Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein

(SEUNG K. KIM AND DALE KAISER)

Departments of Biochemistry and Developmental Biology, Stanford University School of Medicine, Stanford, California 94305

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**ABSTRACT**

C-factor, a *Myxococcus xanthus* protein that restores the developmental defects of a class of nonautonomous mutants resulting from mutation of the csgA gene, has been purified approximately 1000-fold from starved wild-type cells. The monomeric form of C-factor is a single polypeptide with a molecular mass of 17 kDa that can be solubilized by detergent from membrane components. Characterization by gel filtration and denaturing gel electrophoresis suggests that biologically active C-factor is a dimer composed of two 17-kDa monomers. Antibodies against a form of the *M. xanthus* csgA gene product overexpressed in *Escherichia coli* cell react with purified C-factor.

Multicellular organisms establish cell fate by cell-cell interactions (1–3). One approach to the biochemistry of these interactions begins with purification and characterization of the relevant signal molecules. Biochemical studies of the cell interactions which coordinate multicellular differentiation of the Gram-negative bacterium *Myxococcus xanthus* benefit from that organism's prokaryotic cellular organization and genetic system (4). Starvation of *M. xanthus*, like that of all myxobacteria, activates a multicellular program in which roughly 10^7 cells move coordinately into centers of aggregation where they build a structure having a specific shape called a fruiting body. Some cells lyse, while other cells in the nascent fruiting body differentiate into dormant ovoid spores (5).

C-factor (csg C signal) mutants are non-cell-autonomous developmental mutants that cannot sporulate alone but are rescued to sporulate by development with wild-type cells (6–8). The rescue of sporulation by wild-type cells does not involve genetic exchange but rather occurs extracellularly. All existing members of the csg class have resulted from mutation at a single genetic locus named csgA (8, 9). DNA sequencing of a reading frame identified by codon usage indicates that csgA could specify a 17.7-kDa protein (10).

Under submerged culture conditions (11) csgA mutants fail to construct any detectable multicellular structures, to lyse, or to sporulate (12). Mutations in csgA not only block morphological development but also alter the pattern of developmental gene expression as monitored by *Tn5lac*, a transposable promoter probe which can generate transcriptional fusions of *lacZ* to developmentally regulated genes (13–15). All the developmental defects resulting from mutation of csgA are overcome by development with wild-type cells (15). Thus, the csgA protein may be involved in production or transmission of a developmental signal that is crucial for normal fruiting body morphogenesis, cellular differentiation, and developmentally regulated gene expression.

Rescue of the developmental defects of mutants by admixed wild-type cells has led to the proposal that *M. xanthus* passes signal molecules from cell to cell to coordinate formation of a multicellular fruiting body. If so, csgA mutant cells might be used to monitor purification of molecules from csgA protein(s) for the fruiting body development. Here we describe purification of C-factor and its chemical characterization.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth.** *M. xanthus* strain DK5204, a kanamycin-resistant (Km'), developmentally competent strain which contains Tn5lac at position Ω4435 (14) and was used as a source of C-factor, was grown and harvested as described (20). *M. xanthus* strain DK5233 (csgA, Km'), used as the responder strain in the assay for C-factor development rescuing activity, has been previously described (15).

**Chemicals and Chromatography Materials.** Deoxycholate, cholate, octylglucoside, 3-(N-morpholino)propanesulfonate (Mops), 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), kanamycin sulfate, and gel electrophoresis standards were from Sigma. Dialysis tubing with a nominal molecular mass cutoff of 8 kDa was from Spectrum (Los Angeles). YM10 membranes and a Centricon 10 microconcentrator were from Amicon. FPLC Mono Q HR 5/5 and FPLC Superose 12 HR 10/30 columns were from Pharmacia. *Staphylococcus aureus* V8 protease was from Boehringer Mannheim. Gel filtration standards were from Bio-Rad. Protein concentrations were estimated by the Bio-Rad protein assay according to manufacturer’s suggestions, using bovine IgG as standard.

