (with EDC plus NHS) at room temperature (22–25°C). The reaction was terminated by addition of 5 μl of sodium dodecyl sulfate (SDS) sample buffer (×5 concentrated). The samples were boiled for 10 min, and 22-μl aliquots were used for 15% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained, dried, and autoradiographed. Quantitative analysis of the radioactivity associated with the different cross-linking products was carried out with a Phosphorimag (Fuji, Bas 1000).

CheZ Labeling with Fluorescein—CheZ214FC (100 μg, 200 μg) was incubated with 0.5 mM fluorescein-5-maleimide (Molecular Probes) in Tris-HCl (50 mM, pH 7.9) for 30 min at room temperature (22–25°C). After incubation, CheZ was separated from the unreacted fluorescein by application on to a 0.8-ml G-50 mini-column and briefly spinning at 480 g. The eluted labeled CheZ was dialyzed against Tris-HCl (50 mM, pH 7.9) containing 1.5 mM dithiothreitol. The labeled CheZ was stored at −80°C.

Size Estimation of CheZ by Zonal Centrifugation—CheZ, CheY (14 kDa), CheW (18 kDa), and ovalbumin (43 kDa) samples (150 μl of 2 mg/ml) were loaded onto a 5–20% isokinetic sucrose gradient (5 ml) in Tris-HCl (50 mM, pH 7.9), KCl (100 mM), and MgCl2 (5 mM). The samples were spun at 200,000 × g for 24 h (20°C) in a Beckman SW50.1 rotor. After centrifugation, 130-μl fractions were collected from the top of the gradient and analyzed for protein concentration by the Bradford technique (33). The size of CheZ was estimated by plotting a calibration line of (molecular size)0.67 against the distance traveled by the protein peak (34).

Size Estimation by Size-exclusion Chromatography—CheZ alone or a mixture of CheZ (20 μg), CheY (100 μg), and AcP (18 μg) was run through a size-exclusion HPLC Bio-Sil TSK-250 column (75 × 600 mm; Bio-Rad; 23°C), utilizing HP 1040 A diode array chromatography system, and Waters HPLC system, composed of two pumps (model 510) and an automatic controller. Prior to separation, the column was pre-equilibrated with Tris-HCl (50 mM, pH 7.9), KCl (100 mM), MgCl2 (5 mM), and AcP (18 μg). The flow rate was 1 ml/min.

Fluorescence Depolarization of CheZ—Fluorescence depolarization studies with fluorescence-labeled CheZ214FC were carried out with a Perkin Elmer LS 50 B luminescence spectrometer equipped with a fluorescence polarization accessory. The excitation and emission wavelengths were 490 and 520 nm, respectively (5-nm slit width).
For the phosphorylation site, Asp57 (26, 41), did not promote the oligomerization of CheZ which cannot be phosphorylated due to the substitution of Glu instead of Asp. Furthermore, CheY57DE oligomer was indeed dependent on the concentration of CheY (6 residues and the high number of aspartate and glutamate residues in CheZ (6 residues versus 37, respectively (40)). For this reason the subsequent cross-linking studies were carried out with EDC-NHS.

If the phosphorylation-dependent oligomerization of CheZ is physiologically significant, it should depend on the concentration of CheY –P. As shown in Fig. 3, A and B, the amount of the oligomer was indeed dependent on the concentration of CheY added in the presence of access AcP. Furthermore, CheY57DE which cannot be phosphorylated due to the substitution of Glu for the phosphorylation site, Asp57 (26, 41), did not promote the oligomer formation even in the presence of AcP (Fig. 3C, lane 7). Similarly, depletion of Mg” (necessary for CheY phosphorylation (42)) also prevented the formation of the oligomer (lane 5).

The Amount of CheY in the Oligomer—In order to estimate the relative content, if any, of CheY in the oligomer, we carried out the cross-linking experiment with radiolabeled CheY or radiolabeled CheZ in parallel (Fig. 4). [14C]CheZ cross-linked in the presence of CheZ and AcP (Fig. 4, lane 4) indeed formed a band migrating at the same position as the CheZ oligomer (lane 1). This band was not formed in the absence of CheZ and AcP (lane 3; the faint high molecular size band seen in this lane represents aggregates too large to enter the gel). This result indicates that the CheZ oligomer contains also some CheY cross-linked to it. We quantified the amount of CheY cross-linked to the CheZ oligomer by a PhosphoImager. The result was 0.48 ± 0.18 (mean ± S.D., three independent determinations) molecules of CheY per monomer of CheZ, suggesting a CheZ:CheY ratio of 2:1.

Estimation of the Oligomeric Status of CheZ in the Absence of CheY –P by Sedimentation Analysis—Our observation that, in the absence of CheY –P, CheZ appears as a dimer in cross-linking experiments, is in apparent conflict with Stock and Stock’s (27) observation, reproduced by us, that CheZ behaves as a tetramer when run on an HPLC size-exclusion column. In order to solve this discrepancy we estimated the size of CheZ by zonal centrifugation on a sucrose gradient. As shown in Fig. 5, the CheZ peak appeared between the peaks of CheW (18 kDa) and ovalbumin (43 kDa) at a location which, according to the formula described under “Experimental Procedures,” is the site of a 35 ± 4 kDa (mean ± S.D., two determinations) globular protein. This estimation is closer to the cross-linking results, which indicated that CheZ is a dimer (47.8 kDa), than to the HPLC results which indicated a tetramer. The difference between the size estimations of the different approaches suggests that CheZ is non-globular (see “Discussion”).

