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Purification and Characterization of the CheZ Protein of Bacterial Chemotaxis

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The *cheZ* gene is the most distal of five genes that comprise the Meche operon of the *Salmonella typhimurium* chemotaxis system. We have determined the sequence of the *cheZ* gene along with an 800-nucleotide flanking region at its 3′ end. The flanking sequence contains an open reading frame that probably corresponds to the 5′ end of *flaM*. The *cheZ* coding sequence predicts an extremely acidic, hydrophilic protein with a molecular weight of 23,900. We have purified and characterized this protein. N-terminal analysis of pure CheZ yields an amino acid sequence identical to that predicted by the nucleotide sequence except that the amino-terminal methionine residue is modified by N methylation. The purified CheZ protein exhibits a native molecular weight of 115,000, but in cell extracts the majority of CheZ exists as a much larger aggregate (M_r > 500,000). Under these conditions, CheZ appears to be a homopolymer composed of at least 20 monomeric subunits.

In bacterial chemotaxis, the interaction between the receptors and flagellar motor appears to be indirect, requiring an intermediate signal transduction system composed of at least four cytoplasmic proteins, the products of the *cheA*, *cheW*, *cheY*, and *cheZ* genes (for reviews, see references 4 and 25). The mechanism of signal transduction has not been determined. From genetic studies it has been suggested that CheZ interacts directly with components at the flagellar motor. Thus, the Che$^-$ phenotype of some *cheZ* missense mutations can be suppressed by specific missense mutations in flagellar genes and vice versa (26, 27, 54). Similar results have been obtained with *cheY* mutants (27), and from an analysis of the behavior of an *Escherichia coli cheZ* deletion (33, 34) it has been suggested that CheZ acts to antagonize the effects of CheY. Studies of the behavior of strains with various levels of CheY and CheZ have supported this notion (14).

Here we report the purification and characterization of the *Salmonella typhimurium* CheZ protein, together with the nucleotide sequence of the *cheZ* gene. It has previously been shown that CheZ tends to cosediment with membranes in cell extracts; however, the nucleotide sequence predicts a hydrophilic protein. Since native CheZ appears to exist primarily as a large aggregate (>500 kilodaltons [kDa]), it seems likely that the sedimentation properties of CheZ result from its multimeric structure. Immunoaffinity chromatography with a monoclonal anti-CheZ antibody indicates that no other proteins are tightly associated with this complex.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, exonuclease III, M13 17-mer universal primer, and phosphorylated *Bam*HI linkers were from New England BioLabs, Inc., Beverly, Mass. DNA polymerase I Klenow fragment was from Bethesda Research Laboratories, Inc., Gaithersburg, Md. S1 nuclease was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Deoxynucleotides and dideoxynucleotides were from P-L Biochemicals, Inc., Milwaukee, Wis. [^{35}S]H_2SO_4 (carrier free, 43 Ci/mg) was from ICN Pharmaceuticals, Inc., Irvine, Calif., [^{125}I]NaI (15 Ci/mg) was from Amersham Corp., Arlington Heights, Ill., and [α-^{32}P]dATP was from New England Nuclear Corp., Boston, Mass. Servalyte ampholytes were from Serva, Heidelberg, Federal Republic of Germany. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) was from Sigma Chemical Co., St. Louis, Mo. RPMI cell culture media and heat-inactivated fetal bovine serum were from GIBCO Laboratories, Grand Island, N.Y. IgGsorb was from The Enzyme Center, Malden, Mass. Affi-Gel 10 and DEAE Affi-Gel Blue were from Bio-Rad Laboratories, Richmond, Calif. All other chemicals and materials were obtained from standard commercial sources.

Strains and plasmids. *S. typhimurium* strains were derived from the wild-type LT2 variant, ST1 (1). ST171 (*cheZ221*) was isolated as a tumbly nonchemotactic mutant obtained by diethyl sulfate mutagenesis (1). Strains used in the mapping of the *cheZ221* mutation have been described previously (46). Two *E. coli* strains used as hosts for plasmids, JM109 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) (F′ *traD36 proAB lacI^{q}ZΔM15*) (56) and MM294 *hsdR17* (r_k^- m_k^+) *endA1 thi* (19) with a deletion in *recA* were from J. Messing, Waksman Institute, Rutgers University, New Brunswick, N.J., and B. Bochner, Genentech, Inc., South San Francisco, Calif. The plasmids pGK2 and pGK24 (N. I. Gutterson, Ph.D. thesis, University of California, Berkeley, 1982) are derived from pDK1 (9), a pBR322 derivative containing the *S. typhimurium* Meche operon. The plasmid pGK2 contains a *tar*, *cheR*, and *cheB* insert; pGK24 expresses *cheY* and *cheZ* under control of the *lac* promoter. A CheZ expression vector, pME105, was constructed from pME1, a pUC12 derivative containing an *S. typhimurium* genomic fragment encoding the Meche operon. A 1.8-kilobase *Tth*111I-*Bgl*II fragment (with a *Bam*HI linker attached to the *Tth*111I end) was inserted into the *Bam*HI site in the pUC12 polylinker. The resulting plasmid, containing the distal half of *cheY* and intact *cheZ*, produces CheZ under control of the *lac* promoter.

Preparation of anti-CheZ polyclonal antibodies. *E. coli* MM294 *recA*(pGK24) was grown at 37°C on low-sulfate (0.1 mM) Vogel-Bonner medium (51) containing 1% glycerol, [^{35}S]sulfate, and 40 μg of ampicillin per ml. The cells were harvested by centrifugation at 10,000 × *g*, disrupted by freezing and thawing in lysis buffer (22), and subjected to

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isoelectric focusing in a gradient from pH 4.0 to 6.0 on vertical polyacrylamide slab gels. Protein was visualized by autoradiography, and strips containing CheZ were excised. These were equilibrated for 1 h in 2% sodium dodecyl sulfate (SDS) and then electrophoresed in 15% polyacrylamide by the method of Laemmli (18). Gels were autoradiographed, strips containing CheZ were excised, and CheZ protein was recovered by electroelution. Approximately 0.1 mg of CheZ obtained from 1 g (wet weight) of cells was used to raise rabbit anti-CheZ antisera (50).