**Assay for C-Factor Activity.** C-factor was assayed for its ability to restore multicellular aggregate formation and sporulation to csgA mutant cells. The csgA strain DK5253 was used. Each sample of C-factor was dialyzed against 4 liters of 10 mM Mops/1 mM CaCl₂/4 mM MgCl₂, pH 7.2 (buffer A), containing 50 mM NaCl for 12–18 hr at 4°C. Aliquots of the dialyzed samples were serially diluted, six to eight times, in a 2-fold series. A 400-μl aliquot of each dilution was warmed to 32°C, then added as previously described to responder csgA cells that had been developing in submerged culture (11). One unit of activity is defined as the amount of C-factor that restores wild-type fruiting body formation (200–300 sporulating bodies per 2.5 × 10⁶ input cells) and sporulation (2 × 10⁶ spores per 2.5 × 10⁸ input cells) to csgA mutants developing in submerged culture. Fruiting bodies were scored visually at 6 × magnification by using a dissecting microscope (Wild-Heerbrug, Switzerland). The presence of ovoid refractile spores within fruiting bodies at the bottom of a microtiter well was confirmed with a Leitz inverted light microscope at 40 × magnification. Heat-resistant, sonication-

**Abbreviations:** CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; Km', kanamycin-resistant.

3635
resistant, Km2 spores were quantified as previously described (14).

**Purification of C-Factor.** All manipulations were performed at 4°C. A 100-ml frozen suspension of wild-type cells conditioned on developmental medium (20) was alternately thawed and frozen three times, then sonicated on ice with eight 15-sec bursts (Branson microtip, setting 3). The resulting lysate was centrifuged for 1 hr at 100,000 × g. The supernatant was discarded and the pellet was gently resuspended in 80 ml of buffer A (fraction I).

This resuspended material was brought to 1.2% (wt/vol) CHAPS in 100 ml final volume. A concentration of 1.2% CHAPS was found to be optimal for solubilizing activity. This suspension was agitated gently for 18 hr, then centrifuged at 100,000 × g for 1 hr. The volume of the clear amber supernatant was reduced to 10 ml by ultrafiltration through an Amicon YM10 membrane (10-kDa molecular mass cutoff). This concentrated material is active for several days at 4°C. Yield and purification are based on solubilized activity (fraction II), which is more reliably measured than activity in fraction I.

Ten milliliters of C-factor in fraction II was diluted 3-fold in ice-cold buffer A. Approximately 8 ml of this sample was applied to an FPLC Mono Q HR 5/5 column equilibrated in 20 mM Mops/2 mM CaCl2/0.4% CHAPS, pH 7.0 (buffer B).

The column was washed with 15 ml of buffer B and C-factor was subsequently eluted with a 0–0.35 M NaCl linear gradient (20 ml) in buffer B. C-factor was eluted in a broad peak at approximately 250 mM NaCl (fraction III). Rapid 10-fold dilution in buffer A of material eluted by the salt gradient prevents precipitation of insoluble aggregates which otherwise form over the next several days. In this and all subsequent steps the effluent was monitored by the absorbance at 280 nm, using a variable-wavelength UV detector (Pharmacia LKB).

Active C-factor in fraction III was pooled (12 ml) and concentrated to 800 µl by 2- to 4-hr centrifugation at 5000 × g in a Centricon 10 microconcentrator. The concentrated sample was applied to 100-µl aliquots to a Superose 12 HR 10/30 gel filtration column (previously calibrated with standards ranging from 1.3 to 230 kDa) that had been equilibrated with buffer A containing 100 mM NaCl. Active fractions containing the 17-kDa C-factor polypeptide (partition coefficient Xw = 0.55) were pooled (fraction IV).

The pool of C-factor in fraction IV (8 ml) was dialyzed twice against 1-liter portions of 40 mM Tris-HCl, pH 8.0/4 mM MgSO4/0.4% CHAPS (buffer C). The dialyzed sample was applied to an FPLC Mono Q HR 5/5 column equilibrated in buffer C. The column was washed with 10 ml of buffer C and C-factor was subsequently eluted with a 0–0.35 M NaCl linear gradient (20 ml) in buffer C. C-factor was eluted at approximately 270 mM NaCl (fraction V).

**Molecular Mass Determination.** The molecular mass of purified *M. xanthus* C-factor was determined by gel filtration on a Superose 12 HR 10/30 column. Buffer B was used as the mobile phase. Samples in 50 µl were dissolved in buffer B and the column was eluted at a flow rate of 0.5 ml/min. The eluate was collected in 0.5-ml fractions and UV absorbance was monitored at 280 nm. In addition to UV absorbance, activity measurements were used to confirm the location of the C-factor peak.

