Component C of the Methylreductase System of Methanobacterium*  
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Component C of the methyl coenzyme M reductase system of Methanobacterium thermoautotrophicum has been purified to homogeneity with a 17% recovery of initial units. The native protein has a molecular weight of 300,000 and is composed of three different subunits with masses of 88,000, 45,000, and 38,500. They are present in equal proportion, suggesting a stoichiometry of \( \alpha_2 \beta_2 \gamma_2 \) in the native protein. The amino acid composition reveals a preponderance of acidic amino acid residues. The protein is yellow, having an absorption maximum at 245 nm and a shoulder at 255 nm. Recombination of the methyl coenzyme M methylreductase activity was linearly dependent on concentration, and component C activity. Functions containing Component C have been detected in cell extracts of other methanogens. Component C has been detected in cell extracts of other methanogens.

The terminal step of methane formation requires the participation of three components: component A, an oxygen-sensitive, \( M_r = 500,000 \) protein complex having hydrogenase activity; component B, an oxygen-sensitive, colorless cofactor; and component C, an oxygen-stable acidic protein. Component C has been identified recently as the 2-(methylthio)ethanesulfonic acid methylreductase (2). In the presence of \( Mg^2+ \), ATP, and hydrogen, these components reduce \( CH_3-S-CoM \) to methane and HS-CoM. Here we report the purification and properties of component C; a preliminary report in abstract form has appeared (3).

**Experimental Procedures**

1. The abbreviations used are: CH-S-CoM, 2-(methylthio)ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid; Tris base, trishydroxymethylaminomethane; N\(_2\)-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tea, trisethanolamine; SDS, sodium dodecyl sulfate; DEAE-cellulose, diethylaminoethylcellulose; dscyl, 5-dimethylaminonaphthalene-1-sulfonic acid; Methylmethanopterin, 5-dimethylaminonaphthalene-1-sulfonic acid; and N,N’-methylenebisacrylamide and all protein standards.

2. Portions of this paper (including "Experimental Procedures," Figs. 1 to 8, and Table 1) are presented in mimeo print as prepared by the author. Mimeo is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, Bethesda, Md. 20014, Request Document: No. 80M-2314, cite author(s), and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Vanaray Press.

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Purification of Component C—Component C of the CH3S-CoM methylreductase system of *Methanobacterium thermoautotrophicum* was purified to homogeneity as judged by the behavior upon nondenaturing gel electrophoresis and sedimentation velocity centrifugation. As shown in Fig. 1 when component C was subjected to electrophoresis on polyacrylamide gels of varying porosity at either pH 7 or pH 9, only one major band was detected on gels stained for protein. Sedimentation velocity centrifugation of component C revealed a single, symmetrical sedimenting boundary with a corrected s value of 15.05.

The purification scheme employed for the purification of component C relied heavily upon the use of gradient sievesorptive chromatography (25). Through the use of this chromatographic method, it was possible to remove extraneous proteins and yet maintain an active component C. Conventional methods of protein purification were either ineffective or fatal to the purification of component C. The progress of purification of component C was followed spectrophotometrically as described in "Experimental Procedures." Component C activity was determined on a 25-μl aliquot of each fraction in the standard methyl reductase assay.

| Step or Treatment | Volume (ml) | Total Protein (μg) | Specific Activity (nmol NADH/min/ml) | Recovery (%) |
|-------------------|-------------|--------------------|----------------------------------|--------------|
| 1. Cell-free extract | 100 | 2450 | 1.2 | 100 |
| 2. DEAE-cellulose | 15 | 871 | 2.3 | 81 |
| 3. DEAE Sephadex A25 | 4 | 371 | 6.7 | 92 |
| 4. DEAE Sephadex A25 | 1.8 | 258 | 6.7 | 92 |
| 5. DEAE Sephadex A25 | 2.5 | 50 | 10.0 | 15.7 |

**TABLE I** Purification of Component C From Methanobacterium

![Gradient Sievesorptive Chromatography of Component C](image)

**Figure 2.** Gradient Sievesorptive Chromatography of Component C. Crude component C from DEAE-cellulose column (15 ml) was chromatographed on a DEAE-Sepharose A-25 column containing an 80-ml preformed gradient from 0.1 M NaCl in buffer A as described in "Experimental Procedures." Component C activity was determined on a 25-μl aliquot of each fraction in the standard methyl reductase assay.