Purification of CheZ. *E. coli* MM294 *recA* containing the plasmid pGK24 was grown at 37°C to a density of approximately 10^9 cells per ml in L broth (20) containing 25 μg of ampicillin per ml. The cells were harvested, and 50 g (wet weight) was suspended in 150 ml of 0.1 M sodium citrate–1 mM EDTA, pH 6.5, and lysed in a Raytheon model DF101 sonicator. This step and all subsequent procedures were done at 4°C. Double diffusion immunoassays (23) were used to monitor CheZ protein throughout the purification. Membranes and large debris were removed by centrifugation at 100,000 × *g* for 90 min. The supernatant fraction was titrated to pH 4.5 with 1 M HCl, and the precipitate was collected by centrifugation at 12,000 × *g* for 15 min. The precipitate was washed by suspension in 0.1 M citrate, pH 4.5, and collected by centrifugation. The pellet was suspended in 60 ml of 0.1 M sodium citrate, pH 6.5, and then titrated to pH 6.3 with 1 M NaOH, and the suspension was incubated for 12 h. Ammonium sulfate (saturated solution at 0°C, pH 7.0) was added dropwise to a final concentration of 29% saturation. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min, suspended in 60 ml of 0.1 M sodium citrate–1 mM EDTA, pH 6.5, incubated for 12 h, and then dialyzed against 10 mM piperazine hydrochloride–100 mM NaCl, pH 6.0. The protein was applied to a DE-52 cellulose (Whatman, Inc., Clifton, N.J.) column (2.2 by 48 cm) equilibrated with 10 mM piperazine hydrochloride–0.1 M NaCl, pH 6.0. The column was washed with 300 ml of piperazine buffer and then eluted with a 1.5-liter linear gradient of 0.1 to 0.3 M NaCl in 10 mM piperazine hydrochloride, pH 6.0. Fractions corresponding to a peak of CheZ were pooled, phenylmethylsulfonyl fluoride was added to a final concentration of 0.2 mM, and the pooled fractions were dialyzed against 10 mM sodium phosphate, pH 6.7. The protein was applied to a Bio-Gel HT (Bio-Rad) hydroxylapatite column (1.4 by 17 cm) equilibrated with 10 mM sodium phosphate, pH 6.7. The column was washed with 50 ml of phosphate buffer and then eluted with a 0.2-liter linear gradient of 10 to 200 mM sodium phosphate, pH 6.7. Fractions corresponding to the peak of CheZ were pooled, dialyzed against 10 mM sodium phosphate, pH 6.7, and then concentrated by application to a 1.2-ml Bio-Gel HT column and elution with 4.0 ml of 0.2 M sodium phosphate, pH 6.7. The protein was chromatographed over a Sephadex G-150 (Pharmacia, Inc., Piscataway, N.J.) column (2.2 by 45 cm) equilibrated with 0.1 M sodium phosphate, pH 6.5. Fractions corresponding to the peak of CheZ were pooled and concentrated by using a 1.2-ml hydroxylapatite column as described above.

Preparation of monoclonal anti-CheZ antibodies. Purified CheZ protein (15 μg) in Freund complete adjuvant was injected intraperitoneally into 6-week-old female BALB/c mice. After 2 weeks, mice were boosted with another 15 μg of CheZ in Freund incomplete adjuvant. Mice showing an immune response after 1 week were boosted again with 15 μg of CheZ and 3 days later were sacrificed. Lymphocytes, obtained from the spleen, were fused with NS-1 myeloma cells in the presence of polyethylene glycol. Hybridomas were selected by growth on HAT medium (RPMI–10% heat-inactivated fetal bovine serum containing 14 μg of hypoxanthine per ml, 0.18 μg of aminopterin per ml, and 3.8 μg of thymidine per ml). Cell lines were screened for anti-CheZ antibody secretion by enzyme-linked immunosorbent assays by using protein from sonic extracts of MM294 *recA* containing the plasmid pGK24 immobilized on plastic plates and goat anti-mouse IgG peroxidase conjugate. Hybridomas that screened positive were subcloned two to four times until it was certain that a single clone had been obtained. Cells from these clones were injected intraperitoneally into Pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Inc., Milwaukee, Wis.)-primed mice, and ascites fluid was collected after the development of tumors.

Immunoaffinity chromatography. IgG was purified from mouse ascites fluid by DEAE–Affi-Gel Blue (Bio-Rad) chromatography (5) followed by precipitation in 50% ammonium sulfate at 0°C. Purified IgG (5 mg/ml) was dialyzed against 0.1 M sodium bicarbonate, pH 8.5, mixed with Affi-Gel 10 (Bio-Rad) at a ratio of 10 mg of IgG per ml of Affi-Gel 10, and incubated for 12 h at 4°C. The immunoaffinity column was equilibrated with 0.1 M sodium phosphate, pH 7.3, and used for the purification of CheZ directly from cell extracts. Cell extracts, prepared by sonic disruption, were applied to the column, the column was washed with 50 to 100 column volumes of phosphate buffer, and bound protein was eluted with three column volumes of 0.1 M glycine hydrochloride, pH 2.7, and immediately neutralized by the addition of 0.1 volume of 1 M dibasic potassium phosphate. Yields of approximately 2 mg of CheZ per ml of immunoaffinity resin were common.

Nucleotide sequence determination. The *S. typhimurium* DNA of pME105 was inserted in both orientations into the polylinker region of bacteriophage M13mp10 producing two recombinant phages, M13me2A and M13me2B. Sets of phage with deletions extending processively through the insert were prepared from the two parent phages by using a modification of the exonuclease III procedure (12). The nucleotide sequence was determined on both strands by the dideoxynucleotide procedure (32) by using DNA polymerase I Klenow fragment, [α-^{32}P]dATP, an M13 universal primer (17-mer), and the deletion phage plus strand DNA as the template. Sequences were assembled and manipulated by using the computer programs of Staden (43) and those available through BIONET National Computer Resource for Molecular Biology (Biomedical Research Technology Program, Division of Research Resources, National Institutes of Health, Bethesda, Md.). Analysis of the region beyond *cheZ* was done by using the coding region locator program of Pustell and Kafatos (28). The NUCALN program of Wilbur and Lipman (53) was used to align nucleic acid sequences.

RESULTS

Purification of CheZ and preparation of anti-CheZ antibodies. CheZ has been identified on two-dimensional electrophoretograms as a species migrating with an apparent isoelectric point of approximately 4.6 and a molecular weight of 29,000 (A. Stock, E. Schaeffer, D. E. Koshland, Jr., and J. Stock, J. Biol. Chem., in press). It is one of the most acidic proteins in *S. typhimurium* or *E. coli* and is cleanly separated from other cell proteins by this procedure (Fig. 1A). By beginning with cell extracts from an *E. coli* strain containing a multicopy plasmid that expresses *S. typhimurium* CheZ as approximately 1% of the total cell protein, it was possible to

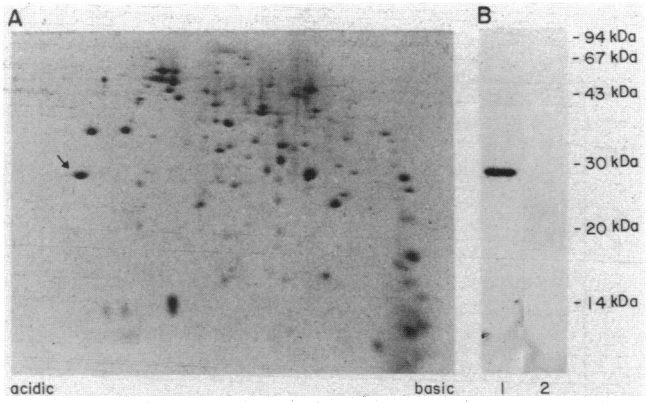

FIG. 1. (A) Two-dimensional gel electrophoresis of a cell extract prepared from *E. coli* MM294 *recA* containing the plasmid pGK24 that expresses *S. typhimurium cheY* and *cheZ*. Cells were grown on L broth containing 40 μg of ampicillin per ml to a density of 10^9 cells per ml and then precipitated in 10% trichloroacetic acid at 0°C. The precipitate was collected by centrifugation at 13,000 × *g* for 10 min, suspended in acetone at 0°C, centrifuged again, and suspended by repeated freezing at −70°C and thawing at 30°C in lysis buffer (22). Approximately 250 μg of protein was subjected to isoelectric focusing in polyacrylamide tube gels by using a gradient from pH 4.5 to pH 6.5 (22). The gel was equilibrated in 2% SDS, electrophoresed in 15% polyacrylamide (18), and then stained with Coomassie blue. The position of the CheZ protein is indicated by an arrow. (B) Autoradiograph of immunoprecipitated CheZ. *S. typhimurium* ST1, a wild-type strain, and ST426, a Fla^- strain with a deletion through *cheZ*, were grown in low-sulfate (0.1 mM) Vogel-Bonner citrate medium containing 1% glycerol and $^{35}SO_4$ to a density of 10^9 cells per ml, harvested by centrifugation at 10,000 × *g* for 10 min, lysed by heating at 95°C for 5 min in 1% SDS in phosphate-buffered saline (12.5 mM sodium phosphate–0.2 M NaCl, pH 7.5), and diluted 1:30 into phosphate-buffered saline containing 1% Triton X-100. These extracts were used for immunoprecipitation with rabbit anti-CheZ antisera and IgGsorb *Staphylococcus aureus* cells as previously described (47). Immunoprecipitates were electrophoresed in SDS–15% polyacrylamide gels.