**Other Methods.** C-factor purification was analyzed by SDS/PAGE using 15% acrylamide and 0.12% bisacrylamide (16). Samples and a set of reference proteins were reduced and denatured by heating for 90 sec at 100°C in 1% SDS/0.24 M dithiothreitol prior to electrophoresis. Silver staining was performed as previously described (17) except that 0.004% KMnO4 was substituted for K2Cr2O7. Proteolytic cleavage of C-factor with *S. aureus* V8 protease was performed under conditions described by the distributor, Boehringer Mannheim. Western blotting analysis was performed (18) with primary rabbit antibodies to the fusion protein encoded by lacZ-csgA; the antibodies were provided by L. Shimkets (University of Georgia). Secondary goat anti-rabbit-IgG antibodies conjugated to alkaline phosphatase were from Bio-Rad.

**RESULTS**

**Purification of *M. xanthus* C-Factor.** Table 1 summarizes the purification of C-factor from *Myxococcus xanthus* cells conditioned on solid starvation medium. The overall purification in four steps is over 1000-fold and the yield is 10%. To monitor purification, the activity that rescues multicellular aggregation of *csgA* mutants and differentiation of *csgA* mutant cells into spores was measured by bioassay. The bioassay response, as illustrated in Fig. 1, is not linear with respect to the amount of C-factor added. Nonlinearity was also observed in cell–cell mixtures of intact wild-type cells and *csgA* mutant cells (data not shown). Accordingly, the assay was conducted by end-point dilution in 2-fold steps over a 500-fold range of concentration. Nonlinearity was observed at all stages of purification. The response to C-factor from the last purification step is shown in Fig. 1.

Activity present in untreated cell lysates made from starved wild-type cells (fraction I) was found to be entirely sedimented after centrifugation for 1 hr at 100,000 × g. A key step that allowed subsequent chromatography of C-factor was solubilization of active material by the detergent CHAPS (Fig. 2). Solubilization of activity was optimal at 1.2–1.5% (wt/vol) CHAPS, with loss of biological activity at higher detergent concentrations. Activity was not solubilized after similar treatment of cell lysates with 1.0 M NaCl and 1.0 M KCl, and the yields of activity solubilized with the detergents deoxycholate, cholate, and octyl glucoside over a wide range of concentrations were relatively low (data not shown). Ultrafiltration eliminated most impurities (fraction III), with molecular masses below 10 kDa (fraction II). Because the material in fraction II had a tendency to aggregate in the absence of CHAPS, all subsequent chromatography steps were preceded by extensive washing of column matrices with buffer containing CHAPS (see Materials and Methods). C-factor appeared among the proteins adsorbed on an FPLC Mono Q anion-exchange column and was eluted near 0.25 M NaCl by a linear salt gradient at neutral pH (fraction III). C-factor was present in the major peak of protein eluted from a Superose 12 gel filtration column (fraction IV) and was separated from the bulk of remaining high molecular weight

![Table 1. Purification of *M. xanthus* C-Factor](image_url)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Purification step</th>
<th>Protein, mg</th>
<th>C-factor, units</th>
<th>Specific activity, units/mg</th>
<th>Purification, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sonication</td>
<td>48.3</td>
<td>1000</td>
<td>20.7</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>CHAPS solubilization, ultrafiltration</td>
<td>16.0</td>
<td>600</td>
<td>37.5</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>Mono Q anion exchange, pH 7</td>
<td>1.15</td>
<td>480</td>
<td>417</td>
<td>11</td>
</tr>
<tr>
<td>IV</td>
<td>Superose 12 gel filtration</td>
<td>0.072</td>
<td>240</td>
<td>3200</td>
<td>90</td>
</tr>
<tr>
<td>V</td>
<td>Mono Q anion exchange, pH 8</td>
<td>0.0024</td>
<td>100</td>
<td>41600</td>
<td>1110</td>
</tr>
</tbody>
</table>

See Materials and Methods for details.
contaminating proteins (Fig. 3b). The final step in the purification was anion-exchange chromatography at pH 8.0 on an FPLC Mono Q column (Fig. 4a). C-factor was recovered as a single sharp symmetric peak on the final step of ion-exchange chromatography (fraction V). Analysis of C-factor by denaturing SDS/PAGE and silver staining showed that purified C-factor is a single species with a molecular mass of 17 kDa which coincided with the peak of rescuing activity eluted during this last chromatographic step (Fig. 4b).