Estimation of the Oligomeric Status of CheZ in the Presence of CheY –P by Fluorescence Depolarization—To confirm the oligomerization of CheZ in the presence of CheY –P, we initially attempted to use size-exclusion chromatography. Running a mixture of CheZ, CheY, and AcP through the HPLC column yielded only two peaks, eluted at retention times identical to those obtained when CheZ and CheY were run separately. This could mean either that, in contrast to the cross-linking results, the CheY –P-induced oligomerization does not occur at all, or that the oligomer is unstable and readily disso-
The major finding of this study is that, in the presence of CheY-P, there is further oligomerization of the CheZ dimer. However, it is not possible to determine, on the basis of the results, whether CheY-P is an integral part of the oligomer at a CheZ:CheY-P ratio of 2:1, or whether it is just bound to an oligomer comprising CheZ alone. The results of the fluorescence depolarization implied that the oligomer is about 4-5 times larger than the CheZ dimer formed in the absence of CheY-P. This suggests that the size of the oligomer is in the order of 200 kDa. This size is about at the top of the size range observed in the cross-linking experiments (Fig. 2B). The size estimation of the oligomer from the fluorescence depolarization
is only a first degree approximation, because it was based on the assumption that both the oligomer and the CheZ dimer are spherical (43, 44), an assumption which, according to our own results, is probably incorrect. Determination of the exact size or shape is beyond the resolution of the applied methods. A better estimation of the size of the oligomer is rather difficult by currently available methods because, at this stage, there is no obvious way to separate the oligomer from CheY–P and maintain it in a stable form. For example, techniques in which the shape contribution can be estimated (e.g. light scattering) cannot distinguish between the oligomer and CheY–P in the mixture. Nevertheless, these difficulties in size and shape estimation do not affect the conclusions reached in this study, as neither the exact size nor the shape of the oligomer are necessary for the conclusions. It should be noted that the CheZ oligomer, observed in this study, is different from the CheZ homopolymer (27) and the CheZ-CheA₅ multimeric complex (49), observed earlier, in the sense that the oligomer of this study is not stable and it readily dissociates in the absence of CheY–P.

In the fluorescence depolarization experiments, the anisotropy of fluorescein-labeled CheZ was increased to a large extent in the presence of CheY–P (Fig. 7A). This observation could, in principle, be attributed either to a significant increase in the molecular volume of CheZ, or to a large conformational change at the vicinity of the fluorescein moiety that restricts its free rotation. The following observations strongly suggest that a significant increase in the molecular volume of CheZ, i.e. CheZ oligomerization, is the mechanism responsible for the anisotropy change. (i) To a first approximation, the Perrin plot (Fig. 7B) is composed of two distinct rotations: a fast rotation of the probe, and a slow rotation of the whole protein. To determine the rotational freedom of the probe, we extrapolated the straight line of the Perrin plot to 1/anisotropy = 0. Since the straight line shown in the figure represents the rotation of the protein, the extrapolated value represents the hypothetical case in which the protein rotation is frozen but the probe rotates freely. As shown in Fig. 7B, both the dimeric and the oligomeric forms of CheZ fall in the same anisotropy range (1/anisotropy values of 4.0 and 4.8 for the dimer and oligomer, respectively). This indicates that the rotation of the probe itself is not significantly affected by the oligomerization, and therefore that the anisotropy change is not the result of a change in the probe rotation. (ii) An increase in anisotropy could, in principle, be due to a decrease in the lifetime of the excited state of the probe, reflected in a reduced efficiency of the fluorescence. However, the large oligomerization-dependent change in anisotropy was accompanied by only a minor reduction (7%) in the fluorescence efficiency. The lack of substantial changes in the fluorescence efficiency and in the motional freedom of the probe, is supported by the observation that both the 214F → C substitution (Fig. 6) and the conjugation of this cysteine with fluorescein maleimide didn’t affect the activity of CheZ.

It is well known in a variety of systems, including bacterial signal transduction systems (50, 51), that oligomerization regulates protein activity. Accordingly, it is conceivable that the oligomerization may either activate or inhibit the phosphatase activity of CheZ. In the first possibility, a burst of CheY phosphorylation will activate CheZ and will thereby promote faster deactivation of CheY. Fast deactivation of CheY will prevent non-beneficial too long periods of tumbling. If the other possibility is correct and oligomerization inhibits the phosphatase activity of CheZ, the oligomerization may serve as an amplification step in which phosphorylation of CheY leads to oligomerization of CheZ, inhibition of its phosphatase activity, and, consequently, further increase in the level of CheY–P. The results described in the subsequent paper (28) suggest that the first possibility is the correct one.

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