obtain sufficient pure CheZ protein from preparative two-dimensional gels to raise rabbit anti-CheZ antibodies (Fig. 1B). By using immunoassays to monitor the protein, CheZ was then purified to apparent homogeneity by conventional chromatographic procedures under nondenaturing conditions (Fig. 2).

CheZ exhibited heterogeneity throughout its purification. As reported previously (30), approximately half of the CheZ present in cell extracts sediments with membrane fragments and other large cellular structures during centrifugation at 100,000 × *g*. In our experiments, the partition between pellet and supernatant varied from 30 to 70%. This distribution was not consistently affected by altered ionic strength (up to 2 M KCl or 1 M $MgCl_2$) or by the addition of detergents (0.1% Triton X-100 or 30 mM octylglucoside). Moreover, neither levels of CheZ production (wild-type cells versus cells with multicopy plasmids that express Chez at high levels) nor the presence of other components of the chemotaxis-flagellar system (expression in Fla^+ versus Fla^- cells) had any significant effect. These and further results (see below) suggest that CheZ is not a membrane protein nor is it bound to a membrane-associated component of the chemotaxis system.

The precipitation and column chromatography steps also produced approximately twofold losses in CheZ. The protein precipitated throughout a wide range of acid and ammonium sulfate concentrations and eluted from DEAE-cellulose as a broad peak that trailed off with no defined endpoint. As the purification progressed, CheZ became more homogeneous, and the elution profiles from the final hydroxylapatite and gel filtration chromatographic steps exhibited better-defined peaks.

The heterogeneous behavior during purification and the resulting low yield raised the possibility that a discrete subset of the total CheZ protein had been isolated. To obtain a more efficient and rapid purification, monoclonal antibodies were generated by using the purified CheZ protein as the antigen. Two hybridoma cell lines that secrete anti-CheZ immunoglobulins were obtained. Antibodies purified from mouse ascites fluid were covalently coupled to an agarose gel bead support, and the resulting immunoaffinity resin was used to purify CheZ directly from extracts prepared by sonic disruption of cells. The immunoaffinity columns routinely allowed a single-step purification of CheZ (>90% pure with a yield of approximately 100%) even from cells that do not overproduce the protein (Fig. 3).

Characterization of CheZ. Analysis of CheZ by molecular sieve chromatography indicated a variety of multimeric forms. CheZ purified by conventional precipitation and chromatographic procedures eluted as a single peak with an apparent molecular weight of 115,000 (Fig. 4A). Since the monomer molecular weight of CheZ predicted from the nucleotide sequence of the *cheZ* gene is 23,900 (see below), the purified protein appears to exist as a multimer, possibly a tetramer.

CheZ purified from wild-type *S. typhimurium* cell extracts by immunoaffinity chromatography exhibited a complex profile when subjected to molecular sieve chromatography (Fig. 4B). Three peaks were observed with apparent molec-

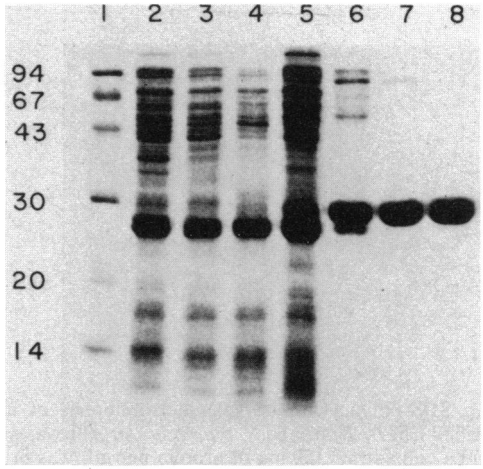

FIG. 2. SDS-polyacrylamide gel electrophoresis of fractions from the CheZ purification (described in Materials and Methods). Lanes: 1, molecular-weight standards; 2, 75 μg of cell extract from MM294 *recA* containing plasmid pGK24; 3, 75 μg of the supernatant centrifuged at 100,000 × *g*; 4, 75 μg of the acid precipitation pellet; 5, 75 μg of the ammonium sulfate precipitate; 6, 50 μg of pooled fractions from DEAE-cellulose chromatography; 7, 25 μg of pooled fractions from hydroxylapatite chromatography; 8, 25 μg of pooled fractions from Sephadex G-150 chromatography. The samples were electrophoresed in 15% polyacrylamide, and the gel was stained with Coomassie blue. It should be noted that the major band apparent in lanes 2 to 5 (M_r = 27,000) is not CheZ and does not cross-react with anti-CheZ antibody. Numbers at left indicate molecular weight (10^3).

ular weights of greater than 500,000, 315,000, and 115,000. A similar profile was obtained with CheZ from *E. coli* JM109(pME105). Moreover, when the peaks were analyzed by SDS-polyacrylamide gel electrophoresis, each was found to be composed of a single protein that comigrated with CheZ. No cellular proteins associated with CheZ in a stoichiometry of greater than a few percent could be detected in any of these fractions or in the primary eluate from the immunoaffinity column. Thus, it seems unlikely that the observed aggregation is caused by association of CheZ with other cellular components.

To investigate the nature of CheZ in cell extracts, freshly prepared sonic extracts of wild-type *S. typhimurium* were fractionated by molecular sieve chromatography, and CheZ protein was detected by immunoblotting (Fig. 4C). The majority of CheZ protein was eluted in the excluded volume, although smaller amounts of CheZ were detected at later elution times. Thus, the large aggregates observed with immunoaffinity-purified CheZ appear to reflect the state of CheZ in cell extracts. We conclude that the 115-kDa form represents a minimum state of CheZ aggregation. In vivo, CheZ may exist primarily in aggregates consisting of 20 or more monomers. This fits the observation by Chelsky and Dahlquist (6) that CheZ in intact cells is efficiently cross-linked by dithiobis(succinimidyl propionate). The apparent association of CheZ with membranes reported here and