**Stability and Inactivation of C-Factor.** Purified C-factor (0.5 µg/ml in 40 mM Tris-HCl, pH 8.0/4 mM MgSO4/250 mM NaCl/0.4% CHAPS) was stable for at least 1 month at 0°C. Storage at −80°C for a similar period resulted in formation of an insoluble precipitate and loss of 90% activity. Purified C-factor diluted to less than 50 ng/ml was stable for several months at −80°C. At less than 5 ng/ml, C-factor retained spore and aggregation rescuing activity after 10 min at 100°C. As shown in Table 2, C-factor activity is completely eliminated by treatment with *S. aureus* V8 protease, but not by heat-inactivated protease, demonstrating that the isolated factor is a polypeptide.

During the course of purifying C-factor we observed that rescuing activity in our submerged culture assay required the presence of Mg2+. Our standard assay conditions for activity include incubation of responder csgA mutant cells in 1 mM Ca2+ and 4 mM Mg2+. Earlier studies (11) showed that *M. xanthus* absolutely requires Ca2+ for development. Ca2+ will not substitute for Mg2+, even when the Ca2+ concentration is increased to 4 mM, suggesting that C-factor specifically requires Mg2+ for activity.

**FIG. 1.** Concentration response of the C-factor assay used to detect restored developmental sporulation and multicellular aggregation of csgA mutant cells. At 6 hr after starvation, when the morphological defects of csgA mutants are first manifest, fractions to be assayed are diluted in a dilution series to a confluent mat of 2.5 × 108 cells. Heat-resistant, sonication-resistant spores and darkened, aggregated mounds of cells called fruiting bodies which appear after 30 hr are counted. Wild-type strain DK1622 forms 2.5 × 106 spores per ml and 300 fruiting bodies under similar conditions.

**FIG. 2.** The detergent CHAPS solubilizes C-factor activity. Aliquots of fraction I containing equal amounts of C-factor activity were gently agitated in the presence of CHAPS at the indicated concentrations (wt/vol) at 4°C for 12 hr prior to centrifugation at 4°C and 105,000 × g for 1 hr. Pelleted material was resuspended in a volume of buffer A equal to the volume of the supernatant. Resuspended pellets and supernatants were dialyzed and assayed. Each data point at the indicated CHAPS concentration represents the percent activity in either supernatant fraction or pellet fraction relative to the sum of activity present in both fractions.

**FIG. 3.** Chromatography of solubilized C-factor. The absorbance profile at 280 nm is shown by the thin unbroken line. Activity, in units, is indicated by open squares. (a) Mono Q anion-exchange chromatography of detergent-solubilized C-factor at pH 7.0 as described in the text. The linear gradient from 0 to 0.35 M NaCl is indicated by the broken line. Only the gradient elution profile is shown. (b) Superose 12 gel filtration of C-factor. Active fractions eluted from anion-exchange chromatography at pH 7.0 were pooled, concentrated, and applied to an FPLC gel filtration column.
**Molecular Mass Determination of Active C-Factor.** The molecular mass of purified, biologically active C-factor was determined by gel filtration on a Superose 12 column. Using standards ranging from 158 to 1,352 kDa, we estimated the molecular mass of active C-factor to be 35 kDa (Fig. 4). As described above, this purified material was composed of a polypeptide with a molecular mass of 17 kDa as estimated by denaturing SDS/PAGE on a 15% acrylamide gel. Behavior under non-denaturing chromatographic and denaturing electrophoretic conditions suggests that C-factor exists in solution as a dimer of 17-kDa monomers and that this form is biologically active.

**Purified Antibody to csgA Product Recognizes Purified C-Factor.** The *M. xanthus csgA* open reading frame has been recently fused at its 5' end to the *Escherichia coli* lacZ gene and overexpressed in *E. coli* (L. Shimkets, personal communication). Antibodies which were raised and affinity-purified against an overexpressed lacZ-csgA fusion protein were tested for their ability to react with purified C-factor. As shown in Fig. 6, purified antibodies to csgA product were found to react with purified C-factor (lane a) and with purified lacZ-csgA fusion protein (lane b). Higher molecular weight forms of both C-factor and lacZ-csgA fusion protein are detected by this "Western" analysis. The size of each higher molecular weight form is approximately twice that of the lowest molecular weight, and presumably monomer, forms.

**DISCUSSION**

Protein fractions solubilized from starved *M. xanthus csgA* cells but not csgA mutants contained an activity called C-factor that allowed csgA mutant cells to complete fruiting.