![Molecular Weight Determination of Component C by Polyacrylamide Gel Electrophoresis](image)

**Figure 3.** Molecular Weight Determination of Component C by Polyacrylamide Gel Electrophoresis. The relative mobility of component C and various proteins of known molecular weight to bromphenol blue were determined by electrophoresis on 10, 8, 7 and 5% polyacrylamide gels at pH 7. The slope obtained for each protein was plotted according to the method of Hedrick and Smith (12). The standards are indicated.

The amino acid composition of component C has been examined; based on an M, of 300,000 the moles of amino acid mol of protein values were: Asp, 272; Thr, 126; Ser, 117; Glu, 347; Pro, 149; Gly, 259; Ala, 272; Val, 214; Met, 60; Ile, 138; Lys, 45; Trpy, not determined; Cys, 29. The most unusual feature of the amino acid composition was a 2:1 preponderance of acidic amino acids to basic amino acids, a fact supporting the observation that the protein complex bound rather tightly to anionic ion exchange resins. The ratio of polar to nonpolar amino acids was 1.36, r calculated from the amino acid composition was 0.71.

The subunit composition of component C after SDS-gel electrophoresis is shown in Fig. 5. The native protein is composed of three different subunits: M, = 68,000, M, = 45,000, and M, = 38,500. The estimated molecular weight of the two smaller subunits varied slightly, i.e. ±3,000. The fact that each subunit was present in equal molar amount in component C is supported by densitometer scans. Graphical integration of each peak area gave the following molar subunit stoichiometries on a total mass basis: 2.1 for the M, = 68,000 subunit; 1.9 for the M, = 45,000 subunit, and 2.15 for the M, = 38,500 subunit in the native protein of M, = 300,000. Thus, the native protein contains two copies of each subunit.

Evidence to support the presence of three different subunits in the native protein was obtained through NH2-terminal analysis. Component C was separated into its constituent subunits by SDS-gel electrophoresis, the band corresponding to each of the subunits was excised, eluted, and subjected to electrophoresis again to assure its purity. These separated subunits were dansylated, hydrolyzed, and then chromatographed on polyamide sheets with the appropriate standards. Methylene was the NH2-terminal amino acid of the M, = 68,000 subunit whereas the two smaller subunits contained...
alanine at this position. Treatment of the native protein with dansyl chloride followed by hydrolysis and chromatography gave only two spots on polyamide sheets, corresponding to methionine and alanine. The alanine spot had greater fluorescence intensity when viewed under long wavelength ultraviolet light. Thus, only three subunits were found for component C.

The protein had a characteristic nonfluorescent, yellow color, the spectrum of which is shown in Fig. 6. The protein had an absorbance maximum at 425 nm and a shoulder at 455 nm. There were no peaks in the 300 nm region as would be expected for a typical flavin. Over a 2-h incubation period, none of the common reductants such as ascorbate, dithionite, or sodium borohydride or oxidants such as ferricyanide brought about any significant change in the visible spectrum of the protein. Attempts to perturb the visible spectrum of component C by addition of either hydrogenase or hydrogenase and component B were not successful.

Reconstitution of CH$_3$-S-CoM Methylreductase System—The ability of purified component C to reconstitute the CH$_3$-S-CoM methylreductase system is shown in Fig. 7. The rate of methanogenesis was linearly dependent on added component C over a 5-fold concentration range, i.e. 133 nM to 670 nM. The slight amount of activity seen in the absence of added component C was due to residual enzyme adhering to the much cruder hydrogenase. This was readily demonstrated by immunodiffusion experiments where both hydrogenase and purified component C were allowed to diffuse toward antibodies to component C. The immunoprecipitation line obtained for the hydrogenase fused with that found for the purified protein (data not shown).