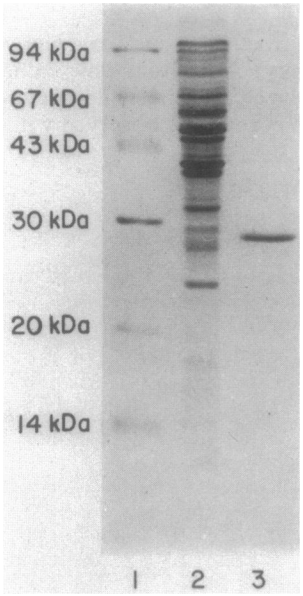

FIG. 3. SDS-polyacrylamide gel electrophoresis of immunoaffinity-purified CheZ. Wild-type *S. typhimurium* ST1 was grown in L broth, and a cell extract (50 mg of protein per ml) was prepared by sonic disruption. The extract (12.0 ml) was applied to a 1.1-by-1.0-cm anti-CheZ monoclonal immunoaffinity column equilibrated with 0.1 M sodium phosphate, pH 7.0. The column was washed with 100 ml of phosphate buffer, and bound protein was eluted with 10.0 ml of 0.1 M glycine, pH 2.7. The eluant was collected in 1.0-ml fractions and neutralized by addition of 0.1 volume of 1.0 M K_2HPO_4. Fractions 2 to 8 were pooled, protein was precipitated by addition of trichloroacetic acid to 10% at 0°C, the precipitate was collected by centrifugation for 15 min at 13,000 × *g*, and the pellet was suspended in cold acetone, centrifuged again, and finally suspended in 2% SDS. Samples were electrophoresed in 15% polyacrylamide, and the gel was stained with Coomassie blue. Lanes: 1, molecular-weight standards; 2, 75 μg of ST1 cell extract; 3, 5 μg of immunoaffinity-purified CheZ.

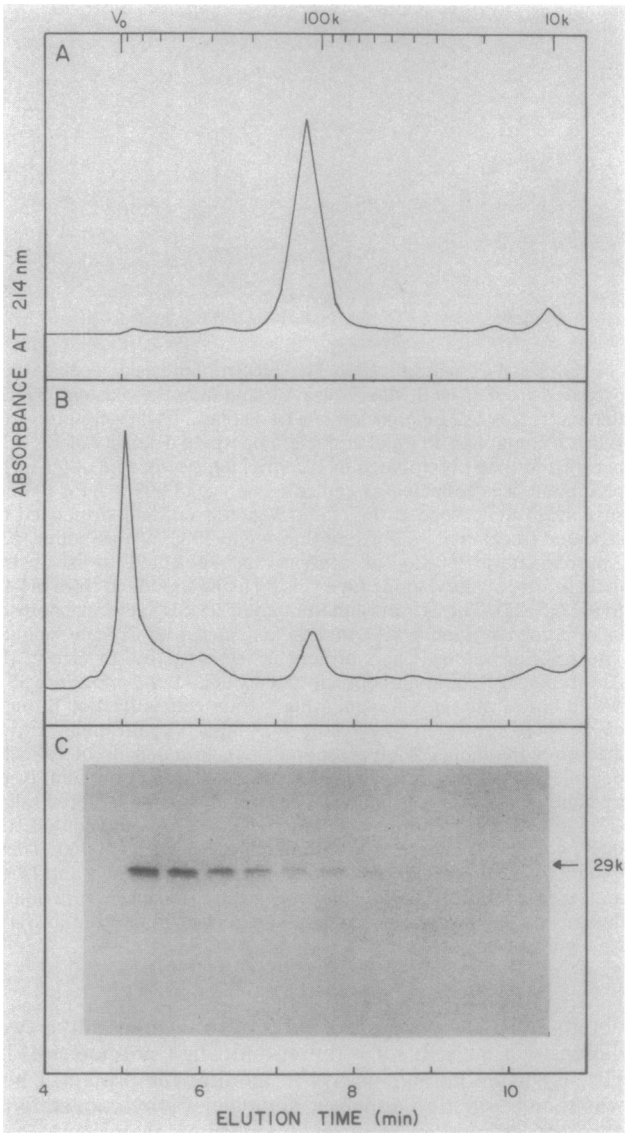

FIG. 4. Molecular sieve chromatography of pure CheZ and CheZ in cell extracts. Samples were applied to a Waters Protein-Pak 300 SW column equilibrated with 0.1 M sodium phosphate, pH 7.0. The flow rate was 1.0 ml/min at room temperature, and detection was at 214 nm. The molecular-weight scale at the top of the figure was determined from calibration of the column with protein standards: equine spleen ferritin, 440 kDa; bovine immunoglobulin G, 158 kDa; bovine serum albumin, 67 kDa; chicken egg albumin, 43 kDa; bovine pancreas α chymotrypsinogen, 25 kDa; and equine skeletal muscle myoglobin, 17 kDa. V_0, Void volume; k, kilodaltons. (A) CheZ, purified from MM294 containing the *cheZ* expression vector pGK24 as described in Materials and Methods. (B) CheZ, purified from wild-type *S. typhimurium* ST1 by immunoaffinity chromatography as described in the legend to Fig. 3. (C) A cell extract of wild-type *S. typhimurium* ST1 (100 μl) was chromatographed as described in the legend to Fig. 3, and fractions were collected at 0.25-min intervals beginning at time zero. A 50-μl sample of every other fraction was electrophoresed in 15% polyacrylamide and analyzed by immunoblotting with polyclonal anti-CheZ antibody and [^{125}I]protein A as described previously (38). Even fractions 18 to 40 corresponding to 0.25-min intervals from 4.5 to 10 min are shown on the autoradiogram.

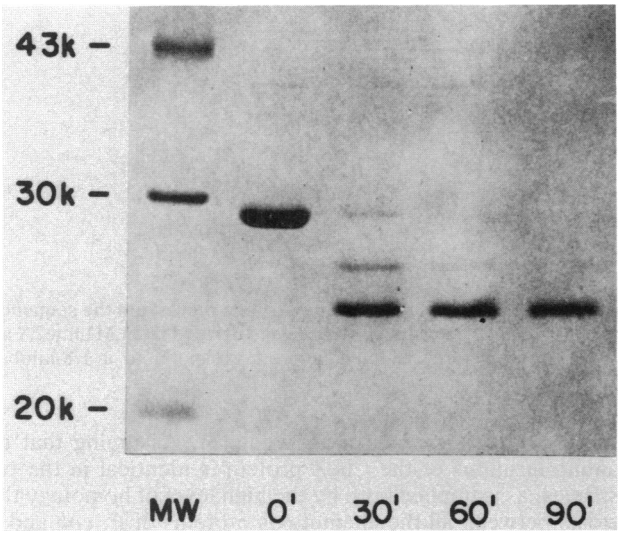

FIG. 5. SDS-polyacrylamide gel electrophoresis of pure CheZ digested with a limiting quantity of trypsin. Pure CheZ (50 μg), obtained by immunoaffinity chromatography, was incubated with trypsin (0.12 μg) at 30°C. At the indicated times (given in minutes), samples were removed and the reaction was quenched by heating in 2% SDS at 100°C for 10 min. Samples (10 μg) were electrophoresed in 15% polyacrylamide, and the gel was stained with Coomassie blue. k, kilodaltons.

elsewhere (30) probably results from cosedimentation of large CheZ aggregates with these fractions.