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**Table 2.** C-factor activity is protease sensitive and magnesium dependent

<table>
<thead>
<tr>
<th>C-factor treatment</th>
<th>Spores, % of wild type</th>
<th>Fruiting bodies, % of wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>V8 protease</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>Inactivated V8 protease</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>1 mM CaCl₂/no MgCl₂</td>
<td>&lt;0.02</td>
<td>0</td>
</tr>
<tr>
<td>4 mM CaCl₂/no MgCl₂</td>
<td>&lt;0.02</td>
<td>0</td>
</tr>
<tr>
<td>1 mM CaCl₂/1 mM MgCl₂</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>4 mM CaCl₂/4 mM MgCl₂</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

Two units of C-factor was added to responder strain DK5253, which is csgA. Buffer includes 1 mM Ca²⁺ and 4 mM Mg²⁺ unless otherwise noted. *S. aureus* V8 protease was inactivated by heating at 100°C for 5 min. Sporulation tests were performed as described previously (14). Wild-type strain DK1622 forms 2.5 × 10⁶ spores per ml and 300 fruiting bodies per culture well under these conditions, and these reference values were used to calculate the percent of wild type in columns 2 and 3.
lacZ-csgA fusion is (10) predicts activity, mutants. coconjugate (19) magnesium is two activities show from chromatography ovoid movement polypeptide sesses both of approximately of identical revealed for Mg2+ polypeptide Purificationplementation of indicated lane alkaline phosphatase this antibody and with antibody and with goat anti-rabbit-IgG antibodies conjugated to alkaline phosphatase (18). Positions of protein markers (sizes in kDa) are indicated on the left. Lane a, ~100–200 ng of purified C-factor; lane b, 1.1 μg of purified lacZ-csgA fusion protein.

body morphogenesis and sporulation. This extracellular complementation of csgA mutants provided an assay for C-factor purification. Purification over 1000-fold to apparent homogeneity revealed that C-factor is a single species composed of a polypeptide with a molecular mass of 17 kDa that requires Mg2+ for activity. Active C-factor is likely a dimer composed of identical 17-kDa monomers as judged by behavior during gel filtration and denaturing gel electrophoresis and by detection of dimer-sized species on a Western blot with an antibody that recognizes C-factor. Active at a concentration of approximately 1 nM, C-factor is, to our knowledge, the first polypeptide isolated from _M. xanthus_ with morphogenetic properties.

Several observations indicate that the same molecule possesses both an activity which allows coordinated multicellular movement into centers of aggregation and an activity which allows rod-shaped cells to differentiate into dormant ovoid spores. First, both activities copurify in all three chromatography steps. Second, both purified activities are coeluted from a Superose 12 gel filtration column. Third, _S. aureus_ V8 protease inactivates both activities. Fourth, the two activities show identical resistance to heating. Finally, magnesium is required for both activities. These properties also distinguish C-factor from peptidoglycan (12) and a glycoconjugate (19) which were previously reported to restore some of the defects of the csg class of developmental mutants.

Several observations contribute to demonstrate that C-factor is encoded by the _csgA_ gene. All known csg class mutations lie in the _csgA_ gene (8). Wild-type cells produce C-factor activity, but _csgA_ cells do not (20). The _csgA_ DNA sequence (10) predicts a primary translation product with a molecular mass of 17,700 Da, in close agreement with the observed molecular mass of purified C-factor polypeptide (Fig. 4b). Polyclonal antibodies raised and purified against a lacZ-csgA fusion protein expressed in _E. coli_ react with monomer and dimer-sized forms of purified C-factor (Fig. 6). Finally, a partial amino acid sequence of purified C-factor matches the predicted sequence of the _csgA_ gene product (10, 20).

This work also begins to address the question of how C-factor is transferred. Cell-mixing studies with csg mutants indicated that this activity is extracellular, suggesting that starved _M. xanthus_ cells normally pass extracellular C-factor to synchronize and coordinate both aggregation and sporulation. Restored _csgA_ mutant development resulting from addition of 1–2 nM exogenous solubilized C-factor argues that this molecule normally acts at low concentration on the cell surface. That C-factor is normally associated with membrane components suggests that only cells immediately contiguous with a cell producing C-factor may respond. The ability to detect and to purify biologically active C-factor should help reveal its mode of action.

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