Immunodiffusion: Detection of Component C in Other Methanogens—Immunodiffusion experiments were undertaken to determine whether antibodies to component C of M. thermoautotrophicum would react with extracts of other methanogens to produce immunoprecipitation. Clarified cell-free extracts of the following methanogens were prepared: M. thermoautotrophicum; Methanospirillum hungatei; Methanohacterium formicicum; Methanohacterium htyantii str. MOHG; Methanobrevibacter ruminantium and Methanogenium marisnigri. All methanogens tested gave immunoprecipitation lines that fused with component C except M. marisnigri and M. ruminantium even though these extracts had fully active methylreductase activity.

Immunoreplicate Electrophoresis—Because of the tendency of component C to associate with other proteins in cell extracts, it seemed wise to examine the cross-reactivity of these methanogen cell-free extracts after resolution by non-
dissociating gel electrophoresis on a 10% polyacrylamide slab. The results are shown in Fig. 8. Clearly, M. marinus and M. ruminantium have protein bands with the same mobility as purified component C, but as described above, these failed to cross-react with antibodies to component C.

**DISCUSSION**

Since component C is an acidic protein, ion exchange chromatography appeared to be a logical technique to use for purification. Chromatography of component C on DEAE-cellulose under a variety of conditions gave negligible purification. The use of the stronger ion exchange resin DEAE-Sephadex A-25 resulted in nearly total inactivation of recoverable component C activity. Poor purification was obtained with Sephacryl S300 or Bio-Gel P200. A method of purification was needed that would remove contaminating proteins and still yield an active component C; gradient sievesorptive chromatography fulfilled these requirements. Furthermore component C emerged from these columns in a concentrated band of activity and its behavior was reproducible from column to column. This technique may be ideal for the purification of other large, multisubunit proteins where stability is a problem.

The low increase in specific activity upon purification of component C is puzzling. However, as seen in Fig. 7, component C is one of the major soluble proteins released upon cell breakage. Whether or not it is 12% of the total protein remains to be seen. It also is possible that one or more of the proteins removed during the purification of component C may play a role in efficiently integrating component C into the methylreductase system. Experiments to clarify this possibility are in progress.

When it became evident in 1967 (26) that the major metabolic system used by most methanogens was an anaerobic respiration in which hydrogen was oxidized and carbon dioxide was reduced to methane, we concluded that ATP synthesis in these organisms must occur by electron transport phosphorylation; ATP pools and the effect of uncouplers were studied (27, 28). Recently, excellent evidence has been presented by Doddema and Vogels (29, 30) as well as by Sauer et al. (31, 32) that intact vesicles of methanogens oxidize hydrogen, producing ATP by electron transport phosphorylation with the reduction of carbon dioxide to methane. The membranous vesicles of Sauer et al. (32) produced only a slight increase in methane formation when ATP or CH2-S-CoM were added, and only a fraction of the methyl moieties of added CH2-S-CoM was converted to methane by these intact vesicles. To us there appears to be a reasonable explanation for these results; the membranous vesicles may represent a highly integrated system in which ATP and CH2-S-CoM could be generated in nearly saturating amounts either on or inside the membrane environment. Thus, externally added ATP or CH2-S-CoM, both highly charged molecules, may not penetrate readily to the appropriate enzyme sites in the membrane.

To understand the enzymology of this multi-enzyme, multienzyme system, we have elected to study specific reactions of the components of which can be fractionated in solution. (Whether or not these proteins are truly soluble is another question.) We have focused on the CH2-S-CoM methylreductase, and by providing ATP and CH2-S-CoM have simplified the system, component C being the first protein to be purified to homogeneity. The native protein as purified contains six subunits having a stoichiometry of \( \alpha \beta \gamma \delta \). The molecular weight of about 300,000 is slightly greater than twice that reported by Gunsalus and Wolfe (1). Although no evidence of an M, = 135,000 component C was found during any stage of purification; it is possible that their protein was a trimer composed of \( \alpha \beta \), and \( \gamma \). Component C has a distinct nonfluorescent, yellow color which can be attributed to an acid- or heat-extractable chromophore. Neither the structure nor function of this chromophore is known at the present time. Preliminary observations suggest that the chromophore is the nickel-containing factor Fe,N (33).

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