The purified CheZ protein migrates with an apparent molecular weight of 29,000 during SDS-polyacrylamide gel electrophoresis (Fig. 2). This value differs significantly from the molecular weight of 24,000 reported by Silverman and Simon (37) in their original characterization of the products of the cloned *E. coli* chemotaxis genes. In those studies, another protein with an apparent molecular weight of 29,000 was identified as the product of the *cheR* gene. Later work with the cloned *S. typhimurium* genes identified a 29-kDa species as CheZ, while a minor species with an apparent molecular weight of 30,000 was associated with CheR (9). Both *E. coli* and *S. typhimurium* CheZ proteins exhibited apparent molecular weights of 29,000 in immunoblot analyses of extracts from wild-type strains. The previously identified 24-kDa species appears to be a proteolytic fragment of CheZ. In our initial attempts to purify CheZ, conversion of the 29-kDa protein to a fragment with an apparent molecular weight of 24,000 was observed. Addition of phenylmethylsulfonyl fluoride prevented this process. Moreover, this fragment was obtained when purified CheZ was subjected to limited proteolysis with trypsin (Fig. 5). The truncated form is relatively stable to further proteolytic degradation and probably represents a distinct domain of CheZ structure. N-terminal amino acid analysis of the fragment produced by limited tryptic digestion yielded a sequence of 14 amino acids that was identical to that obtained from the intact protein. Thus, it appears that the cleavage occurs near the C terminus of CheZ. The apparent native molecular weights of the proteolytically degraded pure CheZ protein were approximately 75,000, 185,000, and >500,000. Thus, the cleaved C-terminal region appears to be peripheral to the complex.

The amino acid composition of the purified CheZ protein corresponds well to that predicted from the nucleotide sequence of the *cheZ* gene (Table 1). The amino acid composition is not unusual except for the absence of cysteine residues. N-terminal analysis of the purified CheZ protein yielded a 15-amino-acid sequence that matched that predicted by the nucleotide sequence of the gene (see Fig. 7). The first cycle of Edman degradation produced an additional phenylthiohydantoin derivative in an approximately equimolar amount to phenylthiohydantoin-methionine. This was shown to be phenylthiohydantoin-*N*-monomethyl-methionine (Stock et al., in press). Thus, CheZ is covalently modified at its amino terminus by N methylation of a methionine residue.

Nucleotide sequence of the *cheZ* gene. The *cheZ* gene is located at the distal end of an operon, designated Meche, that encodes the genes for a chemoreceptor, *tar*, and four cytoplasmic chemotaxis proteins. A fragment of the cloned operon was used to determine the sequence of *cheZ* and the region beyond the operon. A 1.8-kilobase *Tth*111I-*Bgl*II fragment containing *S. typhimurium cheZ* was inserted in both orientations into the polylinker region of phage M13mp10, and two sets of phage with processive, unidirectional deletions through the insert were generated with exonuclease III. By using the deletion phage as templates, the nucleotide sequence of *cheZ* and flanking regions was determined on both strands by the dideoxynucleotide chain termination procedure (32) (Fig. 6). The nucleotide sequence of *cheZ* together with the predicted amino acid sequence of the gene product are shown in Figure 7.

The initiation codon of *cheZ* is positioned just 10 bases downstream of the termination codon of the preceding gene, *cheY*. In fact, the termination codon of *cheY* overlaps by two bases the Shine-Dalgarno sequence (35, 44) GAGG that is probably the ribosomal binding site for translational initiation of *cheZ*. The three Met codons AUGAUGAUG, five bases downstream from this site, provide some ambiguity about the actual start of *cheZ* translation. Although the

TABLE 1. Amino acid composition of CheZ

Amino acid	Residues/molecule	
	Predicted[a]	Obtained[b]
Ala	23	23.6 ± 0.4
Arg	15	16.5 ± 0.8
Asx	28	27.9 ± 0.8
Cys	0	ND[c]
Glx	35	35.5 ± 1.2
Gly	9	10.2 ± 0.6
His	2	2.4 ± 0.2
Ile	12	10.8 ± 2.3
Leu	23	21.0 ± 1.0
Lys	6	6.1 ± 0.3
Met	10	6.8 ± 1.1[d]
Phe	5	4.6 ± 0.2
Pro	9	10.3 ± 0.3
Ser	13	13.8 ± 0.4
Thr	9	9.8 ± 0.1
Trp	2	ND
Tyr	1	0.7 ± 0.3
Val	12	11.1 ± 1.1

[a] Predicted from the nucleotide sequence of the *cheZ* gene given in Fig. 7.
[b] Samples of purified protein were hydrolyzed in 6 N HCl at 110°C under vacuum in a Waters Pico-Tag work station (Waters Associates, Inc., Milford, Mass.). Amino acid derivatives were prepared with phenyl isothiocyanate and were quantified by high-pressure liquid chromatography (with detection at 254 nm) against an amino acid standard by the procedure of Bidlingmeyer et al. (3). Values are given as averages ± standard deviation after 18, 48, and 72 h of hydrolysis.
[c] ND, Not determined.
[d] The low value probably reflects oxidation of Met during the hydrolysis step.

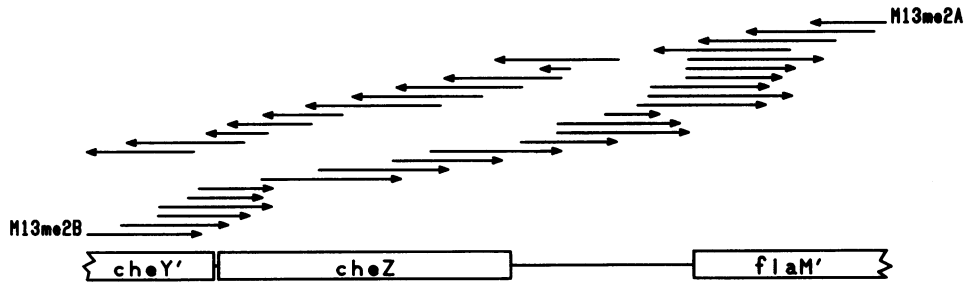

FIG. 6. Deletion phage used in determination of the nucleotide sequence of *cheZ* and flanking regions. Each line represents the sequenced region of a phage insert; the arrowheads indicate the direction of sequencing. Sequences determined from phage derived from M13me2A and M13me2B correspond to the coding and noncoding strands, respectively. The genes diagrammed below correspond to a 1.8-kilobase *Tth*111I-*Bgl*II fragment subcloned from the pUC12-*S. typhimurium* plasmid, pME1.

second AUG codon is spaced most optimally relative to the Shine-Dalgarno sequence, the first and third codons do not fall outside the range observed for translational initiation sites. N-terminal analysis indicates two methionine residues at the amino terminus of the mature protein. This eliminates the third AUG and favors the second as the initiation codon. Comparison of the *S. typhimurium cheZ* initiation region with the corresponding region of the *E. coli* sequence (21) strongly supports designation of the second AUG as the initiation codon. The region extending from the *cheY* termination codon to the putative *cheZ* initiation codon shows identity at 14 of 16 positions (asterisks indicate lack of identity):

S. typhimurium UGAGGAUGCGAUGAUG
* *
E. coli UGAGGAUGCGACUAUG

The two bases which differ correspond to bases of the first AUG in the *S. typhimurium* sequence. Assuming that the amino terminus of the CheZ protein is identical in the two species, as is implied both by the high level of homology that exists between all the chemotaxis proteins of *E. coli* and *S. typhimurium* and by the occurrence of the same covalent modification at the N-terminal residue of CheZ in both species (36; Stock et al., in press), the second AUG is most likely the site of translational initiation of *S. typhimurium cheZ*.

The predicted amino acid sequences of *S. typhimurium* and *E. coli* CheZ are highly homologous, with only 15 differences among 214 residues (Fig. 8). Although the two proteins exhibit 93% identity, the genes which encode them exhibit only 83% identity. This value is similar to that predicted for complete randomization at the nucleotide level given the constraints imposed by conservation of amino acid sequences and bias in codon usage. Thus, it seems likely that the differences between the proteins of the two species are

```
                                                                    -1
                                                TGAGAAACTGGGCATGTGAGGATGCGATG
                                                CheY-->LeuGlyMet
                                  54                                108
ATGATGCAACCATCTATCAAGCCTGCTGATGAAGGCTCAGCAGGAGACATCATTGCGCGCATCGGTAGTCTGACCCGAATGCTGCGCGACAGCCTGCGTGAACTGGGG
MetMetGlnProSerIleLysProAlaAspGluGlySerAlaGlyAspIleIleAlaArgIleGlySerLeuThrArgMetLeuArgAspSerLeuArgGluLeuGly
 * * * *   * * * * * * * *   * *
                                  162                               216
CTGGACCAGGCGATTGCCGAAGCGGCGGAGGCGATCCCTGATGCGCGCGACCGTCTGGATTATGTCGTCCAGATGACGGCGCAGGCGGCGGAACGCGCGCTAAACAGT
LeuAspGlnAlaIleAlaGluAlaAlaGluAlaIleProAspAlaArgAspArgLeuAspTyrValValGlnMetThrAlaGlnAlaAlaGluArgAlaLeuAsnSer
                                  270                               324
GTCGAAGCCTCGCAGCCGCATCAGGATGCGATGGAAAAAGAGGCGAAAGCGCTAACCCAACGTTGGGACGAGTGGTTTGACAATCCGATCGAGCTTTCCGACGCCCGT
ValGluAlaSerGlnProHisGlnAspAlaMetGluLysGluAlaLysAlaLeuThrGlnArgTrpAspGluTrpPheAspAsnProIleGluLeuSerAspAlaArg
                                  378                               432
GAACTGGTGACGGATACGCGCCAGTTTCTCAGGGATGTCCCGGGCCATACCAGCTTTACTAACGCGCAACTGCTGGACATCATGATGGCGCAGGATTTCCAGGATCTG
GluLeuValThrAspThrArgGlnPheLeuArgAspValProGlyHisThrSerPheThrAsnAlaGlnLeuLeuAspIleMetMetAlaGlnAspPheGlnAspLeu
                                  486                               540
ACGGGTCAGGTGATCAAACGCATGATGGACGTGATTCAGGAGATTGAACGCCAGTTGTTAATGGTGCTGCTGGAAAACATCCCGGAACAGAGCGCGCGACCAAAACGC
ThrGlyGlnValIleLysArgMetMetAspValIleGlnGluIleGluArgGlnLeuLeuMetValLeuLeuGluAsnIleProGluGlnSerAlaArgProLysArg
                                  594
GAGAACGAAAGCTTGCTCAACGGCCCGCAGGTGGATACCTCGAAAGCTGGCGTCGTTGCCAGTCAGGATCAGGTGGACGACCTGCTGGACAGTCTTGGCTTTTAA
GluAsnGluSerLeuLeuAsnGlyProGlnValAspThrSerLysAlaGlyValValAlaSerGlnAspGlnValAspAspLeuLeuAspSerLeuGlyPhe
```

FIG. 7. Nucleotide sequence of *cheZ* and the predicted amino acid sequence of its protein product. The nucleotide sequence is numbered relative to the first base of the *cheZ*-coding region. The termination codon of *cheY* is underlined, and a possible ribosomal binding site preceding *cheZ* is boxed. The amino acid sequence of pure CheZ protein determined by sequential phenyl isothiocyanate degradation with an Applied Biosystems Gas Phase Analyzer is indicated by asterisks; serine residues were not identified because of the relative instability of the phenylthiohydantoin-serine derivatives.

FIG. 8. The nucleotide sequences of *S. typhimurium* and *E. coli* (21) *cheZ* genes were compared by using the NUCALN program (53). Differences in corresponding bases are indicated by solid lines, and amino acid differences predicted from divergent codons are shown in one-letter code.

not randomly distributed but rather occur at positions at which they can be tolerated, in contrast to positions within conserved regions which are crucial to the function of the protein. In view of this, it is interesting to note the cluster of nonidentities that occurs in the middle of the CheZ protein.

The acidic, hydrophilic nature of CheZ is apparent from its predicted amino acid sequence and composition (Table 1). In general, hydrophilic residues are overrepresented and hydrophobic residues are scarce. A hydrophobicity profile of CheZ generated by the equations of Kyte and Doolittle (17) demonstrates the extreme hydrophilicity of CheZ (Fig. 9). The protein contains very few regions of significant hydrophobic character. This profile argues against CheZ being a weakly anchored integral membrane protein, one of the possibilities previously suggested by the partitioning of CheZ between the cytoplasm and inner membrane fractions of broken cells (30). The predicted isoelectric properties of the *cheZ* gene product correspond well with empirical observations. CheZ is a very acidic protein with a predicted pI of approximately 4.6 and a net charge of −18 at neutral pH. Thus, in addition to being one of the most acidic of all *E. coli* proteins, CheZ also is distinguished by having one of the highest charge densities. The reduced electrophoretic mobility of CheZ relative to its calculated molecular weight may reflect these unusual electrostatic properties.

Nucleotide sequence of the region beyond *cheZ*. Previous studies have indicated that *cheZ* is the most distal gene of the *S. typhimurium* Meche operon (9), and genetic mapping indicates that the *flaM* operon (*flaG* in *E. coli*) lies downstream (13, 15, 16, 55). Approximately 50 base pairs downstream from the termination of *cheZ* is a region with several dyad symmetries (Fig. 10). Formation of stem-loop structures in the corresponding transcript might be involved in termination of the Meche operon. Though the region is relatively rich in T residues (six contiguous residues just 2 base pairs past the cheZ termination codon and another six contiguous residues 28 base pairs downstream from the most distal dyad symmetry), there are no runs of T residues adjacent to the dyad symmetry as would be expected of a Rho-independent termination site. The most extensive dyad symmetry beyond *cheZ* is homologous to the consensus repetitive extragenic palindromic sequence estimated to be present at greater than 500 copies in noncoding regions of the *E. coli* and *S. typhimurium* genomes (45). Whether this palindrome delineates the end of the Meche operon or is otherwise involved in transcriptional regulation remains to be determined.

Just 10 base pairs beyond the TAA termination codon of *cheZ* is the beginning of an open reading frame that extends for more than 800 base pairs, as far as the sequence has been determined. Since extensive open reading frames are uncommon in noncoding regions, it seemed likely that another operon would be positioned relatively close to the termination of *cheZ*. In fact, genetic analysis has indicated a transcriptional promoter positioned between *cheY* and *flaM* (15). It is not possible to identify the beginning of the coding region, but there are several potential translational initiation sites (underlined in Fig. 10). Certain features tend to favor the ATG at nucleotide position 1049 as the most probable site of initiation. Approximately 300 base pairs downstream from *cheZ* are potential −35 and −10 regions of transcriptional initiation (indicated by * in Fig. 10). A CTGATAT and an appropriately spaced TATTAT sequence bear reasonable homology to the classical promoter consensus sequences TTGACAT and TATAAT. Approximately 65 base pairs downstream from this region is a weak Shine-Dalgarno sequence, GATC, followed 5 base pairs later by the translational initiation codon, ATG. An analysis of codon usage supports the designation of the open reading frame beyond this initiation site as a protein-coding region (Fig. 11). The predicted product of this coding region has an extremely hydrophobic amino acid composition and a hydrophobicity profile consistent with an integral membrane protein (Fig. 12).

Reexamination of the evidence for an interaction between CheZ and the CheB methylesterase. *S. typhimurium* ST171 has probably been the most frequently investigated of all chemotaxis mutants (1, 2, 7, 10, 11, 15, 31, 39, 41, 42, 48, 49, 52, 57). The mutational defect in this strain was originally mapped to the *cheZ* gene and designated the *cheZ221* allele (1). ST171 was later shown to be defective in receptor-demethylating activity (48). Since null *cheZ* mutants exhibited wild-type demethylating activity, and it was definitively shown that a different gene, *cheB*, encodes the demethylating enzyme, it was concluded that CheZ functioned to regulate the demethylation reaction (48). To further investi-

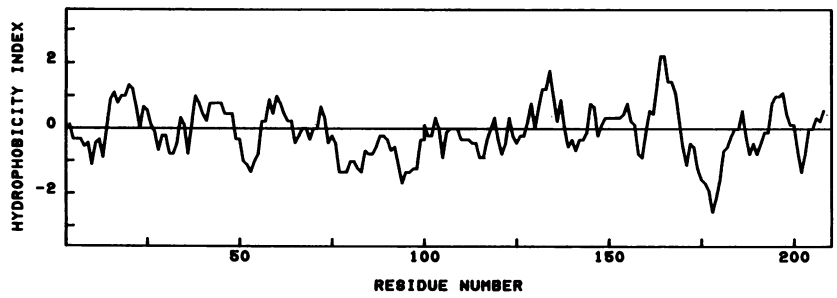

FIG. 9. The hydrophobicity of the CheZ protein was predicted according to the equations of Kyte and Doolittle (17) over a running average of seven residues.

670

TAAGCTTTTTTGACCTGATGGCGCTGCGCTTATCGGGTTCGAATCCGTCAACCCGGCCGGATACGAGACTTTCTTATCGTAT

ProAspGlyAlaAlaLeuIleGlyPheGluSerValAsnProAlaGlyTyrGluThrPheLeuSerTyr

778 832

CCGGCTATCTCTATTAACGCCATAAACCCCGCCTTTTTTACCGCTTACTCTGCCTATTGGCGTAAAGCGGTTCTGGCATCATTCTCTCTTATCAATCTATCCAGGGTT

ProAlaIleSerIleAsnAlaIleAsnProAlaPhePheThrAlaTyrSerAlaTyrTrpArgLysAlaValLeuAlaSerPheSerLeuIleAsnLeuSerArgVal

886 940

TGCTGCGTGGCAGAAGAGAGCGACGACGACAAAACAGAAGCCCCCACACCCCACCGACTTGAAAAAGCGCGGGAAGAAGGGCAGATCCCCCGTTCCAGAGAACTGACC

CysCysValAlaGluGluSerAspAspAspLysThrGluAlaProThrProHisArgLeuGluLysAlaArgGluGluGlyGlnIleProArgSerArgGluLeuThr

******* ****** 994 1048

TCACTGCTGATATTGCTGGTGGGCGTTTGTATTATTTGGTTCGGCGGCGAGTCGTTAGCGCGGCAGCTGGCGGGAATGCTCTCAGCAGGCCTGCACTTCGATCACCGT

SerLeuLeuIleLeuLeuValGlyValCysIleIleTrpPheGlyGlyGluSerLeuAlaArgGlnLeuAlaGlyMetLeuSerAlaGlyLeuHisPheAspHisArg

1102 1156

ATGGTGAACGATCCTAACCTGATCCTGGGGCAGATAATTTTGCTGATTAAAGCGGCGATGATGGCACTGCTACCGCTCATCGCGGGCGTGGTGCTGGTGGCGCTTATC

MetValAsnAspProAsnLeuIleLeuGlyGlnIleIleLeuLeuIleLysAlaAlaMetMetAlaLeuLeuProLeuIleAlaGlyValValLeuValAlaLeuIle

1210 1264

TCGCCGGTTATGCTTGGCGGCCTGATTTTTAGCGGTAAGTCGCTACAGCCAAAATTTTCTAAATTAAACCCGCTGCCGGGAATTAAGCGCATGTTTTCGGCGCAGACC

SerProValMetLeuGlyGlyLeuIlePheSerGlyLysSerLeuGlnProLysPheSerLysLeuAsnProLeuProGlyIleLysArgMetPheSerAlaGlnThr

1318 1372

GGCGCGGAATTGCTAAAAGCGGTGTTGAAATCCACGCTGGTCGGCTGCGTTACCGGCTTTTATCTCTGGCATCACTGGCCGCAAATGATGCGCCTGATGGCGGAGTCG

GlyAlaGluLeuLeuLysAlaValLeuLysSerThrLeuValGlyCysValThrGlyPheTyrLeuTrpHisHisTrpProGlnMetMetArgLeuMetAlaGluSer

1426

CCGATCGTCGCAATGGGGAATGCGCTGGATCTGGTTGGACTCTGCGCGTTACTGGTGGTACTGGGCGTGATTCCGATGGTGGGATTTGACGTGTTTTTCCAGATC

ProIleValAlaMetGlyAsnAlaLeuAspLeuValGlyLeuCysAlaLeuLeuValValLeuGlyValIleProMetValGlyPheAspValPhePheGlnIle

FIG. 10. Nucleotide sequence of the region beyond *cheZ*. Numbering of the nucleotide sequence corresponds to that presented in Fig. 7. Downstream from the termination codon of *cheZ* (underlined) are regions of dyad symmetry (opposing arrows) which may be involved in termination of the Meche operon. An amino acid sequence derived from the nucleotide sequence is indicated below the entire open reading frame. All potential translational initiation codons (ATG and GTG) are underlined, and sequences homologous to the *E. coli* −35/−10 transcriptional promoter consensus are indicated (*).

gate the role of CheZ in demethylation, we undertook an extensive analysis of the ST171 defect. There had been no evidence of a direct interaction between the CheZ and CheB proteins (38, 40). Nevertheless, we subjected extracts of ST171 and wild-type cells to molecular sieve chromatography, and in both cases the CheB methylesterase, detected by immunoassay, eluted with an apparent molecular weight of approximately 36,000, cleanly separated from the CheZ protein. We could find no condition that restored demethylating activity to ST171 extracts, nor could we detect any inhibitory effects of the mutant extracts on the wild-type enzyme. Addition of purified CheZ or anti-CheZ antibodies during assays of either the methylating (CheR) or demethylating (CheB) activities in extracts of wild-type cells had no effect. When a plasmid encoding the wild-type *tar*, *cheR*, and *cheB* genes (pGK2) was introduced into ST171, the resulting construct, ST171(pGK2), produced levels of

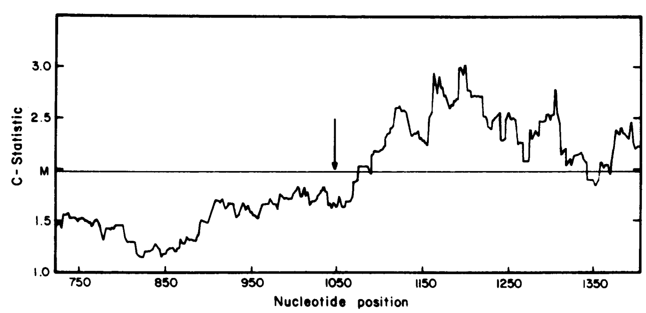

FIG. 11. Identification of a protein-coding region beyond *cheZ*. The region beyond *cheZ* was analyzed by using the protein locator program of Pustell and Kafatos (28). The program was run with a 50-codon window and a codon bias table constructed from the *S. typhimurium* sequences available in the September 1985 Genbank DNA sequence release and our sequences of the *S. typhimurium* Meche genes. Nucleotide positions correspond to those presented in Fig. 10. The C-statistic is a measure of the codon bias; a value of 1.0 corresponds to an unbiased region, and M indicates the minimum value measured for any of the sequences used to construct the codon bias table. Values higher than M generally indicate protein-coding regions. The arrow marks the position of the ATG codon at nucleotide position 1049 of Fig. 10.

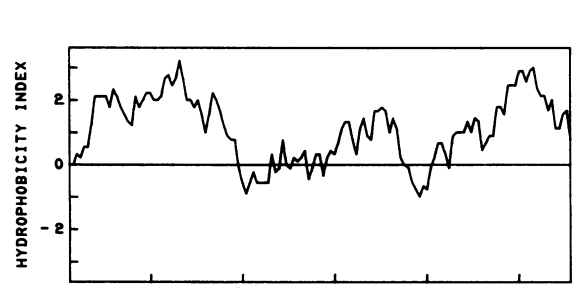

FIG. 12. The hydrophobicity of the predicted gene product encoded by nucleotides 1049 to 1477 of Fig. 10 was determined by the method of Kyte and Doolittle (17) over a running average of seven residues.

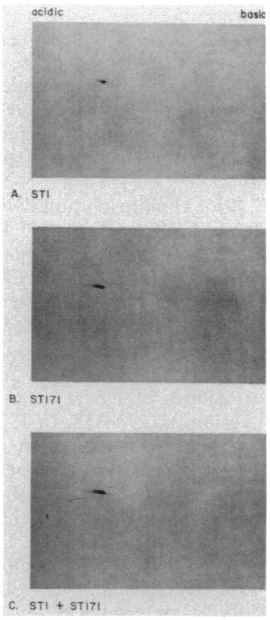

FIG. 13. Two-dimensional gel analysis of CheZ proteins immunoprecipitated from ST1 and ST171. Immunoprecipitates from wild-type ST1 and *che* mutant ST171 were prepared as described in the legend to Fig. 1B. Immunoprecipitates were solubilized in lysis buffer (22) by repeated freezing (−70°C) and thawing (30°C), subjected to isoelectric focusing in a pH gradient of 4.0 to 6.0 in polyacrylamide tube gels, equilibrated in 2% SDS, and electrophoresed in SDS–15% polyacrylamide slab gels. The dried gels were autoradiographed.

esterase activity that were comparable to those produced by pGK2 in a wild-type *S. typhimurium* strain. At this point, we began to consider that the *cheZ221* lesion had been misclassed and was instead a mutation in *cheB*. CheZ from the mutant and wild type showed identical mobilities during two-dimensional polyacrylamide gel electrophoresis (Fig. 13). Furthermore, complementation and recombination analyses with a set of previously defined deletions of the *S. typhimurium che* region (46) showed that ST171 both complemented and gave Che^+ recombinants with deletions extending through *cheZ* into *cheY* but was unable to complement or yield Che^+ recombinants with *cheB* deletions (Table 2). From these results, we conclude that ST171 contains a mutation in *cheB* and not in *cheZ*.

TABLE 2. Genetic mapping of *che221* mutation in ST171

Tester strains[a]	Relevant genotype	Complementation[b]	Recombination[c]
PS221/1100	Δ*flaCflaM*	+	+
PS1278/1103	Δ*flaC-cheZ*	+	+
PS1280/1259	Δ*flaC-cheY*	+	+
PS1275/1109	Δ*flaC-cheB*	−	−
PS1276/1250	Δ*flaC-cheB*	−	−
PS1277/1051	Δ*flaC-tar*	−	−

[a] Tester strains were deletions described previously (46). The two strains of each set differ only in that the first contains a *recA* mutation. These were used for complementation tests, while the corresponding $RecA^+$ variants were used to score for Che^+ recombinants.

[b] Complementation was assessed by streaking *recA* tester cells with P22HT phage grown on ST171 across a soft agar plate by the procedure of Aswad and Koshland (1).

[c] Recombination analyses were done by transducing each of the Tet^s deletion mutants to Tet^r by using P22HT phage grown on PS108 *che-221 zea-2*::Tn*10*. In each case, more than 200 recombinants were individually screened for swarming ability. A minus score indicates that no Che^+ recombinants were detected.

DISCUSSION

We have sequenced the *S. typhimurium cheZ* gene and have purified and characterized its protein product. The sequence of the *cheZ* gene predicts an acidic, hydrophilic, 23,900-molecular-weight protein, properties that correlate with those of purified CheZ. N-terminal amino acid analysis of the purified protein yields a 15-residue sequence identical to that deduced from the nucleotide sequence of the *cheZ* gene. However, the first amino acid of CheZ, a methionyl residue, is modified by N methylation in approximately 50% of the CheZ monomers (Stock et al., in press). The apparent molecular weight of CheZ determined by SDS-polyacrylamide gel electrophoresis is 29,000, but a much larger molecular weight is observed under native conditions. Molecular sieve chromatography indicates a variety of aggregated states with a minimum molecular weight of approximately 115,000 and a maximum exceeding 500,000. CheZ is cleaved specifically by a variety of proteases yielding a stable N-terminal product that migrates with an apparent molecular weight of 24,000 during SDS-polyacrylamide gel electrophoresis. This protease sensitivity suggests a domain structure within the CheZ protein. Although limited proteolysis causes an apparent decrease in the size of CheZ homopolymers, the multimers do not dissociate. Thus, it appears that the C-terminal region of the protein is not the locus for interaction between CheZ monomers.

CheZ appears to be an essential component of the chemotaxis system, although its molecular role is unknown. Strains with mutations in *cheZ* generally, but not always (54), exhibit a tumbly behavior. This contrasts with the smooth-swimming phenotypes of mutants with defects in the other *che* genes required for signal transduction, *cheA*, *cheW*, and *cheY* (24, 52). Moreover, *cheZ* mutants respond to chemoeffector stimuli, whereas *cheA*, *cheW*, and *cheY* mutants do not. Previous reports of *cheZ* mutants with methylesterase defects suggested an involvement of CheZ in the receptor methylation system, presumably through interaction with the CheB methylesterase. Our findings support those of Kutsukake and Iino (15) that *S. typhimurium* ST171 is not a *cheZ* mutant and that the lack of esterase activity results from a mutation in *cheB*.

Suppressor mutations of genes required for flagellar motor function (*S. typhimurium flaAII.2* and *flaQ* and *E. coli flaA*) have been found to map to *cheZ* (26, 54), and conversely *cheZ* suppressor mutations have been mapped to the *E. coli flaA* and *flaB* genes (27). These data have led to the suggestion that CheZ interacts directly with the flagellar motor to bias the direction of flagellar rotation. Other observations (33, 34) have indicated that CheZ inactivates the receptor-generated signal for tumbly behavior since *cheZ* mutants exhibit increased response latencies to receptor stimulation. It is thought that CheY acts directly at the level of the flagellar motor to effect tumbly behavior (8, 27, 29), and recent results suggest the CheZ may function to counteract the effects of CheY to bias the motor toward smooth swimming (14). The unusual physical properties of CheZ, specifically, its high charge density and tendency for self-aggregation, suggest that CheZ may be a structural component of the switching apparatus at the flagellar motor.

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